Vineet Kumar
Indu Shekhar Thakur *Editors*

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*Dedicated to my teachers and mentors, from whom I continue to learn, and to my family for their support, blessings, motivation, and love.*
***Vineet Kumar***

*Dedicated to my family especially my wife without whose support this book would not have been possible.*
***Indu Shekhar Thakur***

# Preface

The book, *Omics Insights in Environmental Bioremediation,* is a meticulously sketches in-depth information of omics technologies developed in past few years and are used in the bioremediation of contaminated environments that are polluted with various inorganic, metals, organometallic, and organic hydrocarbons in a coherent manner related to different cutting-edge. Inorganic chemicals, organic hydrocarbon, metals, nonmetals, metalloids, radionuclides, pharmaceutical substances, polyaromatic hydrocarbons (PAHs), organic solvents, persistent organic pollutants (POPs), emerging contaminants, and eco-estrogens are increased in the soil, water, and other components of the environment as a result of human activities, rapid industrialization, and growing populations. The chlorinated, and polyaromatic hydrocarbon not degraded easily into the environment by physico-chemcial and biological means that are persistent, recalcitrant, and highly toxic to human and other biological entities. The POPs transported by wind and water, which have become a local, regional, and global problem, have high bioconcentration, toxicity, and long-distance mobility. They can bioaccumulate in animal tissues and biomagnify along food chains and food webs and pass from one species to the next tropical level. The toxic nature of these chemicals adversely affects human health, environment, and wildlife. Some of the refractory and noxious organic compounds are formed unintentionally once used in agriculture, disease control, manufacturing, or industrial processes which are highly toxic and persist in the soil and aquifers for several years. Degradation and remediation of such organic compounds are urgent need to reduce the burden in the environment. The use and production of these compounds are banned over five decades ago, but they are still present in the environment, a risk to human health and ecosystems, and pose a threat to food safety.

Biodegradation and bioremediation are important strategies to degrade refractory and toxic pollutants and to reduce the risk of contamination in the environment. Microorganisms having catabolic gene and proteins to degrade such organic compounds but are not successful at open contaminated sites. The numerous technologies being developed for this purpose are bioaugmentation, biostimulation, bioslurping, bioventing, and biopiling and others in which pollutants-degrading bacteria are introduced from an external site into a contaminated site and are expected to provide a safe and inexpensive *in situ* strategy that does not adversely affect soil function. However, pollutants-degrading bacteria

rarely exist in nature, and their enrichment and isolation have been extremely difficult in laboratory conditions. The application of omics technologies to bioremediation and biodegradation research provides a greater understanding of the key pathways and new insights into the adaptability of a wide range of microorganisms that are key player in the removal of chemical contaminants from polluted environment. Molecular techniques, such as transcriptomics, metagenomics, proteomics, and related omics approaches have led to new environmental management approaches and provides an opportunities to researchers to decontaminate water, soil, and aquifers. The methods and techniques have sped up the investigation of culturable and unculturable microorganisms including organization of microbiome which is reliant on culture technology. This also helps to understand emerging genetic diversity including emerging DNA (eDNA) and RNA (eRNA) in the environment which can be exploited for ex situ and in situ bioremediation purposes.

Metagenomics research methods and approaches have phased out molecular cloning techniques and paved the way for a new comparative metagenomics strategy for structural and functional genomics. Advances in high-throughput sequencing technology have changed the traditional microbiology tools and techniques used for environmental applications. Metagenomics has emerged as a recent field of study that aims to recognize microbial diversity and functions, their activities, interactions, cooperation, and growth in a variety of contexts. The other omics approaches, such as transcriptomic technologies are used to investigate genome-wide transcriptional activity of an organism's transcriptome, the sum of all of its RNA transcripts. The information content of an organism is recorded in the DNA of its genome and expressed through transcription to understand comparative genotyping in a variety of microbiological functions and applications. The functional insights into the activities of microbial communities can be evaluated by analyzing mRNA transcriptional profiles and meta-transcriptomics technologies. Expression of proteins by the genome of an organism is studied by the proteome which involves translation of these RNA molecules into protein. The complete set of all the ribonucleic acid (RNA) molecules expressed in some given entity, such as a cell, tissue, or organism, is transcriptomics which encompasses everything relating to RNAs. This includes their transcription and expression levels, functions, locations, trafficking, biodegradation, and bioremediation of persistent toxic compounds in the environment.

In view of above, the book entitled *Omics Insights in Environmental Bioremediation* is formulated with four major parts containing latest and updated information on various aspects of omics and environmental bioremediation. The four parts are spread over 29 chapters, contributed by leading workers, drawn from the world over, in their field, providing latest research and development in different aspects of omics technologies for the ecological restoration and safety of public health. In this book, expert authors critically review the most important current research and development in this exciting field and further address different topics related to basic and advanced knowledge on omics-based microbial technologies for the bioremediation of environmental pollutants.

*Part I* Bioremediation and Biodegradation mainly comprises 6 chapters. Chapter 1 provides an overview of metagenomic approaches and discusses how they have been utilized to better understand damaged ecosystems and guide

environmental rehabilitation best practices. Chapter 2 gives a brief overview on the impact of metabolomics on bioremediation. In addition, a detailed application of metabolomics combined with microbial biodegradation has been explained with respect to some of the earlier research. A another omics approach, such as metabolomics can be used to better insight the breakdown of different complex compounds and their metabolites. Chapter 3 describes how the metagenomics approaches are utilized in monitoring the contaminated environment and bioremediation to solve the pollution problems, and discussed its potentialities, and current challenges. Moreover, the role of metagenomics in apprehending the microbial community diversity and functions involved in bioremediation has been discussed. This chapter also discusses the analysis of microbial diversity in divergent environments and mostly employed sequencing platforms. Chapter 4 highlights the utilization of the combined system of plant and microbes enhancing the removal of contaminants from the polluted environment and describes how they play a vital role in the remediation of chemical contaminants. Plant-associated microbes has been considered as a promising approach in the remediation and ecorestoration of polluted environment. Chapter 5 discusses the various bioremediation techniques and their mechanisms used for the removal of toxic heavy metals from the polluted sites. The chapter also presents recent advancement in the field of bioremediation in terms of use of plants and their metabolites, plant growth promoting rhizobacteria (PGPR), and nanoparticles for the efficient removal of heavy metals from contaminated sites. Chapter 6 conveys the most delegate chemicals and their bioremediation by the action of cytochrome P450, lipases, proteases, dehydrogenases, dehalogenases, hydrolases, and laccases, which have shown promising tools for possible degradation of polymers, fragrant hydrocarbons, halogenated compounds, colors, cleansers, and agrochemicals. The significance of biocatalytic tools for future advancement in natural biotechnology has also been discussed.

*Part II* Environmental Pollution and Wastewater Treatment comprises 8 chapters. Chapter 7 explores and discusses the sources of microplastics in wastewaters, their properties, ecotoxicity, health risks, and numerous approaches that are already in use and those that are being developed for characterization of microplastics in wastewater, and bioremediation strategies for the removal of microplastics from wastewater for pollution prevention and control. Chapter 8 mainly deals with the microbial community and composition present in activated sludges. The seasonal modulation of the microbial communities and antibiotic-resistant genes present in activated sludge has also been discussed in detail along with the abundance of different microbial groups, their role, and physiological activities in activated sewage sludge. Chapter 9 provides an overall information of various parameters of discharged effluents to determine the role and mechanisms of microorganisms that are used in different types of aerobic and anaerobic treatments. Besides, controlling parameters of wastewater treatment plants, like solids retention time, hydraulic retention time, and other attributes are discussed in order to obtain reusable water as per the permissible limit norms set by the various environmental regulatory agencies. In addition, various microbial-based aerobic techniques for

decontamination of wastewater from various industries, such as the textile industry, paper-based industry, petroleum industry, food and dairy-based industries, brewery industries, and miscellaneous industries has been explained in detail. Chapter 10 focuses on the important aspects of omics, and their applications, limitations, challenges, and futuristic approaches related to industrial wastewater treatment. The diverse composition of industrial effluents and the prevailing treatment methodologies are addressed, and the omics-based approaches in industrial wastewater treatment and the futuristic approaches are critically discussed. Chapter 11 discusses the various microalgae's involvement in wastewater treatment, ranging derived from degree of microalgal bioremediation to environmental enhancement via microalgal biomass productivity and $CO_2$ fixation. This chapter also covers biological and technical aspects for modifying algae-based wastewater systems and increasing biomass output for value-added products and biomaterials for future uses. Chapter 12 describes the role of phytoremediation technologies used in the removal of toxic heavy metals, such as lead, cobalt, nickel, and cadmium from the environment, and emphasis is given to hyperaccumulator plants and their role in heavy metals removal. In the removal of these heavy metals, aquatic hyperaccumulator plants determined today are put forward and recommendations are made to solve these problems in wastewater. Chapter 14 highlights the use of microbes having effective potential to degrade noxious compounds contaminating groundwater and soil.

*Part III* Omics Approaches in Environmental Remediation comprises 8 chapters. Chapter 15 provides information on metagenomics, and other recently developed omics technologies that are a very much reliable, sensitive, accurate, and more rapid approach for the detection of microbes and provide a complete view of the microbial community. These approaches helps in the identification of unknown microbial communities and microbes which cannot be cultured in laboratory and can help in distinguishing microbial communities. It relies on much information from the databases to compare the observed information and the existing ones. This not only helps in speeding up the biodegradation process but also can identify new species or enzymes and even complex metabolic pathways which can help in further researches. Chapter 16 provides insights about genes and genomes that play a role in building heavy metal and metalloid tolerances that will help scientists to develop more efficient microorganisms to employ in the bioremediation of soil and water ecosystems. Chapter 17 conveys metagenomics research for evaluating microbial community composition in polluted areas to select effective microbes helping in heavy metals remediation. This chapter also discusses microbial interaction with heavy metals as well as different bioremediation strategies, microbial community analysis of contaminated areas through metagenomic library construction and different screening approaches, and how different bioinformatics tools help to analyze the metagenomic data. Chapter 18 highlights the combinational use of genomics, proteomics, metabolomics, along with different omics approaches and the promises they hold for the advanced bioremediation processes. Chapter 19 highlights the application of omics technologies in the study of microorganisms adapted to cold environments and the potential application of these or their constituent biomolecules

in the bioremediation of contaminated environments, to provide a framework for understanding the main metabolic traits developed by these extremophilic microorganisms to adapt to cold in polar or oceanic environments, as well as their potential use in bioremediation processes. Chapter 20 describes the functional role of microbes using omics approach and its limitation in industrial application. Omics also deciphers the potential connection between the genetic and functional similarity among numerous microbes which helps in hastening in situ bioremediation and minimizing the pollution load. Chapter 21 focuses on the metagenomics principle along with recent advances in this field. The main focus of this chapter is on exploring degrading enzymes by metagenomics and their applications in microbial biodegradation of environmental pollutants. How these approaches accommodate to find out the role of involved microorganisms in the biodegradation of pollutants in the contaminated sites is summarized. Chapter 22 discusses biofuels, their generations, and how they are normally produced. This also explores the application of microorganisms and algae in the production of biofuels and their advantages, and how the OMICS techniques can be used, helping in the choice of microorganisms or plants or modifying them to obtain higher yield in the production of renewable fuels.

*Part IV* Recent Trends and Development in Omics Technologies comprises 7 chapters. Chapter 23 puts forward an overview of brief history of nucleotides sequencing technologies emerged over recent times with a light on different platforms of sequencing available. It also deals with bioinformatic analysis and tools available for interpretation of the obtained sequencing databases. The application of high-throughput sequencing (HTS) platforms in algal research is also covered in this chapter. Chapter 24 discusses in detail the computational strategies for identifying possible enzyme candidates from shotgun sequencing data and experimental strategies to characterize candidate enzymes once they have been identified. Finally, author review emerging methods for metagenomic enzyme discovery as well as future goals and challenges with an emphasis on metagenomics-based approaches. Ultimately, the chapter present a largely generalized approach to metagenomic enzyme discovery that can be applied in most investigations of the nature, with discussions specific to pollutant degrading enzymes wherever appropriate. Chapter 25 highlights the insights of various metagenomic approaches, such as metatranscriptomics, metaproteomics, metabolomics, and fluxomics used in environmental and microbial analysis. This chapter also elaborates on the high-throughput next-generation sequencing (NGS) technologies and the procedures associated with the library preparation. Workflow of metagenomics which is used as targeted and shotgun metagenomics is discussed. Various examples of microbes in environmental cleanup and bioinformatics tools utilized in the metagenomic data analysis are also elaborated. Furthermore, the challenges associated with the metagenomic approaches are discussed. Chapter 26 provides a fundamental understanding of metagenomics methodology and applications in order to better understand microbial diversity, functions, and structure in contaminated environments. Chapter 27 highlights the recent advancements in this technology that resulted in a breakthrough in tackling it by clustering regularly interspaced palindromic repeats (CRISPR)-assisted three types of remediation. The CRISPR is a game-changing

genomic tool that allows plants, bacteria, and fungi to improve specific characteristics. Authors summarize current progresses in our understanding of CRISPR Cas-based functional gene editing for improvement in microbial bioremediation, phytoremediation, and mycoremediation in this chapter. Furthermore, authors discuss recent strategies involving CRISPR Cas in the improvement of bioremediation for successful waste management. Chapter 28 addresses the importance of studies carried out with DNA metabarcoding technology in environmental samples, aiming the identification of the microbial communities that possibly act in the processes of removal of polluting compounds present in the environment. Chapter 29 highlights the present level of knowledge on data-driven enzyme redesign in order to actively progress new research using artificial intelligence. This review also explains about the artificial intelligence system for stimulating, predicting, and controlling therapeutic processes, as well as for environmental cleaning.

The main aim of the book is to focus on the use of microbial bioremediation approaches in cleaning the polluted environment and make the depleted or degraded fields/water bodies fertile and rejuvenated to maintain sustainability. Thus, in our opinion, this book is extremely useful for scientists, environmentalists, and ecotoxicologists, working in the field of microbial degradation and bioremediation and explicitly targeted as good teaching material for undergraduate, postgraduate, and more seasoned researchers, and recommended reading for everyone interested in environmental microbiology, biotechnology, and molecular biology.

Gurugram, Haryana, India Vineet Kumar
Gurugram, Haryana, India Indu Shekhar Thakur

# Acknowledgments

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Finally, we would like to apologize in advance for any errors that may occur in the text and express our heartfelt embarrassment.

We should be pleased to receive any comments on the content and style of *Omics Insights in Environmental Bioremediation* from students, professionals, environmentalists, and policymakers, all of which will be given serious consideration for inclusion in any further editions.

# Contents

# About the Editors

**Vineet Kumar** is presently working as Assistant Professor in the School of Engineering and Sciences at G D Goenka University, Gurugram, Haryana, India. Prior to joining at G D Goenka University, Dr. Kumar served in various reputed institutes in India like CSIR-National Environmental Engineering Research Institute (NEERI), Maharashtra, India; Guru Ghasidas Vishwavidyalaya (A Central University), Bilaspur, India; Jawaharlal Nehru University, Delhi; Dr. Shakuntala Misra National Rehabilitation University Lucknow, India, etc. He received his M.Sc. (2010) and M.Phil. (2012) degrees in Microbiology from Ch. Charan Singh University, Meerut, India. Subsequently, he earned his Ph.D. (2018) in Environmental Microbiology from Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, India. Dr. Kumar's research interest includes environmental microbiology and biotechnology, bioremediation of hazardous and emerging pollutants, phytoremediation of heavy metals, metagenomics of industrial waste, wastewater treatment, environmental monitoring, waste management, bioenergy, and biofuel production. Presently, his research mainly focuses on the development of integrated and sustainable treatment techniques that can help in minimizing or eliminating hazardous waste in the environment. He has published more than 32 articles in reputed international journals, written 4 Proceeding papers, 45 book chapters, and authored/edited over 18 books on the different aspects of phytoremediation, bioremediation, wastewater treatment, omics, genomics, and metagenomics, with more than 1005 citations and an *h*-index of 18. He has presented several papers relevant to his research areas

at national and international conferences. He is an active member of numerous scientific societies including the Microbiology Society, UK, and the Indian Science Congress Association, India. He serves as an editorial board member and reviewer for many peer-reviewed journals and has received awards for his work, including the Young Scientist Award, National fellowship, and best poster presentation awards. Dr. Kumar has been serving as an associate editor/guest editor and reviewer in many prestigious international. He is the founder of the Society for Green Environment, India (website: www.sgeindia.org). He can be reached at drvineet.micro@gmail.com.

**Indu Shekhar Thakur** is currently working as a Professor & Director in Amity School of Earth & Environment Science (ASEES) and Head of the Centre of Excellence in Bio-Energy at Amity University Haryana, Manesar, Gurugram, Haryana-122413. He was a Professor in the School of Environmental Sciences (SES) at Jawaharlal Nehru University (JNU), New Delhi, India. He has obtained his M.Sc. (1980) in Life Science and M. Phil. (1981) in Environmental Science from JNU, New Delhi, India. At the same institution, he earned his Ph.D. (1986) in Environmental Science. He started his career as Assistant Professor at G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, and developed several courses, viz. Environmental Sciences and Agroecology for undergraduate students and Biodegradation and Waste Treatment Design and Ecotoxicology for postgraduate students. He joined the SES at JNU, New Delhi, in January 2004 as a Professor. He has more than 35 years of teaching, research, and outreach/extension activities experiences in the field of Environmental Sciences. He and his research group are working on bioremediation, biovalorization, and detoxification of natural and organic compounds, developed bacterial consortium by genetic breeding, characterized genes and proteins, proteomics, genomics analysis for $CO_2$ sequestration for biomass, enzymes, biodiesel, bioflocculant, bioplastic, and biomaterials; bacteria augmented municipal sludge scaled to 200 litre bioreactors for cost-effective biodiesel production; biocomposite materials synthesized by enzymes adsorbed on calcite of $CO_2$ sequestrating bacteria and biochar for chromate, arsenite, and heavy metals removal; degradation of

pentachlorophenol in tannery, lignin in pulp and paper mill, melanoidin in distillery, dioxin-like compounds, ecoestrogens, emerging contaminants in solid sludge, landfill leachates, pesticides in the agriculture field, and recovery of nitrogen and phosphorus in wastewater performed. He has published more than 247 research papers, review articles, and technical reports in international and national peer-reviewed journals of repute with more than 7689 citations and an *h*-index of 51. In addition, he has published 4 books and 35 book chapters and completed 22 sponsored research projects funded by various agencies and departments. He has four patents and technologies to his credit. He was a Visiting Scientist and Visiting Professor to Germany, Japan, Switzerland, Canada, France, etc. and as a member of the several committees visited the USA, Italy, Austria, Netherland, Finland, Sweden, etc. He is a member of the editorial board and reviewing committee of several journals and professional societies. He has completed more than 22 research projects as PI, and 28 Ph.D., 2 M.Phil., and 14 Postgraduate thesis/dissertations are completed under his supervision. He developed several courses for undergraduate and graduate students and reactivated ENVIS program of MOEFCC, DSA-SAP-II of UGC, major resource person for transferring academic activities of National Institute of Animal Welfare, MOEFCC, to JNU, New Delhi. Based upon this outstanding contribution in the areas of environmental microbiology and environmental biotechnology, he has been awarded the Fellow of the National Academy of Agricultural Sciences (FNAAS), Fellow of National Academy of Sciences India (FNAS), Fellow of International Bioprocessing Association (FIBA), Fellow of National Environmental Sciences Academy, and Fellow of Biotech Research Society of India (FBRSI). He has been also honored with Malviya Memorial Senior Faculty Award of Biotech Research Society of India. He is also a member in the American Society of Microbiology, USA, and the Association of Microbiologists of India.

# Part I

# Bioremediation and Biodegradation

# 1 Bioremediation and Functional Metagenomics: Advances, Challenges, and Opportunities

Swati Sokal, Preksha Palsania, and Garima Kaushik

**Abstract**

Non-degradable pollutants have emerged as a consequence of industrialization, population increment, and changing lifestyles, endangering human well-being and the environment. Biological techniques based on microorganisms are gaining popularity as an environmentally beneficial and cost-effective way to reduce pollution. Microorganisms may thrive in a variety of environments and create metabolites that degrade and change contaminants, allowing contaminated places to be organically revived. For a greater knowledge of biological and life sciences, multiple technologies have begun to be integrated into metagenomics. Technology such as metagenomics is now being used to develop strategies for studying the ecology and variety of microbes, as well as its application in the environment. Metagenomics is a novel and rapidly expanding discipline of environmental biology that provides a strong tool for accessing information on the genomes of environmental microorganisms and entire microbial communities. The application of metagenomics in environmental surveying and bioremediation is becoming more usual. In recent years, a number of functional metagenomics techniques have been used to investigate a wide range of resistant microbial degradation mechanisms. In a metagenomic investigation, it is critical to identify and screen metagenomes from the polluted location. These procedures are well-known for their effectiveness in eliminating many types of contaminants. These strategies may change rapidly as technology develops, but the once that focus on the best ways to improve bioremediation of the contaminated places will be the most successful. Culture-independent molecular approaches, on the other hand, can

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disclose very relevant information on the metagenome of environmental microorganisms, which play a key role in biogeochemical cycles and the breakdown and detoxification of environmental pollutants. These high-throughput studies would assist in the discovery of novel species for bioremediation, as well as providing new and interesting insights into their primary biodegradative processes at the molecular level. In this chapter, we are attempting to convey an overview that how functional one of the finest bioremediation adaptations that leads to the development of a clean non-toxic environment is metagenomics. We also went through the metagenomics analysis methods with respect to bioremediation. In addition to this, we provide an overview of examples of metagenomics in bioremediation which have recently been reported. Furthermore, our study clarifies the widespread use of metagenomes formed from metagenomics communities, which are capable of comprehending environmental pollutants and poisons.

**Keywords**

Bioremediation · Metagenomics · Biogeochemical · Microorganisms · Genomes

## 1.1 Introduction

Pollution is a worldwide issue, since numerous natural and manmade substances have been introduced into places where they are endangering human and ecosystem health (Fig. 1.1). The destruction, conversion, or stabilization of these substances by organisms, usually bacteria and plants, is known as bioremediation. When natural organisms at a contaminated site remove toxins on their own, the site's toxicity can simply be surveilled as decrement in pollutant or changed into a less hazardous form (Verma and Jaiswal 2016). In many circumstances, though, intervention can speed up the bioremediation process. The most typical methods for aiding clean-up are the insertion of stimulating alterations on site (e.g. micronutrients, organic material) and the transport of contaminates to treatment centres that are not on-site.

Microorganisms are frequently the most important players in bioremediation (Baker and Herson 1994). High-resolution genomic data is required to figure out how toxins and medicines alter the diverse microbial communities that exist in natural environments (Metzker 2009).

Many of the taxa and enzymes involved in bioremediation are unknown, despite the fact that some taxonomic groupings have been related to the presence of specific contaminants. Since there are many different microbial species that can coexist in single gramme of soil, pollution that is chemically similar to that of natural components in soil (where many species compete for carbon, nutrients and energy) consume as a source for survive. If the contaminant is complicated or fabricated in origin, there may be none indigenous strains effective of metabolizing it or decreasing its toxicity straight away (Boopathy 2000).

**Fig. 1.1** The different places depicting soil and water contamination as a result of industries' dumping of inadequately treated or untreated wastewater (Chandra et al. 2015)

Although many bio remediating microorganisms have been isolated from many sources, including contaminated areas, it is now widely recognised that the knowledge gained from these isolates is insufficient to completely understand the functioning of complex microbial communities. More comprehensive genetic data from natural habitats is needed to acknowledge how pollution impacts microbial communities as a whole, and whether bioremediation may be improved further (Lee 2013). Large-scale, culture-independent research, which is essential to attain this aim, is now achievable because of the advent of new high-throughput sequencing methods.

Bioremediation techniques can be divided into three types based on the degree of intervention. Natural attenuation is the least invasive method, in which pollutants are detoxified through natural processes by native species. This method is appealing because it does not require any expensive or perhaps harmful additions. However, natural attenuation rates in many systems may be excessively sluggish and unresponsive to dangers to the environment and health (Kumar et al. 2018). Biostimulation makes use of natural organisms, but it aims to speed up biodegradation by removing some environmental constraints. This is frequently accomplished by supplementing with restricted nutrients. Biostimulation can nevertheless cause sluggish biodegradation in some situations (Jain and Bajpai 2012).

The native microbial community's incapacity to digest the pollutant of concern could be the cause of the poor biodegradation rates. To address this problem, during bioaugmentation, a foreign organism or enzyme may be introduced to a system to increase biodegradation rates. Because a foreign organism is introduced into an

ecosystem, this method is the most invasive. In some cases, however, bioaugmentation has proven to be the most effective method of clean-up. One prevalent worry with bioaugmentation is that foreign species may not be able to thrive in the contaminated environment.

To most effectively drive bioremediation processes, it is necessary to acknowledge the microbial populations entangled in bioremediation, not just the end results and rates of pollutant breakdown. Changes in the characteristics and actions of a microbial community may have an impact on the fate of a pollutant in the environment since microorganisms are the drivers of bioremediation. Recent research has used next-generation sequencing techniques to learn more about the microbial communities participating in various bioremediation strategies (Jilani and Altaf Khan 2004). These methods have substantially increased our knowledge of the bioremediation techniques using microorganisms, as well as the influence of various contaminated clean-up response options. Molecular biology and metagenomics have dramatically advanced our knowledge of the biological systems present in these polluted settings, as well as our understanding of the microbial world in many situations. We intend to give a brief overview of metagenomic approaches and discuss how they have been utilized to better understand damaged ecosystems and guide environmental rehabilitation best practises.

## 1.2 Bioremediation

Bioremediation is a waste management technology in which biological organisms are used to remove or neutralize pollutants in the environment. The "biological" species include microscopic organisms like algae, fungi, and bacteria, as well as the "remediation" of the issue's treatment. Microorganisms thrive in a diverse range of environments across the biosphere. They thrive in a variety of environments, including plants, animals, soil, water, the deep sea, and the frozen ice. Microorganisms are the ideal candidates to function as our environmental stewards because of their sheer numbers and desire for a wide spectrum of pollutants (Kumar et al. 2022).

Bioremediation technologies became widely used and are still increasing at an exponential rate today. Because of its environmentally benign characteristics, bioremediation of polluted places has proven to be effective and trustworthy. Recent advancements in bioremediation techniques have occurred in the last two decades, with the ultimate goal of successfully restoring damaged areas in an economical and environmentally beneficial manner. Different bioremediation approaches have been developed by researchers to recover polluted ecosystems (Jan et al. 2003). Most of the issues related with pollution biodegradation and bioremediation can be solved by indigenous microorganisms found in disturbed areas.

Bioremediation has a number of advantages over chemical and physical remediation approaches, including being environmentally benign and cost-effective.

Bioremediation works by reducing, mineralizing, degrading, detoxifying, or transforming more hazardous contaminants into less toxic ones. Agrochemicals,

chlorinated compounds, dyes, heavy metals, pesticides, nuclear waste, organic halogens, plastics, greenhouse gases, xenobiotic compounds, hydrocarbons, and sludge are among the pollutants that can be removed. Toxic waste is removed from a polluted environment using cleaning techniques. Through the all-inclusive and action of microorganisms, bioremediation is heavily involved in the eradication, degradations, detoxification, or immobilization of various physical dangerous chemicals and chemical waste from the surrounding (Chauhan and Singh 2015).

## 1.3 History of Bioremediation

Bioremediation technique is known since the 1940s. Scientists already knew that certain bacteria have potential to degrade petroleum hydrocarbons.

George M. Robinson pioneered bioremediation in the 1960s. In 1968, while working as an engineer in California, Robinson arranged the clean-up of the first large-scale microbiological oil spill. He employed bioremediation to clean-up sewage, spills, and leach fields, as well as control odours and pests. Microbes are being employed to remediate oil spills, sewage, contaminated soil, and boost crop production. Bug cultures developed by George Robinson or one of his co-workers are used by almost every firm in this market (Taylor and Reimer 2008).

By the 1970s, tremendous progress had been made in this field of study. Nature, microbiologists understood, had an answer. Scientists knew that if the right amount of nutrients, like nitrogen, oxygen, and phosphate, could be added in the contaminated wells, the bacteria would multiply and remove the toxic gasoline faster and more efficiently than physical methods ever could.

Bioremediation has been utilized in a number of well-known clean-ups, including the 1989 Exxon Valdez oil spill in Alaska. Microbes assisted the many volunteers who worked to clean up the 11 million gallons of spilt oil by breaking down the oil as a food source. Engineers can help speed up the process of a pollutant spill by using particular bacteria, reducing environmental damage.

## 1.4 Bioremediation Successes

Bioremediation has been a huge success for the US Geological Survey. Their use of bioremediation has aided in the safe and effective clean-up of several spills, some of which were highly toxic, as well as the improvement of bioremediation knowledge and expertise. The following are a few of their accomplishments.

1. *Chlorinated Solvents, New Jersey*:
   In the heavily industrialized Northeast, chlorinated solvents are a particularly prevalent pollutant. Microorganisms can employ chlorinated chemicals as oxidants when other oxidants are unavailable because their metabolic processes are so flexible. United States Geological Survey (USGS) scientists at Picatinny

Arsenal, New Jersey, have extensively recorded such changes, which can naturally cure solvent contamination of ground water.

2. *Pesticides, San Francisco Bay Estuary*:
   The poisoning of water bodies by pesticides is a major concern across the US. In field and laboratory study in the Sacramento River and San Francisco Bay, the impacts of biological and non-biological processes in degrading commonly used pesticides such as thiobencarb, carbofuran, melinite, and methyl parathion have been proven.
3. *Gasoline Contamination, Galloway, New Jersey*:
   Contamination by gasoline in the US gasoline is arguably the most prevalent contamination of ground water. Rapid microbial degradation of gasoline pollutants has been documented at this location, demonstrating the importance of activities in the unsaturated zone in contaminant degradation.
4. *Sewage Effluent, Cape Cod, Massachusetts*:
   In the United States, sewage effluent is commonly disposed of in septic drain fields. Systematic observations of a sewage effluent plume at Massachusetts Military Reservation provided the first reliable field and laboratory data of how rapidly natural microbial communities reduce nitrate pollution in a shallow aquifer.
5. *Crude Oil Spill Bemidji, Minnesota*:
   A pipeline which carries the crude oil exploded near Bemidji, Minnesota, in 1979, contaminating the underlying aquifer. The harmful compounds seeping from the crude oil were rapidly destroyed by natural microbial communities, according to USGS scientists who studied the site. Significantly, the plume of contaminated ground water stopped increasing after a few years as rates of microbial degradation and contaminant leaching were found to be in balance. This was the earliest and best case of intrinsic bioremediation, in which contaminated ground water is remedied by naturally existing microbial processes without the need for human intervention (Atlas and Bragg 2009).
6. *Agricultural Chemicals in the Midwest*:
   In several Midwestern states, agricultural chemicals have an impact on the chemical quality of ground water. The fate of nitrogen fertilizers and pesticides in ground and surface waterways has been studied in the Midwest. Many common pollutants, such as the herbicide atrazine, are destroyed through microbial degradation and non-biological mechanisms.

## 1.5 Mechanism of Bioremediation

Microbes have great potential to interact with chemicals both chemically and physically, causing structural alterations or total mineralization of the contaminants. The ability to digest and detoxify inorganic and organic contaminants in the environment is possessed by a large variety of bacteria, fungus, and actinomycetes genera (Thapa et al. 2012). Organic contaminants are biodegraded by microorganisms through two processes: (1) primary metabolism and

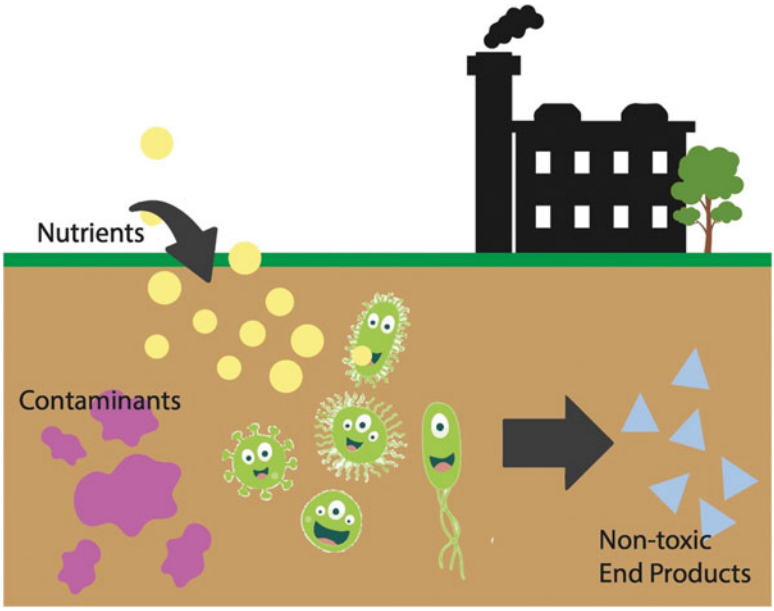


**Fig. 1.2** Mechanism of bioremediation (Boll et al. 2014)

(2) co-metabolism. The usage of the substrate as a source of carbon and energy has been termed as the primary metabolism of an organic substance. This substrate acts as an electron donor, allowing bacteria to proliferate. Organic contaminants are completely degraded as a result of this procedure (Fig. 1.2).

Co-metabolism, also known as gratuitous metabolism, co-oxidation, or accidental or free metabolism, occurs frequently in nature, where degradation activities are accompanied by the transformation of additional molecules, such as xenobiotics. The metabolism of an organic material that isn't a source of carbon and energy or a necessary nutrient and can only occur in the presence of a main substrate is referred to as "co-metabolism." Co-metabolism must be employed to remediate a xenobiotic-contaminated region when a molecule cannot serve as a source of carbon and energy owing to its chemical structure, which does not activate the needed catabolic enzymes. When microbial activity develops at a contaminated environment, it is a common occurrence. The enzymes of developing group of tissues and the manufacture of cofactors required for enzymatic reactions, such as hydrogen donors for oxygenase, are requirements for metabolic transformation (Leitão 2009).

The most typically reported pollutants to co-metabolize are PAHs with more than five aromatic rings, chlorinated biphenyls, chlorine mono-aromatics, and chlorinated aliphatic hydrocarbons. A technique may leverage aerobic or anaerobic metabolism of heterotrophic bacteria, depending on the pollutant of interest and the media. Aerobic metabolism, commonly known as aerobic respiration, is the utilization of oxygen ($O_2$) as a reactant to convert a portion of the carbon in a pollutant to carbon dioxide ($CO_2$), with the remaining carbon being utilized to regenerate new cell mass.

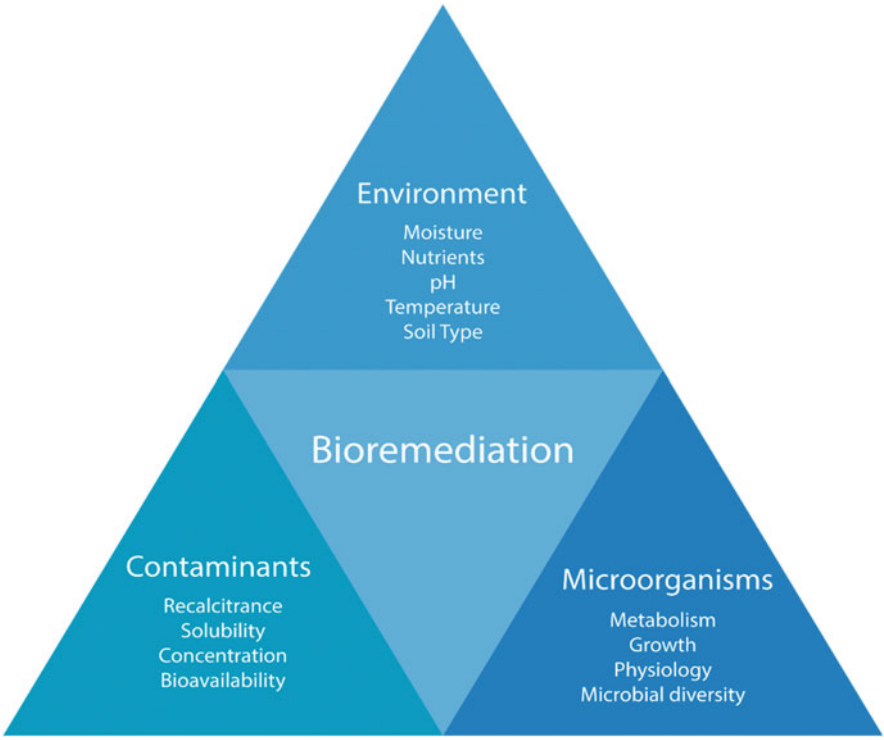


**Fig. 1.3** Major classes of factors influencing the bioremediation (Kumar et al. 2018)

Aerobic metabolism is more extensively employed and can be beneficial for hydrocarbons and other organic compounds.

Indigenous microorganisms in contaminated areas possess great tolerance for harmful contaminants and use them as a nitrogen, carbon, or energy source, degrading them into simple products up to total mineralization (Varma et al. 2011). Contaminated habitats, on the other hand, frequently contain a combination of organic and inorganic substances. If the concentrations of readily biodegradable pollutants in the matrices are too low, they may go undetected or disintegrate very slowly. The octanol water partition coefficient (log $K_{ow}$), acidity constant (pKa), aqueous solubility (Sw), octanol solubility, and pollutant concentration are all physicochemical characteristics that influence the absorption of organic pollutants by plants and microorganisms. The arrival and transfer of organic contaminants inside the cellular state of microorganisms is determined by the octanol water partition coefficient.

Some bacteria are unable to mineralize these contaminants because the enzymes utilized for degradation do not recognize them as a substrate. They may contain substitution groups such as amino, carbamoyl, halogens, methoxy, nitro, and sulfonate, and may be chemically and physiologically highly stable. Furthermore, the

chemicals may be water-insoluble in some situations and remain adsorbed to the soil's exterior matrix. Furthermore, the large molecular size of persistent organic pollutants (POPs) and the absence of permease in cells of microbes may hinder their uptake by cells. As a result, there could be a variety of reasons for ineffective biodegradation in contaminated areas. Microbial bioavailability is a fundamental concept in evaluating all bioremediation strategies. Bioavailability refers to the amount of a pollutant in soil that may be taken up or changed by living organisms. Simply put, a contamination cannot be rectified if it is so tightly bound up in the solid matrix that microorganisms seem unable to access it. The amount of bioavailable pollutant is determined by two main factors: mass transfer and cell intrinsic activity (Sharma 2012). Bioavailability varies by species and organism, and in situ microbial breakdown of organic pollutants is influenced by contaminant bioavailability and microbe catabolic activity. The rate and extent of contamination removal vary depending on the pollutant of concern and site-specific variables. Several factors influence clearance rates, including pollutant dispersion and concentration, contaminant concentrations, indigenous microbial populations and response kinetics, pH, temperature, nutrition availability, and moisture content. Many of these variables are dependent on the site and the local microbial community, making them difficult to control.

## 1.6 Microorganisms Used in Bioremediation

Due to their exceptional metabolic capacities and ability to grow under a wide range of environmental conditions, microorganisms are extensively spread throughout the biosphere. Microorganisms' dietary adaptations might be used to aid pollution biodegradation (Paul et al. 2005). Bioremediators are biological agents that are utilized in contaminated site bioremediation. Bacteria, archaea, and fungus are among the most effective bioremediators. Because of their benefits over traditional remediation processes, microorganisms play an essential role in pollutant removal in soil, water, and sediments (Singh et al. 2014). Microorganisms are helping to restore the natural environment and prevent pollution (Agrawal et al. 2021). Microbes have two characteristics that make them suited for the remediation process: flexibility and biological system. Microbial activity is primarily reliant on carbon. The bioremediation process was carried out in several conditions by a microbial consortia. Nitrosomonas, Achromobacter, Arthrobacter, Pseudomonas, Flavobacterium, Mycobacterium, Alcaligenes, Bacillus, Corynebacterium, Xanthobacter, and other microbes are among them. Bacteria are varied creatures that make great biodegradation and bioremediation players. Because bacteria have few universal toxins, any given substrate is likely to be broken down by an organism if given the correct conditions (anaerobic versus aerobic environment, sufficient electron acceptors or donor, etc.).

### 1.6.1 Fungi

Currently, bacteria are used in most bioremediation applications, with fungi being used in only a few cases. It can be transferred into bioremediation applications that can break down organic molecules and lower metal hazards. Fungi have an edge over bacteria in some circumstances, not only in terms of metabolic variety, but also in terms of environmental robustness. They can oxidize a wide range of compounds and withstand tough environmental conditions such as low moisture and high pollutant concentrations. As a result, fungi have the potential to be a very strong agent in soil bioremediation, and particularly diverse species, such as White Rot Fungi, have been a hot research area. The discovery of the white rot species *Phanerochaete chrysosporium*'s ability to digest many important environmental toxins in 1985 sparked interest in using fungi as a potential therapy for contaminants. The ability of these fungi to digest complicated compounds like lignin is their greatest distinguishing attribute. Other white rot fungus species were later identified to have similar properties. Furthermore, white rot fungi are useful because their hyphal extension degrades lignin extracellularly. This enables them to access soil contaminants that other species are unable to, as well as increase the surface area available for enzymatic interaction. These low-cost fungi can withstand a wide range of environmental conditions, including pH, temperature, and moisture content (Watanabe 2001). While many microbial organisms employed in bioremediation require the environment to be preconditioned before they can thrive, white rot fungi can be used directly in most systems since they disintegrate due to nutrient restriction.

#### 1.6.1.1 Phanerochaete Chrysosporium

The first fungus *P. chrysosporium* was to be linked to the breakdown of organic pollutants. Pesticides, polycyclic aromatic hydrocarbons (PAHs), dioxins, carbon tetrachloride, and a wide range of other pollutants have all been demonstrated to have significant bioremediation potential. Among fungal systems, *P. chrysosporium* has become a model for bioremediation. Other well-known white rot fungus include *Pleurotus ostreatus* and *Trametes versicolor*.

## 1.7 Factors Affecting Bioremediation

Controlling bioremediation process is a complicated system involving numerous variables. These characteristics include the presence of a microbial population capable of degrading pollutants, the availability of toxicants to the microbial population, and environmental parameters (temperature, soil type, oxygen, pH, and nutrient availability). Microbial activity, degradative enzyme activity, and hydrocarbon breakdown in general are all affected by these factors. This suggests that bacterial bioremediation could be more successful and efficient if these variables are tweaked, controlled, and managed (Lee 2013).

### 1.7.1 Biotic Factors

#### 1.7.1.1 The Availability of Bacteria That Degrade Hydrocarbons

In hydrocarbon-contaminated soils, microorganisms that break down these hydrocarbons are abundant and also widespread. The bacteria's capacity to quickly adapt to a hydrocarbon-polluted environment and utilize the pollutant as a sole energy and carbon source for metabolism and development is the reason for this. In a polluted environment, the presence of naturally occurring probable hydrocarbon-degrading organisms has a direct relationship with the efficiency of hydrocarbon biodegradation. These bacteria have metabolic activity and may break down hydrocarbon pollutants in either an aerobic or anaerobic environment. However, the types and nature of hydrocarbon pollutants, as well as the surrounding environmental circumstances, have an impact on their abundance and diversity in terms of species. (Fomina and Gadd 2014). As a result, the presence of naturally occurring appropriate bacteria is critical for microbial therapeutic activities to be implemented.

#### 1.7.1.2 Competition and Cooperation Among Bacteria

Bacterial cooperation and competition could be a key motivator for survival and stability among microbic communities on a specific home turf. Microorganisms that degrade hydrocarbons compete for survival in hydrocarbon-contaminated environments. Microorganism competition, whether interspecies or intraspecies, may, nonetheless, be a limiting factor in biodegradation efficacy. For example, hydrocarbon-degrading fungi and bacteria may compete for the activity of organic compound pollution as carbon sources as well as various limited resources for metabolism and growth. Furthermore, some hydrocarbon-degrading microbic species create compounds that prevent other hydrocarbon-degrading bacteria from growing and expanding. Exogenous bacteria are commonly used in analysis to break down organic chemical pollutants; however, they do not appear to be always efficient. As a result, the interdependency of microbic population is crucial for bioremediation to achieve success. Some hydrocarbon-degrading microbic consortia are incontestible to make synergistic connections for full degradation. Thus, microorganism consortia will give vital info for in place bioremediation of organic compound-related pollutions by providing comprehensive views regarding modification of their hydrocarbon biodegradation capabilities.

#### 1.7.1.3 Exogenous and Indigenous Hydrocarbon-Degrading Bacteria

Bacteria, either naturally existing or introduced, can eliminate hydrocarbon contaminants. Native bacterial populations, on the other hand, are better at mineralizing hydrocarbon pollutants than imported inocula for long-term bioremediation effectiveness. Degrading a wide range of hydrocarbon components from contaminated sites necessitates a microorganism with high stability and physiological adaptations to local nutrition and environmental conditions. When compared to foreign microbial consortia, indigenous bacteria shows higher rate of degradation. This is due to the fact that foreign bacteria cannot withstand abiotic stress and must

rely on the soil environment to survive and multiply. As a result, for reliable and low-cost bacterial bioremediation, native bacterial isolates of certain strains or consortia derived from hydrocarbon-contaminated areas are recommended.

#### 1.7.1.4 Number of Hydrocarbon-Degrading Bacteria

The quantity of bacteria in the soil that digest hydrocarbon pollutants is a significant factor in their decomposition. If the region has not previously been polluted by hydrocarbon pollutants, the quantity and variety of microorganisms that can degrade hydrocarbons are substantially smaller than the total number of naturally accessible microorganisms. Bacteria that degrade hydrocarbons, on the other hand, have a better chance of living in hydrocarbon-polluted environments than bacteria that do not. (Fantroussi and Agathos 2005). This difference is significant since microorganisms that degrade hydrocarbons are known to have efficient hydrocarbon degradative enzymes as well as a variety of metabolic pathways. The higher the population of bacteria that degrade hydrocarbons, the faster they degrade. As a result, determining the quantity of possible microorganisms in contaminated areas is critical to achieving effective bacterial remediation.

#### 1.7.1.5 Redox Potential of the Bacteria

To perform biological functions, maintain cell structure, and reproduce, bacteria require energy. A redox mechanism within bacterial cells produces this energy physiologically. By increasing the electron transfer from electron donors to electron acceptors, bacteria catabolize hydrocarbon contaminants as a source of energy in aerobic or anaerobic bioremediation. Redox potentials are critical for enhancing bacterial growth and facilitating the bacterial energy production (respiration) by oxidation of hydrocarbon-derived contaminants. Many organic contaminants, such as hydrocarbons, have a sluggish rate of degradation due to their high redox potential, according to studies. In aerobic environments, oxygen is required for the enzymatic activation of aromatic hydrocarbons as monooxygenases or dioxygenases, as well as serving as a final electron acceptor. Aromatic compounds are best example for giving electron as it is known to have large Gibbs free energy change of oxidation of aromatic compounds with various electron acceptors required for bacterial growth. Furthermore, chemicals having a high reduction potential (such as perchlorate and chlorate) are good electron acceptors for microbial metabolism in anaerobic bioremediation processes. At extensively polluted areas, electron donors (hydrocarbons) outnumber electron acceptors' oxidation potential. As a result, the availability of certain electron acceptors can affect the respiration and biodegradation processes. As a result of the low redox potential and depletion of electron acceptors, bacterial breakdown may be inhibited. This is due to the reduced redox potential, which causes anoxic conditions and slows biodegradation.

#### 1.7.1.6 Effect of Biosurfactants

Biodegradation becomes a concern when pollution caused by hydrocarbons is dumped into the soil. Because of their high soil adsorption and lack of bioavailability, in that case it shows limited microbial degradation. This problem has been

addressed with the use of inorganic and organic surface-active compounds. Chemically generated surfactants have been worked to increase the solubility of hydrocarbons through a process known as emulsification. Ionic, non-ionic, biological, and combination surfactants were used to remediate hydrocarbon contaminants. Chemically synthesized (inorganic) surfactants, on the other hand, are not suggested for future as they should not be used since they are resistant to secondary contamination, are primarily harmful to the environment, and have little to no influence on the efficiency of hydrocarbon biodegradation. This suggests that biosurfactants derived from natural sources (plants or microorganisms) are appropriate since they are biodegradable and have beneficial characteristics, nontoxic, have a high specificity under difficult conditions, and are better adapted to breakdown. To emulsify and readily absorb the hydrocarbon pollutants, hydrocarbon-degrading bacteria produce such surface-active biological chemicals extracellularly. Biosurfactants include glycolipids, lichenysin, and surfactin, as well as lipopeptides, fatty acids, phospholipids, polymeric biosurfactants, lipoproteins, and neutral lipids. *Enterobacter* sp., *Burkholderia* sp., *Bacillus* sp., *Pseudomonas* sp., *Aeromonas* sp., *Acinetobacter* sp., *Micrococcus* sp., *Rhodococcus* sp., and several halophile species are among the microorganisms that create them. Biosurfactants help hydrocarbon breakdown by assisting in the solubilization and desorption of contaminants, as well as altering the properties of bacteria cell surfaces (Meysami and Baheri 2003). The surface tension and interfacial tension of the water/oil or water/air interaction can be reduced by biosurfactants. As a result, the hydrocarbon substrate's surface area increases, making emulsification simpler, and the entire process increases the substrate's availability for absorption and metabolism.

## 1.7.2 Abiotic Factors

### 1.7.2.1 Contaminant Physical and Chemical Properties

A microbial bioremediation approach is usually necessary to figure out how possible microorganisms interact with hydrocarbon pollutants. The chemical and physical characteristics of hydrocarbon pollutants can impact the biodegradation, transportation, and metabolism of a single strain or consortia of bacteria. This is owing to the contaminated environment's composition, concentration, molecular size, structure, toxicity, and unpredicted hydrocarbon molecules. Hydrocarbon-degrading bacteria's occurrence, stability, and biological activity are all affected by these factors. Polyaromatic hydrocarbons and highly condensed cycloalkane compounds are also more resistant to microbial degradation than unbranched alkanes and lighter PAHs. The contaminant's non-degradation potential is due to their nature, which accounts for their solubility, bioavailability, toxicity to bacteria due to lipid membrane disruption, and inability to split the ring for degradation. To assess the rate of hydrocarbon biodegradation, it is necessary to understand the chemical properties, physical condition, and toxicity of hydrocarbon pollutants, as well as their fate.

#### 1.7.2.2 Hydrocarbon Concentration

The rates of transformation, absorption, and mineralization of bacterial biodegradation are affected by the quantities of hydrocarbon pollutants. Because of the heavy and undispersed nature of high concentration contaminants, they are poisonous and have a severe impact on the growth and production of biomass of degraders, necessitating a protracted treatment period. Furthermore, it was discovered that hydrocarbon concentrations greater than 5% inhibit microbial decomposition and may disrupt the C:N:P ratio and oxygen availability. Similarly, extremely low hydrocarbon concentrations prevent biodegradation by repressing bacterial metabolic genes that produce degradation enzymes, resulting in a lack of carbon supply or availability to support microbial growth. As a result, the presence of an ideal concentration for complete mineralization from contaminated settings is required for bacterial biodegradation of hydrocarbons.

#### 1.7.2.3 Nutrient Availability

Although microorganisms can be found in contaminated soil, they are unlikely to be in sufficient numbers to allow for clean up of the site. To aid indigenous microorganisms, biostimulation generally entails the provision of oxygen and nutrients. These nutrients are the fundamental components of life, allowing bacteria to produce the enzymes required to break down pollutants. Nitrogen, phosphorus, and carbon will be required by all of them.

Furthermore, excessive concentrations of hydrocarbon pollutants may affect the NPK ratio, resulting in oxygen deficiency. In general, the growth of hydrocarbon-using bacteria in the soil is hampered by an insufficient supply (either too much or too little) and/or the absence of mineral nutrients. As a result, ensuring that contaminated soils receive the proper quantity of nutrients (P and N) is crucial for efficient biodegradation of hydrocarbon. As a result, biodegradation of hydrocarbon depends on soil environment modifications, and the maximum rate of breakdown can be accelerated by providing the appropriate nutrients. Carbon is the most basic element in living creatures, and it is needed in higher quantities than other elements. pH, temperature, and moisture all have an impact on microbial growth and activity. Temperature influences the rate of numerous biological reactions, and for every 10 degrees Celsius rise in temperature, the rate of many of them doubles. The cells, on the other hand, perish at a specific temperature. A plastic covering can be used to boost solar heating in late spring, summer, and autumn. To promote microbial activity for hydrocarbon breakdown, sufficient moisture is required in hydrocarbon-contaminated areas (Grath et al. 1994).

#### 1.7.2.4 Oxygen Availability

Bacteria that break down hydrocarbons can breathe both in the availability and non-availability of oxygen. At the terminal, sub-terminal, and bi-terminal levels of aromatic hydrocarbon pollutants, the presence of oxygen in the soil acts as the last chemical reactant and electron acceptor for oxidation and ring breakage. Therefore, it is a limiting factor for aerobic bioremediation. Studies show that destroying 1 mg/mL hydrocarbon pollutants requires 3.2 mg/mL oxygen, even if the total biomass of

potential hydrocarbon-degrading bacteria is not taken into account. Successful biodegradation requires 10–40% oxygen. As a result, aerobic catabolism produces a faster rate of biodegradation than anaerobic metabolism.

#### 1.7.2.5 Moisture Availability

The transportation medium for the nutrients of soil and elimination of bacterial waste products into soil particles is soil moisture and has an impact on hydrocarbon bioavailability, aeration, the character and number of soluble materials, diffusion processes, osmotic pressure, pH, gas transfer, and soil toxicity. The porosity and water holding capacity of soil are diminished when it contains hydrocarbon pollutants. In the end, this environment reduces microbial activity because bacterial and soil water activities are directly related, meaning that as moisture content falls, so do bacterial activities, and conversely, when soil moisture levels are high, oxygen transport is limited. As a result, the availability of water for microbial development and metabolism limits hydrocarbon breakdown in terrestrial environments. As a result, appropriate moisture availability in the ranges of 50–75%, 30–90%, and 50–80% is required for hydrocarbon biodegradation.

Extreme moisture levels, on the other hand, are detrimental to microbial development and metabolism. This is because, rather than creating anaerobic soil conditions, oxygen diffusion in the soil is reduced and aerobic hydrocarbon breakdown is hindered. To promote microbial activity for hydrocarbon breakdown, sufficient moisture in hydrocarbon-contaminated locations is required.

#### 1.7.2.6 Bioavailability

The rate of contaminant absorption and metabolism, as well as the rate of contamination transport to the cell, determines how quickly microbial cells can convert toxins during bioremediation. In most polluted sediments, this appears to be the case. After 50 years, polluting explosives in soil have not degraded. A range of physiochemical processes, including absorption and desorption, diffusion, and dissolution, influence a contaminant's bioavailability. The sluggish mass transfer to the degrading bacteria reduces the bioavailability of pollutants in soil. When the rate of mass transfer is zero, contaminants become unavailable. Aging or weathering is the term used to describe the decline in bioavailability with time. It could be caused by:

1. Contaminants are incorporated into natural organic matter through chemical oxidation reactions.
2. Slow diffusion through very small pores and absorption into organic substances.
3. The formation of semi-rigid films with a high resistance to NAPL-water mass transfer around non-aqueous-phase liquids (NAPL).

The introduction of food-grade surfactants, which increase the availability of pollutants for microbial breakdown, can help solve these bioavailability issues. (Chakraborty et al. 2012).

## 1.8 Bioremediation Types

Bioremediation procedures can be used both ex situ and in situ at the application site. The type of contaminant, the volume and depth of contamination, the ecosystem type, the cost, and environmental policies are all factors to consider when choosing a bioremediation technique. Abiotic factors such as nutrient concentrations and oxygen, pH, temperature, and other abiotic variables impact the effectiveness of bioremediation procedures.

### 1.8.1 Ex Situ Bioremediation

Ex situ approaches entail excavating contaminants from polluted places and bringing them to a treatment facility. Ex situ bioremediation procedures are frequently chosen depending on the depth of contamination, the type of pollutant, the degree of pollution, the cost of treatment, and the location of the contaminated site. Ex situ bioremediation procedures are likewise governed by performance requirements.

#### 1.8.1.1 Treatment in the Solid Phase

Solid-phase bioremediation is a form of an ex situ method that requires digging and stacking contaminated soil. Organic wastes, such as animal manures, leaves, and farm wastes, are included, as well as industrial, household, and municipal wastes. Pipelines positioned throughout the heaps encourage bacterial growth. For ventilation and microbial respiration, air must flow through the pipes. Solid-phase systems require a lot of space and take a long time to clean up compared to slurry-phase techniques. Land farming, biopile, composting, and other solid-phase treatment methods are examples.

#### 1.8.1.2 Slurry-Phase Bioremediation

When compared to alternative treatment methods, slurry-phase bioremediation is a faster technique. In the bioreactor, polluted soil is mixed with nutrients, water, and oxygen to produce the suitable environment for microbes to break down the contaminants in the soil. Separation of rubbles and stones from polluted sediment is part of this process. The amount of water added is determined by the amount of pollutants present, the rate of biodegradation, and the soil's physicochemical parameters. The soil is removed and dried when this process is completed using centrifuges, vacuum filters, and pressure filters. The next step is to dispose of the soil and treat the resulting fluids in advance.

### 1.8.2 In Situ Bioremediation

These methods entail treating contaminated matter at the source of the contamination. It doesn't require any excavation and creates little to no soil disturbance. In comparison to ex situ bioremediation approaches, these procedures should be quite

cost-effective. Bioventing, biopharming, and phytoremediation are examples of in situ bioremediation processes that might be enhanced, but intrinsic bioremediation and natural attenuation may not. Chlorinated solvents, heavy metals, dyes, and hydrocarbons have successfully treated using in situ bioremediation approaches.

## 1.9 Bioremediation Approaches for Environmental Clean-Up

### 1.9.1 Ex Situ Bioremediation Approaches

#### 1.9.1.1 Biopile

Above-ground layering of recovered hazardous soil is followed by oxygenation and nutrient replenishing to improve bioremediation by microbial metabolic activities. Aeration, irrigation, fertilizers, leachate collection, and treatment bed systems are all part of this technology. The cost-effectiveness of this one-of-a-kind ex situ technique, which allows for precise control of operative biodegradation factors including pH, nutrition, temperature, and aeration, is rapidly being examined. The biopile is used to treat low-molecular-weight contaminants that are volatile, and it may also be utilized to clean up contaminated very cold harsh situations which makes it versatile. Additionally, warm air can be fed into the biopile design to give both air and heat, allowing for improved bioremediation. To speed up the restoration process, bulking agents such as straw sawdust, bark or wood chips, and other organic elements have been added to a biopile build. Despite the fact that biopile systems are linked to other methods for ex situ bioremediation in the field, such as land farming, bioventing, and biopharming, engineering, maintenance, and operation that is dependable costs, and a lack of electricity at remote locations, preventing continuous air circulation in polluted heaped soil via an air pump, are all obstacles. Additionally, during bioremediation, excessive air heating can cause soil dryness, which limits microbial activity and favours volatilization rather than biodegradation. On-halogenated VOCs, fuel hydrocarbons, SVOCs, and pesticides are all treated with this system. The efficiency of the method will vary, and it may only be applicable to certain substances within specific contamination classes.

#### 1.9.1.2 Biofilter

A biofilter consists of a huge media bed through which contaminants travel and are digested by microbes. They are one of the oldest methods of environmental clean-up. They are utilized in the waste water treatment and air pollution management. Peat, bark, gritty dirt, or plastic shapes are some of the materials utilized as bed medium. The trickling filter is a common type of biofilter that is used to treat a variety of waste waters, and waste that has been converted into liquid. A trickling filter consists of a vertical tank with a support rack filled with aggregate, ceramic, or plastic media and a vertical pipe in the middle with a rotating connection and spray nozzles on the top end. A spray arm is connected to the rotary connection and has spray nozzles fitted along its length for waste water distribution. On the surface, microorganisms forms biofilm that serve as the packaging for the breakdown of pollutants in the effluent.

Contaminants bind to the surface of the media, where they are destroyed by microorganisms. Specific bacteria strains can be added into the filter, and optimal conditions can be created to break down specific substances more efficiently.

#### 1.9.1.3 Land Farming

Land farming is one of the most successful bioremediation technologies due to its low equipment needs. It is most frequent in ex situ bioremediation, although it may also occur in some in situ bioremediation environments. Because of the treatment site, this element is considered. Contamination depth is crucial factor in land farming, which can be done by ex situ or in situ. Contaminated soils are excavated and tilled on a regular basis in land farming, and the form of bioremediation depends on the treatment site. Because it has more in common with other ex situ bioremediation techniques, ex situ bioremediation happens when hazardous soil is removed and treated on-site.

Excavated contaminated soils are often carefully placed above the ground surface on a fixed layer support to facilitate aerobic biodegradation of the contaminant by autochthonous microorganisms. Overall, land farming bioremediation is a straightforward design and implementation technology that requires little financial investment and may be utilized to repair huge amounts of polluted soil with minimal environmental impact and energy consumption.

##### 1.9.1.3.1 Composting

Composting is a controlled biological process in which organic pollutants are transformed to harmless, stable metabolites by microbes. To adequately compost soil polluted with harmful organic pollutants, thermophilic temperatures must typically be maintained. Temperatures rise due to heat produced by microbes during the breakdown of organic material in garbage. In most situations, this is accomplished by the use of naturally occurring microbes. To enhance the porosity of the mixture to be decomposed, soils are dug and blended with bulking agents and organic additions such as wood chips, animal, and vegetal wastes. Maintaining oxygenation, watering as needed, and regularly monitoring moisture content and temperature all contribute to maximum degrading efficiency. Composting in windrows is generally thought to be the most cost-effective method. Meanwhile, it may be the source of the most fugitive emissions. Off-gas control may be required if volatile organic compound (VOC) or semivolatile organic compound (SVOC) pollutants are present in soils (Perpetuo et al. 2011).

The composting technique can be used on biodegradable organic compound contaminated soils and lagoon sediments. Aerobic, thermophilic composting has been shown in full-scale programmes to reduce explosives, ammonium picrate, and related toxicity to acceptable levels. PAH-contaminated soil can also benefit from aerobic, thermophilic composting. Composting materials and equipment are all commercially available.

#### 1.9.1.4 Bioreactor

A bioreactor is a vessel that uses a series of biological reactions to turn basic materials into specific products. Different operational modes exist in batch, fed-batch, sequencing batch, continuous, and multistage bioreactors. Bioreactors are good for the growth of bioremediation. A bioreactor is loaded with dirty samples for the clean-up procedure. Ex situ bioremediation technologies provide a number of benefits over bioreactor-based treatment of contaminated soil. The use of a bioreactor-based bioremediation technique with greater control of aeration, agitation, pH, substrate, and temperature, and bioremediation time is sped up by increasing inoculum concentrations. The capacity to manage and modify biological reactions in a bioreactor is shown by the ability to regulate and adjust process parameters. Bioreactor designs are versatile enough to allow for optimal biological degradation while minimizing abiotic losses. In soil and groundwater, bioreactors are typically used to treat VOCs and fuel hydrocarbons. Pesticides are less effective in this procedure. The technique was utilized to treat soil that included trinitrotoluene (TNT) and Research Department eXplosive (RDX) in one application. It operated in both aerobic and anaerobic settings in the lab, with a significant reduction in pollutant concentration. In addition, intermediate by-products were degraded.

Hazardous by-products formed during the decomposition of some chlorinated solvents can also be degraded using in situ bioreactors. Adapted bacteria in this sort of bioreactor mineralize the organic molecules of interest. A biological support medium is used to capture the bacteria. A vapour extraction system can be utilized in conjunction with an in situ immobilized bioreactor system. Basic bioreactors are a well-established technique for the treatment of municipal and industrial wastewater. Fuels can be treated in bioreactors, which are commercially accessible. Explosive chemicals have been tested in the laboratory. A novel method for treating halogenated VOCs, SVOCs, pesticides, polychlorinated biphenyls (PCBs), and other chemicals is to sequence anaerobic/aerobic bioreactors. As contaminated groundwater travels through the reactor, these strategies accelerate degradation. This method has been used to remediate organic compounds in various leaking underground storage tanks and industrial locations with great success.

### 1.9.2 In Situ Bioremediation Approaches

#### 1.9.2.1 Bioventing

Bioventing is a potentially innovative way for promoting spontaneous in situ biodegradation of any aerially degradable chemical in soil by providing oxygen to the soil microorganisms already there. Bioventing, in contrast to soil vapour vacuum extraction, employs low air flow rates to give just enough oxygen to keep microbial activity going. The most common way of delivering oxygen is direct air injection into residual pollution in soil. In addition to adsorbed fuel residues, volatile compounds are biodegraded when vapours travel slowly through biologically active soil.

Bioventing techniques have effectively treated petroleum hydrocarbons, non-chlorinated solvents, some insecticides, wood preservatives, and other organic contaminants.

Inorganic pollutants cannot be destroyed by bioremediation, but it may be used to modify their valence state and causing inorganic adsorption, absorption, accumulation, and concentration in micro- and macro-organisms. While still in their infancy, these solutions hold a lot of potential for stabilizing or removing inorganics from soil. Two critical requirements must be satisfied for bioventing to be successful. To maintain aerobic conditions, sufficient volumes of air must circulate through the soil; second, bacteria that degrade hydrocarbons naturally must be available in sufficient densities to accomplish satisfactory biodegradation rates. The initial testing will determine the air permeability of the soil as well as in situ respiration rates. Microbial activity in soil is known to be influenced by basic nutrients, temperature, moisture (e.g. nitrogen and phosphorus), and pH. Despite the fact that soil pH studies show that the optimal pH range for microbial activity is 6 to 8, microbial respiration has been seen at all locations, even in soils outside of this range. The ideal moisture level in the soil is quite soil-specific. Too much moisture in the soil might reduce air permeability and oxygen delivery. A lack of moisture inhibits microbial activity. However, in extremely dry conditions, irrigation or humidification of the injected air may be able to accelerate biodegradation. Bioventing breaks down pollutants more quickly in the summer, although some remediation can occur in soil temperatures as low as 0 °C (Prasad 2014).

The bulk of the necessary gear is easily available, and bioventing is becoming increasingly common. Bioventing is becoming increasingly popular among remediation experts, particularly when combined with soil vapour extraction (SVE). Bioventing, like other biological treatments, takes a long time to remediate a site because of the particular soil and chemical characteristics of the contaminated medium.

#### 1.9.2.2 Biosparging

Biosparging is an in situ remediation approach that involves supplying oxygen and nutrients to polluted soils in order to encourage aerobic biodegradation of contaminants by indigenous microorganisms. In order to induce in situ aerobic biological activity, biosparging involves pumping pressurized air or gas into a polluted zone. This technology targets chemical substances that can be biodegraded under aerobic conditions and is used to treat soluble and residual contaminants in the saturated zone. By giving oxygen to the microorganisms and increasing the interactions between air, water, and the aquifer, the injection of air promotes the development of the aerobic microbial population and thereby enhances the bioavailability of pollutants. The goal of a biosparging system is to increase pollutant biodegradation while minimizing volatile and semi-volatile organic compound volatilization. The air injection flow rate is designed to give the amount of oxygen needed to improve bacterial contamination degradation. However, some volatilization may occur, necessitating air capture and treatment, depending on the operation mode and design chosen. The injection method and gas composition are two of the

most important variables to consider when designing a biosparging system. Vertical or horizontal wells, as well as trenches or reactive barriers, can be used to inject gas. Nutrients are also injected below the water table to boost microbial degradation activity, causing pollutants to be destroyed or transformed. The microbial community adjusts to the changing chemical and geochemical circumstances. When pollutant concentrations meet treatment targets, the treatment is terminated. In situ biosparging has the potential to be used in isolated northern locations where material delivery and injection equipment mobilization are a problem. Biodegradation can be hampered by cold temperatures, and microbial activity may only occur during the summer months, thus treatment could take years. Because temperatures are essentially consistent throughout the year, microbial activity may be possible in deep soil. Treatment time for biosparging is very varied, depending on the qualities of the contamination, the natural bacterial population, and the physical and chemical characteristics of the contaminated site. Under ideal circumstances, treatment times of 6 months to 2 years are common.

#### 1.9.2.3 Bioslurping

Bioslurping combines bioventing with vacuum-assisted free-product pumping to extract free-product from groundwater and soil, as well as to bioremediate soils.

Bio-slurping comprises employing vacuum-enhanced extraction/recovery, vapour extraction, and bioventing all at the same time to cope with light non-aqueous phase liquid (LNAPL) contamination. Vacuum extraction/recovery removes free product and some groundwater from the vadose zone, while vapour extraction removes high volatility vapours from the vadose zone, and bioventing improves aerobic biodegradation in the vadose zone and capillary fringe.

The bioslurping system consists of a well into which an adjustable-length "slurp tube" is attached. The slurp tube is lowered into the LNAPL layer and connected to a vacuum pump, which starts pumping to remove free product as well as some groundwater (vacuum-enhanced extraction/recovery). The vacuum-induced negative pressure zone in the well aids LNAPL flow toward the well, dragging LNAPL trapped in microscopic pore gaps above the water table. When the LNAPL level lowers somewhat in response to pumping, the slurp tube begins to suck in and remove vapours (vapours extraction). The removal of vapour enhances air movement across the unsaturated zone, increasing oxygen concentration and improving aerobic bioremediation (bioventing). When mounding occurs, the slurp returns to sucking LNAPL and groundwater since the introduced vacuum raises the water table a little. The liquid from the slurp tube (both product and groundwater) is sent to an oil/water separator, while the vapours are sent to a liquid vapour separator. Aboveground water and vapour treatment systems may be incorporated if necessary. In other cases, however, system design adjustments have made it possible to release groundwater and vapour collected by bioslurping without treatment. LNAPL and vapour recovery are directly connected with the degree of vacuum, according to field tests of bioslurping systems. When bioslurping was compared to traditional LNAPL recovery methods, it was shown that bioslurping outperformed both skimming and dual-pump methods. Bioslurping has been said to have cheaper project costs

(because of less groundwater extraction and the fact that vapour and groundwater may not require treatment) and less aquifer "smearing" than other LNAPL recovery/treatment processes. Potential "biofouling" of well screens owing to active aeration is highlighted as a disadvantage of bioslurping, as is the lack of treatment of residual LNAPL contamination in saturated soils.

## 1.10 Metagenomics

As the name indicates, metagenomics is concerned with the metadata of many genomes in order to offer rapid and exact information on the composition and dispersion of an interacting microbial community in an environment, as well as their evolutionary history. Metagenomics has been referred to as environmental DNA library (eDNA library), community genomics, environmental genomics, soil microbial DNA library, community genome analysis, whole-genome shotgun sequencing, and a variety of other terms.

Metagenomics adds to the toolkit for studying non-cultured species (Tringe and Rubin 2005). This new field proposes a method for examining microbial communities as a whole rather than individual members. Metagenomics is the process of extracting DNA from a community in order to pool all of the organisms' genomes. These genomes are typically divided and cloned into a culturable organism to produce metagenomics libraries, which are then analysed based on DNA sequence or functionalities given on the surrogate host by metagenomics DNA (Garfield and Merton 1979).

Targeted metagenomics and shotgun metagenomics are two common types of metagenomic methods. The diversity of a single gene is investigated in targeted metagenomics or microbiomics to determine the entire sequences of a specific gene in a given environment. The most typical use of targeted metagenomics is to look at the phylogenetic diversity and relative frequency of a single gene in a sample. To understand the taxonomic richness of an environment, microbial ecologists frequently use small subunit rRNA sequencing. It can also be used to explore how environmental toxins affect the structure of microbial communities. Environmental DNA is isolated for targeted metagenomics, and the gene of interest is PCR (polymerase chain reaction) amplified with primers designed to amplify the largest diversity of sequences for that gene.

Next-generation sequencing is used to sequence the amplified genes. Next-generation sequencing, which can explore hundreds of samples at once, generates thousands of small subunit rRNA reads per sample. The universality of the PCR primers employed for the investigation limits targeted metagenomics, which captures the diversity of a specific gene of interest. In addition, different bioinformatics analyses have the ability to distort total diversity estimations. Targeted metagenomics provides the benefit of giving a comprehensive inventory of the microbial species present in a collection of samples, as well as in-depth analyses of microbial diversity changes before and after a disturbance. Shotgun metagenomics uses genomic sequencing to analyse an environmental community's

whole genetic complement. In this procedure, environmental DNA is extracted and subsequently fragmented to form sequencing libraries. After that, the libraries are sequenced to ascertain the sample's entire genetic content. Shotgun metagenomics is a strong tool for determining a microbial community's functional potential. The depth of sequencing is generally the most limiting factor in shotgun metagenomics. To get a comprehensive inventory of the genes in an environmental sample, deep sequencing is typically necessary. A thorough study of a community's functional potential necessitates a thorough examination of every creature's genetic material. Shotgun metagenomics frequently over-samples the dominant bacteria in a community while sparingly sampling the genetic content of the community's low-abundance members.

A phylogenetic anchor is used in several studies to relate a functional gene to a taxonomic categorization. With metagenomics sequencing, this can be challenging until enough sequencing depth is reached and the reads can be effectively assembled into suitably lengthy contigs. Several recent reviews have attempted to highlight the major phases in metagenomics as well as the numerous potential hazards (Kumavath and Deverapalli 2013).

## 1.11 Metagenomics in Bioremediation Process

Metagenomics is an approach for analysing directly extracted genetic material in environmental samples. Metagenomics study gives information on non-cultivable species' microbial populations in a niche habitat using sequence and function-based research approaches (Kumar et al. 2020, 2021). Gene clusters for pollutant-degrading enzymes may be encoded, and novel bacteria can be identified (Table 1.1). Metagenomics techniques can also be used to detect and monitor

**Table 1.1** Examples of metagenomics analysis of contaminated site (Zhang and Bennett 2005)

| Most abundant organisms | Analysis | Contaminated site |
|---|---|---|
| *Arcobacter butzleri*, *Aeromonas hydrophila*, and *Klebsiella pneumonia* | Metagenomics | Advanced sewage treatment systems |
| *Proteobacteria*, *Thiobacillus*, and *Sulfuricella* | Metagenomics | Cadmium-contaminated soil |
| *Thermi*, *Gemmatimonadetes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* | Metagenomics | Hydrocarbon-contaminated soil |
| α-*Proteobacteria* and y-*Proteobacteria* | Metagenomics | Petroleum-contaminated soil |
| *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Synergistetes*, and *Acidobacteria* | Metagenomics | Pharmaceutical-enriched wastewater |
| *Pseudomonas* species, *Gammaproteobacteria*, *Actinobacteria*, and *Alphaproteobacteria* | Metagenomics | Diesel-contaminated Arctic soil |
| *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, and *cyanobacteria* | Metagenomics | Cu-contaminated sites |

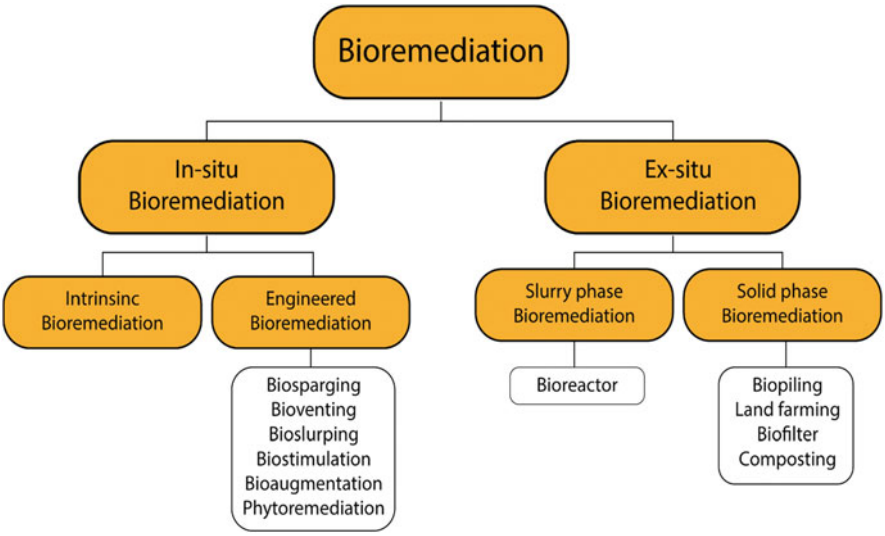


**Fig. 1.4** Bioremediation approaches for environmental clean-up (Harekrushna and Kumar 2012)

microbes. Metagenomics is a technique for studying DNA taken from ambient sources without isolating and cultivating microbes. This method was first used to find new microbial products and to sample microbial diversity from various habitats in the environment. To remove pollution from the environment, microbes are used in bioremediation. This method, which uses microorganisms to clear hazardous pollutants, is both ecologically friendly and practical (Shah et al. 2011).

Microorganisms not only help to regulate biogeochemical processes in the environment to keep humans healthy and to alleviate and stimulate plant diseases, but they also assist in the removal of poisons from the environment. The goal is to study uncultivated microorganisms in order to get a better understanding of the genuine microbial community, including their functions, collaboration, interactions, and adaption to new environments. Metagenomics is a topic of study that is continually expanding and evolving. Sequence-based and feature-based metagenomic techniques are the two most common types. Currently, these tactics have increased our understanding of the environment of non-culturable bacteria, giving us a better understanding of the microbial world. The function-focused metagenomics method encourages the identification of novel genes and enables for genetic analysis, both of which are intriguing prospects for uncovering new ecosystems. Metagenomics research includes gene recognition, explanation of entire metabolic processes, genome assembly, and sequence-based identification of species from diverse populations (Chen and Murrell 2010). Microbial diversity and particular genes found via metagenomics research that have the potential to act as pollution indicators are also included in the bioremediation process. Several microbes play a crucial role in pollution clean-up thanks to bioactive compounds and enzymes

discovered utilizing metagenomics methods. Bacteroidetes, Firmicutes, Actinobacteria, Spirochetes, Chloroflexi, Proteobacteria, Acidobacteria, and Patescibacteria are some of the most common microbial groups that can withstand high metal concentrations (Watanabe 2001). Microbial enzymes, metabolites, and bioactive chemicals, all of which have a role in water treatment, can also be discovered using metagenomics approaches. Using metagenomics approaches, many enzymes were discovered in chlorinated biphenyl-polluted soils, activated sludge, oil-contaminated water, wastewater, cow rumen, chemically contaminated soils, and compost wastewater. Alkane enzymes, carboxylesterases, dioxygenases, esterases, laccases, monooxygenases, phenol-degrading enzymes, polyaromatic and hydrocarbon-degrading enzymes, trichlorophenol hexadecane hydrolyzing enzymes, and trichlorophenol hexadecane hydrolyzing enzymes are some of the enzymes that remove toxins. The metagenomic analysis looks for novel bacteria-producing genes and assists in the identification of new processes and approaches to improve clean-up strategies (Wood 2008).

## 1.12 Metagenomics Research in a Contaminated Environment

### 1.12.1 Sampling from Contaminated Site

Biological replicates are necessary for statistical data analysis in order to examine the microbial communities' geographical and temporal variability in diverse settings like soils and sediments. Indeed, physical features might alter locally in the latter, affecting the structure of the microbes. Microbial population is usually substantially less in polluted habitats than in pristine environments, and it is heavily influenced by the type of contamination present and the history of contamination. The complex interaction between nutrients, organic contamination, and hydrological processes generates geographic heterogeneity of resident bacteria in groundwater habitats. The study of fine scale heterogeneity offers a lot of potential for accurately assessing biodegradation rates and designing pollutant fate models (Lovey 2003).

Environmental metagenomics will provide information on community composition and potential activity on the basis of samples obtained at certain date and place, but not on resilience or change resistance. In certain circumstances, a large sequencing depth can compensate for a small number of sequenced samples, resulting in an obvious microbial configuration at the studied site (Röling 2015). A common metagenomic approach is shown as flow chart in Fig. 1.6.

### 1.12.2 Extracting the DNA from Contaminated Samples

In all metagenomics research, DNA derivation is a critical step. Impurities in recovered DNA, natural components of soil/sediment, and soil contaminants should be eliminated to guarantee that it is representative of microbes found in the investigated environment. Stable Isotope Probing (SIP) has been integrated with

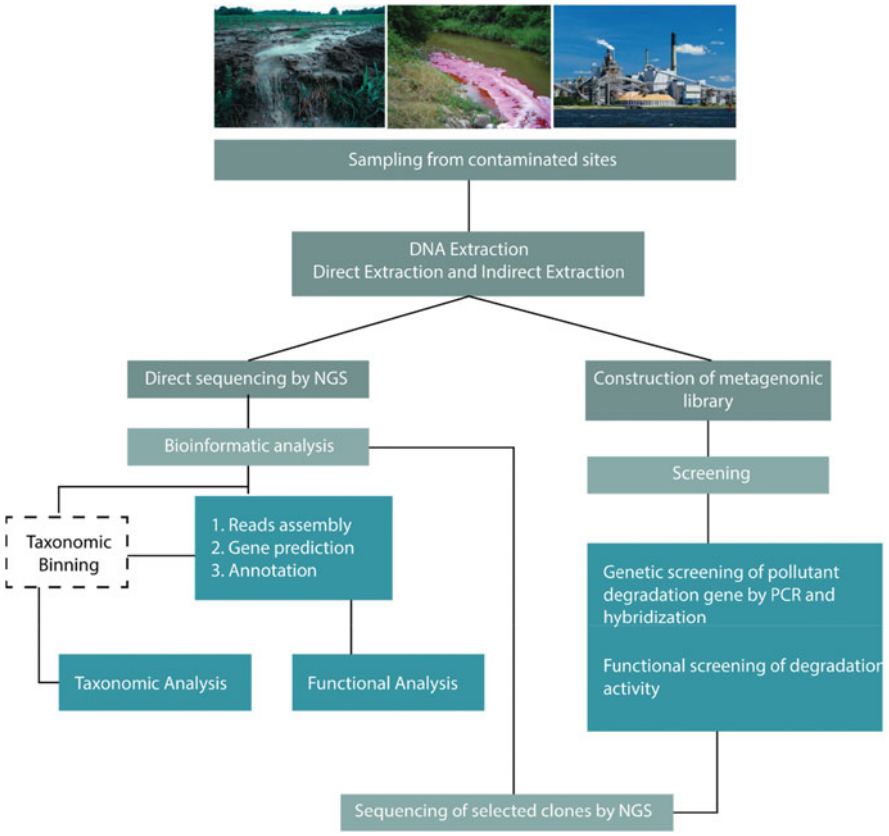


**Fig. 1.6** A common metagenomic approach is depicted as flowchart (Franzosa et al. 2015)

metagenomics in pollution biodegradation research for bioremediation applications. The most essential considerations in deciding which process to utilize for DNA extraction are the number of microorganisms in the sample and the level of contamination. Many approaches for recovering high-quality, high-quantity, and high-molecular-weight DNA from the environment have been developed. Either the cells are lysed in the sample or they are eliminated before lysis. The process is known as direct extraction because DNA is extracted from inside the sample matrix in the first scenario. When used on samples with a low cell density and a low level of contamination, this method provides more DNA than the second. Because DNA is extracted from pristine cells that are not exposed to the sample matrix, the second method is known as indirect extraction. When possible, the latter extraction is favoured since it lowers the simultaneously extraction of inhibitory compounds in the sample, which might interfere with subsequent steps (e.g. cloning, polymerase chain reaction, and sequencing). Due to cell mortality during matrix purification, DNA yield is lower than in direct extraction. Extracting DNA from polluted settings

remains difficult, despite the different technologies available. Metals and aromatic hydrocarbons, for example, have a negative impact on bacterial activity, resulting in lower bacterial biomass and, as a result, a lower yield of recoverable DNA. In this procedure, the phage phi29 polymerase is employed to amplify DNA using random hexamers at a constant temperature.

## 1.12.3 Metagenome Analysis

### 1.12.3.1 Targeted Metagenomics Using a Library

Cloning of ambient metagenomics DNA and screening of the clones for a desired function are the steps in library-based targeted metagenomics. It has been frequently used to pollute settings in recent years in order to identify the gene pool implicated in microbial degradation processes. Library-based targeted metagenomics restricts metagenome analysis to certain tasks, resulting in the context of a larger range of genes associated to the researched ecological role, even if there aren't many of them, when as compared to complete DNA sequencing.

#### 1.12.3.1.1 Creating a Metagenomics Library

The availability of commercial kits that include host cells and vectors with optimized methods for the many types of vectors that could be used in metagenomics has made the construction of metagenomic libraries easier. Multiple copy number vectors enable "massive" rDNA creation for later study. Overexpression of some genes, on the other hand, could be fatal to the host. Vectors with a single copy (e.g. BAC) ensure consistent preservation of a vast number of DNA segments and lower DNA output. Single-copy to high-copy-number vectors that are inducible were developed as a consequence, combining the aid of both types of vectors while also overcoming their limits in a single system.

#### 1.12.3.1.2 Screening of Metagenomic Clones

There are two types of metagenomics library screening approaches: techniques based on sequences and methods based on functions.

##### 1.12.3.1.2.1 Screening Based on a Sequence

This method is based on comparing recovered ambient DNA to sequences currently stored in databases. It is heavily influenced based on what we know about previously found genes. It usually entails utilizing PCR or hybridization-based methods to find clones with a conserved section within the targeted gene family or functional class of proteins. The large number of pollutant degradation gene sequences obtained from in situ has contributed in the development of degenerate probes and primers to help us better understand pollutant degradation processes. The search for sequences encoding a degradative gene of interest in gene databases is the first step in developing such primers. The alignment of the recovered sequences indicates areas that are both conserved and varied (Dettmer et al. 2007). PCR amplicons that have been tagged with radioactive fluorescent (e.g. digoxigenin) or antigenic

(e.g. digoxigenin) molecules are known as hybridization probes. Hybridization conditions are empirically tweaked to uncover alternative sequences deviating from the original probe. Furthermore, in metagenomics libraries, the employment of a group of probes that are all aimed at the same thing, different GOI may have the advantages of

1. increasing the likelihood of finding a single gene,
2. in a single experiment, the identical hybridization conditions are applied to a large number of clones, and,
3. increasing the likelihood of catching interesting target gene sequences.

#### 1.12.3.1.2.2 Function-Driven Sequence

The function-driven screening is not reliant on previously identified sequences. It is solely dependent on the manifestation of a biological characteristic in the host cell. It has a better chance of finding novel and orthologous genes. Screening is typically done in polluted locations by selecting growing clones in minimal media augmented with the pollutant as the carbon or energy source. In this case, screening is necessary based on previous information of the target degradation process, as it is essential to construct a viable genetically modified host (Valiente and Pesole 2012).

To recognize the development of metagenomics clones more quickly, a chromogenic substrate such as MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) can be used in conjunction with the selective medium. Bacterial respiration converts it to the purple chemical formazan. When the target enzyme creates a colourless output, MTT comes in handy. Other substrates, such as indole and catechol, generate a coloured response, making it easy to identify clones that contain enzymes involved in aromatic compound oxidation. Apart from those substrates, scientists have only made intermittent attempts to thrive selective media for recognizing clones that produce degradative enzymes. This is due to a present dearth of knowledge about the substrates (and products) that many of these enzymes can metabolize.

## 1.12.4 Direct Sequencing of Metagenomics

The genetic material found in a sample collected from environmental is sequenced without first constructing a metagenomic clone library. To acquire a better understanding of the ecosystem of microbes with less laboratory work, bioinformatics capabilities and extensive DNA sequence databases are necessary.

The high-throughput sequencing of an individual gene is another technique to examine environmental metagenomes without pre-cloning. This method doesn't allow the retrieval of entire information contained in a metagenome, but it allows for better sequencing depth for the targeted functions. For example, targeted sequencing of 16S rRNA gene amplicons is routinely used in (preliminary) investigations of contaminated environments. 16S rRNA gene surveys can reveal a lot about microbial dynamics in these kinds of places. In comparison to library-based

metagenomics, direct sequencing is becoming more popular in environmental investigations. This is due to the fact that the former has profited from an increased speed and throughput sequencing of DNA by orders of magnitude, while the latter has suffered from relatively slow developments in screening methods (Handelsman 2004).

### 1.12.5 Next-Generation Sequencing

A technological aspect common to all NGS technologies is massive parallel sequencing of clonally amplified or single DNA molecules spatially separated in a flow cell. There are numerous great comparison studies of NGS platforms that provide important information in terms of features of analysis and applications. Illumina just released the MiniSeq which is proved to be cost-effective and user-friendly sequencer. The Illumina and other NGS technologies' short read lengths, on the other hand, are a limiting factor in genome completeness, variant calling, genome de novo assembly, and read mapping to reference genomes (Handelsman 2005). MiniSeq's cheaper cost and speed come at the expense of a little for samples; there is an increase in inaccuracy and underperformance with less diversity and questionable base discrimination. It is due to the fact that the number of scanning channels has been lowered to reduce fluorophore consumption (Liu et al. 2012).

### 1.12.6 Bioinformatics Analysis

NGS sequencing of environmental DNA is becoming faster while also becoming less expensive, and new, superior sequencing equipment is launched almost every other year. NGS technologies generate massive volumes of data, but developments in analysis are lacking. Only dominant strains or tiny genomes could previously be reconstructed from complex metagenomes, but that is set to change according to computational advances that will allow individual genomes of rare species to be reconstructed (Kumar and Khanna 2010). Using metagenomics to answer ecological problems necessitates a thorough examination of the sequences retrieved.

### 1.12.7 Assembly

Assembly is the process of reassembling a genome using sequenced reads that are first turned into contigs and then scaffolds. The two techniques to genome assembly are de novo genome assembly, which reconstructs the genome from read data, and reference-guided assembly /comparative assembly, which assembles sequencing, reads using reference sequences (Tijssen 2002).

### 1.12.8 Binning

Binning is the process of grouping genomic reads/fragments that are presumably from the same organism based on sequence-related signals acquired over time, such as phylogenetic signals. Binning's purpose is to assign sequences to "bins" that match to their taxonomic rank. Binding can also help with the assembly of previously difficult-to-cultivate genomes. To do so, a variety of bioinformatics tools are already accessible. There are two ways to do the binning procedure: supervised and unsupervised. Statistical classification methods for read categorization, such as similarity/distance matrix models or hidden Markov models, are used in supervised approaches for training. Unsupervised approaches, on the other hand, sort items into bins based on their composition, eliminating the necessity for a reference database. These techniques are typically futile since the vast majority of microorganisms discovered in environmental samples are usually unknown or can't be grown.

### 1.12.9 Annotation

The foundation for microbiological sequence functional annotations is BioCyc, COG, KEGG, NCBI genomes, SEED, Pfam, and other databases. Because functional annotations employ homology-based techniques similar to those used for taxonomy assignment, the availability of previously recognized sequences acts as a limitless. Only 20–50% of a metagenomics sequence can be annotated in its entirety, according to current estimates. Smaller datasets, however, can benefit from manual curation to enhance annotation accuracy. The genomic segments are annotated in two steps. Following the identification of genes of interest, taxonomic neighbours and probable gene functions are assigned. In a nutshell, there are two major paths to choose.

## 1.13 Metagenomics in Bioremediation: Current Challenges and Future

NGS appears to be difficult due to a lack of appropriate sequence descriptions from a higher number of microorganisms present in various habitats. Due to varying relative numbers of various community members within a population, certain genomes may be covered by thousands of sequences while others are only covered by a few of sequencing reads or none at all. These considerations, together with the project's budget, help to decide how much sequencing work is done on a project. Millions to billions of short reads are generated by the most prevalent metagenome sequencing technologies now in use (Kumar et al. 2020).

Estimates of community diversity are routinely conducted before metagenomics sequencing experiments. While these efforts (which usually involve rRNA gene amplicon analysis) might be valuable for community study, when it comes to strain level diversification or population heterogeneity, they might be deceiving. Another

challenge with metagenomics assembly is that, despite advances in assembly algorithms and computer hardware technology, the assembly of such enormous volumes of complex data can soon surpass the memory constraints of any machine. The community's natural diversity and variations identified within the population contribute to this problem, which is amplified as there may be inconsistencies in the sequencing (even at very low levels) in the sequencing data. Metagenomics is a systematic approach to genetic analysis of microbial populations. This gives a glance into the "Uncultured Microbiota's" microbial community. Bioremediation has always adapted new scientific and technological breakthroughs to create healthier ecosystems, and metagenomics is one of the best adaptations ever. In a metagenomic investigation, identifying and screening metagenomes from contaminated environments is critical. The second section focuses on recent multiple case studies that illustrate how metagenomics can be used in bioremediation. The third section, as a result, discusses metagenomic bioremediation in various polluted habitats, such as soil and water. Starting with a full understanding of metagenomic screening, FACS, and several advanced metagenomic sequencing methodologies, diverse sequences and function-based metagenomic strategies and tools are available (Joshi et al. 2014). Many experiments that were previously unthinkable are now possible because of advances in technology that promote metagenomic study. Companies like PacBio and Nanopore are working on sequencers that can read Kbs of DNA, enabling for continuous genome construction in mixed populations.

The convergence of a number of high-throughput approaches will allow researchers to look at the relationships between species composition, gene density, gene expression, chemical reactions, and protein production in polluted surroundings. SIP is exposing a substrate to heavy isotope-labeled compounds and allowing microbes to digest it and integrate the labelled atoms into biological components such as DNA, RNA, and phospholipids. To separate the "heavy" (labelled) DNA from the "light" (unlabeled) DNA in DNA-SIP, all DNA from a treated sample is collected and centrifuged in CsCl gradients. This approach has a lot of potential for finding functionally active microorganisms, especially those involved in pollutant degradation, according to a recent assessment. Using SIP-metagenomics analyses of polluted substrates, the active response genes and species may be retrieved from the massive quantity of background genetic information from the original, uncontaminated soil. One of the following big metagenomics projects is expected to be the discovery of a core microbiome. To put it another way, what genes and species can be found in a certain habitat as well as different settings. It will be crucial to establish if there are critical genes and organisms that do indeed respond favourably to the addition of a contaminant in order to accomplish successful clean-up in the setting of bioremediation. Outside of this common core, genes that are favoured must be the result of extra environmental or stochastic factors (Simon and Daniel 2009). Many contemporary genomic investigations depend on snapshots of genetic information in environmental samples, despite the fact that many microbial communities are constantly changing due to microorganisms' rapid pace of development. A metagenomics analysis of metal-contaminated groundwater revealed that pollution has decreased biological diversity and metabolic complexity to near-zero levels after

50 years. Despite the finding of all necessary metabolic pathways, there were 10 times fewer OTUs and a commensurate loss in metabolic complexity compared to a neighboring background site. Long-term monitoring of how evolution selects genes in polluted settings will certainly benefit the research and treatment of chronically contaminated areas, albeit massive volumes of data would demand a solution to the human-processing bottleneck first.

## 1.14 Conclusion

As sequencing costs fall, the value of high-throughput 16S rRNA sequencing and metagenomics grows. These methods allow researchers to investigate the impact of various bioremediation interventions on the native microbial ecosystem in greater detail. This allows these techniques to be fine-tuned for certain microorganism groups that are crucial to the bioremediation process. Metagenomics has the ability to guide the adoption of remediation technologies in order to achieve rapid and minimum invasive pollutant removal. Moving forward, a thorough understanding of the main taxa and routes indulge in many of these processes is critical. The ease with which data may be sequenced has resulted in the identification of several uncultured phyla and gene families with unknown functions. This is also true in contaminated situations. To further understand the bacteria engaged in these processes, metagenomic methods must be combined with traditional culture-based methodologies. Changes in microbial or gene diversity as a result of the reaction to a disturbance are frequently investigated using current techniques. Genetic and biochemical studies on model organisms are required to gain a deterministic view of the community's response or the underlying metabolic mechanisms involved in reacting to these perturbations. The dominating species in these environments are typically distantly related to these model organisms. We will have a better comprehension of the metagenomic data sets obtained from these areas if we can identify ecologically relevant organisms from the ecosystems of interest. The application of 16S rRNA sequencing and metagenomics to guide bioremediation tactics and acquire deep insights into microbial responses to pollution or remediation procedures has a lot of potential. A more comprehensive picture of the bioremediation basis emerges when these approaches are paired with pure-culture analyses of environmental microorganisms.

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# Bioremediation: Gaining Insights Through Metabolomics

2

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**Abstract**

Metabolomics is a tool for analyzing many biological compounds due to its ability to acquire novel approaches to study metabolic pathways. The current chapter focused on the impact of metabolomics on bioremediation. A detailed application of metabolomics has been explained with respect to some of the earlier researches. An approach combining microbial biodegradation with metabolomics can be used to better understand the breakdown of different compounds. Advancement with respect to varied instruments along with computers and software has made the metabolomic approach a very sensitive and specific method. Even future approach towards space debris bioremediation is the current focused research. Thus, metabolomics and bioremediation have a great interaction.

**Keywords**

Metabolomics · Bioremediation · Metabolic pathway · Biodegradation · Biological compound

## 2.1 Introduction

Metabolomics, which is the study of the metabolites present in tissues, biofluids, and cells (Johnson et al. 2016), involves identifying and quantifying all intracellular and extracellular metabolites. Using metabolome analysis to discover novel metabolic pathways and study metabolic networks is now widely recognized as a powerful

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approach. The metabolic-related data represent physiological responses to development, nature, and environment, and thus, has wide application in various fields like drug therapy, enzymatic studies, bioremediation, health studies, etc. (Azubuike et al. 2016; Deidda et al. 2015; Majumder et al. 2021; Wishart 2016).

An industrial revolution and population explosion have caused various pollutants to enter the environment; health and the environment are harmed by these harmful compounds. Bioremediation is best approach towards environmental protection. Bioremediation mediated by microbes possesses great potential for restoring contaminated environments in an ecologically acceptable manner (Arora 2018). Due to the lack of information regarding factors controlling and regulating diversity of microbial communities in contaminated environments, it is often difficult to execute such a plan. But recent research has shown that advanced tools like proteomics, metabolomics, and fluxomics have a key role in designing sustainable, eco-friendly treatment strategies for contaminants (Malla et al. 2018).

As metabolomics has a great role in environmental microbiology, use of metabolomics will help answer any specific question in microbiology that depends on understanding the physiological state (Booth et al. 2013). Applied metabolomics has a great deal to offer the field of environmental microbiology. It has been used to study biofilms, metal resistance, and responses to environmental stress (Garza et al. 2018). Metabolomics helps identify biodegradation pathways involved in the end product's toxicity and the biodegradation pathways involved in their biosynthesis. Thus, it offers environmental microbiologists a means to better understand molecular mechanisms that underlie microbial bioremediation processes, thus improving its effectiveness and improving the design of more technically sound remediation methods (Ma 2012).

## 2.2 Impact of Metabolomics on Bioremediation

In recent years, bioremediation has been of interest because it has the potential to remove toxic contaminants from soils and water at a low cost and with high efficiency. Using this technology would address the problem of industrial production that poses a health risk to humans (Yan et al. 2020). Microorganisms are used in bioremediation to break down hazardous compounds, leaving behind by-products which are no longer toxic in the environment. After the removal of contaminants, microorganisms recolonize the soils to restore nutrients and structure, thus making the land suitable for agriculture and industrial use. As part of an organism's energy metabolism, detoxification mechanisms, or by a fortuitous set of enzymes, metabolism can remove unwanted substances (Ostrem Loss and Yu 2018). Importance of metabolomics has shown in Fig. 2.1.

Metabolomics has a great role in studying varied metabolic activities among wide range of microorganism playing role in bioremediation (Kellogg and Kang 2020). Types like Non-targeted or targeted metabolomics analyses can be carried out (Matich et al. 2019) also shown in Fig. 2.2. As this type has a great application while dealing with bioremediation as target-specific, it can analyze the special

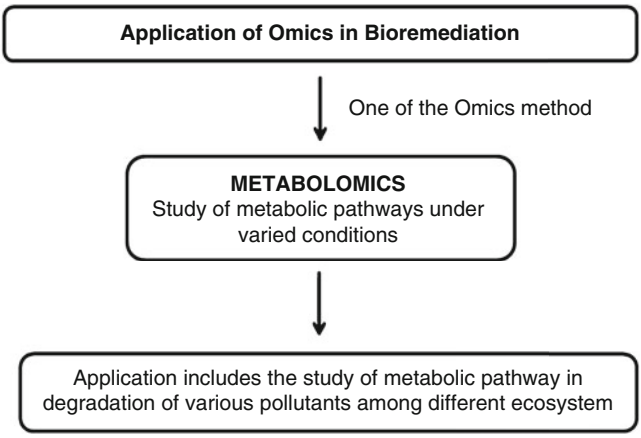


**Fig. 2.1** Importance of metabolomics (Garza et al. 2018)

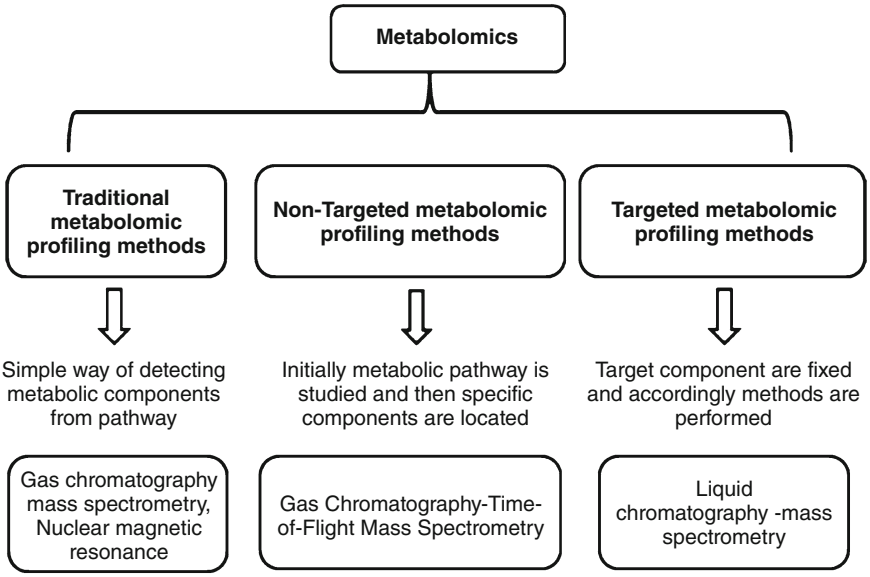


**Fig. 2.2** Types of metabolic profiling methods (Matich et al. 2019)

groups among metabolism. A repressor or suppressor, whether internal or external, will change gene expression and protein production, both of which are controlled by a variety of homeostatic mechanisms (Chandran et al. 2020). Such changes are made more evident at the metabolome level. Metabolomics may therefore be a better option than other omic technologies to detect such expressions (Lankadurai et al. 2013).

Metabolomics can be used to study different metabolic components like amino acids, carbohydrates, enzymes, substrates, precursors, repressors, etc. Many studies have carried metabolomic profiling using different instruments and methods like Gas chromatography, Nuclear magnetic resonance, High performance liquid chromatography, electrophoresis, spectroscopy, etc. But this was always accompanied with low cost, easy handling, sensitivity, and less time-consuming (Arora et al. 2018b).

A study on bioremediation showed by *Scenedesmus* sp. IITRIND2 microalgae that exhibited arsenic tolerance, for this tolerance its arsenic tolerance/degradation pathways were studied for various metabolic components involved in it. Here in the presence of both types of arsenic like oxic pentavalent arsenic, As (V) and anoxic trivalent As (III) (Rahman et al. 2014), microalgae metabolism pathways were studied. Thus, varied changes among pathways were seen during degradation as physiochemical property was altered. This whole metabolic profiling was done by using NMR spectroscopy (Arora et al. 2018a).

Expired pharmaceutical compounds have become major concern of an environment specifically found near beds of river and also the component of sewage treatment plant, as many bacteria have a mechanism for resistance against antibiotics, especially efflux pumps play a very major role in removal of antibiotics from inside cell to external environment. Thus, different metabolic pathways related to pumping process can be studied (Molina-Santiago et al. 2014). *Pseudomonas putida* DOT-T1E strain is found to have three efflux pumps named as TtgGHI, TtgABC, and TtgDEF. This strain was found to be propranolol-tolerant by growing that strain in presence of high propranolol concentration and studying its metabolic profiling by gas chromatography-mass spectrometry (GC-MS), it revealed the formation of ornithine in the presence of propranolol and thus has a great application in bioremediation process regarding pharma waste (Sayqal et al. 2016).

In addition to providing insight into complex molecular networks and metabolic pathways within the soil microbial community, soil metabolomics can be used to evaluate soil functionality (Withers et al. 2020). In addition to agricultural fields, gardens, and homes, N-methylcarbamate insecticides are widely used as household pesticides. The degradation of that component is essential and many microbes can carry out degradation of this component. As degradation of N-methylcarbamates pesticides by *Burkholderia* sp. strain C3 was studied, the metabolomics analysis identified 196 polar metabolites, among that ten medium to long chain fatty acids and one type of polyhydroxyalkanoates (PHA) were playing major role in metabolism pathway of N-methylcarbamates usage as carbon source (Seo et al. 2013). Similar study was done with respect to degradation of phorate by *Lactobacillus plantarum* P9. Phorate is also a widely used chemical pesticide and has been degraded from soil (Chu et al. 2018). Metabolomic study of P9 strain was done by using high-performance chromatography with (time-of-flight) TOF spectroscopy by growing strain in the presence and absence of phorate as alteration in metabolome profile and derivation of new products was found during degradation process (Li et al. 2018). Some pesticides are not harmful exactly, but long usage can lead to accumulation in soil and flow of it in aquatic system during flooding further can affect aquatic organisms. One example includes excess presence of Cyfluthrin in

**Table 2.1** Metabolomic profiling of microbes and respective degraded pollutant

| Sr. No. | Microorganism | Degraded pollutants | References |
|---|---|---|---|
| 1 | Microalgae -*Scenedesmus* sp. IITRIND2 | Toxic pentavalent arsenic (V) and anoxic trivalent arsenic (III) | Arora et al. (2018a) |
| 2 | *Pseudomonas putida* DOT-T1E | Propranolol | Sayqal et al. (2016) |
| 3 | *Burkholderia* sp. strain C3 | N-Methylcarbamates | Seo et al. (2013) |
| 4 | *Lactobacillus plantarum* P9 | Phorate | Li et al. (2018) |
| 5 | *Photobacterium ganghwense* strain 6046 (PGS6046 | Cyfluthrin | Wang et al. (2019) |
| 6 | *Pseudomonas* spp. | BTEX | Li et al. (2020) |
| 7 | *Drechslera* sp. | Methyl tertiary-butyl ether (MtBE) | d'Errico et al. (2020) |
| 8 | *Mycobacterium* sp. DBP42 and *Halomonas* sp. ATBC28 | Plasticizers | Wright et al. (2020) |

aquatic system found to cause ill effects on marine organisms (Rodríguez et al. 2016). *Photobacterium ganghwense* strain 6046 (PGS6046) was found to grow extensively in the presence of cyfluthrin; its metabolomic analysis by gas chromatography mass spectroscopy and ultra performance liquid chromatography mass spectroscopy revealed formation of 156 metabolites during degradation process (Wang et al. 2019). Xenobiotic from soil overly known as BTEX is the most common contaminant from soil. Metabolomic analysis revealed that *Pseudomonas* spp. was found to be potent BTEX degrader among diverse contaminated soil samples (Li et al. 2020).

Fungi have also found to play a major role in bioremediation (Rupcic et al. 2018). In a metabolomic study of endophytic fungi *Drechslera* species, was found to degrade methyl tertiary-butyl ether (MtBE), a major hydrocarbon pollutant. Metabolomic profiling was done using nuclear magnetic resonance and thin layer chromatography; derivative of alkynyl-substituted epoxycyclohexenone and monocerin were two major metabolites found in degradation pathway (d'Errico et al. 2020).

Marine plastic pollution is havoc for its ecosystem. As much research has been done with respect to degradation of those plastic by marine microbes (Paluselli et al. 2019), two marine microbes *Mycobacterium* sp. DBP42 and *Halomonas* sp. ATBC28 were found to grow extensively on plasticizers. There metabolomic profiling found the involvement of various enzymes in β-oxidation pathway for removal of ester side chain from different plasticizers. As this microbe was biofilm producer, this property has great application in plastic degradation (Wright et al. 2020). Metabolic profiling of different microbes along with respective pollutants has been summarized in Table 2.1.

Thus, metabolomics is a great technological advance that gives us a lot of new ways to study various biological compounds following a genome sequence. Analyzing metabolomics facilitates the retrieval of qualitative and quantitative data from varied biological systems. It facilitates the study of the response of various microorganisms to contaminants (Jeevanandam and Osborne 2021).

## 2.3 Application of Computer in Metabolomic Study

Despite machine learning being an ancient scientific field, its application in biological research only flourishes in the recent past because of the availability of sufficiently large datasets and computer including digitalization. This application widely helps in gaining detail data by using varied machines and computer systems with different software (Cuperlovic-Culf 2018). Instrument makers often offer general data management solutions with a user-friendly interface that is designed to fulfil a wide range of client needs. Many advanced metabolomics applications necessitate the development of software by the scientific research community. These tools could enable the development and testing of wholly novel metabolomics methodologies that are not covered by vendor software, or they could handle a specialized area that is not covered by vendor software (Chang et al. 2021). Different data softwares that are used in metabolomics studies have been summarized in Table 2.2.

## 2.4 Application of Metabolomics in Space Bioremediation

Satellite services have become an integral part of our modern lives. It is more likely that a satellite will be smashed into pieces when it launches into space, which increases the likelihood of collisions (Aglietti 2020). Human activities in outer

**Table 2.2** Data software use in Metabolomics

| Software | Working | References |
|---|---|---|
| MS PepSearch | Metabolite identification | (Lowenthal et al. 2013) |
| GNPS | Study of statistics/pathway/ metabolite | Wang et al. (2016) |
| SIRIUS | Molecular fingerprinting | Böcker and Dührkop (2016) |
| Weka | General tool | David et al. (2013) |
| FingerID | Molecular fingerprinting | Heinonen et al. (2012) |
| XCMS online | General tool | Domingo-Almenara and Siuzdak (2020) |
| Mummichog | Pathway analysis | Li et al. (2013) |
| MetaboAnalyst | General tool | Xia and Wishart (2016) |
| MeltDB 2.0 | Metabolomic analysis | Kessler et al. (2015) |
| MS-DIAL | Preprocessing tool | Tsugawa (2015) |

space are directly responsible for space debris. Debris in space is out of control manmade objects that cause more harm than good. Some of them involve tools that astronauts lost during extravehicular missions, some satellites, and fragments from satellites. It is possible for fragmentation events to occur accidentally or intentionally as in an antisatellite weapons test (Haroun et al. 2021).

Natural grey soil containing potentially poisonous metals and compounds, such as perchlorate on Mars, can also be found on other bodies in the solar system. The utilization of microorganisms in bioremediation would allow hazardous or recyclable components to be removed from soil, human waste, and other bodies in our solar system. Bioremediation techniques thus provide an once-in-a-lifetime opportunity to establish self-sustaining human communities beyond Earth (Davila et al. 2013).

## 2.5 Future Advancement

Though metabolomics is still a new science and less developed than other fields, it is the result of cell processes (Pinu et al. 2019). Analyzing environmental samples using metabolome-based approaches has helped us create models to simulate microbial activity under a variety of bioremediation strategies (Malla et al. 2018). Recent research has investigated the biodegradation of manmade pollutants using microbial metabolome analysis. Analyzing microbiome-derived metabolites remains challenging due to their physiochemical heterogeneity and the difficulty of obtaining an accurate sample. Some approaches in metabolomics that are projected to have a positive impact on microbial metabolomics in the future by examining recent tech advancements in metabolomics include identification of all different types of metabolites, so improvement was made in instruments as different combinations were used like GC-MS, LS-MS, IC-MS, HILIC-MS, CE-MS, etc. In future, more sensitive tools can be combined and used. Even separation and extraction of those metabolic components are challenging task, thus advanced structural elucidation, physicochemical studies, and optimization can be focused (Misheva et al. 2021). By advancing omics and genetic engineering tools, we may be able to develop microbe-metabolites capable of a more sustainable bioremediation of the environment (Joshi and Sarma 2021).

## 2.6 Conclusion

Normally, nature keeps the environment in perfect balance by removing impurities, but in the current period of industrialization, the rate at which pollutants are released into the environment has exceeded the environment's threshold level. New methods such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics said to be an omics have recently been added to systems biology applied to microbial consortia in various contexts. Omics techniques show a lot of promise for predicting microbial activity in disturbed settings and using microbial processes to attenuate pollutants and speed up bioremediation. Molecular mechanisms involved in the

microbial transformation of harmful pollutants would aid in tracing the causative species and ensuring that the toxins are efficiently eradicated from the environment using omic approaches to bioremediation.

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# Metagenomics, Microbial Diversity, and Environmental Cleanup

3

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**Abstract**

The rapid industrialization, population surge, and modern lifestyles have led to increasing pollution load at an alarming rate due to persistent and nondegradable contaminants and deteriorating the well-being of humans and the environment. Bioremediation being an environment-friendly and cost-effective biological technique utilizes the microbes for the elimination of contaminants from the contaminated sites, therefore, gaining significant interest as a substitute for environmental cleanup. Microbes can endure divergent environments and can revive polluted sites naturally by degrading and transforming the pollutants into nontoxic metabolites. Nowadays, advanced omics tools such as metagenomics, transcriptomics, proteomics, etc. have been designed for understanding the microbial ecology, diversity, functions, and their utilization in environmental monitoring and cleanup. Metagenomics plays a significant role in finding unseen genetic features of microbes in the divergent environment and in discovering the novel genes, enzymes, pathways, and bioactive molecules for biotechnological applications. Therefore, this chapter talks about the metagenomics approaches utilized in monitoring of environment and bioremediation to solve the pollution problems, its potentialities, and current challenges.

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**Keywords**

Metagenomics · Bioremediation · Shotgun sequencing · Microbial diversity

## 3.1 Introduction

The pollution of water and soil is the utmost concern of today's world and has headed towards harmful effects on the environment and human health. The quality of water and soil has been deteriorated by diverse factors and contaminants such as polyaromatic hydrocarbons, heavy metals, pesticides, dyes, hydrocarbons, etc. (Kaur and Goyal 2019). Therefore, the promotion and implementation of checking pollutant load in the soil are very much important for the prevention of risks related to public health. Various methods such as sorption, ion exchange, chemical precipitation, solvent extraction, photocatalytic degradation, and coagulation have been employed for removing heavy metals and organic contaminants from contaminated water and other polluted sites and these processes have various disadvantages (He et al. 2020). On the other hand, bioremediation is considered an attractive method for removing environmental contaminants as it utilizes natural biological processes to degrade pollutants. The bioremediation process employs microbes for degrading and detoxifying the environmental pollutants. The changes in the microbial composition and activity can significantly alter the contaminant fate in the environment (Chakraborty et al. 2012). The growing consciousness has resulted in many advanced approaches to curtail the pollution load using scientific technology. Microbes such as bacteria, fungi, and yeast are known for the outstanding detoxification of pollutants (Abou Seeda et al. 2017). Microbial bioremediation approaches rely on microbial consortia of a variety of indigenous organisms of the contaminated sites. The isolation of such indigenous microbes will help us to know the microbial metabolites and the degradation processes. Therefore, recent studies have incorporated next-generation sequencing techniques to well understand the microbial populations involved in bioremediation involvements. The advanced and new approaches of molecular biology and metagenomics have greatly expanded the knowledge gap of understanding the biological systems and the microbial world of contaminated environments (Techtmann and Hazen 2016).

Soil-borne microbes have the major biodiversity group on Earth which comprises more than $10^{30}$ microbial cells and have $10^4$ to $10^6$ diverse species per gram of soil (Torsvik and Øvreås 2002). Microbial communities of soil play a dominant role in terrestrial ecosystems owing to their huge count, large biomass, and diverse ecosystem functions (nutrient cycling, plant nutrition, and disease suppression) (Mendes et al. 2017). There is enough information on microbial communities of soils of different environments, but there is a lack of functional responses of the microbes to changes concerning soil management and chemical properties. Therefore, the study of microbial diversity is very significant for understanding microbial ecology of soils and other ecosystems. The major percentage of microorganisms are non-cultivable under in vitro conditions; therefore, the identification of the microbial world remains enormously confounding (Chandran et al. 2020). A very little percentage of

microbes from varied environment samples are culturable and unapproachable for research (Awasthi et al. 2020). Previously, molecular tools and fingerprinting technologies, such as T-RFLP (terminal-restriction fragment length polymorphism), ARISA (automated ribosomal intergenic spacer analysis), and DGGE (denaturing gradient gel electrophoresis), have been linked to classical microbiology techniques for studying ecology, diversity, and richness and abundance of species of microbes of soils. In recent times, with metagenomics practice, one can study the soil microbial communities in terms of diversity as well as functional aspects of microbiological parameters (Mendes et al. 2017).

In 1998, the metagenome term was first introduced by Handelsman and collaborators for describing the significance of soil microbes as potential sources of novel natural compounds. They also hypothesized that novel chemical compounds could be mined from uncultivated microbes which constitute more than 99% of the total microbial diversity (Sleator et al. 2008). Sanger sequencing technology had been utilized for metagenomic studies during the initial progress of this field, but later on the advanced next-generation sequencing (NGS) technologies having the advantage of sequencing millions of DNA fragments at once at the cheap price were started in metagenomics (Alves et al. 2018). At present, metagenomics is categorized into two major methods, one is the structural metagenomic approach and the other is the functional metagenomic approach. The structural metagenomic approach is based on studying the structure of the unculturable microbial inhabitants, i.e., microbial composition and dynamics in a unambiguous ecosystem in presence of different spatiotemporal parameters (Alves et al. 2018). Whereas the functional metagenomic approach is centered on the identification of genes that encode for the important functions of concern, that includes the creation of expression libraries having huge amount of metagenomic clones followed by activity-based investigation (Guazzaroni et al. 2015). With the help of advanced molecular tools such as genomics, transcriptomics, proteomics, fluxomics, metabolomics, etc., we can approach unculturable microbes of diverse environments. Additionally, the commencements of next-generation sequencing techniques and in silico analysis have assisted the scientists to access the uncultivable microbes, their complete data, their enzymes, and metabolic pathways related to bioremediation (Pandey et al. 2019). The metagenomic approach has unlocked the new possible ways to evaluate the microbes, their genetic variety, and metabolic paths related to degradation of xenobiotic pollutants. In the current chapter, the role of metagenomics in apprehending the microbial community diversity and functions involved in bioremediation will be discussed. Further, it will discuss the analysis of microbial diversity in divergent environments and mostly employed sequencing platforms.

## 3.2 Conventional Methods of Gene Sequencing

Molecular biology approaches are used for identifying microbial diversity and for determining the efficiency of the bioremediation process. Various conventional culturable and nonculturable technologies are utilized to determine the structures

of microbes and its composition such as randomly amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR), ribosomal intergenic spacer analysis (RISA), ARISA, amplified ribosomal DNA restriction analysis (ARDRA), temperature gradient gel electrophoresis (TGGE), DGEE, and T-RFLP.

### 3.2.1 Polymerase Chain Reaction (PCR)

This technology amplifies a single DNA copy across several orders to generate millions and thousand copies of a specific DNA sequence. It is a reliable, easy, and cheap technology to replicate DNA of interest and is widely used in molecular biology research. This technique requires primers, DNA polymerase, nucleotides, and DNA templates to link every single nucleotide together for the formation of PCR product which is then analyzed by agarose gel electrophoresis.

### 3.2.2 Fluorescence In Situ Hybridization (FISH)

This technology is regarded as molecular cytogenetic method which exploits fluorescent probes that bind to chromosomes that have high degree of complementarity sequence. This technique has led to in situ enumeration and identification of microbes by whole-cell hybridization (Rastogi and Sani 2011). Numerous molecular probes that target 16S rRNA genes are already conveyed at taxonomic levels. This method is used in combination with high-resolution automated analyzer and flow cytometry. This method has been effectively utilized in studying the bacterial population of soil pickled with herbicide triazine (Caracciolo et al. 2010). However, background fluorescence, inaccessibility of target, and low signal intensity are the main constraints of this method. However, this problem has been resolved by the utilization of brighter fluorochromes and treatment of chloramphenicol to elevate the content of rRNA.

### 3.2.3 Amplified Ribosomal DNA Restriction Analysis

This technology is centered on variations of DNA sequences that exist in amplified PCR of 16S rRNA genes. In this technique, the PCR product amplified from environmental DNA is digested by restricted fragments and endonucleases which are resolved on polyacrylamide gels. However, this method provides less information on the type of microorganism present in the sample. ARDRA is also used in characterizing communities of microbes that are involved in biodegradation of xenobiotic compounds and industrial wastewater treatment (Shah 2014).

### 3.2.4 Ribosomal Intergenic Spacer Analysis

This technique majorly includes amplified PCR for a portion of intergenic spacer region present between large (23S) and small ribosomal (16S) subunits that shows variability of nucleotide sequence and length of various microbes (Ciesielski et al. 2013). This method is also called community fingerprinting as it is used in comparison and characterization of microbes in different environmental conditions. The modified form of RISA that is ARISA includes the utilization of fluorescence-labeled forward primer, and fragments of ISR are detected by a laser detector that leads to simultaneous analysis of various samples.

### 3.2.5 DNA Microarrays

This technique is also called nucleic acid microarrays and uses specific sequences of DNA that are synthesized or deposited in the 2-dimensional array over the surface that leads to the attachment of DNA noncovalently or covalently to the surface. It probes a solution of nucleic acids and hybridization of the targets to probe that measures the nucleic acid concentration in solution (Bumgarner 2013).

### 3.2.6 Randomly Amplified Polymorphic DNA (RAPD) Analysis

This method uses amplified PCR by primers that are 10 nucleotides short and anneals randomly at numerous sites on genomic DNA at low annealing temperatures usually 35 °C. It leads to the formation of PCR amplicons of diverse lengths in an individual reaction separated by polyacrylamide or agarose gel electrophoresis. RAPD provides ease and high speed due to which it is utilized in fingerprinting of microbial diversity and closely related strains.

## 3.3 Next-Generation Sequencing Techniques

Next-generation sequencing denotes technologies permitting millions of sequence reactions in parallel to a solid surface such as glass slide or beads. Therefore, this requires the spatial separation of reactions rather than physical separation. Hence, various million reactions follow at the same time which leads to reduction in labor input and huge cost as compared to conventional methods. The path includes various commercial platforms of NGS on the basis of different technologies and follow general steps or patterns. The steps in DNA sequencing by NGS are as follows (a) preparation of library, (b) library amplification, (c) sequencing via diverse approaches. The results that are generated vary as per the data quality, length read, and data quantity. Classification of various NGS sequencing technologies on the basis of technology type, system of detection used, amplification method, and chemistry is described below and mentioned in Fig. 3.1.

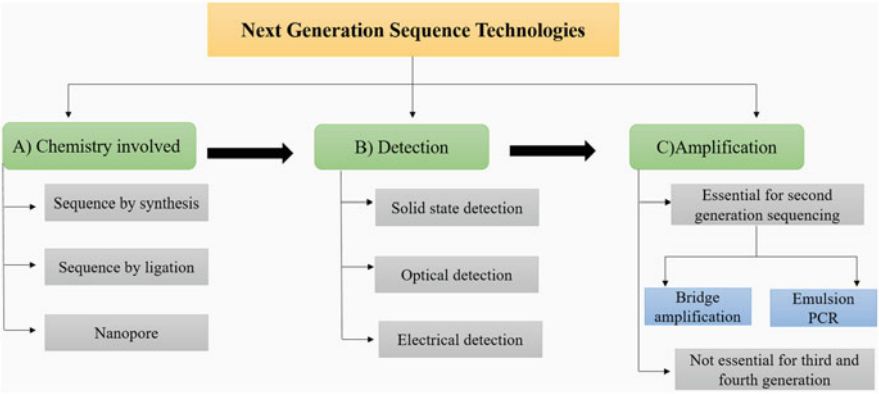


**Fig. 3.1** Classification of various NGS technologies

### 3.3.1 Pyrosequencing Technology

In 1993, Nyren and coworkers introduced pyrosequencing technology through sequencing by synthesis (SBS). In this technique, DNA sequencing is centered on detection of released pyrophosphate molecule during nucleotide addition by DNA polymerase (Ronaghi et al. 1996). The speed of reaction speed is very fast at room temperature, around 3–4 s is utilized for the reaction competition from nucleotide addition to chemiluminescent detection. However, later a US-based Biotechnology Company 454 Life Sciences took up this technique and made it commercially available with slight amendments. This technology utilizes enzyme luciferase from American firefly (*Photinus pyralis*) and recombinant ATP sulfurylase (*Saccharomyces cerevisiae*). This technology includes two different approaches: (a) solid phase in which DNA gets immobilized, and (b) liquid phase in which pyrase (nucleotide degrading enzyme) which excludes the solid support requirement due to which reaction occurs in single tube.

### 3.3.2 Roche 454 (GS FLX Plus)

Pyrosequencing technology sequencer of 454 Life Sciences in 2007 was overtaken by Roche which was later identified as Roche 454. The phases included are nucleic acid fragmentation followed by template strand synthesis with polymerase enzyme assistance. Once new nucleotide is incorporated by polymerase, the pyrophosphate is released. The released molecule changes ADP to ATP in presence of sulfurylase enzyme. Pyrosequencing-based platforms use parallel systems in picoliter volumes for sequencing in microfluid format. The methodology involves DNA fragmentation using a nebulizer (spray method), ligation of adaptors to the fragmentated DNA, and preparation of library followed by library attachment to beads. The bead makes individual compartments called microvesicles or microreactors. In these

compartments, clonal amplification occurs by emulsion PCR, next the emulsion breaks down and beads attach to clonally amplified DNA that are rich in microreactors (Margulies et al. 2005). Entirely, clonal amplified DNA bound beads get distinctly loaded on a picotitre plate with $3.4 \times 10^9$ wells of 55 μm in depth. The slide plate containing picoliter-sized wells is mounted on the flow cell, forming flow channel for sequencing reagents above the wells. GS FLX is a genome sequencer that produces 450 Mb data from a single run as compared to new genome sequencer that produces 700 MB of data from a single run within 10 h. The main advantages are it is a fast technology and produces reliable and accurate results for high-throughput real-time sequencing. Moreover, this technology does not need labeled primers and nucleotides and is appropriate for confirmatory sequencing as well as de novo sequencing (Ronaghi 2001). The major restraint is sequencing the same nucleotide repeat (homopolymer sequencing) and it is costlier as compared to other technologies.

### 3.3.3 Reverse Terminator Technology

This technology was first introduced by Dr. JingyueJu and is based on sequencing by synthesis strategy. The major difference between reversible sequencing and traditional sequencing is that it uses modified nucleotide analogue to end the primer extension reversibly, whereas traditional technology employs ddNTPs to terminate the primer extension (Guo et al. 2010). It is majorly categorized into two types that are 3′ blocked and 3′unblocked reversible terminators. Illuminia Solexa has commercialized this technology due to its acceptance in sequencers of second generations (Bentley et al. 2008).

#### 3.3.3.1 Illumina Solexa

In 1998, Shankar Balasubramanian and David Klenerman introduced the idea of using one sequencing DNA molecule attached to a microsphere with Solexa foundation. In 2006, "Solexa Genome Analyser" was invented which was later occupied by Illumina for clonal amplification of DNA sequencing (Voelkerding et al. 2009). This technology uses flow cell made of transparent optical slides with eight lanes over the surface with oligonucleotides attached on flow cell. The methods of sequencing are template DNA fragmentation and repairing end fragments. 3′ ends are adenylated by the single "A" nucleotide addition to enable ligation at the 3′ end with adaptors carrying overhang "T." As the flow cell and ligated adaptors are complementary, anchors get hybridized. The bounded DNA template to flow cell leads to the formation of a cluster by bridge amplification as compared to PCR emulsion (Adessi et al. 2000). Polymerization is terminated due to the addition of ddNTPs (fluorescent-labeled reversible terminators), and these incorporated nucleotides are detected via capturing fluorescence (Guo et al. 2008). Illumina is the major dominating platform in the high-throughput sequencing market and produces different platforms such as NextSeq, HiSeq, and MiSeq series. Out of this most popular and recognized are HiSeq and MiSeq platforms. HiSeq2500 has

the ability to produce 1 terabytes (TB) of data from a single run in 5–6 days. On the other hand, MiSeq was introduced in 2011 as a tabletop sequencer in which a run is completed within 4 h for the targeted sequencing of bacteria. Recently, Illumina released HiSeq4000 and HiSeq3000 on the basis of the patterned flow cell and their run time and data output lie between HiSeq2500 and HiSeq X Ten (Reuter et al. 2015).

#### 3.3.3.2 Ion Torrent

This technology converts nucleotide sequence directly into digital information over a semiconductor chip (Rothberg et al. 2011). The sequencing reactions of this technology occur in millions of well-covering semiconductor chips encompassing millions of pixels which lead to the conversion of chemical information into sequencing information. The process begins with the fragmentation of DNA into 200–2000 base fragments that are ligated to adaptors. The fragments of DNA adhere to adaptors and beads by complementary sequencing which is then amplified by emulsion PCR. Subsequently, the beads flow through the chips that contain wells, and when nucleotides are incorporated hydrogen ions are given off and the signal is recorded. The advantage of this technology is that there is no requirement for a camera or scanner as direct nucleotide addition is converted into a voltage which is recorded and thus speeds up the process. Ion Torrent is sold by Ion Proton system, IONX5 XL system, and Ion personal genome machine system.

#### 3.3.3.3 Sequencing by Ligation Technology

This technology determines the sequence of DNA by using the mismatch sensitivity of enzyme DNA ligase (Ho et al. 2011). In 2008, this technology was marketed by Applied Biosystems USA. The basis of this technology depends on the varied length of oligonucleotide probes, which are labeled with various fluorescence tags liable to nucleotides aimed for sequencing.

#### 3.3.3.4 ABI SOLiD

In 2005, George Church invented this technology as a detection and small oligonucleotide ligation system. However, later SOLiD was marketed by Applied Biosystems in 2008 which is currently overtaken by Life technologies. The sequencing reaction of this technology is categorized into five steps: (a) DNA library preparation, (b) clonal amplification in microreactors via emulsion PCR, (c) bead adherence, (d) sequencing, (e) primer resetting. The SOLiD500 takes almost 5–6 days to complete a single run and produces data of 120–240 gegabytes (GB) with 75 bases read length, whereas the SOLiD4 platform produces 100 GB data. The major advantage of this technology is 99% accuracy as each nucleotide is sequenced twice (Voelkerding et al. 2009). The main constraints are time taking procedures and less data production as compared to Illumina.

## 3.4 Metagenomics Sequencing and Its Framework

The metagenomic study of the environmental sample (contains whole genomic material), collected straight from surroundings, is known as metagenomics (Schmeisser et al. 2007). Metagenomics is a new field of genomics that uses a variety of ways to channelize microbial groups in environmental samples and to unravel the genomes of unrefined microorganisms, revealing the heterogeneousness of taxonomically and phylogenetically compatible genes, catabolic genes, and whole operons (Riesenfeld et al. 2004**;** Uhlik et al. 2013). Researchers will be able to combine pure culture studies with genomics using the metagenomic data (Yergeau et al. 2017). It uses a diverse variety of microorganism's ambient genomes, which compliments the chances of discovering novel genes and pathways, and new enzymes that are immensely precise catalytic capabilities (Awasthi et al. 2020). Early metagenomic investigations centered on habitats like acidic environment, mining, drainage, and the human colon microbiome due to a shortage of high-throughput sequencing tools and program (Oulas et al. 2015**;** Hodkinson and Grice 2015). Utmost environments with severe temperature, alkality, acidity, less oxygen, deep-sea hydrothermal vents, heavy metal-contaminated soils, and so on have been examined using metagenomics that supply limitless opportunities for bioprospecting and inspect novel biomolecules like proteins, enzymes, and so on, due to the advances of potent software tools and molecular advancements (Scholz et al. 2012). Handelsman (2004) initially proposed the idea of metagenomics in 1998; nevertheless, the earliest evidence of metagenomics comes from Pace et al. (1985), who was the first to do phylogenetic analyses of ambient microbial populations. The separation of nucleic acid (DNA or RNA) from surrounding sources is the beginning step in metagenomic assessment study. Genome improvement measured by metagenome investigation is possibly used to assess vital microbial groups in contaminated environments (Felczykowska et al. 2015). Cataloging using Stable Isotope Probing (SIP) is used to improve RNA, DNA, or phospholipids of vital microbial populations. Phylogenetic markers, conserved genes, expression of particular phenotypes, and other features can all be inspected in the emerging transformants (Handelsman 2004). Two methods of analysis can be used to extract biological data from metagenomic libraries: function-driven study and sequence-driven study. The sequence-driven analysis is centered on the sequencing of clones conserved DNA sequences, whereas function-driven analysis is centered on the identification of clones that express their functional activity (Chen and Murrell 2010; Wong 2018).

The original goal of metagenomics was to identify population present in environment for some activities associated with biology and discovery of genes or gene clusters related with it; thus, function-based screening was coined (Bharagava et al. 2019). In addition, the development of high-throughput NGS technologies [such as 454 pyrosequencing, Illumina (Solexa) sequencing, and SOLiD (Sequencing by Oligonucleotide Ligation and Detection) sequencing] spawned a new approach into metagenomics: sequence-based screening. Metagenomic investigations have been performed by using high-throughput microarrays, with the exception of a

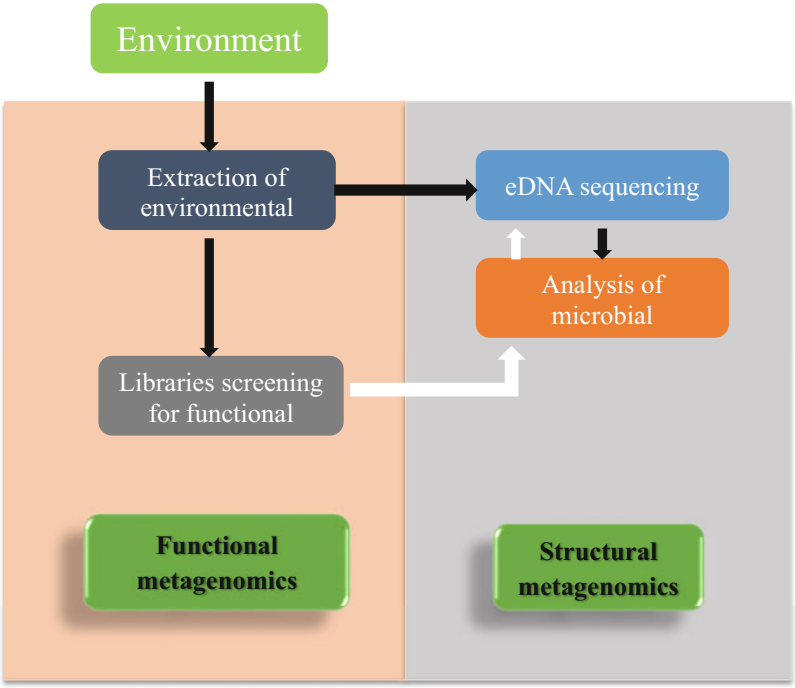


**Fig. 3.2** The two main approaches of metagenomic framework

sequence-based screening of ambient metagenomic libraries (Uhlik et al. 2013). These libraries are used to study microbial populations and take a look on biogeochemical processes in the environment. Currently, GeoChip microarrays contain 83,992 50-mer sequences encoding enzymes involved in biogeochemical (C, N, P, S) cycles, metabolic processes, heavy metal tolerance, antibiotic resistance, and ecological pollutant degradation (Lu et al. 2012). As a consequence, GeoChip microarrays could be used to study microbial communities' organization, behavior, and prospective metabolic functions, as well as their modifications in response to specific disturbances (Brodie et al. 2006). PhyloChip is a domain of microbes and pollutant degradability, a type of microarray used for elevated phylogenetic research of microbial communities (DeAngelis et al. 2011).

Metagenomics is subdivided into two parts, each of which focuses on a different feature of the microbiological population in the area of specific habitat. The first, known as structural metagenomics, focuses on studying the organization of an undeveloped bacterial community that can be broadened to incorporate other aspects like the reconstruction of a complex metabolic pathway among community members (Fig. 3.2) (Handelsman 2005; Tringe et al. 2005). In this context, microbial community configuration states the makeup and development of the population microbes in relation to evolutionary processes and geographic features of a single ecosystem. The research of plant communities gives a better understanding of the relationships

among the numerous components that comprise a community and also identifying environmental or physiological roles among individuals (Tringe et al. 2005; Vieites et al. 2009). The functional metagenomic approach, on the other hand, tries to discover genes that translate for such a desired purpose by creating expression libraries with hundreds of metagenomic clones and then screening them using activity-based methods (Fig. 3.2) (Schmeisser et al. 2007; Guazzaroni et al. 2015).

Structural metagenomics, on another end, tries to explore the elements of a microbial community's genes. As a consequence, the latter method can rebuild the community composition more thoroughly, perhaps revealing whole microbiome metabolic pathways and assigning small or large geoecological functions to community leaders (Alves et al. 2018).

The use of Sanger sequencing technology at the start of metagenomic investigations resulted in significant advances in the area (Gillespie et al. 2002**;** Breitbart et al. 2003; Uchiyama et al. 2005). However, the introduction of next-generation sequencing (NGS) technology capable of simultaneously sequencing millions of DNA fragments at a low cost boosted the field significantly (Sunagawa et al. 2015**;** Klindworth et al. 2013**;** Oulas et al. 2015). In comparison, up to 5000 Mb of Target dna can be recovered utilizing NGS systems every day for approximately 0.50$/Mb, but Sanger sequencing technology can create about 6 Mb of DNA sequence per day for about 1000$/Mb (Kircher and Kelso 2010).

## 3.5 Tools for Metagenomic Data Analysis

Shotgun metagenome in comparison to amplicon may directly offer functional gene profiles and achieve a considerably greater level of taxonomic annotation resolution. However, analysis requires a considerable number of computational resources due to large data size. We advocate deploying metagenomic analytic pipelines using the package manager Conda with BioConda channel (Tange 2018) to make program installation and maintenance easier. Because metagenomic analysis is computationally intensive, it is desirable to execute several projects in parallel with the use of queue management software such as GNU Parallel (Grüning et al. 2018). The HiSeqX/NovaSeMetaBAT2 from Illumina based on tetra-nucleotide frequency and contig abundance, binning methods group contigs into different bins (draught genomes) (Kang et al. 2015). To obtain superior bins, reassembly is undertaken. We advocate adopting a binning pipeline like MetaWRAP (Uritskiy et al. 2018) or DAStool (Sieber et al. 2018) that combines many binning software packages to produce refined binning findings and more complete genomes with less contamination. These pipelines also provide scripts that may be used for evaluation and visualization. Quince et al. (2017) is a good resource for a more in-depth look of metagenomic studies and analysis. For metagenomic sequencing, the q system typically provides PE150 reads, whereas the BGI-Seq500 generates PE100 reads.

The KneadData pipeline (https://bitbucket.org/biobakery/kneaddata) or a combination of Trimmomatic (Bolger et al. 2014) and Bowtie 2 are required for quality control and the removal of host contamination from raw reads, which is required for

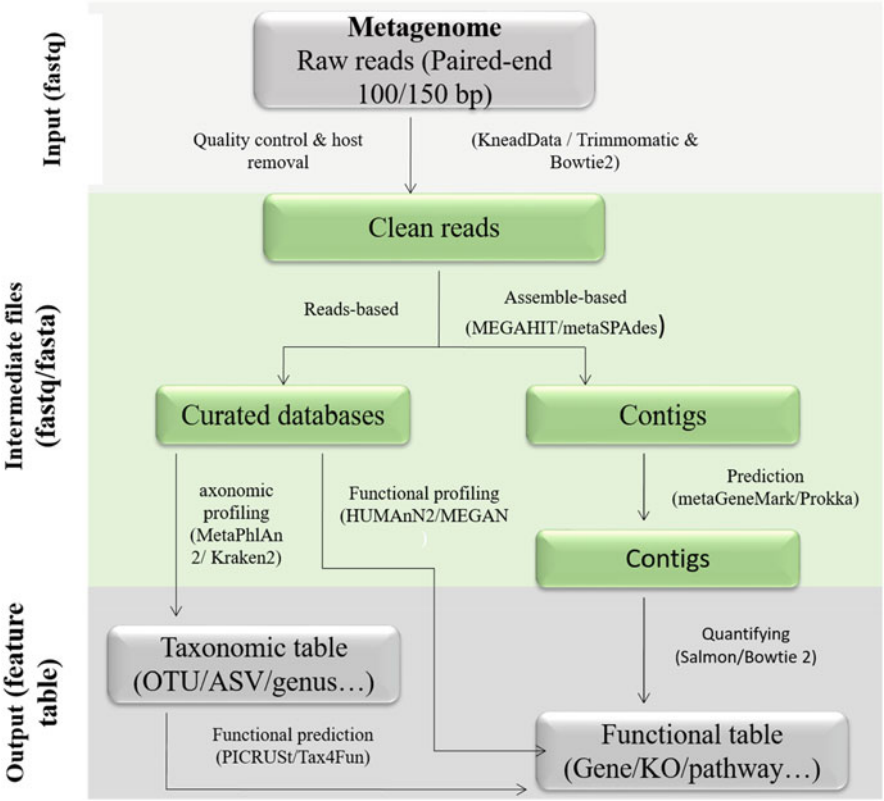


**Fig. 3.3** Flowchart of metagenomic sequencing methods. The text next to the arrow represents methods while parentheses represents frequently used software

metagenomic analysis (Langmead and Salzberg 2012). The conversion of clean data into taxonomic and functional tables utilizing reads-based and/or assembly-based approaches is the most important step in metagenomic analysis. Clean reads are aligned to curated databases using read-based approaches, which provide feature tables (Fig. 3.3). MetaPhlAn2 is a taxonomic profiling program that aligns metagenome readings to a predefined marker-gene database in order to perform taxonomy classification (Truong et al. 2015). Kraken 2 employs lowest common ancestor (LCA) techniques to perform taxonomy classification and does accurate k-mer matching to sequences in the NCBI nonredundant database (Wood et al. 2019). See Ye et al. (2019) for a survey of 20 taxonomic categorization techniques that have been benchmarked. The widely used functional profiling software HUMAnN2 (Franzosa et al. 2018) can also be used to investigate within and between sample contributional diversity (species contributions to a certain function). MEGAN is a crossplatform graphical user interface (GUI) software that performs taxonomic and functional studies (Huson et al. 2016) (Table 3.1). There are other catalogues curated from the human gut (Tierney et al. 2019), the mouse gut (Xiao

**Table 3.1** Tools of metagenomic analysis

| S. No. | Name | Description and advantages |
|---|---|---|
| 1. | Trimmomatic | It is a Java-based software which is used in metagenomic raw reads for quality control |
| 2. | Bowtie 2 | It is a tool for rapid alignment which is used for quantification or to eliminate host contamination |
| 3. | MetaPhlAn2 | Tool for taxonomic profiling having marker gene databank with above 10,000 species. It gives result for the relative abundance of strains |
| 4. | Kraken 2 | A tool for taxonomic classification used for accurate k-mer similarity to NCBI database, fast and accurate classification, and give outputs reads counts for individual species |
| 5. | HUMAnN2 | Centred on the UniRef protein databank, can calculate abundance of gene family, coverage, and abundance of pathway from metagenomic or metatranscriptomic data. |
| 6. | MEGAN | A cross-platform tool for taxonomic and functional assessment of metagenomic data, provides visualizations with metadata (including scatter plot, word clouds, tree maps, grouping, and linkages) |
| 7. | MEGAHIT | Ultra-fast and memory-efficient metagenomic assembler |
| 8. | metaSPAdes | High-quality metagenomic assembler, requires excess time and large memory |
| 9. | MetaQUAST | Assesses metagenomic assemblies quality (including N50, misassemble, outputs PDF, and interactive HTML reports) |
| 10. | MetaGeneMark | Gene prediction in bacteria, archaea, metagenome, and metatranscriptome |
| 11. | Prokka | Offers rapid prokaryotic genome annotation, give output as nucleotide sequences, protein sequences, and annotation files of genes |
| 12. | CD-HIT | Utilized for making nonredundant gene catalogs |
| 13. | Salmon | Offers ultra-fast quantification of reads counts of genes based on k-mer |
| 14. | metaWRAP | Binning pipeline having 140 tools, also supports Conda installation. It provides visualization of refinement, quantification, taxonomic classification, and visualization of bins |
| 15. | DAS tool | It is a binning pipeline which integrates five different binning software packages which is used for refinement |

et al. 2015), the chicken gut (Huang et al. 2018), the cow rumen (Stewart et al. 2019), and the ocean (Salazar et al. 2019). In the right field of study, these customized databases can be utilized for taxonomic and functional annotation, allowing for efficient, precise, and quick analysis. Methods based on assembly MEGAHIT or metaSPAdes can be used to assemble clean readings into contigs (Fig. 3.3). MEGAHIT (Li et al. 2015) is a tool for swiftly assembling huge, complicated metagenome datasets with low computer memory, whereas metaSPAdes can build longer contigs but requires more computing resources (Kanehisa et al. 2016). MetaGeneMark (Zhu et al. 2010) or Prokka is used to identify genes included in assembled contigs (Seemann 2014). Using methods like CD-HIT, redundant genes from separately built contigs must be eliminated (Fu et al. 2012). Finally, using alignment-based tools like Bowtie 2 or alignment-free approaches like Salmon, a gene abundance

table can be created (Patro et al. 2017). A metagenomic dataset typically contains millions of genes. These genes must be grouped together into functional annotations such as KEGG Orthology (KO), modules, and pathways, which is a type of dimensional reduction (Nurk et al. 2017).

## 3.6 Bioinformatics Tools for Functional Analysis of Metagenome

The functional annotation approach identifies the genes of importance as well as the prediction of their functions as per the taxonomy (Almeida and De Martinis 2019). In the metagenomics, the initial stage involves the sequence comparison using available catalogs for finding all of these taxonomies, functional annotation, sequence binning, phylogenomic outlining, and metabolic restoration through freely available software, i.e., MG-RAST (rapid annotation using subsystems technology server) that do the processing and integration of all the metagenomic data. The operators may upload raw data files (FASTA format) on the MG-RAST server which further normalizes the sequences so that operators can do data comparison with other metagenomic data (Meyer et al. 2008).

Mothur server is a different stage of metagenomic sequencing which has several integrated analysis tools like pyrosequencing pipeline (RDP, which cuts and denoise user sequences); NAST, SINA, and RDP aligners (which is used to compare user sequences with available records). On this server, DNADIST (which calculates sequence distances between alignments), DOTUR, and CD-HIT (that relates sequences to OTUs, constructs rarefaction curves, and calculates diversity and richness); $\int$-LIBSHUFF (which test similarity between two communities structure) are available. It also incorporates TreeClimber (which uses the parsimony method to find similarity between two or more communities structure) and UniFrac (to compare the distance between communities through phylogenetic to detect differences in their structure). Only $10^2$–$10^4$ sequences can be analyzed through this server and it cannot be utilized for the investigation of large data. Mothur also comprises more than 25 diversity index calculators, visualization tools, NAST-based sequence alignment, and a pairwise sequence distance calculator (Almeida and De Martinis 2019).

The MEGAN (Metagenome Analyzer) server is another main computational tool that analyzes the metagenomes according to taxonomic and functional classification. The main advantage of taxonomic analysis using MEGAN is nonrequirement of metagenomic assembly. The operation in MEGAN is typically centered on a preprocessing step that compares the contigs against catalogs of identified sequences with the help of BLAST with visualization of output in MEGAN. MEGAN synergistically evaluates and utilizes the taxonomic data of the National Center for Biotechnology Information (NCBI)-based dataset for results and summary. The MEGAN tool works on the simple algorithm which allocates common ancestor (LCA) to each read from its hit by means of a reference taxon (Almeida and De Martinis 2019). The output on MEGAN is in form of a tree that contains species-

specific sequences clustered as terminal branches and highly conserved sequences clustered close to the root along with tree nodes representing different taxonomic levels (Huson et al. 2016).

Kraken is a tool which is used for classification, characterization, and finding an abundance of sequence in accurate speed and accuracy. The speed of Kraken is originated due to precise matches among the k-mer value of sequences and available sequences on databases. This tool can differentiate the sequences at the genus category very accurately and sensitively in comparison to Megablast (Wood and Salzberg 2014).

## 3.7 Application of Metagenomics

Metagenomics does the isolation of genetic material from different environments. Over the past two decades, this approach has evolved to explain and study the microbial communities host occupying a specific niche to apprehend their ecological role, genetic variety, and configuration of the population. Various novel and new molecules with specific applications and functions have been identified by this approach. Recently, various scientists and researchers are involved in this area to untap and reveal genetic resources with the help of government funding. Metagenomics is also used in the area of sustainability, ecology, medicine, and agriculture. This approach assures to provide novel enzymes and molecules with improved features and different functions in the comparison to enzymes isolated from culturable microbes. Apart from revealing novel biocatalysts from nature, metagenomics is also used in fields of medicine, xenobiotic metabolism, bioremediation, and human microbiota.

### 3.7.1 Food Industries

Novel biocatalysts and enzymes isolated from natural sources are mainly used in reactions of food processing. Several enzymes are related to reactions occurring in nature that provides energy for food, but are not able to mimic at the industrial level, e.g., starch degradation. Microbial enzymes are used in several applications such as brewing, processing of food, starch, and fruits, corn syrup and sugar production, flavoring, fermented, and dairy products production. Numerous novel enzymes discovered by functional metagenomics with application in the industrial field are mentioned in Table 3.2.

Esterases and lipases show a major 3part in the pharmaceutical and food industry. Lipases hydrolyze fats into glycerol and fatty acids at the interface of water lipids and reverse the reaction to the nonaqueous phase (Gupta et al. 2004). Dairy industries exploit lipases for milk fat hydrolysis that releases long- and short-chain fatty acids which provide features of cheesiness and creaminess (Hasan et al. 2006). In 2014, Peng and his coworkers discovered novel alkaline lipase from Chinese marine sediments showing its lipolytic activity in *Escherichia coli* host through

**Table 3.2** Novel biocatalysts screened from metagenomic library by functional metagenomics

| Enzymes | Substrate | Host | Environment |
|---|---|---|---|
| Amylase | Pectin | Plasmid | Soil |
| Cellulase | Starch | Fosmid | Soil |
| Endocellulase | Carboxymethyl-cellulose | Plasmid | Soil |
| Endoglucanase | Carboxymethyl-cellulose | $\lambda$ phage | Rice straw compost |
| Lipase | p-Nitrophenyl (pNP) esters | Plasmid | Marine sediment |
| Pectinase | Polygalacturonic acid | Plasmid | Lagoon |
| Protease | Azocasein | Plasmid | Soil |
| Serine proteases | Skim milk agar | Plasmid | Soil |
| Xylanase | Oat spelt xylan | Bacterial artificial chromosome | Compost soil rumen |

metagenomic library screening (Peng et al. 2014). Lipases are also used in baked goods preservation and modification of vegetable oil. Even though in the past decade, lipases that were utilized in food industry were attained from animal sources, recently it has been discovered that microbes hold potential for lipases that can be used in various industries (Table 3.2). Lipases isolated from microbes are used in pharmaceutical industries for the production of antimicrobial and antitumor agents (Kato et al. 1997).

Esterases are involved in the hydrolysis of ester into acid and alcohol in an aqueous solution. The main difference between esterase and lipases is that they hydrolyze short-chain fatty acids rather than a long chain. In food industries, they are utilized in oil, fruit, and fat modifications and alcoholic industries for the production of fragrances and flavors. In 2012, Cheng and his team constructed a metagenomic library from Chinese Holstein rumen of cow microbial content for the identification of feruloyl esterase activity, from which they identified activity of protease insensitive esterase proficient of fatty acid production from wheat straw (Cheng et al. 2012). Examples of a pharmaceutical application include pain reliving medications, inflammation reduction, and chiral drug synthesis (Shen et al. 2002).

### 3.7.2 Novel Bioactive Discovery

The application of microbial enzymes producing pharmaceutical products was formerly synthesized by chemical means. Therefore, it is studied that functional metagenomics can be useful for discovering genes with availability to carry out reactions for the synthesis of bioactive or intermediate compounds in pharmaceutical industries. A study has been done for the expression and identification of biosynthetic microbial pathways for the production of biotin utilized in industrial applications (Entcheva et al. 2001). The utilization of microorganism-producing

**Table 3.3** Bioactive compounds and biosynthetic pathway used in pharmaceutical industries identified by functional metagenomics

| Bioactive compound | Homolog | Host | Environment |
|---|---|---|---|
| Biotin | Identity to proteins from *Erwinia herbicola* | Cosmid | Horse excrement |
| Polyketide synthase genes | Identity to PKS from *Myobacterium avium* | Targeted sequence-based strategy | Marine sponge |
| Pederin | Identity to sequence from *Pseudomonas aeruginosa* | Targeted sequence-based strategy | *Paederus beetles* |
| Borregomycin A | Identity to serpins from *Salinibacter ruber* | Plasmid | Uncultured marine organism |
| Vibrioferrin | Identity to proteins from *Vibrio parahaemolyticus* | Plasmid | Tidal flat sediment |
| Novel salt-tolerant gene | Identity to hypothetical protein of *Caulobacter crescentus* | Plasmid | Fecal sample |

biotin rather than chemical synthesis provides a greener substitute to industries. Various other microbial genes of interest capable of synthesizing bioactive compounds used in pharmaceutical industries for medicine and human health recognized by functional metagenomic methods are enumerated in Table 3.3.

### 3.7.3 Novel Antimicrobials Discovery

Microorganisms have the ability to produce antibiotic molecules that alleviate competitors of natural habitats. In the past, natural sources have proved to be beneficial for the production of antibiotic molecules. Various human bacterial infections are cured with present antibiotic therapies, but in recent years antimicrobial resistance problems have evolved that caused the resistance of antimicrobials. Resistance of antibiotics has confronted various researchers in the clinical area for untreatable serious bacterial infections which have made antimicrobial resistance a thoughtful threat towards the health of humans (World health organization 2014). However, recent advances in metagenomics, metabolic engineering, and high-throughput sequences have provided a new alternative for drug discovery from natural products (Jayasuriya et al. 2007). Thus, the metagenomic approach can be used for the novel antimicrobial compound identification through microbial population screening for studying the activity of microbes against clinically relevant microbes. This method gives variety of antimicrobial compounds as mentioned in Table 3.4. In 2002, Gillespie and his team discovered turbomycin A and B antimicrobial compounds via metagenomic screening from soil sample expressed in *Escherichia coli* host displaying broad-spectrum activity against gram-negative and positive bacteria (Gillespie et al. 2002).

**Table 3.4** Discovery of various antimicrobial compounds by metagenomics

| Antimicrobial compounds | Host | Environment |
|---|---|---|
| Violacein | Cosmid | Soil |
| Fasamycin A and B | Cosmid | Soil |
| Terragine | Cosmid | Soil |
| Beta-lactamases | Fosmid | Soil |
| Turbomycins A and B | Bacterial artificial chromosome | Soil |
| Indirubin | Fosmid | Soil |

### 3.7.4 Xenobiotic Degradation

The study of xenobiotic action, especially antibiotics, on the gut microbiota of humans is crucial to study the drug resistance mechanism and genes accountable to counter the problem of elevating drug resistance and inventing drugs that are effective against pathogens. Thus, studying the role of xenobiotic metabolism and resistance in the human gut microbiome will not provide information into biochemistry and host-microbe interactions but will also provide indications for drug efficiency and toxicity. This issue is being resolved by metagenomics that has enabled the genome analysis of the human gut microbiome. In 2013, Maurice and his team discovered the metabolic activity and gene expression of a distinctive set of gut microbiota that is effective by host-targeted antibiotics and drugs (Maurice et al. 2013). These findings suggest the xenobiotic consequences and indicate microbiota as an alternative factor in developing medicines.

## 3.8 Importance of Metagenomics in Bioremediation of Pollutants

The development of genomic methods has enhanced the management of polluted sites in a sustainable way. The cultural-independent approaches of microbial analysis from the contaminated environment have improved the identification of microbial community dynamics vigorously involved in the bioremediation process that cannot be identified by cultural techniques (Desai et al. 2010). Approaches such as gene amplification through PCR and sequencing methods have been established as extremely beneficial in assessing the microbial population utilized in bioremediation (Gołębiewski and Tretyn 2020). The 16S rRNA sequence analysis is utilized for identification of novel, uncultivable, and phenotypically unidentifiable microbial diversity by amplification and sequencing of hypervariable regions of the 16S rRNA gene (Chandran et al. 2020). 16S rRNA sequencing analysis had been utilized in revealing microbial community diversity and occurrence of dioxygenase genes through the genetics of PAHs-contaminated soil (Haritash 2020). In a study, 16S rRNA gene amplicon sequencing was utilized for studying the microbial community abundance and diversity of the heavy metals polluted soil (Kou et al. 2018).

Metagenomics play a major part in detecting air, soil, and water pollution. Metagenomic analysis reveals the microbial community diversity and explicit genes involved in bioremediation which assist as pollution biomarkers (Kisand et al. 2012). Wang et al. (2015) have reported the 255 taxa and 414 functional modules after analytical and comparative metagenomic analysis of datasets of hydrocarbon-contaminated sites by using hydrocarbon pollution biomarkers. Wang and coworkers used the MetaBoot Software for identifying the pollution biomarkers after relating the metagenomic data of contaminated sites. The comprehensive reading about microbial population of each contaminated site can be analyzed by shotgun metagenomics and the associated environmental pollution monitoring models could be acquired from microbial community structure. Targeted metagenomics is another approach that helps in discovering specific genes within a population serving as probable biomarkers for unambiguous environments. In a study by Techtmann and Hazen (2016), cyclo-alkane degrading genes expression gave the idea that the specific site was polluted with hexane compounds. In a study by Silva et al. (2013), the genes and metabolic pathways of degradation of phenol like aromatic compounds from a petroleum refinery wastewater treatment system were characterized by metagenomics. Metagenome data analysis was performed to find the functional potential of microbes, taxonomic community composition of the microbial community, and genes associated with xenobiotic degradation to understand the metabolic pathways of degradation of compounds such as organophosphorus pesticide and diesel (Garrido-Sanz et al. 2019). The collective physicochemical examination has been done along with metagenomics to elucidate metabolic pathways related to polyurethane degradation (Gaytán et al. 2020). A study by Aubé et al. (2020) has reported the influence of petroleum pollutants on the taxonomy and metabolic composition of microbial mats through metagenomics and metatranscriptomics.

With the help of metagenomics, important bioactive compounds, genes, and enzymes have been also discovered. Several hydrocarbons (alkanes, naphthalene, methylphenanthrene, and aromatics) degrading genes have been identified from contaminated soil and aquatic sites (Guerra et al. 2018). Along with this, many enzymes such as dioxygenases, laccases, carboxylesterases, monooxygenases, hexadecane hydrolyzing enzymes, and degrading enzymes of phenol, polyaromatic hydrocarbon, trichlorophenol, and alkane have also been discovered by metagenomics from the soil, cattle rumen, chlorinated biphenyl polluted soils, activated sludge, wastewater, and oil-contaminated water (Ufarté et al. 2015). The above-mentioned enzymes can degrade several insecticides, pesticides, dyes, diesel components, and plastics (Datta et al. 2020). Biosurfactants are amphiphilic glycolipid compounds which help in promoting emulsification of hydrocarbons in the water, thus utilizing them in the removal of oil spills in aquatic sites. Metagenomics also identifies the genes, reactions, and approaches for high synthesis of biosurfactants in marine bacteria (Williams and Trindade 2017). Biosurfactants such as Palmitoyl putrescine and N-acyl amino acids were identified by metagenomics. Metagenomics also helps in eradicating radionucleotides and toxic metals from contaminated soil and water sites because of their hazard to the

environment and human health (Jackson et al. 2015). With the help of metagenomics, heavy metal and radionucleotide remediating microbes, metal resistant, and radioresistant genes have been extracted from heavy metal and radionucleotide-polluted environments (Xavier et al. 2019). Through metagenomic analysis, it has been found that *Geobacter* species have been found in uranium-contaminated sites (Hoyos-Hernandez et al. 2019). In a stable economy, healthy soil and clean water have huge impacts; therefore, to maintain such a stable system, metagenomics have high role in bioremediation technologies for eradication of contaminants from environments.

## 3.9 Conclusion and Future Perspectives

In this chapter, the effectiveness of metagenomics approaches has been highlighted for understanding the microbial diversity and function of different environmental samples. The advancement of molecular and computational tools along with new next-generation sequencing data has simplified the identification of unculturable microorganisms, their genes, and functions having wide application in public health and bioremediation. Additionally, the characterization of microbial community facilitates a better understanding of the practices influencing the bacterial community structure. Despite new and advanced sequencing technologies, there are enough limitations of these approaches like methodology, sample preparation, DNA extraction, sequencing procedures and analysis error, error in taxonomical and functional annotation, biases, and reference databases gaps. Other limitations are the influences of different methodologies on qualitative and quantitative outcomes of molecular and metagenomics analysis and the unknown function of half of the genes in a metagenome. Further improvements in metagenomics analysis, technology, and bioinformatics tool will help microbial ecologists and physiologists in filling these knowledge gaps. Also, the major challenge of microbiologists nowadays is to find the less biased method facilitating contact to the rare biosphere, distinguishing active microorganisms and dormant cells, and increasing the capacity to relate microbes to their metabolic functions in a community. On the other hand, combining metagenomics and classical microbiology would be improved with information acquired by reference databanks that will lead to precise ecological interpretations. The increased anthropogenic activities and their impacts on the environment have resulted in thinking about novel and effective approaches for bioremediation and cleanup. The current omic techniques such as genomics, metagenomics, transcriptomics, proteomics, and metabolomics have helped in understanding the mechanism and pathways related to microbial bioremediation. These omics approaches have ability to anticipate the microbial metabolism at contaminated sites and find novel microbes and their biodegradation pathways for bioremediation. Therefore, by utilizing the benefits of these multi-omics approaches, new hypotheses, theories, and models of bioremediation of polluted environments can be presented.

With the decreasing sequencing costs frequently, the utility of metagenomics is increasing and the metagenomics technique has enormous potential to gain insight into the proper use of remediation approaches to achieve rapid remediation of pollutants with minimum requirement. This technique enables the systematic understanding and efforts to optimize the efficiency of the bioremediation process so that only the targeted microbial group can be enriched only. There is utmost requirement to completely understand the main taxa, mechanism, and pathways of bioremediation processes. Further, making the sequencing process easy will result in the generation of massive amounts of data for discovering the many uncultured phyla and gene families whose function is not known. There is the utmost requirement to combine the metagenomic technique with cultural-based techniques for apprehending the overall role of microbial diversity in the bioremediation process so that a complete picture of microbial bioremediation may acquire. Also, the systematic knowledge of community response along with biochemical pathways in response to different distresses is needed that is dependent on genetic and biochemical analysis of model organisms. The efforts towards the isolation of relevant microbial taxa from the site of interest will help in fully understanding the metagenomics data of these sites.

On the other hand, due to the dynamic nature of computational tools and softwares, there is a high demand for growing the need of knowing and matching these tools for selecting the right bioinformatics pipeline for analyzing the large sequences obtained from different microbial communities of complex environments. Therefore, microbiologists should have to consult experts from different areas, ecology, molecular biology, bioinformatics, statistics, microbiome, and microbial ecology, about the extraction and process of DNA from different conditions to create a proper pipeline for balanced scrutiny of microbial diversity and maintain decent practices in the study. The right information about designing a project from its sampling point to the bioinformatics pipeline is strongly necessary and suggested so that mistakes can be avoided in data interpretation.

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# Plant–Microbe Associations in Remediation of Contaminants for Environmental Sustainability

4

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**Abstract**

Pollutants are the substances that lead to undesired effects on the environment and pose a threat to all forms of life. The accumulation of these pollutants in the environment causes several diseases which affect both human and animal health. Several methods are implemented to degrade contaminants among which better results are obtained for the bioremediation technique. Plants and microbes are trappers of contaminants and they remove pollutants from the environment in an effective way. When both microorganisms and plants are combined, they showed an increase in their reduction activity compared to other remediation methods. Plant-associated microbes such as endophytes and rhizospheric microorganisms are utilised in the remediation of toxic compounds and are also used to enhance the treatment process. Thus, plant-associated microbes are considered as a promising approach in the remediation of contaminants. A broad knowledge about plant–microbe interactions and the challenges faced during remediation process is more important for the development of new technologies to remove various contaminants. This chapter highlights the need for plant microbes and how they play a vital role in the remediation of contaminants. More approaches should be implemented using plant microbes for the betterment of polluted environments.

**Keywords**

Plant–microbe interaction · Bioremediation · Contaminants · Signalling molecules

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## 4.1 Introduction

Environmental pollution is not a new occurrence, but still it continues to be the world's greatest problem. The most striking reason for environmental pollution is the eradication of the relationship between man and the environment due to the increasing rate of exploitation of natural resources, urban growth, and industrialisation. The source of environmental pollution is not only restricted to deforestation, landfills, dumping of waste into water bodies, population growth, mining but also triggered by the release of harmful substances such as toxic metals, sewage, industrial wastes, and gaseous pollutants into the environment (Kumar and Chandra 2020a; Kumar et al. 2020a, 2021a; Singh et al. 2021). Several physical and chemical techniques are available for reduction of pollutants, but they are expensive and lead to other environmental problems (Appannagari 2017). Thus, the most efficient, eco-friendly, and economical approaches should be considered for removing pollutants. Bioremediation is considered worldwide since it is eco-friendly and feasible (Kumar et al. 2018).

Phytoremediation and microbial bioremediation are the most common type and efficient technique of bioremediation (Chandra and Kumar 2018; Agrawal et al. 2021; Kumar et al. 2022). Phytoremediation is the technology that employs plants to eliminate contaminants from soil. Excessive use of pesticides and fertilisers contaminate soils by releasing heavy metals. This causes several human health problems. Plants can remediate these soils by recycling heavy metals. Phytoremediation inhibits the entry of contaminants into the environment, as they do not allow their entry into groundwater (Chandra et al. 2018). They have the potential to recycle a wide range of contaminants in the environment. The main disadvantage of phytoremediation is it is slower than other techniques and can affect the survival and growth of plants (Kumar 2021). Microbial techniques are also one of the cost-effective and eco-friendly methods of bioremediation. They employ various techniques such as bioreduction, biosorption, bioleaching, and biomineralisation for removal of pollutants. The main disadvantage of this type of bioremediation is it also is a slow process. Unlike phytoremediation, remediation by microbes cannot be monitored by the naked eye. In recent times, cost-effective technologies which employ the use of a combined system of plants and microbes for remediation of pollution has been researched (Rajkumar et al. 2006). The utilisation of combined systems of plants and microbes enhances the removal of contaminants from the environment. This review aims to provide a better understanding of plant–microbe interactions. Various methods using plants and microbes for remediation of polluted sites are discussed.

## 4.2 Plant–Microbe Interaction

Plant–microbe interaction is a complex and continuous process where the microbes have both positive and negative impacts on plants. Thus, understanding plant–microbe interactions would help in differentiating their positive and negative

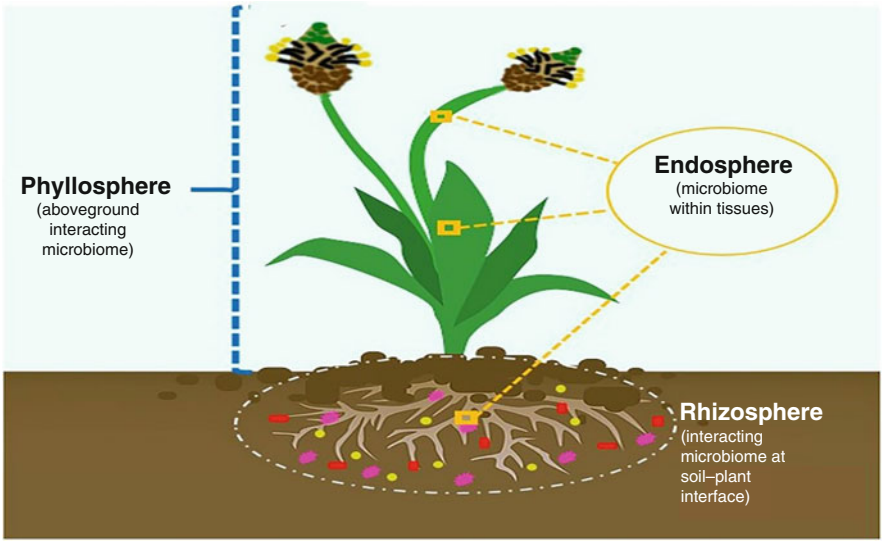


**Fig. 4.1** Three main regions involved in plant–microbe interactions

impacts (Kumar and Chandra 2018a, b; Kumar et al. 2021b, c, d). It is a widely accepted fact that certain plant–microbe interactions help in enhancing the growth of plants, protecting from harmful pathogens, and maintaining soil fertility. The plant-associated microbes leading to positive impacts are grouped into three categories. They are phyllospheric, endophytic, and rhizospheric microorganisms (Fig. 4.1) (Kaul et al. 2021). Phyllosphere is the region that covers the aboveground plant parts. Phyllospheric microbes have the ability to withstand the abiotic stress of UV radiation and high temperatures of 30–35 °C than other plant microbes. They protect crops through various plant growth–promoting (PGP) mechanisms. Some examples of phyllospheric microbiome are *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Agrobacterium*, and *Xanthomonas*. Endophytic microbes inhabit the internal tissue of plants such as root, stem, flowers, fruits, and seeds. They protect the plants from harmful pathogens and increase stress tolerance. Some examples of endophytes are *Enterobacter*, *Achromobacter*, *Streptomyces*, *Pseudomonas*, *Microbiospora*, and *Nocardioides*. Rhizospheric microbes inhabit the area around roots where the microbes around the soil are attracted towards the plants due to the release of root exudates. Some examples of rhizospheric microbes are *Methylobacterium*, *Pseudomonas*, *Rhizobium*, *Arthrobacter*, *Acinetobacter*, *Azospirillum*, and *Bacillus* (Yadav 2021).

### 4.2.1 Endophytic Microbiome

Endophytes are microorganisms that live within the tissue of the plant without causing any diseases. They produce secondary metabolites and protect plants against

various pathogenic microorganisms. Bacterial endophytes enter the plant through roots and are divided into three types: passenger endophytes, opportunistic endophytes, and competent endophytes. Passenger endophytes and opportunistic endophytes have the capability to spread to the root cortex, whereas competent endophytes spread throughout the vascular tissues. Opportunistic endophytes also promote root proliferation. Bacterial endophytes enter into the vascular tissue, where they spread in and colonise the vegetative area of the plants. Microbes that colonise the rhizosphere enhance plant growth and also increase plants' ability to adapt to extreme environmental conditions. A recent study on rice seed colonised by bacterial endophytes has shown an increase in plant growth. Some endophytes have showed antifungal activities against various harmful pathogens present in plants. It has been reported that bacterial endophytes isolated from the wheat cultivar seeds have shown biocontrol activities towards *Fusarium graminearum.* A study conducted on endophytic bacteria isolated from halophytes has shown that they help in reducing plant stress by regulating plant hormones. It is also reported that these bacteria enhance nitrogen fixation and also assist in the uptake of nutritional compounds. Similar to bacterial endophytes, fungal endophytes have also been found in vegetative parts of plants and are categorised into two types: clavicipitaceous and non-clavicipitaceous endophytes. The non-clavicipitaceous endophytes have three subclasses: Class 2 endophytes, Class 3 endophytes, and Class 4 endophytes. Class 2 endophytes spread to rhizomes, roots, and shoots; Class 3 endophytes grow in shoots; and Class 4 endophytes grow in roots of plants. Fungal endophytes also produce secondary metabolites and volatile organic compounds to help plants withstand biotic and abiotic stresses. Fungal endophytes suppress plant pathogen growth by producing secondary metabolites. It has been demonstrated that secondary metabolites released by *Fusarium verticillioides* help reduce the growth of the plant pathogen *Ustilago maydis.* But physiological and environmental conditions are to be considered for the release of secondary metabolites by endophytic fungi. Some endophytic fungi help in plant growth by increasing the efficiency of glycolysis and tricarboxylic acid (TCA) cycle. For example, the endophytic fungus AL12 helps in improving plant growth by increasing the rate of these metabolic pathways in the plant *Atractylodes lancea* (De Mandal et al. 2021).

### 4.2.2 Plant Growth–Promoting Rhizobacteria

Rhizobacteria are the bacterial group present in the rhizosphere which is the region present near the root system of plant. Plant growth–promoting rhizobacteria (PGPR) are bacterial groups which help in plant growth. The rhizobacteria not only stimulate plant growth but also act as an effective system for disease control by direct and indirect means. In direct method, PGPR stimulate plant growth by nitrogen fixation and enhance nutrient production. In indirect method, PGPR stimulate plant growth by exhibiting biocontrol activities towards harmful pathogens (Alotaibi et al. 2021). A study was conducted on PGPR isolated from maize plant to investigate the plant growth–promoting properties. Bacterial strains were isolated from *Zea mays* (maize)

and analysed for phytohormone production. Phytohormones are responsible for plant growth and development. Eighty bacterial strains were checked for production of indole-3-acetic acid (IAA), siderophore, and hydrogen cyanide (HCN) and phosphate solubilisation. The bacteria that show higher plant growth–promoting activities were selected for the study. The bacterial isolates showed higher production of IAA, a majorly abundant auxin phytohormone. Siderophore production was also high in the strains; siderophore increases the production of iron content in plants. Most of the phosphorus remains in insoluble form and solubilisation of phosphorus is essential for plant growth. The selected bacterial isolates showed higher phosphorus solubility. Using 16S rRNA gene sequencing, the bacterial isolates were identified as *Pseudomonas aeruginosa* AK20, AK31; *Pseudomonas fluorescens* AK18, AK45; and *Bacillus subtilis* AK38. Plant growth analysis was checked on *Oryza sativa* (rice plant). After 30 days, plants were harvested for analysing plant growth. All bacterial isolates showed increased growth in the roots and shoots of the plants. The study has provided evidence that PGPR help in plant growth and development, and further research is required for the process to be applied in field conditions (Karnwal 2017). Many studies reported that *Bacillus* sp. and *Pseudomonas* sp. present in tomato, chickpea, and wheat crops act against pathogenic microbes, showing their biocontrol ability towards the pathogens (De Mandal et al. 2021).

The interaction between plants and microbes occurs through various signalling molecules. The interaction starts by exchange of signals produced by the host or microbes, which in turn results in biochemical, physiological, and molecular responses. The signals are recognised by microbes and help form symbiotic relationships with plants through physical interactions using flagella, pili, and adhesion. Various mechanisms of plant–microbe interactions through signalling molecules are discussed here, and the current research focuses on a signal pathway that can recognise rhizospheric microbes. The interaction processes are controlled by several metabolites and exudates which lead to plant growth, protection against pathogens, availability of nutrients, and so on. Some of the plant–microbial interaction signals are illustrated in Fig. 4.2.

### 4.2.3 Plant-Released Signals

Root exudates are organic chemicals that are released through root system for inhibition of harmful microbes and promoting plant growth. Root exudates supply nutrients and other energy source for microbes. Thus, microbes trigger exudation of roots in plants. Root exudates help in phytoremediation process by stimulating the plant to adapt to any metal stress. Some of the studies suggested that in the presence of root exudates, bacterial strains are metabolically active and utilise the compounds present in the exudates. The interaction between plants and arbuscular mycorrhizal fungi (AMF) involves hyphae which promote root colonisation. Certain signals are assumed to be involved before colonisation starts. The AMF interaction is not species specific, so certain plant-released signals and compounds are involved to

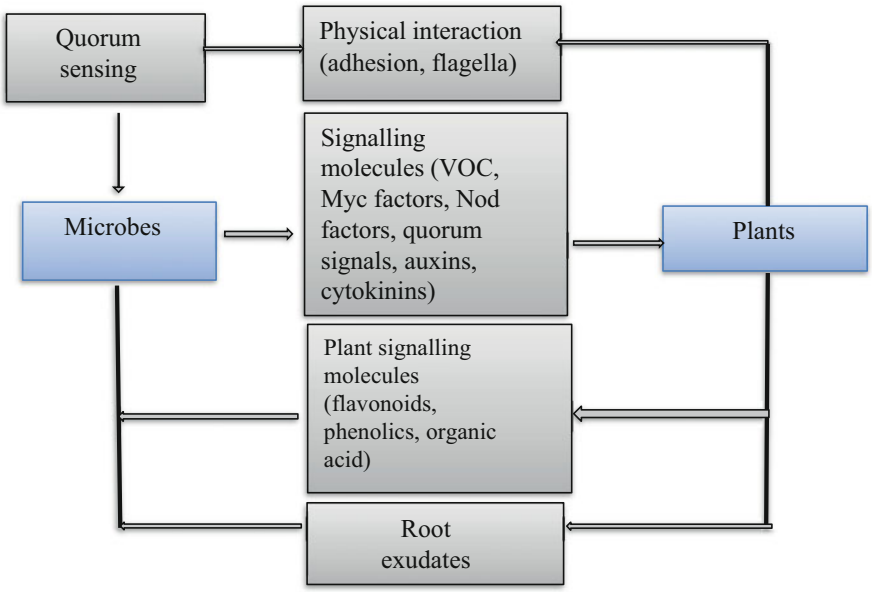


**Fig. 4.2** Various signals involved in plant–microbe interactions

make it species specific. Some of the plant-released compounds are amino acids, sugars, and phenolic compounds which are potential signalling molecules in plant–microbe interaction. Flavonoids present in root exudates act as signalling molecules for plant–microbe interaction. They promote AMF spore germination and root colonisation in AMF. During root colonisation, flavonoids are released by the AMF fungus which promote hyphal growth. Flavonoids are species specific and during plant–AMF interaction their levels change. Flavonoid levels are moderate during root penetration and higher at later stages. After root colonisation the flavonoid levels get changed which play an important role in plant–AMF interaction (Ma et al. 2016). Flavonoids can also show negative impacts on other fungi as they are specific in nature, so their interaction with AMF is still unclear. Flavonoids also act as chemoattractants and help in rhizobia growth. They activate nod genes, that synthesise nod factors which stimulate nodule formulation in the host. Chemotaxis provides the organism with the ability to sense and respond to various signals induced by plants. Due to their specificity rhizobia can easily recognise and attach to the correct host plants. Ponce et al. conducted an experiment to determine the role of flavonoids in the plant–microbe relationship. The flavonoids were isolated from the plant *Trifolium repens* and molecular characterisation was carried out. The plants were allowed to grow in both under the presence and absence of AMF, *Glomus intraradices*. The flavonoid content in roots and shoots in the absence of AMF was analysed. But the role of flavonoids involved in the plant–microbe interaction is unknown. Only in the presence of AMF, the flavonoid are known to exist. Quercetin, a flavonoid, promotes AMF spore germination. Acacetin and rhamnetin showed

ability for inhibiting the eukaryotic topoisomerase. Ponkanetin promotes cytotoxicity and mutagenicity. The study suggested a clear interaction of AMF and flavonoid metabolism in plants. Similarly, plants secrete certain chemicals like phenolic compounds and organic compounds which help in the plant–microbe association. Plants produce phenolic compounds during their growth and development. Phenolic compounds are also synthesised in response to pathogenic infections and environmental stress factors. During a pathogenic infection, plants respond to infection through a series of defence mechanism which leads to the production of antimicrobial compounds. This mechanism is regulated by signalling pathways in which phenolic compounds play an important role. The phenolic compounds undergo changes in soil so that microbes can utilise it as a carbon source. During root and shoot development in *Arachis hypogaea*, the plant releases phenolic compounds, which the rhizobacteria present in the soil use as a carbon source and undergo changes. The responses also facilitate the formation of root nodules of plants. Plants associate with microbes by stimulating catabolic genes in microorganisms which are responsible for root nodulation. Plants and microbes share a mechanism through signals which makes bacteria to enter the root and shoot of plants. The root colonisation by bacteria is said to occur in a series of steps. The first step involves the movement of bacteria towards the root by flagellar activity, which is facilitated by the plant-released compounds. Attachment of the bacteria to the root occurs, after which the interaction between plant and bacteria takes place. In this step, root exudates are released and the bacteria enter the site of damage and promote plant growth. The exudates produced by the plant in response to tissue damage are utilised by microbes as a food source for colonisation. The association between plants and microbes is very specific and most of the studies suggested that the legume plant interacts well with rhizospheric bacteria (Ponce et al. 2004).

### 4.2.4 Microbial Signals

Microbes such as rhizobia and fungi have the ability to respond to plants through various signalling molecules. These signalling molecules can be volatile organic compounds (VOC), nod factors, myc factors, exopolysaccharides, and so on. Both plants and microbes secrete volatile organic compounds and communicate through it. Plants defend themselves from harmful organisms by releasing VOC. Similarly, microbes also emit VOC for communication and attacking. Microbial VOC modulate the activation of plant defence response and stimulate plant growth. Volatile organic compounds present in bacteria help in promoting plant growth and defence by nutrient production. It was first reported in *Arabidopsis thaliana*. After this discovery, a new line of research emerged in the plant–microbe association. Signalling molecules that are produced by myc factors and nod factors are utilised in the formation of new organs and root nodules (Ma et al. 2016). Certain flavonoids can also promote chemotaxis and bacterial growth. Some studies stated that chemotaxis plays an important role in plant root colonisation. De Weert et al. (2002) investigated root colonisation of tomato in which they used selected *P. fluorescens* strains and its

chemotactic mutants to investigate the chemotaxis towards root exudates of tomato. Movement is an important criterion for root colonisation. The movement towards root exudates is checked whether it is facilitated through chemotaxis. Chemotaxis of *P. fluorescens* occurs towards the root exudates of tomato which helps in root colonisation (de Weert et al. 2002). Rhamnolipids present in *P. aeruginosa* inhibit pathogenic fungi and give protection to grapevine. Varnier et al. (2009) conducted an experiment on rhamnolipids, biosurfactant molecules that protect grapevine against *Botrytis cinerea*, a necrotrophic fungus, and are also said to have a direct effect on spore germination and fungal growth. Rhamnolipids were isolated from *P. aeruginosa* and checked for their ability to activate the defence mechanism in grapevine. Different concentrations of rhamnolipids were used, and at higher concentrations, cell death resulted. The rhamnolipids were further tested for the defence mechanism along with chitosan and *B. cinerea*. Chitosan induces $H_2O_2$ production, which is enhanced by rhamnolipids. Oxidative burst was induced in the cells of grapevine by rhamnolipids. Rhamnolipids were tested for fungal activity against *B. cinerea* which results in inhibition of spore germination and growth of mycelium which also protects grapevine from the fungus (Varnier et al. 2009). Furthermore, various studies carried out on different plant species like *Solanum lycopersicum*, *Z. mays*, and *Lactuca sativa* showed response to VOC action. Another study reported that VOC present in *Bacillus subtilis* promoted increased growth in *Cucumis sativus*. VOC of fungal species also promote growth on different plant species. Various studies showed that Bacillus species have a high impact on lateral root development. Microbial VOC can also improve plant stress tolerance. For example, *Pseudomonas chlororaphis* increased the *A. thaliana* tolerance to drought by emitting 2R, 3R-butanediol (Ma et al. 2016). Recent research on microbial VOCs illustrates stimulation of plant growth and bacterial–plant interaction by VOC. However, the mechanism of bacterial VOC is still not clarified. Their signal perception, signal cascades, and cellular responses are still unknown. Thus, further studies are required for employing VOCs in various applications.

### 4.2.5 Quorum Sensing

Quorum sensing is a process which involves signalling for communication between bacterial cells. This bacterial cell–cell communication helps in monitoring the bacterial population density. Both gram-positive and gram-negative bacteria use different signalling molecules. Quorum sensing is a gene regulatory mechanism which involves N-acyl-L-homoserine lactones (AHL), a quorum sensing signal that is present in many gram-negative pathogenic bacteria. Plants can recognise bacterial AHLs, which in turn helps in promoting plant growth and defence mechanism (Ma et al. 2016). AHL signal is an auto-inducer signalling produced by *luxI* type gene. The signalling perception helps the bacterial cell to easily adapt to gene expression on environmental changes. The most recent study suggested that AHLs play an important role in abiotic stress of plant and help plants withstand the abiotic stress. Osmolytes and phytohormones play an important role in salt and drought

tolerance. The auxin indole-3-acetic acid is a key component in water stress tolerance. The bacteria associated with the root of plants secrete phytohormones which are utilised by the plants having low levels of phytohormones. This is successfully used to improve plant growth in salt-affected soils by combining bacterial phytohormones and seaweeds possessing a high level of osmolytes. IAA helps in increasing lateral root surface area, thus improving the facility to absorb more water and minerals. Auxin plays a key role in plant–microbe interaction by promoting plant growth development. IAA, a major naturally occurring auxin, acts as a signalling molecule in the plant–microbe interaction. Over 80% of rhizobacteria are reported to produce IAA. Tryptophan act as a precursor in the production of IAA, which indicates that higher concentration of tryptophan results in a higher production of IAA. Bacterial IAA sets the plants to adapt to metal stress by stimulating changes in cell metabolism (Hartmann et al. 2021). Phosphate-solubilising bacteria potentially reduce metal toxicity and also helps in plant growth. *P. fluorescens* is investigated for plant growth–promoting property and biocontrol activity against *Fusarium oxysporum.* Bacterial strains were isolated from the plant *Vigna mungo*. The metal resistance of the strains was checked against different metals and results revealed their high resistance to copper. The IAA production of bacteria was analysed by using high performance liquid chromatography (HPLC). The biocontrol activity of the strain was demonstrated and the strain showed inhibition against diverse groups of bacteria. Many soil bacteria stimulate plant growth by phosphate solubilisation, IAA production and exhibit certain antimicrobial activity. For example, IAA produced by both rhizobacteria and plants, transmitted as a signal, stimulates the production of antibiotics in *Streptomyces*. The signal perception also inhibits other competitive microbes (Upadhyay and Srivastava 2010). Transcriptional changes in legumes were observed when AHLs communicate with the roots of *Arabidopsis thaliana*. When treated with AHLs, there were changes observed in genes that are responsible for plant growth and genes that regulate growth hormones. The study illustrated that the interaction of N-hexanoyl-DL-homoserine-lactone (C6-HSL) and *A. thaliana* resulted in transcriptional changes in roots and shoots, and also induced plant growth (von Rad et al. 2008). Plant roots utilise water-soluble AHLs through an energy-dependent mechanism and this process occur in plants which have AHL-degrading enzymes such as *A. thaliana*, wheat, or barley. Thus, rhizobacteria provide support to the plant by promoting plant growth and development even under stress conditions. AHLs have provided a promising approach by increasing stress tolerance of plants against various environmental factors (Hartmann et al. 2021). Furanones are AHL mimic compounds which can antagonise AHL type behaviour. They can selectively bind to bacterial AHL receptors just like AHLs and affect the bacterial signalling. Root exudates which are responsible for root colonisation are also involved in quorum sensing. Studies found that some Bacillus species that have the capability to degrade AHLs exhibit biocontrol activity against plant diseases. AHL-degrading bacteria reduced the pathogenicity of plant pathogens. Bacteria were isolated from soils where tobacco grows and 54 bacterial strains were allowed to grow in enrichment media. Among them 25 bacterial isolates showed degradation of AHLs. The bacterial isolates W2

and W3 completely degraded the N-caproyl-L-homoserine-lactone (C6-HSL). W2 isolate showed a degradation efficiency of about 95%. The bacterial isolates belong to *Pseudomonas* sp., *Variovorax* sp., *Variovorax paradoxus*, *Comamonas* sp., *Comamonas testosteroni*, and *Rhodococcus erythropolis*. These bacterial groups were analysed for N-acyl homoserine lactone (N-AHSL)-degrading efficiency. *R. erythropolis* showed higher degradation properties and it is further allowed to interact with quorum signals from other bacteria. Bacteria such as *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, and *Pectobacterium carotovorum* subsp. *carotovorum* were allowed to interfere with *R. erythropolis*. The interaction did not affect the growth of the bacteria. *R. erythropolis* interferes with the violacein production which is responsible for the virulence of bacteria. Violacein is produced by *Chromobacterium violaceum.* A decrease in violacein production was observed. The interaction between *Agrobacterium tumefaciens* and *R. erythropolis* was checked, and it showed reduction in Ti plasmid conjugation. The pathogenicity of *P. carotovorum* subsp. *carotovorum* was analysed in potato plant using *R. erythropolis.* The inoculation of both strains prevented breakdown of tissues of the plant. The study demonstrated that the targeting of quorum sensing (QS) signalling molecules interacts with the pathogenicity of microbes (Uroz et al. 2003). Thus, AHL signalling molecules serve as a promising approach for stimulation of plant growth and abiotic stress tolerance. Studies suggested that plants and microbes associate through various signalling molecules. Further research is essential to select highly specific plant and microbial signalling molecules to uncover novel strategies.

## 4.3 Remediation of Contaminants by Plant–Microbe Combination

### 4.3.1 Removal of Pollutants from Aquatic Environments

Increase of pollutants in environment causes imbalance of ecosystem and serious health problems (Chandra and Kumar 2017a, b). Using plant–microbe interaction, several studies were done on treating pollutants. Here, some studies on cleaning up of pollutants from aquatic environments are discussed. Kristanti et al. investigated the effectiveness of four nitrophenol (NP)-degrading bacteria, namely *Pseudomonas* sp. (strain ONR1), *Cupriavidus* sp. (MFR2), *Rhodococcus* sp. (PKR1), and *Rhodococcus* sp. (DNR2) and the plant *Spirodela polyrhiza* in degrading the nitrophenol from aquatic system. Nitrophenols such as 1-NP, 2-NP, 3-NP, and 4-NP were used for degradation. The experiment was carried out with combinations of strains such as ONR1 with *Spirodela*; DNR2 with *Spirodela*; ONR1–DNR2 with *Spirodela*; and four strains, ONR1, DNR2, MFR2, PKR1, with *Spirodela*; and combinations without *Spirodela*. The degradation efficiency is higher in the presence of *Spirodela* compared to the test without *Spirodela*. Also, ONR1–DNR2 association with *Spirodela* showed greater degradation rate than that of ONR1 and DNR2 individually with *Spirodela*. Then the four strains with *Spirodela* were tested for

degrading the nitrophenol mixtures, which also showed promising results. ONR1 reduced the concentration of nitrophenol to about 92% (2, 4-dinitrophenol), 94% (2-NP), 3% (3-NP), 70% (4-NP), and the degradation efficiency of DNR2 is about 87% (2-NP), 32% (3-NP), and 100% (4-NP, 2,4-DNP). The inoculation of both strains degraded about 100% (2-NP, 4-NP, 2,4-DNP) and 30% (3-NP) over 4 days. In the absence of the bacterial strains, plants can degrade 17% (2-NP), 24% (3-NP), 3% (4-NP), and 9% (2,4-DNP) of pollutants. *Spirodela* without NP-degrading bacteria clean up a minimal quantity of nitrophenol which is stable on repeating cycles. But the degradation in the case of the bacteria along with *Spirodela* is not the same in repeated cycles. NPs often present in mixture, which causes a serious problem in treating them. Thus, studies on treating mixtures of NP are essential and would offer an attractive tool for degradation of NPs. A study analysed the efficiency of plant–microbe combinations in degrading pollutants. But selection of appropriate microbes and plants is essential for efficient degradation (Kristanti et al. 2014). Many industries release organic and inorganic compounds such as phenol and chromium, which cause pollution to the environment. Ontañon et al. conducted an experiment on rhizoremediation of phenol and chromium. Sediment samples were collected from a chemical and petrochemical industry and phenol-, chromium-resistant bacteria were isolated. On the basis of tolerance to the contaminant, a bacterial strain, *Pantoea sp*. FC 1, was selected and confirmed using 16S rRNA gene sequence amplification. The bacterial tolerance against phenol and chromium was investigated individually. FC 1 showed a higher degradation activity for chromium compared to phenol, since phenol affects bacterial growth at higher concentrations. As many authors suggested that hairy roots from different plant species have the capability to degrade phenolic compounds, the study employed association of FC 1 with hairy roots of *Brassica napus* in remediating the contaminants. For degradation process, removal of phenol exhibited with hairy roots alone and with a combination of hairy roots and FC 1. Hairy roots removed 60% of phenol and the efficiency increased on inoculation of FC 1. For chromium, the combined system removed 100% after 3–4 days. This study suggested that association of FC 1 and hairy roots of *Brassica napus* serves as an innovative system in degrading phenol and chromium pollutants (Ontañon et al. 2014). Hu and Li (2021) conducted a study on treating sewage using aquatic plants and microbes. The plants selected for the process were *Eichhornia crassipes*, *Cyperus alternifolius*, *Phragmites communis*, and *Scirpus validus Vahl* and grown in plastic buckets containing sewage. The phytoremediation test is conducted first for a duration of 15 days. The nitrogen and phosphorus contents were checked every 2 days. The nitrogen degradation effects in sewage for the four plants were different. Similarly, the phosphorus content also decreased in sewage treated with different plants. Microbial and plant combination tests were done with the same four plants for treating sewage. Similar to phytoremediation test, the nitrogen and phosphorus contents were analysed for 2 weeks. The nitrogen degradation effect in every test was different. The efficiency of degradation by microbes, plants, and plant–microbe systems were 64.85%, 40.61%, and 77.67%, respectively. Similarly, the phosphorus content degradation efficiency by microbes, plants, and plant–microbe combinations

was 61.56%, 39.23%, and 71.97%, respectively. When comparing all degradation tests, it is quite clear that plant–microbe systems showed better removal compared to plants and microbes alone. Microbial-assisted remediation showed higher efficiency compared to phytoremediation. From the above discussed studies, it is evident that a plant–microbe combined system can enhance the removal of contaminants in an aquatic system.

### 4.3.2 Removal of Pollutants from Terrestrial Environment

There are numerous studies that showed positive results in removal of contaminants from soil and/or sludge (Chandra and Kumar 2017b; Kumar et al. 2020b, 2021e; Kumar and Chandra 2020b). Microbes growing in metal-contaminated soils tend to have a high tolerance towards concentration of metals. Soil and plants are also benefited from these microbes. Among these microbes PGPR show high benefits by altering metal bioavailability. They do this by altering pH, producing phytohormones and thus improving the phytoremediation process. He et al. demonstrated a study in which 11 plant species showing metal tolerance and rhizosphere soil were collected from a copper mine wasteland. Thirteen copper-resistant bacterial strains were isolated and selected based on their different morphological appearance. The characterisation of bacterial strains was analysed by evaluating their production of IAA, acetyl-CoA carboxylase (ACC), and siderophore. The bacterial isolates were tested against different metals. The bacterial strains showed different degrees of resistance towards metals. The bacterial strains *Sphingomonas sp.* YJ3 and *Microbacterium lactium* YJ7 showed resistance towards Cu. The strain *Acinetobacter sp*. SWJ11, *Arthrobacter sp.* MT16, *Azotobacter vinelandii* GZC24, and *Arthrobacter* sp. YAH27 showed resistant towards Ni, Zn, Pb, and Cu when inoculated in *Brassica napus*. The bacterial strains also enhanced root elongation in the plant species (He et al. 2010). *Arabidopsis thaliana* and the bacterium *Sphingobium* are used to remove isoproturon from contaminated zones. In this study, phytoremediation and bioaugmentation processes were combined. The resulting intermediate of isoproturon is treated with *Sphingobium*, which can mineralise it. This resulted in enhanced and complete removal of pollutants. The increase in remediation is due to a synergistic relationship between the transgenic plant and *Sphingobium* (Yan et al. 2018). A study was conducted on *Cytisus striatus* to degrade hexachlorocyclohexane (HCH) with a combination of two endophyte microbial strains *R. erythropolis* ET54b and *Sphingomonas* sp. D4. The study was carried out on two different soils differing in their organic content. The results showed a higher efficiency in remediation of HCH compared to the treatment which does not involve the endophytes, and also enhanced plant growth. The efficiency of degradation in soil depends on the content of organic matter (Becerra-Castro et al. 2013). A 4-month study was conducted on three plant species, namely *Scorzonera mongolica Maxim*, *Atriplex centralasiatica*, and *Limonium bicolor*, in relation to their degradation of crude oil present in the contaminated soil at an oil refinery land farm. The rhizosphere soil adhered to the plant species

were analysed and the microbes present were investigated for metabolic activity. The utilisation of carbon sources by microbes was analysed. The most probable number (MPN) method was used for the identification of the number of microbes that have the capability of degrading petroleum hydrocarbons. The degradation efficiency of the three plant species showed no significant difference. The plant biomass was investigated and the results showed enhanced root system in *A. centralasiatica* compared to other plants. Microbial activity was higher for *A. centralasiatica.* The pH value of the soil of the three species decreased due to production of root exudates and microbial metabolites. The decrease in pH value is due to the increased utilisation of phosphorus, which promotes enhanced plant growth and breakdown of petroleum hydrocarbons. The results showed a decrease in the concentration of petroleum hydrocarbons using the plant–microbe remediation system. Both plants and microbes benefit each other and also enhanced the process of remediation (Ying et al. 2011).

### 4.3.3 Removal of Pollutants from Atmosphere

The major health risk for humans is caused by air pollution which involves ammonia, nitrogen oxides, particulate matter, sulphur dioxide, VOC, and so on. An efficient remediation method is essential to eradicate these pollutants. Selection of appropriate plant and microbial species for remediation is crucial in removing pollutants (Molina et al. 2021). Here, some of the studies on plant–microbe combination to clean up the pollutants are discussed. A study on endophytes confirmed that they have the ability to promote plant growth and also degrade polycyclic aromatic hydrocarbons (PAH). In this study, three willow endophytes, namely WW1, WW3, and WW11, and three poplar endophytes, namely SX61, PD1, and PTD3, were allowed to grow in a medium containing PAH and they showed high growth in the presence of naphthalene. Of all the endophytes, the strain PD1 showed highest growth with the inoculation of PAH. As a result, PD1 was chosen for further studies. PD1 was used to degrade phenanthrene and the results showed that about 60% of phenanthrene was degraded from the medium. Using 16S rDNA sequencing the endophytic bacteria were identified as *Pseudomonas putida*. This study illustrated that the endophyte-assisted phytoremediation showed higher efficiency in degrading PAH (Khan et al. 2014). Yutthammo et al. investigated the activities of the bacteria which have the capability to degrade PAH. The study focussed on the bacteria present in the phyllosphere of ornamental plants. The physical and chemical characteristics of leaves of the ten ornamental plants such as *Ixora* sp., *Murraya paniculata*, *Wrightia religiosa*, *Bougainvillea* sp., *Jasminum sambac*, *Codiaeum variegatum*, *Ficus* sp., *Streblus asper*, *Pseuderanthemum graciliflorum*, and *Hibiscus rosa-sinensis* were investigated. The plant species were compared for the number of phenanthrene-degrading bacteria. A large number of phenanthrene-degrading bacteria were found in *W. religiosa* and *H. rosa-sinensis*, which were further chosen for analysis to check the activities of the bacteria. The results showed higher reduction of phenanthrene level in *W. religiosa* leaves compared to *H. rosa-*

*sinensis*. The bacteria along with *W. religiosa* was then experimented with different compounds of PAH such as acenaphthylene, acenaphthene, and fluorine. The unsterilised leaves showed higher efficiency in removal of PAH than sterilised leaves. The study suggested that the PAH-degrading bacteria are common in phyllosphere and also have the capability to increase the efficiency of the plant leaves, thus playing an important role in removal of urban air pollutants. Each plant in its phyllosphere has a unique bacterial group due to the differences in leaf morphology and chemical compounds present in the leaf. The study demonstrated that the leaves of ornamental plant contain various phenanthrene-degrading bacteria (Yutthammo et al. 2010). An investigation was made on chloromethane-degrading bacteria isolated from *Arabidopsis thaliana.* The study involved isolation of bacterial strains *Methylorubrum extorquens* CM4, *Hyphomicrobium chloromethanicum* CM2 from Russian petrochemical factory soil, and *Hyphomicrobium* sp. *strain* MC1 from industrial sewage. These three strains originated from the leaves of *Arabidopsis thaliana* and a gene, *cmuA*, responsible for dehalogenation of chloromethane was identified. The ability of the three strains to use chloromethane as a carbon source was compared with that of the reference strain. *Hyphomicrobium* sp. showed higher efficiency in degrading chloromethane compared to other strains, since it is well adapted to grow in the presence of chloromethane (Nadalig et al. 2011). The above studies prove that plant–microbe interaction-associated remediation not only degrades the contaminants but also benefits both plants and microbes by producing phytohormones. Even though the method is efficient, the mechanism of remediation by plants and microbes is still not clear. The above-discussed studies on degradation of pollutants by various plant–microbe interactions are illustrated in Table 4.1.

## 4.4 Examples of Bacterial-Assisted Phytoremediation

Phytoremediation is the process wherein plants are used to clean up pollutants, and this process involves various mechanisms such as phytodegradation, phytoextraction, phytostabilisation, and phytostabilisation, as shown in Fig. 4.3. Phytoremediation removes both organic and inorganic pollutants, but there are certain limitations that make the process less efficient. Plants suffer from stress caused by contaminants, which leads to reduction in plant growth, development, and seed germination. These limitations are said to be reduced by rhizobacteria. The microbes in the polluted site easily get adapted and use the polluted substance as a nutrient source. The mechanisms of phytoremediation, when assisted with bacteria, showed higher efficiency in degrading pollutants. Bacteria play an important role in the phytoremediation process by producing siderophores, IAA, ACC, and enhancing the bioavailability of heavy metals by mechanism of precipitation, redox reaction, chelation, and acidification (Gaur et al. 2021). Thus, intensive research on the phytoremediation mechanism helps in understanding the metabolic breakdown of pollutants by plants and microbes. The following are some of the research works carried out on the microbial-assisted phytoremediation process (Table 4.2).

**Table 4.1** Enhanced remediation of pollutants by plant–microbe interactions

| Pollutants | Microbes | Plants | Process | References |
|---|---|---|---|---|
| Nitrophenol | *Pseudomonas* sp., *Cupriavidus* sp., *Rhodococcus* sp., *Rhodococcus* sp. | *Spirodela polyrhiza* | Degradation of nitrophenol in five consecutive cycles | Kristanti et al. (2014) |
| Phenol, chromium | *Pantoea* sp. | *Brassica napus* | Hairy roots of *Brassica napus* and Pantoea sp. degrade 100% of pollutants | Ontañon et al. (2014) |
| Nickel (Ni)<br>Lead (Pb)<br>Zinc (Zn)<br>Copper (Cu) | *Sphingomonas* sp. YJ3, *Microbacterium lactium* YJ7, *Acinetobacter* sp. SWJ11, *Arthrobacter* sp. MT16, *Azotobacter vinelandii* GZC24, *Arthrobacter* sp. YAH27 | *Brassica napus* | Increased plant growth and root elongation; increased resistance towards heavy metals | He et al. (2010) |
| Isoproturon | *Sphingobium* bacterium | *Arabidopsis thaliana* | Combined process of phytoremediation and bioaugmentation; mineralisation of isoproturon | Yan et al. (2018) |
| Hexachlorocyclohexane (HCH) | *Rhodococcus erythropolis* ET54b *Sphingomonas* sp. D4 | *Cytisus striatus* | Increased plant growth and degradation of pollutants based on organic matter content | Becerra-Castro et al. (2013) |
| Crude oil | Rhizospheric microbes | *Scorzonera mongolica maxim, Atriplex centralasiatica*, and *Limonium bicolor* | TPH-degrading bacteria | Ying et al. (2011) |
| Phenanthrene | Poplar endophytes PD1 | *Pseudomonas putida* | 60% degradation of phenanthrene and increased bacterial growth | Khan et al. (2014) |
| Polycyclic aromatic hydrocarbons | PAH-degrading bacteria | *Wrightia religiosa, Hibiscus rosa-sinensis* | Phenanthrene concentration decreases in leaves. | Yutthammo et al. (2010) |
| Chloromethane | *Methylorubrum extorquens*, *Hyphomicrobium chloromethanicum*, *Hyphomicrobium* sp. *strain* MC1 | *Arabidopsis thaliana* | Higher expression level of gene *cmuA* which degrades chloromethane | Nadalig et al. (2011) |

HCH Hexachlorocyclohexane TPH Total petroleum hydrocarbon; PAH Polyaromatic hydrocarbons

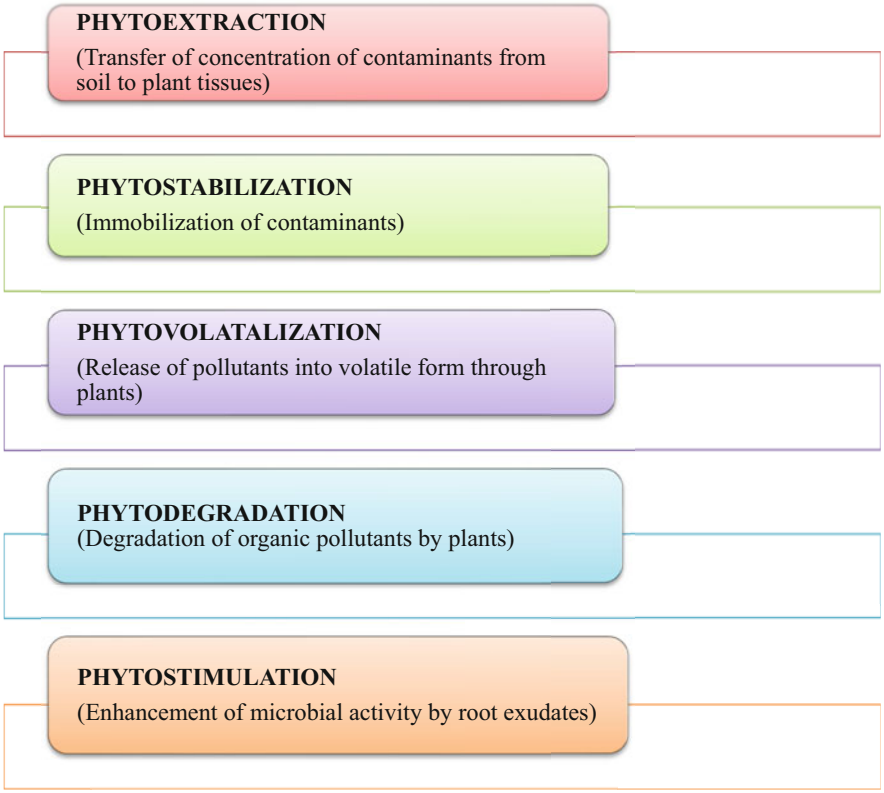


**Fig. 4.3** Various phytoremediation strategies

## 4.5 Microbial-Assisted Phytoextraction

Phytoextraction is the process which involves the use of hyperaccumulating plants to remediate pollutants from contaminated environments. Hyperaccumulating plants are the plants that have the capability to remove metals from the soil. Phytoremediation is best known for its remediation of heavy metals. Various mechanism of phytoremediation involved in metal accumulation is illustrated in Fig. 4.4 (Gaur et al. 2021). Metal-accumulating capability and biomass production are the important factors in phytoextraction process. The optimisation of these factors enhances the phytoremediation strategy. The limitation of this process involves slow growth and their low tolerance to metal stress. Some of the studies illustrated that microbial-assisted phytoextraction has higher efficiency in removal of pollutants. Khonsue et al. investigated *Vetiveria nemoralis* and *Ocimum gratissimum* with cadmium-resistant bacteria. Two cadmium-resistant bacterial strains *Ralstonia* sp. TAK1 and *Arthrobacter* sp. TM6 were isolated from the

**Table 4.2** Microbial-assisted phytoremediation of heavy metals and organic pollutants

| Pollutants | Plants | Microbes | Process | References |
|---|---|---|---|---|
| Cadmium | *Vetiveria nemoralis*, *Ocimum gratissimum* | *Ralstonia sp.* TAK1, *Ocimum gratissimum* | Increased cadmium solubility, increased production of phytohormones (enhanced phytoextraction) | Khonsue et al. (2013) |
| Arsenic | *Betula celtiberica* | *Ensifer adhaerens* 91R, *Rhizobium herbae* 32E, *Variovorax paradoxus* 28EY, *Phyllobacterium myrsinacearum* 28EW | Increased production of indole-3-acetic acid (IAA), aminocyclopropane-1-carboxylic acid (ACC) deaminase, and siderophore, arsenic accumulation (enhanced phytoextraction) | Mesa et al. (2017) |
| 9.Chromium | *Brassica juncea* | *Pseudomonas* sp. PsA4, Bacillus sp. Ba32 | Increased plant growth and inhibitory effects against chromium (enhanced phytostabilisation) | Rajkumar et al. (2006) |
| Pesticides | *Cucurbita pepo* L., *Xanthium strumarium* | *Bacillus vallismortis*, *Bacillus aryabhattai* | Decrease in pollution stress and increased biomass production (enhanced phytostabilisation) | Nurzhanova et al. (2021) |
| Cd, Pb, Zn, Cu | *Hibiscus cannabinus* | *Enterobacter* sp. strain EG16 | Increased phytohormone production, reduction in the concentration of metals and immobilisation of metals (enhanced phytostabilisation) | Chen et al. (2017) |
| Petroleum hydrocarbons | *Lolium multiflorum* | *Pseudomonas* sp. strain ITRI53 *Pantoea* sp. strain BTRH79 | Increased alkane-degrading gene expression level, promotes plant growth (rhizoremediation) | Afzal et al. (2011) |
| Polychlorinated biphenyl (PCB) | *Morus alba* | *Rhodococcus* sp. | Increase the solubility of pollutants, reduction of inhibitory effects of plant growth (rhizoremediation) | Sandhu et al. (2020) |
| Diesel | *Scirpus grossus* | Rhizobacteria | Increase in the reduction of petroleum hydrocarbons (enhanced phytodegradation) | Al-Baldawi et al. (2015) |

(continued)

**Table 4.2** (continued)

| Pollutants | Plants | Microbes | Process | References |
|---|---|---|---|---|
| Petroleum hydrocarbon | *Lotus corniculatus*, *Oenothera biennis* | Endophytic bacteria | Increased phytohormone production and degradation of hydrocarbons | Pawlik et al. (2017) |
| 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (dye) | *Gaillardia pulchella* | *Pseudomonas monteilii* | Decolourisation of dye is enhanced (enhanced phytodegradation) | Kabra et al. (2013) |
| Lead | *Lolium perenne* | *Trichoderma asperellum* | Increased plant growth and dry weight, increased metal extraction from soil (enhanced phytostimulation) | Sun et al. (2020) |
| Arsenic | *Pteris vittata* | *Agrobacterium radiobacter* | Increased phytohormone production and increased metal uptake (enhanced phytovolatilisation) | Wang et al. (2011) |

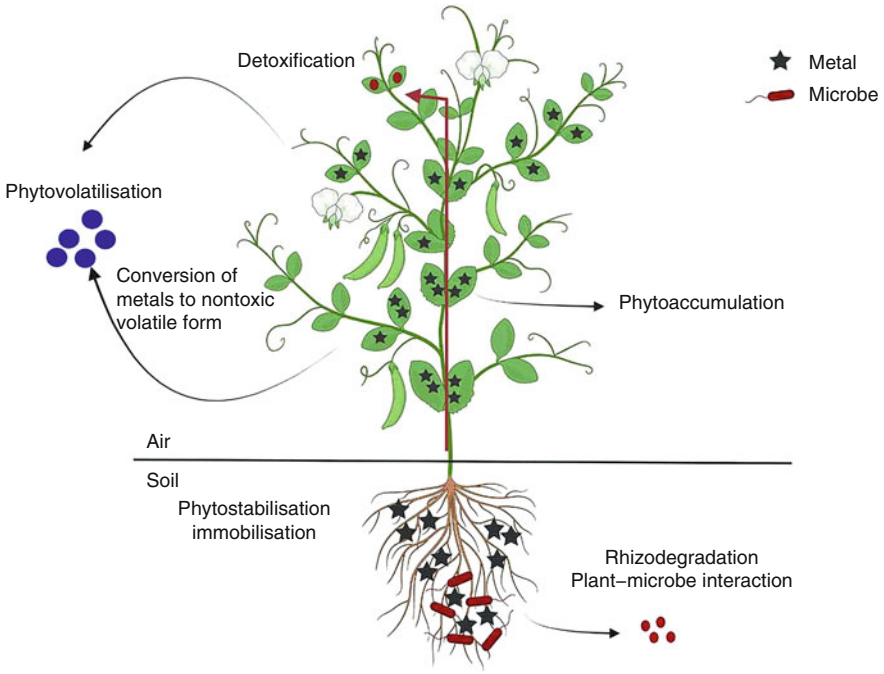


**Fig. 4.4** Metal accumulation in plants and their remediation and detoxification mechanisms

cadmium-contaminated site. Two plants, *V. nemoralis* and *O. gratissimum*, were chosen and transplanted into the cadmium-contaminated soil. The experiment comprised four various treatments of plants: (1) without bacteria, (2) *Ralstonia sp*. TAK1, (3) *Arthrobacter sp.* TM6, and (4) EDTA. The plants were allowed to grow for 1 month. Plant growth and cadmium concentration were analysed. Plants inoculated with bacterial strains exhibited increased cadmium solubility of soil. Increase in solubility increases the mobilisation of heavy metals. An efficient phytoextraction method requires high solubility of heavy metals when it is in contact with plants. The bacterial strains were found to survive in the cadmium-contaminated soil. Cadmium inhibited the growth of root and shoot systems. The bacterial strains increased the production of phytohormones, which stimulated plant growth and development. It is observed that the accumulation of cadmium increases in the plants with bacterial strain inoculation. But cadmium accumulation is higher in root than in shoot, as mostly metal accumulates more in the root system. Cadmium concentration was analysed after 1 month of plant growth. The plant *V. nemoralis* treated without bacterial strains, with *Ralstonia* sp. TAK1, with *Arthrobacter* sp. TM6, and with EDTA showed decrease in cadmium concentration of 24.7%, 28.8%, 32.7%, and 32.2%, respectively. Similarly, in *O. gratissimum*, the decrease in cadmium concentration is 25.5% (Without inoculation), 23.2% (TAK1), 28.7% (TM6), and 32% (EDTA). The study reported that both bacterial strains stimulated the phytoextraction of cadmium. But *O. gratissimum* showed higher efficiency of

phytoextraction with *Arthrobacter sp*. Compared to *O. gratissimum*, the efficiency of accumulation of cadmium in *V. nemoralis* is higher. The findings in the study suggested that the synergistic relationship between microbes and plants in phytoextraction of cadmium-contaminated soils (Khonsue et al. 2013). Mesa et al. demonstrated the bacterial-assisted phytoextraction using endophytic and rhizosphere bacteria. Root samples were collected from *Betula celtiberica* grown on arsenic-contaminated soil. The first step involves isolation and characterisation of endophytic and rhizosphere bacterial communities. Totally, 68% of endophytic bacteria and 53% of rhizobacteria were able to produce IAA. Similarly, 5% of endophytic bacteria and 36% of rhizobacteria were able to produce acetyl-Co A carboxylase deaminase (ACCD). 32% of endophytic strain and 36% of rhizobacteria produce siderophore. A total of 54 rhizosphere bacteria and 41 endophytic bacteria were analysed for metal accumulation in plants and plant growth. Out of these, seven bacteria were considered for arsenic accumulation test. Based on IAA, ACC, and siderophore and arsenic resistance the bacterial strains were selected. The seven bacterial isolates were *Neorhizobium alkalisoli* ZY-4s, *Rhizobium herbae* CCBAU 83011, *Variovorax paradoxus* S110, *Phyllobacterium myrsinacearum* NBRC 100019, *Rhodococcus erythropolis* TS-TYKAKK-12, *Aminobacter aminovorans* LZ1304-3-1, and *Ensifer adhaerens* Sx1. These strains were inoculated into *Betula celtiberica* and allowed to grow for 30 days and analysed for metal accumulation. Out of these, four strains, *Ensifer adhaerens* 91R, *R. herbae* 32E, *V. paradoxus* 28EY, and *P. myrsinacearum* 28EW, were selected for field evaluation. *E. adhaerens* 91R promoted plant growth by increasing root and shoot length, whereas the other three strains were able to increase arsenic accumulation. The use of plants that grow naturally in a particular ecosystem provides an efficient way of phytoextraction by limiting the competition of neighbouring plants. It is noted that most of the endophytes have the ability to produce IAA, and siderophore production is more commonly reported in rhizospheric bacteria (Mesa et al. 2017).

## 4.6 Microbial-Assisted Phytostabilisation

Phytostabilisation is the process that involves immobilisation of the contaminants present in soil or groundwater by chemical compounds produced by plants. The immobilisation of contaminants occurs by absorption by roots and precipitation around the roots, which reduces the mobility of the pollutants. However, it has disadvantages such as existence of residues of pollutants in the soil, overuse of fertilisation, and decrease in plant growth due to exposure to metal stress. The following studies were investigated for bacterial-assisted phytostabilisation to check their efficiency in overcoming the disadvantages. Rajkumar et al. conducted a study on *Brassica juncea* inoculated with two chromium-resistant plant growth–promoting bacteria (PGPB), *Pseudomonas* sp. PsA4 and *Bacillus* sp. Ba32. The soil samples were collected from the metal-contaminated site and chromium-resistant PGPB are isolated. *B. juncea* plants are inoculated with PGPB and allowed to grow for 20 days. The growth parameters of the plants such as shoot length, root length,

and dry weight were measured and accumulation of chromium in roots and shoots was analysed. Sixteen PGPB strains were isolated and the strains *Pseudomonas* sp. PsA4 and *Bacillus* sp. Ba32 were selected as they use acetyl-CoA carboxylase (ACC) as a source of nitrogen. The strain using ACC as a source of nitrogen enhances plant growth. Inoculation of the strain PsA4 in the plants increases 59% root length, 55% shoot length, and 85% vigour index. Similarly, inoculation of Ba32 strain increases 55% root length, 29% shoot length, and 58% vigour index. The bacterial-inoculated and non-inoculated plants were observed. The bacterial-inoculated plant showed increased plant growth in the absence of chromium. PsA4 enhanced plant root length by 73% and shoot length by 28%. Ba32 improved plant root length by 53% and shoot length by 18%. The non-inoculated plants were subjected to different concentrations of chromium, which inhibits plant growth. Plant inoculated with the strains in the presence of chromium showed a moderate activity of increase of 62% root length, 31% shoot length in PsA4. At all concentrations of chromium, PsA4 exhibited higher efficiency in plant growth compared to Ba32. Several strategies were developed for plants to adapt to the polluted environment. But high concentrations of heavy metals restrict the uptake of Fe and P required for plant development. Microbes help by inhibiting the effects of metals and producing more phytohormones, which enhances the uptake of minerals and promotes plant growth. This study illustrated that the inoculation of PGPB bacteria in plants contaminated by chromium showed inhibitory effects against chromium (Rajkumar et al. 2006). Chen et al. carried out a study on the metal-tolerant bacterium *Enterobacter* sp. strain EG16 using *Hibiscus cannabinus* growing in metal-contaminated soil. The *Enterobacter* sp. that is cadmium resistant is isolated from *H. cannabinus* in a metal-polluted site. The bacterial growth is enhanced by the supply of Fe. The effect of cadmium on bacteria was analysed by checking for production of IAA, siderophore, Fe uptake, and the results showed decreased production. The inoculation of bacteria into plants, enhance the root and shoot length. The bacterial strain also stimulates increase of Fe supply in less Fe accumulation plant. The concentration of cadmium also reduces drastically in the plant. Since the contaminated soil was taken from site containing multiple metals, metals such as Pb, Zn, and Cu were also found. EG16 not only reduces the concentration of Cd but also significantly reduces the concentration of Pb and Zn. The Cu concentration remains unaffected by the bacterial strain. Bioavailability and metal mobilisation play an important role in toxicity of plants. The study also illustrated the reduction in bioavailability of Pb, Zn, Cd, and immobilisation of Cu in *H. cannabinus* (Chen et al. 2017). Nurzhanova et al. selected *Cucurbita pepo* L. and *Xanthium strumarium* that are tolerant to dichlorodiphenyltrichloroethane (DDT) and isolated bacterial strains from DDT-contaminated soil. The experiment was carried out in two types of soil. One is naturally obtained from the contaminated site and the other is artificially prepared in laboratory by inoculating DDT. The two soil groups tested here mostly consist of *Pseudomonas* and *Bacillus* dominantly, the next available genera are *Mycobacterium, Arthrobacter*, *Streptomyces*, and the least distributed group is *Micromonospora*. Seeds of the two plant species were transplanted into the two soil types and growth is monitored for 7 months. The

soil adhered to the roots of the plants are taken for isolation of bacterial strains. 580 microbial strains were isolated from the soil and two bacterial strains were selected for further experiments due to their capability to utilise dichlorodiphenyldichloroethylene (DDE). Based on 16S rDNA sequence analysis the bacterial strains were identified as *Bacillus vallismortis* and *B. aryabhattai.* After an incubation of 14 days, it is observed that *Bacillus aryabhattai* consumed around 89.3% of the pesticide and after 21 days, the consumption is around 93.4%. The inoculation of selected microbes and plants resulted in a decrease in pollutant stress and an increase in plant biomass. The bacterial-assisted treatment enhances the phytostabilisation strategy. The rhizosphere contains diverse groups of microbes and in this study the dominant group found in the polluted soil was *Rhodococcus*, which mostly executes dehalogenations, dehydrogenations, oxidation, and so on. Though *Rhodococcus* strains were present abundantly in the contaminated soil, bacterial strains of bacterial genera like *Bacillus* and *Pseudomonas* were present more in both soil types. Thus, two *Bacillus* strains were chosen for further processing in the studies. It is also reported that Bacillus spp. when mixed with fungi accelerate the degradation of DDT, since fungi break down DDT into DDD. The microbes and plants in the contaminated soil were able enhance the degradation process and also reduce plant stress (Nurzhanova et al. 2021).

## 4.7 Microbial-Assisted Phytovolatilisation

Phytovolatilisation is the process wherein plants take up the contaminants and release it to the air through their leaves as volatile compounds. This process helps the contaminants to reduce its toxicity. The disadvantage of this process may be the precipitation of contaminants into lakes or oceans. The phytovolatilisation process is said to be enhanced when it is microbial assisted. Some of the studies suggested the efficiency of microbial-assisted phytovolatilisation is approachable. Wang et al. conducted a study on arsenic (As) degradation by PGPR and *Populus deltoides*, a tree species. *Pteris vittata* plants were collected from arsenic-contaminated region and bacteria from soil adhered to the roots were isolated. Twenty-two As-resistant bacteria were isolated and checked for efficiency of production of IAA and siderophore. The strain D14 showed high resistance towards arsenic and using 16S rRNA gene sequencing, the strain is identified as one of *Agrobacterium radiobacter.* The strain also showed higher production of IAA and siderophore. *P. deltoides* was allowed to grow in the presence and absence of the bacterial strain for 5 months. Without bacterial inoculation, the plants showed a high efficiency in arsenic removal. However, with bacterial inoculation the arsenic removal was enhanced. As the bacterial strain was inoculated, the inhibitory effects of arsenic were reduced. The strain increased plant growth and uptake of the metal. The study evidently showed the capability of plants in metal uptake and the enhancement of the degradation process with microbes (Wang et al. 2011).

## 4.8 Rhizoremediation

Rhizoremediation is the elimination of contaminants around the soil employing microbes that are present in the surrounding soil. Using plant–microbe pairs, the rhizoremediation process is very efficient. The roots produce compounds which are used by microbes as a source of nitrogen, carbon, and phosphorus when attracted to root exudates. The utilisation of these compounds helps in resisting the toxicity of pollutants (Molina et al. 2021). The following are some of the examples of rhizoremediation strategies using plant–microbe pairs. Afzal et al. investigated on the plant–microbe association to clean up the soil contaminated with petroleum hydrocarbons and the activity of plant and microbes. Two bacterial strains, *Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79, were isolated. Seeds of *Lolium multiflorum* were harvested in three types of soil such as sandy soil, loamy sandy soil, and loamy soil. BTRH79 colonised better in the rhizosphere of the plant than ITRI53, which showed better colonisation in the shoot of plant. The bacterial strain expresses the gene which is responsible for the degradation of pollutants. This gene expression level is found higher in loamy soil and also plant growth is efficient. In sandy soil, the survival of the bacterial strain is low, the gene expression level is found only in the shoot region, which is also lower. From the study, it is evident that the type of soil determines bacterial abundance and expression of alkane-degrading genes. The hydrocarbon degradation is evaluated in the presence and absence of bacterial strain. The degradation level is low in plants not inoculated with the strains, and the degradation efficiency is just 12–20% even after 8 weeks; the ones inoculated showed higher degradation of hydrocarbons, and their efficiency is different in different soil types. Hydrocarbons reduce plant growth by hydrophobicity, which limits the plants in taking up nutrients and water. In the bacterial-inoculated plants, the growth of shoot region increased by 8–41%. Loamy soil resulted in higher degradation rate of hydrocarbons of 63% and showed higher plant growth compared to other soil types. The above experiment illustrated that selection of a suitable bacterial strain is essential for improved phytoremediation and in plant growth. It is also evident that the soil type influences plant growth and microbial colonisation (Afzal et al. 2011). Wu et al. did an investigation on sunflower plant and *P. putida* for rhizoremediation of heavy metals. A study was conducted on bacteria that have polychlorinated biphenyl (PCB)-degrading ability and the plant *Morus alba*. The soil sample was collected from the depth of the plant. The bacterial strain was isolated and characterised for biphenyl utilisation. The soil is enriched with four bacterial strains. The bacterial strain MAPN1 showed prominent results and was selected for further studies. Using 16S rDNA gene sequencing, the bacterial strain MAPN1 was identified as one of *Rhodococcus* sp. The bacterial strain is tested for PCB-degrading activity. The strain was allowed to react with naphthalene, anthracene, benzoic acid, salicylic acid, and dibenzofuran. The result was the MAPN1 strain grew on all the tested aromatic substances. The highest growth was observed for the compound anthracene. The strain showed prominent growth using biphenyl as the carbon source and increased the efficiency of phytoremediation strategy. MAPN1 strain has the capability to produce glycolipid

biosurfactants which have the ability to solubilise the pollutants. Plant growth was observed for different concentrations of biphenyl and with inoculation of bacteria. The biphenyl showed an inhibitory effect on plants, in the absence of the bacterial strain. When the bacterial strain is inoculated, the inhibitory effect was reduced and plant growth was enhanced. For 15 days, plant growth was slow. After that plant growth at all different concentrations of biphenyl gradually increased. However, there is no clear study of *Rhodococcus sp*. on plant growth properties, so a better understanding is required to achieve successful enhanced phytoremediation (Sandhu et al. 2020).

## 4.9 Phytostimulation

Phytostimulation is the process which involves promotion of rhizosphere microorganisms by utilising the signalling molecules released by plant roots as a nutrient source. Sun et al. investigated on reduction in lead toxicity on *Lolium perenne* (perennial ryegrass) by *Trichoderma asperellum.* The fungus *T. asperellum* was isolated from the lead-contaminated region. The fungal isolates were cultured in media for 14 days. The experiment was done in four ways: plants were set up in soil which was not contaminated by lead (CK), lead-contaminated soil (T1), lead-contaminated soil with saw dust (T2), and inoculation of fungal isolate with saw dust in contaminated soil (T3). Then the plants were allowed to grow for 28 days. Plant growth was reduced in T1 condition which was exposed to lead; plant height was increased in T2 condition, but dry weight was reduced; T3 condition showed enhanced plant growth and dry weight. A higher concentration of lead was observed in roots than in shoots. The lead-resistant microbes enhanced the extraction of lead from the soil, thus improving remediation of the lead-contaminated soil (Sun et al. 2020). The modulation of signalling compounds in association with plants and microbes was investigated. Seeds of *Withania somnifera* are planted and allowed to grow for 14 days. Four endophytic strains were isolated from the leaves of the plant and tested for the production of IAA, ACC, and ammonia. The fungal isolate *Aspergillus fumigatus* was able to produce IAA, ACC, and ammonia, based on which the strain was chosen for further studies. The endophytic strains, when inoculated into the plants, were able to promote plant growth and colonised effectively. To check the ability of IAA in enhanced plant growth, it is inhibited and plant growth was analysed. The growth of roots was reduced to 66% and with IAA, the growth of maize root was 90%. Thus, it is evident that IAA plays a key role in phytostimulation of plants (Mehmood et al. 2018).

## 4.10 Microbial-Assisted Phytodegradation

Phytodegradation is the process which involves breakdown of organic pollutants either by metabolic activities occurring within the tissue or through enzymatic release from roots. *Scirpus grossus* is used for phytodegradation of pollutants in

contaminated water. The plant was allowed to grow in diesel-contaminated water and analysed. The polluted water, soil and plant samples were taken on 14, 28, 42, and 72nd days to check the effect of the phytoremediation process. The number of rhizobacteria present in the roots of *S. grossus* was estimated by the serial dilution method. The potential rhizobacteria were isolated for running the biodegradation test, which was used to evaluate the plant–microbe interaction. After 72nd day of phytoremediation, the concentration of total petroleum hydrocarbons (TPH) was evaluated. Three plants were used in the experiment and the efficiency of degrading petroleum hydrocarbons was 81.5%, 71.4%, and 66.6%. The degradation test showed the effect of *S. grossus* and rhizobacteria in diesel-contaminated water. There was a difference in the removal of concentration of pollutants on from 14th day to 72nd day. It is due to interaction of plant and microbe. In the presence of a higher population of rhizobacteria, the removal efficiency is higher. The study demonstrated the ability of *Scirpus grossus* to withstand the petroleum hydrocarbons in the concentration of 0.1%, 0.175%, and 0.25%. It is also evident that the interaction between rhizobacteria and *S. grossus* enhanced the removal of diesel (Al-Baldawi et al. 2015). A study on *Lotus corniculatus* and *Oenothera biennis* has shown that hydrocarbon-degrading endophytic bacteria stimulate improved phytodegradation of pollutants. The plants were collected from the hydrocarbon-polluted site and divided into shoots, roots, and leaves. The soil adhering to the roots were taken and endophytic bacteria were isolated from the soil. The production of IAA, ACC and solubility of inorganic phosphate of bacterial isolates were estimated. About 58.33% of bacterial isolates from *L. corniculatus* and 28.7% of isolates from *O. biennis* showed the ability to solubilise phosphate. The capability of bacterial isolates to utilise hydrocarbon as a carbon source was checked. The bacterial isolates were analysed for identification of hydrocarbon-degrading genes. Five bacterial strains, namely *Pseudomonas mandelii* and four strains of *Rhodococcus* sp., showed positive results. All the bacterial isolates are screened for their capability to promote plant growth. All bacterial strains produce IAA and the highest production were observed on *Delftia lacustris* 5FXS, *Delftia lacustris* 6.1XS, and *Rhizobium* sp. 1XS. The highest production of siderophore was observed in *L. corniculatus.* The ability of the bacterial strain in plant colonisation was analysed by checking the cellulase activity. The cellulase production of *O. biennis* and *L. corniculatus* efficiency is 64.29% and 47.67%, respectively. The isolated bacterial strains showed a clear potential to degrade hydrocarbons by emulsification property. Bacterial strains of species such as *Serratia, Delftia, Rhodococcus, Rhizobium, and Pseudomonas, and Rhodococcus sp. 4WK* have the ability to produce biosurfactants which promote emulsification of hydrocarbons, resulting in degradation (Pawlik et al. 2017). Kabra et al. (2013) worked on the treatment of textile effluents by plant–microbe interactions. The soil adhered to the roots of the plant *Gaillardia pulchella* was cleaned and dye was added to it. The dye used in the experiment is 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). The plant inoculated with the dye decolourised it within 72 h. Using 16S rRNA gene sequencing the bacteria present in the roots was found to be *Pseudomonas monteilii*. Seeds of the *G. pulchella* were planted and allowed to grow for 4 weeks. The plant decolourised the dye with an

efficiency of 97% within 72 h, the efficiency of decolourisation by bacteria was 85% within 72 h, and the efficiency of combined system was about 100% within 48 h. The study shows the efficiency of plant–microbe combined system in decolourising the dye is higher than usage of plant and microbes separately (Kabra et al. 2013). From the studies illustrated in the review, it is evident that microbes when interacting with plants help in depleting pollutants and also help plants to withstand the stress caused by pollutants. Microbes provide plants with growth-promoting phytohormones and plants in turn provide carbon, nitrogen, and phosphorus sources to the microbes.

## 4.11 Challenges Faced During Remediation by Plant–Microbe Associations

Even though plant–microbe remediation has a potential ability to degrade pollutants, there are some disadvantages for this combined system, which make it challenging, and only few studies have been conducted on them to improve the efficiency of remediation. Differences in the efficiency of degrading pollutants have been observed, when plants with its associated microbes are used in different combinations. This observation suggests that selection of appropriate microbes and plants is required for remediation of specific contaminants. There is not much research on suitable selection of plant–microbe systems for remediating polluted environments. Some negative impacts on food chain also occur due to the problems faced in disposal of pollutants. Only few details about the composition of the microbes present in contaminated soils or regions are known. In degradation of pesticides, the parent biocompounds used also have toxic effects, which results in harmful impacts on humans. There is no clear understanding of the mechanism of degradation of contaminants, role of rhizospheric microbes, and the interaction of plants and microbes (Kuiper et al. 2004).

## 4.12 Conclusion

From this review, it is evident that plant–microbe interaction-associated remediation is more beneficial compared to other conventional methods. Various regulatory networks in plant–microbe interactions should be investigated. The synergistic relation between plants and microbes, and their role in metal mobilisation and degradation should be analysed clearly. Development of transgenic and recombinant microbes along with plants may also increase the efficiency of the treatment. Plant–microbe interaction-associated remediation will be a promising approach if the limitations are clearly acknowledged and resolved. For utilisation of microbe–plant combined systems to degrade contamination at industrial level, it is pretty much essential to better understand plant–microbe interactions. There is an urgent need in developing novel strategies for pollutant degradation so that we can create a better and safe environment.

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# Recent Trends in Bioremediation of Heavy Metals: Challenges and Perspectives

5

Pooja Arora, Rashmi Paliwal, Nitika Rani, and Smita Chaudhry

**Abstract**

Heavy metal pollution is a matter of serious concern worldwide. Movement of heavy metals starting from the extraction processes to their applications in a variety of industrial activities, results in their indiscriminate release in the environment. Prolonged exposure to these heavy metals can cause detrimental health effects in human as well as other living organisms. Heavy metals include a class of some highly toxic metals such as, Hg, Cd, Cr, Pb, Ni, Cu, and Zn. that are reported to have cytotoxic, carcinogenic, teratogenic, and mutagenic effects. Since, these heavy metals are nondegradable and have a tendency to accumulate in environment, their removal from aquatic and terrestrial system is required. Bioremediation is one of the promising techniques which can be used to remove these contaminants from water and soil using biological agents, including microorganisms (bacteria, fungi, and microalgae) and plants (phytoremediation). Microorganisms and plants are capable of taking up heavy metals from nature and use these toxic contaminants in their metabolic activities, or convert them to less/nontoxic forms. Thus, the microorganism- and plant-mediated treatment processes are widely accepted since these methods are based on natural mechanisms and also reduce the chances of generation of secondary pollutants as in the case of various conventional processes. This chapter thus studies the various bioremediation techniques for the removal of heavy metal from nature and will discuss the mechanisms of different biological agents used for the transformation of toxic heavy metals. Different methods for the assessment of heavy metals have been

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discussed for the effective monitoring of contaminants in nature. The review also presents the recent advances in the field of bioremediation in terms of use of plants and their metabolites, plant growth–promoting rhizobacteria and nanoparticles for efficient removal of heavy metals from contaminated sites.

**Keywords**

Heavy metals · Contamination · Toxicity · Bioremediation · Phytoremediation

## 5.1 Introduction

Rapidity of industrialization, rising demand of energy, mining, and careless exploitation of natural resources in the past many years are the key reasons for rise in environmental pollution which causes serious threats to biodiversity and ecosystem processes. Large amount of various toxic organic and inorganic pollutants causes soil and water pollution. Of these, one of the most toxic pollutants that have an adverse impact on environment is heavy metals (Gautam et al. 2016).

Heavy metals are naturally occurring elements with an atomic number greater than 20 and atomic density above 5 g $cm^{-3}$ and exhibit the properties of metal such as luster, ductility, malleability, and high electric and thermal conductivity. These are one of the most challenging pollutants due to their highly toxic and nonbiodegradable nature. Also, heavy metals have high efficiency to get rapidly bioaccumulated in ecological systems since plants and animals absorb these from the contaminated environment in which they are residing.

Heavy metals can also biomagnify inside the human body due to consumption of bioaccumulated plants, animals, and contaminated water. They pose severe hazardous impacts even at very low concentrations. The natural sources of heavy metal discharge into the environment include geological weathering of bedrocks and volcanic eruptions. The anthropogenic sources include industries (dyeing, tannery, mining, electroplating, paints and pigments, fertilizer, etc.), sewage sludge, waste treatment plants and runoff from agricultural fields. The other sources include electronic waste, personal care products, cosmetics, and medicines. In addition to this, plant and animal waste matter decomposition, plant exudates, forest fire, wind erosion, oceanic spray, and airborne particles from volcanic activity also lead to the addition of heavy metals in environmental components. Emission of heavy metals into the environment also occurs in many other ways such as in air, at the time of combustion, due to extraction and processing of metal-containing ores, to surface waters by means of runoff and also due to releases from storage and transport. Roadways and vehicular emissions are also one of the major contributors of heavy metals in the environment (Jobby and Desai 2017). After their release from the source, these heavy metals tend to remain in the environment for longer time periods attributed to their nondegradable nature. They can also impose toxic and irreversible effects on the associated microorganisms, plants, animals, and human beings (Rahman and Singh 2019).

Currently the term "heavy metal" has been used to explain metallic chemical elements and metalloids which are harmful to the environment and human beings. Of the total 90 naturally occurring elements, 53 are considered as heavy metals. Some heavy metals more common in our everyday life are chromium (Cr), cadmium (Cd), copper (Cu), arsenic (As), lead (Pb), mercury (Hg), nickel (Ni), zinc (Zn), manganese (Mn), iron (Fe), cobalt (Co), silver (Ag), gold (Au), platinum (pt), molybdenum (Mo), tin (Sn), vanadium (V), and titanium (Ti) (Briffa et al. 2020). These metals and metalloids in less concentration play a vital role for tissues and cells of all living organisms because they serve as cofactors for proteins and enzymes. They are also responsible for maintaining the osmotic potential. On the other hand, these metals show highly hazardous effects in high concentration (Tahir et al. 2019). Among all heavy metals, chromium, lead, mercury, cadmium, and arsenic are widely present in the environment. These heavy metals cause various health impacts such as chromium (VI) and arsenic are carcinogenic in nature. High doses of cadmium can cause a degenerative bone disease. Lead and mercury in high concentration cause damages to the central nervous system in the human body (Fatima and Ahmed 2018).

## 5.2 Heavy Metal Pollution

Heavy metals are characterized into three forms which are of great concern, comprising (a) toxic metals (Cr, Hg, Zn, Pb, Ni, Cu, As, Cd, Sn, Co, etc.), (b) precious metals (Pd, Pt, Ru, Au, Ag, etc.), and (c) radionuclides (Th, U, Am, Ra, etc.). In addition of these, some metals are considered to be essential elements (Cu, Ni, Co, Zn, etc.), while others are considered as nonessential metals (Cd, Pb, As, Ag, Au, etc.). Soil contamination with heavy metals is of great concern because it causes adverse effects to humans and the ecosystem directly. Heavy metals from contaminated soil can easily enter the food chain through direct ingestion. Living organisms may also intake heavy metals by means of drinking contaminated groundwater. Metal toxicity in plants results in reduction in food quality and less use of land for agricultural production, ultimately leading to food insecurity and land tenure problems (Jobby and Desai 2017).

Heavy metals enter into the environment through various sources such as industrial wastewater and sewage discharge which are the significant sources of metal pollution in water life. Soils also get contaminated due to the accumulation of heavy metals and metalloids that are emitted from industrial areas, dumping wastes, from leaded gasoline and paints, mine tailings, agricultural activities such as use of fertilizers and pesticides, sewage sludge, irrigation with wastewater, residues of coal combustion, runoff from terrestrial systems, effluents from industrial and domestic sources, accidental leakages spillage, and atmospheric deposition (Table 5.1).

Various adverse impacts of heavy metals are well known. They are very lethal to living organisms even at very low concentrations (Table 5.2). They can cause potential health impacts that can be cytotoxic, carcinogenic, teratogenic, and mutagenic in nature (Fig. 5.1).

**Table 5.1** Sources of some toxic heavy metals in the environment

| Metal | Sources |
|---|---|
| Chromium (Cr) | Mining, road runoff, coolants from industries, leather tanning |
| Lead (Pb) | Lead acid batteries, mining, smelting, paints, e-waste, ceramics |
| Mercury (Hg) | Thermal power plants, fluorescents, dental amalgams, hospital wastes |
| Arsenic (As) | Smelting operations, thermal power plants, fuel burning, pesticides |
| Copper (Cu) | Mining, electroplating, smelting operations, road runoff |
| Nickel (Ni) | Combustion of fossil fuels, electroplating, battery industry, road runoff, thermal power plants |
| Cadmium (Cd) | Ni or Cd batteries, sludge from paint industry, fuel combustion, e-waste |
| Zinc (Zn) | Road runoff, electroplating, smelting |

**Table 5.2** Major heavy metal contaminants, prescribed standards (in drinking water) and their human health effects

| Heavy metal | Maximum concentration levels (mcl) (mg/l) USEPA[a] | Tolerance limits (mg/l) IS:10500, 1992[b] | Potential health effects |
|---|---|---|---|
| Arsenic (As) | 0.05 | 0.05 | Skin lesions and carcinogenic effects |
| Cadmium (Cd) | 0.005 | 0.01 | Kidney problems |
| Chromium (Cr) | 0.1 | 0.05 | Allergic dermatitis |
| Copper (Cu) | 1.3 | 0.05 | Gastrointestinal distress (acute exposure)<br>Liver and kidney damage (chronic exposure) |
| Mercury (Hg) | 0.002 | 0.001 | Kidney and spinal cord ailment |
| Lead (Pb) | 0.015 | 0.05 | Late physical and mental growth, central nervous system (CNS) complications, kidney and high blood pressure issues |
| Nickel (Ni) | 0.1 | 0.05 | Allergic skin diseases, carcinogenic effects, immunotoxic, neurotoxic, genotoxic |

[a]United States Environmental Protection Agency Prescribed
[b]Indian Standard: 10500, 1992

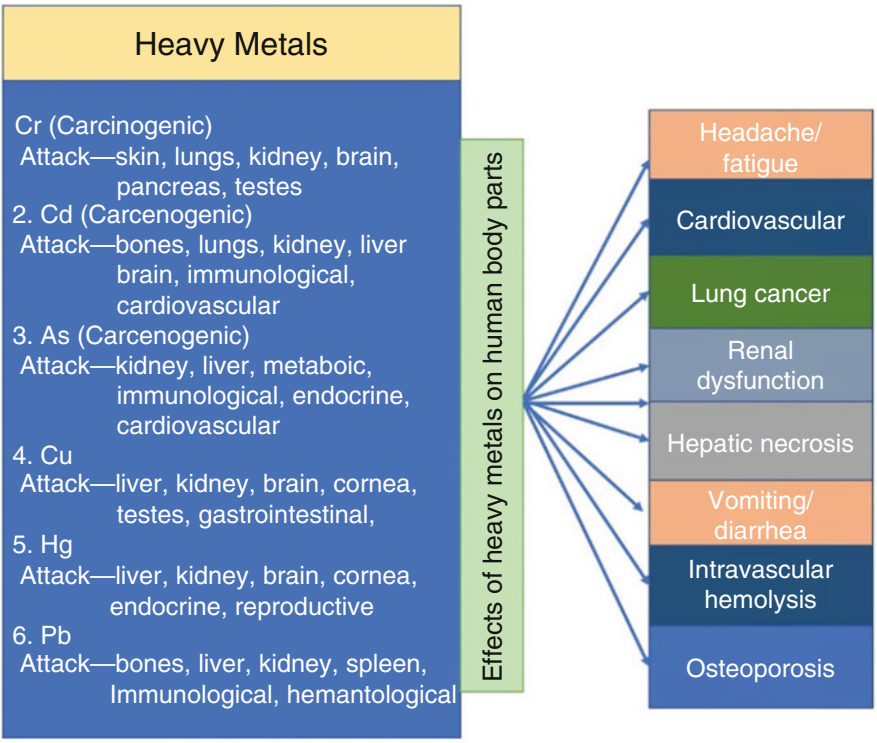


**Fig. 5.1** Toxicity to humans due to heavy metals

## 5.3 Bioremediation of Heavy Metals: Principles, Mechanisms and Factors

Presently a number of methods such as ion exchange, adsorption, chemical precipitation, floatation, electro dialysis, solvent extraction, electrochemical deposition, and reverse osmosis are available in order to eliminate or remove these toxic heavy metals from the environmental components. But these techniques have many limitations such as high cost and very low efficiency. Further, these techniques have the potential to cause deleterious effects on soil and thereby changing its original composition. In order to overcome these drawbacks new eco-friendly methods are being invented and developed which have no such adverse effects. These methods use microorganisms such as bacteria, fungi, or plants to remove heavy metals either by absorbing or by changing the valency of metal element and make them less toxic (Pratush et al. 2018). These methods are collectively recognized as Bioremediation techniques. Eco-friendly and cost effectiveness are more advantageous features of bioremediation as compared to other chemical and physical methods of remediation (Azubuike et al. 2016).

Bioremediation is an ecologically sound and up-to-the-minute technique which by means of natural biological processes completely removes toxic contaminants from the environment. It can be any process which with the help of microorganisms (bacteria, fungi), green plants, or their enzymes brings the modified and contaminated environment into its natural original condition. The time period between the late 1980s and the early 1990s was the golden period for bioremediation (Mani and Kumar 2014).

### 5.3.1 Principles of Bioremediation

Principle of bioremediation includes the use of microorganisms to destroy the harmful contaminations or convert them into less toxic form. Three essential components of bioremediation are (a) microorganisms, (b) food, and (c) nutrients, which are together known to be the bioremediation triangle. The effective role is of microorganisms which metabolizes the chemical compounds to produce water, carbon dioxide (in aerobic conditions) or methane (in anaerobic conditions), microbial biomass and metabolites (Paul et al. 2021).

In bioremediation, the native microflora predominates in the contaminated site and suitable conditions such as more food for suitable growth is provided to them to make them grow to their full potential. This helps in the production of more enzymes as secondary metabolites which have potential to break down the complex contaminants into simpler constituents more efficiently. The process of bioremediation takes place through the breakage of chemical bonds and release of energy. This release energy is again used by the microorganisms for their metabolism and growth. The microbial species used for heavy metal transformation can either be isolated from aerobic or anaerobic or both of the environments. However, the microorganisms isolated form aerobic environments are mostly exploited for the process of bioremediation as compared to the ones isolated from anaerobic environment (Pratush et al. 2018). Complete breakdown of pollutant needs the action of several microbes. The process of biodegradation depends on suitable environmental conditions, type of the pollutant and its solubility, and the bioavailability of the pollutant to the microbial population in order to achieve fast and effective biodegradation. Environmental conditions are also manually controlled or manipulated to facilitate sufficient microbial growth (Tyagi and Kumar 2021).

### 5.3.2 Mechanisms of Bioremediation

Microorganisms are widespread and easily convert heavy metals from toxic form to nontoxic simpler forms. In the process of bioremediation, the organic pollutants or contaminants are converted into carbon dioxide ($CO_2$) and water ($H_2O$), and/or to other metabolic intermediates by microbial activity and the converted materials are utilized as primary substrates required for cell growth. Microbial communities are capable of two-way protection. Firstly, they produce degradative enzymes for the

**Table 5.3** Microorganisms used for heavy metal removal from contaminated sites

| Microorganisms | Heavy metals | Reference |
|---|---|---|
| *Bacillus polymyxa*<br>*Pseudomonas aeruginosa* | Cu, Zn | Philip et al. (2000), Gunasekaran et al. (2003) |
| *Saccharomyces cerevisiae* | Pb, Hg, Ni | Chen and Wang (2007), Talos et al. (2009), Infante et al. (2014) |
| *Pseudomonas aeruginosa*, *Aeromonas* sp. | U, Cu, Ni, Cr | Sinha et al. (2011) |
| *Geobacter* spp. | Fe, U | Mirlahiji and Eisazadeh (2014) |
| *Bacillus safensis* (JX126862) strain (PB-5 and RSA-4) | Cd | Priyalaxmi et al. (2014) |
| *Aerococcus* sp., *Rhodopseudomonas palustris* | Pb, Cr, Cd | Sinha and Paul (2014), Sinha and Biswas (2014) |
| *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* | Fe, Zn, Pb, Mn and Cu | Paranthaman and Karthikeyan (2015) |
| *Lysinibacillus sphaericus* CBAM5 | Co, Cu, Cr, Pb | Peña-Montenegro et al. (2015) |
| *Microbacterium profundi* strain Shh49T | Fe | Wu et al. (2015) |
| *Aspergillus versicolor*, *A. fumigatus*, *Paecilomyces* sp., *Trichoderma* sp., *Microsporum* sp., *Cladosporium* sp. | Cd | Soleimani et al. (2015) |

degradation of target pollutants and secondly, they become resistant to relevant heavy metals. Diverse types of mechanisms including bioaccumulation, biosorption, biomineralization, biotransformation, metal–microbe interactions, and bioleaching are utilized for bioremediation. Microorganisms use chemicals for their growth and development in order to remove the heavy metals from the contaminated site. The metals get dissolved, reduced, or oxidized through microbial activity. Microorganisms restore the contaminated environment into its original form by oxidation, immobilization, volatilization, transformation, and binding of heavy metals.

The success of bioremediation in a particular location can be ensured by thorough understanding of the operating mechanism, controlling activity, and growth of microorganisms, enhancing their response through metabolic capabilities to environmental changes. Organic contaminants tend to disrupt cell membranes. However, microbial cells have the potential to develop defense mechanisms which comprise the formation of outer cell-membrane-protective material, often hydrophobic or solvent efflux pumps. The prevalent example includes the formation of plasmid-encoded and energy-dependent metal efflux systems. These systems include ATPases and chemiosmotic ion/proton pumps and are reported for resistance against Cd, Cr, and As in many bacteria (Dixit et al. 2015). The selection or choice of microorganism, however, depends on the nature of contaminant or pollutant material to be degraded and environmental conditions. Certain microorganisms which have been implied for heavy metal removal are given in Table 5.3.

### 5.3.3 Factors Affecting Bioremediation

The efficiency and potential of bioremediation techniques depends on physicochemical characteristics of the environment, concentration and chemical nature of the pollutants, and their bioavailability to existing microorganisms (El Fantroussi and Agathos 2005). Important factors which are important for the growth of microorganisms in order to remove the heavy metals from the contaminated sites include the following.

1. *Nutrients*: Availability of nutrients are less at the contaminated sites because of organic pollutants which are more at contaminated site and get depleted or degraded during microbial metabolism. So an additional supply of nutrients like nitrogen (N), phosphate (P), and potassium (K) is given from outside to the affected or contaminated site to stimulate the growth and cellular metabolic activities of microorganisms and thereby increasing the rate of bioremediation process. The higher efficiency of bioremediation can be achieved by improving the bacterial C:N:P ratio (Abatenh et al. 2017).
2. *Nature of Pollutants:* Bioremediation depends upon the type or state of pollutants such as solid, semisolid, liquid, and volatile in nature.
3. *Soil Structure*: Soil structure comprises different textures depending upon variable contents of silt, sand, and clay. Powdery, well-structured, or well-maintained soil helps in the effective supply of nutrients, water, and air to microbial consortia for in situ bioremediation.
4. *pH*: Optimum range of pH for the microbial growth and degradation of the contaminants is 5.5–8.0. Higher or lower pH values may slow down the removal process due to high susceptibility and sensitivity of metabolic processes to even minor changes in pH levels (Wang et al. 2011).
5. *Moisture content:* Water acts as a primary and important factor to determine the dielectric constant of soil and other such mediums. The moisture content of soil for efficient bioremediation should be in the range of 25–28%.
6. *Microbial Diversity*: The presence of various types of microorganisms at contaminated site such as *Flavobacteria, Pseudomonas, Chlorobacteria, Aeromonas, Corynebacteria, Mycobacteria, Streptomyces, Acinetobacter, Arthrobacter, Aeromonas, Bacilli,* and *Cyanobacteria* favors the remediation process.
7. *Macrobenthos Diversity*: A consortium of aquatic plants *E. crassipes*, *S. molesta*, *C. demersum* with aquatic animals *A. woodiana* and *L. hoffmeisteri* is very effective to degrade organic and metal load in domestic wastewater.
8. *Temperature:* The optimum temperature ranges from 15 to 45 °C for efficient bioremediation. Temperature influences the physiology of microorganisms resulting in speeding up or slowing down of the bioremediation process. The rate of microbial activities firstly increases with increase in temperature and achieves its maximum level at an optimum temperature. After then, the rate declines suddenly with any further increase in temperature and ultimately stops after specific temperature (Abatenh et al. 2017).

9. *Oxygen*: Oxygen is utilized for the initial stages of breakdown of the hydrocarbons present at the contaminated sites. The presence of oxygen determines whether the process of bioremediation will occur under aerobic or anaerobic conditions.

## 5.4 Techniques for Detection and Assessment of Heavy Metals in the Environment

Heavy metals are the contaminants of greatest concern due to their adverse effects on living organisms. Human and other living organisms can get exposed to the elevated concentration of heavy metals from contaminated soil, water, groundwater, and plants. Therefore, appropriate methods are essentially important for accurate detection and assessment of heavy metals in various environmental samples. Highly sensitive instrumental techniques such as flame atomic absorption spectroscopy (FAAS), graphite furnace atomic absorption spectroscopy (GFAAS), inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), and inductively coupled plasma atomic emission spectrometry (ICP/AES) are widely used to assess the qualitative and quantitative analysis of heavy metals in various samples from the environment. However, rapid detection techniques, for example, X-ray fluorescence spectrometer (XRF), for assessing the heavy metals in variety of environmental samples are most preferred nowadays. Other techniques such as X-ray absorption spectroscopy (XAS) and X-ray diffraction (XRD) have been used to study the heavy metals and their interaction within biosystems such as soil and plants.

*X-ray Fluorescence (XRF):* It is a nondestructive method of analysis which involves the emission of X-ray photon followed by atom ionization through a primary X-ray beam. The primary X-ray beam upon hitting the sample interacts with the electron and removes this it from its inner shell forming. This process creates voids in the inner shell that exhibit an unstable state of the atom. The voids get rapidly filled by electron of the outer shell accompanied by emission of X-rays with a specific wavelength. This X-ray of specific wavelength is the measure of elemental composition of a sample (Meirer et al. 2010).

*X-ray Absorption Spectroscopy (XAS):* X-ray absorption spectroscopy (XAS) gives a researcher a detailed information on the environmental coordination of metals absorbed by plants. XAS is used to investigate the atomic geometry of heavy metals and their interactions within biosystems, that is, soil and plants (Gardea-Torresdey et al. 2005).

*X-ray Diffraction (XRD):* X-ray diffraction (XRD) is a fast technique used for the phase identification in crystal material in order to analyze the structure of the material (minerals, inorganic compounds, etc.). In the recent years, XRD is being widely used to heavy metal remediation studies. Shao et al. (2020) used XRD to confirmed the formation of a stable mineral pyromorphite ($Pb_5(PO_4)_3Cl$) in the soil containing Pb metal after treating with low-cost phosphorous-containing amendments.

## 5.5 Techniques of Bioremediation

The process of bioremediation can be used for soil and water contaminated site through in situ and ex situ techniques (Kapahi and Sachdeva 2019). Both "in situ" and "ex situ" bioremediation approaches involve microbial metabolism. In situ methods are to restore the soil and water contaminated without excavating the sample from contaminated sites while ex situ methods are to degrade the chemical pollutants of excavated samples (Fig. 5.2). Ex situ techniques are more expensive as compared to in situ techniques.

### 5.5.1 In Situ Techniques

In situ bioremediation techniques involve biological degradation of contaminants at the site in natural conditions. In addition to the removal of heavy metals, "in situ" bioremediation is also used for the treatment of chlorinated solvents, dyes, and hydrocarbon polluted sites. To make "in situ" bioremediation more successful, several factors such as oxygen supply, moisture content, pH, temperature, and nutrient supply are needed to be made suitable for potential microbial growth. Of these factors, the availability of molecular oxygen is the one of the major problems which needs to be tackled. Various in situ techniques are discussed below.

1. *Bioventing*: It is a technology to stimulate on site natural degradation of organic compounds which gets adsorbed on soil particles in the unsaturated zone. The process is basically accomplished by inducing air or oxygen to existing and introduced microorganisms into the unsaturated zone of soil to favor their growth.
2. *Biostimulation*: In biostimulation, the indigenous microorganisms are provided with rate limiting nutrients such as nitrogen, phosphorus, oxygen, electron

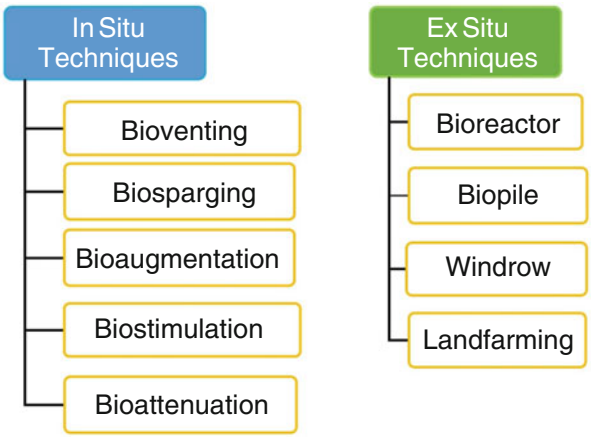


**Fig. 5.2** Types of bioremediation techniques for various contaminants

acceptors, and adequate amounts of water to accelerate their growth and bioremediation potential.

3. *Bioaugmentation:* Bioaugmentation is the introduction of specific indigenous or nonindigenous microorganisms that may be autochthonous, allochthonous wild type or genetically modified to the contaminated sited to remove the target compounds. This technique is aimed at increasing the gene pool and genetic diversity at the site to accelerate the rate of degradation of hazardous substances.
4. *Bioattenuation*: This technique involves the removal of heavy metals without human interference in passive mode suitable for both biodegradable and intractable contaminant. It includes aerobic and anaerobic types of degradation comprising physicochemical methods, namely dispersion, dilution, and ion exchange. The process involves the removal of chemicals by means of tiny bugs or microbes which eat and then digest the contaminants to convert them to water or less toxic forms.
5. *Biosparging*: In this technique, injection of air is given into saturated zone or subsurface soil in order to improve the rate of biodegradation of contaminants by naturally occurring bacteria. Nutrients may also be added to enhance the microbial growth. Pollutant biodegradability and soil permeability plays important role in the effectiveness of biosparging. In stressed conditions, metal-adsorbing materials are produced by bacteria. These materials chemically interact with contaminants and pollutants and cause their precipitation. During biosparging, the oxygen supply produces aerobic condition which are quite appropriate for the degradative action of native microbes.

### 5.5.2 Ex Situ Techniques

Ex situ techniques involve the removal or transportation of contaminated environmental component or site to another site for remediation. The location and environmental conditions of the contaminated site, cost, type of the pollutant, and level of pollution are the main criteria for "ex situ" bioremediation techniques. The "ex situ" type bioremediation techniques are comparatively easier to regulate and control the processes. These are useful to treat a wider range of contaminated soils and toxins. In this mixing of material is done to have a good supply of air and nutrients so that degradation of contaminants is much faster as compared to "in situ" techniques. The various ex situ techniques are described below.

1. *Bioreactor*: in bioreactor technique, large vessels are used to remove the pollutants from wastewater by means of microbes. The different operating modes of the bioreactor are (a) batch, (b) fed-batch, (c) sequencing batch, (d) multistage, and (e) continuous. Temperature, pH, moisture, concentration of substrate, agitation rate, and aeration rate are the important parameters required for working of bioreactors. Due to certain limitations, bioreactor-based bioremediation is not suitable for removal of heavy metals. Firstly, it requires more man power. Also, there is requirement of high cost for the transfer of pollutants from

the contaminated site. Secondly, various bioprocess variables are involved in bioreactor technique. If any variable remains uncontrolled, it turns out to be a limiting factor and leads to reduction of microbial activities and hence makes the technique very less effective.

2. *Biopile*: Biopile-based bioremediation involves conversion of contaminated soils into piles. The pile formation is followed by application of nutrient and aeration to make bioremediation effective by increasing microbial activity. Different terms are used for of biopiling such as bioheaps, biocells, and biomounds for alleviating the problem of contamination from soils and sediments. Temperature, pH, moisture, and nutrients are important parameters to accelerate biodegradation in biopiles. Biopiles are useful to remove heavy metals from soil, and they are a better pollutant removal strategy as compared to other techniques such as land farming and composting which are based on bulk transfer of nutrients, water, and air.
3. *Windrow*: Windrow includes the turning of polluted soil for increasing and improving aeration along with the application of water, uniform distribution of nutrients, contaminants, and microbial degrading activities. The process occurs through assimilation, mineralization, and biotransformation. This treatment is not suitable for remediation of soils which are polluted with toxic volatile compounds since it involves periodic turning.
4. *Landfarming*: Less Equipment is required during landfarming technique operation. However, production of leachate take place during landfarming operation which should be taken care of to prevent the groundwater contamination. Tillage and irrigation with appropriate biological activity enhances the rate of bioremediation by enhancing heterotrophic bacterial counts. The enzyme microbial dehydrogenase, a good indicator of biostimulation, is used in landfarming. Landfarming is the simplest bioremediation practice. However, there are certain limitations to it such as it requires a large operating space, requirement of additional and high cost during excavation, reduction in microbial activity due to unfavorable environmental conditions and less effectivity in removal of inorganic pollutant. These limitations make this technique more time-consuming which in turn makes it less efficient.

## 5.6 Plant-Mediated Heavy Metal Removal

Plants are well known to remove metal contaminants from environment. Plant-based remediation technologies, also known as phytoremediation, have been widely studied for extracting and accumulating the heavy metals significantly. Phytoremediation is an ecological remediation technology where plants are used as an important source for the removal of contaminants whether, organic or inorganic. In the process of phytoremediation plants remove contaminants through different mechanisms such as extraction, sequestration, and detoxification. Plants use different approaches for the removal of heavy metals from the ecosystem, which include phytoextraction, rhizofiltration, phytovolatilization, and phytostabilization. Thus, the techniques of

phytoremediation can be further classified as phytodegradation, rhizofiltration, phytostabilization, phytoextraction, and phytovolatilization (Fig. 5.3).

Heavy metals, for example, Cu, Zn, Fe, and Mo, make an integral part of many enzymes and participate in redox reactions, electron transfer, and also in nucleic acid metabolism, and thus are considered as essential for plants. These metals act as cofactors and activator of important enzymatic activities, thus playing an important role in the formation of enzyme–substrate metal complex (Nagajyoti et al. 2010). Zn, acting as cofactors for over 300 enzymes and 200 transcription factors involved in maintaining membrane integrity, auxin metabolism, and reproduction (Singh et al. 2016). Heavy metals at microlevels are important for plant growth, but at elevated concentrations can exert toxic effects in plants. The most common toxic effects of essential and nonessential heavy metals on plants include inhibition of growth and photosynthesis, chlorosis (loss of the green coloration of leaves), low biomass accumulation, altered nutrient assimilation and water balance, and senescence, which ultimately leads to plant death. There are four proposed mechanisms for the toxicity of heavy metals on plants (Singh et al. 2016).

1. Similarities to nutrient cations, lead competitive uptake on root surface; for example, As and Cd compete with phosphorus (P) and Zn, respectively, for their uptake.
2. The heavy metals directly interact with the sulfhydryl (-SH) group of functional proteins. This interaction disturbs their structure and function, thereby rendering them inactive.
3. Movement of essential cations from particular binding sites causes functional collapse.
4. Reactive oxygen species (ROS) generation as a result damages the macromolecules.

Plants have potential adaptive mechanisms for tolerance toward the high concentrations of heavy metals, their extraction, and accumulation in the above ground parts (Singh et al. 2010; Pal and Rai 2010). Plants having potential to uptake or tolerate a large amount of heavy metals known as hyperaccumulators make them unique to be used as a tool for the remediation of heavy metal. Such plants are also known as "metallophytes." Over 400 plant species vary from annual to perennial herbs, shrubs, and trees belonging to over 45 families have been identified to accumulate significant amount of heavy metals (Giri et al. 2015). Baker (1981) recognized the types of plant–soil relationships, that is, accumulators, indicators, and excluders (Fig. 5.4) growing on metalliferous soils, as discussed below.

1. *Accumulators:* These plants concentrate the toxic metals in their aboveground parts.
2. *Indicators:* Uptake and transportation of metals to the shoots of plant is regulated in a manner where internal concentration reflects the external levels.

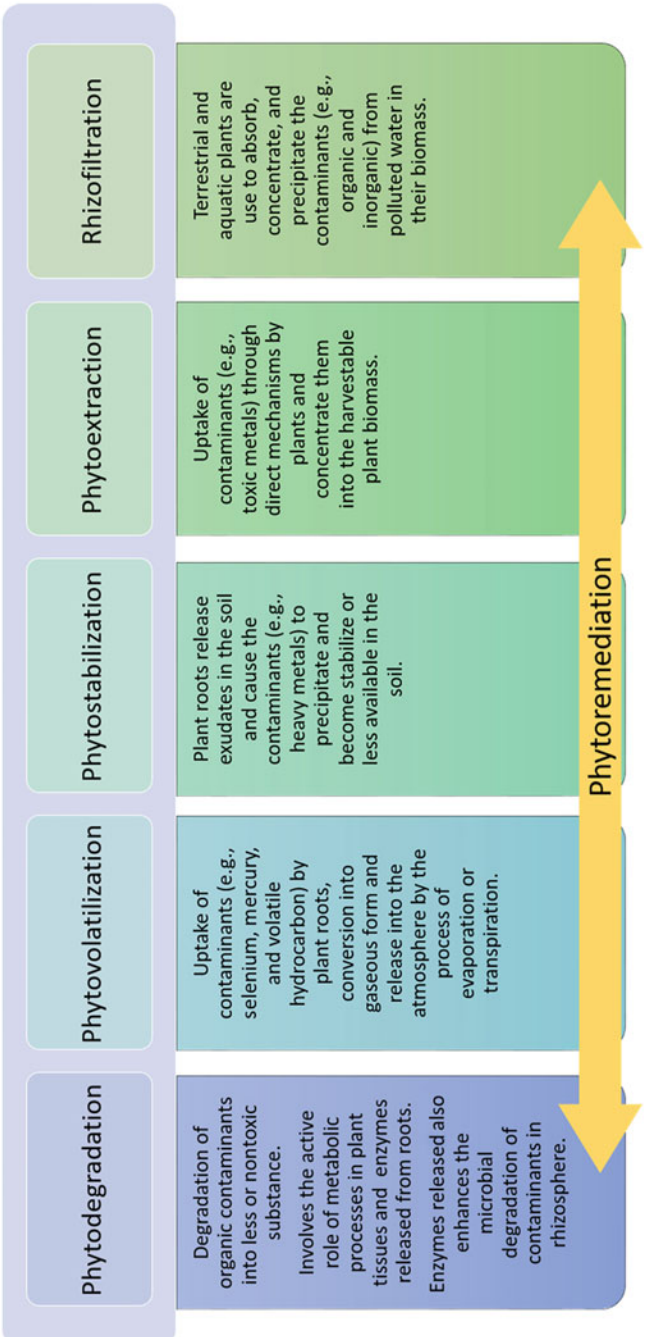


**Fig. 5.3** Different processes of phytoremediation

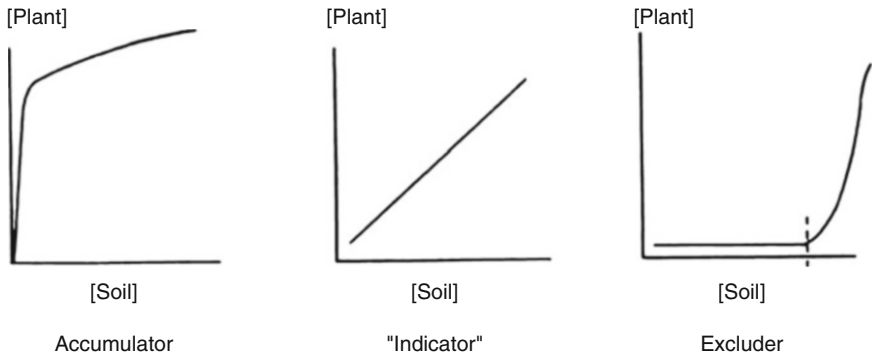


**Fig. 5.4** Three ways in which the response of plants to increasing soil metal levels may be reflected by the metal concentrations in aerial plant parts. Reproduced with permission from Baker (1981). (Copyright 1981, Taylor & Francis Group)

3. *Excluders:* Plants maintain toxic metal concentrations in their parts as constant up to a critical soil value and above which unrestricted transportation of contaminants occur.

Plants use their root system to absorb the ionic compounds present in soil. Plants develop a rhizosphere ecosystem by extending their root system into the soil matrix. The extensive root system of plants helps them to accumulate heavy metals and regulate their bioavailability. In this way, plants not only reclaim the contaminated soil but also stabilize soil fertility (Jacob et al. 2018; DalCorso et al. 2019). Plant roots release some exudates into the soil and enhance the bioavailability of heavy metals by modifying soil pH. Root exudates are primary metabolites (sugar, amino acids, and organic acids) released from plants' root tip and play a crucial role in shaping the interaction between plants and soil, especially nutrient mobilization in rhizosphere soil (Canarini et al. 2019). Apart from root exudates, pH of rhizosphere also influences heavy metal uptake by plants/hyperaccumulators. It has been reported protons released in the rhizosphere by roots enhanced metal dissolution (Singh et al. 2016). Plant roots follow either of the two pathways for nutrient and heavy metal uptake, that is, apoplastic pathway and symplastic pathway. The apoplastic pathway is the passive diffusion of soluble metals through the space between cells, whereas the symplastic pathway is active transport of nutrients/soluble heavy metals against electrochemical potential gradients and concentration across the plasma membrane (Peer et al. 2005).

For successful implementation of the process of phytoremediation plants must possess the heavy metal detoxification mechanism. Plants with constituent and adaptive mechanisms to extract, collect, and tolerate high concentrations of their rhizospheric contaminants are preferred for the application of phytoremediation procedures. Plants have developed a range of potential mechanisms for tolerating and avoiding the toxic effect of high concentrations of metals. By avoidance and tolerance strategies, plants are able to keep cellular concentration of heavy metals

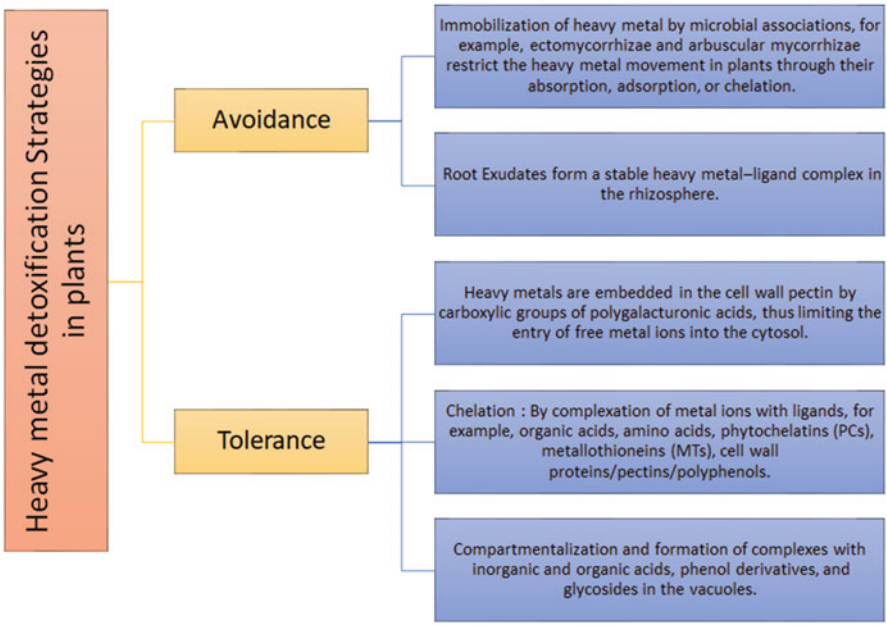


**Fig. 5.5** Avoidance and tolerance strategies used by plants against heavy metal toxicity

below toxicity thresholds (Hall 2002). Avoidance is the first defense mechanism used by plants, whereby the entry of heavy metals into plant tissues is restricted by roots, whereas tolerance is the second- and intercellular-level approach adopted by plants to deal with the accumulated heavy metal ions inside plant cells (Dalvi and Bhalerao 2013). Various avoidance and tolerance approaches adopted by plants against heavy metal toxicity are depicted in Fig. 5.5.

***Plants tolerate the toxic metal concentration in the cytoplasm by complexation and chelation of metal ions with organic acids, thus reducing their bioavailability. Plants accumulates various metabolites in their cytoplasm in order to tolerate or detoxify the effects of high heavy metal concentration. Different metabolites and their roles are discussed in Table 5.4. Kramer et al. (2000) reported chelation of nickel (Ni) by citrate and accumulation in the vacuoles of leaves of the hyperaccumulator *Thlaspi goesingense*. Similarly, chelation and accumulation of Cd in the leaves of *Solanum nigrum* by acetic acid and citric acid was reported by Sun et al. (2006). Sun et al. (2011) observed a positive correlation between the Cd concentration and both tartaric and malic acids in the leaves of *Rorippa globosa* and in *Rissopsetia islandica* the rise in acetic acid levels was observed with Cd concentration, thus suggesting that the accumulation of Cd is associated with tartaric and malic acids in the leaves of *R. globosa* and acetic acid in *R. islandica* (Sun et al. 2011). Similarly, accumulation of amino acids such as proline is one of the strategies used by plants to avoid environmental stress (e.g., heavy metals, salt, water, UV radiation, and excess and deficiency of minerals). It has been observed that oxidative

**Table 5.4** Metabolites and their role in plants to avoid toxic effects of heavy metals

| Metabolites | Role in plants to avoid toxic effects of heavy metals |
|---|---|
| Organic acids (such as citric acid, oxalic acid, and malic acid) | Chelates metalloids inside the cells and reduce their toxic effect |
| Amino acids (proline, asparagine, cysteine, etc.) | Proline works as an osmolyte, radical scavenger, and macromolecule stabilizer<br>Asparagine plays key role in metal–asparagine complex and reduce heavy metal stress<br>Cysteine is a key metabolite in antioxidant defense and metal sequestration. Cysteine is also required in methionine and glutathione (phytochelatins) synthesis |
| Heat-shock proteins (HSPs) | HSPs are expressed or produced in response to stress like high temperature and heavy metals. Work to protect and repair the proteins under stress condition. HSPs also protect the membrane against metal damage |
| Betaines | Betaines (glycine betaines) observed to accumulate under water stress and in metal stress also |
| Metallothioneins (MTs) | Intracellular complexation. Metallothioneins are cysteine-rich metal-binding proteins/peptide ligands |
| Phytochelatins (PCs) | Intracellular complexation. Phytochelatins are also metal-binding polypeptides/peptide ligands, help to sequester and detoxify toxic metal ions |

stress is one of the most common effects of heavy metal toxicity in plants; thus, enhanced antioxidant capabilities of hyperaccumulators make it possible to tolerate high heavy metal concentrations (Peer et al. 2005).

Chelation of toxic metal ions followed the compartmentalization of the heavy metals in the vacuoles in order to reduce their toxic effects on other cell functions (Sheoran et al. 2010). Various studies mentioned certain secondary metabolites and high molecular weight compounds released from root which influence the root–microbe relation (Ahmed et al. 2018). Thus, heavy metal uptake by plants involves a series of processes which starts with heavy metal mobilization followed by root uptake, xylem loading, transportation from root to shoot, cellular compartmentation, sequestration, and volatilization (Peer et al. 2005).

## 5.7 Role of Microbes in Heavy Metal Removal

Microorganisms such as bacteria, fungi, yeasts, and microalgae possess great potential for the remediation of heavy metal–contaminated sites. Microorganisms possess certain resistance mechanisms against the metal toxicity which allow microbes to survive in heavy metal–contaminated environment (Fig. 5.6). The metal resistance mechanisms include (a) exclusion by permeability barrier, (b) intracellular sequestration by protein binding (cysteine-rich metal-binding protein, e.g., metallothionein), (c) extracellular sequestration, (d) active transport/efflux system, (e) enzymatic reduction to less toxic forms, and (f) reduction in the sensitivity of

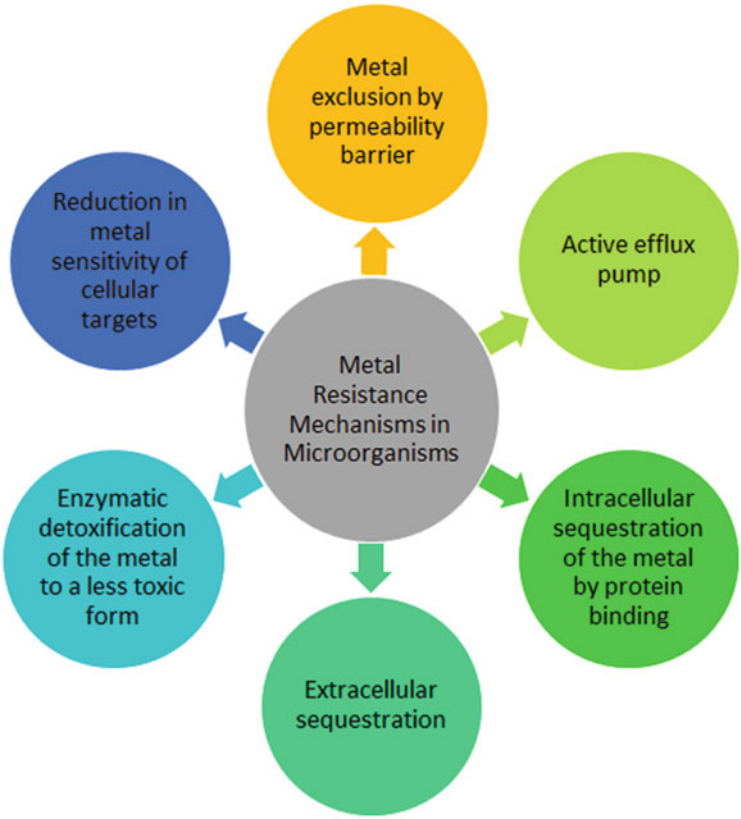


**Fig. 5.6** Mechanisms of metal resistance in microorganisms

cellular targets to the metal ions (Ji and Silver 1995; Bruins et al. 2000; Ramasamy et al. 2007). Microbes play a vital role in modifying the bioavailability of heavy metals simply by solubilizing and/or immobilizing them, hence can be exploited for the treatment of heavy metal–contaminated sites (Ramasamy et al. 2007). Microbes interact with heavy metals through different mechanisms, namely biosorption and bioaccumulation, biomineralization, bioleaching, and bioimmobilization (enzyme-catalyzed transformations), which can be used to remediate the heavy metal–contaminated sites.

Biosorption is a passive mechanism of heavy metal sequestration, which uses living or dead cell biomass. In the process of biosorption the metal ions are stick through surface complexation onto the cell surface. Heavy metals interact with different functional groups available on microbial cell surface. Bacterial cell surface possesses variety of anionic ligands such as carboxyl, amine, hydroxyl, phosphate, and sulfhydryl groups are known to bind heavy metals. Living microbial cells are preferred by many workers for the biosorption of heavy metal due to their continuous metal uptake and self-replenishment characteristics (Hajdu et al. 2010; Shamim

2018). Microalgae can also biologically sequester heavy metals in aquatic environment. Microalgae possess great potential to bind metals on their cell surface and also to intracellular ligands. The availability of large surface-to-volume ratios, the presence of high-affinity groups and metal-binding groups (amino, sulfate, and carboxyl groups) are important features that enable microalgae to sequester metals. The distribution and abundance of cell wall components may vary across different groups of algae; as a result, the types of functional groups also vary.

Bioaccumulation is an active process in which microorganisms built up the heavy metals metabolically into the cellular interior. Heavy metals transport through microbial cell wall into the cytoplasm and becomes immobilized in the cell (Ramasamy et al. 2007). Biomineralization is the process of mineral formation associated with microbial transformation of metal ions into amorphous or crystalline precipitates. Dhami et al. (2017) studied two isolates of ureolytic fungi, namely *Aspergillus* sp. UF3 and *Fusarium oxysporum* UF8 for their biomineralization and metal recovery potential. The two isolates showed significant production of calcite and a coprecipitation of Pb and radionuclide strontium (Sr) in the form of carbonates (Dhami et al. 2017). Microbes by the processes of leaching, chelation, and redox transformation mobilize heavy metals from the contaminated site. Bioleaching is the process microbial extraction/leaching of metals from their ores. Many microorganisms through the enzymatic and nonenzymatic process reduces the heavy metals and other trace elements. The enzymatic reduction uses the metals as electron acceptors. The oxidized metals are highly soluble and have a potential to contaminate the water, while reduced metal forms are insoluble. A wide range of metal reducing bacteria can reduce the chromate ions (soluble) to Cr(III), which precipitate as $Cr(OH)_3$ (Ramasamy et al. 2007).

Microorganisms in the rhizosphere also play an important role in phytoremediation of heavy metals. These microbial communities are classified into two groups, namely, mycorrhizal fungi and plant growth–promoting rhizobacteria (PGPR). Mycorrhizal fungi such as arbuscular mycorrhizal fungi (AMF) exhibit mutualistic association with most plants and benefits them (Marques et al. 2009). Plant growth–promoting rhizobacteria can be classified into two major groups: (a) symbiotic and (b) free living rhizobacteria. PGPR enhance the tolerance in plants against the various stress, such as flood, water deprivation, and salt stress. According to the relationship of PGPR with plants, PGPR can be broadly classified into two major groups, namely (a) symbiotic rhizobacteria, also known as intracellular PGPR (e.g., nodule bacteria), and (b) free-living rhizobacteria, also known as extracellular PGPR (e.g., *Bacillus, Burkholderia, Azotobacter*, and *Pseudomonas*). The symbiotic PGPR invade the interior cells of the plants and survive there, while the free-living ones exist outside the plant cells. Nutrients (for example amino acids, organic acids, and sugar) exuded from the plants' roots influence the healthy concentration of rhizospheric bacteria. Plant growth-promoting rhizobacteria produces different growth-regulating compounds. The low molecular weight (400–1000 K Dalton) organic compounds produced by PGPR are known as siderophores that helps to solubilize or chelate unavailable forms of heavy metals by complexation reaction and make them available for microbial and plant cells (Pal and Rai 2010). Various

**Table 5.5** PGPR and their associated growth-regulating compounds

| Plant growth-promoting rhizobacteria (PGPR) | Plant growth-regulating compounds |
|---|---|
| *Pseudomonas fluorescence* | Siderophores |
| *Bacillus, Azotobacter, Pseudomonas, and Azospirillum* | Indole acetic acid (IAA), and phosphate (P)-solubilization |
| *Micrococcus luteus* | IAA and P-solubilization |
| *Variovorax paradoxus*, *Flavobacterium*, and *Rhodococcus* sp. | IAA and siderophores |
| *Bacillus subtilis* | IAA and P-solubilization |
| *Brevibacterium* sp. | Siderophore |
| *Brevibacillus brevis* | IAA |
| *Pseudomonas and bacillus* | Siderophores, IAA, and P-solubilization |
| *Bacillus* spp. | IAA, siderophores, P-solubilization, HCN, and ammonia |
| *Azotobacter, Pseudomonas fluorescens*, *and Bacillus* sp. | IAA, siderophore, ammonia, HCN, and P-solubilization |
| *Pseudomonas* spp., and *Bacillus megaterium* | IAA, siderophore, and P-solubilization |
| *Pseudomonas chlororaphis* and *Arthrobacter pascens* | P-solubilization |
| *Achromobacter xylosooxidans* | IAA, P-solubilization |
| *Pseudomonas* sp. | IAA, P-solubilization, and siderophores |

PGPR and their associated compounds that promotes the growth of plants are discussed in Table 5.5.

PGPR influence the growth of plants and their efficiency to accumulate heavy metals in various ways, as discussed in Fig. 5.7. PGPR like *Pseudomonad* and *Acinetobacter* have been reported to increase the phytoremediation efficiency of nonhyperaccumulating maize (*Zea mays* L.) plants by improving the growth and biomass of plants (Lippmann et al. 1995). Different microorganisms use different mechanisms for plant growth and tolerance of high heavy metal concentration. Thus, it may be advantageous to design phytoremediation processes in conjunction with appropriate microbial consortia.

## 5.8 Recent Advancement in Heavy Metal Removal Techniques

In the last few years, research on bioremediation of heavy metal has gained much attention to understand the pathways (molecular and biochemical) of heavy metal movement (i.e., uptake, transport, and storage) in plants (Giri et al. 2015). In the recent years, work has been extensively done to improve the process of bioremediation by implementing the genetic engineering tools to the agents (plants and microorganisms) used for removal of heavy metal. Thus, with genetic engineering methods appropriate genes or hyperaccumulation traits can be transferred to the plants. Heavy metal detoxification system has also been explored at molecular levels in microorganisms such as yeast and bacteria. Transfer or overexpression of genes

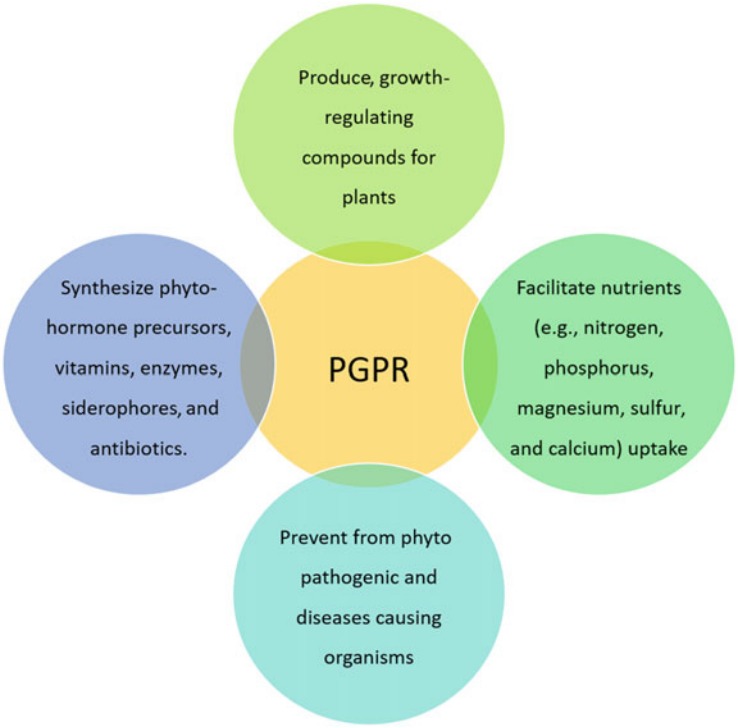


**Fig. 5.7** Different advantages of PGPR for promoting plant growth

from microorganisms into plants is being done to improve the remediation potential of plants. Such genetic manipulations in plants have already yielded promising results. Some of the genetic modifications for enhanced metal tolerance include modifications in oxidative stress–related enzymes, overexpression of glutathione-S-transferase, peroxidase, and aminocyclopropane-1-carboxylic acid (ACC) deaminase (Eapen and D'Souza 2005).

The plants with high biomass production have been proven a good candidature for successful hyperaccumulation of heavy metals and genetic manipulations. Some of the high biomass producing plants are Indian mustard (*Brassica juncea*), tomato (*Lycopersicon esculentum*), sunflower (*Helianthus annuus*), and yellow poplar (*Liriodendron tulipifera*) (Eapen and D'Souza 2005). Chemically treated stems of *H. annus* have been used to optimize the adsorption of Cd (II) ions from water and statistical results confirmed 99.8% removal efficiency under optimized conditions (Jain et al. 2021a). Plants like *B. juncea*, *Nicotiana tabacum*, and *Populus angustifolia* have been extensively studied for genetic modification to enhance the heavy metal accumulation compared to their wild type. Van Huysen et al. (2004) reported the enhanced affinity for selenium (Se) uptake in transgenic *B. juncea* overexpressing ATP sulfurylase (APS transgenics) and cystathionine-gamma-synthase (CGS) than the wild variety. The two Indian mustard plants, with

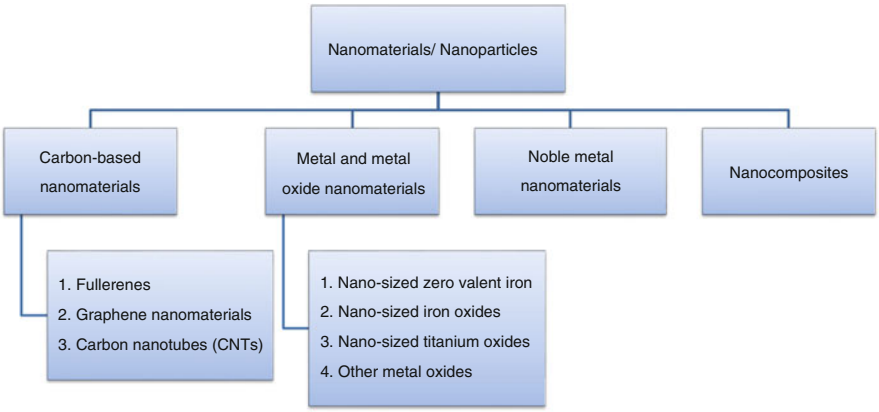


**Fig. 5.8** Classification of nanoparticles

overexpressed genes encoding selenocysteine lyase (cpSL) and selenocysteine methyltransferase (SMT) enzymes were observed to possess great potential for the accumulation of Se from the contaminated soil (Bañuelos et al. 2007). Transgenic plants have proved to be a promising biotechnological approach for the bioremediation of heavy metal–contaminated soil.

In recent years, nanotechnology has also received considerable attention for its application in heavy metal remediation technologies. Nanomaterials are unique in their characteristics, that include nano size (≈1–100 nm size), high mobility in solution, high surface area-to-volume ratio, and high adsorption capacity and reactivity that make them suitable for use in remediation technologies (Yu et al. 2021). Nanomaterials can be of a variety of shapes, sizes (on nanoscale), and functions. Compared to conventional treatment processes, application of nanomaterials possesses various advantages over the conventional treatment practices, that includes enhanced reactivity, unique surface chemistry (i.e., target specific functional groups on surface), and physical properties of nanoparticles. Various nanomaterials can be grouped into carbon based, metal oxide based, noble metal nanomaterials, and nanocomposites (Fig. 5.8).

Graphene oxide is a carbon-based nanomaterial comprises a variety of functional groups (hydroxyl, carboxyl, carbonyl, and epoxy group) for the adsorption of metal contaminants (Lü et al. 2012). Many workers reported graphene oxide for its heavy metal adsorption potential. Ding et al. (2014) studied the adsorption capacity of graphene oxide layered fixed bed sand column for the removal of heavy metals (Cu (II) and Pb(II)) from aqueous solution. Nano-sized metal oxides have also been reported for their remarkable affinity toward the heavy metals such as Pb(II), Cu(II), Ni(II), Mn(II), Ni(II), Cd (II), and Cr(VI) (Engates and Shipley 2011). Jain et al. (2021b) studied the efficient removal of divalent nickel ions from aqueous media through adsorption by copper oxide nanoparticles and inferred that the technique can be utilized for effective sequestration of Ni (II) ions from wastewater. Nanoparticles can be used in a variety of approaches to remediate contaminated environment.

Different approaches used for the treatment of inorganic and organic contaminants include adsorption, separation, catalysis, photocatalysis, and disinfection, as discussed below.

1. *Adsorption:* Adsorption is a surface phenomenon of the adsorbent. Nanomaterials have unique features such as high adsorptive capacity, specific affinity toward the contaminants, and large surface area for adsorption, which make them a good candidate to be applied in treatment plants. The adsorptive capacity of nanomaterials can be enhanced by some structural improvements.
2. *Separation:* This includes processes filtration, size exclusion, and reverse osmosis. Nanofiltration membranes are especially designed to remove inorganic and organic contaminants from wastewater. Properties of nanofiltration membrane include high flux, high retention of anionic salts, and low maintenance and operational cost.
3. *Catalytic and photocatalytic activity:* Nanocatalysts and photocatalysts improve the chemical reactivity by enhancing the production of oxidative species at the material surface. $TiO_2$ is the most extensively studied nanophotocatalyst.
4. *Disinfection:* Nanoparticles can possess the properties of pollutant remediation as well as disinfectants. The carbon-nanofiber composite $TiO_2$/ZnO has been observed to treat toxic chemical dye and microbial contamination such as *Escherichia coli* (*E. coli*). The nanoparticles showed excellent antimicrobial activity along with fast adsorption and methylene blue degradation ability (Pant et al. 2013).

Various nanoparticles utilized for wastewater remediation are carbon–$TiO_2$ nanotubes, carbon–ZnO, graphdiyne–ZnO, graphene–$SiO_2$/$Cu_2O$, graphene–$SiO_2$ nanoplatelets, multiwalled carbon nanotube–metal-doped ZnO nanohybrid, carbon nanoparticles–gold, platinum nanoparticle, carbon aerogel–$TiO_2$, carbon nanotube-$Ag_3PO_4$ in Pickering emulsions, multiwall carbon nanotube–$TiO_2$–$SiO_2$, carbon–nitrogen-doped $TiO_2$–$SiO_2$, carbon nanofibers–Ag–$TiO_2$, carbon–Ag–$TiO_2$, silver nanoparticles, and so on. Development of new nanomaterials has advanced the present treatment techniques, but more research is still needed to make the process sustainable.

## 5.9 Advantages and Limitations

Bioremediation techniques are more economical than conventional methods because low installation and maintenance cost. The most of the pollutants get treated on the site of contamination which reduced the exposure risk to other biotic and nonbiotic components of the environment. The technique is more publicly accepted since it is based on natural attenuation. Further, bioremediation has the potential to eliminate or degrade a wide variety of pollutants completely and permanently. It can be operated on larger scale and can easily be coupled with other physical and chemical methods of remediation. It also does not let the transfer of contaminants from one

environmental medium to other. Therefore, bioremediation offers a less energy-intensive, cost-effective, and yet efficient option to clean the environment. Advantages of phytoremediation includes recovery of precious metals, improvement in soil fertility, and decline in metal leaching and erosion.

Along with large number of benefits, some drawbacks are also associated with bioremediation. The process can be effective under certain environmental conditions which are required to be manipulated for enhanced microbial growth and faster degradation rate. There are also some compounds which are resistant to microbial attack such as chlorinated organic pollutants, high aromatic hydrocarbons, and radionuclides. The time scale of the process is relatively long, and also, the appropriate residual levels of contaminants may not always be achieved. The implementation of technique requires huge experience and expertise. Sometimes small-scale laboratory studies are required to be done before actual implementation in the field. The limitations associated with phytoremediation includes longer time scales, concentration of pollutants or contaminants and their bioavailability to plants, toxic effect of pollutants on plants and inability to degrade organic contaminants due to lack of specific degradative enzymes.

## 5.10 Application and Future Prospects of Bioremediation

Bioremediation technologies are more appropriate and offer many advantages compared to traditional treatment methods, such as cost-effectiveness, high efficiency, and reduced secondary waste production. These techniques also provide flexibility to work continuously, regeneration of biomass and metals recovery. Bioremediation with the recent advancement is becoming a widely acceptable and economically viable technology. Over the last decade the scientific community gathered information on potential modification of remediation processes for heavy metal removal on large scale. These processes include identification of low-cost and commercially applicable biosorbent, and development of transgenic plants and nanomaterials for remediation of heavy metals. Biosorption has been proven as low-cost technology to remediate the heavy metal–contaminated effluents and has received a great attention. Inexpensive biosorbents have been used to detoxify effluents from the metal plating, extraction, and ore-mining operations, as many research works have demonstrated the biosorption as an advantageous alternative to traditional treatments methods (Vijayaraghavan and Yun 2008). However, optimization of the process is required in order to understand the metal–microbe interaction, and regeneration of the material (biosorbent) for effective removal of the contaminants. The nanotechnological approach has contributed an extraordinary adsorption capacity and reactivity to the adsorbent that promotes heavy metal removal. Microbes are pervasive and grow rapidly, becoming habituated to varying concentrations of different toxic metal ions. Genetically engineered microbes (GEMs) have made the microbial remediation more effective, but their applications on the ground have their own concerns such as legality, ethics, and biosafety. Efforts are under

way to achieve a better molecular understanding of mobilization, absorption, translocation, and accumulation of metals in plants.

For efficient phytoremediation of soils contaminated by heavy metals, the activity of plant symbionts in rhizosphere is necessary. Application of mycorrhizal fungi and plant growth-promoting (PGP) bacteria would benefit plant growth and facilitate the mobilization and bioavailability of heavy metals. Pramanik et al. (2017) reported the *Klebsiella pneumonia* K5, a PGPR strain highly resistant to cadmium, possessed several PGP characters, such as nitrogen fixation, phosphate solubilization, and indole acetic acid (IAA) production and also confirmed multiple resistance to heavy metals such as lead and arsenite. Mitra et al. (2018) also characterized a highly Cd-resistance strain *Klebsiella michiganensis* MCC3089 that exhibited many PGP traits such as IAA production, phosphate solubilization, nitrogen fixation, and reduction of oxidative stress. Nitrogen fixation is a common mechanism in the genus because *Klebsiella* species are well-known free-living nitrogen fixers. In a recent work, the phytoremediation potential of *Zea mays* inoculated with AMF *Claroideoglomus etunicatum*, bacterial diversity (*Microbacterium, Agrococcus, Lysobacter, Planomicrobium, Streptomyces, Saccharothrix*), and various unclassified bacteria and fungi was assessed by Hao et al. 2021. The results showed that arbuscular mycorrhizal fungi (AMF) facilitate the revegetation of heavy metal–contaminated soils through interacting with the rhizosphere microbiome (Hao et al. 2021). Thus, rhizosphere microbes are the important partner for stress tolerance in plants and bioaugmentation with AMF, and growth-promoting bacteria can be applied as a beneficial strategy for reclaiming the soil contaminated with toxic metals.

There is no question that molecular knowledge and nanotechnology have helped to explore new avenues to remediate heavy metal–contaminated sites. But more research is still needed to identify new strategies of heavy metal remediation concerning the issues relating biosafety, emerging pollutants, and efficiency of genetically engineered microbes, and transgenic plants. Future research is required aiming at the experimental approach for data collection from multidiscipline and mathematical modeling to achieve better prediction. And for better environmental application, the generated experimental data need to be integrated into different approaches to test the bioremediation effectiveness.

## 5.11 Conclusions

Bioremediation proves to be a fruitful and attractive approach to clean, manage, remediate, and recover the contaminated sites through indigenous or extraneous microbial activity. In recent era, where other physical, chemical, or mechanical methods are very costly and tedious to be put into implementation, bioremediation offers a low-cost and efficient approach toward a cleaner and greener environment. The effectiveness of the technique however depends on thorough understanding of microbial communities, their response to natural and contaminated environment, knowledge of genetic capabilities of microbes to degrade toxic pollutants. Also, the

success requires frequent cost-effective field trails on sites specifically dedicated for research purpose. The speed of the process both in situ and ex situ is determined by competition within biological agents, adequate supply of essential nutrients, other environmental or abiotic factors such as oxygen supply, temperature, pH, moisture, and bioavailability of the contaminants. Therefore, to be more successful, bioremediation is carried out in manipulated environments rather than natural environments. Further, this review provides an insight in to the recent technologies such as use of nanoparticles for heavy metal removal from environmental components. More research is needed in the areas of commercially acceptable biosorbents, development of transgenic plants and advancements in nanotechnology to efficiently remediate the heavy metal–contaminated effluents. Regardless of certain limitations, the more advantages of bioremediation technology make it an acceptable, efficient, cost-effective, and green approach toward a clean environment.

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# Enzyme Technology for Remediation of Contaminants in the Environment

6

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**Abstract**

Enzymes are essential components that help in maintaining proper climate in numerous ways. They are used for natural purposes in various ventures including agro-food, oil, creature feed, cleanser, mash and paper, material, calfskin, petrol, and strength substance and biochemical industry. Proteins likewise help to keep an unpolluted climate through their utilization in squandering the board. Compounds have an incredible possibility to adequately change and detoxify dirtying substances since they have been perceived to have the option to change toxins at a recognizable rate and are conceivably reasonable to reestablish contaminated conditions. Compounds are utilized to make and work on almost 400 regular purchaser and business items. They are used for processing many types of foods and beverages, animal nutrition, materials, household cleaning, and fuel for automobiles and the energy age. The most important enzymes used in bioremediation include cytochrome P450, lipases, proteases, dehydrogenases, dehalogenases, hydrolases, and laccases. These enzymes have demonstrated promising potential for degrading polymers, fragrant hydrocarbons, halogenated compounds, colors, cleansers, agrochemicals, and other chemicals. Research areas in enzyme development and their significance for future advancement in natural biotechnology are discussed.

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**Keywords**

Bioremediation · Contaminants · Pollutants · Enzymes · Cytochrome P450

## 6.1 Introduction

Several compounds that have a high potential for contamination are present in the environment and have an impact on soil, air, water, and living things like plants, animals, and people. They may be taken from one or every natural compartment (Singh and Walker 2006). These potential contaminants are always present as mixtures of different standard mixes that are identical to common and inorganic ones. Current activities like mining and metal managing, petrochemicals and modern structures, effluents, the production of chemical weapons, experiences with paper and ink, concealing associations, and current social events stand out as the origins and wellsprings of contamination. Anthropogenic activities like traffic, plant practices, and others also contribute to contamination (Karigar and Rao 2011). Toxins might affect the thriving of people, creatures, and conditions for quite a long time. Bioremediation is a microorganism mediated biodegradation and/or transformation of toxic compounds into nonhazardous or less-unsafe compounds compared to parental compounds. The employability of various customary parts like living things, parasites, green new development, and plants for persuading bioremediation regarding noxious substances has been represented (Singh and Walker 2006). The use of plants in the remediation of toxic substances is called phytoremediation. It is a promising and environment frienldy phytotechnology that works with the ejection or pollution of unsafe fake materials in soils, sediments, wastewater, sludge, and air. Additionally, internally structured plants are used in bioremediation process (Karigar and Rao 2011). In this context, arsenic is phytoremediated by normally changed plants, for example, *Arabidopsis thaliana* which passes on two bacterial qualities. One of these characteristics allows the plant to change arsenate ($As^{5+}$) into arsenite ($As^{3+}$) and the subsequent one ties the changed $As^{3+}$ and stores it in the vacuole (Singh and Walker 2006).

## 6.2 Enzyme as Contaminant Sterilizing Agent

Enzyme-based remediation of pollutants is a more common and optimistic technology than microbial remediation, which has advantages over conventional advancements Compounds are not limited by inhibitors of microbial metabolism (Haritash and Kaushik 2009). They can be used under trivial conditions which limit the growth and development of microorganisms. They are strong at low contamination process and dynamic when observing microbial antagonists or trackers (Burns et al. 2013). They act against a given substrate (microorganisms may lean toward more adequately degradable blends than the contaminants) and are more adaptable than microorganisms because of their more unobtrusive size. This huge number of

characteristics renders substances eco-friendly catalysts as well as enzymatic methods harmless to biological system operations (Kurtzman et al. 2011; Chandra et al. 2017). It may act both extracellularly and/or intracellularly. Hydrolases, dehalogenase, transferase, and oxidoreductases are the most specialist enzymatic classes. Their essential creators are bacteria, fungi, plants, and microbial-plant associations (Burns et al. 2013).

## 6.3 Pollutants

Pollutants are components, atoms particles in pollution-life can be affected when presented to these materials, and the impacts of them on people and plants are notable. Toxins can be brought into the climate in numerous ways, both normally and by people. The type of pollutant determines what happens to poisons whenever they are radiated into the air, soil, or water supply (Thatoi et al. 2014).

1. Organic Pollutants
2. Inorganic Pollutants

### 6.3.1 Organic Pollutants

Organic contamination is biodegradable toxins in a climate. These sources of contamination are normally found and brought about by the climate (Solís et al. 2012).

1. Nitro compounds
2. Dyes
3. Organophosphorus hydrolase
4. Cytochrome P450 monooxygenase

#### 6.3.1.1 Nitro Compounds

There are two specific enzymatic pathways for the contamination of nitrile, any organic chemcials that have a $-C\equiv N$ group. One is a two-experience degradation including nitrile hydratase and anúdase through an amide as a transitional (Haritash and Kaushik 2009). The second process, which is catalyzed by nitrilase, is the quick hydrolysis of nitriles to the associated acids and stomach settling agent. Nitrilases, a member of the nitrilase superfamily's setup branch 1 enzyme, fuse non-peptide carbon/nitrogen (C/N) bonds that have escalated (Kurtzman et al. 2011). They are tested on by the brief animals, for example, *Nocardia* sp., *Rhodococcus* sp., and animals, as *Aspergillus niger* or *Fusarium solani.* A piece of the nitrilases is really great for hydrolyzing nitriles sound structure unequivocally. There is a lot of information available regarding the structure and breaking point of bacterial nitilases, but less information is available regarding nitilases from filamentous living things. One of the advantages of parasitic nitrilases is their high unequivocal

improvement toward organic contaminants, for example, benzonitrile and analogs, 3-and 4-cyanopyridine, and in addition, a few medium chain length aliphatic nitriles mulled over their exceptional substrates. For example, as a segregrated and bacterial nitilase, a niger nitrilase made the choice to convert an enoromous amount of chemicals quickly (Terry and Banuelos 2020). The biotechnological effect of nitrilases lies in their ability to perceive a wide degree of alicyclic and aliphatic nitriles; to hydrolyze nitriles in fragile environment, with astounding regio- and enantioselectivities from time to time; to show high movement, ampleness, and thermo-steady quality. This in turn creates blend phenomenal doors for biodegraders of nitrile new substances (Burns et al. 2013).

#### 6.3.1.2 Dyes

Azo dyes are broadly used in material, agro-food, medication, textiles, and restorative endeavors. Standard degrees of progress would have terrible and harmful effects if they were continuously used to decolorize in a manner similar to crash azo-shadings. Consequently, the use of a compound prepared for oxidizing and moreover discarding azo shades is a partner with choice rather than these limited and unsafe prescriptions. Unquestionably, white-rot living things help to approach and deal with the issue. They are hiding degraders, and particular powerful friendly orders have the entrancing brand name for conventional utilization of making different profiles of lignin-mineralizing mixes and instances of their appearance depending upon the planned new development and significant gatherings of the tones being demolished.

#### 6.3.1.3 Organophosphorus Hydrolase

Organophosphates are chemical substances that have been used extensively as insecticides, in manufacturing, and even as medicines since 1937. They are highly toxic to neurons, and finally, they were more than that soil microbiota could fix every one of them. Organophosphorus hydrolase (regardless called phosphotriesterase) is one of the updates that can serve for organophosphorus raises bioremediation (Chandra and Kumar 2015). Although its parasitic strategy is passed on to *Penicillium lilacinum* and *Aspergillus niger*, it is generally removed from *Pseudomonas diminuta*. It can return again to P–F, P–O, and P–S, bonds. This compound has zinc ions ($Zn^{2+}$) as a cofactor in its close by game plan, while tests showed that replacement of $Co^{2+}$ gives the utmost uncommon movement against paraoxon. This protein has the quickest synergist rate and is the most supporting accumulate for getting sorted out movement against organophosphates (Thatoi et al. 2014).

#### 6.3.1.4 Cytochrome P450 Monooxygenase

A group of heme-containing enzymes known as cytochrome P450 monooxygenases (CYP; EC 1.14.14.1) catalyzes a variety of reactions, including the hydroxylation of C–H bonds, oxidative dehalogenation, O-dealkylation, N-dealkylation, and N-hydroxylation. CYP gathers tremendous electrons for responses from NADPH-cytochrome P450 reductase, and the last protein gets electrons from air oxygen. Therefore, the presence of a master that is depleting, such as NAD(P)H or FAD, is

crucial. CYPs are versatile proteins introduced in different sorts of microorganisms, improvements, plants, and creatures. Around 7000 clear CYPs have been found till date (Kurtzman et al. 2011).

#### 6.3.1.5 Peroxidase from Horseradish

Horseradish peroxidase (HRP) is a peroxidase protein that is generally taken out and cleaned from the horseradish root (*Armoracia rusticana*). C isoenzyme is the most adequate isoenzyme perceived in horseradish root (HRPC) (Terry and Banuelos 2020). HPRC is a heme-containing glycopeptide having an iron piece in the ferric state in protoporphyrin IX, 308 amino acids, and two calcium molecules in the focal zone that has an atomic stack of 44 kDa. HRP is a compound that catalyzes the oxidation of $H_2O_2$. Right when $H_2O_2$ is free, a two-electron oxidation moderate is produced. Compound I is then lessened to uplift II by an oxidizing substrate. These responses are utilized to recover the first compound (Kaur et al. 2016).

### 6.3.2 Inorganic Pollutants

Inorganic toxins are substances or mixtures that are found in water sources and may be naturally occurring due to geopgraphy or caused by human activity in the form of mining, industry, or horticulture.

1. Mercury
2. Lead
3. Chromium
4. Arsenic

#### 6.3.2.1 Arsenic

Arsenic (As) is a toxic metal that exists on earth in run of the mill and inorganic plans. The inorganic plans $As^{3+}$ and $As^{5+}$ are harmful and can reason gangrene, keratosis, hemolysis, carcinoma, impulse inactivation, and cardiovascular and neurological sicknesses (Reddy and Mathew 2001). $As^{3+}$ and $As^{5+}$ convert with the associate of utilizing arsenate reductase and arsenite oxidase through redox responses. $As^{3+}$ is a more perceptible cell and destructive. $As^{5+}$ is the terminal electron acceptor withinside the lack of oxygen ($O_2$) and diminishes to $As^{3+}$. Ferredoxin or glutathione will be the electron supply. This technique enhances As's capacity to dissolve and helps with soil drainage (Couto and Herrera 2006). Unquestionably the last $As^{3+}$ is delivered through efflux siphons, ArsB and Acr3. Arsenite oxidase changes $As^{3+}$ to less noxious $As^{5+}$ for utilizing both supplemental power supply or as an electrom advocate for carbon dioxide ($CO_2$) obsession. Naturally, the final $As^{5+}$ is still present and may be maintained with the aid of silt (Ullah et al. 2000).

The methylated condition of As is dangerous and might be lost from the soil. Unusually, in methanogenic microorganisms, methylation is joined with methane

biosynthesis and may detoxify soil through this framework. Coenzyme M is the biocatalyst of this cleansing system (Kurtzman et al. 2011).

#### 6.3.2.2 Chromium

Due to its high oxidative potential, which has been shown to have teratogenic, mutagenic, and cell-damaging effects, chromium (Cr) is a hazardous crucial metal. The wide usage of Cr and its mixes and mining applies this poison to soil and water (Haritash and Kaushik 2009). Bioremediation of hexavalent chromium ($Cr^{6+}$) mainly involves the transformation of $Cr^{6+}$ to trivalent ($Cr^{3+}$) species as non or less toxic form of Cr. *Enterobacter*, *Escherichia*, *Bacillus*, and *Pseudomonas* are several genera that are impervious to Cr and can decrease it. Consumption of anaerobic pathway may result in decrease in $Cr^{6+}$. Under anaerobic condition, dissolvable cytoplasmic blends are involved and decline $Cr^{6+}$ in two stages (Hermansyah et al. 2007). created substances offered a clarification to have $Cr^{6+}$-diminishing movement. Additionally, $Fe^{2+}$ and S2-transmitted in immediate second normal portions can lower $Cr^{6+}$ much more quickly than chromate-diminishing microorganisms (Cheung and Gu 2007).

#### 6.3.2.3 Mercury

The poisonous metal mercury (Hg) causes harm in both organic and inorganic structures, but the normal course is more dreadful. Hg harming inclination would cause neuro- and nephrotoxicity, responsive qualities, and shortcoming to talk (Rezende et al. 2005). Hg is an essential component of the Earth's body, but it also distributes and enriches water and soil due to activities like gold mining, indisputable evaluation devices (such as checks, thermometers, and manometers), lights, fluctuating fungicides, the paper industry, and battery cells. Hg exists in three plans: mercuric ($Hg^{2+}$), mercurous ($Hg^{+1}$), and metallic mercury ($Hg^{0}$) structures (Reddy and Mathew 2001). The most ruinous sort of Hg is mercuric chloride. Normal mercury has a tendency to accumulate in living things and is fond of the sulfhydryl social relationships of proteins. Inorganic mercury has the most insignificant danger considering its low dissolvability and high smoke pressure. Hg-safe microorganisms can diminish risky conventional sorts of Hg to less hazardous metallic Hg (Canfora et al. 2008). Mercuric reductase is the central compound that lessens Hg. The mer operon is the arrangement of mercury-resistance characteristics mentioned inside seeing an inducible centralization of Hg. Mercuric reductase assist NADPH and FAD, as electron sources, that diminishes $Hg^{2+}$ to $Hg^{0}$. The last metallic mercury is flighty and spreads to the air. Similarly, dimethylmercury is temperamental and biomethylation can fill in as a technique for Hg bioremediation (Burns et al. 2013).

#### 6.3.2.4 Lead

Lead (Pb) was found in a common aggregate in nature before industrialization. However, over time, by gas eating up, various Pb salts start to enter and contaminate the air, soil, and water. Pb perniciousness may result in whitening, difficulty, and neurological, gastrointestinal, and conceptual problems (Xu 1996). Organic leads,

especially tetraethyl and tetramethyl Pb used in gas, are hazardous sorts of Pb. They are fragile to photolysis and volatilization and rascal to dialkyl species. All things considered, a few microbes can destroy Organo leads using bioremediation techniques. *Cupriavidus metallidurans* can dispose of $Pb^{2+}$ with P-type ATPase and produce inorganic phosphate to sequester $Pb^{2+}$ in the periplasm (Cipollone et al. 2006). Whereas *Staphylococcus epidermidis* can biomineralize $Pb^{2+}$ through carbonate. Urease compound arrangement organizes carbonate glasslike $Pb^{2+}$. It will generally be mineralized as oxalate and pyromorphite, as well. *Aspergillus terreus*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Penicillium chrysogenum*, *Penicillium canescens*, *Rhizopus nigricans*, and *Agaricus bisporus* are among biochanging living creatures. Moreover, it is tended that *Phaeolus schweinitzii* and *Arthrobacter* can destroy trimethyl lead cations (Ullah et al. 2000).

## 6.4 Microbial Enzymes in Bioremediation

### 6.4.1 Microbial Oxidoreductase

The detoxification of harmful typical mixes by different minute animals and advancements and higher plants through oxidative coupling has interceded with oxidoreductases (Cheung and Gu 2007). Microorganisms separate energy through energy-yielding biochemical responses interceded by these stimuli to segment designed insurances and to help the exchanging of electrons from a decreased customary substrate (ally of) another substance compound. During such oxidation-decay responses, the toxins are at last oxidized to innocuous blends (ITRC 2002) (Couto and Herrera 2006). The oxidoreductases examine the humification of various phenolic compounds that are produced when lignin is debilitated in a muddy environment. Basically, oxidoreductases can detoxify pernicious xenobiotics, like phenolic or anilinic compounds, through restricting to humic substances, copolymerization with different substrates, or polymerization or microbial combinations that taken advantage in the degradation azo dyes (Coppella et al. 1990).

Different life forms lessen the radioactive metals from an oxidized dissolvable plan to a decreased insoluble development. All through energy creation, bacterium takes up electrons from typical mixes and utilizes radioactive metal as the last electron acceptor (Terry and Banuelos 2020). With the aid of a midway electron ally, some bacterial species degrade radioactive elements in an indirect manner. At long last precipitant can be viewed as the aftereffect of redox responses inside the metal-lessening microorganisms (Kaur et al. 2016).

The plant social events of Solanaceae, Gramineae, and Fabaceae are found to pass on oxidoreductases which partake in the oxidative contamination of express soil constituents (Ullah et al. 2000). Phytoremediation of normal pollutions has been generally speaking rotated around three classes of blends: oil hydrocarbons, explosives, and chlorinated solvents (Cheung and Gu 2007).

### 6.4.2 Microbial Laccases

Laccases (*p*-diphenol: dioxygen oxidoreductase; EC: 1.10.3.2) are a collection of multicopper oxidases produced by unequivocal plants, parasites, terrible tiny animals, and tiny living things. They catalyze the oxidation of a wide range of reduced phenolic and non-phenolic compounds with effective reduction of atomic oxygen to water. Laccases are known to occur in various isoenzyme shapes which are all encoded by a substitute quality, and, now and then, the attributes have been bestowed diversely relying upon the chance of the inducer (Filazzola et al. 1999). Various microorganisms secrete intra- and extracellular laccases that fit for catalyzing the oxidation and depolymerization of lignin, melanoidin, polyamines, polyphenols, aminophenols, ortho and paradiphenols, and aryl diamines (Rezende et al. 2005; Kumar and Chandra 2018; Kumar et al. 2022). These proteins are secured with the depolymerization of lignin, which accomplishes an assortment of phenols. According to the educated experts, laccases address a hypnotic combination of inescapable oxidoreductase catalysts that demonstrate affirmation of supplying the amazing potential for bioremediation and biotechnological applications (Thatoi et al. 2014; Agrawal et al. 2021).

### 6.4.3 Microbial Oxygenases

Oxygenases have a spot with the oxidoreductase social event of driving forces. They investigate the oxidation of substrates by moving $O_2$ from sub-atomic oxygen ($O_2$) using NADPH/NADH/FAD as a cosubstrate (Canfora et al. 2008). Oxygenases are assembled into two groups; the monooxygenases and dioxygenases subject to incorporation of number of $O_2$ molecules during oxidation of chemcial compounds. They acknowledge an essential part in the absorption of typical blends by developing their reactivity or water dissolvability or achieving cleavage of the sweet-smelling ring (Hermansyah et al. 2007). Oxygenases have a broad substrate range and are dynamic against a wide degree of chemical blend, 1′ including the chlorinated aliphatics. By and large, the presentation of $O_2$ molecules into the ordinary particle by oxygenase accomplishes cleavage of the sweet-smelling rings. For what it's worth, the most centered around compounds in bioremediation are bacterial mono- or dioxygenases. A no-nonsense assessment of the gig of oxygenases in degradation process is accessible (Xu 1996).

Due to their extensive employment as plasticizers, herbicides, insecticides, fungicides, water-driven and heat-moving liquids, and intermediates for planned amalgamation, halogenated ordinary mixes have the best concentrations of organic poisons (Durán and Esposito 2000). The corruption of these pollutions is refined by express oxygenases. Oxygenases comparably intercede dehalogenation responses of ethylenes, ethanes, and halogenated methanes in relationship with multi-functional enzymes technological and bioremediation applications (Coppella et al. 1990).

#### 6.4.3.1 Monooxygenases

Monooxygenases allow the smallest amount of oxygen molecules into chemical compounds. Monooxygenases are depicted into two subclasses subject to the presence of cofactor: P450 monooxygenases and flavin-dependent monooxygenases. Flavin-subordinate monooxygenases contain flavin as prosthetic collecting and require NADP or NADPH as a coenzyme (Kim et al. 2002). P450 monooxygenases are heme-containing oxygenases that exist in both prokaryotic and eukaryotic creatures. The monooxygenases contain a flexible superfamily of blends that catalyzes oxidative responses of substrates going from alkanes to complex endogenous atoms like steroids and unsaturated fats. Monooxygenases go about as biocatalysts in biodegradation coordinated effort and created science due to their essential region selectivity and stereoselectivity on a wide degree of substrates (Kaur et al. 2016). There are some monooxygenases that function without a cofactor, despite the fact that most monooxygenases that are anticipated in advance have cofactors. These combinations require essentially sub-atomic oxygen for their exercises and use the substrate as a diminishing specialist (Reddy and Mathew 2001).

#### 6.4.3.2 Microbial Dioxygenases

Dioxygenases are multicomponent compound frameworks that bring atomic oxygen into their substrate. Hydrocarbon dioxygenases, which have a pleasant scent, coexist with the monster Rieske nonheme iron oxygenases. These dioxygenases catalyze the oxygenation of a variety of substrates in an enantiospecific manner. Dioxygenases essentially oxidize fragrant mixes and, therefore, have applications in natural remediation (Solís et al. 2012). All individuals from this family have a couple of electron transport proteins going before their oxygenase parts. The important stone advancement of naphthalene dioxygenase has attested the presence of a Rieske ($2Fe_2S$) pack and mononuclear iron in every alpha subunit (Reddy and Mathew 2001). The catechol dioxygenases fill in as a piece of qualities system for spoiling sweet-smelling atoms in the Environment. They are found in the dirt moment living creatures and related with the distinction in fragrant antecedents into aliphatic things. The intradiol removing proteins use $Fe^{3+}$, while the extradiol dividing stimuli use $Fe^{2+}$ and $Mn^{2+}$ in a few cases (Cheung and Gu 2007).

## 6.5 Strategies for Overcoming Difficulties Associated with the Enzyme Technology

It is feasible to utilize a protein with the expectation that the outcome of the intentionally interfered reaction will be less harmful than the substrate. Additionally, given that detoxification involves a multistep cycle, such as the action of many proteins, fundamentally unambiguous microorganisms are appropriate for attaining cleaning (Thatoi et al. 2014). Whether or not proteins need cofactors, their utilization may be hazardous, adjacent to a status containing both the substance and the specific cofactor is used. Another issue in the usage of upgrades to detoxify average dirtied

soil is given by the quick pollution of the free substance by proteases passed on by soil microorganisms. The use of proteins for in situ rehabilitaion of contaminated situations may be limited due to a number of drawbacks (Kim et al. 2002). This helps to assist in destroying the reactant farthest reaches of enzymatic central objectives may depend on both the poisons to be changed and the motives in typical conventional ecological elements. In a polluted area, mixtures or made-up blends of numerous common new compounds, rather than just poison, are exposed, and the confounding idea of contamination may consolidate potential adverse or advantageous, synergistic impacts on the protein capabilities (Cipollone et al. 2006). Proteins may decrease or even lose their progression in the wake of ruining change or they may present low security and consistency under dependably coldblooded normal conditions. If their rehashed use is required, boosts may present low reusability, thus reducing the accommodation of the whole treatment. Also, at whatever point restricted mixes are used, the cost of protein segment and cleaning astoundingly hampers their judicious application, basically not actually permanently established overseeing is required. Syringaldehyde and acetosyringone showed to be the best local area individuals (Cipollone et al. 2006).

Another strategy for regulating work on the introduction of driving forces in the detoxification of contaminations is the usage of mixes immobilized on the norm and arranged sponsorships of different nature and through different immobilization frameworks. Immobilized impetuses typically have a really lengthy useful endurance and are completely consistent with physical, chemical, and common denaturing arranged specialists. Moreover, they may be reused and recovered around the culmination of the cycle (Canfora et al. 2008).

AliKhan and Husain (2007) utilized a potato polyphenol oxidase adsorbed on Celite for the efficient remediation of wastewater/disguising meandering aimlessly debased with responsive material and non-material tones, Reactive Blue 4/Orange 86, and isolated its ability and adequacy and the free compound (Coppella et al. 1990). The immobilized protein showed a higher limit in decolorizing individual material tones, presently moreover their tangled mixes (containing an organized blend of as much as four tones) and disguising profluent as isolated and the dissolvable substance. Other than greater resistance to a few degrading situations and overall more decolorizing development than the free improvement toward non-material tones, immobilized main thrust displayed superior performance (Kaur et al. 2016).

An amazingly entrancing immobilization method was executed with lacease. Enzymatic nanoreactors were contracted through noncovalent envelopment of the enzymatic protein by amphiphilic straight dendritic AB or ABA copolymers (Ullah et al. 2000). The glycoside sections in the nearby compound filled in as anchor fights for the straight dendritic copolymers, as pondered by control tests completely finished the DE glycosylated protein. The immobilization further empowered the reactant improvement isolated and the nearby main thrust (77–85 nkat $mL^{-1}$ versus 60 nkat $mL^{-1}$, exclusively). Likewise, the immobilized upgrade was steadier at raised temperatures up to 70 °C and prepared to sufficiently oxidize phenolic

compounds (Syring aldazine) and hydrophobic polyaromatic hydrocarbons (benzo [a]pyrene and anthracene) (Terry and Banuelos 2020).

By naturally occurring methodologies such as regular site worked with mutagenesis and various DNA-refreshing systems (for example, the enthusiastic break of a general public of eccentricity ascribes of a particular family followed by unexpected reassembly (Cipollone et al. 2006) degradative proteins with new or further created activities and boldness can be produced under chosen environmental conditions. Mixtures can be altered through site-specific mutagenesis or directed evolution to enhance already-existing biodegradation pathways to promote or to promote biocatalytic cycles for the synthesis of other things. It is possible to create unique pathways for the degradation of persistent mixtures where no known normal pathways exists by joining pathways "tapes" from diverse unalienable sources (Ullah et al. 2000).

Similarly, a public advantage of passing on GEMs into the environment has actuated ludicrous rules by government bodies (EPA). In like manner, scarcely any separated microorganisms have shown at the hour of field application (Couto and Herrera 2006).

## 6.6 Plants and their Associated Enzymes: A Agents for Decontamination

Phytoremediation is a viable option for reducing some of the negative effects associated with the use of improvements for in situ removal of contaminated environments. It is the in situ use of plants, their enzymes, and associated microbes to break down, detoxify, gather, or transform chemical pollutants found in various matrices (soil, wastewater, water, and air) (Cheung and Gu 2007).

Concerning their quick circumstances in remediation processes, plants may utilize various constructions to capably take out both organic and inorganic destructive substances from a dirtied environment: (a) rhizofiltration; (b) concentration and precipitation of basic metals by roots; (c) phytoextraction, for instance, extraction of harmful substances from contaminated environment in plant tissues including roots and leaves; (d) phytodegradation, for instance, degradation of bewildering customary particles in $CO_2$ and $H_2O$ and their partaking in plant tissues; (e) rhizodegradation or plant-assisted bioremediation with signaling microbial and parasitic debasement by the presence of root fake materials and exudates in the rhizosphere; and (f) phytostabilitation, for instance, adsorption and precipitation of toxins (fundamentally metals) with an in the wake of diminishing of their compactness (Cipollone et al. 2006). The rhizosphere, or soil environment affected by plant roots, is referred to as a beautiful brand name because of the synergistic link between planrs and microbes that specifically happens here (Xu 1996).

Since plants may be lacking in catabolic pathways for the immovable degradation of poisonous substances isolated and microorganisms, research tries have been given to configuration plants with characteristics that can introduce them extra and further made contamination limits. By highlighting the traits associated with the intake, uptake, or transport of unmistakably harmful compounds in plants,

phytoremediation can certainly be made more effective. Additionally, the roots may be provided with the proper features to renew the rhizodegradation and prevent the toxins from being permanently built up (Mousavi et al. 2021).

For instance, research is regularly established on changing fabricated combinations that can play out a reaction like the best one, yet it might be difficult to apply biomolecular meaning to the bioremediation of novel poisons, which are not known to be biodegradable (Rezende et al. 2005). Considering everything, it might be possible later on when our understanding into the protein structure-work, imploding, instrument and parts will be on a very fundamental level improved.

Whether or if a genetically organized microorganism (GEM) capable of producing the optimum protein and having energized cutoff points is properly created through biomolecular process, it still has to contend with several fundamental obstacles when it comes to use. Given the increased energy demands imposed by the presence of newly acquired material in the cell, their enzymatic section that have been released into the environment may not be as prosperous or functional(Cheung and Gu 2007).

A public advantage of passing on GEMs into the ecosystem has instigated crazy standards by government bodies, i.e. U.S. Environmental Protection Agency (U.S. EPA). In like manner, scarcely any separated microorganisms have shown at the hour of field application (Hiner et al. 2002).

## 6.7 Conclusion

Another technique for directing work on the showing of powers in the remediation of pollutants is the usage of mixes immobilized on the norm and arranged sponsorships of various nature and through numerous immobilization structures. Immobilized impetuses have regularly a somewhat long utilitarian reliability (Coppella et al. 1990), being absolutely unsurprising toward physical, substance, and normal denaturing arranged specialists. Moreover, they may be reused and recovered close to the completion of the cycle. Taking everything into account, limits are accessible for applying biomolecular organizing strategies (Cheung and Gu 2007). For instance, research is regularly established on changing made combinations that can play out a reaction like the best one, yet it might be very problematic to apply biomolecular aiming to mitigation of toxic substances, which are recalcitrant to natural biodegradation processes. Considering everything, it might be possible later on when our knowledge into the protein structure-work, imploding, instrument, and parts will be on a very major level improved. Whether or not a genetically organized microorganism (GEM) with revived cutoff points and passing on the ideal protein is adequately made by biomolecular orchestrating, it genuinely faces assorted central targets concerning its application (Filazzola et al. 1999). Also, a public advantage of passing on GEMs into the environment has actuated crazy standards by government bodies (EPA). In like manner, scarcely any separated microorganisms have shown at the hour of field application (Rao et al. 2010).

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# Part II

# Environmental Pollution and Wastewater Treatment

# 7 Environmental Toxicity, Health Hazards, and Bioremediation Strategies for Removal of Microplastics from Wastewater

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**Abstract**

Microplastics (MPs) are minuscule plastic particles smaller than 5 mm in length that have become a significant threat to because of their toxicity in our natural environment and detrimental impacts on our water resources, aquatic life, and humans. Physical, chemical, ecological, and biological impacts are all possible ways of causing dangers posed by MPs. Microplastics also sorb and collect potentially toxic contaminants in aquatic environments. As a result, ingesting polluted microplastics may expose marine species and even the food chain to hazardous contaminants. However, wastewater treatment plants (WWTPs) are the primary source of microplastics that enter marine ecosystems. Microplastics in aquatic environments must be controlled to protect the environment and human health. This chapter examines the sources of microplastics in wastewater, their properties, ecotoxicity, and health risks, existing and newly developed methods for characterization of microplastics in wastewater, and for pollution prevention and control, bioremediation techniques for the removal of microplastics from wastewater have been developed.

**Keywords**

Wastewater · Microplastics · Toxicity · Health hazards · Characterization · Bioremediation

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## 7.1 Introduction

Industrial waste is the principal source of environmental pollution because of the existence of nutrients of environmental concerns, potentially toxic heavy metals, organic pollutants, and emerging contaminants that pose major ecotoxicological risks and environmental dangers (Chandel et al. 2022; Chaturvedi et al. 2021; Saxena et al. 2016, 2020a, b, c, d; Deb et al. 2020; Kumar et al. 2020; Bharagava and Saxena 2020; Mulla et al. 2019; Bharagava et al. 2017a, b, c, 2018; Goutam et al. 2018; Gautam et al. 2017; Saxena and Bharagava 2015, 2017). Among the environmental contaminants, the release of emerging contaminants along with industrial effluents is a major environmental concern. The extensive use of plastic goods in today's world eventually leads to emission of minute plastic particles into the environment. The diameter of the microplastic particles (MPs) is less than 5 mm (GESAMP 2015). The exact level of microplastics in the environment, including unidentified microplastics, is considered to be substantially higher than what uncontrolled plastic product flows predict (Kim et al. 2015). The microplastics (MPs) amount in seawater has been continuously increasing over the last decade, with a growing trend along the shorelines (Barnes et al. 2009), MPs pollution is a relatively new issue in the world, due to the growing use of plastics in practically all aspects of human activities and there is a lack of appropriate treatment of domestic and industrial wastewater (Bui et al. 2020).

At present, with the widespread use of MPs, particularly in the marine environment, marine life is unsheltered to MPs with broad range of effects which depends on the presence toxic chemicals from plastic additives and adsorbed pollutants such as pesticides, persistent organic pollutants, or metals leaching into the environment, particularly in the marine environment (Van Emmerik et al. 2018; Fossi et al. 2014). MPs are hazardous and can also serve as pathogen reservoirs, putting marine life in danger (Kor and Mehdinia 2020). MPs are found largely in coastal habitats, and their exact influence on human health has yet to be identified. Marine life, on the other hand, is at the centre of the food chain and provides a significant portion of the nutrients consumed daily by human beings (Bui et al. 2020). The growing presence of MPs in the environment and biota has attracted the curiosity of scientists and the general public, with emerging evidence of microplastics' detrimental effects (de Sá et al. 2015; Jeong et al. 2016). Surface runoff, wind advection, and WWTPs effluent are just a few of the ways MPs enter water bodies (Dris et al. 2015). Thousands of microplastic particles are deposited in WWTPs every day (Okoffo et al. 2019). Although there is no direct link between MPs concentrations and population density in WWTPs intake streams, agriculture and industrial activities appear to be important factors (Long et al. 2019). To determine the amount of microplastics that enter and exit WWTPs, it is essential to develop a precise and repeatable experimental approach for counting the microplastic particles in sewage influent and effluent.

MPs have been removed using a variety of processes, including grit chamber and primary sedimentation, coagulation, sand filtering, dissolved air floatation and fast (gravity) sand removal (Wang et al. 2020; Hidayaturrahman and Lee 2019; Chen et al. 2018; Bayo et al. 2020; Lares et al. 2018; Murphy et al. 2016). According to a

study, microplastics concentration in the WWTP influents was found to be in between 15 and 640 particles $L^{-1}$, while it was significantly lower in case of the effluent, although varied over four orders of magnitude (Kang et al. 2018). As a result, it is unclear if the discrepancies in microplastic concentrations in wastewater are related to variances in plastic pollution levels or differences in sampling and analytical procedures (Kang et al. 2018).

MPs are currently identified and/or quantified using scientific analytical techniques such as spectroscopy, microscopy, and/or thermal analysis. The most common characterization methodology mentioned in the literature is the use of spectroscopic techniques such as Raman spectroscopy (Peñalver et al. 2020) and Fourier transform infrared (FTIR). MPs have been characterized using scanning electron microscopy based techniques such as, SEM-energy dispersive X-ray spectroscopy(SEM-EDS) and other techniques like Environmental Scanning electron microscopy-EDS (ESEM-EDS) (Rocha-Santos and Duarte 2015). Microplastics thermal analysis is a new technology for MPs characterization. This method is based on identifying the polymer based on the degradation products it produces pyrolysis gas chromatography–mass spectrometry (py-GC-MS), thermogravimetry (TGA), hyphenated TGA such as TGA-differential scanning calorimetry (DSC), TGA–thermal desorption–gas chromatography–mass spectrometry (TGA-TD-GC-MS), TGA–mass spectrometry (TGA-MS), and DSC are some of the other techniques used to characterize (Peñalver et al. 2020).

MPs traversed by the stream eventually enter the sea; hence, WWTPs that discharge their effluents into rivers contribute to ocean pollution. On the other hand, river mouths are the major area for MP contamination (Leslie et al. 2017). To avoid marine MP contamination, it is critical to find effective and environmentally benign methods of removing MPs in WWTPs. Biological methods using bacteria, fungi, and lower eukaryotes have been the focus of most investigations for MPs removal (Masiá et al. 2020). It is still difficult to use living organisms in MPs bioremediation. The key issue with these microscopic creatures is containing them within WWTPs to avoid inadvertent introduction of these organisms in the ecosystem (Nuzzo et al. 2020). Larger organisms, for instance, higher eukaryotes, may be simpler to contain in theory, but their practical use in MPs bioremediation is currently a niche market (Masiá et al. 2020). This chapter examines the sources of microplastics in wastewaters, their properties, ecotoxicity, and health risks, approaches that are already in use and those that are being developed for characterization of microplastics in wastewater, and bioremediation strategies for the removal of microplastics from wastewater for pollution prevention and control.

## 7.2 Sources of Microplastics in Wastewater

Microplastics are produced from a variety of land-based sources and eventually end up in wastewater treatment plants, which are thought to be the link between contaminants and natural habitats (Rochman et al. 2015). Primary microplastics are those that have been made intentionally, whereas secondary microplastics are

those that have been produced by a different type of physical, chemical, or biological degradation (Cooper and Corcoran 2010). Microplastics discovered in wastewater treatment plants primarily consist of fibres and microbeads. Microbeads with a size of 250 μm are found in around 0.5–5% of cosmetics (Bowmer and Kershaw 2010). Exfoliants and toothpaste have been shown to release 4500–95,500 microbeads and 4000 microbeads, respectively, with each use (Carr et al. 2016). Synthetic textile washing, on the other hand, releases around 35% of fibre microplastics into the oceans (Boucher and Friot 2017). A load of roughly 5–6 kg, for example, was found to release 6,000,000–700,000 fibres from polyester and acrylic fabrics, respectively (Boucher and Friot 2017). The number of liberated fibres, however, is dependent on the washing conditions, textile qualities, use, and softener and detergent type (Cesa et al. 2017).

Other domestically produced consumer goods, such as contact lens cleaners and jewellery, have also been found to leak MPs. On the other hand, non-domestic sources, have been reported to leak MPs, including (a) air blasting, (b) transportation and manufacturing, (c) Styrofoam products, (d) textile sector, and (e) dust from the drilling and cutting plastics (Prata 2018). Bayo et al. (2020) recently revealed that seasonal variability is also a significant influence, with the highest amounts of MPs seen during warmer periods, as temperature accelerates plastic degradation and fragmentation. Furthermore, due to urban runoff, large amounts of microplastics have been detected during rainy events (Masiá et al. 2020).

## 7.3 Properties of Microplastics

Microplastics are a polymer blend that comes in a variety of shapes. Microplastics' form is a key criterion for classification. Microplastics in nine different shapes were identified in the influent and effluent of WWTPs: rod, fragment, film, pellet, foam, ellipse, line, and flake (McCormick et al. 2014). Pellets can be cylindrical, circular, flat, ovoid, and spheruloids, while fragments can be rounded, subrounded, subangular, and angular, to name a few. Microplastics, on the other hand, have uneven, elongated, deteriorated, rough, and broken edges as their most common morphologies. MPs in the environment are shown in terms of their sources, transport, accumulation, and fate in Fig. 7.1. MPs are non-biodegradable, water-insoluble synthetic polymers with a high proclivity for fragmentation and microbial ingestion (Beiras et al. 2018). MPs are bioaccumulated by bacteria, fungi, phytoplankton, and zooplankton in many ways in both terrestrial and marine environments (Paul-Pont et al. 2018). Bioadsorption, biouptake (cellular uptake), and biodegradation are the three main mechanisms through which MPs interact/accumulate in microorganisms (MOs) (Avio et al. 2017). MP bioaccumulation has been shown to alter the growth and metabolism of microorganisms (fungi, bacteria, phytoplankton, and zooplankton) (Xu et al. 2019; Sun et al. 2018).

MP bioaccumulation is a serious concern since if swallowed, it can destroy aquatic life. Because of their minute size, microplastics are easily eaten by different marine organisms (Ferreira et al. 2016). Because microplastics are disseminated at

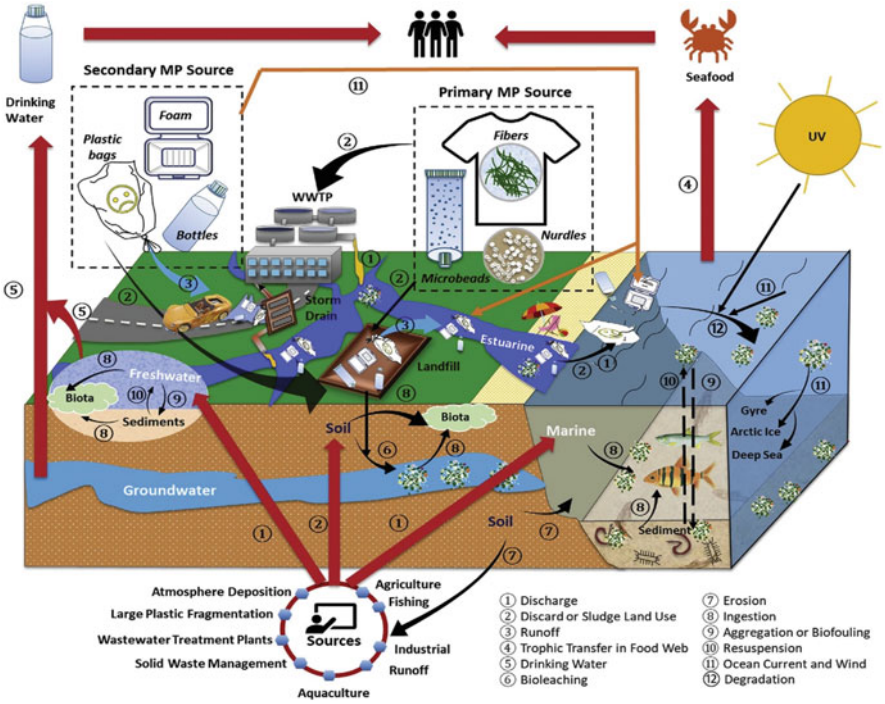


**Fig. 7.1** Sources, transport, accumulations, and fate of MPs in the environment (adapted from Wu et al. 2019)

diverse trophic levels, microplastic concentrations in the body may grow as a result of bioaccumulation at higher trophic levels. Microplastics penetrate the food chain and eventually reach humans (Nelms et al. 2018). This shows that the most serious consequences of microplastic poisoning may be experienced by people. There is currently minimal knowledge about the effects of microplastics on food webs, and no laboratory trials on bioaccumulation toxicity induced by microplastics at higher trophic levels have been conducted (Anagnosti et al. 2021). As a result, whether or if any size of plastic may be transmitted to higher trophic levels is unknown. Many persistent organic pollutants (POPs), such as dioxins, polybrominated diphenyl ethers and PCBs have been well-documented occurrences of trophic transfer within marine food webs (Ogata et al. 2009; Hu et al. 2005).

Biological availability refers to the small percentage of the total number of particles/chemicals in the environment that are accessible for absorption by an organism. Because smaller particles have a larger volume ratio, stronger penetrating power, and greater ability to be taken up by marine species, MP bioavailability is known to improve as particle size decreases (Botterell et al. 2019). Microplastics density in the water column may alter their bioavailability. Low-density plastics like PE on the sea surface, for example, are likely to come into touch with filter

planktivores, feeders, and suspension feeders in the upper water column (Kooi et al. 2017). Other factors influencing microplastic bioavailability in aquatic habitats include colour, shape, ageing, and abundance (Wright et al. 2013; Crawford and Quinn 2017). The binding affinity of MPs particles with other pollutants has an impact on their bioavailability (Bhagat et al. 2020).

Bioaccessibility and bioavailability are critical principles for calculating the risks of exposure to environmental pollutants. The bioavailability of MPs affects their overall effects on organisms (Cole and Galloway 2015). MP bioavailability to be directly absorbed by a wide spectrum of species is enhanced by their small size (Law and Thompson 2014). A planktivore may confuse MPs for natural food during normal eating behaviour, since their size % is comparable to that of planktonic organisms and sediments (Wright et al. 2013). Scherer et al. (2017) discovered that *C. riparius* can uptake 90 μm MP particles is much lower than that of 10 μm MP particles, despite intraspecific variability in feeding rates $(p < 0.01)$. As a result, it was found that as MP size drops, their potential bioavailability in the food chain increases.

Microplastics can function as vectors for harmful chemical pollutants, and because they are most exposed in the marine ecosystem, many marine species inadvertently consume them (Fred-Ahmadu et al. 2020). PAHs, for example, have high partition coefficients when it comes to plastics, indicating that they have a significant affinity for polymers (Fred-Ahmadu et al. 2020). Because some of the most often observed environmental plastics have a lesser density than seawater (density 1.02 g/cm$^3$), they float in water bodies' surface microlayers and may sorb contaminants (SML) (PerkinElmer 2019; Sundt et al. 2014). The contaminant-laden plastics floating in the water can be eaten by marine creatures and seabirds in the epipelagic zone. Even when additive effects are taken into account, polymers like PS, PVC, and PU, as well as plastics with fouling surfaces, have a higher density than seawater or freshwater. The process of "microbial fouling" aids the adsorption of various contaminants onto the surface of microplastics in confined lakes (Neto et al. 2019). As a result, contaminant-sorbed microplastics fall to the bottom of the ocean, where they are available for ingestion by benthic creatures (Teuten et al. 2007).

Chemical contaminants that have been absorbed by microplastics can desorb and biomagnify their way up the food chain, from lower trophic species to fish (Bakir et al. 2014; Rochman et al. 2013). Sorbed pollutants on microplastic particles are easily leached by digestive juices (Voparil and Mayer 2000). MPs that have been ingested for a longer period of time are more effective. remain in an organism's intestines, the more likely pollutants may translocate into bodily tissue. Polybrominated diphenyl ethers (PBDEs) smeared on microplastics were discovered to be incorporated in the tissue of marine amphipods (Chua et al. 2014). As a result of being near to the sources and consumption of these chemicals, the adsorption of various POPs such as polychlorinated biphenyls, hexachlorobenzenes (HCBs), heavy metals and PBDEs to Hydrophobic plastic particles with a large surface area to volume ratio is more prone in freshwater ecosystem than in marine ecosystem (Dris et al. 2015). Freshwater organisms may thus be exposed to increased levels of

contaminants, particularly in areas near industrial and populated areas, where increased level concentrations of hydrophobic pollutants, as well as a higher presence of microplastics, may exist, and in areas near agricultural areas, where both POPs (i.e. pesticides) and plastic products are used.

## 7.4 Ecotoxicity and Health Hazards of Microplastics

Marine animals such as zooplankton (Desforges et al. 2015), mussels (Qu et al. 2018), oysters (Leslie et al. 2017), corals (Hall et al. 2015), and microplastics in the environment may be consumed by fish (Collard et al. 2015). Once swallowed by marine species, microplastics constitute a threat to them. Health hazards posed by MPs to aquatic biota are presented in Table 7.1. Physical, chemical, ecological, and biological impacts are all possible ways of causing dangers (Provencher et al. 2018). Microplastics cause mechanical damage to organisms. Microplastics, for example, have the capacity to block the intestines and cause harm to the gut (through villi cracking and enterocyte splitting), and even affect organism filtering activity and phagocytosis, resulting in organism death (Canesi et al. 2015; Lei et al. 2018). Furthermore, MPs could build up in food web as a result of predation. Microplastics, for example, were discovered to be fed through the pelagic food web by Satlewal et al. (2008), from zooplankton to mysid shrimps. MPs were also observed to move from algae to zooplankton to goldfish, according to Cedervall et al. (2012).

Microplastics would sorb and collect contaminants in aquatic environments chemically. As a result, ingesting polluted microplastics may expose marine species and even the food chain to hazardous contaminants (Santana et al. 2017; Brennecke et al. 2016). In this case, microplastics act as conduits for hazardous pollutants (Carbery et al. 2018). However, little evidence of the influence of trophic transfer of microplastics and pollutants from the food chain on human health exists at this time, necessitating additional investigation. According to Koelmans et al. (2016), the proportion of total hydrophobic organic contaminants (HOCs) deposited on microplastics was modest in contrast to other media in marine ecosystems, and ingestion of microplastics by marine animals may not provide a HOC risk. According to Wang and Wang, PHE sorption capabilities on PE, PS, and PVC microplastics were higher than sorption capacities on sediment samples (Wang and Wang 2018).

Microplastics can also serve as a microbe's artificial substrate in addition to serving as carriers of linked chemical burdens to aquatic species. This has sparked concerns about the biological consequences for freshwater ecosystems as they provide important advantages and services, including as habitat for a diverse range of native plants and animals, drinking water, and recreational opportunities (Meng et al. 2020). In terms of ecology, this might have a significant influence on how microplastics interact with freshwater biotas, such as colonized creatures floating over greater distances and microplastics becoming vectors for poisonous bacteria/algae, diseases, and even invading species. The taxonomic composition of bacterial assemblages colonising microplastics in a heavily urbanized river in Chicago,

**Table 7.1** Studies on toxic effects of microplastics on aquatic biota

| Species | Class | MP type | Size (μm) | Concentration | Exposure | Effects | Reference |
|---|---|---|---|---|---|---|---|
| *Dunaliella tertiolecta* | Microalgae | PS | 0.05–6 | 25 and 250 mg/L | 72 h | Algae growth is significantly slowed, but photosynthesis is unaffected | Sjollema et al. (2016) |
| *Skeletonema costatum* | Microalgae | PVC | 1 | 0–50 mg/L | 4 days | Algal growth inhibition; chlorophyll concentration and photosynthesis | Zhang et al. (2017) |
| *Chlorella pyrenoidosa* | Microalgae | PS | 0.1 and 1 | 0–100 mg/L | 30 days | Unclear pyrenoids, deformed thylakoids, reduced photosynthetic activity and the suppression of algal development from the lag to the early logarithmic stages results in damaged cell membrane | Mao et al. (2018) |
| *Scenedesmus obliquus* | Microalgae | PS | ~0.07 | 44–1100 mg/L | 72 | Algal growth inhibition; Chlorophyll-a content reduction | Besseling et al. (2014) |
| *Pseudokirchneriella subcapitata* | Microalgae | PS | 0.055 and 0.1 | 0.1–1 mg/L | 72 | Algae growth is slowed | Casado et al. (2013) |
| *Centropages typicus* | Copepod | PS | 7.3 | 4000–25,000 beads/mL | 24 h | Algal intake is significantly reduced | Cole et al. (2013) |
| *Nephrops norvegicus* | Crustacea | PP | 3000–5000 | 5 fibres per 1.5 g feed | >8 months | The rate of feeding, body mass, metabolic rate, and fat catabolism all decrease | Welden and Cowie (2016) |
| *Tetraselmis chuii* | Microalgae | PE | 1–5 | 0.046–1.472 mg/L | 96 h | Microalgae population expansion is being slowed | Davarpanah and Guilhermino (2015) |
| *Daphnia magna* | Crustacea | PS | 0.05–10 | 5 mg/L | 14 days | The bioaccumulation of phenanthrene was enhanced by 0.05-m PS, whereas 10-m PS had no impact | Ma et al. (2016) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Arenicola marina* | Annelida | PS | 400–1300 | 0–100 g/L sediment | 28 days | Body weight, feeding activity, and energy efficiency are all reduced | Besseling et al. (2013) |
| *Perna viridis* | Mollusca | PVC | 1–50 | 21.6–2160 mg/L | 2 h/day for a period of 91 days | Filtration behaviour, respiration rate, and byssus production all decreased significantly<br>With increased pollution, survival time has decreased | Rist et al. (2016) |

*PP* polypropylene, *PS* polystyrene, *PVC* polyvinylchloride, *PE* polyethylene, *PA* polyamide, *HDPE* high-density polyethylene

Illinois, differed significantly from those colonising suspended organic matter and water column, and that various taxon, such as pathogens and plastic-decomposing organisms, were more abundant on microplastics, according to McCormick et al. (2016). Several research works have looked into the impact of MPs on marine animal reproduction in the ecosystem (Sharifinia et al. 2020). Sussarellu et al. (2016) describe an emerging perspective that MPs reduce reproductive output by altering organism food consumption and energy allocation. According to Lei et al. (2018), MP particles from various sources, such as (Polyamide, Polyethylene, Polypropylene, Polyvinyl Chloride) PA, PE, PP, and PVC, considerably lower reproductive success in the nematode *Caenorhabditis elegans*, although only PE- and PVC-MPs had a significant impact on brood size.

Microplastics are biologically sensitive to colonization by microorganisms. Microplastics may influence microbial community evolution and gene exchange (such as antibiotic resistance genes and metal resistance genes) among bacteria (Yang et al. 2019). The antibiotic resistance gene profile is determined by the microbial community composition, according to Yang et al. (2019). Freshwater invertebrates, water fleas (*Daphnia magna*), and various fish species all actively feed on microplastic particles (1–100 μm), according to laboratory research, and MP particle intake causes critical immobilization of these animals (Besseling et al. 2019; Oliveira et al. 2013; Rehse et al. 2016), as well as affecting predator-prey relationships (Besseling et al. 2019; Oliveira et al. 2013; Rehse et al. 2016; Rochman et al. 2017). However, several studies have revealed that MPs have no effect on ecosystem processes (Krause et al. 2020), making predictions about ecosystem-level consequences more difficult. Furthermore, intergenerational effects on *Daphnia magna* revealed no impacts in the first generation, while neonates exposed to the same concentration of MPs were extinct after two generations (Martins and Guilhermino 2018). Many freshwater benthic consumers (e.g. Oligochaeta worms, Chironomidae larvae, gammaridae, and amphipods) act as ecosystem engineers in sediments and are heavily exposed to MPs, chemical additives, sorbed pollutants, and possible microbial diseases (Frère et al. 2018; McCormick et al. 2014), posing a serious risk of broad range of impacts, particularly on benthos (Izvekova and Ivova-Katchanova 1972; Ward and Ricciardi 2007). For example, lugworms (*Arenicola marina*) that ate MPs had less bioturbation, which decreased the primary productivity of bioturbated substrate and changed lugworm respiration (Wright et al. 2013; Green et al. 2016). PVC microplastics were found in the diet of African freshwater catfish in a new study (Iheanacho and Odo 2020a, 2020b). In this case, the microplastic caused neurotoxicity, oxidative stress, and lipid peroxidation, all of which had an impact on the fish's physiological status. The majority of Microplastics are found in waterways surrounding big cities, particularly in poorer nations with inadequate waste management systems (Xu et al. 2020).

MPs have been found in a variety of places where, all kinds of marine life exists ranging from microscopic species (such as phytoplankton and zooplankton) to enormous predators (mammals and fish) (Anagnosti et al. 2021; Wang et al. 2020). Microplastics have been demonstrated to alter the reproduction, mortality, development, cellular response, behaviour, life span, assimilation efficiency,

regeneration, oxygen consumption, egestion, metabolism, nutrition, neurotoxicity, carcinogenicity, and gene expression of aquatic creatures (Haegerbaeumer et al. 2019; Xu et al. 2020). Ingestion of microplastics has a direct impact on small creatures at the bottom of the food chain, producing malnutrition and the inability to eliminate microplastics causes mechanical stress called saturation (Cole et al. 2013; Wright 2015). Microplastic absorption by phytoplankton has been shown to disrupt photosynthesis and, as a result, organism growth (Kalčíková et al. 2017; Bhattacharya et al. 2010). MPs long-term exposure caused considerable alterations in energy stores in two sediment-dwelling bivalve species, *Abra nitida* and *Ennucula tenuis*, but did not affect burrowing activity survival, condition index (Bour et al. 2018). The size as well as number of particles were connected to the outcomes, with larger particles and higher concentrations having more severe consequences. At greater concentrations, microplastics caused oxidative stress; damage to the gut, liver, and gill tissues; increased heart rate; and impeded development and motility in goldfish larvae, resulting in oxidative stress; damage to the intestine, liver, and gill tissues; and increased heart rate (Yang et al. 2019).

Because agricultural plastic film and plastic particles are used extensively in industrial production, MPs pollution on land could be more problematic than in the marine environment (Ramos et al. 2015). MPs also represent a threat to terrestrial creatures, as well as human health, through the food chain and other channels (Sharma and Chatterjee 2017). Plastic films or irrigation water containing MPs can both introduce MPs into the soil system (Rillig 2012). MPs have been found in some studies to have an impact on soil organisms, such as altering the isotopic composition of soil collembolans and perturbing the microbiome (Zhu et al. 2018). Earthworms in the soil can be harmed by polystyrene MPs, which can even kill them (Cao et al. 2017). These findings imply that MPs pollution in soils is harmful to soil organisms and that MPs pose an ecological danger in terrestrial ecosystems. In mice, polystyrene MPs were found to cause dysbiosis of the gut microbiota, intestinal barrier failure, and metabolic problems, according to a study (Jin et al. 2019; Lu et al. 2018). MPs could be consumed by micro- and mesofaunas such as mites, collembola, and enchytraeids, accumulating in the soil detrital food web (Rillig 2012). After seeding and planting *Lolium perenne* (perennial ryegrass) in soils containing MP-clothing fibres, shoot lengths, dry root biomass, dry root–shoot ratio, and chlorophyll a–b ratio high-density polyethylene (HDPE), biodegradable polylactic acid (PLA), and all altered dramatically hence concluded, In the presence of MP-clothing fibres or PLA, seed germination was lower than in control soil (Boots et al. 2019).

Water and nutrient absorption by plant roots is also hampered by the presence of MPs. Plant biomass, root characteristics, tissue elemental composition and soil microbial activity have all been shown to be strongly affected by soil MPs, according to current research (de Souza Machado et al. 2018). Humans consume a wide range of plant and animal products that may include MPs, posing a variety of health hazards. Microplastics are mostly absorbed by ingestion, inhalation, and skin contact (Prata et al. 2020). Microplastics have been detected in beer (Liebezeit and Liebezeit 2014), seafood (Smith et al. 2018), honey and sugar (Liebezeit and Liebezeit 2013),

sea salt (Kim et al. 2018), and drinking water (Mintenig et al. 2019). On average, humans consume 4000 MPs each year from water to drink, 37–1000 microplastics from edible sea salt (Van Cauwenberghe and Janssen 2014; Kosuth et al. 2017) and 11,000 microplastics from shellfish. Microplastics (specifically nanoplastics) might reach agricultural fruits/seeds and consequently goes inside the human body through food consumption, according to studies by Sun et al. (2020). Eventually, plant uptake of microplastics may impact human health as well as food security and safety. MPs can also be inhaled through the respiratory system. Airborne microplastics are the most common cause of respiratory exposure. According to a study (Vianello et al. 2019), humans can absorb up to 272 particles each day from indoor air.

The length of time inhaled airborne microplastics travel through the lungs is determined by their size (Enyoh et al. 2019). MPs with a diameter of less than 2.5 m will settle in the lungs first, allowing them to penetrate past the respiratory barrier. Inhalation for a long time Low-level exposure to tiny particles can potentially result in gene mutations (Kingsley et al. 2017). After 10–20 years of being exposed to polypropylene fibres, synthetic textile workers had a higher cancer incidence rate. Workers who worked with polyvinyl chloride had a higher risk of lung cancer as they got older, worked more years, and spent more time in the factories (Prata et al. 2020).

Another form of exposure is dermal touch, but this is a less important route (Prata et al. 2020). Because only particles smaller than 100 nm can be absorbed directly via the skin due to stratum corneum penetration, most microplastics are difficult to absorb (Revel et al. 2018). Microplastics are resistant to chemical breakdown in vivo when they reach the body (Wang et al. 2020). The inhibition of acetylcholinesterase by microplastics could also lead to neurotoxicity (Jeong and Choi 2019). According to a simulated digestion research, microplastics might affect lipid digestion after being consumed by humans by forming microplastics-oil droplet heteroaggregates and inhibiting digestive enzyme activity (Tan et al. 2020), providing a threat to human digestion health.

Microplastics can also be absorbed by human tissues via endocytosis (gastrointestinal tract and airway surface) and paracellular persorption, which is influenced by surface charge, microplastic size, surface functionalization, generated protein corona, and hydrophobicity, among other factors (Wright and Kelly 2017). Increased permeability of the gastrointestinal mucosa can be caused by malnutrition and diets containing high-fructose carbohydrates (due to alterations in the flora of the intestine) and high saturated fats (West-Eberhard 2019). Inhalation and ingestion of MPs in rats resulted in microplastics being discovered in the circulation as well as liver and spleen are examples of distant tissues (Eyles et al. 1995; Jani et al. 1990). A placental perfusion model in humans indicated that 240 nm particle size can cross the placental barrier (Wick et al. 2010). Damage to the DNA replication and repair machinery, as well as DNA damage caused by ROS or particle translocation into the nucleus, can all contribute to MP particle genotoxicity (Rubio et al. 2020).

Microplastics disrupts nuclear membranes, causes oxidative stress, produces damage-related molecular patterns, and activates downstream inflammatory and

apoptotic/necrotic pathways in mammalian cells (Yong et al. 2020; Hwang et al. 2020). According to relevant animal model research, these MPs can be transported from living cells to the circulatory systems and lymphatic, where they can gather and harm the cells and immunity of humans (Brown et al. 2001; Browne et al. 2008). Tissue distribution in mice demonstrated MPs accumulation in the kidney, stomach, liver and also the symptoms of energy balance disruption, oxidative stress, and neurotoxicity, after oral administration of fluorescent 5 and 20 m particle sizes at 106 and 104 mg/mL, respectively (Deng and Zhang 2019). After exposure to particulate matter, in vivo neurotoxicity has been reported, possibly due to oxidative stress and activation of the brain's microglia (immune cells) from direct contact with translocated particles or the action of circulating pro-inflammatory cytokines (from other inflammation sites), resulting in neuron damage (Mohan Kumar et al. 2008).

Several studies have linked microplastics to abnormalities in energy homeostasis. Microplastics, for example, may decrease energy intake (a) by causing a decrease in feeding activity (e.g. in crabs, marine worms, and clams) (Xu et al. 2017; Watts et al. 2015); (b) due to decreased predatory performance (e.g. in fishes) (Wen et al. 2018); and (c) due to alterations in digestive enzymes, thereby causing a loss in digestive capacity (Wen et al. 2018).

Additives and monomers from the microplastics matrix may seep into the body, leading to exposure of tissues to endocrine disruptors including phthalates and bisphenol A, which interfere with endogenous hormones even in minute amounts (Cole and Galloway 2015). Changes in the gut microbiome could have negative consequences, such as the spread of dangerous bacteria, a rise in endotoxemia and intestinal permeability (West-Eberhard 2019). Human's inhale, ingest, and eat microplastics through the air (Gasperi et al. 2018), bottled water (Zuccarello et al. 2019), seafood and table salt (Nelms et al. 2018; Zuccarello et al. 2019). Recent studies have shown microplastics in excreta of humans (Yong et al. 2020), indicating that microplastics have been eaten. Plastic toxins were found in every human tissue analysed from Alzheimer's patients in a recent study, which linked toxicity and neurological impairment to lifelong exposure to microplastics (Manivannan et al. 2019).

## 7.5 Factors Affecting Toxicity of Microplastics

Plastic toxicity varies depending on the polymer type. Polyurethane, PVC, polyacrylonitriles, styrene-based copolymers and epoxy resins, categorized as the most dangerous (category 1A or 1B mutagen or carcinogen) because of the hazard division of monomers (Lithner et al. 2011). It is crucial to keep in mind that the higher toxicity of smaller particles is not always apparent, since it depends on a variety of elements such as exposure time, charge, cell type, dose, and polymer type. Larger particles necessitate the use of specialist cells to phagocytose them (Alberts et al. 2002). Endocytic and passive uptake mechanisms can take up smaller particles. Particle size and toxicity are usually inversely proportional. The toxicity of 500 nm PS particles (IC50 12.6 g/mL) was found to be higher than that of 50 nm (IC50 >

100 g/mL) in NIH/3T3 and ES-D3 mouse embryo cultures, for example (Hesler et al. 2019). Because of their small size and high surface–volume ratio, MP/NP can absorb additional contaminants such as heavy metals, persistent organic pollutants (POPs), and viruses (de Souza Machado et al. 2018; Yu et al. 2019). Plastics can have persistent organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, DDT), heavy metals (Cd, Cr, As, Hg, As, Br, Zn, Cu, Sb, Sn, Ti, Mn, Co, Ba), and pathogenic *Vibrio* spp. (Campanale et al. 2020; Brennecke et al. 2016; Prinz and Korez 2020; Kirstein et al. 2016; Velzeboer et al. 2014; Rodrigues et al. 2019).

The absorption, transport, and toxicity of particles can all be influenced by the surface charge of MPs (Yacobi et al. 2010; Fröhlich et al. 2012; Loos et al. 2014a, 2014b). Plasticizers, stabilizers, dyes, lubricants, and flame retardants are among the leachates/plastic additives, which account for around 4% of MPs content and potentially pose health hazards (Bouwmeester et al. 2015; Campanale et al. 2020; EFSA CONTAM Panel 2016). Hahladakis et al. (2018) show that the existence and release of additives, on the other hand, does not always imply a health risk, as toxicity is dictated by the plastic composition and the rate of leachate migration, as well as the amount and solubility of leachate in the surrounding environment. The migration of additives is in large amounts from plastics in fatty foods and when stored at high temperatures or for long periods (Hahladakis et al. 2018).

Chemical adsorption on MPs can be influenced by some circumstances. MPs type, size, environmental salinity and pH, and plastic ageing are only a few of the variables (Mammo et al. 2020). For the same size (200–250 mm), different kinds of microplastics, such as PP, PVC, polyethylene terephthalate (PET), and PE, have varied surface areas and distribution coefficients (Teuten et al. 2007). At differing pH levels, the sort of charge on microplastics surface and chemicals influences whether adsorption increases or decreases. Adsorption is enhanced when the MP surface and chemicals have opposite charges, but adsorption is reduced when the MP surface and chemicals have identical charges (Karlsson et al. 2017). According to Seidensticker et al. (2018), due to repulsion between comparably charged polar compounds and plastic surfaces, non-polar molecules have stronger sorption on PE and PS than polar compounds. The influence of salinity on chemical sorption on MPs can be assessed using changes in the partition coefficients of a chemical with a change in salinity. Log KMP-SW in saltwater and log KMP-W in the same chemical water are different, according to Wang et al. (2020), suggesting that salinity impacts chemical sorption on MPs.

Weathering or ageing of MPs has been reported to increase the rate of chemical sorption (Endo et al. 2005; Rios et al. 2007). Due to environmental interactions such as long-term exposure to the sun, which can cause photo-oxidation, aged plastics have rough surfaces. This causes plastics to degrade into smaller sizes, increasing their surface area and sorption capacity (Brennecke et al. 2016). Adsorption is linked to several sorption sites that are dependent on crystallinity (Joshi et al. 2017). Higher crystallinity produces a clean surface with fewer sorption sites, lowering adsorption. Previous research has found that the crystallinity of MPs influences HOC partitioning, which affects adsorption (Guo et al. 2012). Guo et al. (2012) found

that lowering the crystallinity of PE from 59 to 26% increased the sorption of phenanthrene, naphthalene, and lindane. Liu et al. (2019) studied the differences in ciprofloxacin sorption on PVC (low crystalline) and PS (high crystalline) and found that ciprofloxacin sorption on PS was lower than that on PVC.

## 7.6 Techniques for Characterization of Microplastics in Wastewater

The sample of wastewater is the initial step in its characterization, and it has a direct impact on the MPs study's outcomes. Filtration collection is the most popular method for taking samples from wastewater in WWTPs (Kang et al. 2020). Input and effluent samples, on the other hand, can provide information on microplastics sources, total MP removal rate, and pollutant loading. After sampling, the sample must be predigested to remove contaminants and increase extraction efficiency, as well as to minimize MP loss and damage to the greatest extent possible. Purification is another crucial stage in MPs characterization since it demands the removal of the largest amount of organic materials while causing the least amount of damage to MPs. The digestive regents utilized and their concentration, as well as reaction variables such as temperature and duration, might affect the purifying effect (Kang et al. 2020) MPs must be removed from pre-treatment samples after purification to be detected and analysed. Flotation (Imhof et al. 2013) and Elutriation (based on an upward gas or liquid flow to separate MPs) (Mahon et al. 2017) are two unique procedures that have been invented but not generally implemented. Density separation and filtration are two popular ways of extracting MPs (Kang et al. 2020).

Microplastics analysis can be bifurcated into two categories: chemical characterization and physical characterization. Chemical characterization, on the other hand, is primarily used to investigate the composition of microplastics. Several analytical techniques are currently being used to characterize microplastics, including polarized light optical microscopy (PLOM) (Sharifinia et al. 2020), energy-dispersive X-ray spectroscopy (EDS) (Li et al. 2018; Mahon et al. 2017), Raman spectroscopy, and Fourier transform infrared spectroscopy (FTIR) (Sun et al. 2019). The term "physical characterization" refers to the process of determining the distribution of size of microplastics and also other physical characteristics, for instance colour and shape. Several studies performed by authors on the detection and characterization of MPs using different analytical techniques are listed in Table 7.2.

## 7.7 Bioremediation Strategies for Microplastics

### 7.7.1 Bacterial Degradation of Microplastics

Numerous investigations on the use of microbes for MP breakdown are now underway. A list of microorganisms reported for the degradation of MPs is presented in Table 7.3. The characteristics features of candidate microbial species for

**Table 7.2** Microplastics are detected and characterized using analytical methods

| Country | Habitat | Size | Abundance | Sampling | Extraction | Quantification | Reference |
|---|---|---|---|---|---|---|---|
| UK | Estuarine/ subtidal sediment; beach ($n$ = 17) | >1.6 mm | NA | Strandline, subtidal; $n = 5$ at each location | Separation by density (1.2 kg $L^{-1}$ NaCl); stirring; filtration (Whatman GF/A 1.6 mm) | Polymer identification and visual counting/ sorting (FTIR) | Thompson et al. (2004) |
| Singapore | Beach ($n = 8$) | >1.6 mm | NA | 1 cm surface ($n = 4$); sub-surface (10–11 cm, $n = 4$) | Separation by density (hypersaturated solution, 1.2 kg $L^{-1}$); filtration (1.6 mm) | Polymer identification (FTIR) | Ng and Obbard (2006) |
| India | Marine sediment (10 locations) | >1.6 mm | 81 mg plastics per kg sediment | Subtidal strandline; $n = 5$ at each site Surface (10–11 cm, $n = 4$); subsurface (10–11 cm, $n = 4$) ten sample sites; a) (between high tide a low water mark) (each site) 10 kg samples; depth (0–5 cm); sieved | Separation by density (1.2 kg $L^{-1}$ NaCl); stirring; filtration (1.6 mm Whatman GF/A) Separation by density (hypersaturated solution, 1.2 kg $L^{-1}$); filtration (1.6 mm) Separation by density (30% NaCl); filtered (1.6 mm) | Polymer identification and visual counting/ sorting (FTIR) Identification of Polymers (FTIR) Counted and sorted visually (optical microscope); polymer identification (FTIR); surface characterization (SEM) | Reddy et al. (2006) |
| England | Estuary (3 locations, 2 sites) | <1000 mm, 1–10 mm | NA | Strandline; randomly placed quadrates (5 replicates 50 cm 50 cm); depth (0–3 cm) | Density separation (saturated NaCl) | Polymer identification (FTIR) | Browne et al. (2010) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Brazil | Beach (1 location) | >500–1000 mm | NA | Strandline; 100 m transect; depth (0–2 cm); 9 replicates; quadrat (988 cm); wire cloths field sieving (0.5, 1 mm) | Density separation (filtered seawater) | Visually counted/ sorted | Costa et al. (2010) |
| Belgium | Marine sediment (three harbours, 3–4 sites at each) | >63 mm | 170 (49–390) pieces per kg | 3 sampling points (strandline, intertidal, subtidal zone; parallel); sediment cores (2–7 cm) | Density separation, 1 kg sediment, 3 L conc saline solution; stirred; settle 1 h; sieved (38 mm) | Visually counted/ sorted (binocular microscope); polymer identification (FTIR) | Claessens et al. (2011) |
| Portugal | Beach (5 locations) | >1 mm | 190 (29–393) pieces per $m^2$ | Strandline; two quadrats (50 × 50 cm; 2 × 2 m) in duplicate; depth (0–2 cm); samples sieved in situ (2.5–3.5 mm) | Density separation, concentrated NaCl 140 g $L^{-1}$, stirred vigorously; filtered (Whatman GF/C 1 mm) | Polymer identification (m-FTIR) | Martins and Sobral (2011) |
| Canada | Freshwater sediment | <5 mm | NA | Parallel to shoreline; 60 m transect; visible debris sampled (within 1 m); two replicates | Separation into three categories (<5 mm, >5 mm and PS); ultrasonicated in DI water for 4 min | Polymer identification (FTIR); surface characterization (SEM) | Zbyszewski and Corcoran (2011) |
| Germany | Beach ($n = 2$) | >0.45 mm | NA | 2 random samples; depth (0–2 cm) | Density separation, NaCl (1.2 g $cm^3$); manual shaking; filtered (0.45 mm nitrocellulose) | Pyr-GC combination with mass spectrometry (MS), Stereomicroscope; SEM | Fries et al. (2013) |
| South Korea | Beach ($n =$ 10) | >2 mm | 980 pieces per $m^2$ | Strandline line; 50 m transect; 10 replicates; quadrat (50 cm); depth (0–5 cm) | Field sieved (2 mm) | Visual identified and sorting | Heo et al. (2013) |

(continued)

**Table 7.2** (continued)

| Country | Habitat | Size | Abundance | Sampling | Extraction | Quantification | Reference |
|---|---|---|---|---|---|---|---|
| Canada | Freshwater lake (3 lakes; 26 locations) | <10 cm | NA | Parallel to shoreline; 60 m transect; 10 m interval; visible <10 cm particles collected | Cleaned (ultrasonic bath, DI water); separated by hand into four categories | Surface characterization (SEM); visual counting/sorting; polymer identification (FTIR) | Zbyszewski et al. (2014) |
| Hong Kong | Beach ($n$ = 25) | 315–5000 mm | 5595 (106–15,554) pieces per $m^2$ | Tide line; parallel; 30 m transect; depth (0–4 cm); quadrat 50 cm | In field sieve (315 mm); density separation (tap water, sonication); wet sieved (315 mm) | Visually counted and sorted (microscope); gravimetrically | Fok and Cheung (2015) |
| South Africa | Beach sediment (21 sites) | 65–5000 mm | 670 particles per $m^2$ | Strandline; triplicate composite samples; depth (0–5 cm); 1200 mL subsample taken | Density separation; saturated saline solution; repeated five times | visual sorting (fragments and fibre, colour; visually counted (dissecting microscope) | Nel and Froneman (2015) |

**Table 7.3** Microorganisms reported for the degradation of microplastics

| Microbes | Habitat | MP type | Exposure and degradation | Reference |
|---|---|---|---|---|
| *Bacillus* | Mangrove sediment | PP | 40 (d) and 4.0% weight loss | Auta et al. (2018) |
| *Rhodococcus* | Mangrove sediment | PP | 40 (d) and 6.4% weight loss | Auta et al. (2018) |
| *Bacillus gottheilii* | Mangrove ecosystems | PE, PET, PP, PS | 40 (d) and 6.2, 3.0, 3.6, 5.8% weight loss | Auta et al. (2018) |
| *Enterobacter asburiae* | Plastic-eating waxworms | PE | 28 (d) and 6.1 ± 0.3% weight loss | Yang et al. (2014) |
| *Bacillus* | Plastic-eating waxworms | PE | 28 (d) and 10.7 ± 0.2% weight loss | Yang et al. (2014) |
| *Aspergillus tubingensis* | Marine coastal area | HDPE | 30 (d) and weight loss; VRKPT1 was found to be effective at degradation of HDPE; virgin polyethylene was used as a carbon source | Devi et al. (2015) |
| *Aspergillus flavus* | Marine coastal area | HDPE | 40 (d) and a weight reduction of 4.0%<br>40 (d) and a weight reduction of 6.4%<br>40 (d) and a weight decrease of 6.2, 3.0, 3.6, and 5.8%<br>28 (d) and a weight reduction of 6.1, 0.3%<br>VRKPT1 was found to be successful at degrading HDPE; virgin polyethylene was utilized as a carbon source.<br>30 (d) and weight loss; VRKPT2 was found to be effective at degrading HDPE; virgin polyethylene was used as a carbon source | Devi et al. (2015) |
| *Penicillium simplicissimum* | Soil and leaves | PE irradiated for 500 h with UV light | Polyethylene with molecular weights ranging from 4000 to 28,000 showed after 3 months, the molecular weights of polyethylene were reduced, and functional groups added to the polyethylene assisted biodegradation | Yamada-Onodera et al. (2001) |
| *Penicillium pinophilum* | Purchased from a strain centre | LDPE powder | The biologically treated MPs exhibited substantial morphological and structural changes after 31 months, including 0.37% mineralization | Volke-Sepúlveda et al. (2002) |

(continued)

**Table 7.3** (continued)

| Microbes | Habitat | MP type | Exposure and degradation | Reference |
|---|---|---|---|---|
| *Zalerion maritimum* | Marine | PE pellets | 28 days and molecular modifications; Z. maritimum was able to use PE, resulting in a reduction in pellet bulk and size | Paço et al. (2017) |
| *Bacillus* sp. and *Paenibacillus* sp. | Municipal landfill sediment | PP | 60 (d) and 14.7% weight loss | Park and Kim (2019) |
| *Bacillus cereus*, *Arthrobacter* and *Bacillus pumilus* | Soil beds | HDPE/ LDPE | 14 (d) and 21.7–22.41% weight loss | Satlewal et al. (2008) |
| *Exiguobacterium* | Plastic-eating mealworms | PS | 28 (d) and 7.4 ± 0.4% weight loss | Yang et al. (2015) |
| *Bacillus sphaericus* and *Bacillus cereus* | Marine | Nylon 66 and nylon 6 | 3 months and 2–7% weight loss | Sudhakar et al. (2008) |

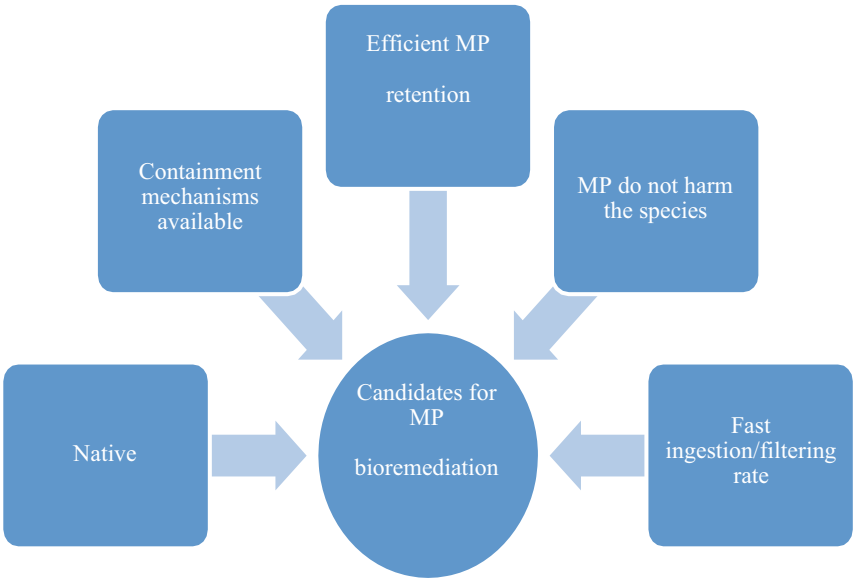


**Fig. 7.2** Characteristics features of candidate microbial species for bioremediation of MPs in WWTPs (adapted from Masiá et al. 2020)

bioremediation of MPs are depicted in Fig. 7.2. Pure bacterial cultures have been employed in studies on the breakdown of Microplastics by microbes in the laboratory (Yuan et al. 2020). Enrichment culturing is usually used to isolate microbial

cultures from silt, sludge, and wastewater. Pure strains have the benefit of being a simple approach for examining metabolic pathways or assessing the influence of various environmental factors on MP degradation in MP degradation studies.

Furthermore, the whole MP breakdown process, as well as variations in MPs, can be precisely tracked by functional bacteria (Janssen et al. 2002). Auta et al. (2018) identified two pure bacterial cultures from mangrove silt and utilized them to break down PP MPs. The weight loss of PP MPs induced by *Bacillus* sp. strain 27 and *Rhodococcus* sp. strain In MP degradation investigations, metabolic pathways or analysing the effect of various environmental conditions on MP degradation are both important. 36 was 4.0 and 6.4%, respectively, after 40 days of incubation. Following considerable study, it was revealed that the bacterium is responsible for altering the appearance of microplastics and their functional group structures and other features (Auta et al. 2018). To summarize, future studies are required to optimize techniques and enhance bacteria strains to increase their speed arbitrate the degradation process and increase the pace of MP breakdown (Yuan et al. 2020).

### 7.7.2 Fungal Degradation of Microplastics

Along with bacteria, fungi can get attached with and use Microplastics (Mitik-Dineva et al. 2009). Fungi can make MPs less hydrophobic by increasing the formation of chemical bonds like carboxyl, carbonyl, and ester functional groups. Until recently, however, there was little research on the fungal-arbitrated elimination of MPs in the literature. Using ectopic screening, the problems of obtaining fungal strains with strong MP-degrading activity were demonstrated (Yuan et al. 2020). Research into the breakdown of microplastics by fungi in various environments is still underway, but some progress has been made. *Penicillium simplicissimum* YK was identified by Yamada-Onodera et al. (2001) for application in PE biodegradation. Surprisingly, the aforementioned strain was able to grow better on a solid medium added with 0.5% PE after 500 h of irradiation than on unirradiated media. Dantzler et al. tested two different isolates of *Pestalotiopsis microspora* for their ability to degrade polyurethane (PUR) to determine if the fungus can degrade a range of MPs in another research (Russell et al. 2011). They discovered that a serine hydrolase was revealed to be the reason for the breakdown of PUR, suggesting that fungus-secreted enzymes can help with MP biodegradation. Devi identified two isolated fungus strains capable of degrading HDPE (*Aspergillus tubingensis* VRKPT1 and *Aspergillus flavus* VRKPT2) (Devi et al. 2015). Hydrolyzable polymers can also be degraded by fungi. Deguchi et al. (1997) were the first to report oxidative assault on nylon-6,6 in the white-rot fungus, IZU-154, *Phanerochaete chrysosporium* and *Trametes versicolor*. These findings indicated that these fungal strains have a high ability to break down MPs in vitro. To improve the rate of fungal-mediated MP degradation, future investigations should employ genomes and proteomics approaches.

### 7.7.3 Microalgal Degradation of Microplastics

The microalgae and plastic interaction waste can substantially alter the properties of these polymers, affecting their fate in aquatic ecosystems (Yokota et al. 2017). Among primary producers, filamentous cyanobacteria of the genus *Phormidium* are known to break down hydrocarbons (Oberbeckmann et al. 2016). It has been well known that species from this genus can be found on plastic surfaces. This raises the intriguing possibility that *Phormidium* is hydrolysing the plastic in the plastisphere actively (Yokota et al. 2017). The advantage of increased sunshine exposure on floating plastic pieces may be the real source of plastic trash enrichment as cyanobacteria are photosynthetic in nature (Roager and Sonnenschein 2019). Microalgae were exposed to high-density polyethylene microplastics at a concentration of 1 g $L^{-1}$ and 400–1000 μm diameter polypropylene (Lagarde et al. 2016); 2 mm polystyrene at 3.96 g $L^{-1}$ (Long et al. 2017), and microbeads from cosmetic products at around 4000 microbeads (Long et al. 2017); and 2 mm polystyrene. Long et al. (2015) explored this interaction in a lab experiment using various aggregates generated from two different algae species (the cryptophyte *Rhodomonas salina*, the diatom *Chaetoceros neogracile*, and a mix of both) and 2 m polystyrene microbeads. The experiment revealed that the microbeads were enriched in all three forms of aggregates. Once absorbed, the microbeads increased aggregate sinking rates to several hundred meters per day, a substantial increase over loose beads' sinking rate (less than 4 mm $day^{-1}$). These results are evidence to the idea that the aggregates of phytoplankton can act as an MP sink. Furthermore, when an aggregate splits, more surfaces and macropores become available for microbeads to cling to, allowing microbeads to be incorporated not just at the aggregate surface or in macropores, but throughout the aggregate (Long et al. 2015).

### 7.7.4 Microbial Consortia in Microplastics Degradation

Numerous investigations have revealed that when an axenic bacterium (pure bacterial cultures) biodegrades organic substances, hazardous end products are produced that impede the growth of microbes (Dobretsov et al. 2013). Using a mixture of bacteria to establish an intact community of microbes that have the ability to assist minimization of harmful metabolite effects on microplastics-degrading bacteria when compared to axenic bacterial cultures. In addition to that, a microbe's poisonous metabolites can frequently be used as a growing substrate of different microorganisms. Consortia bacteria have synergistic symbiotic and mutual relationships, which allows them to be more tolerable and active during pollutant treatment (Singh and Wahid 2015). Park and Kim (2019) looked at how a mesophilic mixture of bacteria generated from trash debris broke down PE MPs. Both *Paenibacillus* sp. and *Bacillus* sp. were plentiful in the mixture of bacteria and decreased the dry weight of Microplastics particles (14.7% after 60 days) as well as the mean diameter of Microplastics particle (22.8% after 60 days). Earthworms (*Lumbricus terrestris*) degrade LDPE, according to Huerta Lwanga et al. (2018).

The earthworm's intestinal bacteria absorbed and destroyed LDPE MPs after they were consumed. This shows that MP-degrading microbes are present in the earthworm's digestive system. Lwanga also looked at the role of bacteria in gut of earthworms in the decomposition of microplastics. The most common isolates from the earthworm's gut were members of the genera Firmicutes and Actinobacteria. Researchers have discovered that when isolated strains were tested for microplastics degradation, the particle size of LDPE-MPs was drastically decreased in the presence of bacteria. There are also eicosane, tricosane, and other volatile chemicals present. Only the treatments that included both LDPE-MP and bacteria produced docosane, indicating that these long-chain alkanes were produced as a result of bacterial-mediated LDPE-MPs breakdown. Due to interactions between many microorganisms and various enzymes, the breakdown and usage of microplastics by mixture of bacteria is a baffling process. As a result, it will be critical to in the future to develop in-depth research on the influencing factors (Yuan et al. 2020).

### 7.7.5 Microbial Biofilm in Microplastics Degradation

MPs are exposed to inorganic particles, organic matter, and microbes in aquatic settings (Parrish and Fahrenfeld 2019). Microorganisms of many forms and sizes, including bacteria, algae, viruses, protists, and fungi can cling at the surfaces of Microplastics as a result (Oberbeckmann et al. 2016). Biofilms, which are complex ecosystems made up primarily of microbes, organic and inorganic particles, cell secretions, and other materials, can form as a result of the colonization of these bacteria (Flemming 1998). Microplastics with rough or smooth surfaces, low or high densities, and a wide range of chemical compositions can be used as a biofilm development substrate. Biofilms change and degrade MPs' physical qualities, according to growing research (Rummel et al. 2017). Lobelle and Cunliffe (2011) studied the development of early biofilms on PE MPs surfaces for three weeks. One week later, biofilms were easily visible on PE surfaces, and they remained intact to proliferate for the next 14 days. The total number of heterotrophic microorganisms on the lowered MPs increased as well, Week three saw an increase in cell count from $1.4 \times 10^4$ cells $cm^{-2}$ to $1.2 \times 10^5$ cells $cm^{-2}$. Over the course of 3 weeks, As the contact between seawater and air became more hydrophilic, the MPs began to sink. Under the influence of biofilms, there was some damage and certain changes that occurred to PE MPs. Miao et al. (2019) investigated the microbial community in various substrates using high-throughput sequencing and generated community metrics such as evenness, diversity, and species richness. Natural materials have less bacteria connected to plastic degradation than MPs, according to the researchers. MPs were shown to have higher levels of Phycisphaerales, Pirellulaceae, Roseococcus, and Cyclobacteriaceae than originally occurring substrates, demonstrating that microplastics are microbial specific. Biofilms utilizes microplastics as carbon sources, energy sources, and adhesion media in conjunction with microbes and enzymes, attacking and degrading them. Biofilms' destruction of MPs, on the other hand, is extremely difficult and has yet to be thoroughly

researched. MP breakdown products, for example, have not been effectively collected and studied. Furthermore, routine data analysis for weight loss and cosmetic alterations has aided in the present understanding of biofilms' impacts on MPs. More controlled study and product tracking will be carried out in the future to better understand these relationships and degradation behaviour.

### 7.7.6 Bioreactor Systems for Microplastic Removal from Wastewater

The majority of microplastics were eliminated from the bioreactor system by bacterial consumption and the formation of sludge aggregates. Domesticated activated sludges, in particular, have been reported to increase microplastic build-up in WWTPs. During the subsequent secondary settling operation, microplastic-containing sludge was eliminated (Jeong et al. 2016). In WWTPs, the A2O bioreactor system is the most extensively utilized (Liu et al. 2021). Due to the sludge return, however, it has a low microplastics removal effectiveness. Microplastics that had been absorbed by the sludge would return to the aqueous phase in a small percentage (20%). Furthermore, the breakdown of microplastics in A2O is relatively sluggish (Liu et al. 2021). As a result, the typical activated sludge method of removing microplastics from WWTPs is inefficient.

Membrane bioreactor or MBR technology has lately gained popularity WWTPs treatment method. Because of the high concentration of mixed-liqueur suspended particles, it has an exceptional performance in removing microplastics (removal efficiency of 99.9%) (range from 6000 mg $L^{-1}$ to 10,000 mg $L^{-1}$) (Dvořák et al. 2013; Talvitie et al. 2017). Membrane separation and the classic activated sludge technique were combined in MBR technology. The bulk of microplastics were maintained on the MBR system's biofilm carrier side. This revealed that the adsorption effect is one of the most important factors in microplastic elimination in the MBR system. The pore size of the membrane employed in MBR systems is typically 0.1 m (Atasoy et al. 2007). Biofilter technology is employed as a major technology following the bioreactor system. MPs with the small size of particles and lesser density are flooded into the biofilter treatment unit. The removal of MPs became more challenging as a result of these factors. Biofilter technology, on the other hand, has the best microplastic removal performance (Lei et al. 2018). The major strategies for removing microplastics are biofilm filtration and adsorption, and biofilter technology combined physical and biological purification processes (Liu et al. 2021). Because microplastics are considered microbe transporters, their presence will have an impact on the microbial activity and community. According to Li et al. (2020), the number of functioning units of taxonomy dropped from 1665 to 1533 after the addition of PVC. As a result, there was an increase in the number of functional units of taxonomy to 1735. As a result, the presence of microplastics PVC did not result in a significant decrease in operational taxonomic units and had no effect on microbial community structure. It is also reassuring to learn that virgin microplastics had no effect on phosphorus-accumulating organisms, nitrite-oxidizing bacteria, or

ammonia-oxidizing bacteria (Liu et al. 2019). As a result, the microplastics effect on the functioning of bioreactor system must not be overestimated. On the other hand, the additives toxicity in MPs to microorganisms was unknown. The influence of microbe-containing MPs on traditional pollution remediation should be studied in the future.

## 7.8 Challenges and Future Perspectives

Multiple knowledge gaps and areas of disagreement must be addressed before the number of MPs in WWTPs can be estimated. To have a better picture of annual MP deposits into WWTPs, temporal and regional trends in MPs must be investigated. An estimate of the annual variance of MPs in wastewater and the ability of WWTPs to handle such flows have yet to be explored, but it is crucial for gaining a better grasp of worldwide MPs wastewater trends. Furthermore, nothing is known about MPs' ability to store and transfer chemical and microbiological contaminants across the landscape (including diseases). Treatment plants contain a wide range of dangerous pollutants and pathogens, but little is known about MPs' ability to adsorb them at all phases of the treatment process and thereafter. Existing studies of microplastics in WWTPs have some flaws that need to be addressed in future research. The establishment of standardized sampling and analysis methodologies to understand the fate of microplastics better in WWTPs or any other media of environment should be the centre of attention of future research. Standardization of reporting units for MP concentration is required. Furthermore, because size of MP particle ranges from 100 nm to 5 mm, mass units are the most accurate depiction of MP contamination within a given sample, allowing for more efficient comparisons between sampling locations. Simultaneously, additional study into specific microplastics should be prioritized, particularly in industrial zones. Hydraulic retention time, salinity, and dissolved organic matter, all of which influence the treatment processes' ability to eliminate microplastics in WWTPs, deserve further investigation. Furthermore, the microplastic removal effect of reaction intermediates, removal of contaminants and their toxicity created by the current treatment technique was unknown. Several MPs-degrading functional microbes have been identified, and several methods for characterizing them have been established. To garner a decrease in MPs, functional microbial agents must be explored and even genetically engineered. The investigation of MP degradation mediated by microbes is a significant undertaking. Greater formation of microbial potential and their use in MP treatment will be required in the future to reduce MP pollution.

On average, a total of 70% of MPs were eliminated during primary treatment. The role of dissolved air flotation (DAF) in the removal of MPs was the most evident at this stage (Bui et al. 2020). The membrane bioreactor (MBR) system is currently the most outstanding treatment technique for secondary treatment, with a success rate of over 99% (Lares et al. 2018). This study indicates that integrating primary treatment units with MBR procedures improves MP removal from wastewaters. One of these technologies' disadvantages is that there are still few studies testing MBRs' efficacy

on MPs; the influence of MPs on the cost–benefit analysis and membrane fouling, have gotten little attention. Furthermore, the operating parameters and environmental elements of MBR had a considerable impact on MP removal efficiency. And the research has not focused on these difficulties so far. As a result, it is critical to pay more attention to future investigations on Microplastics removal in various bioreactors, particularly MBRs.

It is also worth noting that the lack of consistency in analytical investigations leads to a wide range of outcomes. Ignorance of small MPs (20 m) and lack of MPs mass estimation are just a few of the significant issues with the analysis approach. Despite purification and clean treatment, it is impossible to eliminate contaminants from MPs samples, resulting in an unspecific spectrum that is difficult to differentiate from the library's standard spectrum. Long-term data is required for the accurate assessment of MPs concentrations in WWTPs throughout the year.

## 7.9 Conclusion and Recommendations

Despite the fact that WWTPs are not specifically designed to remove MPs, millions of MPs are discharged into the environment every day, both from treated water outflow and sewage sludge used for soil augmentation. As a result, these facilities are seen as a potential source of MPs entering aquatic environments. However, all MP study results are based on laboratory circumstances that can never match the actual environment, hence absolute choices based on laboratory research on how MPs function in an organism's natural habitat are unreliable, and therefore, extensive future research is required. This chapter's conclusions and recommendations are as follows:

a. WWTPs should be prioritized as hotspots for preventing microplastics from entering the environment.
b. More emphasis on improving and implementing advanced tertiary treatment techniques to remove more MPs from treated water is needed.
c. Depending on the species, bioremediation could be a viable option for degrading or accumulating microplastics in wastewater treatment.
d. Research into new methods and biotechnologies for removing MPs from sludges with high efficiency is required.
e. Evaluation of candidate species' ability to retain MPs in realistic environmental concentrations should be carried out.

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# Microbial Community Composition and Functions in Activated Sludge Treatment System

8

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**Abstract**

Activated sludge is the most popular biological method for treatment of wastewater. This process has successfully eliminated detrimental environmental impacts, such as toxicity, persistent organic materials, depletion of oxygen, and formation of algal blooms. However, it is often considered as economically and environmentally unsustainable wastewater treatment technology. The advent of latest technologies and improvements in metagenomics and metaproteomics study has provided a detailed insight into the microbiome of activated sludge treatment system. The present chapter mainly deals with the microbial community present in activated sludges and its composition. The seasonal modulation of the microbial communities in activated sludge is also discussed in detail along with the abundance of different microbial groups and their role and physiological activities in activated sewage sludge are reviewed. Antibiotic resistance genes present in activated sludge have also been discussed in detail.

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**Keywords**

Activated sludge · Wastewater treatment plants (WWTP) · Biological properties · Antibiotic resistance gene

## 8.1 Introduction

A huge amount of wastewater is produced continuously by urban, agricultural, and industrial sectors. This wastewater is characterized by elevated levels of nitrogen, carbon, and other organic elements which leads to the eutrophication of aquatic bodies. The inputs of the wastewater vary greatly leading to a constant change in the composition of wastewater (Kumar and Thakur 2020; Kumar et al. 2021a, c, 2022a, b). Chemically, the wastewater composed of organic and inorganic components is very complex in nature and in any wastewater system only 16% of the water is reused and only 35.8%, 35.8%, and 35.7% of organics, ammonical nitrogen ($NH_4^+$-N), and total phosphate (TP) can be recovered. Thus, detoxification of both domestic and industrial wastewater is considered as a crucial step for protection of environment. The activated sludge technique is currently the widely accepted process for biological treatment of wastewater which is effective for removal of organic pollutants and petroleum product, benzopyrene, and toluene. The activated sludge process is a favored process for the treatment of wastewater as it is considered to be very cost effective and the microbes in the sludge helps in pollutants removal and detoxification. Activated sludge is characterized by the presence of a wide range of bacteria, archaea, viruses, and protists which have very closely interconnected trophic interactions. Since its proposal by Arden and Lockett in 1913, this process has undergone several changes and has been extensively remodeled. The process is broadly divided into two phases including the aeration phase and sludge settlement phase. Settlement is not allowed during the first phase and the wastewater is passed from primary settlement tank into the aerobic tank which is characterized by wide range of microbial population. The aerobic tank is mainly aerated by surface agitation or addition of oxygen via diffuser which is essential for the growth of aerobic microorganisms in the reactor. This oxygen is vital for the maintenance of the microbial flocs and maximizes the contact time between the surface of floc and wastewater. Moreover, oxygen facilitates mass transfer and efficiently dissipate the metabolic products trapped in the flocs. The main function of this activated biomass is the production of a wide range of enzymes which helps in the degradation of the organic pollutant and also perform ammonification, nitrite and nitrate oxidation, and denitrification process which help in a considerable reduction in the nitrogen content. In the second stage, flocculated biomass settles to form sludge which clears the effluent from solids and is discharged as the final effluent. In an activated sludge process, for every kilogram of biological oxygen demand (BOD) removed around 0.5 kg and 0.8 kg dry weight (DW) of sludge is produced. Most of the activated sludge is then returned to maintain a sufficient microbial population to oxidize the upcoming wastewater. The

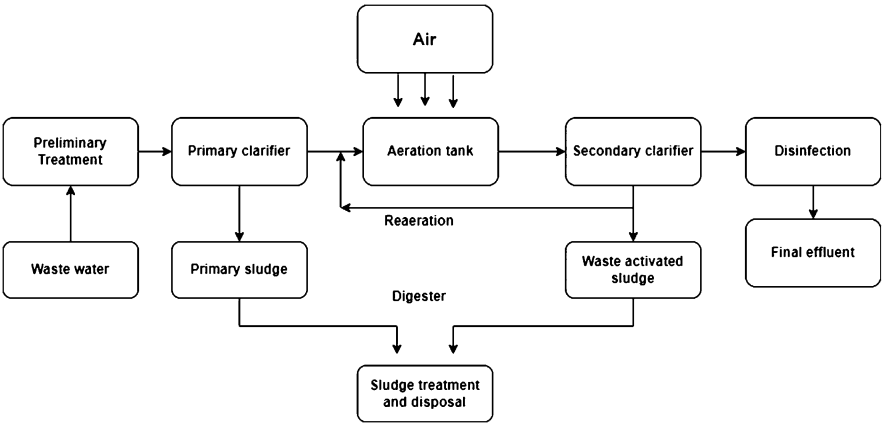


**Fig. 8.1** Flow diagram for the activated sludge process

maintenance of microbial flocs is very crucial in any activated sludge process. They not only help in the adsorption of organic matter but also help in the rapid and effective separation of effluent in sedimentation tank itself. The detailed flow diagram of the activated sludge process is presented in Fig. 8.1 which explains the main two stages. The main component of activated sludge consists of flocculant suspension bacteria, other microfauna, and microflora along with adsorbed particulate matter. It is noted that any changes in the operation parameters may alter the nature of microbial floc which may generate turbid effluents due to scanty settlement leading to a subsequent loss in biomass. Activated sludge works efficiently in food limited conditions and each microbe uses its own cellular content and reduces the biomass produced. The two principles for removal mechanism in an activated sludge process are assimilation and mineralization. Assimilation process is carried out by utilizing the waste materials to create biomass associated with the rapid removal of BOD. Mineralization occurs by conversion of waste material to inert end products that are left in solution in the effluent and requires longer aeration times.

The present chapter mainly deals with the functions and composition of the microbial community present in activated sludge. The seasonal variation of the communities of microbes in activated sludge has been discussed in detail along with the abundance of different microbial groups, their role, and physiological activities in activated sewage sludge were reviewed. Antibiotic resistance genes present in activated sludge have also been discussed in detail.

## 8.2 Characteristics of Activated Sludge

Carbohydrates, lipids, and proteins are the chief organic components present in municipal wastewater, which provide nutrients to the bacterial community and help in floc formation. The inputs of the wastewater vary greatly leading to a

constant change in the composition of wastewater. Chemically the wastewater is composed of organic and inorganic components which is very complex in nature and it is difficult to completely define it. In several research works, it was found that carbohydrate was associated with particles of size greater than 63 μm (Sophonsiri and Morgenroth 2004). Huang et al. (2010) also reported that size fraction lesser than 0.1 μm contribute to nearly 62% of the total organic carbon (TOC) which is mainly complexed with proteins or carbohydrates. Nitrogen can be present in both inorganic forms that is in form of ammonium or nitrate or else present in organic forms. Generally, nitrate is presence in activated sludge are in a soluble form which is the most concern for groundwater pollution. On the other hand, inorganic nitrogen in the form of ammonium is volatile and is lost. Organic nitrogen found in activated sludge can be considered as inert and needs to be degraded by microorganisms, or mineralized to inorganic ammonia ($NH_4^+$ and $NO_3^-$). Some other sludge constituents, including calcium (Ca), magnesium (Mg), phosphorus (P), and iron (Fe), are known to form insoluble compounds with sludge solids, and are present at high concentrations. Other sewage sludge constituents, such as potassium and sodium, being water-soluble, are normally discharged with the treated wastewater. Suspended solids present in activated sludge mostly comprise 70% organic solids and 30% inorganic solids which includes food particles, fecal matter, garbage associated with sand, grit, and clay, which can only be removed from the wastewater using physical or mechanical processes, such as sedimentation or filtration. Other compounds, such as surfactants, humic acids, tannic acids, volatile fatty acids (VFAs), amino acids, RNA, and DNA, has been recorded in activated sludge.

## 8.3 Microbial Diversity in Activated Sludge

Activated sludge is constituted of a plethora of anaerobic and aerobic bacteria, fungi, archaea, and protists which are able to degrade organic pollutants and also reduce toxic metals to its related nontoxic forms. Activated sludge is considered as a complex medium having interconnected trophic relationships between microorganisms. Activated sludge harbors great biodiversity having a functionally important population. In complex ecosystems, bacteria accounts for nearly 95% of the total microbes, which play a crucial role in wastewater treatment. The microbial community of activated sludge was previously studied by culture-dependent methods (Zhang et al. 2018a, b; Yang et al. 2020); however, it does not give a thorough idea due to the incapability to grow most of the microbes in any specific culture conditions. With the advent of different molecular biology methods, the domain of microbial diversity has been revolutionized. Different techniques such as PCR-based techniques provide detailed information on the expression and diversity of ribosomal as well as protein coding genes in the activated sludge environment.

The advent of the "-omics era" has been considered as a breakthrough in the study of microbial diversity, both phylogenetically and functionally. High-throughput sequencing (HTS) using 454-pyrosequencing and Illumina has generated millions of sequence reads in a cost-effective way for superior understanding of the microbial

diversity and their genomic-potential in environmental samples (Kumar et al. 2020, 2021b). Also, methods like DNA-fingerprinting, clone-library, quantitative polymerase chain reaction (qPCR), and fluorescence in situ hybridization (FISH) studies based on functional genes or 16S rRNA gene segments have helped in developing idea on the microbial community of the activated-sludge (Johnston et al. 2019).

Normally sludge is characterized by the presence of floc made up of highly complex microbial communities comprising of archaea, bacteria, and viruses. The bacterial population plays a crucial role in the degradation of nutrients and organic pollutants containing both phosphorus and nitrogen. Moreover, they have the ability to tolerate adverse environmental impact, toxicity, and oxygen depletion. Metabolically they are diverse and perform a crucial role in biological nitrification and oxidizes ammonia to nitrate and nitrite then to nitrogen via denitrification and was found to be dominated by both ammonia-oxidizing bacteria (AOB) (Park et al. 2006; Gao et al. 2014; Pang et al. 2016) and nitrite-oxidizing bacteria (NOB) (Lucker et al. 2010). Research has been conducted on ammonia-oxidizing microorganisms, nitrite-oxidizing bacteria, denitrifiers (Zielinska et al. 2016; Pang et al. 2016), and phosphorus-accumulating organisms (PAOs) (Mielczarek et al. 2013). They have several biomarker genes such as ammonia monooxygenase (*amo*) (Ye et al. 2011) and nitrite reductase subunits (*nirK* and *nirS*) (Geets et al. 2007).

The activated microbial-community comprises Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes along with the presence of Actinobacteria, Chloroflexi, Planctomycetes, Acidobacteria, and Verrucomicrobia (Gao et al. 2016). Yu and Zhang (2012) in their study suggested that bacteria were dominant accounting for nearly 92% and 69% of DNA- and cDNA sequences, respectively, whereas eukaryotes account for approximately 43 and 30.97% of the total sequences in DNA and cDNA, respectively. They also reported that the bacterial community was dominated mostly by Proteobacteria, followed by Actinobacteria, Bacteroidetes, and Firmicutes, representing nearly 22%, 15%, 6%, and 3% of small subunit ribosomal DNA (SSU rDNA) reads, respectively. Both *Verrucomicrobia* and *Nitrospirae* exhibited high occurrence in protein-coding DNA reads. Among Archaea, Euryarchaeota also represented a very high amount of SSU rDNA (19.38%). Actinobacteria, Firmicutes, Planctomycetes, and Euryarchaeota showed a % SSU rRNA–% SSU rDNA ratio of less than one. Bacterial SSU rDNA and rRNA sequence reports show a high abundance of Proteobacteria which was followed by phyla, such as Bacteroidetes, Verrucomicrobia, and Actinobacteria. The main genera occurring in activated sludge are *Nitrosomonas*, *Nitrosospira*, *Methylocystis*, and *Methylosinus* having high ammonia monooxygenase activity. Similarly, *Nitrosomonas*, *Nitrosospira*, *Methylocystis*, and *Anaeromyxobacter* account for the activity of nitrification enzyme. Genera like *Acidovorax*, *Cupriavidus*, *Leptothrix*, *Alicycliphilus*, *Paracoccus*, and *Escherichia* were also reported which have high hydroxylamine reductase activity. On the other hand, *Riemerella*, *Dyadobacter*, *Dechloromonas*, *Candidatus accumulibacter*, and *Acidovorax* reported high nitrous oxide reductase activity. The wastewater treatment plants contain *Curvibacter*, *Azoarcus*, *Thauera*, *Zoogloea*, and *Accumulibacter*, which are mainly denitrifiers, *Tetrasphaera* and *Accumulibacter*, which are reported

to be phosphorus-accumulating organisms. Filamentous bacteria such as *Microthrix parvicella* and *Gordonia* was also abundant.

Zielinska et al. (2016) identified the presence of 38 orders from microbial consortia of wastewater treatment plants (WWTPs) which include Anaerolineales, Burkholderiales, Rhodocyclales, Planctomycetales, Rhizobiales, and so on and six core genera, such as *Prosthecobacter*, *Ferruginibacter*, and *Zooglea*. The presence of denitrifying populations, such as *Azoarcus*, *Thauera*, *Curvibacter*, and *Dechloromonas*, was also evident (Thomsen et al. 2004, 2007). *Candidatus accumulibacter* belonging to the family Rhodocyclaceae were designated as phosphorus-accumulating organisms were also identified along with *Pseudomonas* having denitrifying properties. *Halomonas* was also present in large numbers comprising 5% of the microbes (Nguyen et al. 2012). Kristiansen et al. (2013) reported Tetrasphaera of family Intrasporangiaceace which contained functional genes for denitrification. Similarly, Nielsen et al. (2009) reported the abundant presence of *Dechloromonas* spp. which was also a denitrifier and a putative PAO. Moreover, Acinetobacter (*Moraxellaceae)* was also an abundant bacterial genus which is strictly aerobic and chemoorganotrophic in nature with oxidative metabolism (Vandewalle et al. 2012).

Later in a study, Zielinska et al. (2016) reported the presence of Alphaproteobacteria, and Betaproteobacteria. Among Alphaproteobacteria, Rhizobiales, and among Betaproteobacteria, Burkholderiales were present. Their findings also show lesser presence of gamma (6.5%) and deltaproteobacterial sequences (9.9%) compared to the previous studies conducted by previous researchers. Apart from them, bacterial reads belonging to the order Rhodobacteriales, Rickettsiales, and Rhodocyclales were also reported in this study. The microbes were reported to contain genes coding for periplasmic nitrate-reductase (*napA)* and a gene coding for membrane-bound nitrate reductase (*narG*) (Heylen et al. 2006). However, Actinobacteria accounts for nearly 11% narG.

In a much later study, Zhang et al. (2019) reported the presence of bacterial operational taxonomic unit (OTU) assigned to 14 different phyla including Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Firmicutes, Chlorobi, Planctomycetes, Verrucomicrobia, Saccharibacteria, and Proteobacteria. Proteobacteria consisted of a total of 47% of the OTUs, followed by Bacteroidetes (30%), Firmicutes (7%), Acidobacteria (2.2%), and Chlorobi (1.2%). Among Proteobacteria, classes Gammaproteobacteria (25%) and Betaproteobacteria (24%) were the most prominent. In addition, Flavobacteriia (18%) and Cytophagia (13%) were also significantly abundant. Gammaproteobacteria, being more sensitive to antibiotics, was present in much less quantity (Novo et al. 2013).

Core-microbial OTUs existing in activated sludges were studied and identified by the Global Water Microbiome Consortium (GWMC) (http://gwmc.ou.edu/) which reports the presence of 28 core taxa; however, nearly half of them are annotated only at genus or family level. Song et al. (2020) reported OTU_16 of *Betaproteobacteria* could not be annotated to any taxa. While working they isolated 830 isolates of which Strain SJ-1 was characterized and reported as a novel species, *Casimicrobium huifangae*, of the novel family *Casimicrobiaceae*.

Johnston and Behrens (2020) reported the core microbial community largely comprises Saprospiraceae, *Trichococcus*, *Microthrix*, *Tetrasphaera*, and Fibrobacteraceae. However, only constant activity was visible in Bacteroides, *Hypnocyclicus*, and *Tolumonas*. *Kouleothrix*, *Chloroflexi*, and *Gordonia* showed extensive growth in activated sludge, which is associated with sludge bulking and degrading various xenobiotic compounds. Apart from them *Leptotrichia*, *Arcobacter*, and *Acinetobacter* were also reported which are enteropathogenic bacteria related to human infections.

The details of the microbial community available in activated sludges obtained from different studies are presented in Table 8.1.

## 8.4 Enzyme Activity and Associated Physiological Function of Microbiome in Activated Sludge

A wide range of enzymatic activity was seen by the microbial community in the wastewater. In earlier research done by Nybroe et al. (1992), it was reported that esterase and dehydrogenase activities were correlated with the presence of heterotrophic bacteria. In activated sludge, they did an extensive study in which four different enzymes including α-glucosidase, alanine-aminopeptidase, esterase, and dehydrogenase were obtained from different types of wastewater. The enzyme profile showed the existence of a diverse group of bacteria with a wide range of activities. Konneke et al. (2005) and Park et al. (2006) reported the presence of diverse bacterial communities which perform a vital role in different types of nitrogen metabolisms. Most of the microbes perform a crucial role in ammonification, nitrite and nitrate oxidation, and denitrification, which help in a considerable reduction in the nitrogen content of the wastewater.

With the advent of modern technologies and metaproteomic study, it has helped in providing a more detailed insight of the microbial community and helped in detection of different types of enzyme variants, which indicated the degree of genetic diversity in sludges. Metaproteomic study of the extracellular polymeric substances present in activated sludge also revealed the presence of several cytoplasmic proteins, which may play various roles in the treatment of activated sludge biomass.

The process of nitrification is carried out by two diverse domains of microbes: ammonia-oxidizing microorganisms (Konneke et al. 2005; Park et al. 2006), which oxidize ammonia into nitrate, and nitrite-oxidizing bacteria, which oxidize nitrite into nitrate (Lucker et al. 2010). Ammonia-oxidizing microorganisms lead to the complete oxidation of ammonia (comammox), which oxidizes ammonia via nitrite to nitrate (Jiang et al. 2020). Under anaerobic conditions, denitrifying bacteria reduce nitrite to gaseous-forms like nitrous-oxide and dinitrogen gas which in turn may reduce the wastewater nitrogen concentration. These group of bacteria is represented by bacteria *Curvibacter* within Comamonadaceae, apart from which genera like *Azoarcus*, *Thauera*, *Dechloromonas*, and *Accumulibacter* (Zielinska et al. 2016).

Both DNA and cDNA show the presence of a wide range of ammonia assimilation, nitrite/nitrate ammonification, denitrification, and nitrogen fixation–related

**Table 8.1** Microbial abundance in activated sludge

| Phylum | Class | Genera | Method of study | References |
|---|---|---|---|---|
| Halobacterota<br>Micrarchaeota<br>Nanoarchaeota<br>Proteobacteria<br>Bacteroidota<br>Patescibacteria<br>Myxococcota<br>Actinobacteriota Planctomycetota<br>Chloroflexota<br>Acidobacteriota<br>Firmicutes | – | – | – | Ye et al. (2020) |
| Proteobacteria<br>Nitrospirae<br>Chloroflexi<br>Chlamydiae<br>Chlorobi<br>Chloroflexi<br>Elusimicrobia<br>Ignavibacteriae<br>Latescibacteria<br>Parcubacteria<br>Spirochaetae<br>Armatimonadetes<br>Bacteroidetes<br>Chlamydiae<br>Chlorobi<br>Cyanobacteria<br>Fibrobacteres<br>Verrucomicrobia | – | *Nitrosomonas* sp., *Hyphomicrobium* sp., *Nitrospira* sp., *Bdellovibrio* sp., *Thauera* sp., *Halochromatium* sp., *Terrimonas* sp., *Ferruginibacter* sp., *Dechloromonas* sp., *Hyphomicrobium* sp., *Phaeodactylibacter* sp., *Parafilimonas* sp., *Nitrosomonas* sp., *Nitrospira* sp. | Illumina HiSeq 2500 | Yang et al. (2020) |

| | | | | |
|---|---|---|---|---|
| Proteobacteria<br>Actinobacteria<br>Acidobacteria<br>Bacteroidetes | | *Sphingopyxis* sp., *Bradyrhizobium* sp., *Candidatus* sp., *Saccharimonas* sp., *Mesorhizobium* sp., *Bosea* sp., *Niastella* sp., *Acidovorax* sp., *Alicycloiphilus* sp., *Sphingomonas* sp., *Thauera* sp., *Azoarcus* sp., *Candidatus Contendobacter* sp., *Candidatus Competibacter* sp., *Pyrinomonas* sp., *Piscicoccus* sp., *Kineosphaera* sp., *Microlunatus* sp., *Dehalobacter* sp., *Nitrospira* sp., *Tetrasphaera* sp., *Nakamurella* sp., *Propionicella* sp., *Friedmanniella* sp. | Illumina sequencing | Ai et al. (2019) |
| Proteobacteria<br>Bacteroidetes<br>Firmicutes<br>Chlorobi<br>Chloroflexi | Alphaproteobacteria<br>Betaproteobacteria<br>Albirhodobacter | *Dyadobacter* sp., *Variovorax* sp., *Nitrosomonas* sp., *Nitrosospina* sp., *Brocadia* sp., *Nitrobacter* sp., *Nitrotoga* sp. | Illumina MiSeq sequencing system | Johnston et al. (2019) |
| Acidobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Chlorobi, Saccharibacteria, Verrucomicrobia, and Proteobacteria | Gammaproteobacteria (25%)<br>Betaproteobacteria (24%)<br>Flavobacteriia (18%)<br>Cytophagia (13%)<br>Clostridia (18%)<br>Sphingobacteriia (12%)<br>Epsilonproteobacteria<br>Mollicutes<br>Bacteroidia<br>Chloroflexia<br>Cytophagia | *Flavobacterium* sp. (13%), *Pseudomonas* sp. (8%), *Alcaligenes* sp., *Acinetobacter* sp., *Legionella* sp., *Acidovorax* sp., *Dokdonella* sp., *Bacillus* sp., *Lactobacillus* sp., *Terrimonas* sp., *Pseudofulvimonas* sp., *Nitrospira* sp., *Nitrosococcus* sp., *Nitrosomonas* sp., *Brevundimonas* sp., *Pedobacter* sp., *Chryseobacterium* sp., | Illumina Miseq sequencing platform | Zhang et al. (2019) |

(continued)

**Table 8.1** (continued)

| Phylum | Class | Genera | Method of study | References |
|---|---|---|---|---|
| | Nitrospira<br>Negativicutes<br>Ignavibacteria<br>Spartobacteria<br>Spingobacteriia | *Comamonas* sp., *Archrobacter* sp., *Stenotrophomonas* sp., *Dyadobacter* sp., *Caulobacter* sp., *Cohnella* sp., *Massilia* sp., *Exiguobacterium* sp. | | |
| Proteobacteria, Acidobacteria, Chloroflexi, and Bacteroidia<br>Actinobacteria<br>Synergistetes and Thermi | | *Caldilinea* sp., *Dechloromonas* sp., *Thiobacillus* sp., *VadinCA02* sp., *Thauera* sp., *Nitrospira* sp. | Illumina shotgun DNA library | Zhang et al. (2018a, b) |
| Proteobacteria<br>Nitrospirae<br>Bacteroidetes<br>Actinobacteria<br>Firmicutes<br>Euryarchaeota | Betaproteobacteria (46.19)<br>Gammaproteobacteria (11.14)<br>Alphaproteobacteria (8.19)<br>Deltaproteobacteria (1.51)<br>Epsilonproteobacteria (0.07)<br>Nitrospira (15.4)<br>Flavobacteriia<br>Sphingobacteriia<br>Cytophagia<br>Bacteroidia<br>Ignavibacteria<br>Actinobacteria<br>Gemmatimonadetes<br>Acidobacteriia<br>Solibacteres<br>Clostridia<br>Bacilli<br>Negativicutes | *Rhodobacter* sp., *Caulibacter* sp., *Pseudomonas* sp., *Geobacter* sp., *Rhodopseudomonas* sp., *Thauera* sp., *Dechloromonas* sp., *Bordetella* sp., *Nitrosomonas* sp., *Nitrospira* sp., *Mycobacterium* sp., *Placntomyces* sp., *Bradyrhizobium* sp., *Burkholderia* sp., *Xanthomonas* sp. | Illumina HiSeq. 2000 | Guo et al. (2017) |

| | | | | |
|---|---|---|---|---|
| Actinobacteria<br>Proteobacteria<br>Nitrospirae<br>Bacteroidetes<br>Firmicutes<br>Verrumicrobia<br>Spirocheate<br>Cyanobacteria<br>Deinococcus-Thermus<br>Chloroflexi | | *Nitrospira* sp., *Nitrosomonas* sp., *Pseudomonas putida* sp., *Nitrosovibrio* sp. | GeoChip 4.2 | Xia et al. (2016) |
| Actinobacteria<br>Proteobacteria<br>Verrumicrobia<br>Firmicutes<br>Bacteroidetes<br>Fusobacteria<br>Nitrospirae<br>Planctomycetes<br>Deinococcus-Thermus<br>Chloroflexi<br>Tenericutes<br>Euarchaeota | Deltaproteobacteria<br>Epsilonproteobacteria<br>Betaproteobacteria<br>Gammaproteobacteria<br>Alphaproteobacteria<br>Dienococci<br>Clostridia<br>Thermomicrobia<br>Methanobacteria<br>Methanomicrobia | *Acinetobacter* sp., *Akkermansia* sp., *Acidaminococcus* sp., *Cloacibacterium* sp., *Megasphaera* sp., *Prevotella* sp., *Streptococcus* sp., *Trichococcus* sp., *Gemmatimonas* sp. | GS Junior system (Roche) | Shchegolkova et al. (2016) |
| Actinobacteria<br>Proteobacteria<br>Nitrospirae<br>Bacteroidetes<br>Firmicutes<br>Verrumicrobia | Betaproteobacteria<br>Gammaproteobacteria<br>Alphaproteobacteria<br>Deltaproteobacteria<br>Flavibacteria<br>Nitrospira | *Mycobacterium* sp., *Clostridium* sp., *Hyphomicrobium* sp., *Tissierella* sp., *Sphingomonas* sp., *Desulfobacterium* sp., *Kribbella* sp., *Deinococcus* sp., | Metagenomics-rapid annotation using Subsystem Technology server (MG-RAST) | Yadav et al. (2014) |

(continued)

**Table 8.1** (continued)

| Phylum | Class | Genera | Method of study | References |
|---|---|---|---|---|
| Spirocheate<br>Cyanobacteria<br>Deinococcus-Thermus<br>Chloroflexi<br>Tenericutes<br>Verrumicrobia | Bacteroidia<br>Solibacteres<br>Chloroflexi<br>Clostridia<br>Cytophagia<br>Deinococci<br>Sphingobacteria | *Saccharopolyspora* sp., *Myroides* sp. | | |

genes. DNA sequences related to a wide range of enzymes such as hydroxylamine reductase, ammonia monooxygenase, nitrate reductase, hydroxylamine oxidase, nitrilase, formamidase, carbamate kinase, nitrous oxide reductase, nitrite reductase, nitric oxide reductase, and nitrogenase were obtained. Ammonification genes such as *amoCAB* which encodes enzyme ammonia monooxygenase increase with the rise in temperature from 20 °C to 35 °C, which was associated with a concomitant reduction in enzymes related with denitrification. At much lower temperature (20 to 5 °C) the genes connected to nitrogen metabolism were increased. Moreover, at lower temperature genes related to carbamate kinase, glutamate dehydrogenase, and glutamine synthetase were increased. Enzyme nitrite reductase (*nrfA*), associated with reduction of nitrite to ammonia, along with hydroxylamine reductase (*hcp*), associated with reduction of hydroxylamine to ammonia, was increased.

Yu and Zhang (2012) also reported the abundance of hydroxylamine reductase (*har*), ammonia monooxygenase (*amo*), nitrate reductase (*nar*), hydroxylamine oxidase (*hao*), nitrite reductase (nir), nitrous oxide reductase (*nos*), nitric oxide reductase (*nor*), and nitrogenase (*nif*) genes. In a 2.4 Gbp DNA *nir* gene was found in abundance, followed by *nor* and *nos* coding gene sequences. The prevalence of nitrification enzyme coding gene sequences along with *amo* and *hao* was found to be the lowest. Nitrifying virus was expressed in a higher amount than that of denitrification enzymes. In the case of hydroxylamine oxidase, the cDNA–DNA ratio was around 0.09. Nitrification enzyme genes, such as *amo*, showed much higher expression activities in activated sludges, which was mainly due to the higher concentration of ammonia in sewage.

Xia et al. (2016) reported a total of 528 genes which showed phosphorus utilization activity including polyphosphate kinase (*ppk*; 37.3%), exopolyphosphatase (*ppx* 57.6%), and *phytase* (5.1%). Exopolyphosphatase (*ppx*) was found to be highly capable of catalyzing the anaerobic hydrolysis of terminal residues of long-chain polyphosphate to inorganic phosphate (Pi). Apart from this, the genes related to a wide number of functions like carbon, phosphorus, and sulfur cycling, and also of organic pollutant remediation were reported. The genes related to processes such as denitrification, ammonification, nitrogen fixation, assimilatory, and dissimilatory nitrogen reduction were also found.

According to studies made by Song et al. (2020), they reported a novel species, *Casimicrobium huifangae*, which belonged to the core microbial community of activated sludge. The isolate was found to reduce nitrate into nitrite but neither into ammonia or into $N_2$, NO, and $N_2O$. Genes encoding nitrogen regulation sensor (*ntrB*), nitrate transport (*nasD* and *nrtA*), nitrite reductase (*nirBDS*), nitrate reductase (*narGHV*), and other proteins (*narJKL*) were annotated which was associated with nitrogen metabolism. This strain also has a wide range of phosphate transporters and conversion genes, such as *pstABCS* and *phnEC* for removal of phosphorus. Apart from them, one *ppx*, two *ppk*, and one poly(3-hydroxyalkanoate) polymerase gene (*phaC*) are also present which may help in phosphorus accumulation. Moreover, this isolate was also able to tolerate a wide range of heavy metals and have genes for p-type ATPase for efflux of metals and multidrugs (*mrcA, acrAB,* and *oprM*).

The GWMC recorded the universal occurrence of *Nitrospira* in a global survey of wastewater treatment plants. *Nitrotoga* and *Nitrobacter* were the most abundant nitrite oxidizers. Similarly, *Nitrosomonas* was also present which is the most prevalent ammonia-oxidizer. *Nitrosomonas*, *Nitrotoga*, and *Nitrobacter* were the nitrification bacteria.

## 8.5 Antibiotic Resistance Genes of Activated Sludge

Antibiotic resistance has been considered as a global problem and in developing nations like India, poor waste management and inadequate sanitary practices leads to the further spread of antibiotic resistance genes (ARGs) in environment. They are mostly persistence nature, have slow decaying rate, and are reckoned as chemicals of upcoming concerns or as potent pollutants. Wastewater treatment plants contain microbes from both human and environmental sources and can be a rich source of ARGs, which are developed by natural selection or by adaptation in bacteria due to constant exposure to antibiotics. Moreover, wastewater treatment plants receive water from households, and pharmaceutical industries which contains antibiotic residues and antibiotic-resistant bacteria at higher concentrations. All these exert a selective pressure on antibiotic-resistant bacteria and expression of ARGs (Nnadozie et al. 2017; Karkman et al. 2017), thus acting as a hotspot for the spread of antibiotic resistance in different groups of bacteria. Activated sludge, being rich in nutrient concentration, is ideal for bacterial growth and facilitates horizontal (lateral) gene transfer. Mainly resistance against antibiotic classes, such as β-lactams, fluoroquinolones, tetracyclines, and macrolides is most prevalent (Almakki et al. 2019).

Mobile genetic elements, such as a plasmids, transposons, and integrons, contribute largely to the dissemination of ARGs. However, till now very few studies have been conducted on the host cells which harbor such ARGs. As much as thirty ARGs encoding resistance to quinolones, sulfonamides, tetracycline, or macrolides were identified in activated sludge of two wastewater treatment plants of China by Mao et al. (2015). Mao et al. (2015) reported a significant enrichment of 10 ARG including *sulI, sulII, qnrB, tetG, tetB, tetS, tetH, tetX, tetT,* and *ermC*.

In a recent study by Liu et al. (2019), they have identified around 22 bacterial phyla which can act as a putative host for these genes. Genera, such as *Mycobacterium* and Burkholderiaceae family harbors around 14–50 ARGs. Metatranscriptome analysis showed nearly 65.8% of the identified ARGs were being expressed showing that they are transcriptionally active in the bacterial population of which most were plasmid associated rather than being within bacterial chromosomes. Several researchers like Bengtsson-Palme et al. (2016), Karkman et al. (2016), and Yang et al. (2014) showed the presence of antibiotic resistance genes associated with beta-lactam, sulfonamide, vancomycin, and tetracycline. Metagenomics analysis was found to be the most efficient method for the analysis of antibiotic resistance genes by researchers like Pal et al. (2016) and Van Goethem et al. (2018). Liu et al. (2019) in an extensive study on ARGs in activated sludge reported 24 different

classes of antibiotics in activated sludge and genes associated with antibiotics like acriflavines, aminoglycosides, betalactams, bacitracin, multidrug resistance (MDR), daunorubicin, macrolide–lincosamide–streptogramin (MLS), polymyxin, and sulfonamide. Inactivated sludge multidrug resistance genes were most abundant followed by betalactams, macrolide–lincosamide–streptogramin, and bacitracin. A similar research carried out by Zhao et al. (2018). Yang et al. (2013) reported aminoglycosides and tetracycline resistance to be most prominent in activated sludge. Twenty different antibiotic resistance genes, such as bacitracin (*bacA*, *bceA*), acriflavine (*acrB*, *acrF*), bleomycin (*ble*), beta-lactam (*pbp2*), fosmidomycin (*rosA*), kasugamycin (*ksgA*), daunorubicin (*drrA*), MDR (*mdtC*, *mdtB*, *mexK mexW*), polymyxin (*arnA*, *arnC*), sulfonamide (*sul1*, *sul2*), MLS (*macA*, *macB*), and trimethoprim (*dfrA3*), accounted for nearly 70% of the total types of ARGs, of which Gene *macB* (macrolide resistance gene) was very predominant in nature. Several genera of antibiotic resistance bacteria have also been reported in activated sludge, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* spp., Enterobacteria, *Pseudomonas*, and *Acinetobacter*, among others (Bouki et al. 2013; Figueira et al. 2011). Typically, members of Enterobacteriaceae reported resistance to 13 different antibiotics (Amador et al. 2015). Apart from them, opportunistic pathogenic bacteria such as *Enterococcus faecalis*, *Pseudomonas aeruginosa*, Enterobacteriaceae, *Staphylococcus aureus* with ARGs were also reported (Alexander et al. 2015).

Korzeniewska and Harnisz (2018) reported resistance to cefotaxime which is a relatively new antibiotic and its resistance was easily transmitted in Gram-negative *E. coli*.

Song et al. (2020) reported a novel species, *Casimicrobium huifangae*, carrying resistance to a wide range of antibiotics which included bacitracin (uppP), tetracycline (typA and lepA), streptogramin (vat), macrolides (macB), polymyxin (yfbG), kasugamycin (rsmA), aminoglycosides (aacA), and β-lactams.

Qi et al. (2021) reported different functional microbiomes, one associated with complete catabolism of sulfamethoxazole, and the second one was associated with complete catabolism of phenyl part of sulfamethoxazole (SMX). They also reported *Paenarthrobacter* and Nocardiodes as primary degraders of sulfonamide functional group (–C–S–N– bond) and (3-amino-5-methylisoxazole) (3A5MI). Yan et al. (2022) reported SMX and ARGs from both autotrophic and heterotrophic microorganisms. It was found that heterotrophic bacteria contributed crucially to SMX degradation; however, ammonia-oxidizing bacteria displayed a superior metabolic rate and contributed much to SMX removal by cometabolism.

## 8.6 Future Challenges and Opportunities

The activated sludge microbiome consists of a plethora of bacteria, archaea, viruses, and protists which play a crucial role in the degradation of toxic organic pollutants. Most of these microbial communities are interconnected at trophic levels and also related in their degradation and metabolic pathways. Earlier, it was always difficult

to assess them using culture dependent methods. With the advent of omics technology and the availability of the metagenomics and metatranscriptomic datasets, it has become possible to assess the whole community composition of activate sludge in detail. Moreover, the identification and assessment of ARGs present and actively transcribed have increased our understanding the fate of highly expressed ARGs and multidrug-resistant hosts from wastewater treatment plants. Also, both metagenomics and metatranscriptomic datasets have provided us with ample information on the influence of environmental factors in the activated sludge process. It has provided us with a detailed idea on the shift of alpha and beta community diversity due to variations in temperature, which is considered crucial for the effectiveness of the activated sludge process. We could also assess the key functional groups present in activated sludge, which largely include ammonia-oxidizing bacteria, denitrifiers, and nitrogen-fixing bacteria and their potential role in activated sludge. Further research on the microbial community of activated sludge will broaden our knowledge and help in better application and further modification of the process.

## 8.7 Conclusion

The activated sludge process is a process of biological treatment of wastewater which is popular all over the world. This entire process can be divided into the aeration phase and sludge settlement phase. The wastewater from the primary settlement tank is passed into the aerobic tank having a wide range of microorganism populations. The aeration phase helps in the maintenance of microbial flocs and maximizes the oxidation of the contaminant which is followed by sludge formation and separation. The advent of omics technology has helped us to gain a wide knowledge of the microbial community present in activated sludge. This bacterial community is a repository of many antibiotic resistance genes. Moreover, this microbial community has several physiological functions, performs several types of biogeochemical cycles, and sequestration of nutrient from the sludge. A detailed understanding of the microbial community assembly will help us to develop deeper understanding on the microbial-ecological theories.

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# 9 Decontamination and Management of Industrial Wastewater Using Microorganisms Under Aerobic Condition

Anamika Sharma, Shalini Sharma, Chaudhary Shalu Singh, and Vineet Kumar

**Abstract**

Water is required for the sustainability of life by all living beings. It is used for domestic as well as commercial purposes by human beings in daily activities. As the population is increasing the demand for potable water is also increasing accordingly. Although the resources of potable water are limited, water management is a requirement of the hour throughout the globe. Wastewater management practices reduces the burden on potable water sources available on the planet and enable to recycle and reuse of this treated water for different applications. We require affordable and durable technologies for the proper treatment of wastewater so that usable water will be available to all. From the ancient times of lagoons to the present, water recycling and management play a vital role in conservation of water. In the treatment of wastewater, various microorganisms play an important for the removal of organic and metallic content from wastewater. These microbes decontaminate effluents that lead to less hazardous effluents from various sources. Microbial treatment lowers biochemical oxygen demand and chemical oxygen demand and also reduces the organic content present in the wastewater. Various

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parameters of effluents determine the role and mechanisms of microorganisms that are used in different types of aerobic and anaerobic treatments. These will be discussed in the present chapter for recycling and reuse of water. Besides, controlling parameters of wastewater treatment plants like solids retention time, hydraulic retention time, and other attributes will be discussed in order to obtain reusable water as per the permissible limit norms set by the law of the land.

**Keywords**

Water management · Microorganism · Chemical oxidation · Pollution control board

## 9.1 Introduction

Natural water present in the form of frozen ice and running freshwater streams are generally considered as pure and fit for human consumption, that is, drinking and cooking purposes (DDWS 2011; Gautam and Saini 2020). But surface water from various resources which diffuse from upper surface to underground may get contaminated due to various chemicals or pollutants present on the earth surface, which leads to change in the chemical composition with altered values of pH, total dissolved solids (TDS), and other anionic and cationic values, making it unfit as potable water (WHO 2017). Freshwater gets contaminated or polluted by various industrial and domestic activities of human beings (Li et al. 2019; Liu et al. 2017; Chandra and Kumar 2017a; Kumar et al. 2021a). The scarcity of water is increasing day by day due to various reasons, so there is a huge demand of potable water resources leading to the way for proper decontamination of water and its management for meeting the demands (Barber 2014; Chávez et al. 2019). Water management practices are not new to humans as all major human civilizations bloomed near water bodies (Kumar 2018; Kumar and Katara 2020). The Indus Civilization witnessed the proper management of water through its town planning. For example, Lothal witnessed an underground water system, and Kalibagan also has evidences of a well-functioning irrigation system (Kumar and Katara 2020). Water temples, stepwells, johads, bawdis, and percolation-cum-storage wells are a few examples of preserving water in the last centuries that are evident today too as Agarsen ki bowli in Delhi (Kumar and Katara 2020). These all are examples of open and closed water systems where microorganisms perform the major function of decontamination of polluted water; thus, these microbes are playing a key role in the management of water (DDWS 2011; Gautam and Saini 2020; UNICEF 2008). Domestic wastewater is generally composed of high organic load, while industrial waste compositions are dependent on the source of their origin (UNICEF 2008; DDWS 2011; Nahiun et al. 2021). There are various approaches depending on the composition of wastewater which leads to proper management and sustainable usage of industrial and domestic wastewater (Kumar 2018; Shingare et al. 2019; Kundu et al. 2014; Gautam et al. 2007). Basically, the decontamination of wastewater depends on its composition and best-suited strategies are employed as per the rules and

**Table 9.1** Permissible limits of physical and chemical attributes by the Bureau of Indian standards (BIS) as per the IS 10500:2012 guidelines and World Health Organization (WHO)

| Wastewater parameter | Permissible limits of the parameter as per BIS, IS 105000:2012 | Permissible limits of the parameter as per WHO (2018) standards | Remarks on maximum acceptability according to BIS, IS 105000:2012 |
|---|---|---|---|
| Odour | Odourless | Odourless | Odourless |
| pH | 6.5–8.5 | 6.5–8.5 | 6.5–8.5 |
| Total dissolved solids (mg/L) | 500 | 500–1500 | 2000 |
| Alkalinity as $CaCO_3$ (mg/L) | 200 | 75–200 | 600 |
| Nitrate (mg/L) | 45 | 50 | No relaxation |
| Sulphate (mg/L) | 200 | 200–250 | 400 |
| Fluoride (mg/L) | 1 | 1–1.5 | 1.5 |
| Chloride (mg/L) | 250 | 200–250 | 1000 |
| Turbidity (NTU) | 5 | 5 | 10 |
| Arsenic (mg/L) | 0.01 | 0.01 | 0.05 |
| Copper (mg/L) | 0.05 | 2 | NR |
| Cadmium (mg/L) | 0.03 | 0.03 | NR |
| Lead (mg/L) | 0.01 | 0.01 | NR |
| Ferric ion ($Fe^{2+}$) (mg/L) | 0.03 | Not mentioned | NR |
| Zinc (mg/L) | 0.05 | Not mentioned | NR |
| Chromium (mg/L) | 0.05 | 0.05 | NR |
| Mercury (mg/L) | 0.01 | 0.01 | NR |

*NR* no relaxation

regulations of the land. In India, the Central Pollution Control Board (Government of India) monitors and controls quality of all water related issues. As per the report of CPCB, 2020–2021, there are more than 1600 sewage treatment water plants with a capacity of ~36,000 MLD covering all the states and union territories in our country (NISTP 2021). All standards measured for treating wastewater are given in Table 9.1. Water for drinking usage must contain no amount of heavy metals and

should meet permissible limits set by the regulating agencies (BIS 2012; WHO 2017). There is not a specific approach for achieving the desired results set by the government laws but a set of methods based on the chemical and physical attributes of the wastewater. We will discuss various microbial-based aerobic techniques for decontamination of wastewater from various industries as the textile industry, paper-based industry, petroleum industry, food and dairy-based industries, brewery industries, and miscellaneous industries.

## 9.2 Physical and Chemical Attributes of Wastewater

Physical and chemical properties of water have been changed after the discharge from various domestic and industrial sources (NISTP 2021). Physical attributes include the change in colour, taste, TDS, total organic count (TOC), hardness, and so on. This is primarily treated to obtain the desired limits and various methods are deployed for it. Change in the chemical composition includes alteration in pH, dicationic metallic ion concentration, and phosphate ion concentration, nitrate concentration which are removed by various physiochemical methods. Attempts are made so that treated water can be in the set permissible limits standardized by the Bureau of Indian standards as per the IS 10500:2012 guidelines as listed in Table 9.1. The colour and odour of the treated water should be colourless and odourless. Total dissolved solid includes the mass present in a water sample in a suspended or dissolved state, it may be volatile or non-volatile and it is measured by gravimetric analysis while total alkalinity of water is measured by titration method. Coagulation and flocculation agents include the use of natural coagulants, for example, *Moringa oleifera*, *Cicer arietinum* (Gautam and Saini 2020), while alum, poly-aluminium chloride, aluminium ferric sulphate, Aquacura-IWT, BCC-2810A (Periyasamy et al. 2018; Rizzo et al. 2019; Nahiun et al. 2021) are commercially available products for coagulation-flocculation in primary wastewater treatment.

## 9.3 Biological Parameters of Wastewater

Despite the presence of various inorganic and organic contaminants, various microbes have been reported in wastewater depending on the sources from which it originates (Gunatilake 2015; Liu et al. 2017). The major aspect of controlling biological parameters such as chemical oxygen demand (COD) and biological oxygen demand (BOD) is dissolved oxygen. Then total bacteriological count is done to ensure bacterial-free potable water as enlisted in Table 9.2.

**Table 9.2** Biological attributes as per standards as per Bureau of Indian Standards as per IS 10500: 2012

| Biological parameter | Permissible limit | Maximum limit |
|---|---|---|
| Dissolved oxygen (ppm) | 4 | 6 |
| Biological oxygen demand (ppm) | 30 | 100 |
| Chemical oxygen demand (ppm) | 80 | |
| Faecal coliform (CFU/100 mL) | Nil | Nil |
| *Escherichia coli* (CFU/100 mL) | Nil | Nil |
| Planktons, zooplanktons, algae | Nil | Nil |

## 9.4 Aerobic Treatment of Wastewater

The aerobic treatment of wastewater includes the process to degrade pollutants in the presence of oxygen, that is, microorganisms use free elemental oxygen and organic matter together with other trace nutrients for the depletion of higher molecules into small and stable molecules (Daigger 2007; Burch et al. 2013; Cyprowski et al. 2018; Do et al. 2018). This leads to the generation of small molecules and carbon dioxide and microbial population to increase. Since aerobic wastewater treatment plants (AWTP) use microbial populations, once installed they work easily at a low operation cost (Burch et al. 2013; Do et al. 2018). In primary treatment, the suspended particles of wastewater are removed by screen bars, which results in elimination of debris, grease, solids, and oil using physical and chemical methods (Chávez et al. 2019). To obtain physical and chemical parameters in a permissible range various method including filtration, separation based on affinity, that is, coagulation/flocculation, distillation, ion exchange, and so on are deployed (BIS 2012; Do et al. 2018). The pH adjustment is made by the addition of citric acid and soda ash as per desired (BIS 2012; Anjum et al. 2016). Coagulation/flocculation results in the lowering of solids suspended in wastewater ~70% of both industrial and domestic wastewater, and production of primary sludge takes place (Daigger 2007; Chávez et al. 2019; Crini and Lichtfouse 2019). After this primary treatment, the secondary treatment is done according to the chemical composition of the wastewater (Kumar et al. 2020). Depending upon the chemical and physical makeup of wastewater, the secondary treatment strategies are decided and the biological wastewater treatment system might be composed of different processes and numerous types of microbiological processes (Gunatilake 2015; Dutta et al. 2021; Lado et al. 2021). It provides a successful degradation of biodegradable molecules and is considered heart of the wastewater treatment (Liu et al. 2017). High levels of BOD indicate a heavy load of biodegradable substances present in wastewater discharged from paper industry, dairy industry; fertilizer run-off; and domestic waste, which is a major cause of the introduction of pollutants (BIS 2012; Gunatilake 2015; Liu et al. 2017; Chandra and Kumar 2017a, b; Kumar 2018; Kumar and Chandra 2018; Dutta et al. 2021; Lado et al. 2021).

It also requires a specific operational procedure which depends on the demographic and geographic conditions of the AWTP which is needed for the biological growth of a specific microbial population for lowering down biochemical oxygen demand (BOD) of the wastewater (Morgenroth et al. 2002; Liu et al. 2017; Li et al. 2019). Since we use wastewater in definite volumes, they function as bioreactors in which bacterial growth takes place to disassociate decontaminants into smaller stable products by the virtue of their physiology (Li et al. 2019). The biochemical reaction of aerobic treatment is considered to take place in four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis, respectively (Do et al. 2018; Newhart et al. 2019; Rizzo et al. 2019; Zhao and Chen 2019; Nahiun et al. 2021). These reactions are faster and lead to lowering BOD. At the end of reactions secondary sludge generated is separated (Do et al. 2018). Recently, activated sludge treatment methods have drawn attention throughout the globe as it provides high-strength sludge for generation of biogas.

## 9.5 Advanced Biological Wastewater Treatment Technologies

These combine conventional suspended microbial growth, activated sludge with the uses of membrane filtration rather than sedimentation to separate and recycle suspended solids (Do et al. 2018; Rizzo et al. 2019; Zhao and Chen 2019; Guo et al. 2020; Nahiun et al. 2021). Membrane bioreactors are operated with much higher mixed liquor suspended solids (MLSS) and longer solid retention time (SRT) producing significantly smaller residues with a huge amount of effluent in comparison to conventional methods (Morgenroth et al. 2002; BIS 2012; Do et al. 2018). According to the geometry of membranes used in bioreactors, it may be spirally wound, plate and frame modules, tubular membranes, and hollow fibre membranes (Obotey Ezugbe and Rathilal 2020; Nahiun et al. 2021). Based on porosity of the membrane, it may be one of the following:

- Porous or
- Non-porous: as reverse osmosis membrane is non-porous and are widely used for water treatment.

A generalized scheme of typical advanced wastewater treatment processes is shown in Fig. 9.1. It may consist of the following:

1. Aerobic treatment tanks.
2. An aeration system.
3. Mixers.
4. Membrane tanks.
5. A clean in place system.
6. Hallow fibre/flat sheet membranes filled of polygel matrix.

As per usage of the membranes used in the secondary treatment of the process, it can be divided into two parts:

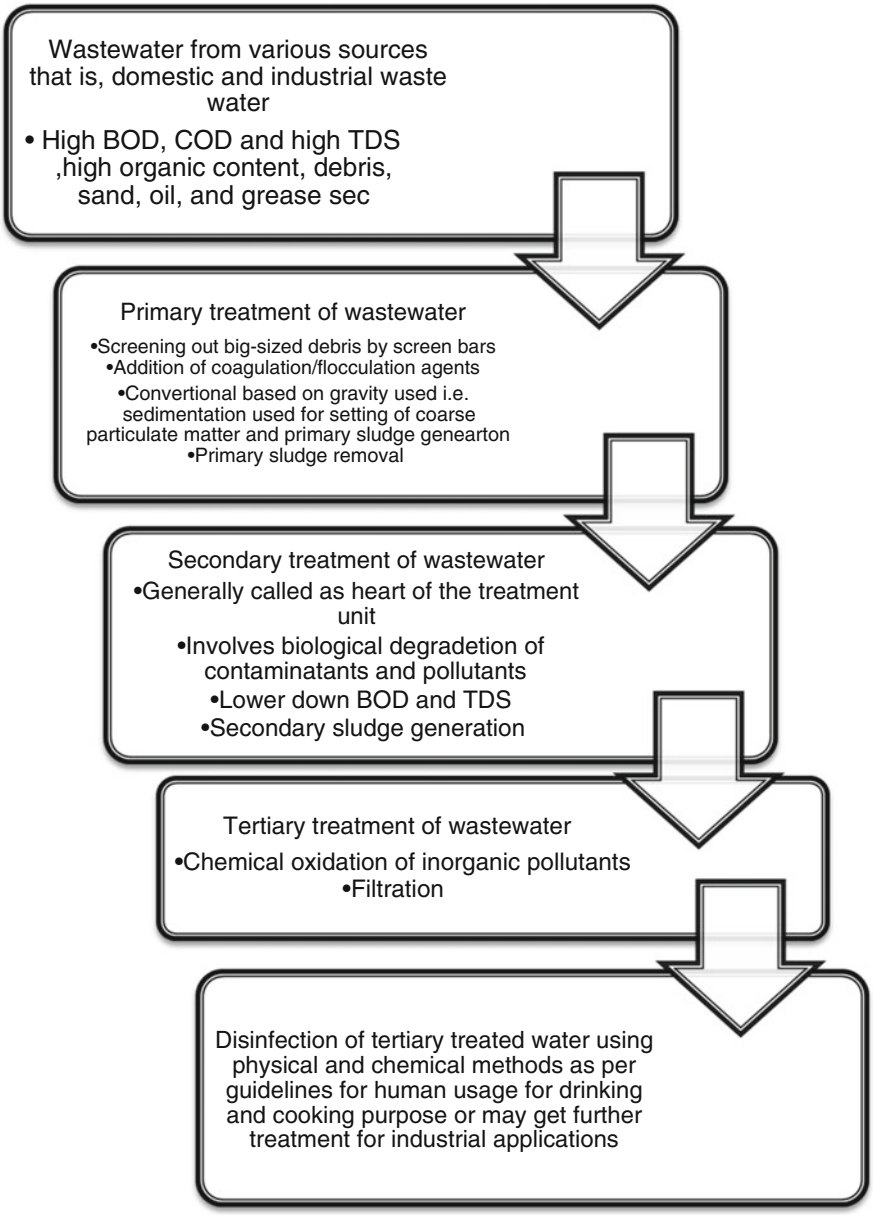


**Fig. 9.1** Different stages of wastewater treatment

- Fixed membrane bioreactors.
- Moving membrane bioreactors.

But the common thing in both systems is to provide a matrix to the advanced biological process for various groups of bacteria that perform nitrification, denitrification, desalination, and anammox reaction for treatment. Li et al. (2019) suggested major groups of bacteria participating in the reaction include *Proteobacteria*, *Bacteriodetes, Acidobacteria, Chloroflexi, Planctomycetes*, and *Verrucomicrobiota*. After secondary treatment for removal of the inorganic compounds, oxidation–reduction process is used and BOD of water is monitored for obtaining permissible limits as listed in Table 9.1. The major aim of this tertiary treatment is to degrade residual phosphates and nitrogen present after bacteriological reaction and other dimetallic ions (Periyasamy et al. 2018; Nidheesh et al. 2020). Two approaches are widely used for achieving it.

**Chemical Oxidation** In the chemical oxidation method, oxidizing agents (e.g. sodium hypochlorite [NaOCl], potassium per magnate [$KMnO_4$], ozone [$O_3$]) are used (Klamerth et al. 2010; Nidheesh et al. 2020; Lado et al. 2021). Because of low cost and ease of use, it is widely used, but its effectiveness is questionable at high concentrations of pollutants (Klamerth et al. 2010). Another approach using chemical oxidation is advanced oxidation using Fenton's reagent. Fenton's reagent is a solution of hydrogen peroxide ($H_2O_2$) and ferrous ion (Lado et al. 2021). Typically, ferrous sulphate ($FeSO_4$) acts as a strong oxidizing agent and is used for a variety of aromatic, amines, pesticides present in wastewater (Nidheesh et al. 2020; Lado et al. 2021). One of the benefits is that in these methods no energy input is required for the activation of hydrogen peroxide.

**Membrane Filtration** Other than this, membrane-based methods are also deployed using nanofiltration and reverse osmosis.

## 9.6 Treatment of Sludge After Treatment of Wastewater

Sludge generated due to different steps of treating wastewater in AWTP plants, which is composed of complex materials, organic pollutants, inorganic pollutants, and pathogenic microbial population containing bacteria, fungi, algae, and viruses depending on is origin (Anjum et al. 2016; Chandra and Kumar 2017c; Periyasamy et al. 2018; Guo et al. 2020; Kumar et al. 2021b). Several approaches are used for treating the sludge as it is a hazard to the environment. Depending on its removal process it may be primary or secondary sludge. Sludge generation depends on the solid retention time and BOD of the wastewater (Anjum et al. 2016). It contains high amounts of energy which can be used for the generation of biogas (methane and carbon dioxide) and solids that can be used as biofertilizers and for landfilling purposes (Guo et al. 2020). Prior to disposal of sludge, it should be treated to avoid its harmful impact on the environment (Rizzo et al. 2019). Sludge press can be used for compression of the sludge to remove out the water from it before further treatment of the sludge (Awuchi et al. 2020). The numerous physical, chemical, and biological approaches are used for the stabilization and detoxification of sludge.

Treatment includes digestion in the presence or absence of oxygen, which is then followed by thermal-microwave digestion, ultrasonic disintegration, and photocatalysis of sludge (Liu et al. 2012; Anjum et al. 2016; Awuchi et al. 2020).

## 9.7 Management and Regulation for Quality Control and Quality Assurance of WTPs

Ensuring management and security of natural equities i.e. pure water which is given by nature to humankind different international and national organizations have made standard operating procedures and laws for protection (Kumar 2018). Environmental standards are used for helping organizations and industries to minimize their negative impact of operations on the environment (APHA 2006). It is enforced and monitored by law's regulations and other environmentally oriented requirements by providing certification to the client and conduct audit time to time for controlling quality and assuring the regulation are followed for safe environment (Cullet and Gupta 2009; Cullet and Koonan 2018). In India, we have Central pollution control board (CPCB), Delhi as a central body which make rules and regulations for controlling the impact of wastewater. Various states have their own state pollution controlling board to follow and implement the rules set by CPCB. The Water Prevention and Control of Pollution Act 1974 was enacted for prevention and control of water pollution and maintaining and restoring of wholesomeness of water bodies. Central and State pollution control boards (SPCB) have been constituted under section 3 and 4 of the acts, respectively. The Act was amended in 1978 and 1988 to clarify certain ambiguities and to vest more powers to the pollution control board. Prior consent is mandatory from SPCB to establish a new industry, operation, process, and so on, which is likely to dispose of their waste into the environment. Contravention leads to imprisonment for a term between 18 months and 6 years as well as a penalty. Apart from the Water Prevention and Control of Pollution Act another environmental act was introduced in 1986 known as the Environment Protection Act which ensures the regulation and mitigation of pollutant form environment and penalty provision for defaulters according to the Indian Penal Code (Cullet and Gupta 2009). Furthermore, the Government of India also introduced Environment Impact Assessment Rules (EIA) 1994, the Public Liability Insurance Act (PLIA) 1997, Coastal Regulation Zone Notification 2011 for prevention of water pollution and hazardous effect of pollutants to environment (DDWS 2011; Cullet and Koonan 2018). Besides these powers to CPCB and SPCB, there are limitations such as low rate of compliance, inadequate auditing, very low penalties to be paid by defaulters for violation of rules, making it less adequate to follow (Cullet and Koonan 2018).

## 9.8 Conclusions

Decontamination of different organic or inorganic contaminants is required to provide an adequate supply of water throughout the globe for various activities of all living beings. The use of conventional, as well as advanced methods, leads to the migration of contaminants in sequential methods. It does not only reduce the risk of spreading waterborne disease but create a good balance of nutrients in the ecosystem. Proper management and auditing of AWTPs are required for quality assurance of potable water as well as sludge. New technologies that are eco-friendly and less hazardous to the environment should be introduced to the AWTP for better treatment. Regulatory compliances should be strictly adhered to by users, that is, domestic and commercial, for a safe environment. Complying authorities should be responsible for regular monitoring and auditing of AWTPs in order to assure the needed products as per guidelines set by BIS. Strict following of rules and continuous education programs are suggested for better waste management of decontaminants for safe water sources throughout the globe.

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# Omics in Industrial Wastewater Treatment 10

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**Abstract**

The scientific revolution in omics technologies has paved the way for emerging technologies in wastewater treatment. These approaches have been adopted to investigate the metabolic potential, diversity, and spatiotemporal dynamics of microorganisms in wastewater systems. The prokaryotic, eukaryotic diversity can be utilized in industrial wastewater systems for higher performance. Industrial wastewater contains high concentrations of organic and inorganic pollutants, exerting a huge pressure on the environment. The adverse effects on biodiversity, soil, natural water bodies, and groundwater emphasizes the urgent need for proper wastewater treatment techniques prior to its disposal to the environment. Wastewater treatment via omics technologies offers advantages namely enhanced nutrient removal, cost reduction, wide applicability, the possibility of biofuel/bioenergy production, etc. The availability of diverse microbial communities can be expected in biological wastewater treatment plants, and they possess different metabolic capabilities which could be harnessed in the wastewater treatment process. Microorganisms can play a key role in the performance optimization of wastewater treatment plants. Coupling microalgae and cyanobacteria, production of microbes-based nanomaterials, use of bacterial and algae symbiotic

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systems for wastewater treatment have been recognized as promising techniques in biological wastewater treatment. The chapter focusses on the important aspects of omics, applications, limitations, challenges, and futuristic approaches in omics technologies related to industrial wastewater treatment.

**Keywords**

Algae · Cyanobacteria · Microbes · Omics · Wastewater treatment

## 10.1 Introduction

Increasing pressure on environment due to the inevitable pollutant loads generated by industries is a significant concern of the modern world. The need of managing water resources sustainably goes hand in hand along with rapid urbanization and industrialization. The health hazards and environmental pollution associated with the improper wastewater discharge calls for better remediation techniques. Though the need of cost-effective wastewater treatment is a global need, the technical barriers, elevated costs of treatment, and poor infrastructure often makes the industrial wastewater treatment a tedious task. The removal breakdown, or transformation of pollutants into different particles is involved in wastewater treatment (Ekwanzala et al. 2021). Among the physical, chemical, and biological methods that are used for wastewater treatment, the biological methods are favored due to the cost-effectiveness and low post-remediation environmental burden.

The technological advancement in the biological sciences has opened a new landscape towards a molecular technology termed as "omics". Omics aid in understanding the microbial interactions and detailed study of genes, proteins, and metabolites. Designing frameworks to harness microbes for desired beneficial outcomes has led to the advancement of omics technologies. Incorporating physiologies and dynamics of microbes to enhance wastewater treatment, therefore, has become a recent trend. Application of omics from laboratory scale to mass scale has paved sustainable and economically sound opportunities for cutting edge omics-based wastewater treatment (Kumar et al. 2020). Processes namely activated sludge, anaerobic digestion is highly dependent on microbial communities and the modern metagenomic approaches are used for current wastewater treatment (McDaniel et al. 2021). A single "ome" would not be sufficient in characterizing microbial consortia to be utilized in wastewater treatment as optimizing biotechnologies for water and wastewater remediation need to be diversified with multiomics approaches (Muller et al. 2014; Sheik et al. 2014). Detailed knowledge on microbial functionality has been addressed by 16S rRNA gene-based omics technologies as shown in Fig. 10.1 for industrial wastewater remediation (Kumar et al. 2021a, b).

Nitrogen, phosphorous and carbon consuming microalgae and cyanobacteria can be utilized in omics technologies in wastewater treatment (El-Sheekh et al. 2021). The treatment of wastewater via approaches of metagenomics has been oriented towards utilizing both culturable and unculturable microbes. Understanding of the

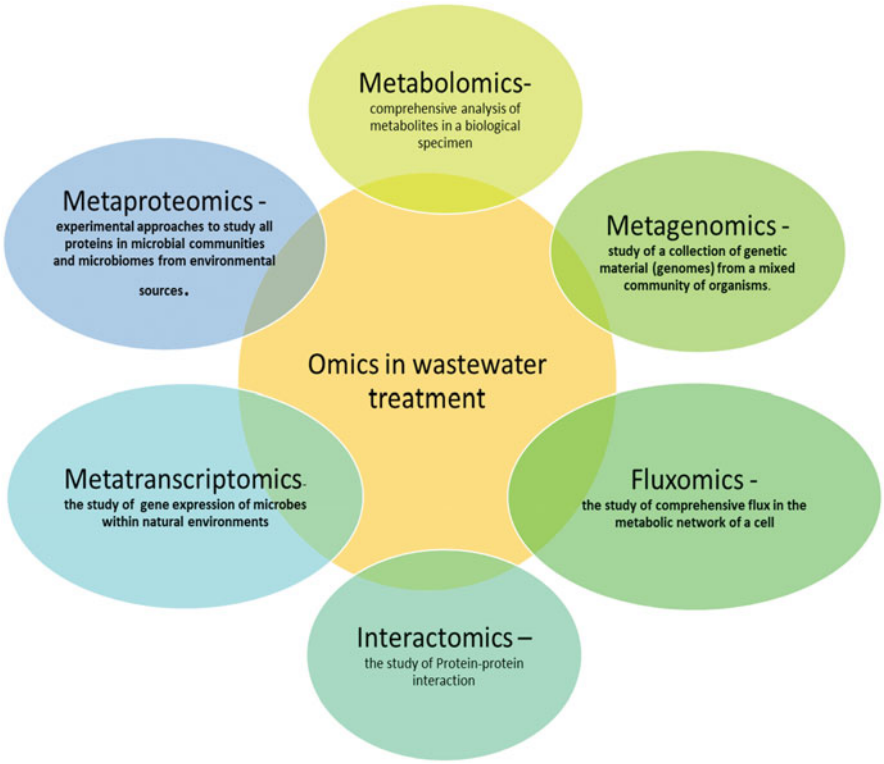


**Fig. 10.1** Possible areas of omics in wastewater treatment

physiology of the microbes along with their functionality has been made possible by metagenomics (Agrawal and Verma 2021). Meta transcriptomics provides insight to functionality, active pathways, enzyme profiling of microbes in wastewater treatment (Ekwanzala et al. 2021).

The objective of this chapter is to streamline the cutting-edge omics technologies in industrial wastewater treatment. The diverse composition of industrial effluents, the prevailing treatment methodologies will be addressed and the omics-based approaches in industrial wastewater treatment and the futuristic approaches will be critically discussed.

## 10.2 The Composition of Industrial Wastewater

Wastewater can be categorized into three key groups based on their source of origin. The wastewater only produced from households is termed as domestic wastewater while wastewater produced by industries is termed as industrial wastewater. Effluents produced in agricultural activities is termed as agricultural wastewater (Hanchang 2009; Manasa and Mehta 2020). Industrial wastewater treatment has

caught the attention of environmentalists, policy makers due to the issues namely high costs, poor infrastructure, technical barriers etc. Meanwhile the release of untreated industrial wastewater possesses a significant threat to the environment (Dutta et al. 2021). Industrial wastewater is classified as inorganic industrial wastewater and organic industrial wastewater. Inorganic wastewater is characterized with significant amounts of suspended matter, and these are produced mainly in the steel and coal industries. Wastewater coming from industries producing pharmaceuticals, soaps and detergents, paper and pulp, pesticides, herbicides, tanneries, leather, textile, fermentation and brewery, and oil-refining factories commonly contain organic substances (Hanchang 2009). The complex nature of industrial wastewater is mainly due to its varied manufacturing processes and different types of industries (Shahedi et al. 2020; Singh et al. 2021). Industrial wastewater varies in its quality and volume, based on the source of origin. Materials which are biodegradable, non-biodegradable and substances recalcitrant to treatment can also be present in industrial wastewater (Kumar et al. 2021a; b). The presence of organic contaminants consisting of organic/organic synthetic substances, inorganic substances, pesticides, insecticides, antibiotics, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), microplastics, heavy metals, carbon, phosphorous, nitrogen, xenobiotics etc. possesses a significant health and environmental threat (Molazadeh et al. 2019). Industrial wastewater contains a varied number of constituents depending on the source of origin, as shown in Fig. 10.2.

Industrial wastewater includes conventional pollutants derived in their manufacturing processes while non-process effluents arise from cooling, backwashing, and boiler blowdown. Waste effluents discharged from selected manufacturing industries have been discussed below.

### 10.2.1 Food and Dairy Industry

Food industry covers a wide spectrum of operations in conglomerate of industries namely beverage, brewery, dairy, confectionery, vegetable oils etc. Considering the dairy industries, cleaning, and washing processes in milk processing plants basically generates wastewater. The wastewater is characterized with elevated levels of biological oxygen demand (BOD), chemical oxygen demand (COD), turbidity, nutrients, soluble organics, solids, chloride, sulphate, oil and grease, elevated sodium contents, lactose, and sanitizing agents (Shete and Shinkar 2013). Non-treated diary waste effluent can create anaerobic conditions, release odorous gases, promote eutrophication, and reduce the aesthetic value, and predominantly aquatic systems can get heavily disturbed.

### 10.2.2 Paper and Pulp Industry

The paper and pulp industry are considered as a prime industrial wastewater producer and an intensive water consumer. Constituents namely lignin, phenols,

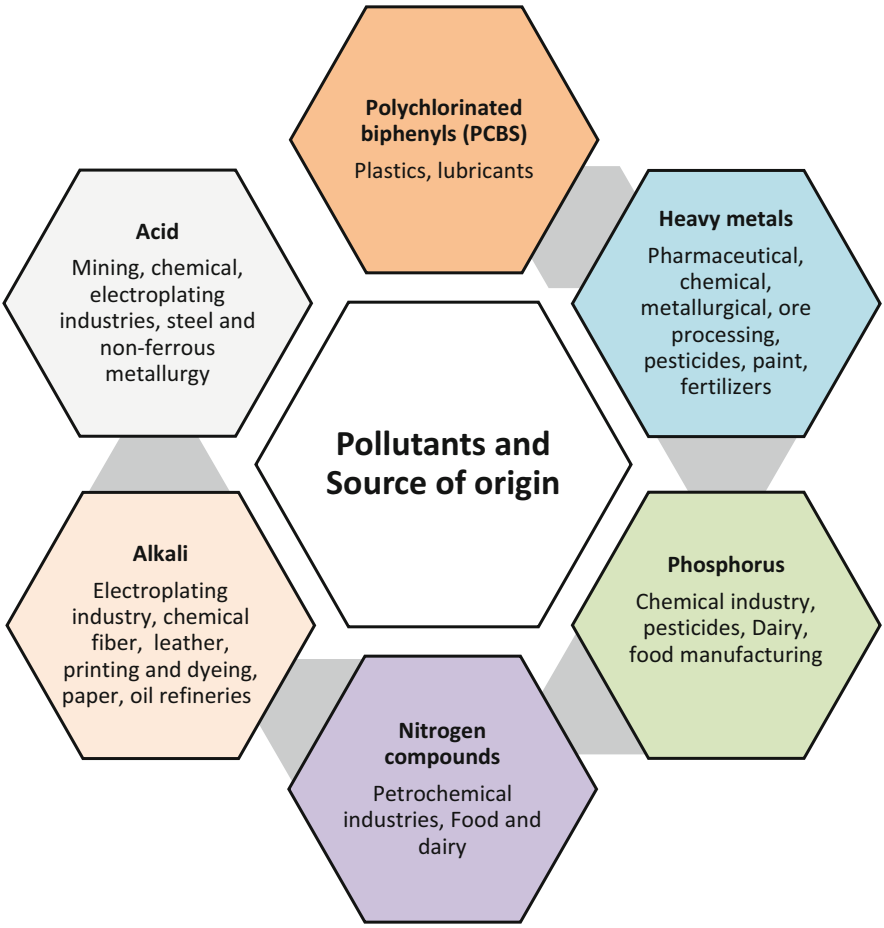


**Fig. 10.2** Key industrial wastewater pollutants and their sources of origin

cellulosic biomass, sulphides, resin acid, wood extractives, tannin resins, organic halides and other inorganic substances are present in its effluent. Elevated BOD, COD, colour, and suspended solids can typically be observed in the paper and pulp industrial wastewater (Brink et al. 2018).

### 10.2.3 Textile Industry

The quality and quantity of wastewater generated by textile industry vary drastically between processes and with reference to the raw material of choice (Holkar et al. 2016). Processes namely yarn manufacture, weaving and singeing generate low quantities of effluents while the heavily concentrated effluents are produced in processes namely sizing. In contrary scouring, bleaching, dying processes accounts

for elevated quantities of wastewater. Industrial dyes, suspended solids, detergents, chemical substances, nitrates, phosphates, metals, oil and grease, surfactants, sulphur compounds, chlorine etc. are characteristic constituents in textile wastewater (Lemlikchi et al. 2012).

### 10.2.4 Mining and Quarry Industry

Generation of high quantities of wastewater with complex chemical composition can be observed in mining industries. Open pit mining generally leads to inorganic nitrogen pollution (Glushankova et al. 2021). Mineral processing, slurry transport and dust suppression are key activities in mining and quarrying where wastewater is extensively generated (Hoekstra 2015). The effluent is characterized with suspended solids, turbidity, colour, oils and grease, soaps and detergents, dyes and phenolic compounds, heavy metals (Cr, Hg, Cu, Cd, Pb, Zn, Ni, etc.) acids, alkalis, cyanides, and dissolved salts (Hagare et al. 2009).

### 10.2.5 Chemical Industry

Chemical industry is a diverse field having manufacturing industries namely pharmaceuticals, petrochemicals, specialty chemicals, inorganic chemicals, etc. (Aftalion 2001). The occurrence of inorganic, organic, and toxic pollutants can be observed in wastewater generated by chemical industries. The quality and quantity of the wastewater can be varied due to the processes, but the presence of mutagenic and carcinogenic materials, surfactants, emulsifiers, and hydrocarbons have been recorded (Nasr et al. 2007).

### 10.2.6 Leather and Tannery Industry

Solid waste and wastewater generated from leather industry is loaded with volatile organic carbons (VOCs) and toxic chemicals (Barik 2018). Raw materials are derived from slaughterhouses and meat industries for the manufacturing processes while usable leathers are then produced in tanneries with the raw materials. Chemical usage in bulk quantities can be observed in leather tanning industry for the purpose of production of the final product. The production of toxic and hazardous chemical in the production process is a significant as nearly 250 kg of tanned waste comprises of 3 kg of Cr while 50,000 kg of wastewater effluent comprises of about 5 kg of Cr (Sivaram and Barik 2019). Basically one metric ton of raw material yields only 20% of final leather product while the waste effluent consists with Cr for more than 60% (Huffer and Taeger 2004).

## 10.3 Industrial Wastewater Treatment Methods

Industrial wastewater treatment technologies can be divided into physical, chemical, and biological processes. Over the years, many advancements and new developments in wastewater treatment processes have evolved, including hybrid methods that incorporate several processes together to increase treatment efficiency (Dutta et al. 2021; Mao et al. 2021). This chapter focusses only on biological processes, particularly the application of omics technologies in biological treatment methods. However, a summary of all three methods is discussed here to provide an overall understanding of the industrial wastewater treatment methods. The following section highlights the conventional processes used in industrial wastewater treatment.

### 10.3.1 Physical Wastewater Treatment Processes

Physical processes are the first step in the treatment process to separate solid particles and other large materials from the wastewater stream. This includes screening, filtration, sedimentation, flotation, and adsorption (Woodard and Curran Inc 2005). Physical methods use naturally occurring forces, such as gravity, electrical attraction, van der Waal forces, as well as mechanical devices such as screens and filters. As such, it does not lead to any changes in the chemical structure of the pollutants. Physical processes make use of a physical barrier that does not allow the target pollutants to pass. These barriers range from bar racks to more advanced filtration systems. Bar racks and screens are usually part of primary treatment, while filters, micro screens, and membranes are used in secondary or tertiary treatment (Ng 2006).

Screening is the first step of industrial wastewater treatment. The process helps remove large solids entering a wastewater treatment plant, thus protecting the downstream equipment from possible damage and blockages (Ng 2006). A grit chamber is often included in a wastewater treatment plant to remove grit, sand, and other non-putrescible materials that may clog pipes. In some plants, primary clarifiers include mechanical skimmers for oil and grease removal. Another commonly used method for the removal of fats, oil, grease, and suspended solids from wastewater is flotation using dissolved air flotation (DAF) units (Kyzas and Matis 2020).

Sedimentation is another physical process where heavier particles and sludge are removed through gravity settling. Sedimentation occurs in different places throughout the wastewater treatment, such as grit and particulate matter removal in the primary settling tank and biological-floc removal in the activated-sludge settling basin. It is also common to use a combined physical-chemical treatment method such as adding coagulants and removing the chemical flocs through gravity settling (Mao et al. 2021). In addition, using different porous or non-porous membranes to filter out the pollutants from the wastewater has also gained attention in recent years. Membrane filtration methods include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO) (Obotey Ezugbe and Rathilal 2020).

### 10.3.2 Chemical Wastewater Treatment Processes

Chemical treatment can be defined as treating wastewater by a process involving chemicals. The most widely used chemical treatment processes are neutralization, chemical precipitation, adsorption, disinfection, and ion exchange (Woodard and Curran Inc 2005; Samer 2015).

Industrial wastes often contain acidic or alkaline components which require neutralization before discharge or treatment. Neutralization of wastewater is carried out to adjust the pH value of the effluent. In neutralization, acid wastewaters are treated by increasing the pH using waste alkaline streams, lime (CaO), dolomite, caustic soda, or sodium carbonate, whereas alkaline wastewaters are treated with an acidic waste stream, sulphuric acid, or hydrochloric acid (Samer 2015).

Chemical precipitation through flocculation and coagulation are used in industrial water treatment processes for solids removal, water clarification, lime softening, sludge thickening, and solids dewatering. Coagulants neutralize the negative electrical charge on particles, destabilizing the forces keeping colloids apart. Flocculants gather the destabilized particles together and cause them to agglomerate and settle down (Sahu and Chaudhari 2013).

Adsorption is another chemical treatment process where an adsorbent removes soluble molecules. Adsorbents such as activated alumina, hydroxides, resins, and activated carbon have a very high specific surface area, allowing the molecules to be attached to their surface (Dutta et al. 2021). Disinfection, which is the final step in conventional wastewater treatment, is a chemical process. It aims to substantially reduce the number of microorganisms in the water to be discharged back into the environment. Chlorine, UV, and ozone treatment are widely used methods in disinfection (Samer 2015).

### 10.3.3 Biological Treatment of Wastewater

Biological wastewater treatment involves the use of active microbial biomass to degrade soluble organic carbon, nitrogen, and phosphorus compounds (Samer 2015). Microorganisms such as bacteria, fungi, algae, etc. play an important role in treating industrial wastewater (Kumar and Chandra 2020; Kumar et al. 2022). These microorganisms can convert dissolved organic matter into simpler forms that can be separated by sedimentation. The biological wastewater treatment processes include aerobic systems such as oxidation ponds, aeration lagoons, aerobic bioreactors, activated sludge, trickling filters, and rotating biological contactors (Dutta et al. 2021). Anaerobic treatment methods include anaerobic bioreactors and anaerobic lagoons (Van Lier et al. 2008).

A number of factors such as temperature, pH, dissolved oxygen, nutrient concentration, and toxic materials affect microbial activities and biochemical reactions. It is important to control these factors in a biological treatment system to optimize microbial growth to increase the efficiency of the treatment process. The biomass may consist of aerobic and/or anaerobic microorganisms and can be deployed either

as suspended or fixed biofilms to address the requirements of different industries (Chan et al. 2009).

#### 10.3.3.1 Aerobic Treatment

In the aerobic treatment of wastewater, aerobic microorganisms use oxygen to degrade organic pollutants into carbon dioxide and biomass (Samer 2015; Kumar and Chandra 2018). An air blower or compressor carries out aeration of the wastewater to create an oxygen-rich environment. Aerobic treatment technologies can be used to treat anaerobically pre-treated wastewater further to reduce BOD and remove total suspended solids. It can also be used as a biological nutrient removal system to remove nitrogen and phosphorus. Some of the commonly used aerobic methods in wastewater treatment are discussed below (Samer 2015).

*Conventional activated sludge:* Organic matter is broken down by aerobic microorganisms in an aeration tank. This forms biological flocs, which are then settled in a sedimentation tank as sludge.

*Moving bed biofilm reactor (MBBR):* A biofilm on plastic carriers suspended and circulated in an aeration tank.

*Membrane bioreactor (MBR):* An advanced technology combining the activated sludge process with membrane filtration.

*Biological filters:* Biological filters such as biological aerated filters (BAFs) and the percolating (trickling) filters are the most commonly used types.

#### 10.3.3.2 Anaerobic Treatment

In anaerobic wastewater treatment, microorganisms degrade and remove organic contaminants from wastewater in the absence of oxygen (Van Lier et al. 2008). Anaerobic treatment systems utilize bioreactors capable of maintaining an oxygen-free environment needed for anaerobic microorganisms. This process is typically used for wastewater streams with high concentrations of organic materials, often prior to aerobic treatment.

The anaerobic wastewater treatment process consists of an acidification phase followed by a methane production phase (Van Lier et al. 2008). In the acid-forming phase, microorganisms break down complex organic compounds into simpler, volatile organic acids. This is followed by the methane-production phase, where microorganisms synthesize organic acids to form acetate, hydrogen gas, and carbon dioxide and then convert these to form methane gas and carbon dioxide (Chan et al. 2009). These by-products can be collected as biogas. The mostly used anaerobic systems are the up flow anaerobic sludge blanket (UASB) reactor, the expanded granular sludge blanket (EGSB) reactor, the internal circulation (IC) reactor, the static granular bed reactor (SGBR) and anaerobic biofilm reactors (Rodríguez et al. 2015).

## 10.4 Application of Omics in Biological Treatment

Techniques of omics have provided imminent array of sustainable biological treatment of industrial wastewater in recent years. Omics based applications are useful in identifying the ample opportunities to facilitate the biosynthesis, degradation of wastewater pollutants and microalgae (El-Sheekh et al. 2021). Toxicity analysis of contaminants may need combined multiomics approaches and analytical techniques. Eucaryotes and prokaryotic cyanobacteria having tolerance to wastewater toxicity, high growth rate and elevated photosynthetic efficiency are a felicitous candidate for wastewater treatment in degrading nitrogen, phosphorus, and metal species. A wide array of microbes can be utilized in wastewater treatment along with omics approaches, as shown in Fig. 10.3. The ability of microbes to utilize inorganic and

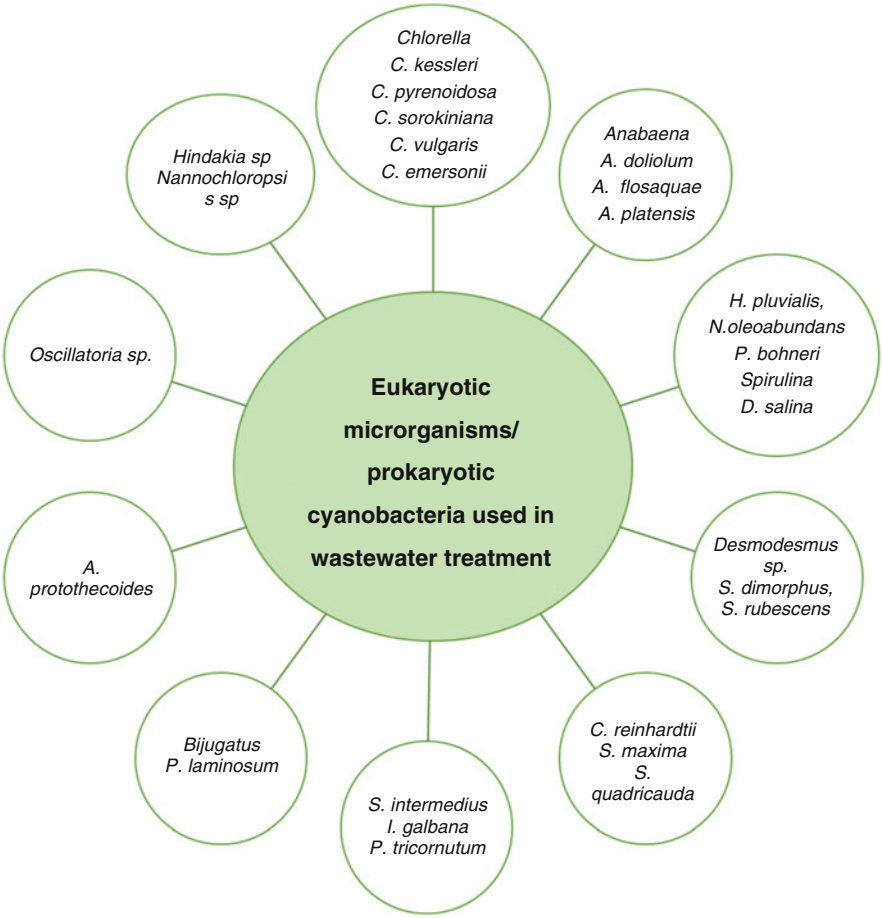


**Fig. 10.3** Selected microbial communities utilized in omics technologies for the treatment of industrial wastewater (Cai et al. 2013)

organic pollutants can be utilized in wastewater treatment and the readily available nutrient in wastewater is an inexpensive growth medium for them.

### 10.4.1 Omics Approaches in Wastewater Treatment

Omics approaches can clearly outline the changes of microbes in protein and DNA level utilized in biological treatment processes. Bioadsorption and bioaccumulation are the main mechanisms involved in pollutant removal which are economically and environmentally sound. Application of omics technologies with microalgae and cyanobacteria can be widely seen. The secondary pollution generated from other physiochemical methods can be eliminated as the use of microalgae removes the toxic metal and organic compounds while generating biomass which can later be utilize for biofuel production. The heterotrophic and mixotrophic microbes rely on organic matter and nutrients which can be used for wastewater treatment (Salama et al. 2019). Among them, the diverse nature of morphology, gene content, and physiology of microalgae and cyanobacteria is significant in wastewater treatment. Different types of industrial wastewater namely brewery, pharmaceutical, palm oil mill, slaughterhouse, textile, agro-industrial, food processing, paper and pulp, sand and olive refineries have integrated microalgae with the treatment processes. Molecular phylogeny coupled with genomic and phenotypic traits with omics results enhanced pollutant removal mechanisms. The initial attempt of incorporating metagenomics in a phytoremediation in system has recorded the removal of nitrogen and phosphorous in a swine lagoon wastewater with Chlorella (Ye et al. 2016). Development of mutualistic photoautotrophic–heterotrophic niches have further studied in efficient nutrient removal from wastewater.

Combining omics techniques to algae termed as "algomics", unlocks information on metabolic pathways, its dynamics, protein synthesis mechanisms, metabolism conditions and pathways of bioremediation. Omics consists with genomics, transcriptomics, metabolomics, and proteomics. Identification of genes, their arrangements, comparison of genome can be summarized via genomics of microbes while transcriptomics aid in studying the microbial gene structure, genomic level regulations and expression. Protein involvement in metabolic pathways and protein trafficking can be identified via proteomics. Quantitative and qualitative investigation of metabolites in microbes is addressed by metabolomics. Increasing flux rate pathways, toxicity mitigation, deletion of competitive pathways can be carried out by metabolic optimization (Anand et al. 2019). Genetic engineering techniques go hand in hand with omics technologies as the transformations in nuclear, mitochondrial genes are essentially utilized in genetic engineering. Nuclear genome encoded secondary products are produced by the enzymes for specific targets. Genome editing tools are used namely clustered regularly interspaced short palindromic repeats associated protein Cas9 (CRISPR-Cas9), transcription activator–like effector nucleases (TALEN), and zinc-finger nucleases (ZFN) for gene modification while gene interfering tools include CRISPR-dCas9, microRNA (miRNA), and small interfering RNA (siRNA) are used for either suppressing or activating genome

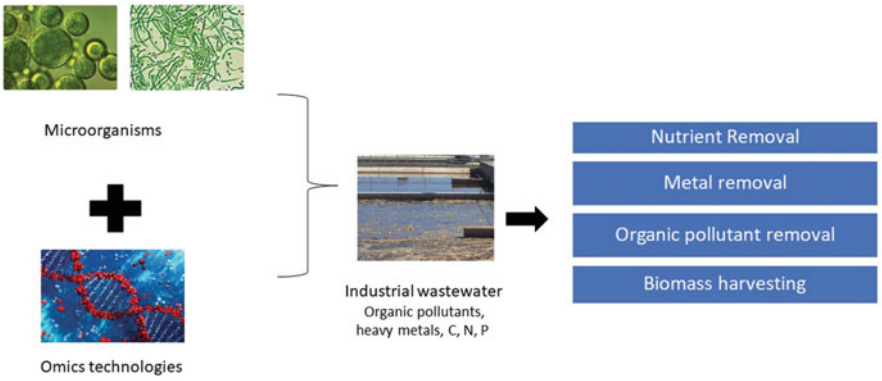


**Fig. 10.4** Pollutant removal of Industrial wastewater treatment with omics technologies

performance (Ng et al. 2017). Figure 10.4 graphically illustrates the application of omics in the industrial wastewater treatment.

### 10.4.2 Omics in Remediation of Organic Pollutants

A transcriptome analysis of *Chlorella vulgaris* F1068 has shown promising results in ammonia uptake from pharmaceutical wastewater (Liu et al. 2015), while *Phanerochaete chrysosporium* has been tested on phenol wastewater via metagenomic sequencing to trial out the microalgal augmentation effect (Hailei et al. 2017). Microalgae can remove organic compounds, namely, surfactants, tannins, phenolic compounds, etc. Varied microbial species have been tested for the remediation of textile wastewater streams. Metabolically, *Phormidium* sp. and *Synechocystis* sp. of cyanobacteria have shown better removal of reactive dye and the efficiency has been increased by adding a plant metabolite namely triacontanol (Karacakaya et al. 2009). Dye types CI Basic Blue and CI Basic Red have been effectively biosorbed by *Chlorella lentillifera* and azo dye breakdown has been reported by *C. vulgaris* (Wang et al. 2016).

### 10.4.3 Omics for the Remediation of Metal Species

Industrial wastewater comprises of diverse range of metal species regarding its source of origin. Heavy metals, namely, Cr, As, Cd, Pb, and Hg have been remediated with the aid of omics technologies in such wastewaters. The removal efficiencies have been recorded as 12.5–81.8% for Cu, 11.8–33.7% for Co, 26.4–100% for Pb, and 32.7–100% for Mn with biomixture of *Nostoc muscorum* and *Anabaena subcylindrical* (El-Sheekh et al. 2021). Comparative two-dimensional gel electrophoresis (2-DE) proteomics approach has been used in studying the effect of Cu on brown algae *Sargassum fusiforme* which has resulted in identifying varied

algal responses to the stress of Cu (Zou et al. 2015). Molecular level detailing can be achieved via metabolomics and the mechanisms useful in toxicity tolerance in the microbes can be identified via the process. Integration of nuclear magnetic resonance spectroscopy (NMR) and metallomics aid in understanding interaction between metals and algal species. Combination of transcriptomics, NMR, and gas chromatography–mass spectrometry (GC-MS) reveals the metabolic pathway variation in relevant to the heavy metal stresses. A panorama of metal remediation avenues can be achieved with multiomics techniques.

### 10.4.4 Genomic Information for Industrial Wastewater Treatment

Recently algal genomes, namely *Chlorella* sp. NC64A, *Phaeodactylum tricornutum*, *Coccomyxa* sp. C-169, and *Botryococcus braunii* UTEX 572, have been sequenced for genome analysis for the purpose of comparison, identification of microbial population dynamics and studying of genetic variations (Mishra et al. 2019). Studying properties of genomes can be utilized for the analysis of mRNA expression which is the foundation for proteomic and metabolomic methodologies. Similar studies have been carried out for diatoms namely *Phaeodactylum tricornutum* and red alga *Cyanidioschyzon merolae* (Nozaki et al. 2007). The performance of microalgae and bacteria consortium is well studies in relevant to omics technologies as optimum nutrient removal from wastewater can be highlighted via functional genomics. Significant improvements have been recorded during the past years using culture independent molecular method for the metagenomic exploration resulting species profiling and distribution dynamics. Insights to resource recovery can be achieved via metabolomic studies as certain experiment have already shown increased microbial production at high (e.g. *tolytoxin*) and low phosphate (e.g. *merocyclophane C*) levels (Mishra et al. 2019). Microalgal responses to ammonia and nitrogen sources, metabolism, carbohydrate synthesis and utilization can be identified via proteomic studies. Ammonia oxidation pathways by algae and bacteria have been studied using proteomic tools to understand the protein functionality under different environmental stresses in wastewater (Perera et al. 2019). Varied responses for transcriptomics are received in relation to the contaminant type and the stresses induced by the pollutant. Proteomics and metabolomics too can be used in these kinds of situations. In understanding the mechanism of metal toxicity, the diverse range of data received via omics can be highly beneficial. Initial addition of pollutants to a microbial system may cause the destruction of the population but gradually the resistance of selected species is developed against the pollutants. The selection of microbes with the potential of survival and remediation of toxic metals in wastewater is a challenge. Proteomics is beneficial in the identification of the suitable candidates.

Symbiotic associations can be observed in cyanobacteria and microalgae with bacteria, and it has shown advantages to reduce environmental impact, increased nutrient recovery, low-cost oxygenation, and ability of production of metabolites, namely proteins and pigments. Combination of omics along with biochemical and

microbiological analyses can provide reliable information about the effect on physiochemical factors on genes, proteins, transcriptomes, and metabolites (Cooper and Smith 2015).

## 10.5 Challenges, Limitations, and Futuristic Approaches

The potential of microbial remediation of wastewater has been discussed for many years. However, there are still limitations and challenges that need to be addressed. Biological processes are sensitive to environmental conditions, including toxic contaminants, overloading, or limiting nutrients. These conditions could lead to poor effluent quality and affect the microbial culture, resulting in poor settling and high effluent suspended solids. When the microbial culture changes, it may take several weeks to bring the operation to its previous conditions (Schroeder 2003). It is essential to understand the metabolic pathways of microorganisms to survive in their physical environment to develop better biological wastewater treatment processes. Omics technologies could help reveal the phenotypic and genotypic traits responsible for higher microalgal growth and nutrient removal, thereby increasing the treatment efficiency. Omics approaches could help better understanding of the remediation mechanism, develop cost-effective and sustainable systems for different industrial wastewater requirements (Chan et al. 2009; El-Sheekh et al. 2021). In addition, omics approaches provide an excellent platform to understand the toxicity mechanisms of the contaminants. However, complications in monitoring metal toxicity in the environment is still a limitation (Mishra et al. 2019).

Using microalgae in industrial wastewater treatment is more efficient and cost-effective than conventional techniques. In addition to removing pollutants, microalgae application also leads to biofuel generation (Mishra et al. 2019). However, several bottlenecks in microalgae cultivation need to be addressed. Challenges reported when cultivating microalgae and cyanobacteria in wastewater include high turbidity levels and total solids in wastewater. These conditions could lead to poor light penetration during microalgal growth. Moreover, the presence of organic carbon in wastewater is another limiting factor, as this could support the growth of other competing organisms and hinder microalgal growth (El-Sheekh et al. 2021). Limitations occur due to the gap of knowledge about diverse microbial consortia and the high throughput measurement methods. The need for hypothesis validation via research and development is an urgent need. More research is required to develop new microalgal strains that show multiple characteristics, such as higher biomass production and efficient pollution remediation. Economical and sustainable biofuel and large scale biomass production is an important consideration in renewable biofuel production (Tripathi et al. 2019). There is an increased research interest to develop technologies to produce renewable biofuels from microalgae. More attention to gain understanding of gene function via RNA transcripts analysis and genetic alteration of microalgae for efficient biofuel production is highly recommended. In-depth knowledge on genetic functionality, physical and biological potential of microbes over spatiotemporal arena gives promising opportunities to utilize

candidate microbes in biological wastewater treatment. Insights on promising area in omics technologies in wastewater treatment by the application of biosurfactants has come to limelight. Future research could use renewable, cheap substrates for biosurfactant production by using newly developed strains to deliver customized molecules for specific applications. For example, biosurfactants produced from some marine microorganism species could be effectively used to remediate oil spills in marine ecosystems (Gaur et al. 2022).

Integrated omics technologies can be beneficial in optimizing microbial functionality under different physicochemical conditions with relevant to the type of industrial wastewater, in streamlining the metabolic pathways and in developing bioengineered value-added products from mix substrates. The futuristic opportunities in developing tools for bioprospecting can be highlighted with omics technologies as it serves as a linking agent between genetic potential and phenotype. Elucidating opportunities of integrated omics technologies in pollutant removal can be further elaborated if microbial community dynamics, abundance of metabolites in microbes and their gene expression is studied and tested from lab scale to industrial scale.

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# Microalgae in Wastewater Treatment and Biofuel Production: Recent Advances, Challenges, and Future Prospects

11

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**Abstract**

Microalgae have become indispensable in the treatment of wastewater. Microalgae have proved to have a substantial potential as a long-term and cost-effective wastewater treatment technology when used in the biological purification of wastewaters from various sources using wastewater as a growth substrate. Biomass from microalgae is an alternate treatment option for removing nutrients, contaminants for example nitrogen compounds, heavy metals (HMs), and toxic chemicals. Wastewater treatment by using microalgae is a cost effective as well as a practical approach for $CO_2$ fixation, aside from being a renewable resource, supply of biomass. Lipids, proteins, and carbohydrates are abundant in microalgae. They can be put to use as value-added goods and biomaterials following wastewater treatment. This chapter discusses the various microalgae's involvement in wastewater treatment, ranging derived from degree of microalgal bioremediation to environmental enhancement via microalgal biomass productivity and $CO_2$ fixation. This chapter also covers biological and technical techniques for modifying algae-based wastewater systems and increasing biomass output for value-added products and biomaterials for future uses.

**Keywords**

Wastewater treatment · Microalgae · Environment · Remediation · Biofuel · Value-added products

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## 11.1 Introduction

In the previous century, water pollution has become an increasing concern as urbanization and industrial activity have spread around the globe. The surface of Earth is covered by 71% of water and is being contaminated by human activities as more garbage is thrown into rivers and beaches without being treated first (Sonune and Ghate 2004). By 2030, the globe will have a 40% water shortage, posing a serious societal and economic burden (Sun et al. 2016). Water resources appear to be under severe qualitative and quantitative threats today, despite that strategic importance of freshwater is now more well understood than ever before, with issues of sustainable water management appearing on nearly every social, scientific, and political agenda around the world.

However, owing to the exhaustion of fossil fuels and the global expansion of diverse industrial operations, another important problem in the current context is meeting energy demand while lowering the environmental impact of industrial gas emissions. Due to the high volumes of wastewater generated from large quantities of water utilized for municipal, agricultural, and industrial uses, there are concerns. Eutrophication in lakes can be caused by excess nutrients in wastewater, such as phosphorus and nitrogen, causing ecosystem disruption (Chandel et al. 2022). Since the mid-twentieth century, eutrophication has become a severe environmental issue. Annual availability per capita, which peaked at over 3300 $m^3$ in 1960, has declined by 60% to under 1250 $m^3$ in 1995, the world's lowest, and is anticipated to drop another 50% to around 650 $m^3$ by 2025 (Abdel-Raouf et al. 2012). Agriculture consumes the most water (87%), while home and industry supplies account for 7% to 8%, respectively (Samhan 2008).

Wastewaters contain substantial volumes of inorganic and organic nutrients, which promote ecological disparity due to their high chemical and biological oxygen demands, as well as other undesirable contaminants (Chai et al. 2020). Excess nutrients, such as nitrogen and phosphorus, promote eutrophication in aquatic bodies, causing water system disruption (Chai et al. 2020). Heavy metal ions present in industrial effluents, like copper, lead, zinc, cadmium, and nickel, pollute the environment (Saxena et al. 2016, 2020c, d; Rabbani et al. 2021; Zainith et al. 2021). The contamination of rivers, lakes, and seas is also caused by heavy metal ions and leftover nutrients in home and agro-industrial wastewaters (Saxena et al. 2020a, b; Goutam et al. 2018). Heavy metals can accumulate in aquatic life, producing serious health issues in humans (Bharagava et al. 2017a, b, c, 2018; Gautam et al. 2017). Humans can be harmed by heavy metals, even at small doses. Lead, for example, is extremely hazardous for the brain, kidneys, and vitamin D metabolism, particularly in youngsters (ATSDR 2007).

Algae-based wastewater treatment systems are appealing because they are good at removing inorganic contaminants like heavy metals and carbon dioxide (Suganya et al. 2016; Li et al. 2020). Microalgae have been utilized to treat a variety of industrial and domestic wastewaters in the past including POME (palm oil mill effluent), sago starch wastewater, heavy metals, rubber mill wastewater, and textile wastewater (Hadiyanto et al. 2013; Kamaruddin et al. 2013; Lim et al. 2010).

Microalgae include blue-green algae, eukaryotic protists, and prokaryotic cyanobacteria. There are around 72,500 species in this complex collection of creatures, although only 44,000 people have been explained so far (De Clerck et al. 2013). In association with heterotrophic bacteria, microalgae can remove inorganic nutrients by using oxygen ($O_2$) produced by microalgae during photosynthesis (Passos and Ferrer 2014). Other contaminants, such as heavy metals (Aksu 2001), nitrogen compounds, and hazardous chemicals, can be taken up by some microalgae species. The ability of microalgae to produce useful products is well-known and they are regarded as a formidable biotechnological platform for commercializing these goods. Biofuels, Pigments, bioplastics, aquaculture feed, and a variety of bioactive medicinal substances are among them (Wolf et al. 2015). Microalgae applications are becoming more well recognized in the scientific community.

From the degree of microalgal bioremediation to environmental betterment, this chapter covers the numerous advantages of microalgae over wastewater treatment through utilization of carbon dioxide fixation, microalgal biomass productivity. This chapter also covers biological and technical methods for modifying algae-based wastewater systems and increasing biomass output for value-added products and biomaterials for future uses.

## 11.2 Benefits of Using Microalgae for Environmental Applications

Microalgae are green photosynthetic organisms that grow faster than plants and take up less space (Chiu et al. 2008). Microalgae, such as eukaryotic algae and cyanobacteria, are a more long-lasting energy-intensive environmentally friendly, and conventional biological treatment techniques (Oswald 2003; Singh et al. 2015). Algae granules used in synthetic wastewater is extremely effective at removing phosphorus and recovering and reusing it derived from resulting P-rich algae biomass (Cai et al. 2019). The use of microalgae for wastewater treatment is cost-effective and practical approach for $CO_2$ fixation, in addition to being a renewable supply of biomass (Almomani et al. 2019). Microalgae biomass can absorb up to 1.8 pounds of $CO_2$ from the atmosphere for every pound of biomass (Elrayies 2018).

In terms of generation of biomass and $CO_2$ reduction, microalgae outperform terrestrial plants. Microalga biomass is a useful resource because it can produce fatty acids and triacylglycerols in higher concentrations (Sibi et al. 2016) and high-value chemicals, which are used to make biofuels (Kuo et al. 2017). Microalgae take 49 to 132 times less room to be cultivated than other crops (Khoo et al. 2020). Microalgae account for only 0.5% of total plant biomass, but they provide over 75% of the oxygen required for life (Wolkers et al. 2011). Microalgae have the advantage of thriving in tough environments like wastewater treatment, efficient land area for production, resulting in reduced land rivalry with agriculture, human (residential area), and animal farms (Tang et al. 2020; Chew et al. 2018).

Microalgae cultivation necessitated same amount of carbon dioxide, water, sunlight, and nutrients as traditional food crop cultivation but microalgae grow far faster than other plants. Microalgae cultivation can provide ecological services while having no negative environmental effects (Amini et al. 2020). The biomass of microalgae has doubled after only 13 h of cultivation (Massimi and Kirkwood 2016). The adoption of phosphorus, nitrogen, and carbon from the growth medium such as $NH^{4+}$, $NO^{3-}$, $HCO^{3-}$, $NO^{2-}$, or organic nitrogen is necessary for microalgae to produce protein, lipids, and carbohydrates. Phosphorus uptake will be utilized exclusively for lipid synthesis (Okoro et al. 2019). As a result, microalgae treatment operations emit fewer greenhouse gases; for example, rather than being transformed to nitrogen oxides, the bulk of nitrogen is digested by the microalgae (Mohsenpour et al. 2020). Microalgae and associated microbes in wastewater treatment have been shown to emit insignificant amounts of $N_2O$, according to various research (Guieysse et al. 2013; Fagerstone et al. 2011). A microalgae wastewater treatment method has 0.0047% g $N_2O$-N $g^{-1}$ N-input emission factor according to (Alcántara et al. 2015). Overall, providing dissolved $O_2$ to wastewater via microalgae photosynthesis is a surefire way to save a lot of energy and corresponding greenhouse gas emissions reductions.

## 11.3 Techniques for Microalgae Culture

Microalgae cultivation necessitates certain reaction circumstances such as temperature, light intensity, mixing conditions, gas exchange, and nutritional content. Varied species of microalgae, on the other hand, have different cultivation needs. Because carbon is essential for the creation of biomass in microalgae, $CO_2$ is the most prevalent carbon source for their growth (Liu et al. 2011). Varied growing settings result in different microalgae growth characteristics and compositions, as is well known. The choice of a microalgae cultivation system is critical for optimum nutrient removal and biomass production. Algae farming systems can be divided into two categories: open systems and closed systems (photobioreactors). Open and closed hybrid systems can be used to create high biomass production while simultaneously eliminating a large amount of nutrients. Both open and closed systems can be used to cultivate microalgae (those that have contact with the atmosphere) (Ting et al. 2017).

Because open systems are easier to build, operate, and maintain than photobioreactors, they are typically marketed on a high scale (Cai et al. 2013). However, they require a vast area to develop, have low biomass output, are more sensitive to biological pollutants, and so have a restricted number of microalgae species (Eboibi et al. 2014). To prevent protozoa and other microalgae species from contaminating the water, open systems necessitate extremely selective conditions. Monoculture production is achievable under harsh conditions, albeit only a few strains are acceptable. Raceway ponds (stirred ponds), which are open and shallow and designed with one or several channels in a closed-loop where water is circulated by a paddlewheel, aid in keeping cells floating and hence higher biomass yield

(Razzak et al. 2013). The most common large-scale production systems in use today are HRAPs (High-rate algal ponds) and raceway ponds. This is a typical open system for algae growth that is only around 0.3 m deep to offer enough sunlight for photosynthesis by microalgal cells that are mixed with nutrients and cycled on the raceway track by paddle wheels (Chisti 2007). Raceways ponds are relatively inexpensive to construct and operate, but their productivity is often low due to dark zones, pollution, poor mixing, and inefficient $CO_2$ consumption (Mata and Martins 2010). To accomplish so, depths of 0.15 to 0.45 m, usually 0.30 m, are employed to ensure light penetration, and a paddlewheel is used to promote circulation during the operation (Singh et al. 2015). HRAPs' main purpose is to maximize algal biomass production from process effluent. Nutrients present in anaerobically digested effluents, agricultural wastewater, and municipal wastewater are all eliminated using HRAPs (Munoz et al. 2018). HRAPs for wastewater treatment are usually part of a complex pond system that consists of a series of ponds.

Closed reactors are more efficient at photosynthesis than open ponds because they prevent water evaporation and contamination. Flat plate reactors, tubular photobioreactors, and bag systems are some of the most common closed reactors (Hoffmann 1998). Closed pond systems, also known as bioreactors, can alleviate or eliminate many of the issues that plague open-pond systems. Closed systems have the most advantages in terms of reducing water evaporation and reducing polluting species. The growth conditions are tightly controlled in closed systems, often known as photobioreactors (PBRs). It is also possible to cultivate a single species (Chisti 2007). Researchers have concentrated their efforts on developing photobioreactors capable of producing large amounts of microalgal biomass because they overcome numerous challenges associated with open cultivation. In general, these reactors are made to allow more light into the system. They also allow for ideal mixing, allowing for optimal light for cell growth and improved gas exchange (Rinkevich 1999). To circumvent the aforementioned challenges, closed systems have been developed. Photobioreactors (PBR) are transparent systems that are meant to help photosynthetic organisms grow more efficiently. Each project's economic and operational conditions have been enhanced. Tubular photobioreactors, for example, are often composed of glass or clear plastic with a diameter of less than 0.1 m, allowing light to pass through even at high microalgae concentrations (Chisti 2007). PBR, unlike HRAP, allows for the growth of pure microalgae species; nevertheless, pure species production is problematic when sewage is employed as a growing medium due to the existence of indigenous microalgae in the wastewater that could thrive. Photobioreactors (PBRs) are a type of microalgae growing system that is meant to eliminate the problems that come with large open systems. Bubble column, air-lift, tubular and flat-panel are just a few of the shapes and configurations available (Handler et al. 2012). Cross-contamination and unequal light intensity are two drawbacks of open pond systems that can be solved using PBRs. Photobioreactors are frequently built to provide specific optimum culture conditions to photosynthetic strains that have been determined to be rich in value-added compounds such as carotenoids for mass synthesis of the desired product (Acien et al. 2012). PBR

provides several advantages, including a low degree of contamination, efficient use of cultivation space, and a high rate of gas transfer. However, it has been suggested that it will not be suitable for commercial use unless sufficient energy management improvements are made (Lam and Lee 2012). Compared to photobioreactors, HRAP is better suited for low-temperature operation (Arbib et al. 2013). HRAP systems are particularly useful in treating wastewater, and they can also aid with $CO_2$ sequestration by growing microalgae.

## 11.4 Techniques for Harvesting of Microalgal Biomass

Algae biodiesel manufacturing continues with the harvesting process. The microalgae harvesting process is complicated by the small size of algal cells (typically 1–20 mm) and the need to manage large liquid volumes due to the low density of algal cells maintained in open ponds. Due to a lack of efficient and cost-effective harvesting systems, algal-based wastewater treatment is not commonly used in the wastewater business (Lam and Lee 2012). Bulk harvesting (flotation/gravity sedimentation, flocculation) and thickening (filtering, centrifugation) are the two main types of microalgae harvesting techniques (Brennan and Owende 2009). Before using any of the harvesting methods, many aspects of microalgae, form size, surface charge, and specific gravity as well as its cell density in the medium, should be addressed (Mathimani and Mallick 2018; Kadir et al. 2018). The most common type of sedimentation is gravity sedimentation straightforward and cost-effective mechanical approach available. The size of the cells causes sedimentation in this approach. This approach is best for mixed cultures containing huge, heavy cells that sediment due to their size. Tsioptsias et al. (2016, 2017) employed a 1-h sedimentation process to extract sludge from molasses wastewater that has been activated by microalgae. In comparison to that obtained through centrifugation, the biomass collected via gravity sedimentation is diluted. This has an effect on the downstream process, as well as cost of heat drying. A mix of harvesting methods, on the other hand, might be able to help solve the problem (Mata and Martins 2010).

Microalgae harvested with nanoparticles ($Fe_3O_4$) under in-situ magnetic separation yielded a maximum recovery efficiency of more than 98% at an as tiring speed of 120 rpm. *Botryococcus braunii* produced 55.9 mg-dry biomass mg-particles-1, while *Chlorella ellipsoidea* produced 5.83 mg-dry biomass mg-particles-1 (Xu et al. 2013). Organoclays (aluminium and magnesium-based amino saline clay) have been used to harvest microalgae with 100% efficiency in 30 min under neutral pH (Lee et al. 2013b). Although centrifugation is the most efficient and dependable method of harvesting algae, it is a time-consuming and expensive operation (Christenson and Sims 2011). For the harvesting of different microalgae species such as *Coelastrum proboscideum*, *Nannochloropsis*, *Scenedesmus*, mixed microalgae consortia, and *Arthrospira platensis* various types of centrifuges such as self-cleaning disc stack, nozzle discharge, spiral plate, continuous flow and decanter bowl have been reported. It is commonly employed to separate microalgae biomass from APPIW at the laboratory scale (Tan et al. 2014; Yang et al. 2015). However, the process uses

a lot of energy and costs a lot of money up front and in the long run. The US DOE (Department of Energy) also indicated that using a centrifuge on a large scale is financially prohibitive (Mathimani and Mallick 2018; U.S. DOE 2010). Centrifugation aids in the recovery of more than 90% of suspended algae, resulting in a solids concentration of 12–22%; yet, it necessitates a large initial investment and ongoing costs (Vonshak and Richmond 1988). Low-cost filtering techniques are commonly employed to harvest filamentous algae strains, with tangential filtration providing 5–27% solid concentration after harvesting, allowing 70–90% of algal biomass to be recovered (Vonshak and Richmond 1988). This technique is based on the gravitational force concept, which accelerates the sedimentation rate. The centrifugation harvesting process varies depending on particle size. The most efficient way for harvesting is tubular bowl centrifugation; however, it has a very limited capacity (Yaakob et al. 2014). As a result, this technique may be better suited to a small-scale laboratory application.

Filtration is the process of passing microalgae-containing effluent through a porous membrane of a predetermined size. The porous membrane traps the microalgae slurry, while the filtrate is made up of the remaining effluent. Various filtration technologies, including dead-end filtration, pressure filtration, vacuum filtration, membrane filtration (macro filtration N10 μm), ultra-filtration (0.02–0.2 μm), microfiltration (0.1–10 μm), and reverse osmosis (0.001 μm), are used to harvest microalgae biomass (Mathimani and Mallick 2018). The micro-strainer device is another successful method for separating microalgae biomass from water. A backwashing stream dilutes the captured biomass in the micro-strainer, making filtration easier (Sim et al. 1988). Filtering unicellular microalgae has some limits. Given that these cells are tiny and roughly spherical, this is the case. Other extracellular elements may be present in these cells, making the filter media insufficient (Benemann and Oswald 1996). In the mining business, this strategy is highly prevalent. Air is injected at the vessel's bottom to aerate the water. As a result, microalgae float on the top foam section's liquid surface. The process of froth flotation is complicated, and it is influenced by several factors including medium concentration, media pH, oxygen content, temperature, aeration rate, and bubble size (Smith et al. 1968). The technology offers several benefits, including a small footprint, a short operation duration, 50–90% biomass recovery, and the capacity to scale up to industrial levels. The main disadvantage of this method is that it consumes a lot of energy and uses chemical flocculants, which might cause problems with downstream processing (Mathimani and Mallick 2018). The process by which particles in a solution clump together to form flocs, which aid in settling, is known as flocculation (Park et al. 2011).

Inorganic and organic flocculants are common in chemical approaches for harvesting microalgae cells. Using different coagulants or flocculants to cause microalgae cells to aggregate in the culture medium and settle down into flocks as a consequence of sedimentation is the flocculation method. In wastewater treatment, coagulants like alum and ferric chloride are frequently used. However, investigations have demonstrated that collecting algae–bacterial flocs with commercial polymers at varying concentrations affects flocculation efficiency (Farid et al. 2013). Electrolytes

and synthetic polymers such as aluminium ferric cations, aluminium sulphate, and ferric chloride are commonly used in chemical flocculation and coagulation to enhance the particle size of algal cells. As a result, this technique focusses on the suspension 20–100 times as a pre-flotation stage (Christenson and Sims 2011). Chemical coagulants, albeit effective at full scale, turn a potentially useful resource into trash sludge that must be discarded (Hoffmann 1998). Inorganic flocculants have the greatest negative influence on microalgae biomass and metal salt contamination. Unlike inorganic flocculants, organic flocculants are non-toxic and do not pollute microalgae biomass. Chitosan, Drewfloc 447, cationic starch, Chemifloc CV/300, Flocudex CS/5000, and other organic flocculants are examples (Mathimani and Mallick 2018; Fazal et al. 2018). Biodegradability reduced dosage requirements, and other flocculant effects such as chitosan are just a few of the advantages. Doses of 5–100 mg/L are effective in achieving a harvesting efficiency of 20–100%, depending on the microalgal species and the type of organic flocculants utilized (Mathimani and Mallick 2018).

Bioflocculation (autoflocculation) and bioflocculation (bioflocculation) are the most often employed biological technologies for biomass collection in recent years. The process of autoflocculation is triggered by incorporating alkali chemicals to the medium, which results in an alkaline pH (Mathimani and Mallick 2018). The active engagement of fungi and bacteria in the wastewater causes bioflocculation, which is the result of wastewater treatment with microalgae (Lee et al. 2013a). Because polyelectrolytes are non-toxic and biodegradable, they can also be employed as flocculants to collect microalgae (Granados et al. 2011). The bioflocculation technique involves co-culturing bioflocculants (oleaginous bacteria and fungus) with microalgae. Co-cultivation causes a microalgal–fungal pellet to develop, which speeds up biomass harvesting. The creation of microalgal–microbial flocs settles more quickly than microalgae cells alone, resulting in the natural bioflocculation phenomena. This is necessary for effective biomass gathering. Secreted biopolymers, such as those generated by bacteria (EPS), can also cause bioflocculation (Chen and Walker 2011). Due to the presence of nutrients essential for the growth of flocculating microorganisms, microbial flocculants have been widely used in wastewater treatment (Chen and Walker 2011). Harvesting microalgae could be cheaper and more energy-efficient with bioflocculation. Because to autoflocculation, the cost of harvesting microalgae biomass is also lowered. There is yet to be a single harvesting method that has been proven to be both cost-effective and efficient. As a result, an optimal mix of microalgae cell harvesting technologies must be established in order to assure maximum biomass recovery while reducing operational costs and energy consumption.

## 11.5 Microalgae in Wastewater Treatment

Wastewater treatment is an important task that must be emphasized in order to contribute to the society and the future. Contaminants in wastewater are eliminated, especially domestic wastewater to create a solid waste or waste stream that can be

released or reused. Untreated wastewater contains a lot of organic matter, a lot of pathogenic microbes, and a lot of nutrients and harmful substances that have to be removed.

Wastewater treatment is an important task that must be emphasized for the sake of society and the future (Mantzavinos and Kalogerakis 2005). Microalgae has long been recognized as a low-cost, ecologically friendly wastewater treatment alternative (De la Noue et al. 1992) (Table 11.1). Except *Anabaena flosaquae*, most microalgae had a relatively high BOD and COD removal efficiency (>80%) (Elsadany 2018). In batch reactor trials, another study looked at COD eliminate by *Selena strumgracile*, *Scenedesmus quadricauda*, and *Chlorella vulgaris* (Lee et al. 2016). Microalgae in wastewater could have a number of advantages, such as removing coliform bacteria, nitrogen, phosphorus, and heavy metals, as well as lowering biochemical and chemical oxygen consumption (Luo and He 2016; Leong et al. 2013). Microalgae remove pathogens, heavy metals, pesticides, and dyes, as shown in Fig. 11.1. Microalgae use the Calvin cycle to convert inorganic carbon to organic carbon. The Calvin cycle converts inorganic carbon to organic carbon by using the reductant NADPH (nicotinamide adenine dinucleotide phosphate) oxidized and energy from ATP hydrolysis generated in the photosynthetic electron transport chain (Falkowski and Raven 2007). Microalgae cells can absorb and consume nitrates and phosphates for growth, lowering the N and P content of wastewater are improving the quality of wastewater discharge (Emparan et al. 2019). The relative removal efficiencies for nitrogen, phosphorus, and COD were 79.96%, 93.35%, and 90.02%, respectively, with effluent values of 15.69, 1.03, and 90.24 mg/L. When it comes to wastewater treatment, high salinity is a serious issue. It slows the absorption of nutrients and accelerates the development of algae (Church et al. 2017). Microalgae may use both inorganic ($NO_3$, $NH_4^+$ and $NO_2$) and organic (urea, purines, amino acids, and nucleosides) nitrogen sources (Ross et al. 2018). When it comes to inorganic nitrogen, microalgae prefer $NH_4^+$ since it is more energy efficient to assimilate and incorporate (Perez-Garcia et al. 2011). For a long time, it has been advocated to grow microalgae on wastewater to provide energy (Mallick 2002). Microalgae cultivation from wastewater takes advantage of the nutrients in the wastewater to assist its growth, lowering manufacturing costs and reducing greenhouse gas emissions. Microalgae will act as a wastewater remediator and carbon sink while consuming far less energy than typical wastewater treatment methods (Zhang et al. 2019).

## 11.6 Microalgae in Heavy Metals Removal from Wastewater

Biosorption and bioaccumulation are the two ways by which microalgae extract HM ions from wastewater. Biosorption is a metabolic process that happens in both non-living and living cells. Attaching HMs ions to functional groups on the cell surface is accomplished through ion exchange, chelation, microprecipitation, and complexation (Park et al. 2016). According to research, algal cell wall components with important functional groups, such as alginate and fucoidan, are primarily responsible for HM ion biosorption (Zeraatkar et al. 2016). Biosorption is a

**Table 11.1** Studies on the applications of microalgae in wastewater treatment

| Microalgae species | Wastewater | Contaminants | Removal efficiency (%) | Biomass | References |
|---|---|---|---|---|---|
| Algal–bacterial symbiosis *(Chlorella + Nitzchia)* | Settled domestic sewage | COD, BOD, N and P | COD (87), BOD (97), N (92), and P (74) | N/A | Wang et al. (2010) |
| *Auxenochlorella protothecoides* | Concentrated municipal wastewater | TP, TN and TOC | TP (13.5), TN (9.8), and TOC (16) | 0.193 | Renuka et al. (2015) |
| *Chlamydomonas Mexicana* | Piggery wastewater (filter sterilized) | TP, TN and TOC | TP (1.4), TN (3.12), and TOC (1.45) | 0.028 | Renuka et al. (2015) |
| *Chlamydomonas polypyrenoideum* | Dairy industry wastewater | $NO_3^-$, $Fl^-$, $PO_4^{3-}$, TDS, TSS, $Cl^-$, $NH_4^+$ and COD | $NO_3^-$ (62), $Fl^-$ (66.6), $PO_4^{3-}$ (69), TDS (89.8), TSS (91), $Cl^-$ (78), $NH_4^+$ (63), and COD (80) | 2.2 g/L | Umamaheswari and Shanthakumar (2016) |
| *Chlamydomonas* sp. | Industrial wastewater | $NH_4^+$ and $PO_4^{3-}$ | $NH_4^+$ (100) and $PO_4^{3-}$ (33) | 1.34 g/L | Umamaheswari and Shanthakumar (2016) |
| | Industrial wastewater | $NH^{4-}N$, $NO^{3-}N$ and $PO^4P$ | $NH^{4-}N$ (10), $NO^{3-}N$ (10), and $PO^4P$ (3.3) | 0.134 | Renuka et al. (2015) |
| *Chlorella pyrenoidosa* | Soybean processing wastewater | TP, TN, $NH^4N$ and COD | TP (70.3), TN (88.8), $NH^4N$ (89.1), and COD (77.8) | 0.64 | Renuka et al. (2015) |
| | Piggery wastewater | N, P and $NH^{4-}N$ | N (75.7–82.5) and $NH^{4-}N$ (62.5–74.7 > 9) | 0.04 | Umamaheswari and Shanthakumar (2016) |
| | Settled domestic sewage | N and P | N (93.9) and P (80) | N/A | Wang et al. (2010) |
| | Domestic sewage and industrial wastewaters | BOD, P, COD and N | BOD (80–88), P (50–60), COD (70–82), and N (60–70) | N/A | Wang et al. (2010) |
| *Chlorella sorokiniana and aerobic bacteria* | Industrial wastewater | COD, N and P | COD (84.8), N (>95), and P (80.7) | N/A | Bhatt et al. (2014) |
| *Chlorella* sp. | Municipal wastewater concentrate | TN and TP | TN (6.3) and TP (5.7) | 0.07 | Wang et al. (2010) |
| | Digested dairy manure wastewater | TN, $NH^{4-}N$ and TP | TN (75.7–82.5), $NH^{4-}N$ (100), and TP (62.5–74.7) | N/A | Li et al. (2019) |

| | | | | | |
|---|---|---|---|---|---|
| *Chlorella vulgaris* | Tertiary wastewater by forward OMPBR (osmosis membrane photobioreactor) | TN and TP | TN (86–99) and TP (100) | 5 g/L | Yu et al. (2017) |
| | Textile wastewater | TP, COD and TN | TP (33.1–33.3), COD (38.3–62.3), and TN (44.4–45.1) | 0.73 g/L | Bhatt et al. (2014) |
| | Brewery wastewater | TN and TP | TN (87.27) and TP (79.75) | 2.28 g/L | Umamaheswari and Shanthakumar (2016) |
| | Citric acid effluent | BOD, COD, TP and TN | BOD (95.7), TP (90.6) COD (94.9), and TN (94.4) | 0.765 g/L | Umamaheswari and Shanthakumar (2016) |
| | Pig excrement that has been diluted (0.2% suspended solids content) | TN, BOD and TP | TN (54–98), BOD (98), and TP (42–89) | N/A | Satpal and Khambete (2016) |
| | Domestic wastewater | NO2-N, NO3-N, NH4N and PO4-P | NO2-N (3.26), NO3-N (1.52), NH4N (2.17), and PO4-P (2.6) | N/A | Renuka et al. (2015) |
| | Tertiary municipal wastewater | TN and TP | TN (25) and TP (24) | 0.04 | Renuka et al. (2015) |
| *Cyanobacteria* | Settled swine wastewater + secondarily treated domestic effluent | N and P | N (95) and P (62) | N/A | Satpal and Khambete (2016) |
| *Galdieria sulphuraria* | Primary effluent | BOD, P and NH3-N | BOD (16.4), P (1.41), and NH3-N (6.26) | N/A | Tchinda et al. (2019) |
| *Gloeocapsa gelatinosa* | Wastewater from the Periyor that is chemical (based products) | $PO_4{}^{3-}$, $NO_3$- and $NO_2^-$ | $PO_4{}^{3-}$ (75), $NO_3$- (80.9), and $NO_2^-$ (100) | N/A | Emparan et al. (2019) |
| *Nannochloropsis oculata* | Aquaculture system with wastewater recirculation (RAS) | TN, $PO_4{}^{3-}$ and $NO_2^-$ | TN (78.40), $PO_4{}^{3-}$ (14.7), and $NO_2^-$ (84.38) | N/A | Emparan et al. (2019) |
| *Oedogonium* sp. | Digested piggery wastewater | TP and $NH_4{}^-N$ | TP (13.2) and $NH_4{}^-N$ (13.7) | N/A | Renuka et al. (2015) |

(continued)

**Table 11.1** (continued)

| Microalgae species | Wastewater | Contaminants | Removal efficiency (%) | Biomass | References |
|---|---|---|---|---|---|
| *Pithopora* sp. | Thermal wastewater gathered from the power station | COD, BOD, $PO_4^{3-}$ and $NO_3^-$ | COD (87.75), BOD (88.23), $PO_4^{3-}$ (89.37), and $NO_3^-$ (23.07) | N/A | Emparan et al. (2019) |
| *Scenedesmus acutus* | Wastewater from a city's activated sludge plant | COD, $PO_43^-$, $NO_3^-$ and $NH_4^+$ | COD (77.3), $PO_4$ $3^-$ (66.2), $NO_3^-$ (71.1), and $NH_4^+$ (93.6) | N/A | Emparan et al. (2019) |
| *Scenedesmus obliquus* | Piggery effluent | TN, TP, $NH_4^+$, TP, TN and $NO^{3-}$ | TN (58), TP (24), $NH_4^+$ (57) and TP (83), TN (60) and $NO^{3-}$ (84) | N/A | Emparan et al. (2019) |
| | Brewery effluent | TN and TC | TN (1.48), and TC (4.37) | 0.1 | Renuka et al. (2015) |
| | MPBR with hollow-fiber microfiltration of Polyvinylidene fluoride (PVDF) for aquaculture wastewater | P and N | P (82.7) and N (86.1) | 0.0426 | Yu et al. (2017) |
| *Scenedesmus quadricauda* | Sewage wastewater treatment plant domestic wastewater | COD, BOD, $PO_4^{3-}$ and $NO_3^-$ | COD (70.97), BOD (89.21), $PO_4^{3-}$ (81.34) and $NO_3^-$ (70.32) | N/A | Emparan et al. (2019) |
| *Scenedesmus* sp. | Photomembrane bioreactor modified wastewater treatment plant effluent | N and P | N (46), and P (100) | N/A | Bhatt et al. (2014) |
| | Photomembrane bioreactor modified wastewater treatment plant effluent<br>Outdoor flat-plate bioreactor effluent from pre-treated sewage | N and P | N (5.84) and P (0.85) | 0.0523 | Yu et al. (2017) |
| *Spirulina* sp. | Wastewater from a dairy industry was collected | COD, $NO_{3-}$ and $PO_4^{3-}$ | COD (77), $NO_{3-}$ (80), and $PO_4^{3-}$ (72) | N/A | Emparan et al. (2019) |
| *Synechocystis salina* | Chemical wastewater (based products) collected from the Periyor | $NO^{3-}$, $NO^{2-}$ and $PO_4^{3-}$ | $NO^{3-}$ (82.5), $NO^{2-}$ (96.23), and $PO_4^{3-}$ (64.52) | N/A | Emparan et al. (2019) |
| *Synedra affinis* | Sewage wastewater collected from the drain opens into river, Yamuna | $NO^{3-}$, $NO^{2-}$ and $PO_4^{3-}$ | $NO^{3-}$ (100), $NO^{2-}$ (100), and $PO_4^{3-}$ (100) | N/A | Emparan et al. (2019) |

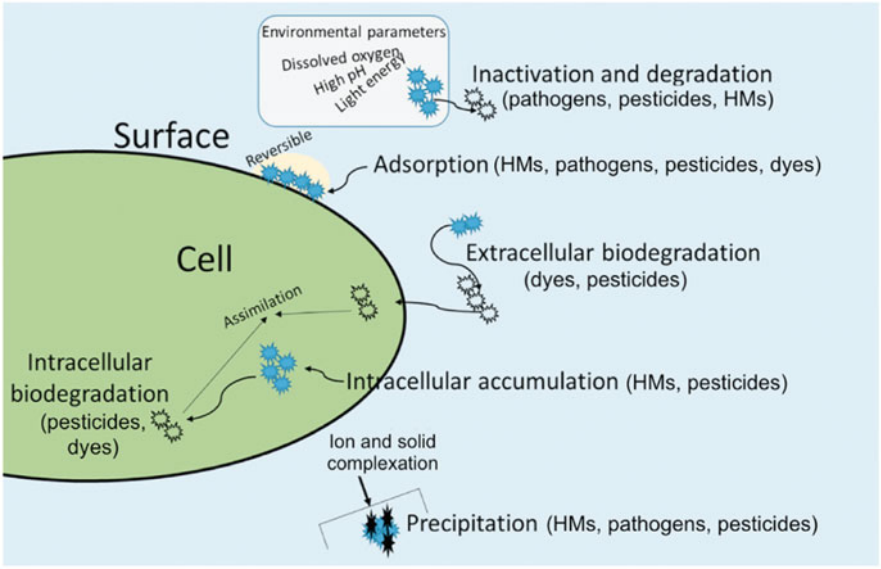


**Fig. 11.1** Removal mechanism involving pathogens, pesticides, dyes, and heavy metals by microalgae (adapted from Markou et al. 2018)

cutting-edge approach for extracting heavy metal ions from wastewaters that depends mostly on non-living algae and inactive biomass. Because heavy metal ions can damage living cells, live algae with a low sorption ability have only been used in a few cases (Lamaia et al. 2005).

There are several microalgae strains that have been reported in heavy metal remediation (Table 11.2). Heavy metal uptake by microalgae is facilitated by both passive and active biosorption by dead biomass and living microalgae cells. The surface of the microalgal cell contains functional groups such as hydroxyl (-OH), carboxyl (-COOH), amino ($-NH_2$), and sulfhydryl (-SH), and cationic metal ions are physically adsorbed across it (-SH). Metal ions pass through the cell membrane into the cytoplasm during active biosorption (Chalivendra 2014). Different functional groups are formed as a result of component variations in cell walls across diverse algal species. Pb, Cd, Ni, and Zn were used to measure the metal uptake of the biosorbent and matrix system, and the results were consistent with the Langmuir isotherm model (Aziz et al. 2016). The capacity of metal ions biosorption by various algal strains would vary due to the varying distribution and abundance of cell wall components in distinct algal strains (Romera et al. 2006). In brown algae, alginate polymers are the principal mechanism of heavy metal ion sorption, and their biosorption capability is proportional to the presence of binding sites on this polymer (Romera et al. 2006). Several heavy metal's removal systems, including ATS (algal turf scrubbers) and HRAP (high-rate algal ponds), has been endorsed for use in real-world situations all around the world. These technologies, on the other hand, are insufficient for large-scale adoption. Phytoremediation is a cutting-edge clean-up

**Table 11.2** Studies on the applications of microalgae in heavy metals removal

| Microalgae species | Heavy metal | Removal efficiency (%) | Wastewater | Reference |
|---|---|---|---|---|
| *Scenedesmus* sp. | Fe | 59.33 | Wet market wastewater | Jais et al. (2015) |
| *Pseudochlorococcum typicum* | Cd | 86 | Aqueous solution | Shanab et al. (2012) |
| *Spirulina maxima and Chlorella vulgaris* | Zn | 94.1 | Municipal wastewater | Chan et al. (2014) |
| *Spirulina platensis* | $Ca^{2+}$ | 98 | Wastewater | Al-Homaidan et al. (2015) |
| *Scenedesmus* sp. | Cu | 100 | Wet market wastewater | Jais et al. (2015) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | La | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Scenedesmus* sp. | Cu | 73.2–98 | Tannery wastewater | Ajayan et al. (2015) |
| *Pseudochlorococcum typicum* | Pb | 70 | Aqueous solution | Shanab et al. (2012) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | Fe | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Scenedesmus* sp. | Pb | 75–98 | Tannery wastewater | Ajayan et al. (2015) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | Al | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Scenedesmus* sp. | Zn | 65–98 | Tannery wastewater | Ajayan et al. (2015) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | Mn | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Scenedesmus* sp. | Cr | 81.2–96 | Tannery wastewater | Ajayan et al. (2015) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | Ba | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Scenedesmus* sp. | Zn | 79.65 | Wet market wastewater | Jais et al. (2015) |
| *Chlorella pyrenoidosa* | Fe | 32 | Dairy wastewater | Kothari et al. (2012) |
| *Spirulina maxima* and *Chlorella vulgaris* | Cu | 81.7 | Municipal wastewater | Chan et al. (2014) |
| *Scenedesmus incrassatulus* | Cr(VI) | 43.5 ± 1.0 | Simulated wastewater | Jácome-Pilco et al. (2009) |
| *Scenedesmus obliquus CNW-N* | Cd | 100 | Aqueous solution | Chen et al. (2012) |

(continued)

**Table 11.2** (continued)

| Microalgae species | Heavy metal | Removal efficiency (%) | Wastewater | Reference |
|---|---|---|---|---|
| *Synechocystis* sp. | Zn | 40 | Aqueous solution | Chong et al. (2000) |
| *Chlorella* sp. | Cu | 97.78–99.26 | Acid mine drainage | Choi (2015) |
| *Spirulina maxima* and *Chlorella vulgaris* | Cu | 81.7 | Municipal wastewater | Chan et al. (2014) |
| *Chlorella* sp. | Zn | 97.78–99.26 | Acid mine drainage | Choi (2015) |
| *Pseudochlorococcum typicum* | Hg | 97 | Aqueous solution | Shanab et al. (2012) |
| *Chlorella* sp. | As | 97.78–99.26 | Acid mine drainage | Choi (2015) |
| *Pavlova lutheri*, *Tetraselmis chuii*, *Chaetoceros muelleri*, and *Nanochloropsis* | Ce | <95 | Municipal leachate | Richards and Mullins (2013) |
| *Chlorella* sp. | Cd | 97.78–99.26 | Acid mine drainage | Choi (2015) |
| *Tetraselmis suecica* | Cd | 60.1 | Simulated wastewater | Pérez-Rama et al. (2002) |
| *Chlorella* sp. | Fe | 97.78–99.26 | Acid mine drainage | Choi (2015) |
| *Spirulina platensis* | $Cu^{2+}$ | 91 | Wastewater | Anastopoulos and Kyzas (2015) |
| *Pterocladia capillacea* | $Cr^{3+}$ | 20–100 | Wastewater | El Nemr et al. (2015) |
| *Chlorella* sp. | $Ca^{2+}$ | 56 | Wastewater | Raikova et al. (2016) |
| *Cystoseira stricta* | $Pb^{2+}$ | 10 | Aqueous solutions | Iddou et al. (2011) |
| *Cladophora fracta* | $Cu^{2+}$<br>$Zn^{2+}$ | 99<br>85 | Oil sands tailings<br>Pond water | Mahdavi et al. (2012) |

technique that primarily relies on algae's biosorption and bioaccumulation capacities are the most important in the bioremediation process, with biosorption taking the lead (Furey et al. 2016).

HM ions are bioaccumulated within cells after being transferred across living cell membranes in a variety of methods (including active and passive transport systems). Heavy metal removal from industrial effluents and household wastewater is one of the primaries focusses for microalgal use in biotechnology using high-rate algal ponds (Oswald 1988). These systems work because they transform hazardous metals into non-toxic ones (Mantzorou et al. 2018). The use of metabolically active

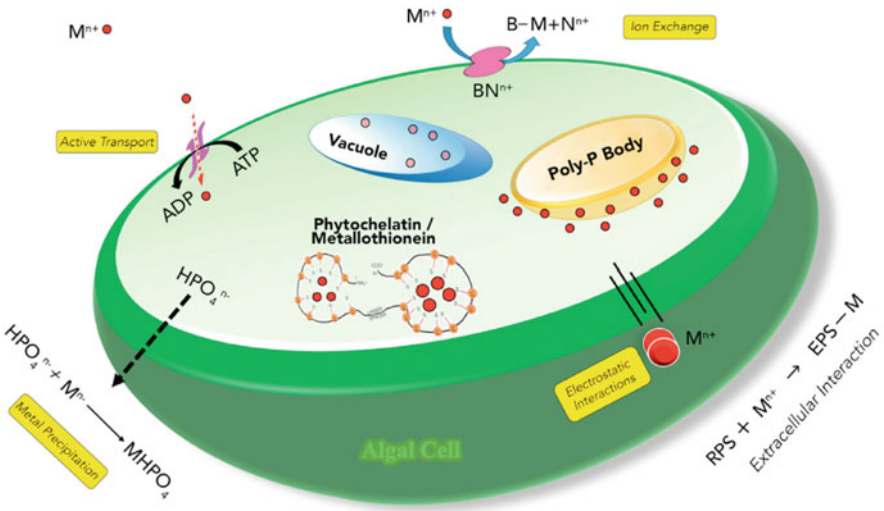


**Fig. 11.2** Metal binding sites of a microalgal cell (adapted from Salam 2019)

immobilized microalgae for detoxification processes and metal recovery is particularly appealing in applications where extremely low levels of residual metal ions are required (Wilde and Benemann 1993). Because a considerable amount of the metal attached to living or dead cellular surfaces, the principal binding sites of microalgae, and immobilizing matrices may be desorbed and later recovered by acid treatment, this is a possibility (Donmez and Aksu 2002). The metal binding sites of a microalgal cell are depicted in Fig. 11.2. Pb, Cd, and Hg bioconcentration factors varied from 600 to 2300. All metals that were extracted from the solution were collected in the biomass (Henriques et al. 2017).

## 11.7 Microalgae in Organic Pollutants Removal from Wastewater

Because of biodegradation and biosorption, microalgae may absorb a wide range of organic contaminants, including pesticides, as an energy source for their development in wastewater. Biosorption is the mechanisms that occur in both living and dead cells' walls include absorption, surface complexation, adsorption, precipitation, and ion exchange. Biodegradation, volatilization, photodegradation, and sorption to the biomass are the most important contaminant-removal processes in microalgae PBRs (Bilal et al. 2018; Tolboom et al. 2019). Other short- and long-term trials were conducted in order to ascertain the percentage of chemicals removed by microalgae (Hussein et al. 2016). Molinate, isoproturon, simazine, atrazine, carbofuran, propanil, pendimethalin, dimethoate, metolachlor, and pyproxin were the pesticides employed in the experiment. The pesticides employed in the trial were molinate,

atrazine, simazine, carbofuran, isoproturon, propanil, and dimethoate. Microalgae have been used in the past to remove colour and vinyl sulfone dyes from textile industrial effluent due to their binding affinity and high surface area (de Andrade and de Andrade 2018). The dye removal method in microalgae cell walls includes electrostatic biosorption, attraction, bioconversion, and complexation. Dye ions cling to the surface of algal biopolymers and then convolute into biopolymer's solid phase. Extracellular polymers with functional groups will assist the biosorption of dye molecules into the polymer surface (Kumar et al. 2014). Furthermore, pH of the amount/composition the extracellular polymeric molecules in PBRs may influence the ability of microalgae to absorb MPs (Cheng et al. 2019). Organic MPs are removed from the aqueous phase by microalgae by biodegradation, which is thought to be the most efficient method. During biodegradation, microalgae use a variety of enzymes to interact with MPs, including carboxylation, hydroxylation, hydrogenation, oxidation, demethylation, and ring cracking (Ding et al. 2017).

Spirogyra biomass has been shown to be a useful biosorbent in the removal of reactive dyes (Aksu and Tezer 2005). *Caulerpa lentillifera* and *Caulerpa scalpelliformis* biomass can biosorb basic colours (Chai et al. 2020). *C. vulgaris is frequently used as a biosorbent to remove reactive dyes like Remazol Black B* (Aksu and Tezer 2005). By converting a mono-azo dye to aniline, *Chlorella vulgaris* can diminish the colour by 63–69% (Chai et al. 2020). In addition, the effectiveness of five microalgal strains like *N. elepsosporum A. flosaquae*, *N. linkia*, *C. vulgaris* and *A. variabilis* in removing red colouring from the effluent of textile industries were evaluated (Elsadany 2018). The investigation revealed the strains of microalgae examined were capable for removing the red colour from the treated wastewater effluent of textile industries with varying rates of reduction. Complete dye removal was obtained by *N. elepsosporum* (96.16%), *N. linkia* (79.03%), *A. variabilis* (88.71%), and *A. flosaquae* (50.81%). The complexity of textile wastewater with interwoven compositions incorporating other substances such as heavy metals does not appear to alter the efficacy of colour removal by microalgae.

## 11.8 Microalgae in Emerging Removal of Contaminants from Wastewater

Emerging contaminants are of great environmental concern and have been reported to cause several toxic effects in living beings (Chaturvedi et al. 2021). Out of a great number of microalgae species, only a few have been thoroughly investigated for their potential to bioremediate emerging contaminants (ECs) (Table 11.3). As a result, the selective removal of ECs by distinguishing microalgae species requires screening methods to examine and validate. Biosorption, biodegradation, bioaccumulation, volatilization, and photodegradation are some of the mechanisms involved in the elimination of emerging contaminants by microorganisms (fungi, bacteria, or microalgae) (Maryjoseph and Ketheesan 2020). Microalgae choose a

**Table 11.3** Studies on the applications of microalgae in emerging contaminants removal

| Microalgae species | Emerging contaminant | Removal efficiency (%) | Removal mechanism | Reference |
|---|---|---|---|---|
| *Chlamydomonas* sp. *and Chlorella* sp. | 7-aminocephalosporanic acid | 100 | Adsorption > hydrolysis, photolysis | Guo et al. (2016) |
| *C. pyrenoidosa* | 7-ACA | 96.1 | Biodegradation, adsorption | Yu et al. (2017) |
| *Mychonastes* sp. | 7-aminocephalosporanic acid (7-ACA) | 100 | Photolysis, adsorption > hydrolysis | Guo et al. (2016) |
| *C. pyrenoidosa* | Amoxicillin | 100 | Algal action | Li et al. (2015) |
| *S. obliquus* | Amoxicillin | 98.5 | Photodegradation, biodegradation, and hydrolysis | Yang et al. (2017) |
| *C. pyrenoidosa* | Cefradine | 7–23 | Algal action | Li et al. (2015) |
| *C. vulgaris* | Cefalexin | 97.15 | Photodegradation | Liu et al. (2017) |
| *C. pyrenoidosa* | Cefalexin | 99.23 | Photodegradation | Liu et al. (2017) |
| *C. pyrenoidosa* | Cefalexin | 94.9 | Algal action > self-degradation | Guo and Chen (2015) |
| *Nonliving lipid-extracted Chlorella* sp. | Cefalexin | 71.2 | Adsorption | Angulo et al. (2018) |
| *C. pyrenoidosa* | Cefradine | 89.9 | Algal action > self-degradation | Guo and Chen (2015) |
| *Chlamydomonas Mexicana* | Ciprofloxacin | 13 | Biodegradation, accumulation, and adsorption | Xiong et al. (2017c) |
| *Nannochloris* sp. | Ciprofloxacin | 100 | Direct photolysis | Bai and Acharya (2017) |
| *S. obliquus* | Enrofloxacin | 23 | Biodegradation > accumulation, adsorption | Xiong et al. (2017a) |
| *Chlorella PY-ZU1* | Tilmicosin | 90.2–99.8 | Adsorption, photodegradation, and biodegradation | Cheng et al. (2017) |
| *Nannochloris* sp. | Trimethoprim | 11 | Biodegradation, photolysis | Bai and Acharya (2017) |
| *C. vulgaris* | Levofloxacin | 91.5 | Accumulation and biodegradation | Xiong et al. (2017b) |
| *C. vulgaris* | Metronidazole | 100 | Adsorption | Hena et al. (2021) |

| | | | | |
|---|---|---|---|---|
| *Chlorella* sp. | Florfenicol | 97 | Bioaccumulation, biodegradation, and adsorption | Song et al. (2019) |
| Algal–bacterial consortium | Ciprofloxacin | 97.46 | Sorption and photodegradation | Hom-Diaz et al. (2017) |
| *Fischerella* sp. | Methyl parathion | > 80 | Biodegradation and sorption | Tiwari et al. (2017) |
| *Selenastrum capricornutum* | β-Oestradiol | 100 | Adsorption and biodegradation | Hom-Diaz et al. (2015) |
| *Selenastrum capricornutum* | 17α-ethinyloestradiol | 95 | Biodegradation and adsorption | Hom-Diaz et al. (2015) |
| *Scenedesmus dimorphus* | 17 α-Oestradiol | 85 | Biodegradation | Zhang et al. (2014) |
| *Chlamydomonas reinhardtii* | β-Oestradiol | 100 | Biodegradation and adsorption | Hom-Diaz et al. (2015) |
| *Spirogyra* sp. | Sulfathiazole | 36 | Indirect and biodegradation photodegradation | Garcia-Rodríguez et al. (2013) |
| | Sulfapyridine | 15 | Indirect and biodegradation photodegradation | Garcia-Rodríguez et al. (2013) |
| | Sulfamethoxazole | 14 | Indirect and biodegradation photodegradation | Garcia-Rodríguez et al. (2013) |
| | Tetracycline | 89 | Photodegradation | Garcia-Rodríguez et al. (2013) |
| | Oxytetracycline | 93 | Biodegradation and hydrolysis | Garcia-Rodríguez et al. (2013) |
| *Chlorella vulgaris* | Norfloxacin | 36.9 | Photodegradation | Zhang et al. (2012) |

specific or, better still, a combination of mechanisms to eliminate these pollutants from the water as a survival response due to differences in the physicochemical properties of ECs. For example, in a High-Rate Algal Pond (HRAP) system (Matamoros et al. 2016) 4-octylphenol (OP) and galaxolide only demonstrated volatilization due to high Henry law constant values, but *Chlorella vulgaris* bioaccumulates and biodegrades levofloxacin (Xiong et al. 2017a). Adsorption of metronidazole onto exo-polymeric components of Chlorella vulgaris was recently confirmed (Hena et al. 2021). According to Yu et al. (2017), the removal of micropollutants by microalgae entails fast adsorption owing to physicochemical interactions between the cell wall and the pollutants, comparatively slow molecular transference across the cell wall, and subsequently bioaccumulation, biodegradation, or both. Hansda and Kumar (2016) found that the surfaces of microalgal cells contain a variety of functional groups such as hydroxyl, sulfate, carboxyl, and other charged groups that have varying affinity, selectivity for various inorganic and organic substances.

Bioadsorption reactions, chelation, ion exchange reactions with functional groups on the microalgal surface, microprecipitation, and surface complexation reactions are a few of the chemical reactions that take place at the cell surface during bioadsorption (Schmitt et al. 2001; Donmez et al. 1999). Adsorption became the main mechanism as a result of the shading impact of microalgal biomass, which blocked light from penetrating deeper into the bioreactor as biomass increased. A single component cannot determine the adsorption method of micropollutants onto microalgae according to the preceding talks. However, EC adsorption rates ranges from 0% to 100%. Adsorption rates of six pharmaceutical medicines onto the cell surface of the green microalga *Chlorella sorokiniana* were determined to be 20% by De Wilt et al. (2016).

Microalgae eliminate micropollutants from the aquatic phase as well as biomass through biodegradation, which is one of the most efficient methods (intracellular or intercellular). Biodegrading compounds in the intercellular or intracellular phases bioaccumulate and adsorb onto the biomass, respectively. Extracellular enzymes produced by microalgae disintegrate some toxic antibiotics into less or non-toxic intermediates, which are biodegraded and bioaccumulated by intracellular enzymes, according to Naghdi et al. (2018). Ceftazidime and its basic parent structure 7-ACA are degraded intracellularly by *C. pyrenoidosa*, which is an example of this mechanism. Ceftazidime was first adsorbed on algae, then slowly moved through the algal cell wall before being broken down by enzymes (Yu et al. 2017). Intracellular degradation-based antibiotics removal by *S. obliquus* (Xiong et al. 2017b) and *C. vulgaris* are two instances (Xiong et al. 2017a). The antibiotic is demolished by algae metabolites such as extracellular enzymes during the extracellular degradation process, and the biodegraded intermediates/end-products may be metabolized further by algal cells (Naghdi et al. 2018).

## 11.9 Waste Valorization into Biofuels and Other Value-Added Products Using Microalgae

Microalgae have a variety of commercial uses. These are mostly phototrophic, which have major technical and commercial benefits, such as food nutrition. Microalgae cultivated in wastewater offer a large selection of applications in the fuel industry. However, it has a wide range of non-fuel uses, together with cosmetics, fertilizer, and animal feed. Many processes, such as pyrolysis, can be used to produce biochar from microalgae, which has enormous promise for fertilizer and carbon sequestration in agricultural applications (Marris 2006). In the bioenergy conversion process, it can also be used as a process fuel. It reduced carbon emissions by up to 84% for carbon sequestration objectives (Lehmann 2007). As a result, the value of utilizing waste resources is still insufficient to compensate for the high cost of capturing and transporting waste resources. It is critical to increasing productivity to create a financially sustainable algae biofuel sector (Correll 1998). The success of microalgae-based technologies is contingent on the optimization of all of their output. It is possible that the fact that this technology is used for biofuel generation and wastewater treatment isn't enough to make it cost-effective and useful. Eventually, the microalgae can act as a simple and environmentally friendly source of biofertilizer for crops and other plants (Gonzalez et al. 2009). Seed germination, plant development, and fruit yield were all improved when the dry cell contents of a green microalga, *Acutodesmus dimorphus*, were utilized as biofertilizers in Roma tomato plants (Garcia-Gonzalez and Sommerfeld 2016). Seed germination, plant development, and fruit yield were all improved when the dry cell contents of *Acutodesmus dimorphus*, a green microalga, were used as biofertilizers in Roma tomato plants (Garcia-Gonzalez and Sommerfeld 2016). Seed germination, plant development, and fruit yield were all improved when the dry cell contents of *Acutodesmus dimorphus*, a green microalga, were used as biofertilizers in Roma tomato plants (Garcia-Gonzalez and Sommerfeld 2016). Microalgae take nutrients from wastewater with a high efficiency. Many microalgal species flourish in wastewater due to the availability of nitrogen, carbon, and phosphorus, which function as nutrients for the algae. Unicellular algae have shown great nitrogen uptake efficiency and have been shown to dominate oxidation ponds (Pittman et al. 2011).

Biomass is produced on a modest scale in high-rate algal ponds or waste stabilization ponds for wastewater treatment (Johnson and Sprague 1987). It has been recommended that instead of lipid production, which is the present focus in the field of algae-based biofuels industry, algae should be grown for biomass productivity (Griffiths and Harrison 2009). The biological feedstock (potato, barley, wheat, sugarcane, biodiesel, and corn sugar beet) using rapeseed, coconut, palm, animal fats, sunflower, soybeans, Jatropha, Miscanthus or cassava, wood, straw, and grass) is the source material for the end products of biodiesel, ethanol, and methanol in the first and second generations of biofuel production (de Vries et al. 2010; Sims et al. 2010). They necessitate a considerable amount of land to develop these oil crops, which prevents biofuels from replacing fossil fuels because the volumes produced are insufficient to meet world transportation fuel demand (Banse et al. 2011; Secchi

et al. 2011). The cultivation area required for first and second-generation biofuel production can be lowered by many orders of magnitude by employing microalgae as a biological feedstock in third-generation biofuel production to generate the same quantity of biomass (Harun et al. 2011; Ozkan et al. 2012). Biomass may be converted to a variety of energy sources, such as heat, electricity, and biogas. Unlike biomass from crops, microalgal biomass has thick cell walls that prevent intracellular lipids from being released, making lipid extraction challenging (Lam and Lee 2012). To produce a sustainable fuel with the smooth engine running, the viscosity of microalgae oil must be lowered. Transesterification is a typical method for reducing the viscosity of microalgae oil by chemically converting it to FAMEs, often known as biodiesel (Bala 2005). According to the feedstock used, biofuel manufacturing is divided into three generations.

Microalgae biofuels are classed as third-generation biofuels since they contribute considerably to non-edible and edible resources, respectively. Several projects have been launched to increase the potential output of microalgae-based biofuels. When dairy effluent was used as a nutrient medium, *Chlamydomonas* was used to treat wastewater and produce biofuel at the same time, yielding lipid productivity of 87.5 2.3 mg/L/day (ref). C14:0, C16:0, C16:1, C18:0, C18:2, and C18:3 fatty acid chains were discovered, and they were all acceptable for biofuel production (Arora et al. 2016). Algae has a high oil content, making it possible to make high-quality biodiesel from bioresources (Chisti 2007). Chemical (supercritical fluid extraction, Soxhlet extraction, extraction using ionic liquids, and accelerated solvent extraction) and mechanical (microwave-assisted, oil expeller, and ultrasonic-assisted) methods have all been used to improve the biofuel-producing ability of microalgae grown in wastewater (Mubarak et al. 2015). In comparison to supercritical fluid extraction, solvent extraction, microwave extraction, and ultrasonic extraction all have advantages. Because of the cavitation phenomena, sonication causes the cell structure to break down mechanically, increasing the microalgae cells' potential to generate lipids (Drira et al. 2016). Physical procedures or chemical solvent extractions a combination of the two are used to extract lipids. Extraction techniques must be efficient, fast, and non-destructive to extracted lipids, as well as scalable (Medina et al. 1998). At 50 °C, transesterification of *Nannochloropsis oculata* in the presence of CaO and $Al_2O_3$ catalysts yielded a 97.5% conversion (Umdu et al. 2009). At 350–400 °C and 2500 pressure, green algae were transesterified in the presence of titania, zirconia, and alumina catalysts, yielding 90.2% conversion (McNeff et al. 2008). After 12 h of transesterification at 38 °C with 75% lipase (*Candida* sp.), methanol and *C. protothecoides* converted to 98.15% (Cheng et al. 2009).

Patil et al. (2008) show that liquefaction might be able to burn wet biomass and convert it into smaller molecular materials with higher energy densities. Wet-algal biomass is used to make liquid fuel in this method. Mechanically harvested microalgae cells have a high moisture content and are utilized as a feed for liquefaction (Hu et al. 2017, 2019). Bio-oil is a dark brown liquid made from biomass after hydrothermal liquefaction (HTL) and pyrolysis. Pyrolysis was limited to moisture-free 5% biomass, whereas HTL can be applied to any type of biomass (Toro-Trochez

et al. 2019). In the absence of air and at 350–700 °C, algal-biomass may be utilized to produce a wide range of products including biochar, syngas, and bio-oil (Goyal et al. 2008). Because microalgae biomass has a high moisture content, it must be dried before being pyrolyzed (Amin 2009). Fast pyrolysis yielded nearly 18–24% bio-oil for *Chlorella protothecoides* and *Microcystis aeruginosa* (Miao and Wu 2004). Bioethanol generation from algal biomass by anaerobic fermentation is a more straightforward and straightforward process than other fermentation methods. *Chlamydomonas perigranulata*, a genetically modified microalga, self-ferments carbohydrates and produces bioethanol (Daroch et al. 2013). People's interest in biomethane fermentation technology is piqued by the fact that it produces valuable items such as biogas. Biogas is an amalgam of $CO_2$ (25–45%), methane (55–75%) and various other gases produced by anaerobic bacteria which digests microalgal material (Singh and Gu n.d.; Harun et al. 2010). Biomethane is utilized as a source of energy or fuel gas to create power, and leftover biomass can be utilized to make biofertilizers (Singh and Gu n.d.). Other advantages include less energy-intensive reaction conditions, a less alcohol–oil ratio required, and faster recovery of the product (Du et al. 2008).

## 11.10 Challenges and Future Prospects

The most significant barrier/challenge for WWT using microalgae in an open pond system at an outdoor site is the culture's environmental stability. Microalgae growth is best at temperatures of 18–24 °C and light intensity of 80–200 mol/$m^2$/s (Barati et al. 2018). Although microalgal-based wastewater treatment tries to efficiently remove nitrogen and phosphorus, it cannot remove all new pollutants and heavy metals. Microalgae growing in wastewater is a convenient and effective form of WWT although it is not an economically feasible alternative WWT method. Only certain microalgae species and development paths can produce high-quality biomass which can be actually used to extract valuable products, and this treatment method's lower cost is due to the fact that only certain microalgae species and growth modes can produce high-quality biomass that can be transformed into valuable bioproducts. Photobioreactors that are enclosed and also require chemical agents for sterilization and artificial light boost the overall cost of production (Umamaheswari and Shanthakumar 2016). The three most expensive pieces of equipment are freeze-dryers, photobioreactors, storage, and decanters (Acien et al. 2012). Choosing the correct species of microalgae for wastewater treatment is critical. Because of the diverse chemical and physical components of wastewater from various sources, the selected microalgae species should be able to cope with changes in ambient circumstances (Gour et al. 2020; Chew et al. 2018). Furthermore, to cope with stress and survive any undesired species attacks or food shortages, the species must be able to share metabolites (Amenorfenyo et al. 2019). Mixotrophic and Heterotrophic microalgae that are facultative in using organic carbons as their only substrate and cutting off all light sources for growth are also limited. Industrial wastewater has lower algal growth rates due to low levels of N and P-containing compounds and

also high levels of toxins. The potential for broad-scale treatment of industrial wastewater is considerably limited due to the huge quantity of heavy metal ions, rendering algae cultivation and $CO_2$ fixation ineffective (Molazadeh et al. 2019).

Several obstacles stand in the way of developing large-scale algae harvesting and production. Some of the harvesting techniques include centrifugation, flotation, flocculation, gravity sedimentation, filtration, and ultrasonication (Lam and Lee 2011). Harvesting procedures that are less expensive take longer to separate (gravity sedimentation) and produce mixed microalgae–chemical flocculant biomass, whereas more expensive harvesting techniques yield mixed microalgae–chemical flocculant biomass (flocculation). The optimum temperature and light intensity, whether high or low, can alter microalgae species' growth profiles. Photoinhibition occurs when strong light intensity inhibits the action of microalgae's photosystem-II (PS-II), resulting in a reduction in microalgae growth. As a result, microalgae production has become highly challenging, as microalgae development now relies on both controllable (wastewater dilution, substrate concentration, flow velocity, and so on) and uncontrolled (outside light intensity and temperature) aspects. In harsh situations, few studies have looked at the ideal microalgal growth conditions (Gupta and Pawar 2019; Chu et al. 2015). A number of researches have been conducted in order to identify the related procedures and technologies that will allow microalgae to be employed on a large scale in the future. The transition from pilot to full-scale operations; however, usually microalgae are exposed to unfavorable settings, the result of dramatically decreased yields of bioproducts (Matamoros et al. 2015). Novel biotechnological approaches to microalgal cell genome alteration to infuse them with varied properties are fast gaining prominence. However, in some species of microalgae particularly diploid diatoms, the maximum ability of genetic engineering may only be realized if traditional growing techniques are established, allowing to increase performance, practical mutations must be integrated (Zeng et al. 2011).

## 11.11 Conclusions and Recommendations

This chapter pays more attention has been placed on WWT with the aid of microalgae, taking into account its many applications, issues, and potential. Microalgae can assist remove a number of organic and inorganic pollutants from wastewater, as well as produce a variety of useful products. Furthermore, it appears that the generation of microalgae biomass in both industrial and domestic wastewater is a more promising and viable wastewater treatment approach. As a result, wastewater treatment using microalgae has received a lot of interest recently.

More research is needed to make use of EC bioremediation capabilities of microalgal species, boost biodegrading enzymes, increase bio adsorption and optimize growth conditions. Treatment of EC using microalgae may be a cost-effective solution when combined with nutrient removal, such as HRAPs and a feasible strategy for lowering pollutant pollution in rivers. Microalgae will be an ideal solution for energy-related issues because they can be utilized not just for biodiesel,

but for a variety of other things like biomethane and bioethanol. Biofuels, on the other hand, have been employed as a substitute for minimize fossil fuel usage while simultaneously providing wastewater treatment. The fuel obtained from microbial biomass has the possibility to be a conventional fossil fuels substitute, but their high cost precludes them from becoming a competitive competitor. The most serious issues can be addressed mixing wastewater treatment with algal-based biofuel generation. To establish the economic feasibility of large-scale manufacture of biodiesel generated from microalgae, extensive research and development is required.

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# Removal of Cobalt, Nickel, Cadmium, and Lead from Wastewater by Phytoremediation

12

Sevinc Adiloglu and Semin Duban

**Abstract**

Today, heavy metals, which seriously affect ecosystems, are one of the most important problems to be solved. To remove heavy metals, the phytoremediation method, which is one of the easy and applicable methods, comes to the fore. To get the highest yield from the unit area in agriculture, many implementations like treatment sludge, chemical fertilizers, soil conditioners, hormones, pesticides and using wastewater in irrigation, are carried out. The rapidly and unevenly increasing world population, malnutrition, unplanned urbanization with inappropriate land use, perilous wastes, fast-disappearing forests and green areas, consumption and senseless energy production, industrialization, heavy metal–laden products caused by industrial factories and mineral deposits, the negative effects created by human activities and many other similar ones are the most important environmental problems experienced today. On the other hand, to solve the wastewater problem in the world and our country, by using the most appropriate technology, with minimum cost, a healthy study should be carried out so that it can be used again, especially in agriculture. Removing heavy metals and organic pollutants that may arise from industrial wastewater by aquatic plants is becoming common. In particular, studies and researches on determining the accumulation capacity of aquatic plants used in wastewater treatment and which families will be the solution to this issue should be increased. Studies have shown that heavy metals cause corruption in many physiological events like transpiration, stomatal movements, protein synthesis, photosynthesis, water absorption, enzyme activity, sprouting, membrane stability, and hormonal balance in plants because of their venomous effects. For this reason, plants that can accumulate heavy metals and

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plant assets that adapt according to climatic changes will increase the applicability of the phytoremediation method. In this study, phytoremediation methods used in the removal of common lead, cobalt, nickel, and cadmium heavy metals and hyperaccumulator plants will be emphasized. In the removal of these heavy metals, aquatic hyperaccumulator plants determined today will be put forward and recommendations will be made to solve these problems in wastewater.

**Keywords**

Remediation · Wastewater · Heavy metal · Aquatic plants · Hyperaccumulator

## 12.1 Introduction

With the rapid passage of time, increasing toxic heavy metals amounts caused by the developing industry and urbanization have threatened ecosystems. Heavy metals such as lead (Pb), nickel (Ni), chromium (Cr), cobalt (Co), copper (Cu), and cadmium (Cd) are exported at greater levels. Untreated wastes generated as a result of production processes have detrimental causes on the environment (Gavrilescu 2004; Chandra and Kumar 2017a; Kumar et al. 2021a). In addition to the numerous benefits that technological developments and industrialization contribute to mankind, heavy metal pollution, which is undesirable and disrupts the ecological balance, is increasing day by day. This formation is important environmental pollution and heavy metals sometimes cause toxicity even in very small concentrations. Heavy metals emitted to the environment, especially from domestic or municipal waste, industrial activities, mining, agricultural activities, unplanned urbanization, accumulate in nature day by day (Kumar 2018). Although some pollutants are found in low amounts in air, water, and soil, the phenomenon of increasing concentration in consumers in successive rings of food chains is called bioaccumulation (Adiloğlu 2021).

Today, environmental pollution is a problem that is emphasized the most but is the least recommended for its solution. The main elements of environmental pollution are generally domestic and industrial wastes (Kumar and Thakur 2020; Kumar et al. 2020a, b). When these wastes are given directly to nature without any treatment, they are called "waste." Environmental pollution can be minimized by using the wastes in other places in a way that does not create environmental pollution, or by giving them to nature by breaking down, and it can already be cleaned by natural processes in such a small amount of pollution. While protecting the environment and natural resources from pollution is extremely important in terms of preventing environmental pollution, cleaning contaminated areas is also of great importance in the solution of existing environmental pollution.

The problem of water pollution caused by heavy metals has recently attracted the attention of the world. Studies on heavy metal pollution in ecological parameters have given attention to the resources and behavior of heavy metals, effects on public health and the environment, investigation and examination of contaminated sites,

remediation management, risk assessment, and techniques. The evaluation of plants that provide regional and climatic adaptation of many different hyperaccumulator plant families in the remotion of the aforementioned heavy metals comes to the fore in the application of phytoremediation technologies (Chandra and Kumar 2018; Kumar and Chandra 2018). In this study, phytoremediation technologies, some heavy metals, and accumulator plants used for removing these heavy metals, which are the most inexpensive and easily applicable methods, although there are many methods for eliminating water pollution, were evaluated.

## 12.2 Phytoremediation Technologies

Phytoremediation, which is an economical and accepted technology used in the removal of pollution from ecological stakeholders, is illustrated (Fig. 12.1).

### 12.2.1 Phytoextraction

It is generally used as the accumulation of organic or inorganic pollutants, which are pollution factors in the soil, in the aboveground and underground pieces of the plant for soil, water, and sediment reclamation contaminated with heavy metals (Chandra et al. 2018a, b). Harvesting above the surface of the ground part of the hyperaccumulator plant grown in the polluted area or removing its roots by removing these factors from the area and removing the pollution factors in different areas is also a valid practice (Chandra et al. 2018b; Kumar et al. 2021b, c). The reuse of aboveground and underground parts of plants can retain a hundred times more polluting factors than other plants. These parts, which are pruned and mown, can be obtained again as heavy metals and used as fertilizers. Nickel and gold are recovered in this way in the USA (EPA 2000; Pivetz 2001; Adiloğlu and Göker 2021).

The phytoextraction method converts pollutants into portable form by the plant and ensures their accumulation in the plant. It is an economical and applicable method, although it does not have a toxic effect on public health and in ecological terms (EPA 2000).

Phytoextraction, a new biotechnological method, can accumulate 100 times more pollutants in the plants that are used in phytoextraction in comparison with other plants. For this method, it has been revealed by research that there are about 400 plants that can accumulate heavy metals, mainly the plant families Brassicaceae, Euphorbiaceae, Asteraceae, Lamiaceae, and Scrophulariaceae, as hyperaccumulators. After the application of the herbal extraction method, the hyperaccumulator stubbles are mostly recycled by drying-burning-composting and recycling (Memon et al. 2000).

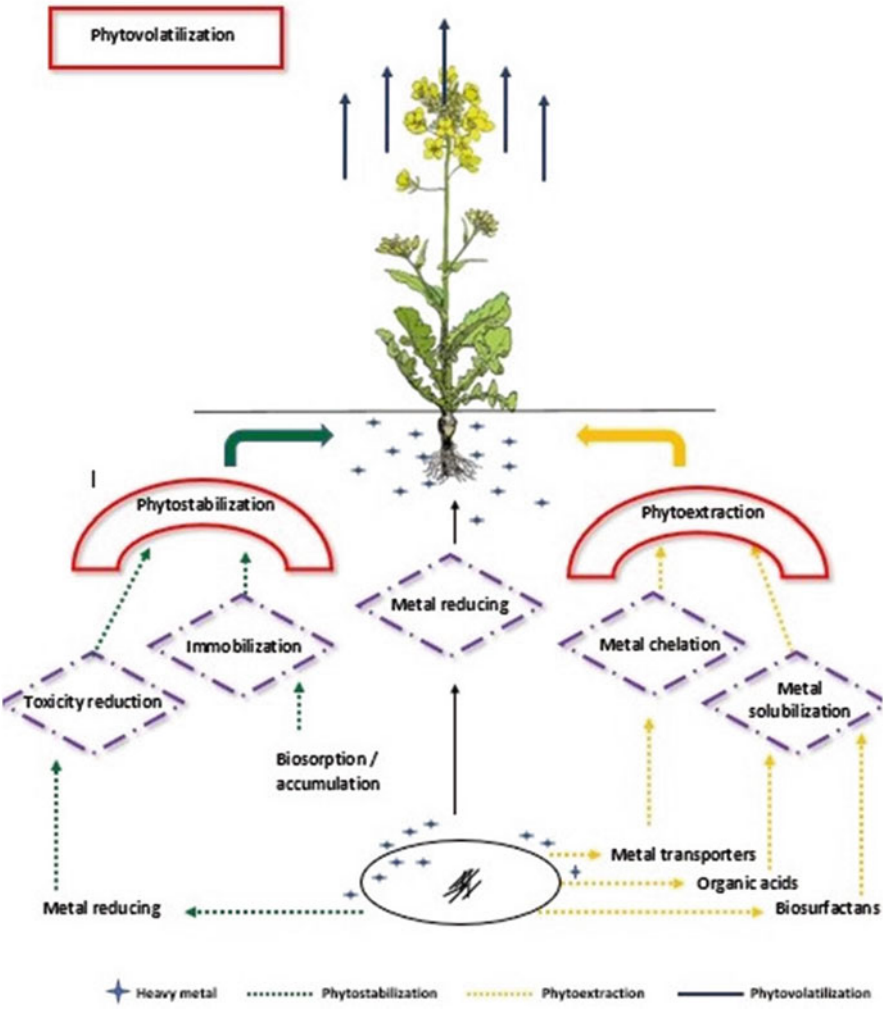


**Fig. 12.1** Phytoremediation technologies

### 12.2.2 Phytodegradation

The phytodegradation method is a biotechnological method used especially in the removal of organic pollutants. It is a method that provides the degradation of the pollutant parameter with enzymes that function in the biochemical and physiological processes of plants. In other words, vegetative degradation is mostly used to remove soil, sediment, sludge, and groundwater pollutants from ecological parameters (Kumar et al. 2021b). The most important advantage of the method is that deduction or deterioration events appear in the plant body in line with physiological events. The disadvantage of the method is that while the process takes place within the plant,

the risk of the formation of toxic products cannot be determined in this case (Pivetz 2001).

In the phytodegradation method, the uptake of the pollutant is limited to the rhizosphere region. The plant type of compounds in an organic form that can be taken into the plant structure depends on how long the pollutant can stay in the soil, its solubility, and its physical and chemical structure. The important advantage of this method is that reduction or degradation takes place in the plant without being dependent on microorganisms. The disadvantage of this method is that it is difficult to detect negative physiological events in the plant during degradation (Pivetz 2001; Adiloğlu and Göker 2021).

### 12.2.3 Phytostabilization

Phytostabilization is a method used for soil stabilization. Plants can tolerate high metal concentrations in the phytostabilization method and can also immobilize metals by sorption, complexation, precipitation, or reduction of metal valences in soil. Pollution factors in the soil in which the plant grows occur by immobilizing the pollutants around the plant roots, accumulating by the roots, sticking to the surface of the roots, or precipitation in the root zone of the plant (Türkoğlu 2006).

In Wang et al. (2007), different doses of Cu were applied to maize (*Zea mays*) plant inoculated with the arbuscular mycorrhizal fungus *Acaulospora mellea* in the laboratory environment, and the development of the plant and Cu uptake were investigated. As a result of the study, they attributed the low plant uptake in pots containing high concentrations of Cu to the acidity-basicity degree of the soil. The data obtained from this study showed that the structure and concentration of organic acids such as malic, citric, and oxalic acids in the soil were changed by fungi. In their study, they have been determined that *A. mellea* did not play an active role in the phytoextraction of copper in maize. However, they stated that the plant would be more favorable for phytostabilization under the high Cu concentrations in the roots of the plants treated with mycorrhiza. On the other hand, the transport of pollutants by wind, water erosion, washing, and soil dispersal is averted. In the system, which is closely concerned with the microbiology and chemistry of the root environment of the plant, the plant can change the structure of the pollutant in a water-insoluble, nontransportable way (EPA 2000; Yıldız 2008).

### 12.2.4 Phytovolatilization

Root depth is very important in the vegetative evaporation method. In the groundwater in question, the roots of the plants must be deep. To clean the dirty groundwater, shallow plant roots can also be taken by pumping the water to the surface. This method's most important advantage is the conversion of highly toxic composites (such as mercury-containing compounds) into less toxic forms. Nevertheless, it is also a disadvantage that these very harmful and toxic materials can be released into

the atmosphere (EPA 2000). From the plant system, pollutants can be separated or evaporated by transpiration. As it is known, water moves from the underground parts of plants to the leaves through the plant's vascular system. Thus, pollutants mix with the air surrounding the plant by evaporation and volatilization. An example of this mechanism is poplar trees (EPA 1995).

Ghosh and Singh stated that some plants (e.g., *Brassica juncea* [brown mustard] and *Arabidopsis thaliana* [thale cress]) absorb heavy metals and turn these metals into gaseous form and release them to the atmosphere (Ghosh and Singh 2005).

Some tree species are frequently used in the phytovolatilization method due to their ability to take pollutants such as *Populus* and *Salix* with phytoremediation (Pulford and Watson 2003).

In another study, it was stated that plants such as thale cress and brown mustard have grown in a nutrient medium including selenium can generate volatile selenium in the form of dimethyl selenide and dimethyl diselenide (Banuelos 2000; Terzi and Yıldız 2011).

### 12.2.5 Rhizodegradation

If the degradation by roots occurs by enriching the microorganisms in the root zone of the plant or by the effect of the plant roots, this event is expressed as rhizodegradation (Kumar and Chandra 2020). The decomposition of organic pollutants in the soil with the help of microorganism activities in plant roots is called rhizodegradation. There are organic and fatty acids, amino acids, sterols, sugars, factors for growth, nucleotides, flavanones, and enzymes that change the microbial activities in the plant root region and are released from the roots. Organic compounds that cause pollution are also in this environment. The most important benefit of degradation by roots is the elimination of pollutants in the natural environment (EPA 2000; Yıldız 2008).

Pollutants removed by rhizodegradation method; pesticides (herbicide, insecticide), benzene, toluene, ethylbenzene and xylene (BTEX), total petroleum hydrocarbon (TPH), polycyclic aromatic hydrocarbon (PAH), surfactants, solvents containing chlorine (TCA, TCE), pentachlorophenol (PCP), and polychlorinated biphenyl (PCB). The plants used in the rhizodegradation method are red mulberry (*Morus rubra* L.), mint (*Mentha spicata*), water cane (*Typha latifolia*), and alfalfa (*Medicago sativa*) (Farrell et al. 1999; Söğüt et al. 2002; EPA 2000; Vanlı 2007).

### 12.2.6 Rhizofiltration

In root filtration, pollutants adhere to the plant roots or are taken up into the roots, depending on biotic and abiotic proceedings. In the course of the occurrence of these periods, pollutants can be received and carried into the plant. The main thing here is to ensure the immobility of the pollutants on or inside the plant. The pollutants can

then be removed from the plant in various ways. Rhizofiltration method is used in groundwater, surface water, and wastewater (Söğüt et al. 2002; Vanlı 2007).

This method is used for removing radioactive matters or metals from polluted water. In rhizofiltration methods, plants are not planted directly in the polluted area, primarily the rapport of the pollutant is provided. Until the plants have a large root system, they are grown hydroponically in clean water, not in the soil medium. For this plant with a developed root system to develop under new conditions, it is taken from the water source where it was grown and transferred to the contaminated water source. The roots are harvested when they are saturated. The advantage of this system is that it allows the use of terrestrial and aquatic plants. On the other hand, the system can be applied in natural environments as well as in artificial areas such as pools, tanks, and ponds (EPA 1995; Türkoğlu 2006).

### 12.2.7 Phytohydraulic Control

Hydraulic control is the prevention of accumulation and transport of pollutants in groundwater or taking them under control with the use of plants. That operation can be used in underground waters and also surface waters. The phytohydraulic control method's most important advantage is the widening of the breeding effect area due to the spreading of the roots over a wide area without any artificial system being established. The most important disadvantage is that the water intake of the plant changes depending on the season and climate. A schematic of the hydraulic control method is given in Fig. 12.2.

Pivetz (2001) stated that a 5-year-old Populus tree can absorb 100–200 L of water per day. It means that the amount of transpiration of a single willow tree is 5000 gallons of water per day. The primary factor affecting the usability of the plant in this method is the fact that the plant water intake varies from season to season and climate. It highlights the importance of using the water-using abilities of these plants in the hydraulic control method. Willow, hybrid poplars, and Eucalyptus species are also employed in phytohydraulic control. Hydraulic control is preferred for water-soluble organic and inorganic pollutants in general (EPA 2000).

## 12.3 Vegetative Cover Systems

Thanks to a self-improving plant system, the vegetative cover method, which takes pollutants under control, takes a long time to develop on the soil surface (Kumar et al. 2021c). As a long-lasting and self-renewing structure, vegetative cover systems grow in or on materials with environmental risks and require minimal maintenance. In the mechanism, there can be different categories of green improvement, including water uptake, root microbiology, and plant metabolism factors, and hydraulic control in the system. In various applications, the vegetative cover is usually formed as a barrier to prevent the dispersal of pollution (EPA 2000) (Fig. 12.3).

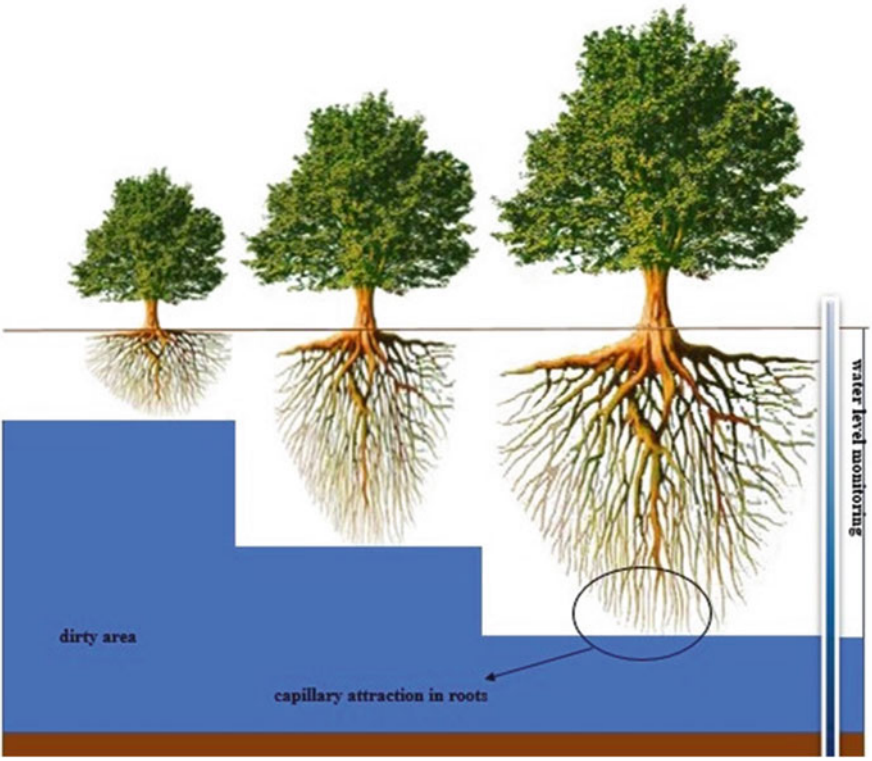


**Fig. 12.2** Phytohydraulic control

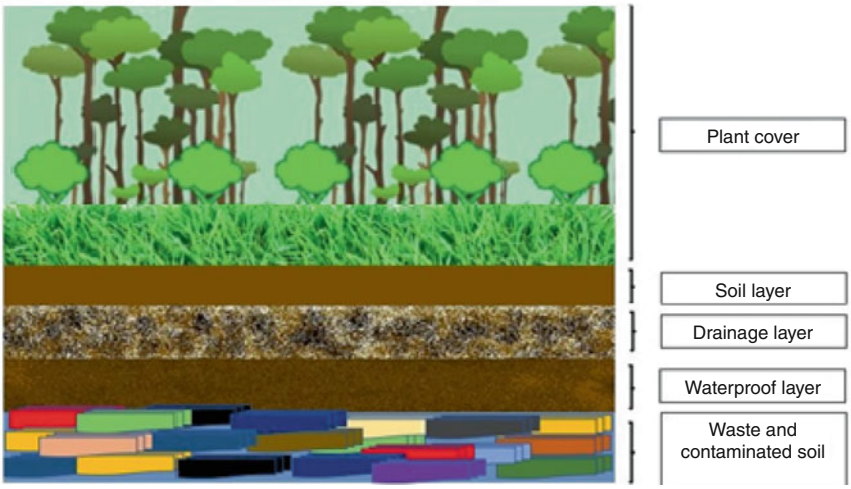


**Fig. 12.3** Vegetative Cover Systems

## 12.4 Buffer Strips and Riparian Corridors

Coastal buffer strips involve planting suitable plants in strips along the banks of the streams along the stream to lift the pollutants in the underground or surface water flowing toward the streams (Pivetz 2001; Vanlı 2007) (Fig. 12.4).

It ensures that the pollution does not spread to the environment and does not mix with the groundwater. Shore buffer strips also reduce sediment by controlling erosion. In studies conducted in Canada, it has been determined that these system applications reduce erosion by 90% and herbicide flow between 42% and 70% (Gabor et al. 2001). For the removal of fertilizers and pesticides, the coastal buffer strip method has been used the most. Poplars are among the plants most commonly used for this purpose (EPA 2000).

## 12.5 Cobalt Removal from Wastewater

In plant production, cobalt (Co), aluminum (Al), nickel (Ni), sodium (Na), vanadium (V), and silicon (Si) constitute functional plant nutrients and are considered essential for plants. Studies on functional nutrients continue today. However, the venomous causes of heavy metals are seen in plants when they are above the need level. These metals, which cause toxicity in all parameters of the environment, contribute to plant production in case of adequacy (Karaman et al. 2012).

Heavy metals in wetlands all over the world; It is present in large quantities in a direction increasingly diffused by anthropogenic (human origin) interventions like

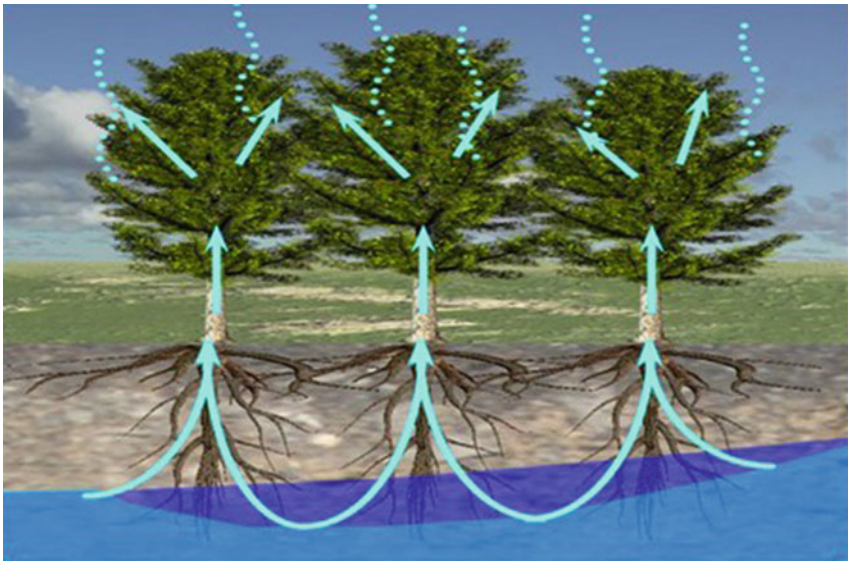

**Fig. 12.4** Shore buffer strips method

mining, industry, agriculture, and construction activities, amongst other aspects. When heavy metals are evaluated in terms of public health, their accumulation in various organs and accumulation in high amounts can cause serious damage to the human body. Cobalt is one of the heavy metals with the most polluting effects on the environment (Forster and Wase 1997). The amount of cobalt is approximately 1.1 mg and is found in muscles, bones, and tissues. In addition, vitamin B12 has 4% Co in its structure and functions in the synthesis of hemoglobin. In the excess of cobalt element, diseases such as loss and function disorder in the heart and lungs and, rise of the blood sugar level, fat levels and cholesterol, cancer, misbirth, and infertility are observed (Akpınar 2005; Dissanayake 1991).

Cobalt is absorbed by plants in the form of $Co^{2+}$ ions. When the cobalt level is high in the soil, a large amount of $Co^{2+}$ is taken up by the plants and transported by transpiration and accumulates at the leaf margins. Since cobalt is an immobile element in the plant, it accumulates in the old leaves and its deficiencies are seen primarily in the young leaves. However, its deficiency is not a common condition. Cobalt uptake increases in the plant when a chelator is used (Karaman et al. 2012; Adiloğlu 2016).

In heavy metals pollution removal, Gupta et al. (2012) water lettuce (*Pistia stratiotes*) was grown in a pollution study. It has been tried to determine that the plant is an accumulator against heavy metals. They stated that the roots of *P. stratiotes* collect high levels of Zn, Ni and Cd and can be used in cleaning these contaminants.

## 12.6 Nickel Removal from Wastewater

Nickel distribution in the soil profile varies depending on the soil parent material, organic matter, amorphous oxides, and clay fractions. The highest Nickel amounts are generally seen in clay soils. Nickel toxicity can be seen in soils rich in serpentine minerals and may cause groundwater pollution depending on soil properties. Nickel, which is a mobile plant nutrient in plants, can accumulate in both seeds and leaves. Nickel is actively absorbed by plants in the form of $Ni^{2+}$ and is transported through special carriers. Due to its high mobility and chelating feature in the plant, it can easily replace the elements found in enzymes and physiological active centers. Sewage sludge and some phosphate fertilizers can also be sources of Nickel. Especially in sewage sludge, Nickel is in the form of organic chelates and can easily pass to the plant and cause toxic effects. It is necessary to add lime, phosphate, or organic matter to reduce the amount of nickel transferred to the plant. However, it also causes pollution in water resources due to industrial and traffic sources (Karaman et al. 2012; Adiloğlu et al. 2016a).

In nickel pollution removal, Manios et al. (2003) examined the heavy materials that *Typha latifolia* takes from water in a study they conducted. With the analyses they conducted in this research, they gave information about the heavy metals (Ni, Cu, Zn) taken up by the root, stem, and leaves of *T. latifolia*. In the analysis, four different plant groups (A, B, C, and D) were irrigated every 2 weeks with water that

contained different concentrations of Zn, Ni, and Cu. After the tenth week, the substrate and plants were analyzed for dried weighed heavy metals.

In consequence of the analyses, the Zn concentration in the leaves, stems, and roots of *T. latifolia* reached 391.7 mg/kg and 60.8 mg/kg in dry weight. In the bottom layer of Group D, all 3 metals were determined at the highest rate. At the end of the linear correlation analysis, they assumed a straight-line relationship between the concentrations of the substrate materials and the concentrations in the solution, and it was stated that the contribution of the plants to the uptake ability of the system was less than 1%.

In a study with plants (*Typha domingensis* and *Chrysopogon zizanioides*), their hyperaccumulator capacity was evaluated by growing them in a wetland resulting from a gold mine waste storage facility seepage. Cultivation of the *T. domingensis* plant produced only 6.65 g biomass per plant during the 75 days. The other plant *C. zizanioides* produced biomass of 12.30–14.18 g per plant. Due to high biomass production, heavy metals Cr, Co, Mn, As, Cu, Zn, Ni, and Pb were found to accumulate 3, 7, 4, 7, 14, 7, 5 times higher than *T. domingensis* plant compared to *C. zizanioides*. For this reason, *T. domingensis* is an accumulator for cleaning these heavy metals from gold mine water seepage, especially in nickel and lead removal (Compaore et al. 2020).

## 12.7 Cadmium Removal from Wastewater

The main parameters that make up the ecosystem are water and soil. Cadmium can contaminate both soil and water sources from many different sources. In particular, contamination from industrial areas and traffic-related toxicity have an important place (Amith et al. 2021; Singh et al. 2021). However, one of the factors that cause the addition of Cd to water and soil with fertilization, which is one of the agricultural activities, is the phosphorus fertilizers used in agricultural production. Because the raw materials of phosphorus fertilizer contain a significant amount of cadmium. A significant amount of Cd accumulates in agricultural soils and underground waters due to the addition of phosphorus, a macronutrient, to the soil with phosphorus fertilizers as a result of incorrect fertilization. Cadmium, which has a toxic effect on plants as a result of agricultural activities, threatens human health with its ecological chain (Karaman et al. 2012; Adiloğlu et al. 2016b).

Öbek (2009) investigated the heavy metals accumulation capacity of the *Lemna gibba* aquatic plant in the wastewater of the wastewater treatment plant final settling pool by using the phytoremediation method in a study he conducted to purify the water, which stands out from ecological parameters, from pollution factors. He reported that the plant accumulated a high amount of Pb, Cr, Cd, Ni, Cu, and Zn within a few days.

*Phragmites australis* is widely used for artificial wetlands for cleaning metallic wastewater. *P. australis* is known to take metals from sediment. He stated that only small amounts of metal were transported from the roots by *P. australis*, but higher concentrations of Zn were found in the aboveground tissues, while other metals were

retained in the roots and rhizomes. It was determined that lead and cadmium were collected only in the roots (Özatlar 2019).

Das et al. (2014) researched the accumulation potential of water lettuce (*Pistia stratiotes*) plant grown at 4 different concentrations of Cd heavy metal (5, 10, 15, and 20 ppm) and stated that this plant is an accumulator against Cd heavy metal.

Khellaf and Zerdaoui (2009) investigated the accumulator capacity of the *Lemna minor* plant in removing heavy metal pollution, and they determined that *L. minor* L. has a high sensitivity to Cd and Cu pollution, but less to Zn and Ni. The same researchers examined the hyperaccumulator capacity of *Lemna gibba* L. in cleaning water contaminated with zinc (Zn) and reported that the plant had the highest uptake capacity at 21 °C.

## 12.8 Lead Removal from Wastewater

When water resources are evaluated in terms of pollution, lead pollution ranks first among the factors that cause environmental pollution, the dimensions of which are increasing day by day in the world. Recycling wastewater to reuse, especially in agricultural areas, is essential. Changing ecological conditions increase the importance of this situation. The history of the use of lead-heavy metal dates back to the ancient Romans. Lead enters the human body, especially through the digestive and respiratory tracts. Lead exists in the atmosphere in solid (dust and especially $PbO_2$ particles) and gaseous (alkyl Pb-exhaust gases) forms. 90% of the lead in the atmosphere is absorbed by the lungs. More than 90% of absorbed lead is collected in red blood cells. Generally, when the lead concentration in the air is at the level of 1 μg/m$^3$, the lead rate in the blood rises to 1 μg/dL. Lead in the body mainly accumulates in the skeletal system. Here, the half-life of Pb is more than 20 years (Castaing et al. 1986; Adiloğlu 2013).

Manios et al. (2002) investigated the effects of some heavy metals on the total protein concentrations of *Typha latifolia* grown on substrates containing wastewater compost. In this, they used areas with full-grown plants in every group, sectioned into five groups in the ponds they selected with a volume of 6.5 L. The fifth of these pools was left empty and solutions including various proportions of Cu, Cd, Ni, Zn, and Pb were applied to the other four for 10 weeks. Consequently, they determined a rise in the concentrations of Zn and Ni in the plant leaves and stems of *Typha latifolia* and observed the identical higher concentrations for the Pb, Cu, and Cd. However, the researchers observed an increase in protein concentration in leaf tissues and did not find any inhibition in the growth and health of plants in three of the four groups. They found that inhibition occurred only in the fourth group because of heavy metal toxicity.

In the research conducted with *Lemna gibba* plant, it was exposed to Pb heavy metal for 7 days in a concentration range of 50–300 mg/L in a certain temperature range. The role of lead on the growth of the *L. gibba* plant was investigated with the same experimental method for 13 days. The highest Pb accumulation of the *L. gibba* plant occurred at the level of 50 mg/L during the third day. It has been stated that lead

has an inhibitory (preventive) effect in duckweed at all concentrations (Miranda et al. 2010).

Bharti and Banerjee (2013) studied the cleaning of elements such as Cd, Zn, Fe, Pb, Cr, Ni, Mn, and Cu in open colliery sewerage in Singrauli District of India by using *Lemna minor* L. and *Azolla pinnata* L. plants. The results showed that both plants significantly accumulated metals in the coal mine effluent. Therefore, they suggested that these two plants be widely used in the treatment of polluted waters.

The phytoremediation method, which is a new and environmentally friendly technique in the treatment of water contaminated with lead-heavy metals, is successfully applied in many countries of the world today.

## 12.9 Accumulator Plants in the Removal of Heavy Metals from Water

Pollution of water with various pollutants has become the most worrying socioeconomically worldwide. Considering the growing shortage of fresh water, it has become mandatory for growers to use wastewater for crop production, especially in semiarid and arid regions. Wastewater contains many essential inorganics and organic plant nutrients, which are generally thought to be essential for plant metabolism (Chandra and Kumar 2017b, c). However, since it contains many toxic elements such as heavy metals, it also causes various hygienic, ecological, and health problems. For this reason, it is necessary to increase the researches on the hyperaccumulator plants used especially for the removal of heavy metals from the aquatic environments and the detection of new accumulator plants on this subject. Water, which is one of the ecological parameters and indispensable for human beings, should be brought to the world economy with economical and easily applicable methods (Yinanç and Adiloğlu 2017; Shahid et al. 2020; Şaşmaz Kışlıoğlu 2021).

Chan et al. (1981) reported that organic and inorganic dissolved and suspended pollutants can be removed by physicochemical mechanisms such as adsorption and precipitation in wetland systems. Researchers reported that in these systems, in addition to soil and microorganisms, especially *Phragmites* (reed) and *Typha* (sedge) wetland plants help in treatment.

Gersberg et al. (1986) stated that plants in wetlands were not efficient in using most of the pollutants at high hydraulic loading, but these plants contributed more to the treatment efficiency at low hydraulic loadings.

Aydoğan and Bektaş (2003) states that the removal of heavy metals and organic pollutants that may arise from aquatic plants used in the cleaning domestic wastewater, which may arise from industrial wastewater, is becoming increasingly common and introduces these systems.

Pulford and Watson (2003) investigated the capacity of trees to assimilate heavy metals in their study. They used *Salix* spp., a fast-growing willow species, in their research. They predicted that the rapid growth of this species and its regular harvesting can take up nutrients quickly, but the same cannot be true for the high

level of heavy metals contamination in the soil. Consequently, they determined that the plant is tolerant of the high amount of nutrients or low concentrations of metal pollution.

Murray (2003) used the phytoremediation method to improve the soils where 1,2-Dichloropropane (DCP) and nitrate accumulated, which were used for disinfection of drinking water in the early 1990s, and to protect the drinking water resources of the region. As a result, they found that grown poplars can metabolize waste nutrients and DCP in the soil. In this study, they showed that hybrid poplars can be used for cleaning shallow groundwater contaminated with organic pollutants and manure.

Scholz (2003) conducted a study to examine the performance of vertical flow artificial wetlands with granular media containing aquatic plants and having different adsorption capacities. The lead and copper sulfate were put into the domestic wastewater being that sample the pretreated mine wastewater. Although it increased the metal loading by 4.6 at the end of the 1-year trial period, it did not observe an increase in metal removal. At the end of the 13-month trial, it was found that aquatic plants and adsorption medium have not caused a significant rise in the removal of the metal. As a result of his correlation analysis, he observed that there is a substantial positive correlation between temperature and conductivity, Dissolved Oxygen (DO), and redox potential. He determined that time-consuming and costly experiments such as biological oxygen demand can be estimated with cheaper experiments such as DO, and temperature.

Researchers noted that water hyacinth (*Eichhornia crassipes*) is highly effective in the San Joaquin River Delta of Sacramento, California. The biggest problem in rooting water hyacinth is that its seeds can live up to 20 years (Khanna et al. 2011; Patel 2012). Despite all the research and work, these notorious seeds have been successful in spreading worldwide. In addition to having high nutrient-rich environmental needs for the development, water hyacinths can also tolerate low nutrient content. The water hyacinth development in seawater is restricted due to salinity rate and therefore they are not common in coastal areas (Priya and Selvan 2014). *E. crassipes* is a perennial and freshwater plant that is upright, round, and brightly colored. An adult water hyacinth consists of a sprout, clump of leaves and fruit, long hanging roots, and stem. The average length of water hyacinths is 40 cm. Sometimes it can grow up to 1 meter. It has 6 to 10 leaves with a circumference of 4 to 7 cm. Some different parts of the plant, for example, stems and leaves, have air-filled tissues that allow the plant to have flayed on the water surface. *Eichhornia crassipes* plants have obvious socio-economic and ecological implications. It is used in the production of biogas, animal feed, fertilizer, water treatment, and firewood (Tham 2012; Günhan 2019).

*Pistia stratiotes*, also called water lettuce, is a perennial, free-floating, stemless, stoned, and fringed plant related to the *Araceae* family. Water lettuce is common in tropical and subtropical waters. Water lettuce grows best in mainly acidic environments but can grow over a wide temperature and pH range. In research, it has been revealed that this plant is used in the removal of As, Cd, Cu, Al, and Pb heavy metals (Günhan 2019).

Metal hyperaccumulator plants 450 types of indoor seeds with accumulative behavior in the context of As, Co, Cd, Mn, Cu, Pb, Ni, Sb, Tl, Se, and Zn (Rascio and Navari 2011). Hyperaccumulation of 317 types of Ni, 28 types of Co, 37 types of Cu, 14 types of Pb, 1 type of Cd, 11 types of Zn, and 9 types of Mn (Baker et al. 2000).

According to Bhargava et al. (2012), there are 320 species of hyperaccumulator plants for Ni, 34 for Co, 14 for Pb, and 4 for Cd. There are at least 500 species that accumulate one of the heavy metal such as (450) Ni, (30) Co, (14) Pb, (2) Cd (Van der Ent et al. 2013).

Şaşmaz Kışlıoğlu (2021) collected naturally grown aquatic plants (*E. cannabinum*, *Juncus*, *Phragmites australis*, *Tamarix tetrandra*, *Xanthium strumarium*, *Salix*, *B. ascbersus*, *Lythrum salicaria*, and *Typha latifolia*). These plants are naturally grown aquatic plants on the sediments in wastewater treatment plant discharge waters, and their capacity to absorb different metals to the roots and stems of these plants was investigated. According to the findings, the following has been defined as accumulator plants:

*For B and Rb, *E. cannabinum* and Juncus
*For Sr, B, Mo, Se, *P. australis*
*For B and Rb, *T. tetrandra*
*For Sr, B, Mo, Se, Rb, Tl, *X. strumarium*
*For B, Cu and Se, *Salix*
*For Sr, B, Cu, and Rb, *B. ascbersus*
*For Sr, B, Cu, Se, and Rb, *L. salicaria*
*For Sr, B, Zn, Cu, Mn, Mo, Se, and Tl, *T. latifolia*.

## 12.10 Bioremediation

Bioremediation is a method of using biological activities to reduce the harmful effects of environmental pollutants in certain regions. Many microorganisms can be used with biological activity such as yeast, algae, fungi, and bacteria (Fig. 12.5).

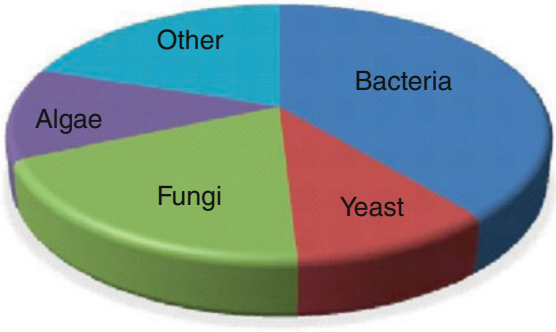


**Fig. 12.5** Microorganism species used in the bioremediation process (Coelho et al. 2015)

I. Not being taken into the cell

II. Retention of metal in the cell by binding to proteins

III. Conversion of metal into a less toxic form

IV. Active transport of metal from microorganisms

V. Keeping metal out of the cell

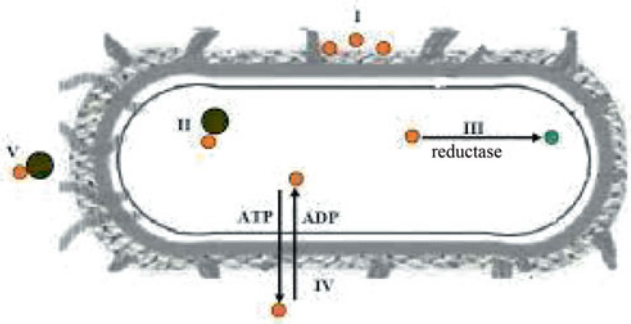


**Fig. 12.6** A schematic representation of metal resistance systems in microorganisms

Prokaryotes (bacteria and archaea) differ from eukaryotes (plants, protists, animals, and fungi) in some aspects. The cellular structures of prokaryotic cells and eukaryotic cells are different from each other. Eukaryotic cells have nuclei and membrane-enclosed organelles, while prokaryotes lack membrane-enclosed organelles and nuclei. Another difference is that the ribosome structure in eukaryotes (80S) is larger than that in prokaryotes (70S) (Killham and Prosser 2007).

The interaction of microorganisms with heavy metal ions varies depending on whether they are eukaryotic or prokaryotic cells. Prokaryotes are less susceptible to metal toxicity than eukaryotes. The way microorganisms interact with heavy metal ions depends in part on whether they are eukaryotic or prokaryotic. Eukaryotes are more susceptible to metal toxicity according to prokaryotes. Thanks to the resistance mechanisms they have developed against heavy metals, microorganisms can maintain much more concentrations of heavy metals. They survive in their environment. These mechanisms developed by bacteria are listed below (Koçberber Kılıç 2008) (Fig. 12.6).

I. Not being taken into the cell.
II. Retention of metal in the cell by binding to proteins.
III. Conversion of metal into a less toxic form.
IV. Active transport of metal from microorganisms.
V. Keeping metal out of the cell.

For effective bioremediation, the microorganisms must attack the pollutants enzymatically. It should also turn them into harmless products. Resistance mechanisms to toxic substances have been developed by bacteria and higher organisms that resist them and render them harmless. The enzymatic degradation process can be carried out by many microorganisms, including aerobes, anaerobes, and fungi. In many bioremediations that take place under aerobic conditions, molecules that are difficult to break down are broken down by microbial organisms. Different types of microorganisms are used to clean areas contaminated with different pollutants (Sharma 2012).

List some of the microorganisms studied and strategically used in purification by bioremediation for heavy metals can be listed as follows.

1. Bacteria:
   **Arthrobacter* spp. (Roane et al. 2001)
   **Pseudomonas veroni* (Vullo et al. 2008)
   **Burkholderia* spp. (Jiang et al. 2008)
   **Kocuria flava* (Achal et al. 2011)
   **Bacillus cereus* (Kanmani et al. 2012)
   **Sporosarcina* (Achal et al. 2012).
2. Fungi:
   **Penicillium canescens* (Say et al. 2003)
   **Aspergillus versicolor* (Tastan et al. 2010)
   **Aspergillus fumigatus* (Ramasamy et al. 2011).
3. Algae:
   **Cladophora fascicularis* (Deng et al. 2007)
   **Spirogyra* spp. (Lee and Chang 2011)
   **Cladophora* spp. (Lee and Chang 2011)
   **Spirogyra* spp. (Mane and Bhosle 2012)
   **Spirulina* spp. (Mane and Bhosle 2012).
4. Yeasts:
   **Saccharomyces cerevisiae* (Machado et al. 2010)
   **Candida utilis* (Kujan et al. 2006).

Microorganisms take part in bioremediation by facilitating the progression of biochemical reactions that reduce the contaminant we want to be cleaned by acting as a biocatalyst through their enzymatic pathways. The purpose of microorganisms is to provide access to a variety of materials that will help them produce nutrients and energy to produce more cells. Only in this case does it act against pollutants. Today, many types of microorganisms provide the removal of heavy metals. Table 12.1 contains the types of microorganisms that are effective on some heavy metals.

The effectiveness of bioremediation depends on the including the chemical makeup and concentration of pollutants, as well as the physical and chemical properties of the environment and their availability to microorganisms (El Fantroussi and Agathos 2005).

**Table 12.1** Some heavy metals and microorganisms used in remediation (Abdulaziz and Musayev 2017)

| Heavy metal | Bacterial species | Fungal species | Algal species |
|---|---|---|---|
| $Cr^{+6}$ | *Bacillus laterosporus*<br>*Staphylococcus xylosus* | *Aspergillus sydoni* | *Chlorella miniata*<br>*Spirogyra* sp.<br>*Oedogonium hatei* |
| Cd | *Bacillus laterosporus*<br>*Plesiomonas shigelloides*<br>*Staphylococcus xylosus*<br>*Exiguobacterium* sp.<br>*Pseudomonas stutzeri*<br>*Rhizobacteria*<br>*Genus desulfovibrio* | *Microsphaeropsis* sp.<br>*Trametes versicolor* | *Sargassum* sp.<br>*Bifurcaria bifurcata*<br>*Macrocystis pyrifera*<br>*Fucus spiralis*<br>*Oedogonium* sp. |
| Pb | *Pseudomonas putida, Bacillus pumilus, Bacillus* sp., *Azotobacter chroococcum XU1, Pseudomonas stutzeri*<br>*Staphylococcus hominis*, *Bacillus simplex*, *Bacillus mojavensis*, *Staphylococcus saprophyticus* | *Saccharomyces cerevisiae*<br>*Trametes versicolor*<br>*Aspergillus niger*<br>*Cephalosporium aphidicola* | *Sargassum* sp.<br>*Fucus spiralis*<br>*Spirogyra* sp. |
| Cu | *Pseudomonas putida*<br>*Rhizobium* | *Aspergillus niger* | *Cystoseira indicia*<br>*Sargassum* sp. |
| Zn | *Thiobacillus ferrooxidans*<br>*Pseudomonas strain* | *Penicillium italicum* | *Sargassum* sp.<br>*Macrocystis pyrifera*<br>*Fucus spiralis* |
| Co | *Pseudomonas stutzeri* | – | *Cystoseira indicia* |
| Ni | *Serpentine rhizobacterium Cupriavidus pauculus KPS 201* | – | *Padina australis*<br>*Sargassum* sp.<br>*Oedogonium hatei* |
| Fe | *Pseudomonas stutzeri* | – | – |

The fact that bacteria and contaminants do not come into contact with each other affects the rate of degradation. Also, germs and pollutants do not spread evenly into the environment (Table 12.2).

Control and optimization of bioremediation processes is a complex system as it is affected by many factors. These factors can be listed as follows:

- Presence of a microbial population capable of degrading pollutants.
- Presence of pollutants in the microbial population.
- Environmental factors (type of soil, temperature, pH, presence of oxygen, presence of other electron acceptors and nutrients).

**Table 12.2** Microorganisms used for utilizing heavy metals

| Microorganisms | Heavy metals | Reference |
|---|---|---|
| *Saccharomyces cerevisiae* | Lead, mercury, and nickel | Chen and Wang (2007) and Infante et al. (2014) |
| *Cunninghamella elegans* | Various heavy metals | Tigini et al. (2010) |
| *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* | $Fe^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $Mn^{2+}$, and $Cu^{2}$ | Paranthaman and Karthikeyan (2015) |
| *Lysinibacillus sphaericus* CBAM5 | Cobalt, copper, chromium, and lead | Peña-Montenegro et al. (2015) |
| *Microbacterium profundi* strain Shh49T | Iron | Wu et al. (2015) |
| *Aspergillus versicolor, A. fumigatus*, *Paecilomyces* sp., *Paecilomyces* sp., *Trichoderma* sp., *Microsporum* sp., *Cladosporium* sp. | Cadmium | Soleimani et al. (2015) |
| *Geobacter* spp. | Fe (III), U (VI) | Mirlahiji and Eisazadeh (2014) |
| *Bacillus safensis* (JX126862) strain (PB-5 and RSA-4) | Cadmium | Priyalaxmi et al. (2014) |
| *Pseudomonas aeruginosa*, *Aeromonas* sp. | U, Cu, Ni, Cr | Sinha et al. (2011) |
| *Aerococcus* sp., *Rhodopseudomonas palustris* | Pb, Cr, Cd | Sinha and Paul (2014) and Sinha and Biswas (2014) |

### 12.10.1 Cobalt Bioremediation by Alphaproteobacterium MTB-KTN90

Studies have shown that a novel magnetostatic bacterium (*Alphaproteobacterium* MTB-KTN90), functioning as a new biosorbent, may have cobalt removal potential. Sensitivity to the pH of the solution was determined in the cobalt removal process with MTB-KTN90. In these studies, higher biosorption capacity was observed in this process when the pH was around 6.5–7.0. Maximum cobalt removal was achieved when conditions were optimum (Tajer-Mohammad-Ghazvini et al. 2016). In summary, Alphaproteobacterium MTB-KTN90 as a new bio sorbent is a very suitable choice for the magnetic removal of cobalt from polluted water environments.

### 12.10.2 Cadmium Bioremediation by *Pseudomonas aeruginosa*

Among the most important bacteria found in most contaminated areas is Pseudomonas aeruginosa. It is a species that can be used as a suitable biosorbent for the removal of cadmium and other heavy metals from wastewater and soil (Chellaiah 2018) (Fig. 12.7).

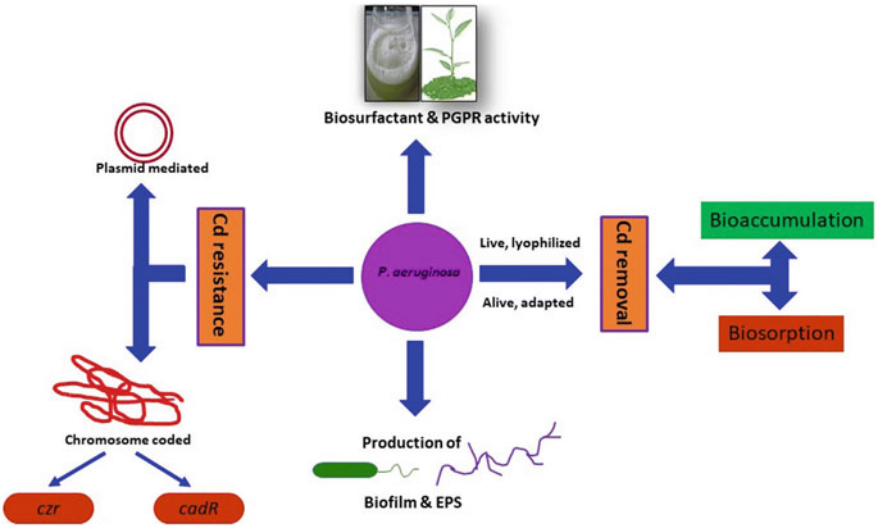


**Fig. 12.7** The relationship of cadmium bioremediation and *Pseudomonas aeruginosa* (Chellaiah 2018)

The important role of pseudomonads in the nutrient cycle and their ability to adapt quickly to contaminated areas make them preferrable for environmentally friendly studies (Chellaiah 2018).

### 12.10.3 Lead and Nickel Bioremediation by *Pseudomonas* spp.

Many experimental and statistical studies show us that it is possible to remove heavy metals from microorganisms. Removal occurs mostly by the way of attachment to the cell surface of the removal route. It has been determined that organisms exposed to the metal before are effective in this regard, and due to the rapid metal retention properties of *Pseudomonas aeruginosa* BK14 and *Pseudomonas stutzeri* BK23 isolates, heavy metals can be removed from wastewater. (Keloğlu et al. 2020).

## 12.11 Genomics–Omics–Proteomics in Bioremediation

### 12.11.1 Genomics

In addition, genomic approaches are helpful to have comprehensive information about the substance transformations performed by the microorganisms and biomolecules such as the enzymes used in this field and to reach new approaches.

For this reason, genomic approaches in bioremediation are rapidly gaining importance.

Accordingly, the discovery of 16s rRNA is a very important progress in bioremediation. With the development of this technique, 16s rRNA can be produced by partially duplicating makes that possible to fully assess microbial diversity. It is a technology that is easy to access with the help of many different bioinformatics tools, and is a high-efficiency but low-cost method. In this way, it has been widely accepted as a preferred technique for identifying various microbial communities (Chandran et al. 2020a).

DNA microarrays, which are a type of DNA dots stored or produced in two-dimensional or three-dimensional arrays on various surfaces, are also one of the widely used methods in genomics. It has been reported that DNA microarrays are used to evaluate the catabolic gene expression identity and physiology of microorganism samples taken from the environment (Schut et al. 2001; Dennis et al. 2003; Chandran et al. 2020b). Functional gene arrays (FGAs), PhyloChip arrays, and GeoChip arrays are some types of microarrays used.

### 12.11.2 Proteomics

Proteins discovered in the field of proteomic with the developing technology have been a guide for bioremediation. Metal-binding proteins (MBPs) are used for phytoremediation of industrial wastewater which is contaminated by heavy metals. MBPs are used to increase accumulation of heavy metals by microorganisms through coupling with protein synthesis. Cd-binding peptides (CdBPs), histidines 2(HP), phytochelatins, metallothioneins, and cysteines (CP) are the most widely known metal-binding proteins (Sharm 2021).

### 12.11.3 Omics

In order to bring the bioremediation process to optimum conditions, it is necessary to apply possible combinations of various complex variables. For this, molecular approaches like transcriptomics, proteomics, metabolomics, genomics, and fluxomics are applied.

With the advent of in silico analysis and NGS methods, environmental microbiologists have focused on these issues and succeeded in opening the microbial "black box" in contaminated environments (Malla et al. 2018; Maphosa et al. 2010) (Fig. 12.8).

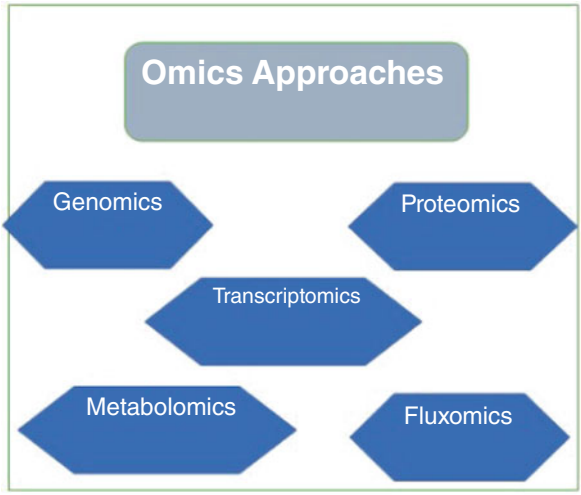


**Fig. 12.8** Omics approaches for analyses of microbial communities

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# Microbial Ecology of Wastewater Treatment Processes: Trends, Challenges, and Perspectives

13

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**Abstract**

Microbial remediation is the most promising, effective, cheapest, and environmentally friendly treatment method for biodegrading a broader range of toxic substrates and metabolites from wastewaters discharged from households, industry, and pharmaceuticals. Currently, micro-organisms must be used as decontamination tools, which in turn reduce the contaminants load of the sewage ecosystem and prevent future detrimental effects on the environment and aquatic ecosystem. Microbial consortia and sewage sludge are completely dependent on the level of water pollution. Many factors can modulate the microbial ecology from autotrophic to heterotrophic bacteria, such as titres of organic and inorganic wastes, hydrocarbons from disposal of solvents, micro-plastics, medicines, fibres, and heavy metal contaminants dissolved in sewage wastewater. The main microbial species found in 95% of polluted and sewage water are *Bacteroidetes*, *Acidobacteria*, *Escherichia coli*, coliforms, *Aeromonas hydrophila*, *Klebsiella pneumonia*, *Vibrio* sp., *Mycobacterium* sp., *Rhodobacter*, *Hyphomicrobium*, *Firmicutes*, *Nitrosomonas* sp., and *Pseudomonas* sp.. Proteobacteria (21–65%) is the dominant class of bacteria found in municipal sewage ecosystem. Beta-proteobacteria, the most abundant class of proteobacteria, also help to break down

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organic wastes and promote nutrient cycling in the ecosystem. When treating biological wastewater systems, the ecology of the microbial communities and their dynamics are always considered. Most genera of sewage decomposer belong to *Betaproteobacteria*, *Acidobacteria*, *Bacteroidetes*, and other genera of aerobic, anaerobic, and facultative microbes such as Flavobacteriaceae, Verrucomicrobiaceae, Pseudomonadaceae, and Comamonadaceae. These bacterial communities are often used in the sewage treatment plants of bioreactors or activated sludge. Molecular studies identified core microbial communities that help to design efficient activated sludge plants. Currently, advanced molecular techniques and/or approaches such as next-generation sequencing, metagenomics, and transcriptomics studies are helping us to determine the metabolic function of microbial consortia through gene sequencing to identify the abundant genera and process critical micro-organisms in activated sludge wastewater treatment systems.

**Keywords**

Microbial consortia · Heavy metal contaminants · Proteobacteria · Next-generation sequencing

## 13.1 Introduction

Due to a boost in the human population, urban expansion, and industrialization with rapidly advancing technology cause a serious threat to the environment and water ecosystem. Accumulation of different toxic contaminants in the ecosystem will in turn causes adverse effects to human health, ecology, and the environment. In developing countries, water wastes composed of 20–30% annual solid wastes. Wastewater is defined as suspended or dissolved organic and inorganic impurities over the layer of water bodies in the form of liquid or solid wastes. The incidence of water pollution is increasing throughout the past couple of decades through micro-pollutants from household products, and industrial by-products like drugs, pesticides, micro-plastics, and chemicals making nuisances within the water bodies and land ecosystems (Daughton and Ternes 1999; Grandclément et al. 2017). These wastes originated from the household and industrial by-products and accumulated in the nearby groundwater ecosystem. Therefore, a number of techniques have been proposed in the recent literature on wastewater treatment methods that can help in the management and control of water pollution through wastewater decontamination and the recycling process (Kumar et al. 2018, 2020; Agrawal et al. 2021).

Among the various wastewater treatment methods, techniques such as the physical, chemical, and microbial treatment of wastewater are continually improving over time. Some of the techniques include physical treatment of wastewater such as artificial aeration, sedimentation, water diversion, and mechanical algae removal (Zhang et al. 2010; Liu et al. 2014) and chemical treatment of wastewater through

chemical oxidation, precipitation, flocculation, adsorption, chelation, and algae removal with chemicals were used (Wu et al. 2018).

Microbial degradation of waste products is the most popular, natural, and standard technique used in the biological remediation process for both terrestrial and aquatic ecosystems. The use of living micro-organisms as a bioremediation technique to break down suspended solids from wastewater was first used by George M. Robinson (Vidali et al. 2002). The microbial degradation of various organic and inorganic wastes takes place with the help of the metabolic activities of various microbial genera (Kumar and Chandra 2018; Kumar and Shah 2021). Water pollution can be controlled through the use of microbial degradation techniques in combination with the degradation activities of various microbial consortia of Betaproteobacteria, Acidobacteria, Bacteroidetes, and other related genera with the help of various biological treatment plants for wastewater recycling. When treating biological wastewater, the ecology of the microbial communities and their dynamics are always taken into account in order to detect the titres of the environmental contaminants' load in particular groundwater and sewage ecosystems.

## 13.2 Composition of Contaminants in Sewage Wastewater

Municipal wastewater consists primarily of 99.9% water with 0.1% suspended contaminants. The current environmental threat to the freshwater and aquatic ecosystem is eutrophication. Most wastewater by-products come from various industrial sewage treatment plants and household products, which consist of organic substances such as proteins, complex carbohydrates, long unsaturated fatty acids, oils, nitrogen, phosphorus, heavy metals, pesticides, various pharmaceutical products, and micro-plastics (Chowdhury et al. 2016; Eerkes-Medrano et al. 2019). A heavy load of nitrogenous compounds, heavy metals, and organic compounds leads to the development of algal blooms above the surface of the water, and some of these inorganic contaminants are non-biodegradable and accumulate in tissues of various living beings (Grizzetti et al. 2012). Traditional water treatment plants are not well equipped to break down the new and emerging pollutants that result from complex wastewater production and hazardous by-products of industry (Norvill et al. 2016). There is, therefore, an urgent need to develop more environmentally friendly methods for water treatment. Some of the most commonly found organic and inorganic pollutants are discussed below.

### 13.2.1 Inorganic Contaminants

Most of the inorganic contaminants many times show high concentrations of toxic metals beyond the recommended limit for drinking water. These inorganic contaminants include dissolved chloride, sulfate, nitrate, nitrite, ammonia, cadmium, lead, mercury, arsenic, phosphate, carbonate, calcium, magnesium, potassium, and various types of nutrients and salts, which generally exist in the form of dissolved

cations and anions in wastewater composing total dissolved solids (TDS) (Ehrlich et al. 1997; Nickson et al. 2000)), including large-scale discharge of traces of antibiotics like ciprofloxacin, erythromycin, trimethoprim, sulfapyridine, and norfloxacin in all the food, faeces, pharmaceuticals flush, and wastewater supplies (Chen et al. 2018). Heavy metal impurities are not biodegradable and therefore increase their chances of accumulating in the living body. These are copper, nickel, zinc, cadmium, mercury, lead and arsenic, barium, beryllium, selenium as well as some of the aromatic compounds and hydrocarbons from the disposal of solvents, micro-plastics, medicines, fibres (cotton swabs, hair, hygiene articles, faeces), oil, soap, grease, and hazardous substances (Lim et al. 2010; Kumar et al. 2012). The accumulation of higher concentrations of these critical pollutants in domestic and sewer bodies causes environmental pollution and a public health crisis. Most of the water polluted with the traces elements like barium, beryllium, selenium, arsenic, and cyanide are responsible for different health crises related to cardiovascular, liver, lung, and bone diseases due to high metal load in drinking water (Wones et al. 1990; Cooper and Harrison 2009). These metal contaminants are also adversely affecting the recycling of bio-solids and chemical waste from contaminated sewage.

### 13.2.2 Organic Contaminants

Emerging organic contaminants that are produced by various industrial chemical reactions such as oxidation, reduction, hydrolysis and their by-products such as volatile organic chemicals (VOCs) include solvents and organic chemicals such as bisphenols, plasticizers/resins, methyl tertiary butyl ether, trichlorethylene (TCE), styrene, benzene, toluene, and vinyl chloride. Some of the organic industrial compounds such as petroleum hydrocarbons, gasoline additives, adhesives, degreasers, fragrances, and fuel additives are included (Pal et al. 2014). Many studies are based on the quality of shallow groundwater, which is heavily contaminated with perfluorooctanoic acid (PFOA), which makes up 80% of all PFAAs found in groundwater. Some of the contaminants of pharmaceutical origin, including carbamazepine, N,N-diethyl-meta-toluamide, sulfamethoxazole, phthalates, and so on, were widespread in untreated groundwater samples for irrigation (Lesser et al. 2018).

## 13.3 Microbial Diversity of Sewage Water

The microbial diversity in wastewater consists of different types of micro-organisms including algae, fungi, bacteria, protozoa, etc. (Cai and Zhang 2013). Bacterial genera make up 90–95% of total wastewater communities. Microbial genera such as Proteobacteria (25–45%) are the dominant strains present in the sewage water as in the order of alpha-proteobacteria, followed by beta-proteobacteria and gamma-proteobacteria (Tanaka et al. 2012; Zhang and Shao 2013). Besides, other dominant groups of sewage water ecosystems are Bacteroidetes (20–40%), Chloroflexi (3–17%), and Acidobacteria (2–15%), and major human bacterial pathogens like

enteric pathogens *Klebsiella* sp., *Vibrio* sp., *Shigella* sp., *Salmonella* sp., and *Escherichia coli* cause gastrointestinal infections, while other related species of bacteria like *Mycobacterium* sp. and *Pseudomonas* sp. are opportunistic bacteria which cause respiratory diseases and immune-suppressive diseases and are the most common inhabitants of wastewater (Cai and Zhang 2013; Anastasi et al. 2012; Levy et al. 2010). Some of the commonly reported bacterial strains in wastewater are listed in Table 13.1.

## 13.4 Wastewater Treatment Methods

There are many qualitative and quantitative methods of monitoring water quality from different supply sources through different biological treatment plants (Fig. 13.1). Test methods and parameters can be divided into three categories.

### 13.4.1 Physical Tests

These tests comprise the water properties judge on the basis of its colour, odour, taste, and turbidity. Change in colour of water is due to the presence of algae, vegetables, weeds, manganese and iron, and other mineral oils. Change in odour and taste of water is due to the presence of decaying organic matter including weeds, algae, and industrial wastes containing ammonia, heavy metals, phenol, long-chain fatty acids, and other hydrocarbons and foul odour is also due to the heavy growth of micro-organisms over the surface of the water. The presence of turbidity in sewage water is due to the presence of suspended solids, colloidal wastes, and soil erosion. High turbidity makes filtration expensive.

### 13.4.2 Chemical Tests

Chemical measurement of the water quality can be analysed through the detection of pH, biocides, toxic chemicals, and heavy metals, and total dissolved biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Measurement of the pH of water for calculating relative acidity and alkalinity of water drinking water must have acidity and alkalinity range between 6.5 and 8.5. In the marine ecosystem, pH values below 4 do not support the growth of living organisms while low pH values help in effective chlorination. Treated wastewater through chlorination typically has higher concentrations of particles (1–10 NTU for secondary treated wastewater). High BOD means low oxygen concentration to support life and indicates high organic pollution.

**Table 13.1** Microbial communities of sewage water

| Microbial genera | Function | Pathogenicity | References |
|---|---|---|---|
| *Proteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* | Dominant phylum of sewage water | Commensal of sewage water plants comprising subdominant abundant groups *Firmicutes, Bacteroidetes, Actinobacteria, and Chloroflexi* | Huang et al. (2018) |
| *Bacteroides* | Common inhabitant of sewage water. Ferment carbohydrates that result in the production of a pool of volatile fatty acids | The fimbriae and agglutinins of *B. fragilis* function as adhesins; the capsular polysaccharide, LPS, and numerous histolytic enzymes are the most important virulence determinants in the bacteria | Wexler (2007) |
| *Enterococcus* | Enterococcus species used as probiotics or in the food industry or as starter cultures enterococci have become nosocomial pathogens causing bacteraemia | Virulence factors include the extracellular protein Esp and aggregation substances (Agg), both of which help in the colonization of the host | Fisher and Phillips (2009) |
| *Faecalibacterium* | An anti-inflammatory commensal bacterium of sewage water | Non-pathogenic, in turn, have a potentially important role in promoting gut health used as a promising probiotic | Sokol et al. (2008) |
| *Acetoanaerobium* | Anaerobic bacteria that produce acetate from $H_2$ and $CO_2$ | Their biodegradation efficiency in wastewater reported | Rainey (2015) |
| *Aquabacterium* | *Aquabacterium parvum* B6, nitrate-dependent Fe (II)-oxidizing bacteria; helps in the improvement of biological nitrogen removal in an up-flow bioreactor for wastewater treatment | *Aquabacterium commune* commonly found in drinking water biofilms | Zhang et al. (2016) |
| *Candidatus Nitrotoga* | Nitrite-oxidizing bacteria (NOB); metabolize nitrite to nitrate, which is removed via assimilation and denitrification processes | Play a key role in contaminants from freshwater | Boddicker and Mosier (2018) |
| *Streptococcus* | Faecal streptococcus is used as the best indicator organism in organic waste | Some species are potentially pathogenic, and in *streptococcus pseudopneumonia* the *ply* gene plays a role in pathogenicity | Jepsen et al. (1997) |

(continued)

**Table 13.1** (continued)

| Microbial genera | Function | Pathogenicity | References |
|---|---|---|---|
| *Subdoligranulum* | Non-pathogenic sewage commensals | Used as probiotic and associated with improved metabolic health | Van hul et al. (2020) |
| *Chryseobacterium* | Phosphate solubilization, plant growth promotion | No pathogenicity but suppresses *Phytophthora* blight | Singh et al. (2013) |
| Comamonadaceae | Widely used in degradation and remediation of wastewater contaminants | Most genera are not regarded as pathogenic | Gumaelius et al. (2001) |
| *Comamonas* | *Comamonas* spp. are capable of assimilatory and dissimilatory nitrate reduction and are considered as denitrifying bacteria. The resultant nitrite can be further converted to ammonia by nitrite reductase (*Nir*) producing microbes | The virulence factors comprise several genes responsible for bacterial motility and adherence | Wu et al. (2018) |
| *Dechloromonas* | Denitrifying bacteria; help in enhanced nitric oxide production under salt or alkaline stress conditions, also able to reduce benzene, perchlorate, and oxidize chlorobenzoate, toluene, and xylene | Non-pathogenic and this organism is used for bioremediation | Salinero et al. (2009) |
| *Geothrix* | Sulphur and Fe-oxidizing autotrophic denitrifying bacteria | Non-pathogenic but used for remediation of nitrate polluted effluent | Zhang et al. (2019) |
| *Nitrosomonas* | Nitrifying bacteria; help in providing nitrogen to plants and limiting carbon dioxide fixation. It converts ammonium ions or ammonia into nitrites | Non-pathogenic | Arp et al. (2002) |
| *Nitrospira* | Nitrite-oxidizing bacteria; help in the conversion/ oxidation of nitrite to nitrate | Non-pathogenic | Daims and Wagner (2018) |
| *OM27 clade* | Non-culturable bacteria, high coral coverage on reefs due to abundances of the OM27 clade | Non-pathogenic | Apprill et al. (2021) |

(continued)

**Table 13.1** (continued)

| Microbial genera | Function | Pathogenicity | References |
|---|---|---|---|
| *Proteocatella rhodoferax* | Ruminal hydrolytic bacteria; help in the hydrolysis of microalgal biomass | – | Carrillo-Reyes and Buitr (2017) |
| *Simplicispira* | Gram-negative bacteria found in activated sludge that help in phosphorus removal | Non-pathogenic, novel denitrifying bacteria found in sludge | Lu et al. (2007) |

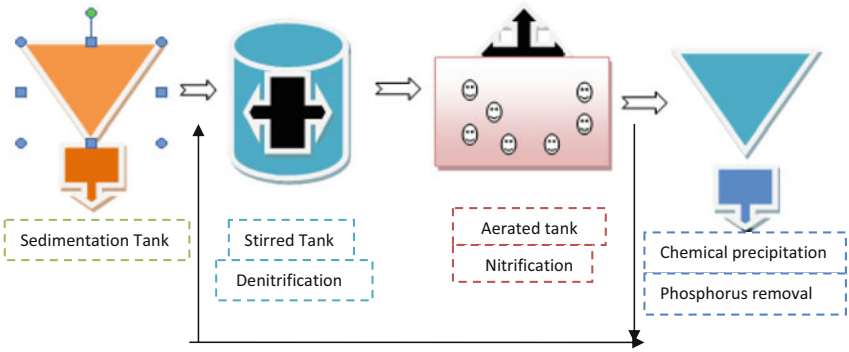


**Fig. 13.1** Tertiary treatment system of wastewater

### 13.4.3 Microbial Tests

Bacteriological analysis helps to study the faecal load and microbial load in a particular water sample using established culture methods. Although it is possible to detect most of the pathogenic microbial contaminants by counting the total bacteria, Coliforms, *E. coli, Salmonella* sp., *Pseudomonas* sp., etc. through different culture tests. Conversely, the lack of faecal commensals suggests that pathogens are also likely to be absent. Using normal intestinal bacteria such as *E. coli* have been used as a bioindicator of faecal burden, it is a well-established principle for monitoring and evaluating the microbial safety of water supplies. Some of the well-known culture methods used for the testing of water quality index are listed below.

#### 13.4.3.1 Direct Plate Count

This method involves plating the drinking or wastewater directly into the nutrient agar or VRBA Agar plate to count different microbial colonies by diluting the original sample so that the colonies are between 30 and 300 per plate of inoculums volume. Typical media include MacConkey agar to count Gram-negative bacteria such as *E. coli* or plate count agar for a general count at 37 °C for 24 h (Gilchrist et al. 1977).

#### 13.4.3.2 Multiple Tube or IMViC Method

It is a group of individual tests (indole, methyl red, Voges–Proskauer, and citrate) known as IMViC that are used to count total coliforms in wastewater or drinking water. The identification of 87 species representing 7 genera in the Enterobacteriaceae family was completed with a typical IMViC test within 48 h after incubation of the culture tubes (Barry et al. 1970).

#### 13.4.3.3 ATP Testing

This test is also known as adenosine triphosphate test, used for the detection of active micro-organisms in water. ATP is released by living cells which can be measured directly by its reaction with the naturally occurring enzyme fireflyluciferase using a luminometer. The amount of light produced is directly proportional to the number of living micro-organisms present in the water samples (Birmele et al. 2010).

#### 13.4.3.4 Membrane Filtration

This method is similar to the conventional plate count method, where membrane vacuum filters are used and these filters are placed on presterilized nutrient medium or Endo Agar within sealed plates. These filters have a millimetre grid printed on them and can be reliably used to count the number of colonies under a microscope (International Organization for Standardization 2000).

## 13.5 Types of Bioreactors Used for Wastewater Treatment

The treatment, purification, and decomposition of the sewage water are carried out with the help of various bioreactors and water treatment systems such as anaerobic sequencing batch bioreactors, fluidized bed bioreactors, bio-augmentation, membrane bioreactors, and activated sludge treatment systems. These biological sewage treatment systems are used for the decomposition of the various household and industrial by-products such as organic and inorganic waste, waste containing sulfur, phosphorus, and nitrogen, heavy metals and other toxic elements, heavy metal pollution of the sewage ecosystem. The type of sewage treatment plant used depends on the composition of the waste products present in the wastewater bodies. The biological removal of water waste (BOD) involves the use of anaerobic processes reactor (Anaerobic Expanded Bed Reactor; AEBR). The second type of anaerobic reactor system is a contact anaerobic process which includes an anaerobic fluidized bed reactor (ANFLOW), an anaerobic up-flow sludge blanket (UASB), an anaerobic sequencing batch reactor (ASBR), etc. However, for the removal of BOD along with the nitrification process biolac-aerated lagoon, optional aerated lagoon, sequencing batch reactor (SBR), cyclic activated sludge system (CASS), etc. are widely used bioreactors. These types of bioreactors are used for selectively performed nitrification, denitrification, ammonification, phosphorus and sulphur removal.

## 13.6 Structure and Function of Microbial Communities in Activated Sludge and Wastewater Treatment Plants

Characterization of microbial diversity and their community structure in the wastewater treatment plants was helpful in order to set up the microbial composition and operational activities of the different bioreactors. Microbial diversities in the treatment plants have been used to set the flocculation, sludge bulking, foaming process of activated sludge plants as bioreactor operational settings. The most commonly reported microbial phylum in the sewage water plants is Proteobacteria, Betaproteobacteria, Acidobacteria, and Bacteroidetes (Meerbergen et al. 2016). These bacterial genera show very high efficiency in the removal of COD (chemical oxygen demand) along with the decomposition of various organic contaminants. Microbial species contain various organic acids, enzymes, antioxidants, and metallic chelates (Freitag and Meihoefer 2000), and these secondary metabolites help in the oxidation of the sulphur, phosphorus, ammonia, nitrogenous wastes containing compounds (Liu et al. 2014). *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* these bacterial strains are able to form biofilms and remove various toxic elements, oil and metal contaminants from waste water treatment plants (Rice et al. 2007; Branda et al. 2006; Zhao et al. 2006). Similarly, cyanobacteria, photosynthetic bacteria found in water bodies, act as an indicator of water pollution, and *Pseudomonas* sp. is able to degrade various polymeric substrates and micropolythenes. Acinetobacter and Arcobacter were dominant genera in sewage treatment plants (Marti et al. 2013). The structures of the algae and bacteria symbiosis are adapted for wastewater treatment.

Another dominant genus in the sewage treatment plants are the *Flavobacteriaceae*, *Verrucomicrobiaceae*, *Pseudomonadaceae*, and *Comamonadaceae*.

These types of bacteria are often used in the sewage treatment plants of bioreactors or activated sludge. The most numerous types of bacteria that are used to treat dissolved organic pollutants belong to genera *Tetrasphaera*, *Trichococcus*, *Candidatus*, *Microthrix*, *Rhodoferax*, *Rhodobacter*, and *Hyphomicrobium*, followed by the archaeobacteria with the Euryarcheota (McIllroy et al. 2015). The process of adsorption followed by degradation was the main functional unit of activated sludge carried out by the microbial species. The growth and activities of these dominant bacterial genera increased from day 1 to day 12, reached the maximum operational taxonomic unit (OTU) number, then decreased slightly, and finally stabilized (Shchegolkova et al. 2016). While some of the associated bacterial genera such as Pseudomonadaceae, Streptococcaceae, and Enterobacteriaceae show increased growth and turbidity from day 1 to day 9, it then gradually decreases and increases as on days 13 and 15 of the incubation time in the bioreactor and then stabilizes. Most sewage treatment plants avoid the use of filamentous bacteria because of their excessive growth behaviour, which leads to foaming and flocculation disorders in the operation of sewage reactors. Hence, the estimation of the microbial community structure in wastewater is by the next-generation sequencing will be the most important parameter of any of the bioremediation treatment facilities.

## 13.7 Molecular Techniques for Next-Generation Wastewater Management

The most intensely explored areas of current research in the field of wastewater treatment are the use of genetically modified micro-organisms and recombinant DNA technology to treat three main groups of wastewater pollutants, namely persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), pentachlorobiphenyls (PCBs), and pesticides. Evaluation of the microbial community structure living in the wastewater ecosystem becomes easier with the help of cutting-edge technologies such as 16S rRNA sequencing, fluorescence in situ hybridization, and gene clone library which in turn assess the diversity of the microbial population due to high levels of contamination and toxicity of environmental pollutants. Therefore, different communities of the bacterial population in the sewage treatment plants can be analysed by 16s rRNA gene sequencing (Guo and Zhang 2012). In order to measure the microbial diversity in the serial passage electro-bioreactor, DNA amplicons were produced after the isolation of the entire genomic DNA from sewage treatment plants. The identification of microbial alpha (α) and beta (β) diversity analyses were performed using statistical tools called QIIMETM (version 1.9.1) (Kuczynski et al. 2011). Likewise, another advanced statistical tool for identifying microbial community structure and genome sequencing called PICRUSt (phylogenetic study of communities through reconstruction of unobserved states) was used together with KEGG (Kyoto Encyclopedia of Genes and Genomes) to identify bacterial communities in wastewater and determine their functionality through dominant OTU gene sequences and another microbial database called the Ribosomal Database Project (RDP) classifier that is used to identify the pathogenic genera that inhabit the sewage treatment plant as microbial communities.

## 13.8 Contribution of Nanotechnology in Wastewater Decomposition

Nanotechnology is the rapidly developing and environmentally friendly process that can be used instead of conventional technologies for water treatment. Manufacture of nanoparticles as a by-product of green chemistry, which leads to less dangerous chemical production when decontaminating water samples or wastewater. Nano-agglomerates from mixed oxides such as iron–titanium, iron–zirconium, iron–manganese, iron–cerium, and other amalgams have been synthesized and successfully used to remove and purify water contaminants. Just like the Nao particles, nanoceramic filters are used to remove viruses and bacteria and to chemically absorb the dissolved heavy metals from the wastewater. These nano-ceramic filters consist of nano-alumina fibres and micro-glass with a high positive charge (Shah and Ahmed 2011). Nowadays use of filtration membranes with hollow tubes along with carbon nanotubes will effectively remove heavy metals and bacteria such as *E. coli* and *Staphylococcus aureus* from contaminated water. Another bioremediation technology called permeable barrier reactor (PBR) helps in the remediation of

organic and inorganic contaminants from biological groundwater. Nanoscale zero-valent iron is a mixture of Fe(II) and Fe(III) oxide with a particle size of 10–100 nm. The nanoparticles help destroy polychlorinated hydrocarbons, arsenates, selenates, pesticides, and dyes (Yukti et al. 2020).

## 13.9 Challenges and Future Outlook

The bioremediation of wastewater through microbial degradation has been the most effective, inexpensive, and environmentally friendly method to date. The biological treatment of sewage treatment plants is a necessary prerequisite for the ecological biological rehabilitation of wastewater bodies. An increasing concentration of toxic metabolites in wastewater can further deteriorate the quality of the groundwater. Various microbial consortia of Betaproteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, *Tetrasphaera*, *Trichococcus*, etc. are used in wastewater treatment, which can help reduce the overall load of organic, inorganic, and heavy metals. The optimization of different microbial consortia and culture parameters leads to an increase in the efficiency of sewage treatment plants and a greater reduction in environmental pollutants from wastewater. Nowadays, various molecular and next-generation sequencing technologies are used to identify the microbial composition of sewage treatment plants, which can be helpful for the future design and modulation of the microbial composition of the different sewage treatment plants.

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# Treatment, Recycling, and Reuse of Wastewater from Tannery Industry: Recent Trends, Challenges, and Opportunities

14

Preeti Chaurasia and Sanjeev Kumar

**Abstract**

Leather industry is one of the greatest economic sectors known as well as one of the highly polluting industries as it generates intolerable solid and liquid wastes. Major pollutants in tannery effluents are sulfides, sulfates, chlorides, tannins, and heavy metals. Hence, tannery effluents are very toxic and have an adverse effect on agricultural lands and water sources. In order to minimize the toxicity, use of microbes to treat industrial effluents has always been an eco-compatible and cheaper method. There are many conventional physio-chemical treatment methods to bring down the influence of discharged effluent on the living but its high operational cost and execution setup are the major drawbacks. In addition to toxic chemicals, several heavy metals are also present in tannery effluents, and are not easily digested by conventional techniques. The present chapter focusses on the use of microbes with potential to degrade noxious compounds contaminating groundwater and soil. The potential microorganisms having degrading property could be isolated and mass multiplied under lab conditions. Potential microbes will selected from effluent affected sites and may further use for the suistanable agricultural practices. Bioremediation and phytoremediation are proven to be effective and is considered as a novel, cost-effective method for the complete removal of toxic chemicals, heavy metals, and dyes originating from tanneries. The treated wastewater can be recycled for irrigation and in industries.

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**Keywords**

Tannery · Wastewater · Toxic effect · Bioremediation · Phytoremediation · Recycling

## 14.1 Introduction

Tanneries are one of the most pollution-emitting industries that are cause for the emergence of effluents with organic as well as inorganic dissolved and suspended solids content. These industries are often responsible for the discharge of toxic elements along with the wastewater into the ecosystem beyond the permissible limit. This increase of toxicity in the effluent is directly discharged into the running water bodies that affects living beings and the environment. Therefore, it has become important for minimizing the major impact of discharged wastewater that affects the lives of living creatures in the environment. As the population is increasing, urbanization is also showing its growth. To achieve the demand of population, industrialization is also at its peak which ultimately gives rise to the detrimental effect on our environment. The leather industry fulfils the demand for footwear, musical instruments, leather items, employment generation, etc. and is one of the leading industries of the world (Durai and Rajasimman 2011). Tanneries consume a large amount of water and in return release the same amount in the form of wastewater after processing. Thus, it is one of the leading contributors to environmental pollution and is a global concern for biodiversity (Kundu et al. 2014; Kumar and Thakur 2020). Because of the inappropriate treatment of the discharged effluent, it is beyond the permissible limit. Tanning is a leather manufacturing process by the treatment of various chemicals on animal skin and hides. During this transformation, a highly coloured, cloudy, and stinky effluent is generated (Dargo and Adhena 2014). The dumped effluent affects the area in their vicinities such as water bodies, adjacent land, and groundwater, and is unfit for irrigation as well as for drinking purposes, imposes adverse effects on the consumers of each trophic level (Ramachandran et al. 2013). The discharged effluent consists of chromium salts, sulphides, inorganic salts, organic salts, chlorides, tannins, heavy metals, dissolved solids, suspended solids, nitrogenous compounds, pentachlorophenol, and gaseous wastes as well (Masood and Malik 2011). Tanneries use tanning agents such as basic chromium sulphate [Cr (III)] in the "chrome liquor" for making leather soft, lightweight, and heat/water-resistant. But chromium (Cr) is a harmful pollutant. Among several forms of Cr, trivalent and hexavalent forms are biologically stable and hexavalent Cr is more soluble hence more harmful than trivalent. Another pollutant that is used for the preservation and treating of leather is pentachlorophenol (PCP), an aromatic compound. It is used as a biocide and also recalcitrant to biological degradation if accumulated in food chains can be the cause of human health issues (Verma et al. 2019).

That is why it is very important to clean tannery wastewater before discarding it into receiving water bodies and land as it can be the cause of pollution. An intensive attempt has been made to treat the effluent discharged. To meet the challenges,

tanneries use conventional techniques to treat the effluent before discarding it to the surrounding environment. Conventional treatment methods require humongous operational cost; require a large area, energy, chemicals, and labour; and also cause further environmental damage by producing a large amount of sludge. So tannery effluents should be treated more precisely before it affects flora and fauna otherwise it will enter the food chain. Therefore, an alternative that is eco-friendly and cost-effective wastewater treatment techniques would be significant. Bioremediation is an emanating technique for the cleaning of noxious pollutants present in the effluent. A holistic approach should be needed to reuse and recycle wastewater for the sustainable development of ecosystem. A multidisciplinary approach not only reduces the scarcity of water but also same time minimize the unfavourable effect of noxious pollutants on the environment and on health. The present chapter focusses on the characteristics of tannery effluents, their detrimental effect, and also its effective remediation methods before its release into the environment.

## 14.2 Characteristics of Tannery Wastewater

Tanning is a chemical process of leather manufacturing that involves the use of different chemicals on animal skin and hides. There are four main steps for the production of leather: (a) beam house, (b) tanyard, (c) post tanning, and (d) finishing operations (Durai and Rajasimman 2011). The process of leather manufacturing and features of wastewater produced is shown in Fig. 14.1. The quality of tannery discharge depends upon the chemicals used, industry size, tanning operations, and amount of water used in the different processes in tanning. The pollution parameters of tannery effluents can be characterized by biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), dissolved solids, heavy metals like chromium, mercury, iron, zinc, copper, cadmium, lead, sulphates, sulphides, and nitrates (Buljan et al. 2011). The pH of the effluent generally ranges from 7.5 to 10 as carbonate, bicarbonate, and hydroxides salts present in tannery effluents give it an alkaline nature and eventually affect the water ecosystem (Kongjao et al. 2008). Moreover, during the production process, effluents contain lime, toxic chemicals, halides, sulphates, ammonia, heavy metals, suspended solids, dissolved salts, oil, grease, and wastewater sludge. This also leads to increasing eutrophication in water bodies. These pollutants are toxic and also carcinogenic to humans as well (Tamburlini et al. 2002; WHO 2002). Because of high contamination, this water is not safe for drinking purposes. The dark-coloured water is itself a pollution indicator and the expulsion of coloured effluents can severely damage the water ecosystem by obstructing light penetration. Further, the addition of salt as preservatives on the skin and organic matter in effluents increase the TDS value with the increase in BOD and COD levels, which decreases the value of dissolved oxygen (DO). Along with the wastewater, solid waste is also generated during leather processing as well as sludge generated during effluent treatment. In leather processing, approximately 85% of mass is generated as solid waste. These solid

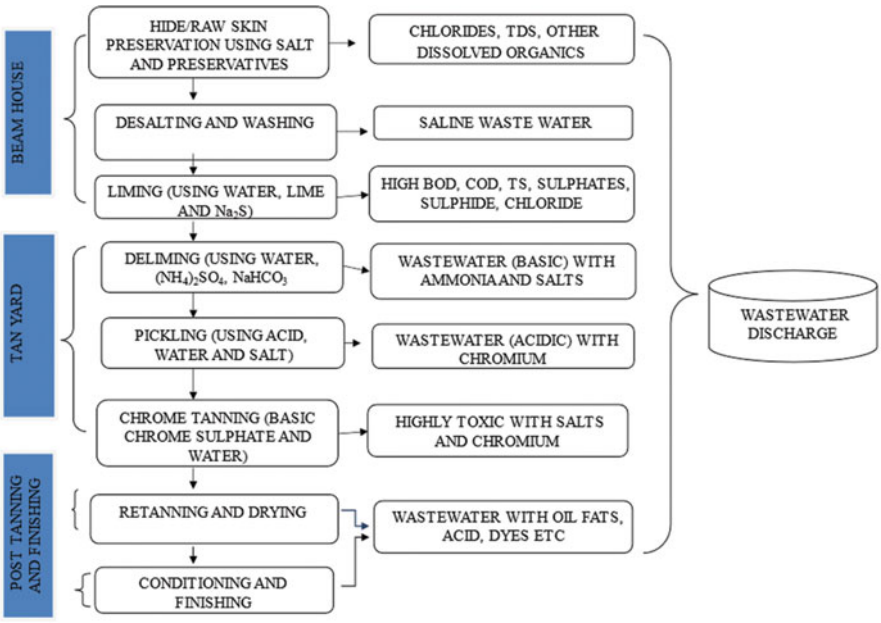


**Fig. 14.1** Steps in leather production process and wastewater generated

wastes are noxious, creating a secondary problem. Furthermore, discharge from leather industry corrupts the soil and groundwater, if not treated (Chung et al. 2004).

A high level of sulphate in the discharged effluent triggers sulphate-reducing bacteria to grow, which in turn produces $H_2S$ gas. $H_2S$ is harmful and lethal too. Delay in disposal and decomposition of solid wastes and wastewater also produces bad odour.

## 14.3 Effect of Tannery Effluents

Tannery industries use various dyes and form dye-based recalcitrant pollutants that affects the ecosystem. Both the biotic and abiotic factors get seriously affected. If the effluent is dumped directly into the water body can be more harmful and cause skin allergies and other diseases. Aquatic plants and animals get affected. Many inorganic metals like Cu, Mg, Ni, Mn, V, $Cr^{3+}$, and Mo present in the wastewater are necessary for metabolic functions but only in trace amounts; some heavy metals such as Pb and Cr (VI) do not play an important role biologically and are noxious for biota. Many humans and animals are dependent on aquatic food so when aquatic life forms on exposure to the dissolved heavy metals such as chromium and harmful pollutants of tanneries, which can lead to the bioaccumulation of heavy metals and so on in the tissues of organisms (Aung et al. 2013). Therefore, it results in biomagnification by entering the food chain and food web.

### 14.3.1 Impact on Humans

Tannery discharge contains metal pollutants that promote diseases to those near the polluted area. Exposure to these contaminants imposes ill effects on skin and mental health too. Mercury, arsenic, and lead can lead to weakness, diarrhoea, anaemia, headaches, brain damage, kidney failure etc. Exposure to mercury can damage the brain and kidneys of developing foetuses permanently (Engwa et al. 2019). Heavy metals in higher concentration can cause headache, anxiety, irritability, abdominal cramps and effects the function of the nervous system, bladder, brain, liver, and kidney. Compounds with hexavalent chromium ions are more soluble in water and can move across biological membranes and interact with intracellular proteins and nucleic acids. Cr (VI) is extremely carcinogenic and mutagenic and can lead to death if infested. It is recommended to remove chromium before being dumped as it can result in oxidative DNA damage which can cause genotoxicity of Cr. As chromate shares a structural similarity with sulphate $(SO_4)^{2-}$, so it can cross the membrane through sulphate transport pathway. This can badly affect human health by causing lung cancer, dermatitis, vertigo, nausea, kidney damage, chronic liver damage, and respiratory infection. Cr also causes ulceration and perforation of the nasal septum, skin lesions, and damage to the respiratory tract (Kumar et al. 2021a, b). PCP also causes many diseases like dermatitis, chronic fatigue, impaired fertility, conjunctivitis lung cancer, intravascular haemolysis, neurological disorder, pulmonary oedema, pancreatitis, liver damage, and kidney failure (Sharma et al. 2009).

### 14.3.2 Impact on Plants

As well-known types of irrigation water affect crop yields and productivity. However, contaminated wastewater from tanneries on disposal with the pollutants into cultivable lands drastically reduced yield and growth of crops. Total suspended solids in effluents makes water become more turbid and lead to poor photosynthetic activity. Plants can be also affected directly and indirectly by the concentration of heavy metals in soils. These noxious metals and chemicals when enter plants thereby alters physiological, genetic, and biochemical functions of the system (Dazy et al. 2008). Greater concentration of heavy metals suppress growth, induce chlorosis, epinasty, poor photosynthetic activity, chromatin condensation, necrosis, and reduced water potential during the vegetative and flowering stage of crops. Higher concentrations of arsenic interrupts the generation of ATP and also oxidative phosphorylation. Lead causes alteration in the permeability of the cell membrane. This can weaken seed germination, transpiration, cell division, root system elongation, etc. Cadmium changes the enzymatic activity involved in the Calvin cycle, carbohydrate metabolism, and carbon dioxide fixation, inhibiting photosynthesis and resulting in a short plant (Gill and Tuteja 2011). Cd toxicity may also alter metabolism in plants which can lead to inhibition of pollen germination, delay in

germination, etc. Ni stress affects photosynthetic pigments and reduces the yield in plants (Kumar et al. 2021a, b).

### 14.3.3 Impact on Microbes

Microbes can use chemical substances as their energy source thereby can degrade naturally as well as synthetic substances in our ecosystem. That's how microbes can reduce the level of pollutants (Pavel and Gavrilescu 2008). Hence this method of microbial degradation of industrial dyes is cost-effective and efficient method (Kumar et al. 2016). But, heavy metals in soil could significantly reduce microbial activity and biodiversity. Soil contaminated with heavy metal showed adverse effects on soil respiration rate, reduced enzymatic activity, the transformation of organic matter, and altering soil pH (Wu et al. 2019). Disposal of sludge adds heavy metals to the soil and negatively affects the growth and reduces the microbial population. These metals in the soil ecosystem create selection pressure that supports specific microbes which can tolerate or develop resistance to the exposed heavy metal. Some microbes have proved themselves as significant and most promising in the bioremediation of heavy metals.

## 14.4 Approaches Towards Industrial Wastewater Treatment

Treatment of wastewater of tanneries is a very challenging task as it contains a high concentration of contaminants and wastes. So, it is very important to cure the effluent before discarding it. The pollutants present gets transformed into a simple and degradable form so it can be disposed of safely without causing any menace to the ecosystem (Kumar et al. 2021a, b). Tannery wastewater is treated through a series of steps.

### 14.4.1 Primary Treatment (Physio-Chemical Treatment)

#### 14.4.1.1 Physical Treatment

The very first treatment step is also known as a mechanical treatment, where raw effluent is screened to remove coarse matter. Grease, sand, oils, fats in the raw waste can be removed by screening. Additionally, it also reduces the chrome and sulphides from the effluent by homogenization and sulphide oxidation.

#### 14.4.1.2 Chemical Treatment

##### 14.4.1.2.1 Coagulation and Flocculation

Chemicals like industrial alum, iron sulphate, iron chloride, and lime which act as coagulants are added to remove colloidal matter. Lime is used as the base to neutralize the acidic effluent (Dargo and Adhena 2014). Coagulants and flocculants

being water-soluble polyelectrolyte help to form clumps of very minute and colloidal particles. For coagulation, a coagulant is mixed rapidly, and then undergoes flocculation process in which particle size is increased by gentle mixing. Size is transformed from clumps to visible suspended solids forming large macroflocs. Mixing velocity and energy should be watched to prevent the destabilization of macroflocs (Ukiwe et al. 2014). Thus, suspended solids such as girt, scum, fatty and nonfatty particles which are still left, settle faster and then can be removed from water by sedimentation. Scum is removed from the top and sludge from the bottom of settling tanks. The disadvantage of this method is the formation of sludge. Sludge is then dried by using sludge thickeners, mechanical dewatering using centrifuges, natural drying.

#### 14.4.1.2.2 Chemical Oxidation

It is a newer technology in which oxidizing agents are used. Ozone, potassium permanganate, and hydrogen peroxide are some oxidising agents which are used to degrade organic pollutants to a manageable level but this method exhibits a lower rate of degradation. In the chemical oxidation process, it is very difficult to remove total organic carbon.

Ozone also acts as a decolourising agent. Ozone is unstable and readily reacts (Dargo and Adhena 2014). Activated carbon as a catalyst enhances sulphide oxidation which is found to be more significant to remove sulphide from wastewater and also lowers COD, BOD, and total organic carbon (TOC) from wastewater.

#### 14.4.1.2.3 Advanced Oxidation Process

AOPs involve technologies such as photocatalysis, Fenton, photo-Fenton, and wet oxidation in which different hydroxyl radicals are used to react for the tannery discharge treatment. Photo (solar)-Fenton process is the most efficient technology which accelerates oxidation of organic compounds in tannery effluents, but it is a much expensive process.

#### 14.4.1.2.4 Ion Exchange

The process involves the replacement of metal ions of a species with an ion of different species attached to an insoluble resin present in the solution. The drawback of this process is that the equipment used is costly due to high operational cost and cannot remove chromium completely. It also produces chemicals not suitable for the environment.

#### 14.4.1.2.5 Reverse Osmosis

In reverse osmosis (RO) heavy metals are removed with the help of semi-permeable membrane. Aromatic polyamide and cellulose acetate are the most commonly used membranes. In this process, the solution is passed with a greater osmotic pressure through a region of higher concentration to a diluted region through a semi-permeable membrane. Earlier this process was used to treat brackish water and for desalination. Nowadays RO is used in wastewater treatment also. But the

disadvantage of this process is its high operational cost. Also, membrane leakage and membrane fouling are the other problems of this method (Ukiwe et al. 2014).

#### 14.4.1.2.6 Electrochemical Treatment

Electrochemical treatment process is an alternative to the conventional coagulation and flocculation processes. This technique involves the use of different electrodes with varying electrolytic property giving oxidation and reduction reactions to remove sulfides, nitrogen, phosphorus, and many toxic heavy metals (Mook et al. 2012). This technology was developed to overcome the problem related to chemicals used in the coagulation/flocculation process and also during sludge formation. Safe disposal of sludge is a major concern. But it is observed that this method is more effective when applied in post-treatment stage rather than treating raw effluent (Goswami and Mazumder 2014).

#### 14.4.1.2.7 Electrocoagulation (EC)

EC is another trendy technique that is the combination of conventional electrochemistry, coagulation, and flocculation techniques. EC involves the use of electrodes made up of iron, steel, or aluminium and submerged in tannery effluents which are to be treated. These electrodes are cheap, available, and effective. EC is efficient in the segregation of suspended solids, grease, fats, etc. It has been found EC as a useful tannery effluent treatment technique depending on the composition of the effluent, material of the electrode, etc. (Yusif et al. 2016). Primary treatment helps to get rid of settleable solids which are organic and inorganic by sedimentation, and also helps to remove floatable materials (scum) by skimming. This treatment can reduce BOD, TSS, and fats (grease) approximately by 25–50%, 50–70%, and 65%, respectively (Buljan et al. 2011).

### 14.4.2 Secondary Treatment (Biological Treatment)

Secondary treatment further lowers down BOD and COD and other parameters that are still present in the effluent even after the primary treatment. Then only it satisfies the standard limit to be discharged into the water bodies. In this process, polluting substances are degraded biologically but in controlled conditions.

#### 14.4.2.1 Aerobic Biological Treatment

As the term implies, oxygen is needed to degrade substrate by using organisms such as bacteria and fungi. Bacteria and fungi release enzymes like oxygenases and peroxidases which help in the oxidation of toxicants in the effluent. These decomposers can obtain energy by decomposing organic substrates (Yusif et al. 2016). Activated sludge reactor and membrane bioreactor are aerobic biological reactors.

#### 14.4.2.1.1 Activated Sludge Reactor

Ardern and Lockett invented this technique to treat effluent and wastewater. The process involves the use of oxygen and floc formed by bacteria and protozoans (known as biological floc). Introduction of biological floc constituting nitrifying bacteria, saprophytic bacteria, etc., and air, to the effluent not only reduces the level of organic matter but also biotransforms ammonia. But this technique always requires sufficient oxygen supply for aeration in the treatment plant. Any shortage in air supply will lead to sludge bulking. This process is costly also (Verma et al. 2019).

#### 14.4.2.1.2 Membrane Bioreactor (MBR)

MBR is a more efficient method in which a suspended growth bioreactor with microfiltration or ultrafiltration using membrane is used. MBR is commonly used in municipal and tannery effluent treatment. For activated sludge, MBR has proven to reduce more organic pollutants and ammonia (Yusif et al. 2016). However, it is also a costlier technique as frequent membrane cleaning and replacement is needed.

### 14.4.2.2 Anaerobic Biological Treatment

The aerobic process has many advantages as it is cost-effective and reliable. It also produces stable end products but the only drawback aerobic method suffers in the treatment of high-strength effluent. Therefore, for tannery effluents, an anaerobic treatment method is followed before aerobic treatment. In this treatment, degradation of organic contaminants in oxygen deficiency by microorganisms takes place. Many anaerobic bacteria like methanogens and acidogenic bacteria convert organic substances into methane, carbon dioxide and hydrogen (Dargo and Adhena 2014). It is more efficient than aerobic degradation as it requires less reactor surface area, low energy use, less use of chemicals, less sludge handling costs; reduces BOD, COD values; and also produces renewable energy, that is, methane. There are two types of anaerobic reactors: up-flow anaerobic sludge blanket reactor and anaerobic filter reactor.

#### 14.4.2.2.1 Up-Flow Anaerobic Sludge Blanket Reactor (UASB)

It is a methane producing digestor used in the treatment of wastewater. In the UASB reactor, the effluent passes at the bottom of the reactor and moves up throughout the granular sludge bed. During up-flow, anaerobic digestion of organic contaminant takes place, and microbes convert organic carbon to methane gas (Verma et al. 2019). The advantage of using UASB reactors to increase the efficiency and stability of this system are as follows: (a) it consists of naturally immobilized bacteria in large volume having remarkable settling properties which could help in the removal of the organic pollutants from the wastewater efficiently; (b) a large concentration of biomass can be achieved without any construction costs.

#### 14.4.2.2.2 Anaerobic Bio-Filter

Anaerobic biofilters are high-efficiency anaerobic treatment systems that involve the use of inert support materials in reactors to provide a stable environment for

anaerobic bacteria to thrive and limit turbulence, allowing detached populations to remain in the system. The following are the primary advantages: (a) The filling provides a greater area for the growth of microorganisms, and also increases the hydraulic residence time of the effluent; (b) Anaerobic biofilters also provides a large surface area between wastewater and membrane interaction. Therefore, the efficacy of this treatment method can be improved. The limitation of this system is that when it comes to high-concentration organic water, the system may collapse, especially in the water intake part (Kassab et al. 2010).

#### 14.4.2.3 Hybrid Treatment

Hybrid or combined reactors are more proficient in the treatment of tannery effluents as compared to the use of either anaerobic or aerobic reactors. Advantages of hybrid systems are as follows: (a) the anaerobic method helps to remove organic matters as well as suspended solids from the effluent, and reduce the organic pollutants from the aerobic degradation as well as the production of sludge; (b) pre-treated wastewater by the anaerobic system is more stable as the anaerobic process could reduce the oxygen demand of aerobic decomposition; (c) the anaerobic techniques modify by altering the biochemical property of the tannery wastewater which is then followed by a better aerobic process. From many reports, hybrid reactors are more stable and efficient for degradation of pollutants and applicable to use potentially. The combined aerobic–anaerobic reactors include an oxidation ditch and constructed wetland (Verma et al. 2019).

##### 14.4.2.3.1 Oxidation Ditch

The oxidation ditch is a process that requires low maintenance. It is a basin formed in a circular shape. In this system, activated sludge is being added followed by microbes, which start acting on the effluent. The rotating biological contactors add oxygen into the passing mixed liquor which is a mixture of raw effluent and sludge, thereby increasing the surface area and its movement in the ditch. When organic matter is reduced, mixed liquor moves out of the ditch where sludge is collected in the secondary settling tank. Aerator pumps are used to thicken the sludge (Yusif et al. 2016). A part of sludge is again used in an oxidation ditch whereas the rest is thrown as waste. This step lowers the concentration of many organic pollutants, phosphorus and nitrogen but also forms sludge bulking and foam expansion.

##### 14.4.2.3.2 Constructed Wetland

A constructed wetland is an artificial wetland behaving as a biofilter. It helps in the removal of sediments, organic matter, and absorption of heavy metals from the effluent. A mixture of water, media, plants, microorganisms, and other animals is used. Plants supply carbon, nitrogen, phosphorus, and oxygen for an aerobic environment through their roots for microbial growth to facilitate degradation of organic matter. In the constructed wetland, approximately 90% of pollutant is removed by microbes and the rest by plants. Thus, it is supposed to be an eco-compatible, easily managed reactor. But, because of some limitations, it is not widely used. As this technique requires a large field area, its efficiency is a little less as compared to other

methods, and also, a selection of plants is available to increase its efficacy (Calheiros et al. 2012).

### 14.4.3 Tertiary Treatment

In most cases still, the effluent does not meet the standard disposal quality after primary and secondary treatment. In such cases, tertiary treatment is used before effluent discharge in the water body. Tertiary treatment is a more refined and expensive method like the Fenton method where organic compounds are treated with hydrogen peroxide in the presence of ferrous sulphate and ozone. These methods help in the removal of residual BOD, COD, and many harmful microbes (Buljan et al. 2011). It removes 90–95% pollutants and thereby treated water can be used for industrial, agricultural, and domestic purposes. Further disinfection methods are also used, like chlorination, ozone, and ultraviolet light, to get good quality potable water, which could be safe for public health.

## 14.5 Bioremediation: A Promising Tool

Bioremediation is a technique that employs biological agents mainly microbes like bacteria and fungi to transform heavy metals to a low-risk phase (Ndeddy Aka and Babalola 2016; Kumar et al. 2018). It is a cost-effective and eco-compatible technique. In other words, bioremediation can help eliminate, attenuate, or transform pollutants by the use of biological agents via various processes. This technique is a rising innovation that utilizes organisms to remediate contaminates sites (Kumar et al. 2020). Some bioremediation techniques require aerobic conditions and some run under an anaerobic environment for the degradation of recalcitrant pollutants to a safer level (Agrawal et al. 2021; Kumar and Chandra 2018). In bioremediation processes, microbes use organic matter or pollutants as their nutrient source to get energy. Many of the studies revealed a great potential of the strains of bacteria and fungi to decolourize the colouring agents, that is dyes from the industrial wastewater (Kumar et al. 2016). However, major issue is with the degradation of heavy metals as they persist or accumulates in the soil and water critically disturbing the environment (Kumar 2018). The adoption of the bioremediation technique has been found to be an efficient method for the cleaning of noxious contaminants present in the effluent by the use of microorganisms to remediate contaminates sites. The microorganisms have the capability of carrying out metabolic machinery for the breakdown of the pollutants of the toxic effluents from the tannery industries. Chemicals have been used for exposing the activities of microorganisms by developing necessary enzymes which aid in metabolizing the compounds in wastewater. Anaerobic reactors are used for treating waste effluents from tanneries using microbial culture. A large amount of microbial biomass is used by wastewater treatment plants, which generally have anaerobic digesters using anaerobic microorganisms. UASB has been used that shows efficacy towards the treatment of strong wastewater in comparison

to conventional reactors. Identification of native microorganisms and consortium that are responsible for efficient reduction of recalcitrant compounds from the wastewater of tannery industries at laboratory scale is a better choice for the environmental clean-up.

The use of microorganisms in bioremediation techniques for treating the wastewater from tannery industries has been effective due to the characteristics of the microorganisms with respect to their size, Supplementation of nutrition, water, and optimum physiological condition for the growth and multiplication of the cells. This treatment of tannery wastewater while using the microorganisms generates the biomass sludge. The decomposition of organic material helps in obtaining the nutrient supplementation for the microorganisms that aids in multiplying the cell. Hence, microorganisms are efficiently used for decaying organic wastes present in the leather waste stream by multiplying their number to show effective degradation of organic matter. Bacterial cells usually undergo biological oxidation that involves degradation of organic compounds in the waste stream and are used as nutritional supplements.

One of the major challenges while using the technique of bioremediation is the degradation of heavy metals as they persist or accumulate in the soil and water, critically disturbing the environment. Hence, different types of bioremediation techniques have been adopted by tannery industries to meet the concerned challenges. Different types of bioremediation techniques are capable of removal of heavy metals and chlorogenic contents from tannery wastewater and establish several other applications in the reuse of the wastewater. Economically valuable metals can be recovered and reused by applying bioremediation for cleaning wastewater from tannery industries. Various types of microorganisms have been proved to be effective for removal of heavy toxins from tannery wastewater. Phytoremediation is another effective method of bioremediation that involves the use of plants for accumulation, hyperaccumulation, and exclusion of heavy metals for remediation of wastewater.

The present chapter thus concludes with the fact that treatment of tannery wastewater involves several challenges concerned with ill-effects of the environment which needs to be addressed on an immediate basis. Hence, the use of eco-friendly and cost-effective techniques such as bioremediation and phytoremediation has been proved to be efficient for removal of toxins and recovery of important metals from wastewater and further capable of making the wastewater available for reuse for several purposes. The treated tannery wastewater is mainly reused for non-potable purposes such as agriculture and leather tanning. Hence, this would prove to be effective in minimizing issues of water scarcity and increase productivity.

### 14.5.1 Types of Bioremediations

Microbes are very efficient; they can transform toxic substances into less harmful intermediates or can completely degrade them into harmless end products. Microorganisms are capable enough to survive at the lowest temperature to extreme

conditions and can exploit contaminants as their sole source of energy. Microbes also restore the original natural surroundings and prevent further pollution. Microorganisms grow and interact with the pollutant depends on environmental conditions like temperature, soil, water solubility, the concentration of pollutant, pH, type, and solubility of a toxic substance. Optimum pH which works best for bioremediation of the pollutant can occur from 6.5 to 8.5 in most aquatic and terrestrial environments. These factors are responsible for the degradation kinetics (Naik and Duraphe 2012).

#### 14.5.1.1 Biosorption

The metals can be held by interactions between the metal and functional groups present on the cell surface of microbes. The various means are adsorption, ion exchange, precipitation, complexation, and crystallization. Several factors like pH, temperature, ionic strength, particle size, and biomass concentration could affect metal biosorption. Living as well as dead biomass are employed for biosorption because of its independence of cell metabolism as metals are taken up passively on the cell wall through surface complexation. The biosorption process needs to be economic as the biomass can be procured from effluent and could be restored for further use. Any biological materials which possess affinity towards metals are referred to as biosorbent. Heavy metals attach to the biomass surface and become loaded with metal ions. Some of the reports have mentioned chromium removal from tannery wastewater by chromate-resistant bacteria through biosorption, such as *Pseudomonas fluorescens* (Bopp and Ehrlich 1988).

Other bacteria such as *Enterobacter* cloacae and *Acinetobacter* sp. also showed biosorption of chromium (Srivastava and Thakur 2007). *Bacillus megatherium*, *Bacillus subtilis*, and some bacterial consortia showed biosorption of other metals like lead and cadmium from tannery effluents (Abioye et al. 2018). Kim reported *Desulfovibrio desulfuricans* for the removal of Ni, Cr (VI), and Cu metals. *Acinetobacter* and *Arthrobacter* have been reported to remove 78% of chromium from the wastewater (Bhattacharya et al. 2014). Fungi also act as biosorbents that uptake metals. *Trichoderma*, *Aspergillus*, *Penicillium*, *Rhizopus*, and *Saccharomyces* species showed remarkable biosorption potential. Approximately 97% chromate at pH 5.5 was removed by *Trichoderma* sp. (Vankar and Bajpai 2008).

#### 14.5.1.2 Bioaccumulation

Bioaccumulation is a system that relies upon diverse physical, chemical, and organic mechanisms and those elements are intracellular and extracellular processes, Bioaccumulation, on the other hand, wishes luxurious price due to the fact the system happens inside the presence of residing cells best as it cannot be reused. Energy demand is also high which is required for cell growth. Heavy metals like chromium, nickel and cobalt were adsorbed on *R. arrhizus*, *A. niger,* and *Saccharomyces cerevisiae* (Gautam et al. 2015). Many dead biomasses of fungi such as *Aspergillus niger*, *Penicillium chrysogenum*, *Rhizopus oryzae*, and *S. cerevisiae* could transform toxic Cr (VI) to less toxic form Cr (III) (Park et al. 2005). Some yeast strains have also been used to convert Cr (VI) to Cr (III), such as *S. cerevisiae*,

*Rhodotorula mucilage*, *Rhodotorula pilimanae*, *Hansenula polymorpha*, *Yarrowia lipolytica*, and *Pichia guilliermondii* (Ksheminska et al. 2008).

#### 14.5.1.3 Methylation of Metals

Metal toxicity is increased by the methylation of metals by enhancing movement across cell membranes. Methylation by microbial cells plays a very important role in metal removal. Usually, methylated compounds are found to be explosive. Some bacteria such as *Bacillus* sp., *Escherichia* sp., and *Clostridium* sp. helped in bio methylation of mercury to gaseous methyl mercury (Ramasamy and Banu 2007).

#### 14.5.1.4 Biostimulation

To stimulate the activity of microbes on infected sites, both soil and water is being injected with supplements (precise nutrients). Addition of stimulators, minerals, and optimizing environmental factors hurry up the microbial metabolism rate. Moreover, addition of biostimulant on suitable microbes may further efficiently degrade the pollutant (Naik and Duraphe 2012).

#### 14.5.1.5 Bioattenuation

It is also referred to as natural attenuation of removing pollutants from the environment. This process takes place either aerobically or anaerobically. It also involves physical processes such as dispersion, diffusion etc, and chemical processes such as ion exchange, abiotic conversion. When the ecosystem gets contaminated with chemicals, nature itself works to clean itself using ways such as (1) soil microorganisms and microbes in groundwater use pollutants as their food, and after decomposing the chemicals, convert them into nontoxic gases and water. (2) Chemicals can adhere to the soil; this method does not clean up the surroundings but will prevent contamination in water. Chemicals hold the soil in a place. (3) This method also prevents the further pollution of clean water.

#### 14.5.1.6 Bioaugmentation

It is a process to create an environment of microbes with augmented biodegradative capacity so that they can act in polluted areas. This method involves natural as well as designed (engineered) microbes making them bio remediators that can rapidly clean up the site with complex pollutants. After the collection of microbes from sites, they are cultured, genetically modified, and then placed back to the location site. A wide range of digesting ability is being proved by Genetically modified microorganisms (GMM) and transforming pollutants into less harmless end products (Sayler and Ripp 2000). GMM is modified by DNA manipulation and is more efficient in comparison to the natural species to break down the contaminate at a faster rate. GMMs have proven capacity for bioremediation of soil, groundwater, and activated sludge filled with pollutants (Thapa et al. 2012).

#### 14.5.1.7 Microbial Technology

Microbial technologies as a remediation technique are very actively growing these days. For depollution, microbe–metal interaction is primarily focussed. Genetic engineering and chemical change ought to modify the additives of cells surface and might correctly enhance the adsorption ability to target metallic species. It is a technique in which a microorganism whose genetic material is changed by recombinant DNA technology. In this procedure, genetically modified organisms are created by improving them either by eliminating toxic genes or adding useful ones under laboratory conditions. In addition, microbes are engineered with favoured traits consisting of the potential to tolerate metallic stress, overexpression of metal-chelating proteins and peptides, and cap potential of metallic accumulation. In genetic engineering techniques, metabolic pathways can be altered so that degradation kinetics can be increased or by modifying enzyme specificity. By using GEMs, hoarding of pollutants can be achieved in a shorter time. With the use of a small amount of biomass, a large area of disposal sites can be treated, decontaminated, and transformed into a purified environment. With several advantages, there are some disadvantages of GEMs. In contrast, genetic manipulation in microbes may alter microbial community resultant of whole ecosystem may affected (Kumar et al. 2021a, b).

## 14.6 Phytoremediation

It is also known as botanical bioremediation, involving the use of plants to depollute soil and water. Phytoremediation is implemented to detoxify the contaminants of the effluent naturally. The ability to hoard heavy metals varies among species primarily based totally on their genetic, morphological, physiological, and anatomical features. The strategy involves the employment of plants are to either accumulate, translocate, stablize, transform or degrade the heavy metals present in the environment (Chandra et al. 2018a, b). Green plants act as lungs which help sanitize atmospheric air by photosynthesis and also degrade toxic metals in soil and water by assimilation, adsorption, and biotransformation process (Chandra and Kumar 2017, 2018). Vascular plants assimilate toxicants either directly from the air through leaves or from soil or water through roots. Several plant species have been identified to absorb and collect certain heavy metals (Lone et al. 2008). The types of phytoremediation are illustrated in Table 14.1. There are some reports on the removal of colours/dyes from wastewater with the help of plants. Protein from plant *Rheum rhabarbarum* showed its capacity to detoxify sulphonated anthraquinones (Aubert and Schwitzguébel 2004). Phytoremediation along with microbial organisms has been a trendy approach towards transforming xenobiotic compounds to less harmful states. Genetically modified plants (GMP) are another innovative approach to detoxify recalcitrant compounds from the fields. The recombinant proteins created by GMP assume a critical part in chelation (e.g. citrate, phytochelatins, metallothioneins, phytosiderophores, and ferritin), osmosis, and layer transport of metals (Vamerali et al. 2010).

**Table 14.1** Phytoremediation and their action

| Types of phytoremediation | Action |
|---|---|
| Phytoaccumulation (Phytoextraction) | The assimilation and uptake of metals is performed by plant roots emulated by their translocation and finally aggregation and fixation over the ground in the ariel parts of plant (shoots). Accumulated metal ions in aerial parts can be removed and disposed of, or burned to recover metals |
| Phytofiltration (Rhizofiltration) | Plant roots are used in this procedure to remove metals from aqueous wastes |
| Phytostabilization | From the soil toxic substances are stored in the rhizosphere which prevents them from leaching |
| Phytovolatilization | It includes the use of plants to remove toxins from the environment such as Se and Hg |
| Phytodegradation | Utilization of plants and related microbes to degrade organic pollutants by using specialized catalysts (dehalogenase, reductase and oxygenase) alternately cofactors for the corruption of contaminants from soil and groundwater |

### 14.6.1 Phycoremediation

Phycoremediation is the process of removing or degrading toxicants using various forms of algae and cyanobacteria. Algae are autotrophic, requires low nutrients, and are very capable to decolourise dyes of the effluent and heavy metals from the effluent. Algal species such as *Chlorella*, *Oscillatoria*, and *Spirogyra* have been mentioned in some reports as decolourisers (Romera et al. 2007). These biosorbents remove heavy metals from the effluent via adsorption or by ion-exchange methods. These functional groups such as sulphonate, sulfhydryl, hydroxyl, carboxyl, phosphate, and amino act as a binding site for metals. The degradation kinetics is dependent on the algal species and the chemical nature of the dye. The algal enzyme azo reductase can break the azo bond in azo dyes and convert them into aromatic amines and then to simpler forms. Green algae, brown algae, diatoms, and cyanobacteria can also decolourize di-azo dye (Dwivedi and Tomar 2018). Some of the organisms showing bioremediation property in removing heavy metals from effluents are listed in Table 14.2.

## 14.7 Conclusion

Several attempts have been made by the tannery industries for treating the discharged effluents which have evolved many challenges in the environment. The use of conventional methods has been proved to be effective with the implementation of physicochemical treatment methods remediating the metal-polluted sites. But due to the high operational costs with the requirement of large area, energy, chemicals, and labour and resulting in environmental damage, these conventional

**Table 14.2** Organisms showing bioremediation

| Organisms | Metal uptake | Reference |
|---|---|---|
| *Bacillus cereus* | Cr | Kanmani et al. (2012) |
| *Bacillus subtilis* | Cr | Kim et al. (2015) |
| *Acinetobacter* sp. | Cr, Ni | Bhattacharya et al. (2014) |
| *Pseudomonas* sp. | Pb, Cu, Cr, Zn, Ni | Kumaran et al. (2011) |
| *Pseudomonas veronii* | Cd, Cu, Zn | Vullo et al. (2008) |
| *Pseudomonas aeruginosa* | Cr | Kumaran et al. (2011) |
| *Pseudomonas fluorescens* | Zn | Uzel and Ozdemir (2009) |
| *Stenotrophomonas* sp. | Cr | Benazir et al. (2010) |
| *Desulfovibrio desulfuricans* | Cr, Ni, Cu | Kim et al. (2015) |
| *Cellulosimicrobium* sp. | Cr, Pb | Bharagava and Mishra (2018) |
| *Methylobacterium* sp. | Pb, Cu | Kim et al. (1996) |
| *Micrococcus* | Cr, Cu, Pb | Congeevaram et al. (2007) |
| *Burkholderia* | Cd, Pb | Jiang et al. (2008) |
| *Agaricus bisporus* | Cd, Zn | Nagy et al. (2017) |
| *Aspergillus versicolor* | Cr, Ni, Cu | Taştan et al. (2010) |
| *Aspergillus niger* | Cr, Ni, Co, Hg | Taştan et al. (2010) |
| *Aspergillus, Penicillium, Mucor*, and *Rhizopus* | Cd, Cu, Fe | Loukidou et al. (2003) |
| *A. foetidus*, *A. niger*, and *Penicillium simplicissimum* | Ni, Co, V, Fe, Zn, Mo, Mn | Anahid et al. (2011) |
| *Aspergillus fumigatus* | Pb | Kumaran et al. (2011) |
| *Penicillium* spp. | Cr | Loukidou et al. (2003) |
| *Ganoderma lucidum* | Ar | Say et al. (2003) |
| *Saccharomyces cerevisiae* | Cr | Parvathi et al. (2007) |
| *Chlorella pyrenoidosa* | U | Romera et al. (2007) |
| *Cladophora fascicularis* | Pb | Aung et al. (2013) |
| *Fucus vesiculosus* | Cr, Cd, Pb | Deng et al. (2007) |
| *Hydrodictyon*, *Oedogonium*, and *Rhizoclonium* spp. | V, As | Murphy et al. (2008) |
| *Spirogyra* spp. and *Cladophora* spp. | Pb, Cu | Lee and Chang (2011) |
| *Spirogyra* and *Spirullina* spp. | Cr, Cu, Fe, Mn, Zn | Romera et al. (2007) |
| *Chlorella vulgaricus* | Cu, Pb Ni, Cd | Mane and Bhosle (2012) |
| *Chlorella miniate* | Cr | Goher et al. (2016) |
| *Melia azedarach, Azadirachta indica*, and *Leucaena leucocephala* | Cr | Sakthivel and Vivekanandan (2009) |
| *Brassica* spp., *Sorghum bicolor*, and *Spinacia oleracea* | Cd, Cu, Cr, Zn | Firdaus and Tahira (2010) |
| *Helianthus annuus* | Cr | January et al. (2008) |
| *Brassica* spp. | Zn, Cu, Ni, Cd, Pb | Purakayastha et al. (2008) |
| *Portulaca tuberosa* and *P. oleracea* | Cr, Cd, As | Tiwari et al. (2008) |

methods need to be replaced with some alternative methods that are eco-compatible and cost-effective for the clean-up of tannery effluents.

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# Part III

# Omics Approaches in Environmental Remediation

# Metagenomics Tools for Assessment of Microbial Diversity in Bioremediation: A Novel Statistical Approach

15

K. Varsha, R. Kirthana, and K. Rajakumari

**Abstract**

Nature has the ability to reduce accumulation of wastes; one of the known processes is using microorganisms. Bioremediation is an upcoming method wherein microbes are used to reduce harmful pollutants. Information related to culturable and uncultured microorganisms is difficult to obtain. Screening of microbiota in effluents can be effectively done using metagenomics which helps in providing information at the genomic level. It helps in overcoming conventional barriers of existing culture techniques by giving insight into any microbial colony. Metabolic degradative pathways can be added to emphasize a more productive way to convert pollutants into useful bioresources. Characterization of unculturable microbes by metagenomics can help to understand their natural ability to break down pollutants. Potent biodegradative genes can be identified in order to make use of a feasible microbial community. Monitoring of metabolic activity or identification of key genes can be done. Though the use of these techniques is at a beginning level, these tools can be applied to attenuate pollutants naturally.

**Keywords**

Metagenomics · Metabolic degradative pathways · Biodegradative · Bioaugmentation · Biostimulation

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## 15.1 Introduction

Pollution arising due to industrial exploitation is increasing gradually and is affecting the overall natural ecosystems and the organisms living in them. Human beings, birds, and animals are also getting affected by various diseases and are getting deprived of fresh natural habitats. The rising population of the world is decreasing the number of exotic species. Getting rid of these contaminants is becoming a greater challenge these days. The management of industrial wastewater is becoming a greater challenge due to rapid urbanization. Many types of effluents are getting released into the water and soil by urbanization activities, chemical industries, agricultural runoffs, textile industries, distilleries, paper pulp industries, improper radioactive wastes disposal, metal manufacturing industries, automobile industries, and many more (Kumar et al. 2020a, 2021a, b; Kumar and Chandra 2020a). Nonpoint source pollution such as agricultural runoffs contribute to pollution of land as well as water and also leads to biological magnification affecting the food chain. New methods must be emphasized to avoid depletion and deterioration of the environment. Breaking down the chemical compounds naturally without the involvement of machines which creates more expenditure is now in high demand rather than filtration along with coagulation, osmosis, and ion exchange methods. Biological treatment of wastes in the soil or water can be done with microorganisms which are more cost-effective (Kumar et al. 2018). A bioremediation process utilizes microorganisms to break down environmental pollutants using their metabolic pathways by breaking them down into smaller parts in order to degrade and detoxify them. Bioremediation is a new method of dealing with environmental contaminants since it involves removing contaminants using natural biological processes (Kumar et al. 2022). Bioremediation provides various benefits and profits to clean up methods and reduces the damage to the environment. In bioremediation underground water and underground soil can be cleaned without affecting the nearby communities. By-products produced by microbes are nonharmful to the environment. This is because the contaminants of the soil or water are converted into water and harmful gases like carbon dioxide. Oxidation–reduction reactions take place in bioremediation. An oxygen electron accepter is added to oxidize a contaminant. An electron donor is added to reduce a contaminant. Bioremediation is less expensive and has more benefits than other remediation alternatives. Additional remediation technique is air striping, thermal deposition, biobleaching, vitrification, soil washing, and rhizofiltration. Bioremediation techniques include bioreactors, land farming, bioaugmentation, bioventing, composting, biostimulation, and rhizofiltration. Are Bioremediation can be done either in situ or ex situ. By treating the contaminated area at the same location, in situ bioremediation is achieved. In ex situ bioremediation, the contaminated place treated some distance away from the site. If the temperature is inappropriate for the microbe to survive or soil is dense with nutrients, then ex situ bioremediation is carried out. Bioremediation is divided into two main types. Microbial bioremediation uses microorganisms to reduce contaminants (Chandra and Kumar 2015; Kumar et al. 2020b; Agrawal et al. 2021). Phytoremediation uses plants to extract contaminants like pesticides, metals,

chlorinated solvents, etc. Several research studies focus on microbial bioremediation (Chandra and Kumar 2017a; Chandra et al. 2018). The rate of contaminant degradation under natural or polluted conditions by microbes is also studied. The advantage of bioremediation is microorganism creates relatively less harmful byproducts and converts contaminants into water or harmless gas. Microbes are essential in the bioremediation process; selecting the appropriate microbe for the degradation of the desired pollutant involves further studies.

Techniques for bioremediation can be classified by the intensity of the intervention into three main categories. Natural attenuation is the least invasive method. Wild-type microorganisms are used here to detoxify contaminants naturally. This method is cost-effective, and the expense of adding additives is also less. However, in many environmental systems, the rate of degradation is extremely slow and can also have health risks. Biostimulation method uses native microorganisms however will increase the biodegradation rate by decreasing environmental constraints by the addition of limiting nutrients. Biostimulation conjointly leads to slow rates of biodegradation because of the lack of the native microbial community to degrade the target contamination. To handle this issue nonnative microorganisms or biocatalysts are often superimposed to the environmental system throughout bioaugmentation to reinforce the rates of biodegradation. This system is that the most invasive, as a nonnative being is superimposed to a targeted contaminated region. Generally, nonnative microorganisms cannot survive in contaminated environments. The microorganisms degrade the substances using a metabolic process or enzymatic process (Kumar and Chandra 2018; Kumar and Shah 2021). It gives rise to complete mineralization of the pollutant which can be of any foundation. They gain energy by breaking the chemical bonds between the atoms of the contaminants and only leaving carbon dioxide, water, or any other useful bioproduct which can be later extracted using various extraction procedures. High resolution of genetic information is required to select the appropriate microorganism which must be employed for the degradation process. Traditional methods involve the collection of samples from the polluted environment and culturing using existing methods and often involve frequent subculturing which creates the risk of losing the required genetic information due to any mutations or contamination. Thousands of microbial species exist in a very single pinch of soil, thus once pollutants area unit similar in composition to nutrients that occur naturally within the environment, an oversized range of species compete to utilize the waste material as a supply of energy. If the pollutant is complex in nature, then local strains cannot utilize the compounds. Many bioremediating microorganisms are isolated from contaminated places; however, today the data obtained from these isolates is to check the structure and dealing of advanced microorganism communities (Chandra and Kumar 2017b, c; Kumar and Chandra 2020). More in-depth genetic information from natural environments is required to understand how pollution affects microbial communities, and whether there is the potential possibility for further optimization of bioremediation strategies. The culture-independent studies are now feasible with the exposure of new high-throughput sequencing technologies which can analyze microbial communities with no rushing (Kumar et al. 2021c). Metagenomics and other omics technologies are a

very much reliable, sensitive, accurate, and more rapid approach for the detection of microbes and give a complete view of the microbial community. It helps in the identification of unknown microbial communities and microbes which cannot be cultured and can help in distinguishing microbial communities. It relies on much information from the databases to compare the observed information and the existing ones. This not only helps in speeding up the biodegradation process but also can identify new species or enzymes and even complex metabolic pathways which can help in further researches. So, without coming in contact with the harmful microbes and contaminants the samples can be analyzed. These techniques are a promising approach that can further be integrated with other technologies for more rapid effective treatment of wastes.

Example for bioremediation—During 1989, an oil spill occured by an Exxon Valdez oil tanker in Alaska. Approximately 11 million gallons of oil was spilled from the tank. Bioremediation was the best method to clean the oil. The different components were tested by EPA and Exxon Mobil Corporation (XOM). The initial test of bioremediation was successfully done. In mid-1992, the oil was cleaned by bioremediation.

## 15.2 Role of Microorganisms in Bioremediation and the Factors Affecting the Process

Bioremediation is the process of degrading, removing, immobilizing, or detoxifying wastes in the environment by using microorganisms such as bacteria and other species such as fungi and plants. Microorganisms react to the wastes utilizing their enzymatic pathways act as biocatalysts and enhance the progress of biochemical reactions that deteriorate the targeted contaminant. Microorganisms can reproduce when they are ready to uptake the contaminants and generate the energy required. Bioremediation depends on many characteristics like the chemical composition and concentration of pollutants, the physicochemical features of the environment, and the pollutant obtained for the respective microbes. When bacteria and pollutants do not come in contact with each other, degradation is slowed down. Environments do not have an even distribution of microorganisms and contaminants. The optimization of bioremediation processes is a complex one due to many factors like the presence of a microbial population which can degrade the pollutants, the availability of contaminants to the microbial population, and environmental factors like the type of soil, temperature, pH, oxygen availability, electron acceptors, and nutrients.

A brief explanation of the role of the factors are given below:

### 15.2.1 Availability of Nutrients

Nutrient additions satisfy the required nutrient balance for microbial growth and development, as well as affect the rate and effectiveness of biodegradation. Biodegradation efficiency can be enhanced by optimizing the ratio of bacterial nutrient

consumption C:N:P by providing essential nutrients such as N and P. Nitrogen increases cell growth rate and decreases the log phase on the microbe. This helps in keeping microbes at the high population in soil and increases the rate of biodegradation. Ammonium salts, urea, and nitrate salts are some sources of nitrogen. The application of appropriate nutrients serves as a necessary precondition for increasing the metabolic activity of microorganisms and hence the biodegradation rate in cold environments (Couto et al. 2014). There are often limitations to biodegradation in aquatic environments due to nutrient availability (Thavasi et al. 2011). In addition to nutrients, oil-eating microbes require adequate nutrients for optimal growth and development, however, the nutrients occur in lesser quantities.

### 15.2.2 Temperature

Temperature is one of the most essential factors in determining the survival of microorganisms and make use of hydrocarbons (Das and Chandran 2011). Enzymes in microbes require an optimum temperature to degrade target pollutants. It can speed up or slow the bioremediation process. If the temperature is more than the optimum temperature then the degradation rate of the contaminant is reduced. If the temperature is reduced to 20 °C to 2 °C then the degradation rate is reduced. In extremely inhospitable freezing environments such as the Arctic, oil degradation is very slow and pushes the microorganisms under more pressure to clean up the spilled petroleum. The polar temperature causes the transport channels which contain water within the microbial cells to shut down or the entire cytoplasm might get a freeze, leaving the most oleophilic microbes metabolically inactive (Woyke et al. 2009).

### 15.2.3 Concentration of Oxygen

Hydrocarbon metabolism is enhanced by the occupancy of oxygen. Degradation of pollutants by the microorganisms can be aerobic or anaerobic. When microbes try to break down environmental contaminants it needs oxygen, so microbes use oxygen to break down environmental contaminants. This is called as aerobic bioremediation. For example, *Bacillus cereus* uses oxygen for removing diesel oil pollution; *Aspergillus niger*, some species of Candida, and *Saccharomyces cerevisiae* use aerobic bioremediation to clean crude oil; *Alcaligenes odorans*, *Bacillus subtilis*, species of Corynebacterium, and *Pseudomonas aeruginosa* use aerobic bioremediation to clean oil. Hydrocarbons and other additional organic compounds, including petroleum hydrocarbons and some fuels, use oxygen for aerobic bioremediation. Oxygen is used as an electron acceptor; in addition, oxygen is less soluble in water and has a low diffusion rate in water and air. Oxygen enhances aerobic biodegradation of petroleum contaminants such as gasoline. Oxygen is the electron acceptor for aerobic bioremediation. Oxygen concentration is increased by increasing ground water level by air sparging. The rate of degradation of bioorganic contaminants by

microbes that occur naturally in the surroundings can increase the oxygen content. Naturally occuring microbes enhance the rate of biological degradation of contaminants and the release of oxygen.

### 15.2.4 Moisture Content and pH

Excess water can adversely affect the rate of microbial degradation. Moisture content in the soil is a very important parameter to be detected. A small amount of water will reduce microbial activity in the soil. A large amount of water will block the pores of the soil and reduce the diffusion of oxygen into the soil. Thus, a sufficient amount of water should be added. Microbial activity is high when there is 60% of water content in the soil. Water gets evaporated during bioremediation, to avoid water reduction; water should be added to the soil with some time interval. The pH of the pollutants or the environment can affect the growth and development of the microbes. For soil degradation, the pH should be slightly acidic or alkaline. For oil degradation pH value should be between 6.5 and 8.0.

### 15.2.5 Trace Elements and Toxic Compounds

Trace elements like zinc, magnesium, manganese act as cofactors for enzymes but a high amount of metal ions can inhibit the enzymes. High concentrations of contaminants whether organic or inorganic can turn out to be toxic for microorganisms (Fig. 15.1 and Table 15.1).

## 15.3 Omics Technology in Bioremediation

Omics focuses to study the properties of the biological system part by part. These studies can help in charting out the bioremediation, bio augmentation and bio stimulation strategies. To identify various regulatory genes involved in degradation process, biomolecules like DNA, RNA, and protein is taken for analysis from the contaminated environment. Omics is a group of technologies which can analyze biomolecules. The study of proteins from anthropogenic activity site is called meta proteomics. The study of proteinaceous substance which produces primary and secondary metabolites is called as metabolomics. Genes which regulate mRNA expression can be studied by transcriptomic techniques. Metagenomics is used analyze DNA from a sample to study its taxonomic features and other potent abilities. Microarrays are mostly used in transcriptomic analysis studies. eQTL studies can be used in transcriptomic analysis for examining dynamic gene structures, such as identifying gene sequences that dominate mRNA expression levels in a given sample collected from any environment. Comparative studies of different omics technology can reveal high degrees of similarities in identified species. In proteomic techniques, the proteins are first digested using enzymes like

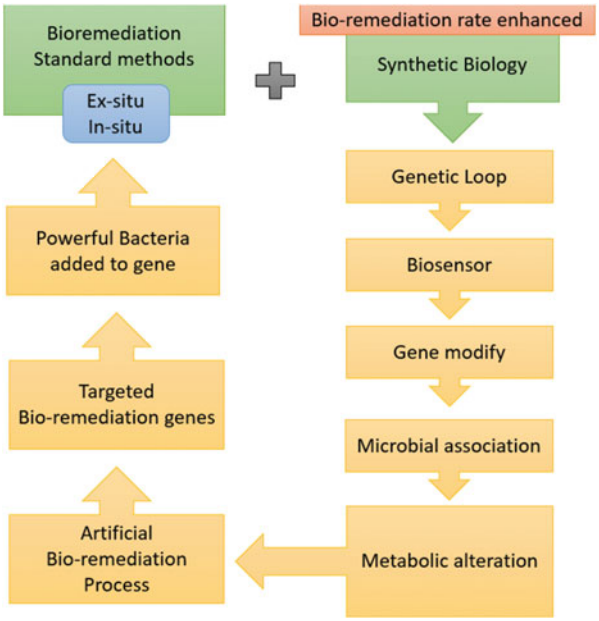


**Fig. 15.1** Role of microorganisms in bioremediation and identification of potential genes can increase the rate of bioremediation

trypsin and then separated by techniques like polyacrylamide gel electrophoresis. With liquid chromatography and MS/MS, the proteases digest the entire proteome and are purified by cation exchange chromatography. It helps in identifying insoluble membrane proteins and other signaling cascades involved in the microbial communities. Metabolomics helps in giving complete information on the metabolites present in the microbe. Metabolomics can be incorporated into other studies to have deeper insight into environmental systems biology. Metabolic components present in the cell can be characterized by many spectroscopic techniques. A bioinformatical tool known as MelonnPan is used to examine the metabolomes from microorganisms in any environment. Fluxonomics is a recently developed technology with focuses on analyzing the metabolic reaction rates in a microbial system. It involves two techniques which is stoichiometric metabolic flux analysis and tracer-based metabolomics. The taxonomic categorization of the microbial communities can be linked with rate of biodegradation process. 16s RNA techniques are used to outline the composition of microbes in a sample. Microarrays can analyze multiple genes without the use of PCR amplification. Xenobiotic compounds can also be identified using these technologies and can estimate the required strategies for growth of a microorganism in a polluted area. Integrative omics approaches can help in developing biotechnological products using identified microbial communities which have potent genes. Advanced metagenomics techniques like the Oxford nanopore technology are technique which operates electrophoresis to pass the DNA/RNA molecule into a nanopore bed by making use of electrolytic solutions kept in a perpetual electric field. Finally, in this method double-stranded DNA is cut off and blunt-ended DNA molecules are established

**Table 15.1** Microbes and compounds which they can degrade

| Microorganisms | Chemical contaminants | Reference |
|---|---|---|
| **(A) Hydrocarbons** | | |
| Species of *Alcaligenes*, *Bacillus subtilis, Corynebacterium propinquum, Pseudomonas aeruginosa* are used | Phenolic compounds | Chandra et al. (2013) |
| *Acinetobacter*, species of *Pseudomonas*, species of *Ralstonia*, and *Microbacterium* | Aromatic hydrocarbon compounds | Simarro et al. (2013) |
| Species of *Gleophyllum* | Striatum pyrene, anthracene, 9-methylanthracene, dibenzothiophene lignin peroxidase chemical | Yadav et al. (2011) |
| Species of *Coprinellus* | Polycyclic aromatic hydrocarbon pollutants | Aranda et al. (2010) |
| *Penicillium chrysogenum* | MAH, benzene and its derivatives, toluene | Ali et al. (2014) |
| **Industrial dyes** | | |
| **Bacteria** | **Chemical compound in the pollutant** | **Reference** |
| *Bacillus firmus* and *Staphylococcus aureus* | Vat dyes | Adebajo et al. (2017) |
| *Pycnoporus sanguineus*, *Phanerochaete chrysosporium* | Industrial dyes | Yan et al. (2014) |
| *Bacillus cereus*, *Exiguobacterium aurantiacums* | Azo dye effluents | Kumar et al. (2016) |
| *Bacillus subtilis* strain NAP1 and NAP2 | Oil paints | Phulpoto et al. (2016) |
| *Micrococcus luteus* | Textile azo dyes | Hassan et al. (2013) |
| **Heavy metals** | | |
| **Bacterial communities involved** | **Metal ions** | **Reference** |
| *Pseudomonas fluoresces* and *Pseudomonas aeruginosa* | Iron, zinc, lead, manganese, and copper | Paranthaman and Karthikeyan (2015) |
| *Pseudomonas aeruginosa*, *Aeromonas* sp. | Uranium, copper, nickel | Sinha et al. (2011) |
| *Aerococcus* sp., *Rhodopseudomonas palustris* | Lead, chromium | Sinha and Paul (2013) |
| *Bacillus safensis* strain (PB-5 and RSA-4) | $Cd^{2+}$ | Rajesh et al. (2014) |
| **Pesticides** | | |
| **Bacteria** | **Chemical compound in the pollutant** | **Reference** |
| *Sphingomonas* sp. | Chlorpyrifos | Li et al. (2007) |

(continued)

**Table 15.1** (continued)

| Microorganisms | Chemical contaminants | Reference |
|---|---|---|
| *Ochrobactrum* sp. | Methylparathion | Qiu et al. (2007) |
| *Micrococcus* sp. | Diuron | Sharma et al. (2010) |
| *Burkholderia* sp. | Fenitrothion | Hong et al. (2007) |
| *Pseudomonas* sp. | Diazinon | Cycoń et al. (2009) |
| **Heavy metals** | | |
| **Bacterial communities involved** | **Metal ions** | **Reference** |
| *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* | Iron, zinc, lead, manganese, and copper | Paranthaman and Karthikeyan (2015) |
| *Pseudomonas aeruginosa*, *Aeromonas* sp. | Uranium, copper, nickel | Sinha et al. (2011) |
| Aerococcus sp., *Rhodopseudomonas palustris* | Lead, chromium | Sinha and Paul (2013) |
| *Bacillus safensis* strain (PB-5 and RSA-4) | $Cd^{2+}$ | Rajesh et al. (2014) |
| *Aspergillus versicolor*, *A. fumigatus*, *Paecilomyces* sp., *Paecilomyces* sp., *Trichoderma* sp., *Microsporum* sp., *Cladosporium* sp. | $Cd^{2+}$ | Mohammadian Fazli et al. (2015) |
| *Microbacterium* strain Shh49T | Iron | Wu et al. (2015) |
| *Geobacter* sp. | $Fe^{3+}$ | Mirlahiji and Eisazadeh (2014) |
| **Oil degradation** | | |
| **Microbial communities involved** | **Oil components** | **Reference** |
| *Alcanivorax, Marinobacter, Thalassolituus, Cycloclasticus, Oleispira* | Petroleum | Yakimov et al. (2007) |
| *Achromobacter xylosoxidans* DN002 | Polyaromatic hydrocarbons | Ma et al. (2015) |
| *Rhodococcus equi*, *S. maltophilia*, *Gordonia* sp. | Diesel | Szulc et al. (2014) |
| *Dietzia* sp. | n-alkanes | Wang et al. (2011) |

with a final repair stage of the process. Shotgun sequencing is a rapidly advancing technique with connects the gap between microbial communities. An effluent discharge can alter the constitution of microbial diversity in an environment, and may also increase biomass, genetic diversity, or activity. A species is enriched that is capable of degrading the contaminant and its intermediate degradation compounds,

at the same time species that are sensitive to the pollutant are diminished. These species have been used to assess contamination sites (Pulleman et al. 2012) because these species are considered bioindicators. Hence, omics technologies can help in estimating the levels of pollution in ecosystems by measuring changes in natural functions.

## 15.4 Metagenomics: A Potential Tool for Bioremediation

The study of recovering genetic material straight from in silico tools the environmental samples is called as metagenomics. Early metagenomic studies used a single PCR product was cloned a bacteriophage and then sequenced on slabs labelled with radioactive phosphorus primers. It includes several procedures which involves the extraction of genetic material, preparation of libraries, sequencing and assembly of genes, annotating the gene sequences and in the end the results are given by methods of statistical and bioinformatical analysis. Large-scale metagenomic market companies include the Novogene Corporation, Danaher, Oxford Gene Technology, and Thermo Fisher Scientific Inc. Majority of the microbes collected from the environment is uncultivable. Metagenomics can help in sequencing the DNA from uncultivable microbes and can give rapid dynamic results. The development of high-throughput sequencing technologies and in silico tools have helped complex metagenomic studies on habitats like acid mine drainage and the human gut microbiome. The development of molecular technologies has enabled the classification of microbes in extreme environments, such as deep-sea hydrothermal vents, heavy metal–polluted soils, and extreme temperature ranges. This method has provided more chances for exploring and discovering novel biomolecules like proteins, enzymes, lipids, and carbohydrates. The metagenomic reference databases are prepared by cloning DNA fragments isolated from samples from microbial habitats and reincorporating them into a host bacterium using a suitable vector, such as the phage, plasmid, or bacterial artificial chromosome (BAC). The successive transformants can later be probed for taxonomic markers, conserved gene sequences region, and expression of a specific gene.

## 15.5 Different Metagenomic Strategies for Analyzing Microbiota in Contaminated Samples

Metagenomics can be used to analyze contaminated samples by two different strategies they are targeted sequencing or shotgun sequencing. The heterogeneity of a gene is examined in targeted sequencing in order to identify complementary sequences of a particular gene in a given environment. Phylogenetic diversity and relative abundance of a particular gene can be analyzed using targeted sequencing as well as the diversity of small subunit rRNA (16S/18S rRNA) in a sample using targeted sequencing. This strategy uses small subunit rRNA sequencing, complete information on taxonomic diversity can be gathered and analyzed, and this can also

be used to investigate the impact of environmental pollutants on microbial community structure. The procedure gets started with targeted sequencing by extracting the environmental DNA and the gene of interest and then are amplified using PCR and specific primers are also designed to amplify the greatest diversity of sequences for the gene of interest (Shakya et al. 2013). The amplified genes are then sequenced using next-generation sequencing. The results give thousands of small-subunit rRNA reads per sample and can examine more than 50 samples simultaneously.

### 15.5.1 Targeted Sequencing

The diversity of a single gene of interest is expressed by targeted sequencing, but is restricted by the universality of the PCR primers selected for the analysis. Recently several computational molecular biology tools have the ability to display the whole diversity. Also, it is impossible to directly assess microbial function. There is not enough resolution for subspecies identification. The genes may also get unequally amplified (Rinke et al. 2013). The pros of targeted sequencing are that it is more precise and provides a reasonably complete data of the microorganism taxa present in a set of samples and allows for detailed identification of changes in microbial diversity before and after a change in equilibrium.

### 15.5.2 Shotgun Sequencing

In shotgun sequencing, the total complementary genes of an environmental group of community are checked through genomic sequencing. The deoxyribonucleic acid obtained from the surrounding is subjected to be broken down to develop sequencing libraries for the genomic data obtained. These libraries are then sequenced to identify the total genomic amount of the given sample. Utilitarian potential of the microorganism community can be obtained by shotgun sequencing. Deep sequencing is required to complete a library of the genes present in an environmental sample. Shotgun sequencing does not give much detail of the sequencing. Complete detail about the genomic content of every organism in the environment is necessary for analysis of the functional importance of a microbial community. In some cases shotgun sequencing in extensive samples, the dominant microbes in a community and only scattered covers the low abundant genomic content members of that community. The process of the analysis of shotgun sequencing data includes accurate annotation of diverse gene sequences in which many sequences have no homolog in the recent sequence databases leading to complexity (Delmont et al. 2012). The ultimate goal of is to link a functional gene with a taxonomic classification. In many cases sufficient sequencing depth is achieved the reads can be correctly assembled into sufficiently long genomes. Nowadays, bioinformatical tools are used to assemble metagenomic sequences into complete genomes to gather complete knowledge of the functional potential of particular microbial species within a habitat. Novel genomic characteristics can be identified using shotgun sequencing technique.

### 15.5.3 Use of Microarrays

A microarray is a collection of spots which is attached to a solid surface. It is used to estimate the formulation of a large number of genes in the same time. In a DNA microarray, each spot which has DNA consists of picomoles of specific DNA sequences called as probes. It uses the principle of hybridization of nucleic acids in which the complementary strands of DNA are joined in conjunction by hydrogen bonds. The biological complexity of most of the genomes can be easily analyzed and studied using these microarrays. Many recent researches have summarized the important measures in metagenomics and many possible hazards in these methods. Several microarray-based methods have been developed along with sequencing-based approaches. The two most frequently used microarray technologies are PhyloChip and GeoChip. PhyloChip is a 16S rRNA-based microarray which examines the diversity of around 10,000 sub-families in more than 140phyla. GeoChip is a utilitarian gene microarray which examines the diversity of approximately 152,000 genes in more than 400 gene categories (Hazen and Sayler 2016). Microarray techniques does not depend on the depth of sequencing of genomes to provide clear cut idea of any microbial community rather they provide accurate elucidation for the numerous taxa/genes present on the chip emphasizing the limitation of the requirement for any homolog in the database to achieve error free classification of genes. Microarray-based methodologies limit the future for uncovering new genes or pathways in a sample because only the genes on the chip can be read. Microarray-based techniques are helpful to complement sequencing-based approaches. All the DNA spots in the micro array contain $10^{-12}$ moles of DNA. These spots are called probes. Short DNA or DNA that hybridize targeted cDNA or cRNA (anti sense RNA) under severe conditions are used in probes. The two DNA complementary strands will get hybridized and strong noncovalent bonds will be formed between the two strands. Nonspecific bonding sequences are removed by washing and only strong bonds will remain hybridized. In probed fluorescent labeled the sequence is bounded and the signals are generated depending upon hybridization conditions (pH). During the process, the strength of the signal obtained and intensity of spot is calculated from spot. This intensity is compared to the same intensity in different conditions. Microarrays can help to find the functions of all genes of microorganisms present in the environment. Some microarrays have smaller platforms; these platforms can be used in specific environments or particular process to find the function of genes in microorganisms. Structure, function, and gene expressing levels of microorganisms can be identified through microarray. The data of many genes in microorganisms in different environments is collected. This information can give a deeper understanding of bioremediation in contaminated soil.

#### 15.5.3.1 PhyloChip Microarray

PhyloChip was developed at Lawrence Berkeley National Laboratory (LBNL). It is used to detect a number of bacteria and archaeal taxa using 16S rRNA gene sequences as a probe. Multiple probes increase the accuracy of identifying the microbes in a sample. Known and unknown organisms are correctly identified and

unknown microbes are classified according to matches and mismatches to known organisms. PhyloChip helps to identify and separate different microorganism from a complex microbial complex. Old method of identifying microorganisms is done using bacterial culture. The culture was exposed to a sample and bacteria were grown. The drawback of this method is 99% of the bacteria die. To avoid this chaos, PhyloChip microarray was developed at LBNL for more accurate result without using culture.

#### 15.5.3.1.1 Applications of PhyloChip Microarray

- The airborne disease microbe which can affect bioremediation can be identified.
- Changes in microbial communities due to climate change in the atmosphere and the soil can be identified.
- Dangerous and useful microorganisms can be identified from soil and water samples.
- Bacterial population in the environment can be identified by environmental bioremediation.
- Pathogen sources can be identified by public health agencies to carry out effective bioremediation.

#### 15.5.3.1.2 Advantages of PhyloChip Microarray

- Microorganisms are more rapidly and accurately identified.
- Several microorganisms can be identified at the same time.
- PhyloChip microarray analysis of rRNA can identify the most active organism.
- Identifies less populated organism in the sample.
- Organism which is more adaptable to the external environment can identify.
- Rate of identifying specific microorganisms is high compared to culture method.
- PhyloChip microarray can be done to any source of samples; such as soil, air, water, and tissue or blood.

### 15.5.3.2 GeoChip Microarray

It is a type of microarray where each probe contains a specific microbial DNA or contains a group of similar nucleic acid. GeoChip contains more than 150 functional groups that take part in nitrogen, and the carbon cycle. It also contains more than 10,000 genes. GeoChip contains 24,243 oligonucleotide probes. In each probe targeted genetic material is being identified. GeoChip was developed to get information of activities of metal reducing bacteria for in situ bioremediation. This helps in the detection of metabolic activities and biogeochemical process of microorganism in sample. The probes are hybridized to detect the homologous genes present in different organisms.

The Probes are classified into two types. They are as follows:

- Gene-specific probes.
- Group-specific probes.

#### 15.5.3.2.1 Gene-Specific Probes

Gene-specific probes should meet the below conditions:

- The genetic material present in a probe should have 90% similarity between the genes.
- Each DNA should contain 20 base pair in continuous stretch.
- Free energy should be less than −35 kilocalorie per mole.

#### 15.5.3.2.2 Group-Specific Probes

Group-specific probes should meet the following conditions.

- The genetic material present in a probe should have 96% similarity between the genes.
- Each DNA should contain less than 35 base pair in a continuous stretch.
- Free energy should be greater than −60 kilocalorie per mole.

The gene probes which are complementary to oligonucleotides are produced and labeled. At 5′ end, Cy5 or Cy3 dye is labeled. The sequence is taken as template and then targeted gene is amplified by the PCR. The PCR product matches the requirements of the GeoChip probe. These DNA extractions are kept in GeoChip Probe. GeoChip microarray produces the data of the genetic material and identifies homologous genes in different organisms.

## 15.6 Cases in Which These Technologies Were Used

A recent study has developed a model which predicts the habitual circumstances according to the structure of habitats of microbes. This model was precisely sketched to identify the ability of microbial communities to predict the presence of particular pollutants like U-238 and $NO^{3-}$ groundwater contamination and contamination due to oil in marine samples (Watson and Morato 2004). The taxonomic diversity of the microbial population using 16S rRNA genes is assessed by using two data sets. The structure of microbial habitat was examined using high throughput 16S rRNA sequencing. The sequencing data obtained was observed and contained the elements to govern the taxonomic composition of the microbes in an environment and the relative abundance of each taxon of microbes. A simulation was built which looked to compare the microbial community composition to the geochemical variables. This model is analyzed against a subgroup of the data and then substantiated against the remaining data. The authentication process involves the process of submitting a microbial community profile to the algorithm and tests whether the model's estimation complemented the measurement obtained from the environment. *Brevundimonas* spp. was displayed as the most effective feature for identifying nitrate-contaminated wells. They have proved to be potent $NO^{3-}$ reducers. Another example for sequencing data using microarrays. Samples were collected from the Gulf of Mexico during the Deepwater Horizon oil spill were tested using the

PhyloChip to examined the taxonomic make up of each sample and the relative abundance of each taxon (Techtmann and Hazen 2016).

### 15.6.1 Sequencing by Ligation Beds

Sequencing proceeded with ligation on beds consisting of more than one sequencing round. The SOLiD technology uses a step-by-step process. Adapters are attached to DNA pieces fastened on 1 μm paramagnetic beads and these DNA fragments are amplified by PCR using oil-water emulsion medium. PCR amplified fragments are secured in beads on a planar surface and are then hybridized using a universal PCR primer coupled with an accessory. During the sequencing process, the position of the nucleotide is observed by ligating universal primer to a fluorescently labeled DNA octamer. The cycle is done till every base pair is sequenced two times which enhances the accuracy of this method of detecting the genomes. Ion torrent sequencing is little bit similar to pyrosequencing technology. It depends on the emission of a proton when a dNTP is added to DNA polymer instead of using fluorescent markers. Adaptors are further added to the broken DNA or RNA strands, and another molecule is rested upon beads and amplified by emulsion PCR and the beads are placed carefully into a single well.

### 15.6.2 First-Generation Sequencing

Sanger sequencing is first-generation sequencing technique which uses denatured DNA template, a labeled primer which is radioactive, DNA polymerase, and dNTPs to generate DNA fragments of various lengths. The DNA fragment size is set on by the incorporated dNTPs. The DNA pieces are then separated based on their lengths on the gel electrophoresis unit and are then later viewed by an UV-light transilluminator system. However, this technique is exorbitant, challenging and does not allow the sequencing of complex genetic sequences, so it is mostly used for single or low-throughput DNA sequencing (Fig. 15.2 and Table 15.2).

## 15.7 Stable Isotope Probing

SIP techniques are employed to recognize the contaminant which can be degraded and also identify the microorganism required for the process. Examples for stable isotopes are oxygen, nitrogen, carbon, sulfur, and hydrogen. A substrate is added with a heavier stable isotope which is later consumed by the microorganism for further studies. When DNA is the biomarker SIP is done using isotopically labeled C, H, O, or N. When the isotope is assimilated the density shifts are a predictable function and can be easily examined. This technique is called as quantitative stable isotope probing. The uptake of nutrients during the biogeochemical cycle of the microorganism can be easily traced. In metagenomic studies, $^{13}C$ is

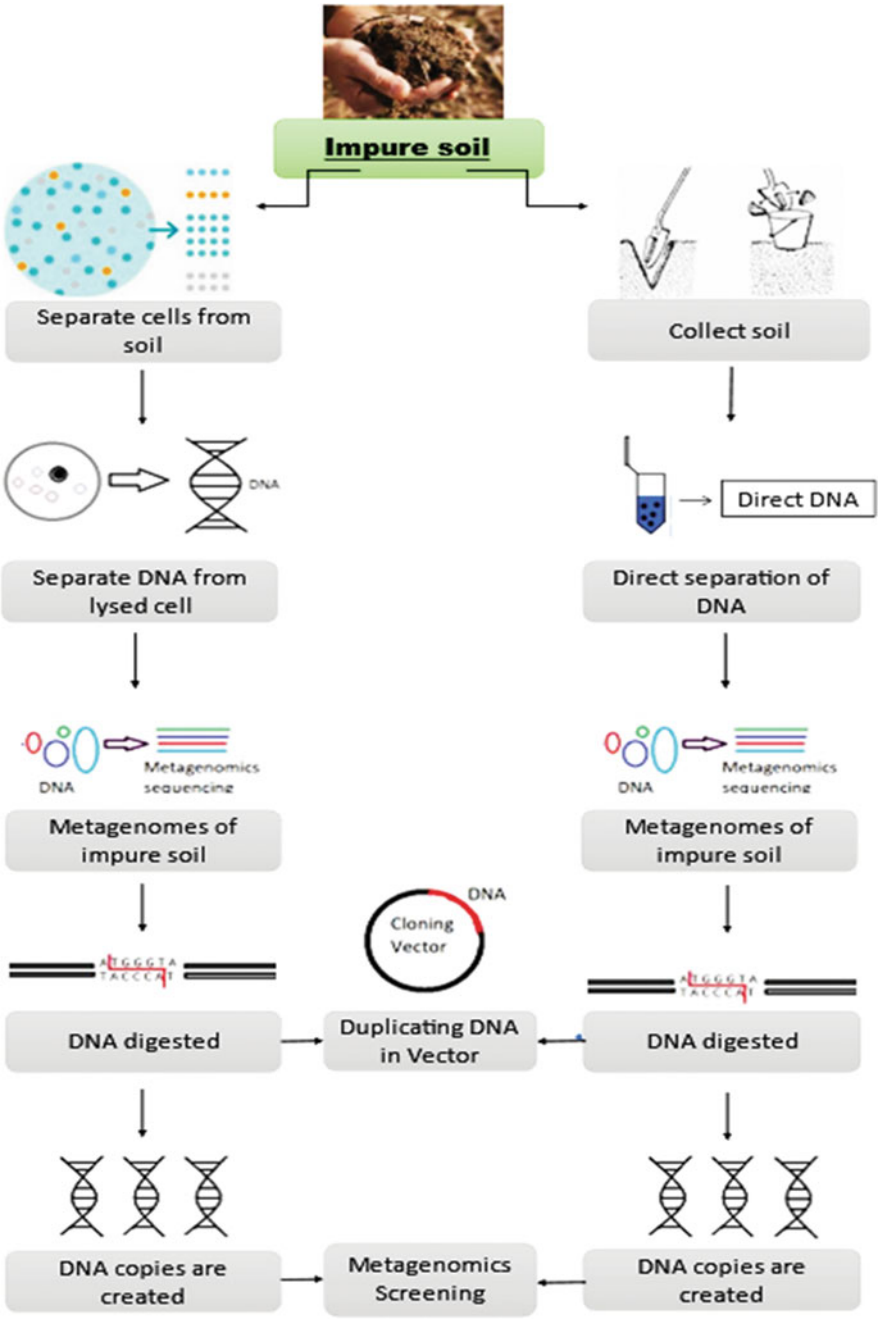


**Fig. 15.2** Analyzing microbial communities from contaminated soil using metagenomics

**Table 15.2** Different metagenomic strategies to analyze microbial communities

| Sample taken from | Contaminant | Gene groups and enzymes examined | Important statement from findings | Sequencing type used | Reference |
|---|---|---|---|---|---|
| *Whole-genome sequencing* | | | | | |
| Soil | Diesel | 16S rRNA, hydroxylases, dioxygenases enzymes | Transfer from gamma proteobacteria to alpha proteobacteria and Actinobacteria approximately after 6 months of bioremediation | Roche/ 454 GS FLX titanium | Yergeau et al. (2012) |
| Groundwater | Heavy metal contamination, organic constituents | 16S rRNA, metabolism and stress response | Drastic mislaying of species and metabolic diversity due to greater than 40 years of contamination | PRISM 3730 capillary DNA sequencer | Hemme et al. (2010) |
| *Functional screening* | | | | | |
| Soil | Aliphatic and aromatic hydrocarbons | Extradiol dioxygenase | High diversity of extradiol dioxygenase genes in contaminated soil | ABI PRISM 3100 Genetic Analyzer | Brennerova et al. (2009) |
| Activated sludge | Several aromatic compounds | Extradiol dioxygenase | Recognized novel arrangements of the extradiol dioxygenase degradation pathway on plasmid-like DNA | ABI 3730xl DNA Analyzer | Suenaga et al. (2009) |

mainly used as a heavy isotope marker. Shotgun metagenomic sequencing of SIP-determined metagenomes can help in identifying proportions of the microbial community which have less quantity but are required for the metabolic operation of interest. The major disadvantage of this methodology is the retrieval of DNA too small in quantities required for shotgun sequencing. Breakthrough in multiple displacement amplification in the upcoming period have helped to get control this crucial step but no bioremediation studies is still under study and experimenting the direct shotgun sequencing of SIP based metagenomes. Still, this methodology has a scope in the future.

The foremost one to correlate SIP with function- and sequence-based metagenomic library screening was Dumont et al. (2006). The soil sample with

incubated with $^{13}CH_4$, $^{13}C$-DNA. Later the results were utilized for the building of a metagenomic library employing a bacterial artificial chromosome (BAC). Also, examining the reserves for key methylotrophy genes led to the discovery of a clone carrying a pmoCAB operon, which encodes for methane monooxygenase. Therefore, the total sequence of the operon was identified by shotgun sequencing. ThepmoA gene which was identified was nearly similar to a *Methylocystis* sp. sequence which was already identified in the same soil (Radajewski et al. 2002). Twelve more different presumed genes were identified on the same clone (Dumont et al. 2006), three of which take part in $C_1$ metabolism. Marked methylotroph communities were also probed taxonomically and genetically by 16S rRNA gene DGGE fingerprints and also sequencing the dominant DGGE bands. In addition to *Methylocystis*, the methanotrophic genera *Methylobacter* and *Methylocella* were also identified together with sequences similar to Bacteroidetes and γ-proteobacteria.

The major disadvantage linked with SIP is observed to be producing excessive concentrations of labeled substrates established during incubations in contrast with concentrations that occur in the sample collection site. High concentrations of these labeled substrates are mandatory to obtain adequate yields of labeled DNA for metagenomic probing studies. One of the main drawbacks of SIP is the restricted availability and high cost of marked substrates. SIP consists of many work-intensive and low-throughput recent techniques. These factors might hinder the examination of an abundant number of replicates to permit statistically valid comparisons among treatments of various environmental samples. The use of robotics and automation could aid in increasing the throughput of SIP gradient partitions and evaluations in the upcoming generations. Another limitation of SIP is the need to integrate many methodologies and technologies from many disciplines of not only omics technology but from many other areas. Fluorescence in situ hybridization (FISH) is an alternative technique frequently emphasized to taxonomically recognize microbial cells using rRNA-targeted oligonucleotide probes and for DNA analysis (Fig. 15.3).

## 15.8 In Silico Tools for Identifying Key Genes from a Microbiota

The amplified sequences can further be utilized for species annotation. In silico analysis of the bacterial genome can predict the required metabolic pathways for the biodegradation of contaminants and helps to give in-depth analyzed data of the metabolic cluster of a particular bacterial community. For instance, the entire genome of *Ralstonia eutropha,* Strain JMP134 (presently called as *Cupriavidus necator* JMP134), which makes use of different kinds of aromatic and chloroaromatic compounds as energy resources, was sequenced and many genes coding the enzymes involved in the degradation of different types of contaminants were found. Next-generation sequencing techniques involve a variety of computational tools for genome sequence assembly. The commonly used gene identification system for microbial communities is GLIMMER (Gene Locator and Interpolated Markov ModelER), which finds the coding region on the genome based on

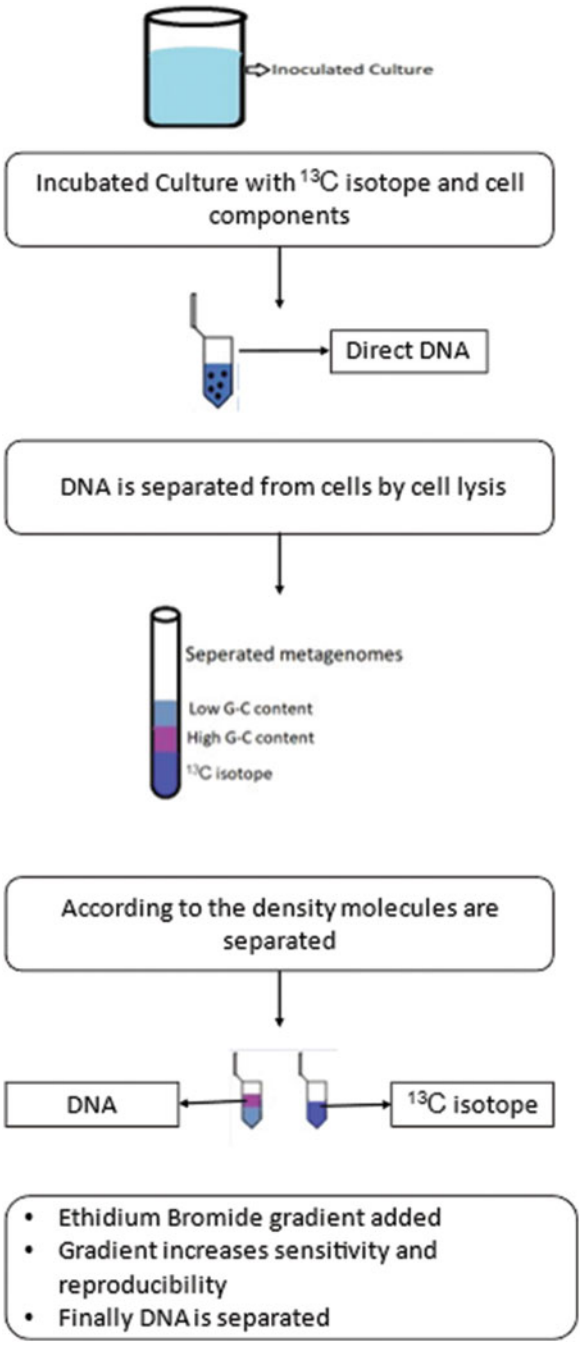


**Fig. 15.3** Metagenomic analysis using stable isotope probing

incorporated Markov models. The identified coding region sequences are examined manually or by automatic annotation software to detect the similar sequences. The common sequence assembly tools are SSAKE, SOAPdenovo, AbySS, and Velvet and automatic annotation software include RAST, BASys, WeGAS, and MaGe/Microscope (Arora and Bae 2014). Computational examination of the genome of few species of *Pseudomonas* showed the existence of the following metabolic pathways for degradation of aromatic compounds are the ortho pathway which facilitates the breakdown of protocatechuate and catechol genes, for the *pha* genes the phenylacetate pathway is executed, and finally the homogentisate pathway which uses *hmg* genes and the gene groups which are categorized for the consumption of N-heterocyclic aromatic compounds that is the nic cluster and a central meta-cleavage pathway which is run by the pcm genes of the genomes in the microorganisms were also examined and identified. Whole-genome sequences can identify genes and their functions and also for discovery of new enzymes. By the combination of genomic and proteomic advents will give new insights into breaking down of substrates in depth. Metabolic, genomic, and proteomic applications were used to build a fully finished and unified pathway for breaking down pyrene in *Mycobacterium vanbaalenii* species and found more than 20 enzymes that were used to construct a pathway for breaking down pyrene. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology is used for working hierarchical divisions. In a study of diesel contaminated soil metagenomic analysis, more than 2500 million sequence reads were obtained, and more than 20 Gb nucleotides were produced. There were more than four million ORFs predicted by the Meta Gene Mark software, and two million ORFs were obtained after the deletion of recurring genes. The complete ORFs of 560 bp (on average) accounted for approximately 20% of the nonredundant open reading frames (ORFs). Around 7 hundred thousand ORFs were categorized into four major pathways like cellular activities, environmental and genetic data gathering, and metabolism. Utilization of carbon, nitrogen, and similar metabolic processes like the xenobiotics degradation lipid, carbohydrate, and amino acid utilization accounted for around 67% of the four main pathways (Kim et al. 2007). In silico tools have helped to identify key genes which can identify microbial communities and its potent degradation pathways. Simplified metagenomics examination Shell for microbial communities is a free metagenomic investigation pipeline that displays the information and execution methods of the construction of a microbe with SmashCell. This can be used for data compiled from the sequencing technology of Sanger. This enables applications like assembly and gene investigation for important metagenomic activities. This also offers methods for quantifying phylogenetic and functional mixtures, comparing different metagenome compositions, and producing working virtual images of these test runs. This offers Arachne and Celera modified variable sets and metagenome for identifying protein-coding genes on the specific proteome. MetLab is a bioinformatics tool which assists in designing metagenomic setup by studying the sequence depth of microbial communities. FAMLI is a free source software package at https://github.com/FredHutch/FAMLI. It identifies protein coding sequences from shotgun sequencing data. The UM-BBD-Pathway Prediction System (PPS) comes under the UM-BBDat http://umbbd.ethz.

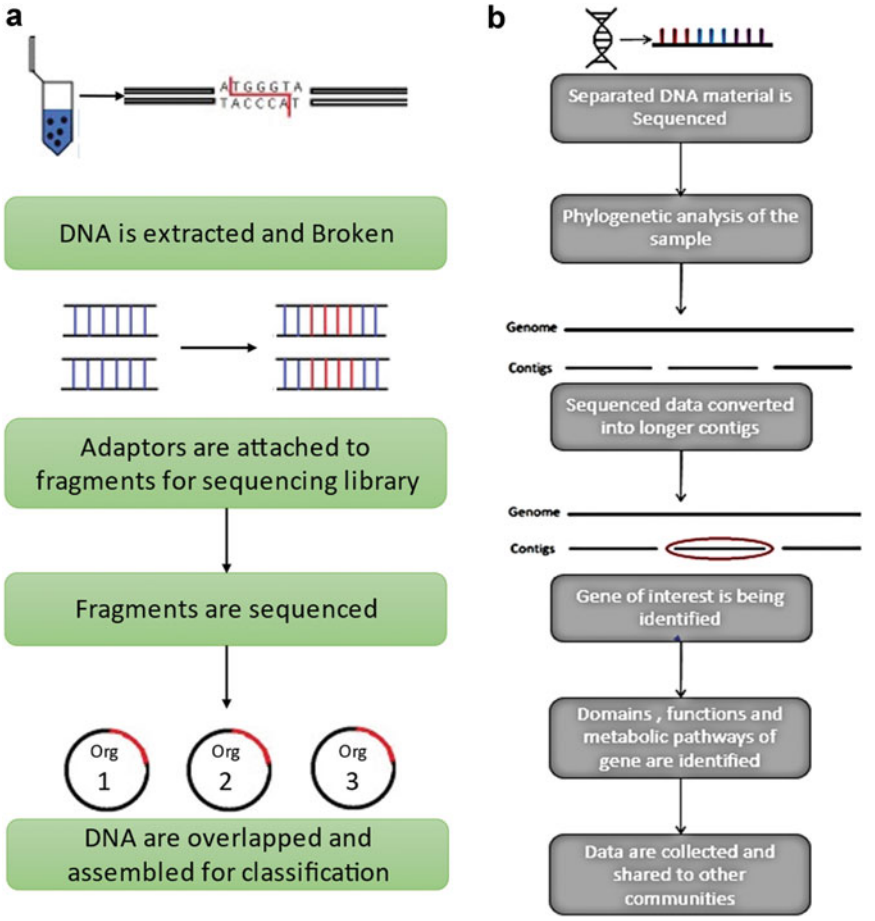


**Fig. 15.4** (**a**) Sequencing of microbial communities which further helps in classifying the sample species. (**b**) A detailed flowchart of analysis genomic and proteomic data using sequencing and in silico tools in metagenomics

ch/predict/. The PPS can be utilized to anticipate metabolic pathways for degradation of pollutants by microbes (Fig. 15.4).

## 15.8.1 Automatic Annotation Software

### 15.8.1.1 RAST

Full form of RAST is Rapid Annotation using Subsystem Technology. RAST is open free server where it shows functional analysis and phylogenetic of metagenomes. This software produces functional sequences that are linked to metagenome by comparing the database with amino acid and nucleotide. This

software analysis the metagenome and also has options to compare targeted metagenome. This software was developed in Argonne National Laboratory by the university of Chicago on 29/11/2016. This software contained more than one million collection data as sets and more than 40 tera base-pairs of data. More than 15,000 data sets are in free access to public. In this software, quality control of metagenome, comparisons among metagenomes, and annotation of metagenome are done by several combinations of bioinformatics tools. Processing of amplicon (16S, 18S) sequences and Metatranscriptome (RNA-seq) sequences can be also done in RAST. RAST software cannot identify noncoding regions in eukaryotic cells therefore RAST software cannot be used for eukaryotes.

RAST can be divided into five stages:

#### 15.8.1.1.1 Data Hygiene

- Quality control is done in this stage. The low-quality area is removed.
- Sequencing error is rectified by DRISEE. The abbreviation is Duplicate Read Inferred Sequencing Error Estimation.
- Bowtie aligner is executed to show a screening of reads.

#### 15.8.1.1.2 Feature Extraction

Gene sequence is being determined. RNA sequence is identified by BLAST search.

#### 15.8.1.1.3 Feature Annotation

Functions and annotation of metagenome are identified. A cluster of proteins is produced by the software up to 90% identity level. The largest cluster of proteins is selected and similarity is analyzed. For rRNA, the cluster is made up to 97% identity.

#### 15.8.1.1.4 Profile Generation

Data is being interpreted by RAST software and the profile of metagenome is produced.

#### 15.8.1.1.5 Data Loading

Data is loaded and displayed.

### 15.8.1.2 BASys

BASys (Bacterial Annotation System) is an open free server used for annotation and comparison of bacterial gene. Using this software four million base pair DNA sequencing can be done in 1 day. Because of this website, a greater number of bacterial genomes were fully sequenced. In 2013, 2700 fully sequenced bacterial gene was deposited in GenBank. BASys was the first open public server with bacterial annotation. BASys was developed in the year 2005. BASys server will accept only raw DNA sequence data or proteomic DNA data. BASys uses methods which fill the remaining annotations of data by submitting the input sequence to other similarity searches of given sequence and analyses of sequences in genome projects.

#### 15.8.1.3 WeGAS

The full form of WeGAS is web-based microbial genome annotation system has tools which includes systems which helps in the prediction of genes, gene similarity search, promoter and protein motif analysis, browsing of different genomes, gene ontology investigation using the COGs and GO, and analysis of anabolic and catabolic pathways with other microbial databases. Primitive data and latterly obtained information from genome analysis plans can be controlled with the WeGAS database system, and analyzed data can used to interpret the results, counting the particulars on each individual gene and concluding genome flowcharts, graphical and pictorial representations, are provided by its imaging software elements. A pie-view browser which shows the round shaped maps of contigs and a COG-GO combination types of browsers are exceptionally useful in analysis of activities. Renowned worldwide open access microbial genome databases can be bought in, explored, and read using the WeGAS database. WeGAS can be directly accessed at website http://ns.smallsoft.co.kr:8051. WeGAS works on the features similar to other different elucidation systems like the Pedant, ERGO, and GenDB. WeGAS was expanded to deeply examine bit by bit and instinctive web portals known to scientists working on genome-based extrapolations. Distinctive features such as a COG-GO mixture of tools displays the ontology inspection results at the same time, and a pie-view browser, which predicts the final blueprint for any genome prediction. WeGAS has been examined through practical genome projects using some bacteria of Vibrio and Streptomyces species and other microorganisms including various practical industrial microbial genome projects was improvised. WeGAS uses a gene-elucidation database provided by Compugen flat file for gene ontology analysis. The WeGAS database consists of data ranging from contigs to practical examination of the gene set for each genome examination activity. The portal of each analysis tool is probed to run each tool and also to visualize the results finally monitoring the progress. The outcome is saved in the WeGAS local directory system.

#### 15.8.1.4 MaGe

Magnifying genomes is a microbial genome elucidation system which is based on relational directories in bioinformatics which consists of data related to bacterial genomes and also acts as a web portal to complete genome elucidation projects. This system allows one to commence the elucidation of a genome at the initial stage of the finishing part of genome analysis. It consists of user interfaces to manually annotate the genome. MaGe is also associated with various well-known biological databases and systems over the world. MaGe can be used in the unification of annotation data from bacterial genomes coupled with gene coding reelucidation operation using other gene models of bacteria, exploration, and reconstruction of metabolic of pathways of any bacterial genome can be done using MaGe. The product produced after gene expression can also be analyzed. MaGe can be obtained at the website http://www.genoscope.cns.fr/agc/mage. The FASTA sequence of the genome is first analyzed to identify the coding region of any metabolic gene expression sequence. The analysis is based on codon utilization detection to frame the gene models. tRNA

gene and rho independent transcription sites can also be identified. The collection of annotated EC characters can be used to frame metabolic pathways. MaGe has many dynamic web pages where required data can be accessed. MaGe also uses comparative genomics which automatic annotation and manual examination have quality checks also. The new variety of the complete genome sequence is always accessible. Metabolic pathways using recognized EC numbers are dynamically drawn using KEGG web database. Human assisted process is also involved to correct mismatched, inconsistent, and pseudogenes. The final chromosome construction is compared with a completely phylogenetically constructed genome. Anonymous users can login as guest to annotate and analyze the genome. Based on the organism's numerous number of functional classifications can be integrated into the database.

## 15.8.2 Sequence Assembly Tools

### 15.8.2.1 SSAKE

SSAKE which is abbreviated as Short sequence assembly by progressive K-mer search and 3′ extension is a tool for de novo assembly of a large number of low reads of genetic materials. It emphasizes a progressive search throughout the title tree for finding the longest overrun between all sequences using a pairwise examination. The output of the assembler is contigs. It collects the entire reads of a genome and is used for gathering variants and also for structural detection. SSAKE was used in profiling T-cell metagenomes and targeted de novo assembly. There are limitations in normal de novo sequencing due to the existence of a huge number of ubiquitous genomes which leads to the inability of characterizing short DNA sequences. In SSAKE a title tree is used to arrange the sequence. The large read depth and short sequence length helps in resequencing where a known quotation is used as a template. Millions of identical short sequences are collected for targeted assembling. The sequences data stored in hash table are cycled through these programs using a title tree which has lengthiest possible k-mer between any two genomic sequences. This minimizes sequence misassembles and the nonrepetitive portions can be represented clearly. For analyzing more complex genomes SSAKE reads can be combined with Sanger reads. This tool can be availed from a bioinformatical company Solexa Ltd. and generates short DNA sequences of 25 nucleotides each. This tool can help in distinguishing overlapping and nonoverlapping genes to identify short sequences in the genome. SSAKE tool has given a deeper and clear insight into complex genomes especially in identifying bacterial communities.

### 15.8.2.2 SOAP De Novo

SOAPdenovo is a new short-read assembly tool which can be used to build a de novo draft assembly for the human-sized large genomes. This application is specifically designed to assemble Illumina GA short-read sequences. It builds fresh pathways for constructing hint sequences and execute perfect inspection of undetermined

genomes in an economically feasible way. The operating procedure of this software is as follows:

- *Accessing global information using configuration file*
  Multiple read sequence files are generated for big genome sequence projects and is generated using multiple sequence libraries. Each library section item must be tagged with these following items.
  - Avg_ins.
  - Reverse_seq.
  - Asm_flags = 3.
  - Rd_len_cutoff.
  - Rank.
  - Pair_num_cutoff.
  - Map_len.
    The file is accepted by the assembler and read using two computational tools FASTA and FASTAQ.
- *Running the program*
  After the assembler accepts the file, the layout file is available to execute the assembler.
- *Manipulation of options*
  The k-mer size is determined and multithreads are used to solve tiny repeats. Low frequency k-mers are removed and the edges are removed. The intra scaffold gap is closed and the high coverage contigs are unmasked before scaffolding process. The length difference is calculated and the filled gaps are also examined. The contig length which has a minimal length is used for the process of scaffolding.
- *Results*
  The output is obtained as assembly outcomes. Contig sequences without using mate pair data are obtained and extraction of final contig sequences by breaking down the scaffolds.
- *Other adjustments of required parameters*
  - *Setting K-mer size*
    Larger k-mers have higher ranges of uniqueness and the k-mers are set between odd numbers like 13–31. There is a requirement for deep sequencing and longer read length.
  - *Setting library rank*
    Pair end libraries are used with insert sizes from larger to smaller to construct scaffolds. The pair in each file provides maximum physical coverage of the genome.

This tool can be accessed at the website (http://soap.genomics.org.cn/soapdenovo.html). SOAP de novo runs on a 64-bit Linux system and can be used to sequence plant, animal, and also bacterial and fungal communities. It reduces memory consumption in graph creation and improves closing for optimizing study of large genomes. Though it is largely used to assemble eukaryotic genes, bacterial

communities like *Staphylococcus aureus* and *Rhodobacter sphaeroides* were analyzed and the results were interpreted.

#### 15.8.2.3 ABySS

ABySS (Assembly by short sequences) is a parallel, paired end, de novo sequence assembler which is used to design shorter reads of genome. This tool can avoid genomic information stack overflow. It consists of bloom filter assembly which can assemble large genomes and also ABySS can eliminate the possibilities of misassembles of genome sequence. Multiple libraries of different fragment sizes can be assembled. Tandem segmental duplications are used to avoid any further misassembles of genome. Total misassembled contigs are only approximately 1% of the whole-genome sequence. The algorithm which is ABySS uses proceeds with two stages.

1. The possible substrings from k-mers are given raised from obtained sequence reads.
2. Mate pair data is used to elongate the contigs by sorting out obscurity in overlaps.

These tools are designed to execute using a single processor. Early features of ABySS include the depiction of a de Bruijn graph which enables for parallel computation of assembly algorithm. The programming language used by this software is C++ and works on the operating system Linux. It is of cost free. Short dead-end branches are eliminated by the algorithm. To disperse the de Bruijn graph the issues which must be resolved are as follows:

1. Determining location of k-mers.
2. Storing adjacent k-mer information.

This tool can be accessed at this website: http://www.bcgsc.ca/platform/bioinfo/software/abyss.

#### 15.8.2.4 Velvet

Velvet is a tool which is used for de novo assembly and short-read sequencing alignments. It mainly focuses on manipulation of de Bruijn graphs for removing errors in genome sequence assembly. This tool was initially released during 2008 and operates on Linux systems. Without the loss of information, the sequences are compressed and simplified using manipulation of de Bruijn graphs. To save the memory costs, the nodes generated during the sequence assembly are merged to represent as one. Velvet recognizes the errors like tips, bubbles, and any misconnections. Based on the parameters given by the user the errors and repeats are removed. More brief information on the errors is as follows:

1. Tips—the nodes are considered as tips and must be eliminated since it disconnects the ends.

2. Bubbles—biological variants can cause creation of two distinct pathways. These errors are removed using the tour bus algorithm.
3. Misconnections—any errors after applying the algorithm are rectified.

The velvet tool applies the following commands.

- *Velveth*—helps to construct dataset for velvetg.
- *Velvetg*—helps to build de Bruijn graph from k-mers obtained from velveth.

These two programs are always used together.
Drawbacks

- Use of command line interface which gives the users with difficulties.
- Has issues with errors and repeats.
- To get more information it must be combined with other software.

Steps involved in annotating sequences using velvet software

1. Paired end files preparation.
   Requires FASTA and FASTAQ datasets to merge the given data as a single file. This step is done to prepare longer contigs in repetitive regions of the genome which is to be probed. This tool can detect and handle unpaired reads.
2. Categorizing the reads.
   The long and short reads are distinguished to reconstruct the path for assembly. The paired end libraries which are of different lengths are distinguished. Short reads can be separated into different categories. The same insert lengths can be categorized together.
3. Hash length selection.
   The single most important parameter which must be chosen carefully. A good hash length is between 21 bp and minus 10 bp.
4. Running velveth and velvetg.
5. Hash length is optimized by repeating step 4.
6. Optimization of other parameters.
   The coverage cutoff, expected insert length, sequence complexity, length, number of reads, and its quality. These are the datasets in which optimization must be performed individually.
7. Collecting data.
   Velvet optimizer prints out the output file which are left out in the final velvet assembly.

## 15.9 Development in Future and Significance of Metagenomics

Metagenomics has given information on the microbial diversity and the metabolic potential of the microbiota can be harnessed to acquire valued bioprocesses. Many industries release contaminants as untreated waste materials into the environment. The harmful chemicals enter into the food chain and water bodies and result in biomagnification of the toxins in the food chain. Different microorganisms which have unique metabolic pathways have been used to utilize toxic materials for cleaning up the environment. Using metagenomics in bioremediation the genes which can breakdown the pollutants can be identified. The complexities of microbial genes are grouped up by metagenomics approaches. Notable genomic, transcriptomic, and metabolomic techniques have been integrated together is identify genes as well as novel findings like the protein, lipids, carbohydrates digesting enzymes and the genes which they encode, and new biocatalysts have been found by applying metagenomic based approaches.

The "Omics" is an upcoming molecular biology technique which has led to the recognition of several microbial processes and metabolism in the aspect of microbes which includes the study of the genome, proteome, the study of pathways and enzymes responsible for metabolism, and the genes which transcribes the enzymes responsible for metabolism. Lack of availability of nutrient resources for the growth of microbes and suitable microbiome, and degrading pathways required to degrade the harmful pollutants might reduce bioremediation success. The remediation process gets increased rapidly by the increase of biostimulation and bioaccumulation in polluted wastewater. Addition of nutrients increases biostimulation and enhances the growth of microorganisms. Microbes are abundantly present in different types of ecosystems. Pollutants which are broken down by microorganisms are present in polluted areas and the consumption of required nutrients like nitrogen, phosphorus, and potassium as growth and development enhancing foods and metabolic pathways of microbes which are going to degrade the pollutant is dependent on the level of contamination in the environment. A microbial community in polluted areas is more efficient than pure isolates.

Combining various high-throughput techniques will get to have a depth view of microbial communities and highlights the connections between species and also provides the maximum information on its diversity, mechanism of gene expression, pathways of protein generation, and chemical transformation in polluted environments. Stable isotope probing (SIP) is an upcoming technique which proceeds by adding heavy isotope-labeled compounds to be degraded and allowing the microorganisms to consume it and incorporates the labeled atoms into the components of cells such as phospholipids, RNA, and DNA. In the method of DNA-SI Probing, all DNA from a sample which heavily treated is obtained by extraction and then centrifuged in CsCl gradient tubes to dissolute the labeled heavier DNA from the unlabeled lighter DNA. This technique has more use by helping in identifying the functionally active microbes, especially which are associated with effluent degradation, and the most recent review provides the study of the potentiality and the power of combining SI Probing technique with

metagenomics. SIP-metagenomic methods of analysis of impure substrates gives way to analyze the species having genes that immediately respond to the contaminants to be separated from the enormous amount of collective genetic data that is obtained from the soil sample. The relationship between the taxonomic lineages and the mechanism of the pathway in which a microbial community functions is being observed by combining SIP and high-throughput sequencing techniques (Bell et al. 2015). The advanced technologies of the RNA-SIP will provide a clear view on how the addition of pollutants affects the rate of transcription. At present, the gradients which contain CsCl helps in the separation of labeled and unlabeled nucleic acids is a difficult procedure and this reduces the number of samples that can be processed within a given time for any study.

In polluted environments, metagenomics is used to examine the level of contamination by cross checking the polluted substrates with nonpolluted reference substrates. These types of comparative studies are used to understand the genes responsible for distinguishing a contaminated environment from other pure systems. The upcoming major projects in metagenomics are the identification of a core microbiota. Genes which are common across any individual environment and also across many numbers of different environments can be identified using metagenomics. Having a clear view on the existence of important micro biomes, different environments the conserved regions can be aligned by sequences and the exact variability between environments can then be examined. In the bioremediation point of view, it is necessary to examine the response of critical genes of the microbial community when a contaminant is introduced to observe successful bioremediation. Genes which get expressed outside the common identified core gene must later be the result of other stimulations from the environment by any other biological or geochemical process.

The upcoming studies on genomes relies on much smaller genetic information in samples obtained from the environment. Evolution of the microbes is observed by the rapid growth of cultures. Studies on groundwater contaminated by metals and other substances showed that more than 20 years of effluent stress has drastically decreased species and metabolic diversity to a very lower level of complexity of microbes. The metabolic pathways which were identified are generally more than five to eight times fewer OTU combined with the decrease in metabolic complexity. These results were obtained from many contaminated sites which are near the industries. Observing evolutionary effect on genes in environments which are polluted over the long term will enable to understand the required treatment of extremely polluted sites, although the explanation of enormous amounts of data of microbial genes will first require sequencing and computational tools. This shows that in-depth and focused metagenomic studies will be a beacon of development of bioremediation technologies.

## 15.10 Conclusion

Anthropogenic activities have led to extreme pollution of the environment and have also given several effects in the food chain, water supply, air quality, soil purity. In order to avoid the drawbacks of the effluents released by the industries, many techniques are recently being employed but still have not reached its mark. Microorganisms have the ability to assimilate complex compounds into simpler ones. This feature can help in breaking down harmful pollutants. Analyzing microbial communities in polluted areas can help us to identify the required metabolic pathways for the degradation of certain pollutants. Metagenomics has proven to replace the old standard methods of analyzing cultures and has provided a more in-depth of view of microbiotas at the genomic and proteomic level. Bioremediation is successful upcoming method which has proved to restore the natural environment only with the use of microorganisms and phytochemicals. Metagenomic analysis can speed up the identification of required microbe for the degradation process. Taxonomic studies are performed with ease with the help of sequencing tools in metagenomics. In silico tools have further improvised the visualization bacterial communities and also can help in the storage of data. These methods resist culturing techniques and use minimal invasive procedures thus helping reduce the cost and time involved in identification. Though high-throughput sequencing tools like the 16s RNA sequencing costs are a bit high it has given in depth view and industries can further rely on these technologies to avoid pollution and identify microbes for bioremediation using these advanced technologies. Thus, metagenomics combined with other omics technologies can help in restoring the polluted environment and give an insight of newer microbial communities to understand evolution and also cut out the major expenses in bioremediation methods.

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# Understanding Bioremediation of Metals and Metalloids by Genomic Approaches

16

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**Abstract**

Several human activities such as urbanization, industrialization, mining activities, agricultural activities, and even transportation lead to the spread and accumulation of many metals and metalloids in soil and water sources directly or indirectly, which harm the health of ecosystems and organisms. Metals and metalloids such as antimony, arsenic, boron, cadmium, chromium, copper, lead, mercury, and zinc appear as contaminants when found locally in concentrations above a threshold level at which they start to exert toxic effects. Bioremediation methods are environmentally friendly, cost-effective, and easy-to-apply and are used to clean up metal and metalloid contaminants from water and soil environments with the help of microorganisms such as bacteria, fungi, algae, and different combinations of such microorganisms. Indigenous microorganisms are especially important as they provide great support to humans in their efforts to remediate the already contaminated ecosystems and to prevent further accumulation of contaminants. Understanding the tolerance mechanisms of microorganisms and the genetic background of the remediation capabilities and discovering novel microorganism species are of importance in the fight against metal contaminants. Genomic approaches shed light on unculturable microorganisms, which have a high potential to accumulate and modify and detoxify metal contaminants in natural environments. Besides discovering tolerant microorganisms, genomic

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studies also reveal genes and their products that contribute to the tolerance and bioremediation processes. Collecting information about genes and genomes that play a role in building heavy metal and metalloid tolerances will help scientists develop more efficient microorganisms that can be employed in the bioremediation of soil and water ecosystems.

**Keywords**

Bioremediation · Metals · Metalloids · Omics sciences · Genomics

## 16.1 Bioremediation

Bioremediation is a process that uses living organisms, especially microorganisms including bacteria and fungi, to degrade or convert toxic materials from contaminated areas into less toxic or nontoxic forms (Chandra and Kumar 2015; Kumar et al. 2018). This process works owing to the natural chemical reactions occurring in organisms, which break down compounds to provide nutrients and obtain energy. The biological mechanisms found in bacteria and plants are used in bioremediation to remove toxic pollutants and restore the ecosystem to its original form (Ayangbenro and Babalola 2017; Kumar et al. 2021a, 2022). Although, nowadays, bioremediation methods have been used for decontamination of several areas such as soils, subsurface soils, groundwater, and surface water, the first improved methods were to remediate petroleum hydrocarbon pollution in order to transform them into less toxic chemicals. Moreover, there are several traditional methods depending on chemical processes, which are used to eliminate contaminated areas, such as high-temperature incineration or chemical decomposition (Kumar et al. 2021b). Generally, at the end of traditional processes, waste products, which again contain toxic materials, are neither cost-effective nor eco-friendly. In this context, biotechnological improvements, especially bioremediation, have immense potential to remediate contaminated ecosystems for pollution protection, waste remedy, and management (Azadi and Ho 2010; Hatti-Kaul et al. 2007).

Processes of bioremediation that depend on technical principles are complex systems for efficient degradation to be sustained. There are three technical principles that affect the rate of bioremediation: bioavailability, bioactivity, and biochemistry of the systems. Bioavailability can be referred to as the amount of pollutants taken up and remediated by microorganisms. During the bioremediation process, microorganisms' contaminant remediation capacity depends not only on their ability to take in contaminants but also on mass transfer. If the mass transfer reaches a limiting factor, then an increase in the microbial transformation capacities does not lead to higher biotransformation (Boopathy et al. 1998).

Moreover, the ability of organisms to effectively decompose various chemicals depends on the type of microorganism and other environmental factors such as temperature, pH, moisture content, carbon and energy sources, contaminated area,

types of contaminants, concentration of contaminants, types of chemicals, and the chemical structures of contaminants that affect the efficiency of bioremediation.

Organisms can biodegrade toxic chemicals into less toxic forms owing to their enzyme systems such as oxidoreductases. Detoxification of toxic pollutants by various bacteria, fungi, and plants through oxidative binding is mediated by oxidoreductases (Gianfreda et al. 1999). Microorganisms convert various toxic compounds into less toxic compounds owing to either aerobic metabolism or anaerobic metabolism, which depends on oxidation and reduction reactions. These reactions are significant for many chemicals to undergo bioremediation. Microorganisms obtain energy to break down chemical bonds and help in electron transfer owing to biochemical reactions in the enzyme system (Chandra et al. 2017; Kumar and Chandra 2018; Kumar and Shah 2021). During oxidation and reduction reactions, eventually, pollutants are converted into nontoxic or less toxic chemicals.

In contaminated environments, such as soil, the level of oxygen might be extremely low. In such a place, oxygen may poorly diffuse to other parts of the soil, and, in this situation, oxygen molecules might be quickly consumed by aerobic microorganisms. If there is a lack of oxygen molecules in the environment, then oxygen can be infused into low-oxygen locations to stimulate aerobic biodegradation. Moreover, in these conditions, biodegradation may happen naturally through the anaerobic metabolism of microorganisms, especially that of many bacterial strains. In an anaerobic environment, for redox processes, bacteria, especially anaerobic bacteria, can use different elements such as iron, nitrate, and sulfate to oxidize chemical contaminants. In addition, some bacteria have not only an aerobic mechanism but also an anaerobic mechanism to survive even in the absence of oxygen. In this manner, they could continue biodegradation.

### 16.1.1 Methods of Bioremediation

In situ and ex situ bioremediation are the two major methods of bioremediation. In situ bioremediation is the use of microorganisms' metabolic activity to remediate a pollutant at a contaminant site (Stroo 2010). Ex situ bioremediation techniques rely on transporting contaminants from a contaminated area to another place for remediation.

In situ, which means in the original place in Latin, is applied if there is no chance to transfer pollutants from a contaminated area, for instance, when pollution affects a wide area (Concetta Tomei and Daugulis 2013; Mosca Angelucci and Tomei 2016; Xu and Lu 2010). Hence, in the case of in situ bioremediation, pollutants in contaminated areas, which are covered with soil or water, are not required to be moved to other places, and, so, this technique does not require excavation processes. This is why in situ bioremediation is mostly the more preferred method than ex situ bioremediation. Besides, in situ bioremediation is of lower cost and is easily applicable. In situ bioremediation methods depend on making conditions available to stimulate bacteria for their growth like other methods of bioremediation (Chandra and Kumar 2017a, b). However, in situ bioremediation has several disadvantages

such as being time-consuming, changing microbial activity depending on uncontrollable environmental factors, and requiring more energy sources for microorganisms to grow. When these requirements are not available, the rate of bioremediation efficiency may reduce depending on the microbial population. During in situ bioremediation, contaminants are consumed by microorganisms that live in the contaminated area as an energy source..

In situ bioremediation that relies on aerobic degradation includes bioventing, which is pumping into a contaminated area either of the following:

(a) Air.
(b) Hydrogen peroxide.

Usually, the bioventing technique is used in contaminated areas with hydrocarbons such as petroleum products (Höhener and Ponsin 2014).

Landfarming, composting, biopiles, and bioreactors are four ex situ bioremediation techniques.

(a) The landfarming technique is a basic process that depends on periodically turning piled contaminated soil to increase the biodegradation efficiency of indigenous bacteria till the contaminants are broken down. Moreover, compared to other techniques, landfarming requires less budget and less equipment for the process.
(b) The composting technique is the integration of contaminated soil with nontoxic organic materials such as fertilizers. Adding other materials such as straw, fertilizers, and agricultural wastes supports the growing bacteria in degrading chemicals.
(c) Landfarming and composting are combined in biopiles (von Fahnestock et al. 1998). This technique relies on several components such as aeration, irrigation, adding nutrients, leachate collecting system, and treatment bed. This technique may be used to remediate environmental pollution caused by hydrocarbons such as petroleum. This technique is a kind of landfarming that checks the physical losses of pollutants by leaching and volatilization.
(d) A bioreactor is the best option of the ex situ bioremediation technique to be applied to heavy metal contamination due to controlled environmental conditions in bioreactors. One of the main benefits of bioreactor-based bioremediation is the ability to precisely control bioprocess parameters such as temperature, pH, and oxygen.

### 16.1.2 Organisms Used in Bioremediation

Many contaminants have been reported to be degraded by microorganisms. Although some algae and fungi can be used for bioremediation, bacteria have gained attention among other microorganisms for their rapid growth and metabolism, lower environmental needs, quick adaptation abilities, and large biodiversity. In the

process of bioremediation, aerobic microorganisms such as *Pseudomonas* spp., *Mycobacterium* spp., *Sphingomonas* spp., and *Rhodococcus* spp. could be used to degrade several contaminants. These contaminants could be used as sources of carbon and energy by these microorganisms. In the first bioremediation of contaminated areas with petroleum, *Pseudomonas putida* was the first bacterium to be employed in 1974 (Prescott et al. 2002).

Cybulski et al. (2003) investigated the ability of different strains of the Bacillaceae and Pseudomonadaceae families to biodegrade hydrocarbons and emulsified hydrocarbons. The results showed that *Pseudomonas aeruginosa* performed the best than did other bacterial strains for biodegradation of hydrocarbons.

Moreover, *Pseudomonas* spp. could use bioaccumulation and biotransformation of not only hydrocarbons but also benzene, anthracene, and polychlorinated biphenyls (PCBs) (Cybulski et al. 2003; Kapley et al. 1999). *Burkholderia cepacia*, *Mycobacterium* spp., and *Rhodococcus* spp., have been reported to utilize benzanthracene, chrysene, fluoranthene, and pyrene as carbon and energy sources (Kanaly and Harayama 2000; Watanabe 2001).

Heavy metals can be found in soil and water because of many human activities, such as electroplating, steel production, and chemical processing, or as natural components (Chaturvedi et al. 2015; Zhou et al. 2016). Many heavy metals such as copper (Cu), zinc (Zn), and cadmium (Cd) have been studied by several researchers since metals and metalloids are not only commonly found in the environment but also necessary for many plants (Chandra and Kumar 2018; Kumar and Chandra 2020a; Kumar 2021; Kumar et al. 2021c, d). At that point, if these metals and metalloids, which are necessary for plants for their metabolisms, occur in larger amounts, then they could be toxic to plants and their consumers. In addition, even at low concentrations, some heavy metal ions could be toxic to humans, for instance, if lead (Pb) accumulates in the human body, then it can harm several systems and organs such as the nervous system and kidneys (Abadin et al. 2007). Other toxic heavy metals such as nickel and cadmium are responsible for carcinogenic and kidney damage, respectively. This is why bioremediation of contaminated areas with heavy metals is significantly important for all living organisms. *Bacillus* spp. have been reported to utilize copper (Cu) and zinc (Zn) as a source of carbon and energy, respectively. *Citrobacter* spp. have the ability to detoxify heavy metals such as cadmium (Cd), uranium (U), and lead (Pb) (Rajendran et al. 2003; Yan and Viraraghavan 2001).

## 16.2 Omics Sciences

The huge diversity and metabolic activities of microorganisms are at the heart of the bioremediation process. Because of their technical limitations, previous studies generally focused on traditional bioremediation methods rather than molecular methods. There are several unculturable organisms in nature. These might be

analyzed owing to the latest improvements in molecular techniques (Handelsman 2004).

Omics sciences have provided more knowledge about microbial communities in their environmental niche using the analysis of DNA, RNA, proteins, or metabolites not only for each organism but also for all the communities at the same time (Rodríguez et al. 2020; Gutierrez et al. 2018; Kumar et al. 2020). Today, researchers have started to study systems biology such as genomics, transcriptomics, proteomics, and metabolomics of the bioremediation systems to clean up contaminated areas owing to improvements in the techniques of molecular biology or systems biology. Systems biology can be defined as the integration of omics-based systems. Systems biology does not aim to replace any of the classical techniques from biochemistry or genetics but instead to provide a set of organizing principles that integrate all these methods. The workflow in systems biology generally consists of repetitive cycles of experiments, data acquisition and analysis, modeling, and computational analysis. Moreover, each of these processes is supported by software tools.

Improvements in molecular techniques such as proteomics, transcriptomics, and metagenomics enable the possibility of development of new strategies and opportunities in environment management (Plewniak et al. 2018; Rodríguez et al. 2020; Kumar et al. 2021e). Genomics, transcriptomics, proteomics, metabolomics, and fluxomics are several types of omics approaches for microbial communities' analysis. Generally, a single omics study cannot show the functional activity of all microbial communities; therefore, multi-omics approaches are commonly used to study microbial communities.

Genomics is an interdisciplinary study based on evaluating the structure and function and mapping of genomes. In short, this is a study of a set of genes, the inheritance substance. The researched areas under genomics include functional genomics, metagenomics, neuromics, personal genomics, and epigenomics. Although there are several advantages of genomic studies, there are also several disadvantages. Although genomics and DNA sequence analysis provide unique data, it is difficult to predict the final biological effect of variations in DNA by only genome analysis because of posttranscriptional and posttranslational changes and epigenetics.

Transcriptomics is an interdisciplinary study of the complete set of RNAs (transcriptome) encoded by the genome of a specific cell or organism at a specific time or under a specific set of conditions. A transcriptome is the total mRNA in a cell or organism and is the template for protein synthesis in translation in a given time. Gene expression microarrays measure packaged mRNA (mRNA with the introns spliced out) as a summary of gene activity.

A proteome is defined as the set of all expressed proteins in a cell, tissue, or organism (Theodorescu and Mischak 2007). Proteomics aims to characterize information flow within the cell and the organism, through protein pathways and networks, with the final aim of understanding the functional relevance of proteins (Vlahou and Fountoulakis 2005). Proteomic approaches could be used to understand the physiological responses to changing temperature, xenobiotics, and many other

stresses of microorganisms (Lacerda and Reardon 2009). Proteomic approaches could be used in the evaluation of physiological changes that microorganisms tolerate during bioremediation.

Metabolites are the end products of cellular regulatory processes. Metabolomics has more advantages than do genomics and proteomics. The levels of metabolites are the final response of biological systems against genetic and environmental alterations. The aim of metabolomics is to study the metabolic components such as enzymes, substrates, and their products.

Metagenomic approaches are now being used to better understand microbial communities, their composition, and their roles in bioremediation in polluted environments. Metagenomic approaches give us reliable knowledge about genes or enzymes, which are part of the detoxification and degradation of environmental pollutants (Kumar and Chandra 2020). Genomic approaches enable unculturable microorganisms to be picked up and cultivated according to their metabolic features. Metagenomics offers significant potential for bioremediation because it allows for the development of approaches in a variety of interconnected ways. Metagenomic approaches could be used to identify potential degrader microorganisms for bioremediation of any contaminant or gene responsible for the detoxification and degradation of any specific hazard. Moreover, metagenomic approaches are used to evaluate a wide range of microbial diversities at different polluted sites affected by specific pollutants. When compared to other traditional bioremediation approaches, metagenomic approaches produce more successful results with higher degradation and detoxification rates (Tripathi et al. 2018). In addition, metagenomics provides metagenomic databases, which will provide a huge supply of genes for the production of new strains of microorganisms for targeted bioremediation.

A short time ago, the genome of *Pseudomonas* spp., which is KT2440-analyzed, showed the presence of genes encoding several enzymes such as oxidoreductases, dehydrogenases, and oxygenases, which play important roles in the degradation of a wide range of chemicals. In addition, many other studies have shown that microorganisms play a role in the bioremediation of heavy metals, relying on the results obtained from microorganisms' whole-genome sequencing (Belda et al. 2016; Dangi et al. 2017).

Extraction of nucleic acid from samples is the first step in every genomic study like metagenomics. Bacteria, archaea, eukaryotes, and viruses are common components of a composite microbial community. Hence, the extraction of nucleic acid includes extracting nucleic acid from all organisms existing in the sample. Studying contaminated areas that contain eukaryotes using metagenomic sequencing is expensive not only because of their massive genome sizes but also because of their low gene coding densities. Hence, choosing a community that does not contain eukaryotic forms of organisms to study metagenomics is a critical point.

There are several studies in the literature using genomic approaches to bioremediation. Genomic methods have been used in identification of members of the community and their relative abundances using the technique of next-generation sequencing (Rothberg et al. 2011). Another study is about the evaluation of the effects of bacterial diversity decrease on soil functions and bioremediation in

diesel-contaminated microcosms using the technique of the MiSeq system (Jung et al. 2016).

### 16.2.1 Genomics in Bioremediation of Metals and Metalloids

Heavy metals include around 30 metals and metalloids such as zinc (Zn), mercury (Hg), lead (Pb), cadmium (Cd), copper (Cu), chromium (Cr), Nickle (Ni), arsenic (As), etc., and some of these, such as Zn, Cu, Mn, Cr, and Ni, provide nutritional benefits to organisms as they can play roles as cofactors. Other heavy metals are not defined to be beneficial to organisms. Although they have benefits, they exert toxic effects when accumulated in high concentrations in organisms. They may accumulate in organisms through air, soil, and water sources, which are contaminated by industrial, agricultural, and mining activities, etc. Depending on the concentrations accumulated in organisms, heavy metals lead to different types of health problems. Heavy metals are nonbiodegradable and hence persist in the environment. Therefore, remediation techniques are required to be carried out to either immobilize or extract them. Bioremediation of heavy metals using microorganisms is a good option to achieve this aim when advantages of microorganism applications are taken into account. In order to understand the tolerance, resistance, and remediation mechanisms of microorganisms, genes and genomes of tolerant and resistant microorganisms should be analyzed. The results would help in the determination of the best microorganisms, target genes, and metabolisms to modify for developing high-performing microorganisms. In this section, genomic studies conducted on the bioremediation of different heavy metals will be mentioned.

#### 16.2.1.1 Copper

Copper is an essential nutrient element for organisms, from the lower to the most developed organisms. It plays a role in metabolism as a cofactor of enzymes, especially antioxidant enzymes that fight against the reactive oxygen species. Despite being a crucial element, it causes toxicity when found in large amounts, as valid for other heavy metals that play nutrimental roles. Toxic levels of copper may result from mining and agricultural and many industrial activities such as electronic production, application of pesticides and fungicides, fossil fuel usage, paint production, etc. Wastewater containing copper may end up in water sources. Ramasamy et al. (2020) studied shallow water hydrothermal vents in Portugal and reported the presence of many heavy metals, where adapted and tolerable microorganisms were inevitably detected. They isolated different heavy metal-tolerant microorganisms and sequenced the whole genome of a new species of the *Alcanivorax* genus via 16S rRNA gene sequencing and reported that this bacterium harbors copper detoxification genes, which make it resistant to copper up to 600 μg/mL. Moreover, the bacteria have genes for resistance to mercury and cadmium. The bacteria of this genus are known for their ability to clean up oil spills in sea water. Such multi-heavy, metal-resistant bacteria are of importance to remediation of water sources as well as soil and wastewater..

Andreazza et al. (2011) discovered Cu-resistant bacteria from different sites directly and indirectly contaminated with copper. One site contained copper mining wastes, which had a Cu concentration of 576 mg/dm$^3$. Two soil samples from vineyards were also analyzed, as they were contaminated with copper due to the application of copper sulfates ($CuSO_4$) as antifungals. The soils of these two different vineyards had copper contents of 142 and 207 mg/dm$^3$, respectively. The researchers examined a total of 106 Cu-resistant bacteria for their copper absorption. Later, they analyzed several isolates from these sites by 16S rRNA gene sequencing. The bacteria determined to have the highest biosorption of copper could remove 80 mg/L Cu in a time period of 24 h. This highly resistant bacterium was found to be 99% similar to *Staphylococcus pasteuri*. The researchers concluded that this specific bacterium isolated from the experimental sites was a potential candidate for Cu bioremediation.

A study conducted in a stream proximal to a potential Cu mining area in Canada revealed that while copper concentration in water near the mineralized area was high, the concentration downstream of the water course decreased, which was detected to be a result of the microbial activity in the water body and not physiochemical processes. Metagenomic analyses conducted clearly showed a correlation between copper concentrations and the water microbiome. The dominant bacteria in the upper stream of the water body where the heavy metal Cu concentration was the highest (1.02 mg/L) were detected to belong to the *Gallionellaceae f*amily (van Rossum et al. 2016).

#### 16.2.1.2 Zinc

Similar to Cu, Zn is a heavy metal having nutritional benefits for organisms. Zinc is especially important in the conformation of DNA and as a cofactor of several enzymes. Although effective for living organisms, it has toxic effects and can be harmful to organisms in elevated concentrations. Due to natural processes and human activities such as steel and iron production, mining activities, and production and usage of pesticides and fertilizers, Zn can contaminate water and soil environments (El Sayed and El-Sayed 2020). Zn contamination is present together with other heavy metals. Studies on the bioremediation of heavy metals and microorganisms that are tolerant and resistant to Zn and other heavy metals to be used for bioremediation have been carried out. Genomic studies are helpful in discovering heavy metal-eliminating organisms. An instance of such studies was the one carried out by Kou et al. (2018). They made use of 16S rRNA gene amplicon sequencing to detect heavy metal-resistant bacteria. They concluded that the presence of heavy metals such as Zn and Cu decreased the number of bacteria belonging to the Chloroflexi, Cyanobacteria, Firmicutes, Latescibacteria, and Planctomycetes genera but increased those belonging to the Bacteroidetes, Actinobacteria, Chlamydiae, Nitrospirae, and Proteobacteria (α, β, δ, and γ) genera. Kour et al. (2019) studied the molecular characterizations of zinc-tolerant bacteria via amplification of different consensus and palindromic sequences of 30 isolates and concluded that the bacterium with the highest zinc tolerance was the *Serratia* sp. Studies such as the abovementioned ones are important to determine

microorganisms with high heavy metal resistance, which can serve as potential candidates for developing microbial formulations to be employed in soil for agricultural purposes besides the remediation of soil and water sources.

#### 16.2.1.3 Boron

Boron is an unprevailing metal found only in a few places on Earth. However, it is highly important in many industrial processes such as production of fiberglass, pharmaceutical and cosmetic materials, and glass and enamel, is utilized for safety purposes in nuclear reactors, photography, in agriculture as fertilizers, and for viscosity control in paints and adhesives (Gürgan and Adiloğlu 2021). Boron can contaminate soil and water sources that are in close proximity to boron mines. Studies have revealed boron-tolerant bacteria around boron mines. A mining site in Turkey was sampled to unveil the microbial flora of the site. The highest boron-accumulating bacteria were determined via a 16S rRNA sequencing study. *Bacillus simplex*, *Bacillus subtilis*, and *Rhodococcus erythropolis* strains were found to be the highest boron-accumulating bacteria, which are important because of their regulatory mechanisms to eliminate the toxic effects of boric acids that are otherwise toxic to microorganisms (Miwa and Fujiwara 2009). Another study revealed bacterial strains belonging to the *Bacillus*, *Halomonas*, *Pseudomonas*, *Enterococcus*, and *Acinetobacter* genera, which were isolated from soil samples obtained from a boron mine drainage in Turkey. At the sampling points, the soil and water had high boron concentrations of 3000–66,000 mg/L and 2800–4900 mg/L boron, respectively. First, a culture-dependent approach was used to narrow down the dominant culturable bacteria to 25 isolates, which were able to grow on 50 and 100 mM of boric acid. Later, a genomic approach was opted for the same area and it revealed that the bacterial compositions changed due to seasonal changes. Yet, the bacteria belonging to the phyla *Proteobacteria*, *Planctomycetes*, *Bacteriodetes*, *Verrucomicrobia*, and *Actinobacteria* were dominant (Aytar Çelik et al. 2021). Another study reported that bacteria that are able to remove boron can also be resistant to other heavy metals (Edward Raja and Omine 2013). The isolated boron-tolerant and boron-accumulating bacteria should be considered for bioremediation of toxic levels of boron and other heavy metal-contaminated sites.

#### 16.2.1.4 Chromium

Cr is one of the widespread heavy metals encountered in heavy metal-contaminated sites. Cr contamination is a result of human and industrial activities such as production of steel, leather, and ceramics, Cr/electroplating, metal processing, and fertilizer applications. Cr contamination is a serious condition as it causes DNA damage and mutations (Viti et al. 2014).

A specific bacterium that was able to remove 80% of chromium in a medium with a starting chromium concentration of 100 μg/mL was determined using a genomic approach of 16S rRNA sequencing. Moreover, this bacterium was able to grow in a medium with many different heavy metals such as, Fe, Mn, Zn, Ni, and Ag (Rahman et al. 2015). The genome of this specific bacterium named *Enterobacter cloacae* B2-DHA was later sequenced with an Illumina HiSeq2500 system (Aminur et al.

2017). In this study, almost 160 genes were determined to play a role in ion transport, binding, and catabolism, therefore shedding light on heavy metal resistance and metabolism-intolerant and metabolism-resistant bacteria. Moreover, two genes for chromium resistance were defined in this bacterium.

In another study, the complete genome of a chromate-reducing thermophilic bacterium *Thermoanaerobacter thermohydrosulfuricus* BSB-33 isolated from a hot spring sediment in India was sequenced. The bacterium was suggested to be a strong candidate for chromium bioremediation due to its distinctive chromium-reducing ability. Results of many intensive studies were collected and evaluated to find the basics of chromate reduction capacity, which resides on oxidoreductases, nitrite reductase, dihydrolipoamide dehydrogenase, and nicotinamide adenine dinucleotide + hydrogen (NADH):flavin oxidoreductase (Bhattacharya et al. 2015).

In addition to the genomic studies to unveil the genetic background and metabolic processes of chromium resistance, new chromium-resistant bacteria have been continued to be isolated from different sites. In one study, tannery industry wastewater was used to disclose those bacterial strains tolerant to chromium already found in the wastewater. A genomic study of 16S rRNA sequencing was conducted on the isolates, and *Klebsiella pneumoniae* and *Mangrovibacter yixingensis* were detected to have Cr reductase genes, and it was suggested that they be used for the detoxification of tannery wastewater (Sanjay et al. 2020).

#### 16.2.1.5 Cadmium

Similar to Cr, Cd is one of the most toxic heavy metals that contaminate the environment through industrial and agricultural applications. It is not an essential element for organisms and affects the growth, quality, and yield of agricultural products through which it enters the food chain and causes respiratory and kidney problems in humans (Thijssen et al. 2007; Haider et al. 2021).

Bioremediation approaches of Cd contamination have revealed some important bacterial strains. One of the species that has gained attention is *Escherichia coli*. Cd resistance of five different *E. coli* strains were determined by Qin et al. (2017). The *E. coli* BL21 (DE3) strain was found to be resistant to cadmium at a level of up to 1 mM. In order to detect the genes playing a role in Cd resistance, a fosmid library was constructed for this strain and different genes were overexpressed to find the relevant gene. Another study made use of the 16S rRNA sequencing approach and found a bacterial strain with a close resemblance to *Cellulosimicrobium* sp., which could remediate six different heavy metals present in a river in New Delhi, India. The bacterium was able to either consume or sequester Fe, Pb, Zn, Cu, Ni, and Cd (Bhati et al. 2019). Such bacteria with resistance to more than one heavy metal are suggested to be considered more for real bioremediation processes. Recent studies have continued to discover new bacterial strains for heavy metal bioremediation by the use of genomic and proteomic approaches (Chakdar et al. 2022).

#### 16.2.1.6 Lead

Together with Cd and Cr, lead has been accepted as one of the priority pollutants in water environments. The risk of lead that causes mental retardation in children

makes it a salient heavy metal in the case of contaminations. Many studies have been conducted for the remediation of lead pollution by biological approaches. Bacterial colonies in industrial effluents of a steel plant in India were investigated (Chatterjee et al. 2012). 16S rDNA universal primers were used for sequencing of the dominant microbial flora. Two potential Gram-positive endospore-forming bacterial strains were identified for having high lead resistance.

Some methanotrophs drew the attention of Faheem et al. (2020) for bioremediation of water environments where Cd, Cr, and lead heavy metals are especially causing pollution. They studied an epipelon biofilm formed by methanotrophs, which possess methane monooxygenases that play a role in bioremediation of heavy metals. This biofilm was successful in complete remediation of lead and cadmium and partial removal of Cr of 50 mg/L each. DNA-based stable isotope probing and $^{13}CH_4$ microcosm resulted in different phylotypes of methanotrophs. Sequencing of 16S rRNA and the methane monooxygenase gene *pmoA* showed that the dominant methanotrophs were *Methylobacter*, *Methylosinus*, and *Methylocystis* during different phases of the process.

Apart from bacteria, yeasts can be used to remediate water sources contaminated by lead. Seven heavy metal-resistant yeast strains were isolated from a lake in Algeria, which is heavily contaminated with lead and Cd (Aibeche et al. 2022). A 26S rRNA sequencing approach concluded that the heavy metal-resistant yeasts were the halotolerant *Rhodotorula mucilaginosa*, *Clavispora lusitaniae*, and *Wickerhamomyces anomalus* species. These yeasts were also found to be resistant to other heavy metals like Cu, Zn, Cd, Cr, Fe, and Hg.

#### 16.2.1.7 Antimony and Arsenic

One of the heavy metals considered to be highly toxic due to its carcinogenic effects is antimony (Sb). Antimony (Sb) is consumed in industrial applications for the production of glass, semiconductors, flame retardants, etc. Its industrial need has led to heavy mining activities, which are the prominent causes of environmental Sb contaminations (He et al. 2019). It is known that inherent soil microorganisms take place in the speciation, mobility, and environmental fate of Sb (Deng et al. 2021). These microorganisms can oxidize toxic heavy metals into less toxic forms. For example, Sb-oxidizing bacteria transform Sb(III) to Sb(V), as the latter is less toxic and more mobile. A genomic study conducted with 16S rRNA sequencing revealed that upon antimony spiking of three Sb(III) and Sb(V) doses (50, 400, and 1600 mg/kg) to different soil samples and after 8 weeks' incubation, the bacterial community composition changed in favor of *Proteobacteria* in toxic Sb(III) spiking. The less toxic form of Sb(V) spiking also resulted in an enriched *Proteobacteria* group by the application of 400 and 1600 mg/kg Sb(V). These results were consistent with the natural environment. Therefore, it is suggested that α-, β-, and γ-Proteobacteria are the most important genera in the transformation of antimony in nature (Wang et al. 2021). It is also suggested that when bacteria are exposed to antimony stress for a long time, they develop resistance (Deng et al. 2021). An aerobic bacterial species named *Roseomonas rhizosphaerae* YW11 was detected to oxidize both the toxic antimony and arsenic forms, Sb(III) and As(III), to less toxic Sb(V) and As

(V) forms, respectively, and therefore enhance the mobility of these heavy metals in the environment. Genomic analyses revealed the presence of As-resistant gene islands, where the genes encoding As(III) oxidase were also induced by the toxic antimony (Sun et al. 2020). The genomic background of such heavy metal-resistant bacteria would enhance the remediation applications.

Similar to Sb, As contamination provokes health risks in many parts of the world. Acid mine drainages are among the sites where high arsenic contamination, besides many other heavy metals, can be encountered. Several *Thiomonas* strains were detected in an acid mine drainage in France, where As(III) concentration was as high as 180 mg/L, by genomic and proteomic approaches. These bacteria were found to possess arsenite oxidase, which is a key enzyme to remediate arsenic in water samples (Hovasse et al. 2016). Many other microorganisms were detected to have this enzyme by many genomic approaches as reviewed by Plewniak et al. (2018).

## 16.3 Conclusions

Many genomic studies have been conducted all around the world to reveal heavy metal-tolerant and heavy metal-resistant microorganisms, which could be good candidates for remediation of the contamination of heavy metals that pose high health risks for the environment, plants, animals, and humans. The development of industrial processes results in more and more effluents rich in heavy metal ingredients every day. Heavy metals such as boron, Cr, cadmium, As, Sb, Pb, and Hg end up in soil and water resources through which health risk potentials come to life. Bioremediation is a cheap and practical alternative for cleaning up many contaminants including heavy metals from soil and water sources. Genomic approaches have a fundamental need at the start of the bioremediation process to find highly resistant microorganisms. The rich data produced by genomic and proteomic studies will help scientists find and even develop the best candidates to be employed in the bioremediation of different heavy metal-contaminated sites.

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# Bioremediation of Heavy Metals by Metagenomic Approaches

17

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**Abstract**

Deposition of heavy metals and other contaminating materials in the environment is a ceaseless and inescapable process. Bioaccumulation of heavy metals is extremely harmful to all domains of life. While dealing with heavy metal pollutions, microbes, especially Proteobacteria and Actinobacteria, acclimatize and adapt themselves to contaminated sites through different metabolic activities. Microbial bioremediation through biosorption, bioaccumulation, solubilization, immobilization, transformation, etc. is an innovative, sustainable, cost-effective, and efficient approach for reducing heavy metal toxicity. Since traditional culturable approaches have limitations, metagenomic approaches help uncover the microbes, associated genes, and their functions in heavy metal bioremediation. Rapid improvements in molecular techniques, such as high-throughput DNA sequencing, amplification, cloning, microarrays, and other "-omic" tools, have shown promise in revealing the metabolic diversity and nutrient versatility of microbes living in contaminated environments. Study site selection, sample collection and nucleic acid extraction, genome enrichment, and metagenomic library construction are the key steps in metagenomic research. Starting with a complete understanding of metagenomic screening, several function-based techniques including phenotype-based screening, substrate-induced gene expression (SIGEX), and metabolite-regulated expression (METREX) are used to discover new classes of genes. Bioinformatic tools like MEGAN, CAMERA, MG-RAST, and IMG/M play a variety of roles in the field of metagenomic bioremediation data analysis. Although new findings overcome major difficulties in microbial community analysis, still many activities lack adequate screening

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methods and have poor expression systems for metagenomes. In spite of these limitations, we could carry out our metagenomic research to evaluate microbial community composition in polluted areas to select effective microbes that can help in heavy metal remediation and to determine how to apply them to enhance the nature-friendly elimination processes.

**Keywords**

Bioremediation · Sustainable · Heavy metals · Metagenomics · Bioinformatics · Microbial community · MEGAN

## 17.1 Introduction

Heavy metal contamination throughout the world and its impact on living organisms is not a new topic of discussion. Enormous numbers of studies have been conducted in different continents in recent decades regarding "ambiguously" defined metals and their perilous effects on different organisms either in low or high concentrations. Loosely classifying these metals on the basis of their toxicity as either significantly toxic (e.g., As, Cd, Pb, Hg, etc.) or certainly toxic (e.g., Zn, Cu, Ni, etc.), we observe that the former (with no biological significance) would affect our health at lower concentrations and the latter (sometimes acting as trace elements) at higher concentrations (Su 2014). Land areas, water bodies, and the atmosphere are now highly under the risk of this pollution due to natural and anthropogenic activities. Rapid industrialization and high chemical production were the highest in China in 2010, followed by the USA, Japan, and Germany, whereas more than 70 million chemicals are produced throughout the world, which eventually get deposited either in water bodies or soil (Wu et al. 2016). Biological organisms encounter these metals directly or indirectly, thus producing reactive oxygen species (ROS). The attraction of heavy metals toward thiol groups hampers proper protein function, which is one of the causes of metabolic impairment.

Microbes, the silent leading scientists with the enormous potentiality to restrict heavy metal pollution on the basis of their natural and/or adapted physical and molecular attributes, are the pathfinders of this warfare through bioremediation, a cheap and environment-friendly technology. Specific bacteria, fungi, and other microbes may be able to transform (keeping in mind that heavy metals cannot be eradicated or degraded totally from the environment) metals from one form to another and alter their bioavailability. Enhancing the solubility of metals by microbes results in metal leaching, whereas decreasing solubility reduces bioavailability. So, both kinds of interactions show positive results with respect to different biological organisms. Biosorption, a potent bioremediation technology adapted by microorganisms, can be divided into metabolism-dependent (transport across the membrane, precipitation, etc.) or metabolism-independent (ion exchange, complexation, etc.) (Ojuederie and Babalola 2017). Extracellular polymeric substances (EPSs), present outside the cells of prokaryotic and eukaryotic organisms, consist

of both micro and macromolecules (polysaccharides, proteins, phospholipids, nucleic acids, etc.) and are responsible for biosorption of metallic compounds using their negatively charged ions, interacting electrostatically with positively charged metal ions (Pal and Paul 2008; Kumar 2018). pH is an important parameter for the biosorption process because altering the pH results in changing the solubility of compounds (Ojuederie and Babalola 2017).

To date, we have been able to study very few microbes because of our lack of knowledge about their environmental niche where they interact with different biotic and abiotic factors, that's why they are unculturable and still play pivotal roles in the environment. Recently, metagenomics opened a new window of hope for us, to gather information about those unculturable microbes with its tremendous applicability. Metagenomics, however, with some limitations, not only gives us insights into finding unculturable microbes but also is a potent technology to decipher genes for proper functioning. Function-, sequence-, and polymerase chain reaction (PCR)-based screenings are well-known methods of metagenomics to study heavy metal bioremediation. Some computational tools like MEGAN, MG-RAST, CAMERA, etc. may be mentioned related to metagenomic analyses (Singh et al. 2009).

In this chapter, we discuss microbial interactions with heavy metals as well as different bioremediation strategies, microbial community analysis of contaminated areas through metagenomic library construction and different screening approaches, and how different bioinformatic tools help analyze the metagenomic data.

## 17.2 Impacts of Heavy Metals on Living Systems

From prokaryotes to higher mammals, all are under the threat of heavy metal toxicity. In human beings, various heavy metals are compartmentalized within different body cells as well as cell organelles, leading to tissue damage, malfunctioned protein production, and damage to organs like the kidneys, lungs, liver, heart, etc. Long-time exposure accompanied by high accumulation of these heavy metals in body cells causes neurotoxicity. The general enzymatic activities of proteins are altered in the case of microbes. These may be involved in the reduction of the diversity of microbes within a contaminated area. However, some microbes are resistant to specific heavy metals, which will be discussed briefly. In the case of plants, photosynthetic enzyme production, photosynthesis, and total metabolic activity are drastically hampered. For crop plants, loss of crops leads to an economic imbalance. Heavy metals accumulate in different tissues of organisms. Meat we eat, like chicken, goat, etc., may also contain heavy metals within their tissues. On consumption, these meats accumulate within our bodies and cause different diseases. Heavy metals have negative impacts, specifically on sex organ development in different animals like birds, snakes, and mammals. Anemia and testicular hypoplasia are common diseases among birds. Due to reduction in the production and functions of ROS scavenging enzymes like superoxide dismutase, peroxidase, catalase, etc., the growth, development, and reproduction of fishes are reduced. It is also related to economic loss in society (Engwa et al. 2019).

### 17.2.1 The General Mechanism of Heavy Metal Toxicity

Reactive oxygen species (ROS) production is one of the important consequences of heavy metal toxicity, which, on entering the nucleus, not only interacts with DNA and damages it but also interacts with the lipid bilayer of the cell and leads to lipid peroxidation. Among the huge numbers of heavy metals, here, we discuss the general impact of cadmium, arsenic, lead, mercury, and copper (a trace element). Interestingly, some metals mimic other metals or compounds, for example, arsenate mimics phosphate and impairs oxidative phosphorylation. Nonessential metallic ions like $Cd^{2+}$ interact with nonmetallic biomolecules and form complexes (Fig. 17.1) that "misbonding" has a negative impact within cells.

Iron and copper are important metals, which have tremendous functions within cells; however, excessive concentrations may produce ROS in the form of hydroxyl ions ($OH^{\bullet}$), which react with biomolecules, such as proteins, lipids, and DNA. It may be responsible for DNA methylation, histone structure alteration, and changes in the expressions of cell cycle-regulating proteins like p53 and p21, leading to tumor formation (Engwa et al. 2019).

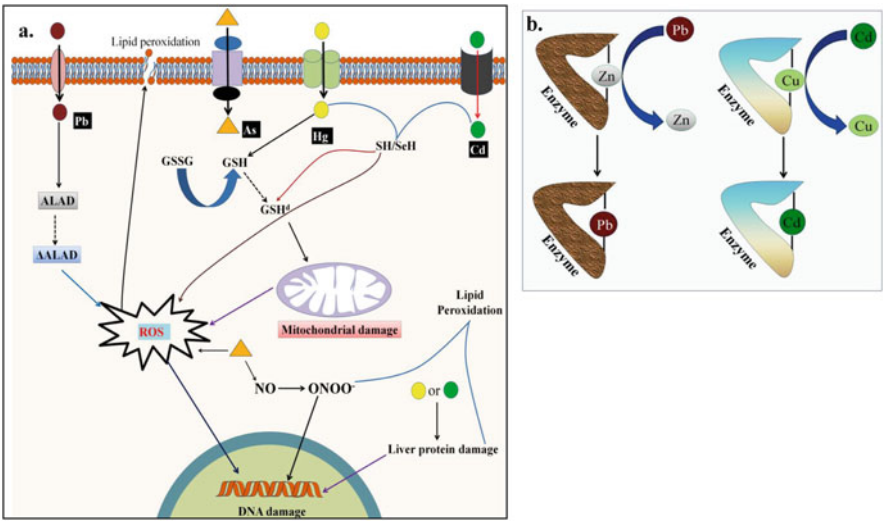


**Fig. 17.1** Heavy metal toxicity within a cell: (**a**) arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) enter through specific channels within the cell. Lead interacts with ALAD and induces the production of the ΔALAD enzyme. By displacing Zn from the enzyme, ROS production is induced. Cd and Hg interact with SH/SeH-containing molecules, which, in turn, induce GSH derivative ($GSH^d$) or ROS production. $GSH^d$ damages the mitochondria. Arsenic induces reactive nitrogen species (RNS) production, and RNS causes DNA damage or lipid peroxidation. Cd and Hg may damage the liver proteins of mammals. ROS may cause lipid peroxidation or DNA damage. (**b**) Pb and Cd mimic Zn and Cu, respectively, and remove functional heavy metals

### 17.2.2 The Global Scenario of Heavy Metal Contamination

It is quite difficult to find out which biosphere is not contaminated by heavy metals. For example, the polar regions are to some extent devoid of anthropogenic activities; however, the Antarctic and Arctic regions of the world are contaminated with a variety of heavy metals. Air-borne heavy metal particles (emitted through industrial fossil fuel burning, forest fires, etc.), suspended in the atmosphere, come down through rain, leading to the contamination of polar earth crusts. Oceans are one of the contaminated zones of heavy metals, where coral reefs are also reported to have high amounts of heavy metal deposition. As usual, urban areas are 10 times more contaminated than are rural ones. For example, the average concentrations of arsenic and copper in the rural and urban areas of the world are 3.2 and 20 $ngm^{-3}$ and 7.9 and 155 $ngm^{-3}$, respectively. Anthropogenic activities contaminate both the atmosphere and aquatic ecosystems. Three decades ago, studies revealed that a total of 19 and 42 and 35 and 112 thousand tons of arsenic and copper were deposited in the atmosphere and aquatic ecosystems per annum, respectively (Nriagu 1990). Recently, Hoang et al. (2021) have extensively studied the impact and contamination of heavy metals in different rivers of the world. They argued that different metals like cadmium, arsenic, lead, copper, etc., which are enormously deposited within rivers, result in chronic toxicity in aquatic plants and animals. Due to increased industrialization, lead poisoning is a major problem in first-world countries and in the marine environments of North America and Western Europe. Arctic regions are under heavy metal stress due to Eurasian industrialization. Many Asian countries like India, Taiwan, Bangladesh, Pakistan, etc. are highly under the risk of arsenic poisoning. Heavy metal-contaminated foods are also one of the greatest concerns for us. Sometimes, heavy metal contaminated foods are also transported to different regions of the world, which also causes health risks to different organisms (Hembrom et al. 2020). After studying the present scenario, we may conclude that there is not a single biosphere where clutches of heavy metal contaminations are not present.

## 17.3 Reciprocal Interaction Between Microbes and Metals

Environmentally ubiquitous microorganisms are leading components that can interact with heavy metals (Rahman and Singh 2020). Excessive concentrations of heavy metals are toxic to microbial cells and could adversely affect the cellular activities of microbes. In microbial cells, heavy metals mimic the essential elements of various enzymes hampering metabolic processes, producing reactive oxygen species (ROS), and, ultimately, altering RNA, DNA, and protein structure and function (Prabhakaran et al. 2016). To overcome such toxic effects of heavy metals, microorganisms have been acquired using various strategies like bioadsoption, bioaccumulation, oxidation–reduction, biomineralization, precipitation, and bioleaching.

In the biosorption process, heavy metals are strongly attached to the outer most structure of the bacterial cell surface with the aid of various functional groups like hydroxyl, carboxyl, amino, ester, sulfhydryl, carbonyl, phosphate, etc. (Ali Redha 2020). These functional groups interact with heavy metals by covalent bonding, electrostatic attraction, van der Waals interaction, ion exchange, complexation, and precipitation. For example, carboxyl, phosphate, and sulfate functional groups present in the polysaccharides and proteins of *Oceanobacillus profundus* KBZ 3-2 could help in the ion exchange and complexation process of Pb (II) and Zn (II) ions (Mwandira et al. 2020). Whereas in bioaccumulation, they are accumulated after entering through various exporters (like ABC transporter, P-type ATPase, cation-diffusion facilitator, etc.), ion channels, ion pumps, and endocytosis. Microorganisms could also produce siderophores and metallothioneins, which help facilitate the intracellular bioaccumulation of heavy metals (Sharma and Shukla 2021).

Microorganisms also catalyze the oxidation–reduction reactions of heavy metals, where electrons are transferred from one elemental state to another (Rahman and Singh 2020). Various heavy metals such as arsenic (As), chromium (Cr), etc. can be effectively removed from the environment through the bacterial oxidation–reduction process. In the case of enzymatic arsenate [As(V)] reduction, arsenate reductase helps convert it to arsenite [As(III)], whereas in chromate [Cr(VI)] reduction, various reducing enzymes including NAD(P)H-dependent chromate reductase, oxidoreductases, nitroreductase, flavin mononucleotide (FMN) reductase, and lipoyl dehydrogenase are involved (Thatoi et al. 2014).

However, in biomineralization, microorganisms naturally mineralize various minerals like silicates, phosphates, sulfates, carbonate, and oxides via different mechanisms. For instance, microbially induced calcium carbonate precipitation (MICP) is a type of biomineralization process in which microorganisms induce calcium carbonate precipitation through different mechanisms; among them, urea hydrolysis is predominant. Various ureolytic bacteria could produce urease enzymes, which help precipitate metal ions through urea hydrolysis and produce carbonate and ammonia. For example, *Sporosarcina pasteurii*, a urease enzyme-producing strain, was used to precipitate lead (Pb), cadmium (Cd), and zinc (Zn) (Jalilvand et al. 2020). In the case of bioleaching, microorganisms can solubilize metal sulfide into sulfate. In this regard, acidophilic bacteria, mostly *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, are generally involved in the oxidation of iron ($Fe^{2+}$ to $Fe^{3+}$) and sulfur ($S^0$ to $SO_4^{2-}$) compounds, which ultimately changes the pH and facilitates the bioleaching of heavy metals.

## 17.4 Bioremediation: A Future Outlook Toward Mankind

Environmental pollution is an increasing concern of mankind. Nonbiodegradable pollutants such as heavy metals, plastics, industrial dyes, pesticides, etc. are detrimental to human health on entering the food chain. Instead of using various physical and chemical conventional techniques, bioremediation is a simple, nonconventional,

renewable, cost-effective, and eco-friendly approach to detoxify heavy metals from contaminated sites (Kumar et al. 2018; Rastogi and Kumar 2020). Bioremediation is not only engaged in environmental cleanup but also involved in wastewater treatment, crop productivity, and production of some value-added products. Similarly, *Pseudomonas* sp. WS-D/183 can be effectively used for textile wastewater treatment as well as for heavy metal removal and plant growth promotion (Hussain et al. 2020). Several recycled microalgal species are capable of pesticide degradation and produce some value-added products like biochar, biodiesel, etc. (Nie et al. 2020). These culturable microbes have been efficiently studied for bioremediation purposes, but the study on unculturable microbes (their growth, metabolism, and function) provides new insights into the future bioremediation strategy. In this regard, "omics" technologies, especially metagenomics and proteomics of the microbial community, could be effectively used for understanding the mechanisms of hazardous pollutant eradication from the environment.

## 17.5 Microbial Metagenomics: A Multi-potential Concept Toward Heavy Metal Bioremediation

It is extremely useful to have prior information of the chemical, physical, and ecological parameters of selected habitats to carry out a metagenomic analysis. The extraction of nucleic acid from the sample site is the first step in any metagenomic study. When a whole community is selected for metagenomic analysis, its species composition must be considered to ensure affordable and high coding gene densities. Therefore, not eukaryotic but only prokaryotic microbial communities gain attention in most metagenomic studies.

### 17.5.1 Unraveling the Structure and Functional Potential of Microbial Communities

Only a small amount of information regarding the indigenous microbial diversity in contaminated sites has been obtained using culture-dependent approaches. We were unable to properly investigate the microbial diversities, metabolic capacities, and plasticity in heavy metal-contaminated locations using traditional culture-based methodologies. To overcome these limitations, a new methodology called "metagenomics" has evolved to investigate the genetic resources of both culturable and nonculturable microbes from any environment. Metagenomics and high-throughput sequencing methods have revealed the abundance, adaptation, ecology, and evolution of microbes surviving in contaminated environments.

**Table 17.1** List of major microbial phyla in contaminated areas

| Dominant phylum | Percentage of presence in community | Metagenomic projects | Source | References |
|---|---|---|---|---|
| Gammaproteobacteria | 22.88 | Metagenomic analysis of soil bacterial community and level of genes responsible for biodegradation of aromatic hydrocarbons | Soil | Czarny et al. (2017) |
| Alphaproteobacteria | 22.83 | | | |
| Clostridia | 11.11 | | | |
| Bacilli | 8.40 | | | |
| Betaproteobacteria | 7.55 | | | |
| Planctomycetia | 5.21 | | | |
| Proteobacteria | 38.56 | Metagenomic analysis of the microbial community and function involved in cd-contaminated soil | Soil | Feng et al. (2018) |
| Acidobacteria | 18.13 | | | |
| Gemmatimonadetes | 9.26 | | | |
| Thaumarchaeota | 5.45 | | | |
| Proteobacteria | 48.63 | Analysis of microbial communities in heavy metal-contaminated soils using the metagenomic approach | Soil | Hemmat-Jou et al. (2018) |
| Actinobacteria | 17.28 | | | |
| Acidobacteria | 14.44 | | | |
| Gemmatimonadetes | 8.92 | | | |
| Bacteroidetes | 4.87 | | | |
| Gammaproteobacteria | 12 | | | |
| Alphaproteobacteria | 3 | | | |

### 17.5.2 Dominant Microbial Community in the Metagenome

Heavy metal-contaminated areas are enriched with some special microbial phyla, and microbial composition varies between contaminated sites. A study by Prakash et al. (2021) reported that contaminated soil was dominated by bacteria (77.50%), followed by unknown organisms (12.50%), and archaea, viruses, and eukarya (<10%). Proteobacteria (Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria) are the keystone groups of bacteria, where the genera like *Nocardioides* and *Pseudomonas*—are dominated. They have great morphological and metabolic diversities that play a pivotal role in heavy metal bioremediation (Xavier et al. 2019). Proteobacteria, Acidobacteria, and Actinobacteria are the major groups of terrestrial bacteria (Hur and Park 2019). From different metagenomic studies (Table 17.1), it was found that contaminated sites are dominated by the same bacterial groups, which postulates that these groups of bacteria are able to handle extreme environments. Alphaproteobacteria and Deltaproteobacteria are able to bioremediate chromium compounds, Betaproteobacteria can degrade aromatic, aliphatic, and hydrocarbon compounds, and Gammaproteobacteria play a role in oxidation of sulfide compounds. Firmicutes under environmental stress like hypersalinity and low oxygen levels can eradicate pollutants. Bacteriodetes can efficiently degrade various organic pollutants under low oxygen concentration. A previous report mentioned that Planctomycetes are able to remove dissolved organic matter, whereas Gemmatimonadetes can

accumulate phosphate and remove phosphate from wastewater. Alphaproteobacteria, Gammaproteobacteria, and Firmicutes possess specific functional genes for sulfate reduction that might help in the conversion of metals into inert metal sulfides. Members of Sphingomonadaceae can remediate heavy metals from sewage. Fungal species reported from contaminated sites have the potential to enzymatically convert toxic compounds, hazardous substances, and metal ions into nontoxic simpler forms. The cell walls of fungi and bacteria interact electrostatically, resulting in biosorption of cadmium, copper, zinc, uranium, and cobalt (Liaquat et al. 2021). Heavy metals such as lead and copper have been found to be tolerated by marine fungi, and filamentous fungi have the capacity to remediate Cd, Cu, and Ni. Enzymes like catalase and peroxidase produced by bacteria and fungi have heavy metal tolerance capacity, whereas phytochelatin synthase (PCS) catalyzes the formation of phytochelatin from glutathione, protecting cells from nonessential heavy metal toxicity. Algal biomass is also used to remove heavy metals through biosorption, an ecologically safe and cheaper process. An earlier report suggested that the Phaeophyceae group of algae contains alginate, which acts as a good candidate for biosorption of heavy metal ions (Shine et al. 2015).

### 17.5.3 The Importance of Metagenomic Library Construction

Metagenomic DNA is directly isolated from the polluted environment using culture-independent methods. Various methods have now been discovered to isolate DNA from environmental samples. To represent all microbial genomes, entire metagenomic DNA must be extracted and purified, followed by construction of a metagenomic library. Isolation of metagenomic DNA from environmental sources has been accomplished using two methods: cell recovery and direct lysis. Both physical and chemical methods, involved in the extraction process, have independent advantages regarding metagenome extraction. In physical methods, more microbial diversities are recovered, whereas in chemical methods, high-molecular-weight DNA is obtained. The choice of cloning vectors is depend on the size of gene of interest. Plasmid cloning vectors need small DNA fragments, whereas larger inserts require cosmids (25–35 kb), fosmids (25–40 kb), or bacterial artificial chromosomes (BACs) (100–200 kb). It is preferable to use clones with high-molecular-weight DNA inserts for the evaluation of genes and metabolic pathways, as this increases the likelihood of obtaining positive hits during library screening. More clones are required for optimal coverage of the metagenome when the cloned inserts are smaller.

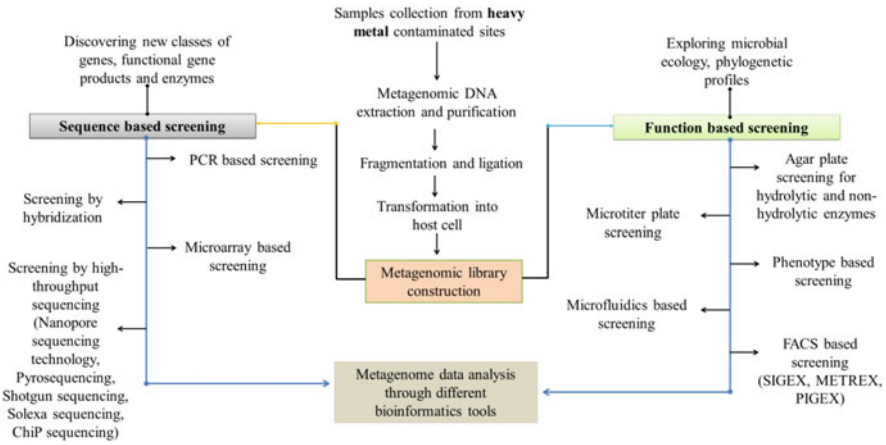


**Fig. 17.2** A workflow of different metagenomic techniques

## 17.5.4 Screening of Detoxifying Genes from the Polluted Environment

### 17.5.4.1 Function-Based Screening

This method is highly useful for discovering new metabolic activities, new classes of genes, functional gene products, and enzymes relevant to bioremediation (Fig. 17.2). Prior sequence information or sequence similarity is not required for the function-based approach. One of the major disadvantages of the function-based approach is that it requires production of functional gene products by the bacterial host (Daniel 2005). To investigate these rich genetic resources, a range of function-based metagenomic screening approaches have been devised, culminating in the discovery of a huge number of new enzymes with distinct metabolic activities.

#### 17.5.4.1.1 Phenotype-Based Screening

Because intact cells and their native cellular milieu are employed, phenotypic screening is usually more biologically realistic and less artificial. In this method, a metagenomic DNA library was created using metagenomes obtained directly from contaminated locations. The primary hits found in phenotypic screening could be used to target a variety of proteins (receptors, enzymes, transcription factors, and so on) as well as signaling cascades. Several detoxifying or biodegradative enzymes such as naphthalene dioxygenase, nitrilase, styrene monooxygenase, and extradiol dioxygenase (EDO) have been identified from DNA libraries through a function-based approach (Pushpanathan et al. 2014).

#### 17.5.4.1.2 Agar Plate Screening

This functional approach is useful to identify novel enzymes (hydrolytic and nonhydrolytic) that function under diverse environmental conditions. This methodology has led to the discovery of a number of new hydrolytic enzymes, including

lipases, esterases, cellulases, proteases, laccases, glycosylases, nitriles, and halides. This method is frequently used to look for genes that cause resistance to harmful elements such as antibiotics, high salt concentrations, high pH, and heavy metals. Enzymes like β-glycosidases, dioxygenases, dichlorophenol hydroxylases, polyhydroxyalkanoate synthases, etc. are identified through this method (Ngara and Zhang 2018).

#### 17.5.4.1.3 Fluorescence-Activated Cell Sorting (FACS)-Based Screening

This technique is useful for library screening based on the size, shape, and fluorescence properties. Because of its excellent sorting properties, FACS is easily related to a number of different high-throughput screening approaches, such as droplet sorting and reporter-based screening. Reporter gene expression is a fundamental step in reporter-based screening where a metagenomic library is transformed into host cells harbouring reporter genes. Substrate-induced gene expression (SIGEX) is a FACS-based intracellular screening method, which sorts green fluorescent protein (GFP)-expressing cells and responds to the presence of aromatic and hydrocarbon degrading genes. It is possible to identify phenol degradative operons and genes involved in benzoate and catechol pathways by this approach. SIGEX has drawbacks, for example, it is sensitive to the structure and orientation of genes associated with desirable attributes. During the screening technique, catabolic genes that are constitutively expressed are missed (Wakamatsu et al. 2018). Another modified reporter-based screening system is called product-induced gene expression (PIGEX), in which a transcriptional activator is highly sensitive to the product of the desired enzyme. Metabolite-regulated expression profiling (METREX) is also a FACS-based intracellular screening strategy that improves sensitivity while screening poorly expressed gene products (Williamson et al. 2005). This system is used to screen biodegradative genes as well as quorum-sensing signal molecules using biosensors from the microbial community.

#### 17.5.4.1.4 Microfluidics-Based Screening

This is a high-throughput screening technology, coupled with FACS, which gives an advantage over other methods due to its appropriateness for cell-based assays, minimal analytical costs, and ease of handling liquids in picoliter amounts. A single droplet functions as a reaction chamber where cells, enzymes, substrates, and products are confined (Ngara and Zhang 2018). The detection method, which is usually confined to a fluorescent signal, is the key limitation of this technology.

In addition to these screening methods, microtiter plate screening, genetic enzyme screening systems (GESSs), microfluidic gel microdroplets (GMDs), and microfluidic absorbance-activated droplet sorters (AADSs) (Ngara and Zhang 2018) are also available for screening microbial communities from the metagenomic library.

### 17.5.4.2 Sequence-Based Screening

This screening-based approach is not only restricted to discovering new classes of genes but also to exploring microbial ecology, transposon in bacteria, and

phylogenetic profiles. A previous knowledge of nucleotide information from databases is required for the screening of detoxifying genes. Massive amounts of sequence data are collected and combined into larger fragments until a sufficient level of coverage is achieved. Degenerate primers or high-density arrays can be used to improve the detection frequency of a variety of target genes. Deep sequencing with high coverage provides great resolution, allowing even rare single-nucleotide polymorphisms (SNPs) to be detected (Pushpanathan et al. 2014). Some of the sequence-based methods include primer-based screening through polymerase chain reaction (PCR), microarray profiling, probe hybridization, and high-throughput sequencing technologies.

#### 17.5.4.2.1 Screening by PCR

This method of approach is useful in microbial community analysis as well as in biotechnological fields for characterization of enzymes, antibiotics, or resistant genes. For the identification of a desired gene fragment, conserved nucleotide sequences are taken into consideration. Using this screening approach, we can identify members of a particular environment, catabolic genes, and their phylogenetic relationships. The advantage of using this procedure is that it can bypass the processes of cloning, transformation, and thus no need of clone maintenance. For proper screening, there are some crucial steps including primer design, annealing temperature, amplification cycles, template concentration, and the DNA polymerase used (Sze and Schloss 2019). Full-length environmental genes from various metagenomic samples have been successfully amplified and expressed using primer sequences based on specific enzyme-encoding sequences. By analyzing transcripts and the quantity of ecologically relevant core genes in environmental samples, quantitative PCR methods can be used to investigate the specific metabolic activity. Although a major drawback of PCR-based strategies is unequal amplification of mixed template DNA, even it sometimes prevents accurate microbial diversity estimation in environmental samples (Kalle et al. 2014).

#### 17.5.4.2.2 Hybridization-Based Screening

In this approach, target-specific probes are also used to screen metagenomic libraries. PCR amplification and further sequencing help recover full-length targeted genes. The amplification of conserved sequences from target genes using biotinylated primers is one of the hybridization techniques that use magnetic bead separation and subtractive hybridization. The probe binds to full-length gene sequences, which are subsequently isolated from the rest of the metagenome using a magnetic field. This screening approach has been used to identify 16S rRNA genes and highly conserved domains like polyketide synthases, gluconic acid reductases, and nitrile hydratases (Daniel 2005).

#### 17.5.4.2.3 Microarray Profiling

Microarray technology has been successfully used to determine the composition and activity of the soil microbial community as well as to identify functional genes. Short oligonucleotide probes are fixed as dots on the surface of a slide in microarrays. As a

result, each point in the array corresponds to the complementary sequence of a specific gene from the genomes and/or transcripts of interest. Only those sequences that effectively hybridize with correct complementary probes are immobilized on the slide after the nucleic acids are extracted and tagged with fluorescent dyes, followed by hybridization and washing procedures. However, gene detection through the microarray approach shows a 100- to 10,000-fold lower sensitivity than that through PCR (Daniel 2005). The "GeoChip" technology has been developed based on the microarray approach to explore direct linkages between biogeochemical processes and functional activities of microbial communities (He et al. 2007). Another microarray-based metagenome profiling has been discovered in which the metagenomic DNA itself can be used to develop the microarray. This gateway does not require any previous sequence knowledge about microbial communities and opens a door to uncover novel detoxifying genes (Pushpanathan et al. 2014).

## 17.6 Bioinformatic Tools in Metagenomic Data Analysis

Several computational tools are now gaining popularity in multiple fields of science. Bioinformatics is used in the field of metagenomic bioremediation to conduct a variety of tasks, the most important of which is the processing of metagenomic data. Multiple metagenomic studies are generating large amounts of sequence data, thus putting pressure on bioinformatics to develop more reliable and accurate methods (Table 17.2) for interpreting metagenomic sequence data.

## 17.7 Other Applications of Metagenomics

### 17.7.1 Fluorescent in Situ Hybridization (FISH)

Fluorescent in situ hybridization is a cyto-molecular technique that usually relies on the principle of binding of a fluorescent probe to highly similar nucleic acid sequences. Small probes are generally 18–30 nucleotides long, capable of easily passing the biological membrane, and flourishing on binding to the sequence of interest. This technique is extremely useful for sorting out a particular group of microbes from environmental samples using FACS. Cells expressing the target gene and the level of expression can also be quantified using FISH. Actively growing microbial populations contain a higher number of ribosomes in comparison to slower growing ones and will have a higher quantity of rRNA. So, if the probe is targeting the rRNA of a microbe, then the growth rate of the community can be quantified by correlating the intensity of the fluorescent under a confocal laser scanning microscope. The abundance of microbial communities and their capability to bioremediate in soil-contaminated areas are reflected using FISH as a key approach (Pushpanathan et al. 2014).

**Table 17.2** List of computational tools for metagenomic data analysis

| Computational tools | Characteristics | References |
|---|---|---|
| MEGAN | 1. Accepts a variety of data (taxonomic and functional) input types and may export analysis (geographical and statistical) results in a variety of text-based and graphical formats<br>2. Focuses primarily on taxonomic profiles | Singh et al. (2009), Bharagava et al. (2019), Logares et al. (2012), Lanzén et al. (2012) |
| SmashCommunity | 1. Suitable for data derived from sanger and 454 sequencing<br>2. Estimates metagenomes' quantitative, phylogenetic, and functional components | |
| CAMERA | 1. Provides a powerful cross-analysis of environmental samples<br>2. A genome analysis tool that allows users to query, analyze, annotate, and compare data from metagenomes and genomes | |
| MG-RAST | 1. Offers quantitative insights into microbial communities<br>2. Accepts different phylogenetic and metabolic data<br>3. Performs protein prediction, clustering, and similarity-based annotation on nucleic acid sequences | |
| IMG/M | 1. Able to handle larger metagenome datasets<br>2. Provides a platform for function-based comparison including abundant protein families, functional families, or functional categories across metagenomic samples | |
| UniFrac | Compares microbial community diversity in a phylogenetic approach | |
| FOAM | 1. Classifies gene functions relevant to environmental microorganisms<br>2. Easy to organize results into different functional categories | |
| JANE | 1. Allows you to quickly get a sense of the genome's structure and functions<br>2. Expressed sequence tags (ESTs) and variable length sequences can be mapped and assembled quickly | |

(continued)

**Table 17.2** (continued)

| Computational tools | Characteristics | References |
|---|---|---|
| COGNIZER | 1. Provides multiple workflow options<br>2. A stand-alone annotation platform that allows users to functionally annotate sequences from metagenomic datasets | |
| RDP | 1. Used in the fields of microbial ecology, environmental microbiology, nucleic acid chemistry, and phylogenetics<br>2. Provides aligned and annotated rRNA gene sequence data to the research community as well as tools to allow researchers to evaluate their own rRNA gene sequences in the RDP framework | |
| myPhyloDB | Microbial community populations may now be stored, processed, analyzed, and distributed more easily | |
| WebMGA | 1. A quick and unique tool as well as a lot of flexibility when it comes to analyzing complex metagenomic data<br>2. ORF calling, sequence grouping, raw read quality control, elimination of sequencing artifacts and contaminations, taxonomic analysis, and functional annotation are only a few of the tools included in WebMGA | |
| WebCARMA | 1. Using short reads that encode for known proteins, this characterizes the species diversity and genetic potential of microbial samples | |
| provide | Used for accurate estimation of viral diversity in metagenomic samples | |
| Treephyler | 1. Used for fast taxonomic profiling of metagenomes<br>2. Applicable to both nucleotide and protein input data | |
| PhyloPhytias | Allows the accurate classification of most sequence fragments across all considered taxonomic ranks | |
| TACOA | The taxonomic origin of genomic segments as short as 800 bp can be predicted with great accuracy | |

(continued)

**Table 17.2** (continued)

| Computational tools | Characteristics | References |
|---|---|---|
| Phymm/ PhymmBL | Phylogenetic classification of metagenomic data with interpolated Markov models | |
| GAAS | 1. In both textual and graphical modes, it delivers improved estimates of community composition and average genome length for metagenomes.<br>2. Produces a more accurate representation of community composition for larger genomes | |
| Meta-pipe | Provides preprocessing, assembly, taxonomic classification, and functional analysis of marine metagenomes | |
| Xipe | 1. Normalizes sequencing efforts by repeated random subsampling of the datasets under scrutiny<br>2. Pairwise tests between all feasible pairs of metagenomes can be used to compare numerous metagenomes | |
| LefSe | 1. Provides a platform for metagenomic biomarker discovery<br>2. Determines features like organisms, clades, operational taxonomic units (OTUs), genes, or functions in an effective manner | |
| STAMP | 1. A comparative metagenomic tool that operates statistical analysis and reporting<br>2. Allows to conduct statistical studies in a graphical environment | |
| AMPHORA | Phylogenetic analysis of metagenomic data | |
| ARB | Metagenomic data analysis (sequence alignment, primary and secondary structure editing, phylogenetic analysis) and maintenance | |
| GOLD | Genetic data management system that stores and monitors huge metagenomic data worldwide | |
| Megx.net | Stores and analyzes marine metagenomic data based on oligonucleotide signatures | |

(continued)

**Table 17.2** (continued)

| Computational tools | Characteristics | References |
|---|---|---|
| RefSeq | Sequences representing genomic data, transcripts, and proteins from more than 2400 organisms are included in this nonreductive collection | |
| SILVA | Contains rRNA gene database from bacteria, archaea, and eukaryotes and provides high-throughput classification of data | |
| SINA | Alignment and taxonomical classification of 16S rRNA gene sequences from metagenomes | |
| XplorSeq | Allows for the easy collection, management, and phylogenetic analysis of DNA sequences | |
| PhyloSift | Phylogenetic analysis of protein coding and RNA sequences in metagenomic datasets | |
| VSEARCH | A versatile tool for processing and preparing metagenomic sequence data by global sequence alignment of the query against potential target sequences | |
| METAREP | Helps analyze and compare annotated metagenomic datasets at various functional and taxonomic levels | |
| MetaBAT | Able to analyze and reconstruct synthetic and real metagenome datasets with accuracy and computational efficiency | |
| Tax4Fun | Based on 16S rRNA information, determines the functional capabilities of microbial communities | |
| CREST | Environmental sequence classification tools (alignment-based) for creating and using custom taxonomies and reference datasets | |
| LotuS | Analyzes microbial 16S data and can generate phylogenetic trees from next-generation sequencing data | |

### 17.7.2 Ribosomal Intergenic Spacer Analysis (RISA)

RISA is a PCR-based application in which the intergenic space sequence and length between the 16S and 23S rDNA is amplified in order to identify the bacterial community in aquatic environments, land systems, and human gut. The length in between the two rRNA genes is significantly variable among microbes and is thus highly useful to generate phylogeny. It is widely used as an important part of metagenomic tools as it has no need to culture bacteria but to generate a library, which is further utilized to reveal unculturable microbes (Mahomed et al. 2021).

### 17.7.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis is a PCR-based DNA fingerprinting technique in which amplification of 16S rDNA-conserved regions is performed. Both culturable and unculturable microbes are subjected to DNA isolation. All samples are amplified by 16S rDNA-specific primers to be digested by restriction endonucleases. Gel electrophoresis is performed to identify the microbial populations that have unique patterns. This technique is of interest nowadays as it can reveal the microbial community of a habitat before and after heavy metal contamination. ARDRA is a useful tool for microbial community typing in copper-contaminated areas. Smit et al. (1997) successfully demonstrated the eubacterial community shifting after copper contamination in the soil.

## 17.8 Conclusions

In microbial ecology, metagenomic and bioinformatic approaches have already been utilized to analyze the genetic resources present in heavy metal-contaminated sites comprising a wide range of uncultivable microbes. Functional analysis focuses on screening libraries that allow identification of novel genes, whereas sequence-based metagenomic analysis rely on comparisons with databases of known genomic sequences. Despite significant breakthroughs in metagenomic approaches, there are a number of constraints that limit microbial community analysis. The two major restrictions are the inefficient expression of some metagenomic genes in the host bacteria used for screening and the lack of effective screening methods for further interpretations. On the one hand, we must take steps to limit anthropogenic activities, whereas on the other, we have to create high-throughput technologies and screening methodologies to investigate the uncultured microbial communities.

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# Proteomic, Genomic, and Metabolomic Understanding and Designing for Bioremediation of Environmental Contaminants

18

Upasana Jhariya and Sukdeb Pal

**Abstract**

Bioremediation is one of the most effective and eco-friendly approaches to remediate contaminants from the environment. Microorganisms possess inbuilt genetic, biochemical, and physiological assets, which often shape them into superlative substitutes for degradation of hazardous pollutants. Since ancient times, traditional culturable approaches have been used to identify potent degraders of pollutants; but, in order to reduce pollution levels, these conventional methods must be upgraded. The interconnection between genes and proteins determines the ability of a microorganism to adapt and control its metabolism and also governs the structure and dynamics of microbiomes in response to a polluted environment. Genomic and proteomic approaches provide a way to explore the interplay between genes and enzymes involved in the degradation pathways. Moreover, the metabolomic approach allows for qualitative and quantitative analysis of intermediates produced by microorganisms and helps understand the microbial physiological conditions. This chapter discusses the combinational use of genomics, proteomics, and metabolomics, along with different omics approaches and the promises they hold for the advanced bioremediation processes.

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Bioinformatics · Genomics · Proteomics · Metabolomics · Bioremediation

## 18.1 Genomics in Environmental Bioremediation

Microorganisms have the ability to thrive in various environmental conditions. They adapt to their surroundings by altering their genetic composition and by expressing genes that are necessary for their existence. The ever-increasing pollution level necessitates an environmentally friendly strategy to treat resistant contaminants and maintain ecosystem balance by restoration. Microorganisms degrade or accumulate contaminants and convert them into nontoxic products such as $CO_2$, $H_2O$, and other cellular biomass (Bharagava et al. 2019; Kumar and Chandra 2018).

The pathways and metabolisms followed by microorganisms for the degradation of contaminants are highly complicated because of numerous gene interconnectedness and their patterns of expression. Genes playing an important role in the catabolic process can originate from either chromosomes or extra-chromosomes and are expressed through the regulation of various physiological and metabolic conditions (Pal et al. 2017). Recent advancements in science and technology have provided a way to better understand the genomic composition, potential, and mobility of catabolic genes. These also aid in exploring the underlying physiology, regulation, and pattern of metabolism of the cell. Development in science and technology has enabled us to understand the genomic potential, genomic contexts of catabolic genes, and mobility of genes, along with the underlying physiology, regulation, and metabolism of the cell. Genomic approaches offer the study of the complete genetic makeup of an organism using a combination of recombinant DNA technology, molecular biology, and bioinformatic tools. Genomic analysis has revealed important details of microbial adaptation in polluted environments and the pathways of pollutant degradation or accumulation and production (Pandey et al. 2019b; Rosanti et al. 2020). A few genomic and other omics approaches used for the degradation of contaminants are listed in Table 18.1.

Genomic approaches have also elucidated that the microbial adaptation ability in a polluted environment is regulated by the genes themselves using two-component networks, thermo sensing, chemotaxis, and nutrient absorption, along with degradation of the contaminant at the end (Das et al. 2015). Various molecular techniques such as 16S rRNA sequencing, whole-genome sequencing (WGS), metagenomics, transcriptomics, and other omics techniques have been investigated and can be considered as powerful tools for the mineralization, removal, and degradation of hazardous pollutants present in the environment (Rawat and Rangarajan 2019; Kumar et al. 2020, 2021).

Choi et al. (2013) performed comparative and functional genomics to study the degradation of aromatic hydrocarbons using *Pseudoxanthomonas spadix* and *Burkholderia xenovorans.* Similarly, genomic and ecophysiological analysis of the crude oil-degrading strain *Franconibacter pulveris* DJ34 showed that the genome of

**Table 18.1** List of different -omics approaches that have recently been investigated for improved bioremediation using microorganisms

| S. no. | Approach | Contaminants | Organisms | Experiments | References |
|---|---|---|---|---|---|
| 1. | Genomics | Polychlorinated biphenyls | *Cupriavidus necator* strain JMS34 | BPH locus of *Burkholderia xenovorans* LB400 was inserted in *Cupriavidus necator* JMS34 through genetic modification | Saavedra et al. (2010) |
| 2. | | Mercury | *Medicago truncatula* | High-throughput degradome sequencing | Zhou et al. (2012) |
| 3. | | Phenanthrene, pyrene, benzopyrene | *Rhodococcus wratislaviensis* | Overexpression of amidohydrolase | Subashchandrabose et al. (2019) |
| 4. | | Lead | *Pseudomonas aeruginosa* N6P6 | Overexpression of *bmtA* gene | Kumari and Das (2019) |
| 5. | | Polycyclic Aromatic Hydrocarbons | *Acidobacterium* spp. and *Collimonas* spp. | DNA-based stable isotope probing | Jiang et al. (2015) |
| 6. | Proteomics | Phenol | *Anoxybacillus rupiensis* 19S | Tandem LC-MS/MS used for phenol degrading protein identification | Jardine et al. (2018) |
| 7. | | 4-Nitrophenol | *Rhodococcus* sp. strain BUPNP1 | 2D gel electrophoresis and peptide mass fingerprinting used for 4-NP degrading protein | Sengupta et al. (2019) |
| 8. | | Uranium | *Geobacter* sp. | C-type heme-containing protein analysis and overexpression of the *GscA* gene | Yun et al. (2016) |
| 9. | | Aluminum and nickel | *Microbacterium* spp., *Bacillus* spp., *Arthrobacter* spp. | High-quality MALDI TOF mass spectra used for the dominant bacterial species | Kopcakova et al. (2014) |

(continued)

**Table 18.1** (continued)

| S. no. | Approach | Contaminants | Organisms | Experiments | References |
|---|---|---|---|---|---|
| 10. | | Copper | *Acinetobacter calcoaceticus* strain KW3 | MALDI-TOF/TOF mass spectrometry used for identification of proteins (CopA) involved in Cu resistance mechanisms | Kang et al. (2020) |
| 11. | Metagenomics | Oil degradation | *Proteobacteria* was dominant in the drill metagenome, whereas *Firmicutes* was enriched in consortia samples | Taxonomic analysis conducted to reveal the predominance microflora | Guerra et al. (2018) |
| 12. | | Polluted Ganga and Yamuna river samples | *Streptomyces bikiniensis*, *Rhodococcus qingshengii*, *Bacillus aerophilus*, *Pseudomonas veronii*, dominant flora | Metagenomic libraries and Next-Generation Sequencing conducted | Behera et al. (2020) |
| 13. | | Hydrocarbons | *Naegleria*, *vorticella*, *Arabidopsis*, *Asarum*, and *Populus* | Amplicon sequencing using Illumina's MiSeq platform | Kachienga et al. (2018) |
| 14. | | Diesel-contaminated microcosms | Community profiling | Metagenomic sequencing and analysis using STAMP, MG-RAST, and M5RNA databases | Jung et al. (2016) |

this strain contains genes responsible for the degradation of different polyaromatic hydrocarbons, metal resistance, biosurfactant-producing as well as chemotaxis genes, etc. (Pal et al. 2017).

### 18.1.1 Unraveling of Phylogeny Through 16S rRNA

Microbial communities play different roles in the environment, and identification of microbes will unravel the important communities (Sharma et al. 2021). The discovery of 16S rRNA sequences that are substantially conserved across all microorganisms was a major breakthrough in microbial research. The phylogenetic characterization and evolutionary relationships of organisms, which make up the microbial community, have been greatly aided by the 16S rRNA genes. The 16S rRNA gene of bacteria consists of 1600 base pairs with 9 hypervariable regions of different conservations labeled as V1–V9. The higher taxa group consists of the most conservative areas, whereas regions prone to quick evolution aid in genus or species identification (Chakraborty et al. 2012).

The phylogenetic ranking of organisms taking part in the bioremediation processes can be evaluated by taxonomic identification indicated by the 16S rRNA method. Sakshi et al. (2020) isolated polyaromatic hydrocarbon-degrading microorganisms (PAHs) from soil and characterized them along with their evolutionary relationships. In this study, *Rhodococcus pyridinivorans* DTU-7P and *Kocuria flava* DTU-1Y were identified using the 16S rRNA technique and showed the presence of catabolic genes/enzymes taking part in PAH biodegradation pathways (Sakshi et al. 2020). Moreover, Tang et al. (2018) investigated the association between nephrolithiasis and gut microflora using 16S rRNA gene sequencing. Results have shown that among the 20 genera, *Dorea*, *Parasutterella*, *Fusicatenibacter*, *Erysipelatoclostridium*, *Ruminiclostridium*, and *Phascolarctobacterium* were related to each other in terms of trace element concentration in the blood, including chlorine, sodium, calcium, and potassium (Tang et al. 2018). Moreover, Lu et al. (2019) identified the crude oil-mineralizing bacteria *Pseudomonas*, *Methylobacillus*, *Nocardioides*, *Methylophilaceae*, *Achromobcter*, *Pseudoxanthomonas*, and *Caulobacter* at the generic level using the 16S rRNA sequencing approach. Additionally, they used quantitative polymerase chain reaction to study the relation between RHDα replication and PAH degradation.

The study of microorganism communities present in the environment by culture-dependent and culture-independent techniques has been improved by 16S rRNA gene analysis. Based on sequence similarity, PCR-amplified 16S sequences were grouped together to produce operational taxonomic units (OTUs) for identifying the taxonomy (Lovley 2003). However, this method is not perfectly capable of analyzing all the crucial physiological features of every microorganism. Moreover, in this approach, the phylogeny-based physiological prediction may be affected by the absence of closely related organisms in the sample. The diversity of microbes is also ignored due to the visualization of the distinct bands shown by the dominant

microbial cultures where a single band can represent more than one microbial member (Green et al. 2010).

### 18.1.2 The Whole-Genome Approach

16S rRNA sequencing cannot anticipate the complete genomic structure of an organism; thus, complete genome sequencing is armed to speculate the entire genetic makeup of microorganisms. A few common types of genome sequencing are shotgun metagenomics, single-cell genome sequencing, amplicon sequencing, and whole-genome sequencing of culturable microorganisms. Advances in WGS (whole-genome sequencing) help reveal insights into the degradation mechanism and adaption of microbes in a contaminated environment. Whole-genome sequencing will help unveil different enzymes involved in the conversion of harmful products into nonharmful compounds (Oyewusi et al. 2021). Gan et al. (2013) identified six bacteria belonging to the genus *Novosphingobium.* It showed evidence of the presence of genes for PAH degradation and salt tolerance using data of whole-genome sequencing. The study by Lasa and Romalde (2017) involved whole-genome sequencing of three *Psychrobacter* strains isolated from Galicia and identified as mercury-remediating and antibiotic resistance genes.

## 18.2 The Proteomic Approach

Metagenomics is a vital tool that plays a significant role in understanding the genetic makeup of a particular matrix, although it only provides partial knowledge about gene expression in the contaminated site. Proteomics helps understand the pathways of the expression of a gene and enzyme population in a polluted environment (Pande et al. 2020). Moreover, other omics approaches such as metabolomics, transcriptomics, and proteomics can elevate bioremediation outcomes. Different streams, such as ecological research, mainly focus on evaluating the types of microbes residing in a harmful environment (Jhariya et al. 2022). Proteomics has been involved in studying the process of adaptation of enzymes in a thermophilic environment. Proteins from microorganisms residing in thermophilic and hyperthermophilic environments have a unique feature to adapt, function under different harsh conditions, and hence are of great importance. The conformational adaptation and protein folding molecular properties can be revealed using proteomic tools. The proteomic approach has also been used for learning the physiological changes occurring in microorganisms during the process of bioremediation (Pandey et al. 2019a).

### 18.2.1 Enzymes and Protein Expression Profiling Using the 2DE Approach

For a very long time, the 2DE technique has been used for separation of proteins. It includes two-dimensional separation, such as essential techniques of IEF in the first dimension and SDS-PAGE size-wise fractionation in the second dimension (Mohanty and Devi 2021). Advanced bioinformatic tools and IPGs have significantly enhanced the reproducibility and potential of the 2DE technique. Various researches have shown that this technique has the potential to detect differences between the proteins of contaminated and contamination-free environments. Yun et al. (2016) used the proteomic approach with gel separation and MS spectrophotometry for ex situ uranium bioremediation of ground water and identified that the *c*-type cytochromes are the key electron transfer factors for the reduction of metal ions in the *Geobacter* species.

According to systems biology, any physiological situation of an organism results in differential gene expression and protein synthesis. This involves a set of interconnected enzymatic reactions to neutralize harmful waste present around the cell. 2DE can delve into the pathways followed by microbes through identifying essential enzymes and proteins from the contaminated site, which is a crucial base of environmental remediation (Granato Villas-Bôas and Bruheim 2007). Pessione et al. (2003) studied the membrane protein of the strain *Acinetobacter radioresistens* S13 and found that a number of external membrane proteins were upregulated, such as trimeric porin, OmpA-like protein, glycosyltransferase, etc. Other techniques like MS-based proteomics are nowadays gaining popularity for profiling distinct enzymes and proteins, including peroxisomal antioxidants, epoxide hydrolases, and sarcosine oxidase-like enzymes expressed in polluted marine environments (Mi et al. 2005). The profiling of cellular lysate of *Mycobacterium* sp. using 2DE gel approach revealed that a 81 kDa protein similar to enzyme catalase-peroxidase was expressed under the exposure of pyrene (Wang et al. 2000). Another study of protein profiling of the *Mycobacterium* sp. showed the induction of two ring-hydroxylating dioxygenases, namely, Pdo1 and Pdo2, under the process of pyrene catabolism (Krivobok et al. 2003). The 2DE protein identification technique can contribute to cell-free bioremediation by providing extracted biomolecules from the cell.

Recent advancements have introduced difference in-gel electrophoresis (DIGE), which employs strongly fluorescent dyes to precisely quantify the essential protein expression in 2DE. DIGE involves staining of proteins with green and/or red dyes and mixed with an internally labeled protein stained by a blue hue. This kind of labeled protein advancement in technique helps avoid any uncertainty in the matching of protein spots (Ohlendieck 2018). Colquhoun et al. (2012) identified changes in protein expression triggered by bacterial growth on dibenzofuran using DIGE and MALDI-MS. Liew et al. (2021) showed a significant difference between environmental and clinical strains via matched 526 protein spots through 2D DIGE and LC-MS QTOF. These findings indicate the DIGE's potential to recognize proteins of interest in bioremediation but fail to analyze the vast variety of bacterial

cell lysates under different stress situations. Therefore, improved separation methods need to be developed for better identification in proteomics (Lim et al. 2016).

### 18.2.2 MS Techniques in Proteomics

Mass spectrometry (MS) advancements have improved amino acid analysis for protein identification and have aided in the development of the area of environmental proteomics. MS can infer the composition of an unknown protein on the basis of peptide mass detection made up of amino acids. Proteins are generally digested with the help of different proteases to break them into small peptides for analysis in MS known as PMF (Tyanova et al. 2015). The process includes a chain of continuous breakdown of proteins into small peptides, or amino acids that differ in mass from each other, which helps in the detection of the amino acid sequence of the peptide. This process is also known as tandem MS or MS/MS because it utilizes one MS analyzer for the selection of ions during fragmentation and the second one to measure the ions of the fragments (Vaudel et al. 2015).

Another technique used for the detection of proteins is MALDI-TOF-MS. It also detects bacteria, viruses, fungi, spores, and low-molecular-weight compounds in environmental samples. In bioremediation, MALDI-TOF has great importance as it allows the identification of bacterial trademark proteins and biomarkers such as primary and secondary compounds from samples of selective contaminated sites for identification of compelling microbes (Greco et al. 2018). Another advanced technique is Fourier transform ICR (FT-ICR) MS that allows a magnified detection limit of ~30 zmol for a ~10 kDa protein (Tucholski and Ge 2022). Hence, these advancements in detecting and identifying proteins and metabolites will ultimately provide a path for cell/cell-free bioremediation.

### 18.2.3 Protein–Protein Interactions

Since the last decade, the demand for microarray techniques has increased to study protein aggregation and protein–protein interactions. The mapping of extensive signaling pathways in bacteria is now possible as it has been found that these metabolic pathways are sequenced by complexes of different proteins during the chemotactic activity of an organism. These protein complexes can be recognized by placing them on a glass slide on a microarray-based system. In the field of bioremediation, the protein array technique can analyze the binding of various inhibitors or enzymes (Singh 2006).

### 18.2.4 Challenges in the Development of Proteomics

Proteomics has proven itself to be a powerful method for characterizing the functional molecules of diverse signaling/degradation pathways in biological

advancements. Still, its use is limited to only a few labs for ex situ applications. Therefore, this technology needs to be developed for environmental remediation in a cost-effective manner. However, proteomics is providing palpable benefits by aiding in understanding key protein production and expression, which can be used for predicting degradation pathways. Moreover, it offers knowledge about the expressed protein structure and functions. Despite this, integrating proteomics with other "-omics" approaches, especially metabolomics, where primary and secondary low-molecular-weight metabolites play essential roles in bioremediation, has become a matter of consideration (Bhende et al. 2022).

## 18.3 Metabolomics

This branch of science allows the identification, characterization, and quantification of metabolites such as intracellular and extracellular metabolites produced by an organism using various analytical tools. Like a transcriptome and a proteome, a metabolome is circumstance-dependent, and the quality and quantity of each metabolite bank on the physiological and growth conditions of the cell of an organism. The intricate nature of metabolic pathways, where one metabolite can be involved in more than one pathway, makes it difficult to interpret the direct relation with the gene. Moreover, metabolomes are made up of a vast range of chemical compounds, making it highly difficult to analyze a complete set of metabolomes simultaneously (Jeevanandam and Osborne 2021).

### 18.3.1 Fundamental Principles and Measurement Strategies for the Metabolome

Metabolites are organic compounds involved in metabolic pathways and used in the proper functioning of the cell of an organism. Various microorganisms produce different metabolites less than 1000 Da molecular weight and are known as primary as well as secondary metabolites. Metabolomics ultimately targets quantification of all types of metabolites produced by a cell. This can be accomplished via metabolite isolation and characterization methods that combine computational methods and nanotechnology, used for sampling, extraction of chemical compounds, sample preparation, and analysis (Pande et al. 2020).

Metabolome-analyzing technologies should be more accurate and advanced with the potential to analyze a large set of metabolites at a time. Significant development has been achieved in designing analytical tools to quantify and evaluate numerous metabolites. However, these advanced technologies still lack simultaneous quantification and interpretation of the complete metabolome due to the vast diversity of chemical composition and low molecular weight of metabolites. Some commonly used tools for studies of metabolomes are liquid chromatography mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy (Rawat and Rangarajan 2019).

### 18.3.2 NMR Spectroscopy and MS in Metabolite Profiling During Bioremediation

NMR spectroscopy was first utilized by Eakin et al. during the investigation of catabolism of $1^{13}C$ glucose in *Candida utilis* live-cell suspension. Since then, significant breakthroughs in NMR equipment and methodologies have been made, but the technology still depends on the concepts established in the preliminary study. The method involves using enriched precursors to improve sensitivity and selectivity, capturing a number of consecutive spectra to estimate kinetic constants and analyzing cell lysates to detect metabolites (Santos et al. 2016).

Soil from a bioremediation site is mostly analyzed by solid-state NMR or high-resolution magic angle spinning (HR-MAS) NMR rather than liquid-state NMR. In contrast, the study of organic pollutants in bioremediation is conducted by liquid-state NMR. Detection of xenobiotic degradation by microbes using liquid-state NMR has been restricted to heteronuclei NMR studies (Rawat and Rangarajan 2019). In an analysis by Arora et al. (2018), identification and quantification of groups of around 45 metabolites, per se, organic acids, sugars, amino acids, nucleotides, osmolytes, phosphagens, etc., using NMR spectroscopy from *Scenedesmus* microalgae during bioremediation of arsenic-contaminated sites was performed. Morgado et al. (2012) observed a series of oxidation processes in NMR spectroscopy for multi-heme protein characterization. They compared isotopically labeled peptides with $^{1}H$-$^{13}C$ HSQC NMR spectra for labeled and unlabeled samples and studied *Geobacter sulfurreducens* heme proteins functionally and structurally. Haque et al. (2021) performed compositional characterization of the biosurfactant $^{1}H$-NMR and ESI-MS analysis for crude oil bioremediation.

An advanced form of NMR, HR-MAS NMR, has various advantages compared to older NMR techniques. For instance, the bioavailability of contaminants can be obtained in detail, a large amount of metabolome can be analyzed, and migrating pollutants can also be easily detected. Cross-polarization magic angle spinning (CP-MAS) NMR, a more advanced kind of NMR, has been used to investigate covalent bonds between organic contaminants and organic materials in the soil (Skorupa et al. 2021).

MS in bioremediation allows for a highly accurate analysis of metabolites with greater sensitivity. The quadrupole (Q) GC-MS in the electron impact (EI) ionization mode is the most widely used technology for the complete assessment of biological matter. The ionization method in GC benefits from the detection of any metabolite eluting from the column of the machine instead of selective detection of cationic or anionic ionization. Other advanced technologies in the analysis of metabolites are ion-trap (Q)-LC-MS, GC-TOF-MS, and MALDI-MS. A vast variety of MS techniques provide detection of a large number of metabolites with high sensitivity. However, some of their disadvantage include difficult sample preparation, less available metabolite loss during extraction, variable ionization efficiencies of metabolites, and the incapability to provide accurate quantification. Certain metabolite detection also becomes difficult because of ionic suppression and the impact of diverse environmental systems (Kuckova et al. 2019).

## 18.4 The Omics Approach for Bioremediation of the Environment

### 18.4.1 Metagenomics in Bioremediation

The complicated microbial communities taking part in the biodegradation of contaminants can be better understood using a metagenomic approach. Generally, metagenomics is introduced as a collection of environmental genomes or eco-genomics, which is used for the study of microbial communities and their applications in bio-decontamination of the environment. Modifications in metagenomics have revealed the characteristics of major genes/enzymes involved in the degradation of contaminants (Nazir Nazir and Nazir 2016). The communities and diversities of culturable and nonculturable microbes have been characterized using different approaches of metagenomics. Along with this, phylogenetically and taxonomically based different operons consisting of genes can also be analyzed using this approach (Uhlik et al. 2013).

Generally, two methods of metagenomics are used for analysis, namely, shotgun and targeted metagenomics. Targeted metagenomics is focused on the identification of all the sequences of a single targeted gene available in an environmental sample. The variety of microbial phylogeny and the presence of specific genes can also be explored in the given sample (Zhang et al. 2019). Thereby, targeted metagenomics detects a significant change in the diversity and morphology of communities before and after exposure to pollutants in the environment. On the other hand, shotgun metagenomics is referred to as the total genomic content of the natural microbial communities. It renders a correlation between taxonomic organization and functional genes. Based on the gene sequence, novel proteins and enzymes are screened. The sequences of probes and primers used in sequence-based screening are designed on the basis of predefined coding genes for enzymes or other necessary bioactive compounds (Kachienga et al. 2018). The PCR amplification technique is employed to identify these genes in the unknown metagenome, which are then sequenced. With the help of other expression models, cloning of these genes can be carried out for further cross-validation and overexpression. Function-based screening is mainly used to recognize and determine genes with the capability to encrypt a novel compound. This process involves slicing the entire DNA into smaller segments, which are then linked to a vector to generate libraries. These clones are then screened for a particular phenotypic activity, which allows for the discovery of molecules with the needed functional activity. Furthermore, the sequencing of 16S rRNA from a given metagenome aids in the identification of the sample's microbial community diversity. The analysis of complete metagenomic 16S RNA provides a better understanding of the culturable and nonculturable microbial populations and their interactions with one another as well as with their particular surroundings (Reiman et al. 2018).

### 18.4.2 Case Studies for Metagenomic Approaches Used in Bioremediation

Metagenomics has enhanced our understanding of bio-decontamination and detoxification mechanisms using microbial communities in the polluted study area. Metagenomics can also identify specific degraders or catabolic genes that contribute more to the biodegradation of both biotic and abiotic xenobiotics. The comparative metagenomic procedures can also identify variations in functional microbial communities presented at different polluted sites by the same pollutants. Advances in sequencing technologies, such as NGS, have permitted research into the deeper covering of the microbial communities and are also critical for providing a fair picture of the taxonomic diversity, abundance, and roles of the microbial population in ecosystems (Zwolinski 2007).

Garrido-Sanz et al. (2019) performed amplicon sequencing of a diesel-polluted site for exploring the microbiome participating in the degradation of contaminants. The study involved amplicon sequencing of 76 variants and concluded that *Pseudomonas*, *Aquabacterium*, *Chryseobacterium*, and *Sphingomonadaceae* are the dominating species in the diesel-polluted site. Later on, they showed that the microbial community changes when grown on different PAHs, proving that different microbes work for degradation of different compounds. Moreover, shotgun metagenome sequencing of the consortium growing on the diesel-polluted sample revealed gene-encoding key enzymes taking part in the degradation of various aromatic and polyaromatic compounds.

A study by Wang et al. (2015) highlighted the predicative and comparative metagenomics to identify the biomarkers of pollution. They used MetaBoot software for recognition of biomarkers for a hydrocarbon-contained environment through a comparative study of metagenomic datasets obtained from a PAH-contaminated field of 255 taxa and 414 functional modules. A comprehensive study was conducted to identify the PAH-degrading gene from soil and water samples and discovered key genes for mineralization of methylphenanthrene, naphthalene, and phenanthrene (Dellagnezze et al. 2014).

Metagenomic profiling of three different sites of a petrochemical plant helps explore the types of genes inhabiting a microbial community. Genes with antimicrobial resistance are found approximately 15 times more abundantly in PAH-contaminated soils than in unpolluted soils (Chen et al. 2017). Raiyani and Singh (2020) performed metagenomic sequencing for taxonomic and functional profiling to analyze a sea-inhabiting bacterial community's structure and functional contours. They used high-throughput sequencing technology to discover the bacterial diversity of microbial functions of two seawater locations. PICRUSt software was utilized for taxonomic classification of metadata to explore metabolic information.

### 18.4.3 Tools Used for Metagenomic Analysis

Nowadays, a vast range of in silico tools, software, bioinformatic pipelines, statistics, and algorithms are being used for interpreting and correlating molecular data to omics approaches. Nonetheless, resources dedicated specifically to microbial bioremediation remain limited (Dudhagara et al. 2015). The former in silico tools that assemble pathways of bioremediation data are a database called the Biocatalysis/Biodegradation Database, designed by the University of Minnesota. This database consists of an inventory of 187 pathways, 1287 reactions, 1195 compounds, 833 enzymes, 491 microorganism entries, and 259 biotransformation rules (http://umbbd.msi.umn.edu/) (Ellis and Wackett 2012). Another application known as MetaRouter keeps track of a wide range of biodegradation and bioremediation data in a system that allows for queries related to updates and modifications. MetaRouter is a consolidated network, which collects data related to chemical compounds, multiple chemical reactions, microorganisms, and enzymes, consequently creating a relational database. It helps identify the interlinked pathways between two different chemical substrates. The search for predicted pathways can be shortened or filtered on the basis of pathway length, specific enzymes for particular microorganisms, and metabolites produced during the process that possess different chemical characteristics such as melting point, solubility, etc. The link for a web search for MetaRouter is www.pdg.cnb.uam.es/biodeg_net/MetaRouter/ and is freely accessible through the Internet (Shekhar et al. 2020). Another tool is MEGAN (MEtaGenome ANalyzer) that aids in the effective analysis of an immense quantity of metagenomic data. This software also allows functional and taxonomical evaluation of a large set of metatranscriptomic data. The application reads data from NCBI databases and maps them to SEED, COG, and KEGG classifications. These programs utilize a variety of visualization schemes, per se, co-occurrence plots, and employ inbuilt statistical tools such as principal coordinate analysis (PCoA) and clustering tools (Huson et al. 2016).

A simple metagenomic analysis shell for microbial communities (SmashCommunity) is another pipeline for annotation and analysis of data retrieved from metagenomic samples. For instance, Sanger data as well as other 454 sequencing techniques have been feasibly performed on it. Metagenomic analyses such as assembly, gene prediction, and quantitative phylogenetic analysis have also been performed using this software. It renders optimized factors via Arachne and Celera for metagenome sequencing. GeneMark and MetaGene are also used in visualizing the coding sequences of proteins in metagenomic data. As a visualization tool, this software employs the interactive Tree of Life (iTOL) web application. This program also analyzes various metagenomic sets of data by clustering profiles using a relative entropy metric to compare quantitative outlines (Arumugam et al. 2010). Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) is a web-based analytical gateway that allows users to deposit, locate, analyze, visualize, and share data regarding microbial life. CAMERA analyzes large amounts of environmental metagenomes, their surrounding characteristics, and

provides tools in the portable mode for cross-evaluation of environmental samples (Gan et al. 2013).

### 18.4.4 Metatranscriptomics

Metatranscriptomics provides information on the expression and control of a composite microbial population observed in nature. In metatranscriptomics, initially, the mRNA is extracted to convert it into cDNA for its sequencing on an NGS platform. The use of stable isotope probing (SIP) in contemporary metatranscriptomic methods could be utilized to recover the transcriptomes of any material (Lueders et al. 2016). Metatranscriptomics includes multiple tools such as AbySS, Trans-Abyss, Scripture, Oases, Cufflinks, and MetaVelvet. A few drawbacks of the transcriptomic approach include less regeneration of high-quality mRNA, fragmentation, and fast degradation of various RNA and mRNA species. Direct uses of cDNA sequencing, as well as the capability of direct transcript quantification, can be used to circumvent these limitations (Simon and Daniel 2011).

## 18.5 Prediction and Reconstruction of Bioremediation Pathways Using Genomic, Proteomic, and Omics Approaches

Microorganisms eradicate pollutants by expressing a set of genes or proteins and simultaneously triggering the next gene or protein. Genes taking part in microbial degradation pathways are frequently located in clusters in microorganisms, and their expression is controlled during biodegradation by specific regulators that operate either by activation or repression. Microbes activate these clusters of genes only when they need them during the process (Pande et al. 2020). Years of research have improved screening, whole-genome sequencing, and database collating and storing information, allowing for a better understanding of the metabolic pathways. These technologies have reframed the original pathways for the conversion of toxic contaminants to nontoxic intermediates. There are two main approaches involved in metabolic pathway reconstruction: (1) an in silico approach along with various computational tools to design and develop the metabolic pathways and (2) an experimental approach: molecular techniques used for cross-verification of in silico designed pathways (Park and Choi 2020).

### 18.5.1 Computational Tools Used for Reconstruction of Pathways

Approaches like whole-genome sequencing and knowledge of natural microbial pathways pave way for their better understanding and modification to achieve improved environmental bioremediation. A few tools and techniques of bioinformatics used in bioremediation are shown in Fig.18.1. This reconstruction approach

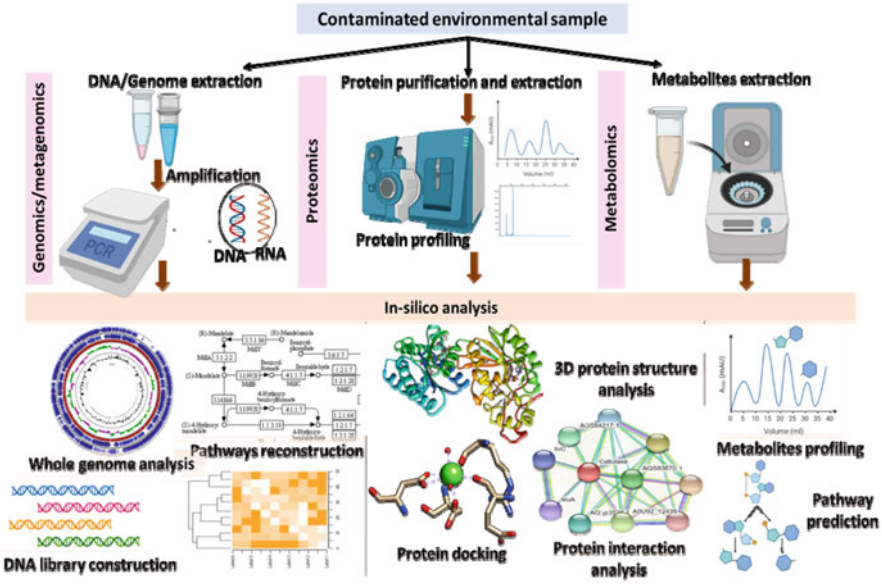


**Fig. 18.1** Tools and techniques used in genomics, proteomics, and metabolomics for analysis of contaminated environmental samples

for pathway modification involves various enzymes from different steps and sets of enzymes encoding genes. There are a few databases such as KEGG (Kanehisa et al. 2016), MetaCyc (Caspi et al. 2018), Rhea (Morgat et al. 2019), and BRENDA (Schomburg et al. 2017) used for the prediction of biochemical pathways employed by different enzymes. Moreover, these databases also provide an inventory of enzymes that helps in the development of new and improved metabolic pathways by amending with the help of supplementary reactions to fill the gaps. These databases only show chemical transformation and do not account for the physiological aspects that bacteria use to encode pathways. BLAST (Torkian et al. 2020) is a sequence alignment program applied for the identification of statistically significant and analytically important similarities among query sequences and for both protein and nucleotide sequences. This technique is based on the assumption that homologous sequences in known and undiscovered pathways are expected to encode functionally similar proteins.

Another tool involved in the modification and reconstruction of metabolic pathways is GSMM, which stands for genome-scale metabolic model. It is an automated tool, based on genotypic features of microorganisms for prediction of their phenotypic characters. A GSMM model utilizes genetic information from the data repository of a specific microorganism for construction of metabolic pathways (Thiele and Palsson 2010). Other software such as BioCyc and KEGG provide all the possible metabolic pathways of a particular microbe of whole-genome data (Caspi et al. 2016), whereas MetaCyc helps construct biochemical networks on the

basis of some special metabolite production. Similarly, KASS is also designed to recreate these microbial metabolic pathways. Constraint-based reconstruction and analysis (COBRA) is another model that provides a platform for optimum genetic modification for significant improvement in the activities of microbes for metabolite production in bioremediation (Heirendt et al. 2019).

Technological advancements have also made it possible to recreate metabolic pathways by employing different bioinformatic techniques. Novel and effective metabolic pathways can be built by anticipating biological reactions using the chemical structures of metabolites formed in the pathways (Kanehisa 2017). For instance, PathPred and the University of Minnesota Pathway Prediction System (UMPPS) are free-to-use tools that help in peculating networks using metabolites' chemical structures (Wicker et al. 2016). These interfaces provide users with a platform for selection of biochemical reactions for modification of pathways and also render information regarding xenobiotic degradation and biosynthesis of useful metabolites. Few advances in computing methods could aid in the simultaneous estimation of thousands of metabolites for improved bioremediation (Heirendt et al. 2019).

## 18.6 Conclusions

Various anthropogenic activities have resulted in environmental contamination and deposition. Till now, bioremediation techniques have developed in various ways, such as enzymatic activity enhancement for degradation. However, in-depth information on genomes, proteins, and metabolites is still lacking for advanced bioremediation application. Gaining better insights into the bioremediation, degradation mechanism of microbial physiological machinery, and type of biomolecule taking part in the different degradation processes is extremely crucial. Continued scientific progress will eventually allow for fully integrated techniques to study an organism's functional physiology employing gene, protein, and metabolite expressions. Genomics will play a vital role in understanding the genetic makeup of an organism that plays a role in environmental bioremediation. Along with genomics, proteomics can help in gaining knowledge about the key enzymes degrading environmental contaminants. Analysis of small organic compounds will provide a way to understand the metabolic pathways of bioremediation. However, successful bioremediation of the environment still needs better understanding and improvements in currently used techniques. A combination of proteomic, metabolomic, and omics approaches along with genomic approaches may be what the future holds for effective bioremediation.

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# Omics Insights into Cold Environments: Cold-Tolerant Microorganisms and their Potential Use in Bioremediation

19

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**Abstract**

The climatic diversity of our planet allows the development of a broad range of living organisms. Cold environments are colonized by organisms, which are able to tolerate low temperatures. Within this group of organisms are microorganisms that have thrived because of their adaptation to these environments. This adaptive capacity of microbes is basically due to the evolution of different physiological processes regulated at low temperatures, which is an adaptive strategy. Bacteria with the capacity to tolerate and colonize cold environments (psychrophilic and psychrotrophic—"extremophiles") regulate the expression of their genetic patterns to overcome the limitations generated by cold. As part of this regulation, biological products or biomolecules are generated that are stable in extreme environments (low temperatures), with these biomolecules being highly appreciated by the scientific community, due to their stability, resistance, versatility, and applicability in different biochemical processes of the industry. In addition, products from the metabolism of microorganisms can be used in the treatment of contaminated environments (bioremediation). Currently, there is a strong interest in exploring the molecular mechanisms of microorganisms

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through current techniques that allow elucidating their composition and functioning. This chapter focuses on the application of omics technologies in the study of microorganisms adapted to cold environments and the potential application of these or their constituent biomolecules in the bioremediation of contaminated environments.

**Keywords**

Cold environments · Transcriptomics · Metagenomics · Psychrophilic · Psychrotrophic · Extremophiles

## 19.1 Introduction

Society's lifestyle, because of industrialization and population growth, has resulted in an increased release of nonbiodegradable pollutants, which deteriorate both the environment and public health (Jeyavani et al. 2021). In this sense, bioremediation is an appealing process for remediating environmental pollutants (Kang 2014) because it uses microorganisms to reduce pollution in an environmentally friendly and cost-effective manner; fungi, yeasts, and bacteria have been considered among these microorganisms (Kumar et al. 2018; Chandran et al. 2020).

Microorganisms can live in harsh environments and start producing metabolites that reduce and transform pollutants (Rampelotto 2010; Chandran et al. 2020). Psychrotrophs and psychrophiles (Furhan 2020) are species that live in cold environments (extreme temperatures) and have adapted to low-temperature environments through various biological mechanisms (Baraúna et al. 2017). The metabolic strategies developed by psychrotrophic and psychrophilic microorganisms allow them to survive in cold environments; examples include (1) increased fluidity of cell membranes; (2) decreased freezing point of the cytoplasmic aqueous phase; (3) production of cold-shock proteins and cold acclimation proteins (CSPs and CAPs, respectively); (4) production of catalases, peroxidases, superoxide dismutase, and oxidoreductases that confer protection against reactive oxygen species; and (5) maintenance of catalytic efficiency in cold conditions (Casanueva et al. 2010).

Owing to their genetic, metabolic, and cellular apparatus, which makes it possible for them to withstand extreme physical and chemical conditions, these microbes are emerging living organisms for environmental biotechnology (Dvořák et al. 2017), allowing for natural recovery of contaminated sites (Abatenh et al. 2017); thus, there is a need to discover new life forms, the pathways underlying adaptability to extreme habitats, and the distinctions between extremophilic and non-extremophilic microorganisms (Giovanella et al. 2020). In fact, genomic and metagenomic sequencing are the most commonly used strategies for biodegradation studies with extremophiles (Krüger et al. 2020), as they provide information on new genes and pathways related to the biodegradation of a given pollutant (Giovanella et al. 2020).

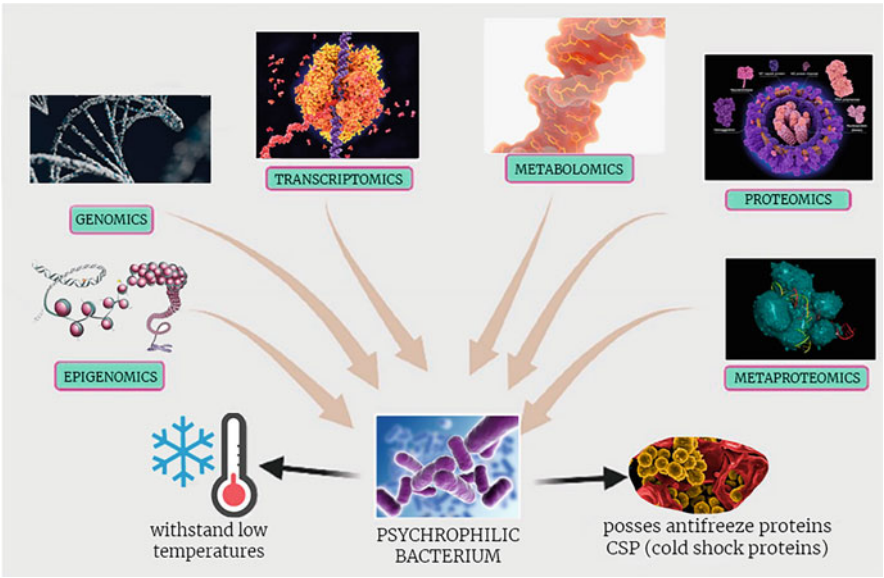


**Fig. 19.1** Omics strategies for the study of psychrophilic microorganisms

Different omics approaches have been used to fulfill our current understanding of microbiological resilience to harsh weather (Fig. 19.1), and metaproteomics has even been used to examine the interactions between microorganisms living in cold environments (Williams et al. 2013), thus trying to fill the identified gaps of these microorganisms' ecology, expression levels, and metabolic activity. However, research incorporating these techniques is still limited, and although extremophilic microorganisms have been identified, they have received little attention in terms of technological applications in environmental biotechnology (Giovanella et al. 2020).

Large amounts of data can be generated by innovative proteomic techniques based on liquid chromatography coupled with mass spectrometry (LC-MS/MS), and these data can help us understand the important aspects of psychrotrophic and psychrophilic microorganisms' adaptation to cold (Karlsson et al. 2012; Baraúna et al. 2017). The analyses of microbial transcriptomes and proteomes through omics have allowed us to obtain enormous amounts of data, which support the reliability, sensitivity, and precision of the analyses, all of which allow us to perform an integrated analysis of the mechanisms of microbial adaptation to extreme environments (Olaya-Abril et al. 2014). Furthermore, the application of bioinformatic techniques has allowed us to improve the study and analysis of omics data obtained from extremophilic microorganisms (Baraúna et al. 2017).

In this regard, national and international scientific programs around extremophiles should be encouraged and sustained to positively contribute to sample collection and analysis in extreme environments. These findings could lead to a better understanding of this microbial community and their biological properties in

bioremediation (Giovanella et al. 2020). As a result, this chapter aims to provide a framework for understanding the main metabolic traits developed by these extremophilic microorganisms to adapt to cold in polar or oceanic environments as well as their potential use in bioremediation processes.

## 19.2 Microbial Biodiversity in Cold Environments

The discoveries and studies of microbial microorganisms found in specific habitats have assisted us in determining the limits of life as well as the processes, development, and evolution that these microorganisms have undergone. All of these refer to the importance of both environmental factors and structural changes in microorganisms, which have overseen providing diverse conditions for various microorganisms to develop properly (Aguilera et al. 2017). In this manner, it has been possible to discover the existence of microorganisms capable of living, adapting, or surviving in large areas of the biosphere where low temperatures are the dominant factor (Gabriela and Cortés 2019).

However, the classification of microorganisms is based on their response to long-term survival in low-temperature environments. Thus, psychrophilic bacteria, also known as cryophilic, cold-loving microorganisms, do not need to make an effort or are not required to live in low temperatures because those temperatures remain constant and can develop in a temperature range between 0 and −15 °C. Furthermore, psychrotolerant bacteria, microorganisms that tolerate or withstand low temperatures, live in environments that change their temperatures on a regular basis, which is why they must tolerate such fluctuations; they can develop in a temperature range of −4 to −42 °C (Bhandari 2020).

The interaction of psychrophilic microorganisms with a low-temperature environment causes a response in cell metabolism; as a result, the bacteria internally synthesize those enzymes that provide them with low temperature resistance. However, if the bacteria are moved to a temperate environment, then they lose the ability to produce these cold-tolerant biomolecules. Similarly, psychrophilic microorganisms maintain active transport with energy expenditure due to a higher number of unsaturated fatty acids in their cytoplasmic membranes (Fig. 19.2), which allows them to maintain the semi-fluid state of the membranes, preventing low temperatures from altering their structure and function (Gabriela and Cortés 2019).

Several studies have reported various genera of psychrotrophic microorganisms with plant growth-promoting properties (*Bacillus*, *Kocuria*, *Pseudomonas*, *Arthrobacter*, *Flavobacterium*, *Hydrogenophaga*, *Burkholderia*, *Enterobacter*, *Janthinobacterium*, *Brevundimonas*, *Serratia*, *Citricoccus*, *Lysinibacillus*, *Clostridium*, and *Exiguobacterium*) (Bhandari 2020; Kumar and Chandra 2020). It should be noted that although both psychrophilic and psychrotolerant microorganisms can provide characteristics that aid in the cultivation of plants that can withstand low temperatures or participate in bioremediation, as stated in the research, based on the study of bacterial strains found in the Peruvian scientific sector “Machu Picchu”

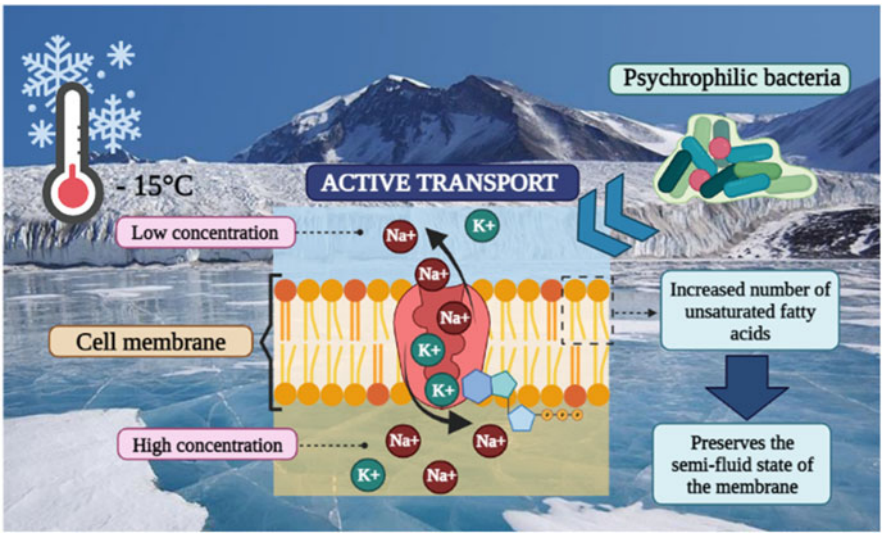


**Fig. 19.2** Cryotolerant strategy of psychrophilic bacteria

(Pretell Masias 2021), greater emphasis has been placed on the investigation of psychrotolerant microorganisms.

## 19.3 Transcriptomics of Psychrophilic Microorganisms with the Bioremediation Potential

Extremophilic microorganisms can survive in hostile environments because they have evolved and adapted various survival mechanisms. These microbes can synthesize enzymes with extreme-condition adaptability, such as psychrophiles, which can survive at temperatures ranging from −10 to 20 °C. The correlation between high catalytic activity and low thermal stability at moderate temperatures is a distinguishing feature of low-temperature stable enzymes. These microscopic organisms produce enzymes such as amylases, proteases, cellulases, xylanases, and lipases, which can be used and applied to remove and biodegrade environmental pollutants (bioremediation) (Gunjal Aparna et al. 2021). The use of lipases from *Moraxella* sp., a microorganism isolated from Antarctic seawater, as well as yeast strains that can be used for oil degradation, is an important fact. These enzymes are used in the treatment of saline wastewater as well as in the biodegradation of pollutant residues in oilfields. Lipases can also be used to biodegrade residual polyester and remove biofilms that accumulate on cold aquatic bodies (Gunjal Aparna et al. 2021). As a result, it is intriguing for the biological and environmental sciences to investigate the composition of the transcriptome of psychrophilic organisms in order to understand the diversity of genes that are expressed under

these extreme conditions and, as a result, to consider their application in bioremediation processes.

Transcriptomic studies, in combination with bioinformatic processing, have been developed in recent years with the goal of studying gene expression profiles through the simultaneous evaluation of multiple genes at a specific point in the development or metabolism of a living being. Essentially, this entails investigating the transcriptome, which is nothing more than a collection of messenger RNA (mRNA) and non-coding RNA molecules found in a cell (Cos 2010). Initially, studies were conducted using techniques such as fluorescence in situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR).

FISH is a technique for detecting nucleic acid sequences in preserved cells using a fluorochrome-labeled probe that is directed at a specific site on the chromosome and emits fluorescence that can be seen with a fluorescence microscope (Cos 2010). RT-PCR identifies, detects, and quantifies RNA by using it as a template to synthesize a complementary molecule (cDNA), which then serves as the template for a real-time PCR reaction. The exponential increase (amplification) in DNA copies is reflected in the appearance of fluorescence, the intensity of which is directly proportional to the amount of RNA (cDNA) in the sample, making this reaction highly sensitive even at low nucleic acid concentrations (Rodríguez Martínez and Suescún Otero 2013).

As they only allow for qualitative or semi-quantitative analysis of one candidate gene at a time, the aforementioned techniques are considered low-throughput. As a result, new quantitative techniques, such as RT-qPCR, were developed over time, improving the methodology and allowing multiple transcripts to be analyzed at the same time. Nowadays, most studies are conducted using microarrays, which involve the hybridization of RNA (converted to fluorescent cDNA) to a chip containing multiple cDNA probes belonging to a specific number of genes of interest, with the caveat that the genome of the organism under study is required. However, new next-generation sequencing (NGS) technologies that allow absolute and reproducible quantification of the total amount of mRNA or microRNA present in a sample have recently been introduced (Gunjal Aparna et al. 2021).

### 19.3.1 Transcriptome Analysis of Psychrophilic Bacteria

The transcriptomic and proteomic responses of *Psychrobacter* sp. PAMC 21119 isolated from Antarctic permafrost soil were studied, contrasting profiles at −5 and 20 °C and discovering a total of 2906 transcripts and 584 genes differentially expressed by RNA-Seq. This research enabled the identification of genes that finalize the translation of other genes as well as those that govern ribosomal structure and biogenesis; however, the expression of genes associated with lipid transport and cellular metabolism decreases at these extreme temperatures (Koh et al. 2017).

The main objective of employing a transcriptomic approach is to collect all the information on the differential expression of genes in a species; however, often, no

clear information is obtained about their specific metabolic capabilities since the function of the identified proteins is unknown.

### 19.3.2 *Pseudomonas* with Bioremediation Capacity

It is well known that psychrophilic *Pseudomonas* can be used in the bioremediation of heavy metal- and hydrocarbon-contaminated environments. Several studies have been conducted to provide quantitative information on their enzymatic degradation capacity using cold-active enzymes (psychrozymes) that catalyze the oxidation stages of p-xylene biodegradation in highly contaminated soils (Miri et al. 2022). Most crude oil compounds contain large amounts of benzene, toluene, ethylbenzene, and a mixture of xylene isomers (BTEX). Owing to their toxicity, these are considered to be a significant class of chemical pollutants. Temperature limits the rate and extent of microbial biodegradation of hydrocarbons in cold regions, and it influences hydrocarbon condensation and viscosity. Pseudomonads, on the other hand, can decode specific genes to produce toluene/o-xylene monooxygenase (ToMO), which has a broad aromatic substrate specificity and can hydroxylate more than one aromatic ring position in two successive monooxygenation reactions. It also produces catechol 1,2-dioxygenase (C1,2D), an iron-containing enzyme capable of cleaving the catechol ring for complete BTEX detoxification (Miri et al. 2022).

*Pseudomonas* strains found in arctic climates have genes and transcripts involved in hydrocarbon degradation. Enzyme-mediated bioremediation has produced promising results with a mixture of bacteria containing the alkane monooxygenase AlkB, which is encoded by the *AlkB* gene. A petroleum hydrocarbon bioremediation assay revealed a 1000-fold increase in AlkB monooxygenase, which has been closely associated with a rise in the depletion of the total aromatic and saturated compounds present in petroleum waste soils, indicating that this technique can be successful in remediating a broad range of liquid fuels in a short period of time (Siddiqui and Bano 2019).

*Pseudomonas pseudoalcaligenes* S1 differential gene expression (transcriptome) studies are conducted using Illumina's Hiseq2000 platform (RNA-Seq). The genes' differential expression revealed that they are involved in heavy metal and redox transport function, regulation, transcription, and translation. Meanwhile, the expression of a ClpB chaperonin significantly reduces the aggregation of folding proteins at low temperatures (Zhang and Hu 2019).

Metagenomic studies have identified a large number of psychrophilic bacteria, including those mentioned above. However, after reviewing the literature on transcriptomic techniques and the information they provide on how they interact with pollutants (hydrocarbons and metals), it is possible to highlight the significance of studies with omics visions of this type. Previously, researchers stated that psychrophilic bacteria have the ability to degrade polymers, the accumulation of which in the seas is a problem for the environment. It would be interesting to delve deeper into the transcriptomes of various psychrophilic bacteria that could be used in the bioremediation of plastic-contaminated marine environments.

## 19.4 Proteomics of Psychrotrophic Microorganisms with the Bioremediation Potential

Proteomic studies in psychrotrophic microorganisms are important for the advancement of bioremediation because they allow knowing and predicting the biodegradation route followed by microorganisms for specific contaminants, as well as distinguishing the changes that occur at the physiological level in relation to proteins, with knowledge of the key enzymes to produce a response to external changes and the composition of the proteins being of great importance (Singh and Nagaraj 2006). Proteomic research has been able to determine changes in protein composition and abundance, while emphasizing the importance of knowing the genomic sequence of psychrotrophic microorganisms, as it allows for the identification of enzymes required for the understanding and prediction of degradation pathways (Seung et al. 2007).

Proteomic studies of *Pseudomonas aeruginosa* with psychrotrophic characteristics show that one-third of the microbial genes encode for membrane proteins and about 78 proteins show significant changes when exposed to contaminated environments, indicating that they play an important role in heavy metal biodegradation and adaptive mechanisms (Wright et al. 2019).

## 19.5 Transcriptomics of Psychrotrophic Microorganisms with the Bioremediation Potential

Advances in industry cause pollution that harms public health and the environment, including the generation of toxic waste that is dispersed through air as fine particles, in parts of soil and in water, eventually entering our food chain and posing high risks, thus necessitating the development of eco-friendly technologies. Bioremediation technology is a potential option because it uses living organisms, which include the use of bacteria, microalgae, and fungi that through specific processes allow the degradation and recovery of environments to their initial characteristics, being a profitable and viable option for the decontamination of the ecosystem (Megharaj et al. 2014).

In general, the microorganisms used in these bioremediation processes belong to the mesophilic group because they have demonstrated an optimal process in the laboratory; however, they are inefficient under various conditions, such as low temperature, high pH, and so on, and thus the bioremediation processes are limited. However, there are indicators of the presence of microorganisms capable of performing these processes at low temperatures. To address this issue, researchers must look for cold-tolerant psychrophilic strains with the ability to biodegrade harmful agents in contaminated environments, as well as understand their molecular mechanisms of action and degradation, in order to develop future biotechnological applications for designing new long-term bioremediation strategies (Olguín et al. 2007).

Psychrotrophic and psychrophilic microorganisms have processes and enzymes for optimal activity development, such as the protein "cold-shock protein A" (CspA) and the RNA helicase protein; these conserved biomolecules are expressed as an adaptive response to low temperatures and have been identified by proteomic studies in both microorganisms. Similarly, an increase in the level of amino acids (glycines) responsible for greater mobility of protein secondary structures has been reported, as has an increase in amino acids such as alanine and asparagine (Rosales and Cecilia 2012).

Transcriptomics is increasingly being used for its ability to study the characteristics of gene expression in cells in a specific manner that is used to study the molecular basis of specific and complex traits such as bioremediation activity, which is becoming known through mapping and identification of the bioremediation genes of interest (Lidder and Sonnino 2012). Using molecular biology methods based on the characterization of the profile of *rRNA* genes and catabolic genes, it is possible to study and develop predictive and functional models of biodegradative action during the bioremediation process, allowing specific bioremediation criteria to be applied (Morelli et al. 2015).

## 19.6 Proteomics of Psychrotrophic Microorganisms with the Bioremediation Potential

At present, significant advances in "omics" are being made, which are directly related to the use of analytical tools to identify high-performance biomolecules (Kumar et al. 2021). This has enabled a thorough understanding of the adaptation mechanisms of psychrophilic and psychrotrophic microorganisms, revealing the adaptability and versatility of life in the so-called "cryosphere" (Aliyu et al. 2017). Psychrophilic microbes have colonized Earth's permanently cold environments, from deep oceans to high mountains and the Antarctic regions of the planet's various continents (D'Amico et al. 2006), stably cold marine environments, including the deep sea and sea ice of the Arctic and Antarctic (Deming 2002), and many of these organisms, based on their optimal growth temperature, have adopted the term "psychrotrophs" or "psychrophiles". Because there may be some confusion when distinguishing between the terms "psychrophilic" and "psychrotrophic," some authors have determined that the distinction should be ignored and that all cold-tolerant bacteria should be referred to as psychrophilic (Chattopadhyay 2006). These microorganisms have a tendency to increase their growth at temperatures above the natural environment (Feller and Gerday 2003); they are cold lovers, with a minimum, optimum, and maximum growth temperatures of 0, 15, and 20 °C or less, respectively, or psychrotolerant, with maximum growth above 25 °C but with the ability to grow at extremely low temperatures (Junge et al. 2018); this response to cold temperatures has been extensively studied in different bacteria such as *Escherichia coli* (Qiu et al. 2006). Considering this, the use of proteomics has proven to be highly effective in identifying proteins involved in cold adaptation because it allows the identification of diverse proteins while also providing a

platform for examining gene regulation at the mRNA level (Goodchild et al. 2004). The adaptation of these microorganisms to cold provides biogeochemical functions, and the production of enzymes and metabolic by-products generates interest in future biotechnological research as well as the development of industrial and biomedical applications (Junge et al. 2018).

### 19.6.1 Changes in the Cell Membrane

Certain psychrophilic and psychrotrophic microorganisms undergo structural changes during cold adaptation, with modification of the cell membrane being one of the most studied cases in these microorganisms; it has been reported that these changes occur in certain psychrophilic and psychrotrophic microorganisms due to an increase in the amount of unsaturated fatty acids of short and branched chains (Dall'Agnol et al. 2014). *Exiguobacterium antarcticum* B7, for example, has a complex regulatory network that governs gene expression, ensuring its survival, which is typical of psychrotrophic organisms (Dall'Agnol et al. 2014). Furthermore, it has been reported that one of the mechanisms used by cells to protect themselves from osmotic imbalance is to accumulate compatible solutes inside the cell to ensure their survival, with cell membranes regulating cellular homeostasis by controlling the function of transport processes. Cold-shock proteins have been discovered to be synthesized in both mesophilic and psychrophilic bacteria, whereas cold acclimation proteins (CAPs) are found only in psychrophilic bacteria (Chattopadhyay 2006).

### 19.6.2 Enzyme Modifications

The relationship between activity, stability, and flexibility is one of the widely accepted hypotheses for cold adaptation, which implies that psychrophilic biomolecules raise the versatility of their configuration to compensate for the "freezing effect" of low-temperature habitats (Johns and Somero 2004). The potential for a low rate of catalysis is among the most observable disadvantages of adaptation to cold temperatures, which can be explained by Arrhenius' law, which states that redox reactions at 0 °C should be 10–60% lower than those at 30 °C, but it is clear that psychrophilic biomolecules also modify to function effectively at cold temperatures (Casanueva et al. 2010). Molecular enzymology with psychrophiles has recently been investigated, primarily because they have higher specific activities than do mesophiles (Feller and Gerday 2003).

## 19.7 Metabolomics of Cold-Tolerant Microorganisms and Their Application in Bioremediation

Over the last few decades, much information has been gathered about bacterial diversity in cold areas, like the Antarctic, Arctic, and Himalayas, as well as the biochemical and genetic basis of their cold tolerance (Rampelotto 2016). Ice

microbes have become important natural resources with impacts on a broad spectrum of biological disciplines due to their highly efficient and power catalytic properties. The valuable potential of psychrophilic microorganism-produced biocatalysts is focused on the production of compounds with industrial applications, the most notable of which are cold-active enzymes, ice-binding proteins, polyhydroxyalkanoates, and surfactants (Shen et al. 2021). Temperatures below 0 °C should be considered because they slow down cellular reaction rates (metabolism) by altering the functionality of molecular structures. This is because these cells not only tolerate this extreme environment but also produce a number of industrially important metabolites (Alcazar et al. 2010). A microbial metabolome is initially defined as a collection of low-molecular-weight molecules that include metabolic intermediates, hormones, and secondary metabolites. The investigation of these molecules is extremely beneficial in providing information about the physiological state of cold-tolerant microorganisms. One disadvantage of microbiology is that the vast majority of polar microorganisms cannot be cultured or isolated, necessitating the development of new strategies and next-generation approaches to increase the likelihood of discovering new genes, proteins, metabolites, and other biomolecules of industrial interest (Garcia-Lopez et al. 2021).

## 19.8 Challenges of the Application of Cryotolerant Microorganisms in Bioremediation Processes

The restoration of contaminated environments is a critical task for long-term development. Bioremediation is an appealing alternative to physical–chemical treatment processes for the cleanup of contaminated sites. Microbes are the primary catalysts in the bioremediation of polluted environments (Orellana et al. 2018; Agrawal et al. 2021; Kumar et al. 2022). Surprisingly, we are only at the beginning of our investigation into the mechanisms, species identification, and natural transformation of these microorganisms in next-generation technologies.

The efficiency of bioremediation is affected by a variety of factors, including biological factors (genomic and phenotypic), nutrient availability, temperature, oxygen concentration, humidity, pH, metal ions, and the presence of toxic compounds (Kumar et al. 2020). In addition to the well-known disadvantages of bioremediation, such as being limited to biodegradable compounds, there are concerns that biodegradation products may be more persistent or toxic than their parent compounds, biological processes are often highly specific, extrapolating from laboratory and pilot-scale studies to large-scale field operations is difficult, and bioremediation that is appropriate for sites with complex mixtures of contaminants is required (Chandra and Kumar 2015). Nonetheless, the benefits outweigh the drawbacks (Abatenh et al. 2017).

In the future, new efficient strategies must be developed to address the limitations of temperature, contaminant bioavailability, nutrient availability, aeration, and humidity so that reinoculation of indigenous psychrophiles, while efficient in contaminant remediation, decreases the limitations of cold regions to optimize the

ability of cryotolerant microorganisms in the bioremediation process, for example, the combinatorial approach of bioaugmentation and biostimulation (Chaudhary and Kim 2019; Jeong et al. 2015; Kim et al. 2018; Akbari and Ghoshal 2014; Dias et al. 2015; Chen et al. 2017; Kumar and Chandra 2018).

The use of recombinant enzymes originally identified and isolated from cold-tolerant microorganisms, which have shown not only high activity but also weak stability, is one of the bioengineering techniques. Some researchers have solved the stability problem through immobilization, which could improve enzyme properties such as thermostability and operational stability as well as, indirectly, substrate specificity and catalytic activity. These favorable characteristics make the process ideal for pollutant biodegradation (Fan et al. 2017). Because of their highly specific activity and catalytic efficiency at low and moderate temperatures, cold-adapted enzymes produced by psychrotolerant bacteria are of significant scientific and industrial interest (Huston 2008).

Recent research has shown that both aerobic and anaerobic biodegradation processes are important in cold climate sites (Miri et al. 2019); however, highly polluting industrial wastes should be treated in an anaerobic reactor due to their high chemical oxygen demand (COD), potential for energy recovery, and low sludge production. However, in practice, anaerobic treatment may exhibit a low microorganism growth rate, a low sedimentation rate, process instabilities, and the need for posttreatment of process effluents, urging researchers to conduct additional research on anaerobic biodegradation that proves beneficial in cold anoxic regions (Chaudhary and Kim 2019).

Another major challenge is the addition of legal constraints to the list of operational limitations of bioremediation techniques that are already subjected to environmental conditions. In the Arctic, remediation is limited by cost and time and is governed by legislation and national guidelines; in the Antarctic, it is limited by cost and environmental policy. Natural attenuation is not always appropriate due to slow velocities, whereas thermal incineration, which is common in temperate remediation, is prohibited in Antarctica and is unfavorable in the Arctic due to the risk of permafrost thermal degradation and downward migration of contaminants (McDonald and Knox 2014).

Furthermore, regulatory restrictions that prohibit the use of foreign microbes pose a challenge; under these conditions, the use of transgenic plants in the cold regions of Earth should be considered for future bioremediation in cold regions (Chaudhary and Kim 2019). The next step should then be to conduct in situ experiments in cold regions such as Antarctica, with the goal of making human presence as inconspicuous as possible while preserving the primordial characteristics of the environment and biodiversity (Martorell et al. 2019). It is important to note, however, that returning these contaminated sites to their "pristine" state is neither practical nor feasible. Antarctic remediation should prioritize risk reduction and aim for appropriate soil reuse within station boundaries. Soil can be reused on site if it is combined with a sound risk management strategy to reduce off-site impacts (McWatters et al. 2016).

Petroleum hydrocarbons, primarily fuels, are the primary source of energy in polar regions, and, as such, their transport, storage, and use can contaminate soil and water. At the same time, while oil has been one of the most studied pollutants in bioremediation in cold climates in recent years with the goal of effective environmental cleanup using an ecological and cost-effective approach, other methods are still being researched, such as the use of biosurfactants, genetically modified bacteria, cold-adapted enzymes, and immobilization methods (Miri et al. 2019). However, bioremediation of petroleum hydrocarbons in cold climates has numerous limitations and challenges. More research and field demonstrations are needed to determine which method is best for biodegrading petroleum contaminants under these conditions. Furthermore, there is a scarcity of information on case studies of large-scale bioremediation projects (Greer and Juck 2017).

Although some progress has been made in recognizing the importance of microorganisms in the decontamination of contaminated waters, some critical issues remain unresolved. Bioremediation efficiency could be improved through genetic engineering of microorganisms with a degradative pathway of a target compound, appropriate approach selection, the use of nanoparticles, and other methods. Further functional genomic studies with similar isolates, and also gene function, will include a deeper appreciation of the biotechnological value of cold-tolerant microorganisms (See-Too et al. 2017). The genome sequences of several new cold-tolerant isolates, which have been recently published, are likely to shed more light on the mechanism of bacterial cryotolerance (Shivaji et al. 2017).

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# Bioremediation Assessment in Industrial Wastewater Treatment: The Omics Approach

20

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**Abstract**

As the number of industries is increasing day by day, the amount of toxic and non-biodegradable pollutants released by them into the environment is also increasing, thus adversely affecting ecosystems. Generally, industries consume various chemicals for the production of different types of materials, and, at the end of the process, different types of noxious wastes are released. Microbial bioremediation has already proven itself to be an economically feasible technology that can help in the removal of contaminants from the environment in an effective manner. In this context, isolation and identification of microbial consortia from pollutant soils may be used as bioremediation agents. A current advancement in the bioremediation of industrial effluents is the omics approach, which helps in exploring the microbial diversity present in contaminated sites. In addition to this, omics helps understand the microbial physiology and regulatory mechanisms. Recent advancements in omics technology have explored proteomics, transcriptomics, metagenomics, and metabolomics through in-depth analyses. Moreover, whole-genome sequence data have revealed microbial diversity at polluted sites. The present chapter investigates the functional role of microbes using the omics approach and its limitations in industrial applications. Omics also deciphers the potential connection between the genetic and functional

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similarity among numerous microbes, which helps in hastening in situ bioremediation and in minimizing the pollution load.

**Keywords**

Bioremediation · Microbes · Proteomics · Metagenomics · Transcriptomics

## 20.1 Introduction

Currently, owing to the huge demand for petrochemicals and other chemicals, chemical industrial parks have been established in a number of countries. Typically, wastewater is collected in parks and then sent to a central treatment facility. Most contaminants in mixed chemical wastewater (MCWW) from chemical industrial parks are poorly biodegradable or highly hazardous, making it a difficult combination to clean up in the environment. In contrast, many trace contaminants in MCWW cannot be effectively removed by typical treatment techniques, even if the effluents' conventional characteristics fulfil the local requirements. Toxins from wastewater treatment plants (WWTPs) are discharged into aquatic environments, where they have the potential to harm the ecology. Reliable information on MCWW and its harmful impacts must be gathered to protect the environment around chemical industrial parks. In spite of this, effective methods and approaches for assessing the multifarious hazards of MCWW and related effluents are missing. Some of the most often used methods for assessing wastewater toxicity are the Ames bioassay, comet assay, and luminous biosensor. Bioassays that concentrate only on a particular end point or mode of action are unlikely to uncover the novel biomarkers or significant mechanisms underpinning the toxicity of mixed effluents. Toxicological examination of MCWW and its WTPE needs more broad and sensitive procedures. Toxicological analyses of a wide range of environmental contaminants are becoming more and more reliant on "-omics" technologies such as transcriptomics and metabolomics. Using transcriptomic and metabolomic techniques, the toxic effects of heavy metals mainly in polluted water were evaluated. Sticklebacks' toxic effects of environmental diphenanthrene were also studied (Williams and Trindade 2017).

Our planet's shifting ecosystems have resulted in a change in microbial communities in polluted environments. Our society's well-being depends on our ability to protect the environment (Varjani et al. 2021). In that view, many limitation has been found in conventional approach for the identification and screening of diverse microbial community (Dhanjal and Sharma 2018). In order to learn more about microbial strains' ability, omics technology was developed. "Metagenomics (whole DNA)", "metaproteomics (total protein analysis)", and "metatranscriptomics (total RNA analysis)", among other terms, were considered.

The "omics" technique used to find unknown bacteria is called metagenomics (Kumar et al. 2020, 2021). Without enrichment or cultivation, the "genetic material" is extracted straight from "ambient samples". What we call metagenomics encompasses everything from library creation and DNA extraction to screening or

data processing. This may be conducted in two ways: "sequence-based screening" that relies on sequences that are already defined and "function-based screening" that is not at all dependent on an existing metagenomic database library. To explore novel patterns, cloning DNA was used to construct sequences with favourable qualities (Datta et al. 2020; Ngara and Zhang 2018). This might lead to the discovery of novel microorganisms and their products. The study and analysis of microorganisms with more genetic information may be possible via metagenomics, according to experts. By analysing the data in metagenomic libraries, researchers may gain deeper knowledge of bacterial communities and their functions (Dhanjal and Sharma 2018). In addition to the fact that "sequence-based metagenomics" cannot detect bacteria that are sparsely populated, metagenomics also cannot distinguish between genes that have been expressed and those that have not been translated (Malik et al. 2021). Metagenomics and transcriptomics are often used jointly to uncover gene expression patterns.

## 20.2 Characteristics of Industrial Effluents

The word "effluent" stands for the liquid form of wastage that is discharged from any industry into rivers or seas. It is not a proper solution to or a scientific method of dumping any waste product. This effluent contains wastewater along with contaminants such as heavy metals, pesticides, phenols, hydrocarbons, detergents, xenobiotic compounds, radionuclides, surfactants, pharmaceutical substances, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), and other organic pollutants of different resources (Sharma et al. 2021). Moreover, all this wastage material disturbs the balance of the aquatic ecosystem and also decreases the oxygen level to a great extent in water. "Biological oxygen demand (BOD)" is significantly decreased, and this means that the oxygen level is also significantly decreased, causing microorganisms and other aquatic organisms to suffer due to a lack of oxygen. This indicates that the water quality is extremely bad and also that it is not safe for use (Varjani and Upasani 2021). Moreover, this type of effluent is more dangerous than other garbage, and almost every agricultural area and other parts of the environment are polluted through this wastage. It disrupts the whole system of nature and has a worse impact, specifically on soil and water. Different types of crops and insects are hampered through these hazards, and excessive amounts of pesticides and oils become a threat to bioaccumulation (Zhi et al. 2017). The pollutants released are directly related to the types of industries. For example, tannery industrial effluents have more concentration of chromium, whereas textile effluents have more colorants, BOD, and COD) (Table 20.1). Wastewater discharge from these industry now treated in effluent treatment plants before discharging water into the adjacent water source. In this current era, one of the major aims of scientists is to remove wastes from the environment and create a proper balance and, through effluent treatment processes, to remove suspended solid particles (Williams and Trindade 2017). For wastewater treatment, microbes' immense catabolic power allows them to modify or eliminate contaminants from

**Table 20.1** Physiochemical parameters of wastewater from industries

| Effluents from industries | Physiochemical parameters | | | | | |
|---|---|---|---|---|---|---|
| | pH | COD (mg/L) | BOD (mg/L) | TSS (mg/L) | TDS (mg/L) | References |
| Tannery industry | 7.6 | 1210 | 800 | 1062 | 450 | Kumar et al. (2019) |
| Textile industry | 8.1 | 1285 | 750 | 1080 | 430 | Kumar et al. (2019) |
| Pharmaceutical industry | 3.7–8.5 | 3500 | 1800 | 1110 | 1500 | Gupta et al. (2019) |
| Pesticide | 12–14 | 8587 | 3688 | 3391 | 1236 | Misra et al. (2013) |
| Distillery | 3.3–5.7 | 8700 | 3850 | 2120 | 650 | Singanan (2017) |
| Sugar mill | 5.7 | 452 | 225 | 1033 | 400 | Kumar et al. (2019) |
| Paper and pulp mill | 6.5 | 320 | 210 | 1137 | 437 | Kumar et al. (2019) |
| Winery | 3.3–6 | 3880 | 1740 | 2900 | 3110 | Garg (2019) |
| Brewery | 5.4 | 643 | 230 | 3010 | 2364 | Garg (2019) |
| Oil refinery | 8.3 | 965 | 6851 | 315 | 6267 | Shabir et al. (2013) |

water. For example, "aerobic processes" are those in which bacteria use $O_2$ as an acceptor of electrons to oxidize organic or inorganic materials. Activated sludge (AS) systems have been around for a long time, but newer designs, such as "membrane reactors, biofilm reactors, and aerobic granular sludge reactors", are becoming increasingly popular. Carbon dioxide and methane are produced in anaerobic processes via a reaction chain that comprises the "hydrolytic, acidogenetic, acetogenic, and methanogenic phases" of mineralization. "Upflow anaerobic sludge blanket (UASB)" is a highly used "anaerobic reactor for wastewater treatment". It was created in The Netherlands in the late 1970s. "Expanded granular sludge blanket (EGSB)", "internal circulation (IC)", "static granular bed reactor (SGBR)", and "anaerobic biofilm reactor" are all examples of anaerobic design concepts that are often used. It is vital to remove not just organic carbon molecules from grey water but also nitrogen and phosphate, which are the key contributors to "eutrophication" in surface waters.

Understanding the microbial communities that convert organic and inorganic compounds is the key to successful biological wastewater treatment (Daims et al. 2006). Microorganisms participating in a number of activities, such as "nitrification, denitrification, phosphate removal, sulfate reduction, methanogenesis, and xenobiotic remediation", may be studied using cultivation methods (Kumar and Chandra 2020a, b). Through whole-genome sequencing (WGS) techniques, we were capable to recognize "functional genes" and "metabolic pathways" of microorganisms that play an important role in the elimination of pollutants from the wastewater system. A large percentage, however, has not yet been cultivated because of laboratory biases or a need for metabolic functions by other organisms. Nowadays, bioengineers and microbiologists are facing a tremendous challenge in the research of "microbial communities" and their link to process performance. Techniques like

"metagenomics", "metatranscriptomics", "metaproteomics", and "metabolomics" allow a cultivation-independent review of an entire community of microorganisms under specific environmental conditions, without the need to grow the organisms themselves. This is a major advancement in the study of microorganisms (Daims et al. 2006). There is a great deal of variety in microorganisms in an environment, which may be discovered and studied using these techniques. Bioreactor design and control, as well as a better understanding of the metabolic pathways involved in wastewater treatment processes, can only be enhanced via the use of cutting-edge techniques. A systems microbiology approach to metabolomics may also be used to build mathematical models for the prediction of the reaction of environmental or man-made systems to external disturbances.

## 20.3 Bioremediation and Its Limitations

Bioremediation is a microbial technology that helps clean up contaminated environments in a proficient and eco-compatible manner (Kumar et al. 2018; Kumar and Chandra 2020a, b). It is an alternative technique to the traditional methods of pollutant removal with the use of bacteria, fungi, algae, yeasts, etc. However, there are some gaps that exist as the growth and functioning of microbial regulatory mechanisms are not well-known. Bioremediation is effective in those compounds that can be biologically decomposed (Chandra and Kumar 2015; Agrawal et al. 2021). However, some of these compounds are not completely degraded such as some highly chlorinated pollutants and high-molecular-weight polycyclic aromatic hydrocarbons. The mechanism of microbial degradation is also widely dependent on the range and types of pollutants. It is also effective in specific molecular entity investigation. A successful degradation also requires additional nutrients and environmental growth factors. Sometimes, nutrient imbalance or toxic intermediate metabolites can negatively affect the rate of mineralization and can persist in the environment (Sharma 2020). The molecular identification is because of the presence of unique conserved sequences in individual species; 16S rRNA gene sequence analysis investigations improve the knowledge as well as evolutionary lineage of every microbe. However, this method is not totally capable of displaying microorganisms' crucial physiological features. The absence of closely related species in the consortium complicates phylogeny-based physiology prediction (Mishra et al. 2021). Microbial communities are also underestimated because dominating microbial species form defined bands, which can disguise a number of microbial individuals (Chandra and Kumar 2017a, b). Identifying the expression of bioremediation-related gene/genes, rather than only assessing the 16S rRNA genes, is likely to be more useful. Bioremediation-related genes, on the other hand, might be present in microbes but not expressed.

Another limitation is bioremediation strategies that are suited for locations with a complex blend of pollutants that are not evenly diffused in the environment. Therefore, it is extremely difficult to investigate the specific severity of pollutants at sites. However, scaling up bioremediation processes with same-time identification

of microbes from batch and pilot size investigations to large-scale field operations is more tedious. More investigations will be needed to develop newer approaches to bioremediation technologies, including the development of technologies that are relevant for locations with composite mixtures of pollutants and xenobiotic compounds that are not unvaryingly scattered in the environment.

The microbial treatment procedure is time-consuming. In comparison to alternative treatment techniques, such as excavation and soil removal from a polluted site, bioremediation takes longer. Regulatory ambiguity is that we cannot state that the clean-up is 100% complete since there is no universally accepted definition of clean. As a result, evaluating bioremediation's efficacy is challenging, and no accepted end point for bioremediation treatments exists (Sharma 2020).

Metagenomic techniques can transcend previous efforts employing microbial bioremediation for addressing industrial pollutants by analysing and exploring uncultured microbiomes existing in the ecosystem in a polluted area. The latest breakthroughs in omics technology, on the other hand, have made it feasible to better comprehend microbial physiology and metabolic activity.

## 20.4 Exploring Microbial Diversity Using the Omics Approach

Through diverse approaches like genomics, proteomics, and metabolomics, omics refers to the comprehensive analysis and quantification of a reservoir of biomolecules that makes up the cellular structure, physiology and metabolism, and dynamics. The name comes from the word "omic", which is appended to the end of the approaches listed above.

Furthermore, the "-omes" of the omics, i.e. genomes, proteomes, and metabolomes, for the multi-omics approaches mentioned previously, are described as the topics of study in each discipline (Fig. 20.1). Genomic studies find great applicability in the study of microbial pure cultures, involved in bioremediation activity. Whole-genome sequencing provides the sequencing of the genomes of these microorganisms, the physiological operational activities of which are unknown. Advancements in various complicated biological interactions are made possible by processing through proteomic assessments, which are simplified by a technique known as mass spectrometry. Metagenomic quantification makes it possible to study microbial diversity that is still unknown to us. Data obtained by different omics tools can now be brought together to better understand the metabolic process of microbes during the bioremediation procedure, which may even lead to the formation of some strains showing the capability of degrading a certain xenobiotic compound (Yunusa and Umar 2021). To foresee microbial activity through different bioremediation strategies, models of pure cultures and environmental samples can be constructed using omics techniques.

The underlying mechanisms of the impact of land use change on germs and ecosystems need to be understood holistically for improved management of the diversity of microorganisms (Zhou et al. 2019). Next-generation sequencing (NGS) is an advanced technology that relies upon microbial sample collection from the

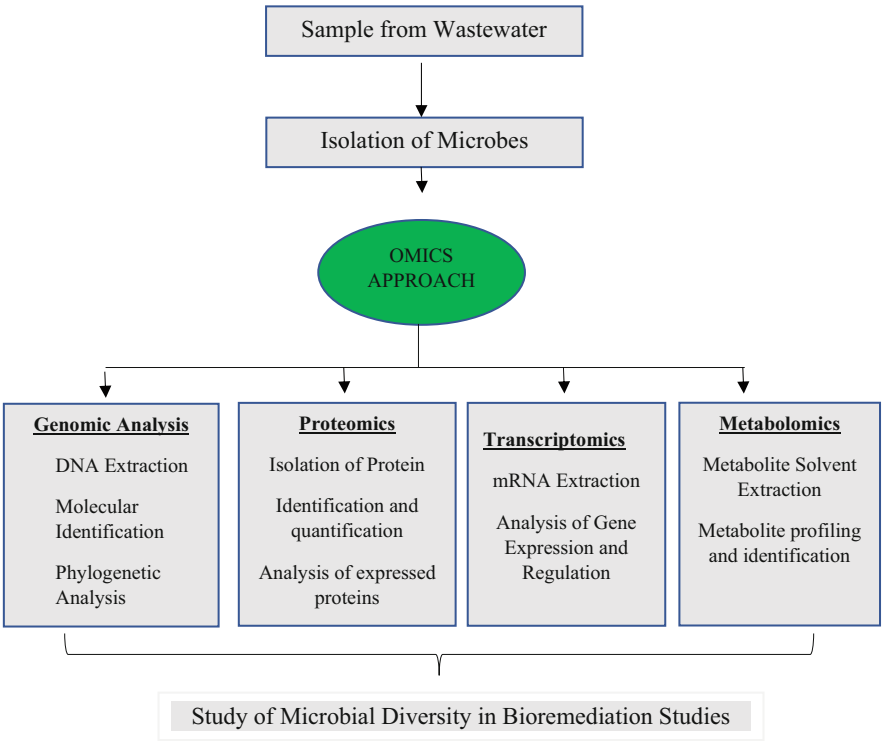


**Fig. 20.1** Omics approach used in environmental sites to study microbial diversity

ecological interactions of the environment, and it also provides biomonitoring with the dynamics of the microbial world. Omics technology expands its features into different parts of public life, and these are agriculture, diversity of microbes, medicine, and evaluation of the microbial world; all these are quite cost-effective and are newer technologies when compared to NGS (Tiso et al. 2020). However, NGS approaches have limitations and are a narrow range-based genome sequencing with strenuous technology. NGS is computer-based and assesses previous biological data for next-generation omics technology with a diversity of ecosystems (Zhou et al. 2019).

### 20.4.1 The Genomic Approach

The genomic approach provide us complete genetic information about the native microbial diversity residing in the effluent sites. Functional genomics involves the study of individual genes and the expression of genes. The relationship between genes and their actions can be easily studied. This method also gives rise to the investigation of those genes whose action is still unknown through various genome sequencing techniques. Metagenomics is a branch of study in which genomic

exploration of environmental samples takes place, whereas pangenomics studies complete genes or genomes that are analogous to a particular species (Singh and Nagaraj 2006). The technique of genome sequencing goes through three steps: first-generation sequencing (complete genome shotgun sequencing), next-generation sequencing (high-throughput sequencing), and third-generation sequencing (single-molecule long-read sequencing).

#### 20.4.1.1 First-Generation Sequencing

The Frederick Sanger and Maxam–Gilbert sequencing strategies have been labelled as the first DNA sequencing technology. In the Sanger sequencing method, a denatured DNA template, a radioactive primer, DNA polymerase enzyme, and chemically changed nucleotides referred to as dideoxynucleotides to generate DNA fragments with diverse lengths are used. The length of a DNA fragment is decided via integrated deoxynucleoside triphosphates (dNTPs). On the basis of their length, DNA fragments are detached on gel electrophoresis and are visible via an X-ray or an ultraviolet (UV) mild imaging system. This method is an old and expensive approach and also consumes a lot of time. It is broadly applied to only single and low-throughput DNA sequencing (Chandran et al. 2020).

#### 20.4.1.2 Next-Generation Sequencing

This is also known as second-generation sequencing. This technology generates high-throughput DNA sequences in less time and at a cheaper cost. It provides an extensively analogous evaluation from a couple of samples in which small DNA fractions are ligated with adaptors for random reads all through DNA amplification, which presents vast statistics inside a quick duration. The NGS era includes preparation of a library, sequencing, base calling, alignment to the installed genome, and diverse annotation. The preparation of a library starts with DNA fragmentation into small sections through sonication, enzymatic digestion, or transposase observed through ligation with adaptor sequences, followed by the amplification of the prepared library by the use of clonal amplification and PCR strategies to generate DNA replicas. These amplified DNA are then sequenced with the utilization of different approaches (Chandran et al. 2020). The omics techniques operated in NGS are (a) pyrosequencing (also known as the Roche 454 platform) that is conducted using pyrosequencers in which the sequence is synthesized by the identification of the pyrophosphate group released after joining of nucleotides in the newly synthesized DNA strand, (b) Illumina (Genome Analyser), which is a sequence-by-synthesis method, (c) Ion Torrent sequencing, which is conducted through the identification of hydrogen ions discharged during DNA polymerization, and (d) the ABI SOLiD system in which the sequencing technology is based on ligation of DNA fragments. Each form of sequencing bases has its peculiar and distinct advantages and disadvantages depending on the price, read length, depth, and accuracy of the sequence. The technique of NGS permits whole-genome sequencing of microorganisms, which are viable but difficult to be cultured from polluted sites. Informational data have revealed sequences of genes that predict how microbes are involved in the removal of contaminants (Fig. 20.2).

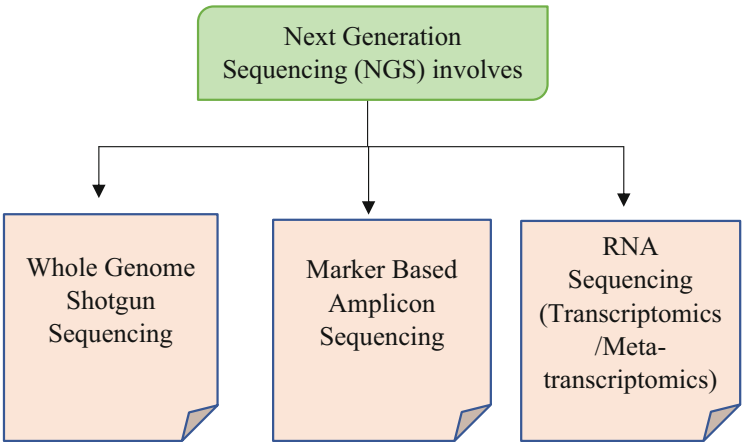


**Fig. 20.2** Methods included in next-generation sequencing

#### 20.4.1.3 Third-Generation Sequencing

Third-generation sequencing does not involve tedious PCR amplification and is not limited to amplification of one gene. At present, the extensively used methods in third-generation sequencing are (a) Pacific Biosciences sequencing technology, which uses single-molecule real-time technology (SMRT) and fluorescent labelled nucleotides, (b) Oxford Nanopore technology, which uses the electrophoresis method in which DNA/RNA passes through nanometre-sized pores under an electric field, and (c) Heliscope technology that utilizes a highly fluorescent detection system. This method can be extensively used in whole-genome sequencing and for studying complex populations, targeted sequencing, RNA sequencing, epigenetic studies, and DNA, RNA, or protein analyses.

Shotgun metagenomic sequencing is an effective method in microbial environments as it provides a lively and predictable assessment of the diversity of a microbiome (Hillmann et al. 2018). It does not involve amplification via PCR and investigates the useful ability and structural composition of cultures. It is an emerging molecular approach that links microbial structures to their useful competence. It also provide oppurtunity to detect of microbial biota grown under alter enviromental condition. Shotgun metagenomic sequencing of genomic DNA from a pattern has been completed using library construction methods. The library planning method, which includes random DNA fragmentation and adaptor ligation, is comparable to that of consistent entire-genome sequencing. To develop a taxonomy profile, a shotgun metagenomic sequencing approach for taxonomy evaluation comprises quality trimming and examination of a reference database containing complete genomes or specifically tailored marker genes. The statistics may be utilized for further studies such as metagenomic meeting and binning, metabolic feature profiling, antibiotic resistance gene profiling, and so on because they include all genetic data in a pattern. For the breakdown of persistent organic pollutants, such as naphthalene, toluene, petrol, xylene, and many other xenobiotic compounds,

whole-genome shotgun sequencing has been successful in identifying taxonomic microbial diversity and their genes isolated from different industrial effluents and soil samples (Chandran et al. 2020).

### 20.4.2 Transcriptomics

Transcriptomics involves the assessment of microorganisms by analysing their RNA sequences. Messenger RNA (mRNA) analysis gives an idea about gene expression, genetic composition, etc. (Singh and Nagaraj 2006). A transcribed gene is known as a transcriptome. Transcriptome analysis is conducted using microarray and sequencing methods. Microarray processes help evaluate the gene expression, whereas sequencing uses next-generation sequencing, which determines the concentration of RNA in a particular sample. This procedure is a cheaper, effective, and advanced technique to study proteins. RNA sequencing is vast because it allows researchers to study many forms of RNA at a high level of coverage and broad findings. Transcriptomics involves the formation of a transcriptome and then its conversion to complementary DNA (cDNA), thereby resulting in cDNA fragmentation to form a library employing RNA sequencing. Transcriptome analysis was applied to activated sludge microbial diversity, which unravelled the role of the nitrifying bacteria in the breakdown of petroleum oil (Sato et al. 2019).

The transcriptomes of *Geobacter uraniireducens* predominatly found in uranium contaminated subsurface sediments. By whole-genome microarray analysis, *Geobacter uraniireducens* was compared with the strain's cultures in defined media, which clearly showed that 1084 genes possessed a higher value of transcripts when grown in uranium-contaminated sites (Holmes et al. 2009). Another comparative transcriptomic study by Yoneda et al. in 2016 also revealed the pathways of phenol degradation utilization and tolerance of phenol by the lipid-accumulating bacteria, *Rhodococcus opacus* PD630. *Rhodococcus aetherivorans* has been found to tolerate a broad range of stress in the presence of antibiotics, heavy metals, and xenobiotics (Cappelletti et al. 2016). From other findings, it was revealed that *R. opacus* R7 expressed 542 genes as stress-tolerant, osmotic regulators with metabolic activity, when exposed to o-xylene, a xenobiotic compound (Zampolli et al. 2020). The relevance of differentially expressed genes associated with crude oil degradation was identified by transcriptome analysis of crude oil-degrading *Pseudomonas aeruginosa* strains (Das et al. 2021).

### 20.4.3 Proteomics

Proteomics helps in deciphering the proteins present in a cell and their response to the environment. This involves the use of two-dimensional gel electrophoresis and mass spectrometry analysis, which provide the details of protein content and the effect of phosphorylation during posttranscriptional changes. The enzymes and proteins that are involved in the degradation of several pollutants can be extracted

from isolated organisms by proteomic studies. Bioremediation determination of small peptide chains is performed using MS. Proteomics helps detect biomarkers for recognizing noxious contaminants, such as polycyclic aromatic hydrocarbons (PAHs) (Aardema and MacGregor 2003). After peptides are extracted from the soil sample, they are identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF), which translates the peptide fingerprints into individual proteins. Another method that is used is liquid chromatography mass spectrometry (LC-MS). It detects contaminants in effluents and analyses the metabolites signalling the decomposition of surfactants, detergents, pharmaceutical wastes, pesticides, etc. (Yunusa and Umar 2021).

Proteomic and metaproteomic method are helpful for the assessment of an anaerobic microbiome having the capability to degrade toluene (Jehmlich et al. 2010). Another study reported protein expression by anaerobic microbes when exposed to trichloroethylene (Siggins et al. 2012).

A change in external stimuli activates the expression of proteins and thus the metabolism of microbes. This change in protein expression is a potential approach to bioremediation. Several investigations have revealed the assessment of proteins that are expressed in the microbiomes residing in marine and soil environments, acid mines, sediments, hot springs, freshwater, and various other places (Wang et al. 2017). Krivobok et al. in 2003 revealed the protein expression of the *Mycobacterium* sp. A group of 20 proteins were observed when another strain of *Mycobacterium*, *Mycobacterium Vanbaalenii*, was cultured in a PAH-rich environment (Kim et al. 2004).

A comparative proteomics was obtained on the basis of the adaptation of the bacteria *Arthrobacter phenanthrenivorans* Sphe3 on supplementing with phenanthrene, phthalate, and glucose as the sole source of carbon and nutrients (Vandera et al. 2015). The report of LC-MS/MS shotgun proteomics showed the degradation of hydrocarbons (*n*-alkanes) at a low temperature by the psychrophile *Oleispira antarctica* RB-8 (Gregson et al. 2020). Another proteome study by Medić and colleagues in 2019 using nano LC-MS/MS revealed the regulation of *Pseudomonas aeruginosa* on exposure to a complex compound, 2,6-di-tert-butylphenol.

### 20.4.4 Metabolomics

Microbes generate metabolites under altered environmental conditions. However, these metabolites are difficult to analyse and quantify using the traditional approach. The identification of an altered environment and microbiota based on the metabolomic approach is accomplished by MS and nuclear magnetic resonance (NMR) spectroscopy. Metabolomics carries several advantages as it indicates the health of the environment as the environment changes with the addition of pollutants. Microbial cells' full metabolic makeup is the focus of metabolomics. All of the cell's metabolites and components are caught in a single image, essentially displaying the cell's metabolic activity and physiological state. Large volumes of information are provided as a result. Using an "untargeted metabolomic method",

Floros et al. (2016) were able to analyse the chemistry of a broad range of marine creatures. Thus, metabolomics is an expanding omics technology, which helps foresee metabolites and their regulatory pathways. With the use of several advanced instruments along with bioinformatics, investigation of metabolites has become an easier and fastidious method. Microbial metabolome investigation has also helped study the breakdown of anthropogenic contaminants. A report on metabolome analysis of the degradation of phenanthrene by *Sinorhizobium* sp. C4 was established (Keum et al. 2008).

Metabolomics also helps understand the pathways for a specific microbial strain or genera, which were previously unknown. Dubinsky et al. (2013) reported the capability of a bacterium species *Cycloclasticus*, which can degrade polycyclic aromatic compounds (PACs) at a low temperature. However, later on, *Cycloclasticus* was also found to aerobically degrade saturated hydrocarbons at varying temperatures (Joye and Kostka 2020).

A metabolomic profile of *Marinobacter hydrocarbonoclasticus* was prepared. This organism showed its growth in varying salinities, utilizing electron acceptors such as oxygen and nitrates (Huettel et al. 2018). Recently, a study on *Candidatus Macondimonas diazotropica*, a bacterium showing degradation of oil spilled on shores, has been conducted. It can fix nitrogen and can easily survive in nitrogen-deficit environments (Karthikeyan et al. 2019).

## 20.5 The Omics Approach to Bioremediation and Pesticide Biodegradation

As part of "biological wastewater treatment (BWWT), the activated sludge process and other approaches are used to remediate urban and industrial wastewater". There are a number of biotechnologically relevant storage compounds that may be amassed by bacteria in biological wastewater treatment facilities supported by diverse and dynamic microbial populations. Owing to their structural and functional variety, wastewater technologies hold a lot of promise for the sustainable production of a broad range of commodities from wastewater and other mixed substrates in the future. "Eco-systems biology" encompasses a wide range of activities, including observation, data integration, analysis, modelling, prediction, experimental validation, and, finally, management of microbial ecosystems (Muller et al. 2012). Techniques like this may help researchers better understand the interplay between organisms and their environment in order to improve biotechnology outputs through a variety of control mechanisms (Muller et al. 2012; Rodríguez et al. 2020).

In both industrialized and developing nations, biological wastewater treatment systems may be found. In contrast to most natural ecosystems, they exist in a highly stable and well-defined physicochemical environment (Daims et al. 2006). "Temperature, pH, oxygen, and nutrient concentrations" are all recorded and tracked on a regular basis, allowing for a quick hypothesis creation and validation. As a result, causal relationships between environmental factors and the "microbial community structure" and/or function may be discovered using temporal sampling. "From

laboratory bioreactors to large scale industries, microbial consortia isolated from treatment plants are highly adaptable for experimental validation".

However, BWWT plants' microbial communities are extremely dynamic, yet they retain a range of diversities/complexities between medium and high, which indicates a baseline stability over time in the form of order of a few quantitatively dominating populations (Muller et al. 2012). It will be simpler to analyse omics data in the future because of these properties. It is possible to use de novo metagenomic assemblers, such as effluent treatment plants' microbial communities, with sufficient sequencing depth to identify intermediate-level communities. Many community members now have access to population-level genomic reconstructions, which are essential for the accurate elucidation of functional omics data. Plant microbes in soil environments and acid mine drainage biofilms share many characteristics, but the BWWT plants' microbial communities are an important bridge/model between the two extremes while maintaining the key characteristics of both extremes, such as quantitative dominance of specific taxa and rapid stochastic environment diffraction (a characteristic of soil environments). Plant microbes of this kind have a variety of characteristics that make them an excellent example for microbial ecology, particularly ecosystematic omics research that employs a discovery-driven proposal (Muller et al. 2012).

Omics tools help understand and take measurements of microbial metabolic pathways in contaminated situations, and it is one of the helpful tools for bioremediation. Moreover, it is an effective pathway to remove pollutants by microorganisms, and it helps manage contaminants and pollutants (Mishra et al. 2021). In this bioremediation, some of the effective pathways are bioinnovation and biosparging that involve high pressure-based air injections that help extract ground-level water. In this context, another important path for bioremediation through omics is bioaugmentation. It is used to add large amounts of indigenous microorganisms to implicating exogenous species in the target site (Moutinho et al. 2021).

Microbial consortia are found everywhere in our surroundings, and they have the ability to degrade xenobiotic compounds such as pesticides. Pesticides on reaching soil seep into groundwater systems, and the microbiomes existing there will start to degrade those pesticides via their diverse metabolic pathways. Microorganisms with the bioremediation potential fail to digest xenobiotics entirely or are inefficient in bioremediation procedures involving pollutant mixtures. To overcome these constraints and successfully apply bioremediation, a deeper knowledge of the parameters involved in the microorganisms' development, dynamics, and functions utilized to remediate polluted areas is necessary (Dangi et al. 2019). In this regard, multi-omics studies are critical for formulating appropriate statistics about the genes and proteins, which participate in the degradation of pesticides, along with the metabolites synthesized by microbial pesticide degradation, and the cellular action to deal with pesticide stress.

Pesticides such as organochlorines and organophosphorus are widely examined utilizing omics methods. Many microbial genomes showing the capability to digest pesticides have been recognized and published so far, mostly bacterial isolates from the families *Burkholderia*, *Pseudomonas*, and *Rhodococcus* (Table 20.2) (Rodríguez

et al. 2020). A combination of two isolates of bacteria, *Sphingomonas* sp. TFEE and *Burkholderia* sp. MN1, showed their ability to degrade fenitrothion, an insecticide. The action of decomposition on complex compounds was made feasible by individuals of the consortium as they possess different metabolic activities (Katsuyama et al. 2009). Thus, to know the molecular processes in microbial bioremediation steps, along with the detailed behaviour of microbes degrading pesticides, omics technology is extremely beneficial. Omics reveals the cell physiology in stress, molecular mechanisms, genes, and proteins involved in mineralization of insecticides and pesticides in addition to the metabolic pathways and metabolites produced during mineralization. In this chapter, multiple omics studies in the field of pesticide bioremediation are reviewed; these studies highlight the potential of systems biology through genomics, metagenomics, transcriptomics, proteomics, and metabolomics to provide an integrative understanding of the whole biodegradation process.

Studies on genes and genomes are an emerging subject in the study of xenobiotic-degrading microbial isolates. In terms of pesticide biodegradation, genomic and metagenomic methods have validated the quantification and analysis of many genes in various microorganisms that might lead to their biotransformation and also help provide integrative knowledge of the complete degradation procedure. A replica of selected genes of bacteria and heterologous protein expressions of their products has revealed a finer and deep knowledge of their catalytic properties and potential for use in bioremediation techniques. Furthermore, the availability of a closed or draft genome of microbes mineralizing pesticides is a must for implementing additional omics investigations (Bharagava et al. 2019). Pesticides and other xenobiotics in the environment influence gene expression levels in exposed microorganisms; this adaptive control in gene expression, also known as transcriptional regulation, is linked to the presence of pesticides, their mineralization, and complete procedure. A transcriptome is referred to as all messenger RNA (mRNA) that are expressed in a cell at a certain developmental stage, physiological state, or in reaction to a specific situation, such as pesticide exposure in the environment. In a variety of microbial species, transcriptomic methods have been utilized to investigate genome-wide transcriptional activity, establish regulons and stimulons, outline operon structures, identify DNA-binding sites, and perform comparative genotyping. Methods such as microarrays and RNA-Seq are currently the most common methods for determining the transcriptional profile of nearly any biological material under a wide range of circumstances. A high-throughput transcriptome study of pesticide degradation in the future will enable the physiological optimization of bioremediation strains as well as the development of novel or more efficient routes for pollutant breakdown (Rodríguez et al. 2020).

The majority of environmental metabolomic research has focused on determining the changes in metabolite concentrations related to exposing model bacteria to hazardous substances known as xenobiotics. Risk evaluation, biomarker research, reactions to the factors that cause environmental stress, and illness diagnosis and monitoring are all possible uses of these investigations. Metabolomics has been used to better understand the microbial biodegradation of a variety of xenobiotics, most

**Table 20.2** Pesticides/herbicides in multi-omics studies on microbes

| Pesticides | Microbes degrading pesticides | Omics methods used in studies | References |
|---|---|---|---|
| Methyl parathion<br>(the organophosphorus family of pesticides) | *Burkholderia* sp. (MP1)<br>*Pseudomonas putida* (MR3)<br>*Burkholderia cenocepacia* (CEIB S5-1, 2)<br>*Burkholderia zhejiangensis* (CEIB S4-3)<br>*Fischerella* sp. | Micro RNA sequencing<br>Ion Torrent Personal Genome machine<br>Genome Analyser Hi Seq Genome analyser<br>RNA sequencing MALDI-TOF-mass spectroscopy (MS) | Liu et al. (2014)<br>Parakhia et al. (2019)<br>Martínez-Ocampo et al. (2016)<br>Castrejón-Godínez et al. (2019)<br>Tiwari et al. (2018) |
| Dichlorodiphenyltrichloroethane (DDT)<br>Hexachlorobenzene<br>Prochloraz<br>(the organochlorine family of pesticides) | *Stenotrophomonas* sp.<br>*Bacillus*<br>*Dehalococcoides mccartyi* CBDB1<br>*Penicillium digitatum* | Micro RNA-Seq<br>Next-generation sequencing<br>Liquid chromatography/MS<br>RNA sequencing | Pan et al. (2016)<br>115<br>Schiffmann et al. (2014)<br>Liu et al. (2015) |
| Glyphosate (herbicide) | *Aspergillus nidulans* | Microarrays | Mesnage et al. (2020) |
| Chlorimuron-ethyl (herbicide) | *Klebsiella jilinsis* 2N3 | RNA sequencing | Zhang et al. (2019) |
| 2,4-Dichlorophenoxyacetic acid<br>4-Nitrophenol | *Burkholderia*, *Sphingopyxis*, and *Variovorax*<br>Microbial community | High-performance liquid chromatography (HPLC), metagenomics<br>Microarray | Wang et al. (2017)<br>Dennis et al. (2003) |
| Diuron | *Arthrobacter sulphovorans*, *Variovorax soli*, and *Advenella* sp.<br>*Pseudoxanthomonas indica*, *Bacillus anthracis*, and *Bacillus cereus* | HPLC, isotope tagging<br>HPLC | Villaverde et al. (2017)<br>Villaverde et al. (2018) |
| Pyraclostrobin | *Proteobacteria*, *Bacteroides* | HPLC-QTOF/MS | Diggle et al. (2007) |

(continued)

**Table 20.2** (continued)

| Pesticides | Microbes degrading pesticides | Omics methods used in studies | References |
|---|---|---|---|
| Neonicotinoids | *Paenibacillus*, *Rhodococcus*, *Microbacterium*, *Paraburkholderia*, *Pseudacidovorax*, *Kocuria* | PCR, LC-MS | Rodríguez-Castillo et al. (2019) |
| Linuron | *Variovorax* sp. *WDLI*, *Hyphomicrobium sulfonivorans*, and *Comamonas testosteroni* | Transcriptomic analysis | Bhatt et al. (2019) |
| Monocrotophos | *Bacillus drentensis ENAI*, *Bacillus aryabhatta ENP3*, *Bacillus firmus ENS*, and *Staphylococcus vitulinus* | LC-MS | Balakrishnan and Rao (2019) |
| Chlordecone | *Citrobacter*, *Dysgonomonas*, *Clostridiales*, *Desulfovibrio*, *Sporomusa*, *Proteobacteria*, *Pleomorphomonas* | Genome sequencing, GC-MS | Bhatt et al. (2020) |
| Quinoline | *Desulfobacteria*, *Rhodococcus* | GC, pyrosequencing | John et al. (2016) |
| 2-Chloroaniline, 2-Chloroanthraquinone graphene oxide | *Lactobacillales*, *Oscillospira*, *Ruminococcus* | GC-MS | Lu et al. (2019) |
| Acetamiprid | *Acinetobacter*, *Afipia*, *Microbacterium*, *Stenotrophomonas*, *Sphingobium* | LC-MS, PCR | Xu et al. (2020) |
| Phenylurea herbicides | *Achromobacter* sp. *ANB 1Diaphorobacter* sp. *LR 2014-1* | Genomics, HPLC | Zhang et al. (2018) |
| Chlorpyrifos | *Pseudomonas putida* NII 1117, *Klebsiella* sp. NII 1118, *Pseudomonas stutzeri* NII 1119, *P. aeruginosa* NII 1120 | LC-MS | Sasikala et al. (2012) |
| Clothianidin | *Acinetobacter johnsonii*, *Ochrobactrum anthropic*, *Pseudomonas* sp., and *Stenotrophomonas maltophilia* | LC-MS | Wang et al. (2019) |
| Lindane | *Proteobacteria*, *Clostridium*, *Sphingobium japonicum* UT26 | Metagenomics<br>Sanger method | Raju and Bidlan (2017)<br>Nagata et al. (2010) |

| | | | |
|---|---|---|---|
| Organophosphorus and benzimidazole, insecticide, Neonicotinoids, fungicides, pyrethroids | *Pseudomonas*, *Rhodotorula*, *Aspergillus*, *Enterobacteriaceae* | HPLC | Li et al. (2020) |
| Phoxin | *Bacillus amyloliquefaciens* | RNA Seq | Meng et al. (2019) |
| Thiobendazole | *Hydrogenophaga*, *Hydrocarboniphaga*, *Methylobacillus*, *Sphingomonas* | q-PCR, LC-MS | Perruchon et al. (2017) |
| *p*-Nitrophenol | *Escherichia coli* | Microarray, proteome, RT-qPCR1 | Zhang et al. (2020a) |
| Paraquat | *Mycobacterium tuberculosis* | RNA Seq | Aparicio et al. (2018) |

notably PAHs. The number of metabolomic research studies on pesticide effects and biodegradation in bacteria has risen dramatically in recent years. Metabolomics is a useful tool for studying the toxicological effects of pesticide exposure on microorganisms; pesticides can exert a variety of effects on physiological processes such as liver function, endocrine and antioxidant systems, and metabolic alteration in amino acid, carbohydrate, fatty acid, neurotransmitter, and osmoregulatory metabolism levels, among others. For assessing biological systems, metabolomic research uses two major methodologies. The first is a broad, untargeted investigation in which no prior understanding of the biological system's metabolic pathways is necessary. This strategy entails recovering and identifying metabolites that are widely present in the sample, resulting in massive data that should be compared and interrelated across different samples in order to determine their bridge in metabolic pathways, according to the phenomenon being studied. Another option is to conduct a focused investigation to recognize specific metabolites or metabolic pathways based on earlier data; this method validates the outcomes or hypotheses produced from untargeted metabolomic studies (Rodríguez et al. 2020).

## 20.6 The Omics Approach to Unravel the Molecular Mechanisms of Microbial Bioremediation

Conventional biomolecular separation and computational analysis of pure isolate cultures (i.e. those designed for mixed microbial communities) are unsuccessful because of the changing degrees of inter- and intra-sample variability (Muller et al. 2012). Roume and collaborators' biomolecular separation technology, on the other hand, is critical to our understanding of microbial communities. The method of multi-omics research enables the extraction of high-quality genomic DNA, RNA, short RNA, proteins, and metabolites from a single, undivided sample. As a consequence, incongruent omics data may be eliminated from the heterogeneous biomass, hence reducing the amount of noise that can be generated during downstream integration and analysis (Muller et al. 2012).

Multi-omics datasets are also generated once biomolecular isolations are carried out in a standardized and methodical manner. After pre-processing and analysis, the multi-omics data are sent to bioinformatics. It is possible to conduct a preliminary assessment of microbial communities based on the structure and functional potential of the various communities using either "high-throughput ribosomal RNA gene amplicon sequencing or shotgun metagenomic analysis". More importantly, "hybrid de novo metagenomic assemblage of metagenomic and metatranscriptomic data guarantees higher quality than conventional de novo metagenomic assembly because of the ability to reconstruct and resolve genomic complements of low abundance but highly active community" (i.e. high metatranscriptomic coverage for expressed genes). After using "binning/classification techniques, such as those established for a single sample or for spatiotemporally defined data, hybrid assemblies allow for high-quality population-level genomic reconstructions". To make short-term evolutionary inferences about population subgroups and to increase

sensitivity for downstream metaproteomic analysis, hybrid metagenomic and metatranscriptomic data arrangements make it possible to replicate genetic variations with greater importance and potency (Muller et al. 2012). A comprehensive understanding of microbiome functions requires a collection of metatranscriptomic and metaproteomic data to be obtained.

As a result, metatranscriptomics' gene activity may be detected in the microbiome. Genes that are over- or underexpressed in response to environmental changes may be found using this method of gene expression profiling (Malik et al. 2021). DNA sequencing utilizing next-generation sequencing is a branch of genomics called metatranscriptomics, which deals with the extraction and enrichment of messenger RNA and the transition of messenger RNA to cDNA (Ranjan et al. 2015). Soil samples collected from the Jundai River in Brazil were used to extract DNA. In a metagenomic library, MBSP1 was discovered through a screening procedure known as 3C6. One of the most well-known hydrocarbon decomposers, *Haloferax lucentense*, has the most similar sequence to MBSP1 (da Silva Araújo et al. 2020). The researchers collected Buffelspoort Lake soil samples. For the metagenomic library, *E. coli*, *Pseudomonas putida*, and *Streptomyces lividans* were used as starting points. An "ornithine acyl-ACP *N*-acyltransferase (olsB)" enzyme from a "*P. putida* clone was identified by paraffin spray studies". It was possible to overexpress the *OlsB* gene even when the "fosmid" was ineffective in *E. coli*, which resulted in the formation of "biosurfactant activity" in the form of "lyso-ornithine lipids" (Williams et al. 2019).

The effects of "L- and D-Leucine" on "*Bacillus velezensis* BS-37", a biosurfactant-producing bacterium that produced "1000 mg/L surfactin" in the presence of glycerol, were studied by Zhou et al. (2019) using a transcriptome method. A "10-mg dose of L-Leu increased production to 2000 mg/L, whereas a 10-mg dose of D-Leu lowered production to 250 mg/L". On RPKM analysis (reads per kilobase per million mapped reads) of the strain "BS-37 transcriptome" disclosed the pathway of amino acid synthesis and glucose utilization, when "L-Leu was used as precursor, whereas D-Leu was a competitive inhibitor". Metatranscriptomics may be used to better understand and create strains that produce a lot of surfactin (Zhou et al. 2019). A genomic study of *Bacillus amyloliquefaciens* MT45 and *B. amyloliquefaciens* strain DSM7T was used to determine which strain generated surfactin more effectively. It was discovered that MT45 generated more surfactin and enabled more carbon metabolism as well as fatty acid production. As a consequence, the strain MT45's expression of the surfactin synthetase was found to be 9–49 times greater than that of DSM7T, resulting in higher amounts of surfactin production (Zhi et al. 2017). Metatranscriptomics cannot be used to analyse gene activity and gene regulation in bacteria because of its short half-life, instability, and sensitivity of mRNA (Ranjan et al. 2015; Zhang et al. 2020a, b). Metaproteomics, a newer omics approach, is employed to address these limitations (Malik et al. 2021).

Analysis of proteins generated by microorganisms found in environmental samples is the focus of metaproteomics. Using this study, functional gene data are analysed and distributed (Malik et al. 2021). As a bridge between genetic and functional information regarding "microbial populations, meta proteomics data

may be used" (Gaur and Manickam 2021). "*Aspergillus terreus* MUT271" and "*Trichoderma harzianum* MUT290" were two fungal strains found in an oil-contaminated marine zone by Pitocchi et al. (2020). In order to find a Cerato Plantain (CP) family member, proteomics was used. The CPs of the two strains tested included a biosurfactant and a bioemulsifier. "CP from *T. harzianum*" has greater surfactant activity and may be employed in commercial applications (Pitocchi et al. 2020). When glycerol was added to "*Burkholderia* C3", the bacteria demonstrated an increase in dibenzothiophene (DBT) degradation and the synthesis of rhamnolipids (RLs). There was an increase in degradation as well as RL biosynthesis, which was "25–30%". Proteomics was employed to detect and analyse enzyme overexpression in RL' and DBT's decomposition (Ramirez et al. 2020).

Researchers may use "metaproteomics", a technique for detecting proteins generated in response to substrate or environmental conditions, to find new functional genes and metabolic pathways. More information is provided by metaproteomics than by metagenomics or metatranscriptomics (Malik et al. 2021). There are various benefits to using the omics technique, but there are also significant difficulties to overcome. To learn more about genes and their structures and functions, we need a multi-meta-omics strategy that incorporates two or more techniques (Malik et al. 2021).

Chemical and enzymatic catalyses, as well as microbial fermentation, need to be given more attention in order to fulfil the market's need for surface-active molecules (Moutinho et al. 2021). Biosurfactants have been shown to be resistant to extreme environmental conditions. To keep manufacturing costs down, synthetic surfactants must replace microbial surfactants. This necessitates a great deal of gene-level investigation. Metabolic engineering holds significant promise for the development of high-yielding strains from a wide variety of strains. An increase in the potential biosurfactant production of 0.47 g biosurfactant/g substrate has been demonstrated using metabolic engineering techniques. When it was discovered that *B. amyloliquefaciens* LL3 has a fully functional surfactin synthesis operon, the researchers created a new, genome-reduced strain GR167 by slicing or tailoring 4.18% of LL3's useless genomic regions. This resulted in faster growth, increased heterologous protein expression capacity, improved conversion efficiency, and higher surfactin production (Mohanty et al. 2021).

The "GR167 genome's iturin and fengycin biosynthetic gene clusters" were spliced together to create the GR167ID strain. A combination of the PRtpxi promoter from strain LL3 and the PRsuc promoter from strain GR167ID was used to create the strains PRsucS and PRsucT. A 678-fold increase in SrfA operon transcription and a "10.4-fold increase in surfactant production (11.35 mg/L) compared to GR167IDD" were reported in this study's best mutant.

Findings include desferrioxamine E and actinomycin D, among other chemical families and industrially important chemicals. Surfactin biosurfactants were discovered via the analysis of other metabolites (Floros et al. 2016). "*B. velezensis* FZB42" was shown to be effective against the "phytopathogen *Xanthomonas campestris*" in a metabolomic study. The researchers discovered that the key components of the killing process were bacillibactin, a siderophore, and lipopeptides. It was observed

that "*B. velezensis*" contained lipopeptides, including fengycin and bacillomycin, in mono- and co-cultures. "Mass spectrophotometric" analysis of the co-culture supernatant identified these four compounds among 24 other forms. Another discovery was that "fengycin and surfactin" have different side chains. In this study, Nguyen et al. (2016) metabolized about 250 *Pseudomonas* strains. Mass spectrometric examination of the strains revealed structural links in the molecular matrix. "Cyclic lipopeptides", "peptide families", including "putisolvins", "xanthosis", and "massetolides", were discovered to have biosurfactant properties (Nguyen et al. 2016). *Serratia marcescens* has also led to the discovery of antibacterial lipopeptides. In this research, the lipopeptides studied include "L-serine residues" connected to "two fatty acid chains (with a chain length of C10–C12) and glucosamine derivatives" (Clements et al. 2021). Anticancer and antibacterial compounds were discovered in *Rhodotorula mucilaginosa* 50-3-19/20B yeast solvent extracts using metabolomic analysis.

Many studies have been conducted on the genomes of aerobic ammonia-oxidizing bacteria (AOB), and the presence of specific genes in some of the studied species could indicate adaptation to conditions such as high ammonia concentrations, stress from ammonia deficiency, high nitrogen oxide concentrations, or even low oxygen concentrations. Another example of adaptation is the NXR found in nitrite-oxidizing bacteria (cytoplasmic or periplasmic) (NOB). In species in which NXR is found in the periplasm, low nitrite concentrations are more common.

There are certain bacterial species with high metabolomic profiles, such as *Marinobacte*r sp. (Huettel et al. 2018), particularly, *Marinobacter hydrocarbonoclasticus*. It has a repertoire of metabolic capacity for growth in high- and low-salt environments, using both oxygen and nitrate or nitrite as electron acceptors and oxidizing both alkanes and aromatic compounds. One of the most frequent organic contaminants in the ocean is oil, which has been affected by oil spills. "Polyaromatic hydrocarbons, monoaromatic hydrocarbons, and heavy metals are the main pollutants released into the environment by the petroleum industry", which harm microorganisms, people, plants, and animals. Through their surface-active properties, biosurfactants aid in the removal of harmful poisons from the environment (Varjani et al. 2021). To avoid harming aquatic life, a bio-based solution should be used instead of chemical dispersants (Dangi et al. 2019). The multidimensional omics approach has also helped in understanding the communities of microbes that are involved in degradation before and after oil spills. Before a spill, microbial communities might be low; however, spills may trigger the fast growth of the microbiomes in the area of pollution and can proliferate the expression of multiple metabolic pathways. These pathways are dependent on the nature of the contamination and several environmental conditions (Rodríguez et al. 2015). It is shown that biomarkers are identified using a multi-omics approach that includes predictive biomarkers (e.g. response to the first responder, potential spills, spills, etc.) in an environment affected by the spill. In summary, microbiologists apply omics tools and microbial interactions in habitats from samples of both oil-contaminated and uncontaminated water and deposits (Yunusa and Umar 2021).

## 20.7 Microbial Bioremediation in the Omics Era: Opportunities and Challenges for Industries

Microbial genomes have substantially improved our understanding of wastewater treatment using microorganisms. Complementary genomics sheds fresh light on the genetic variety of closely related species. In order to get a better understanding of the relationships between a microbial community's structure and function, as well as to evaluate the long-term stability of critical treatment processes, genomic techniques are needed (Daims et al. 2006). Studying the adaptation processes of wastewater microbial strains in response to the changing conditions is critical for WWTPs since various wastewater compositions and operating situations may encourage or impede the development of a certain microorganism. To survive, microorganisms must change their metabolism and physiological functions in response to their environment.

Ecological systems biology will benefit the most from the integrated omics method when it is applied to samples that are separated in time and space. When paired with appropriate statistical and mathematical modelling approaches, "decoding the data will reveal hitherto unreachable visions into the structure and function of microbial populations". These approaches may be used to extract desirable characteristics like known and unknown populations/genes as well as to procure relationships (or linkages) among those required characteristics using correlations, co-occurrences, "mutual information, and hyper-geometric overlap". They can also be used to model the data. Through the "guilt-by-association" concept and the interactions/dependence of community members, these links may interpret gene functions. The ease with which samples can be collected and the frequency with which data can be collected make biological wastewater treatment facilities ideal for establishing links between shifts in community structure and function and changes in environmental conditions. As a result, "systematic omics studies of BWWT plants' microbial communities may reveal (i) the effect of physicochemical variables on the expression of certain genes or phenotypes" and (ii) the relationship of unknown genes with specific metabolites and known and unknown community members. Even if linkages are inferred, more testing in the lab and/or in situ perturbation investigations, followed by further omics data, are required to verify them. Metagenomics provides a platform to face challenges in various fields such as in the detoxification of biomedical wastes, outlining how microbial communities function during detoxification. This approach can lead to conservation of the environment along with zero health hazard properties. Metagenomics also triggers the detection of a versatile microbial population, which can prove itself to formulate newer and holistic products such as health and food supplements, enzymes, biofuels, agricultural supplements, etc. (Fig. 20.3). These novel formulations can help recover a safe environment.

Now, bioremediation is making its mark in the omics era, but the environmental system is far more complicated than lab techniques for bioremediation by microbes. There is an abundance of microbial communities and variations in environmental conditions. So, there are several challenges and opportunities in omics. Meta-omics

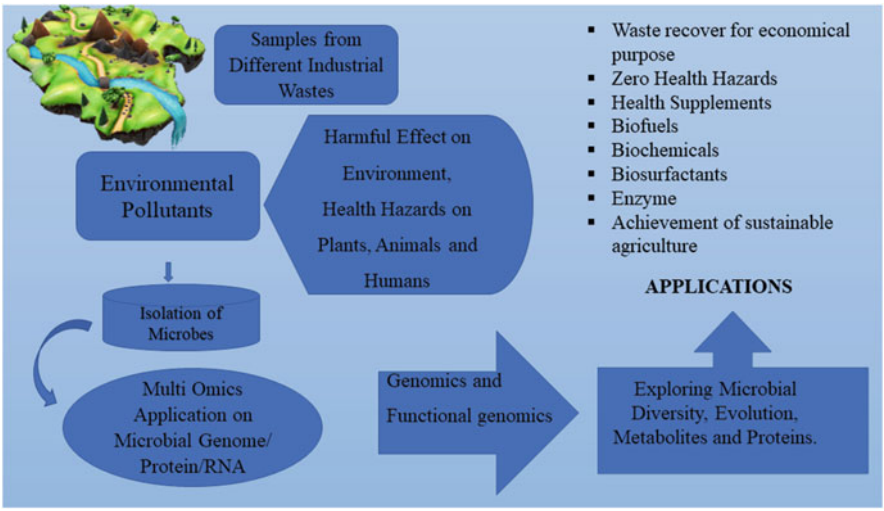


**Fig. 20.3** Application of Omics in various fields

helps investigate the bioremediation mechanisms very deeply. Some of the challenges as mentioned in Yunusa and Umar (2021) include,

(a) In developing countries, there is a need for more computational support. In these countries, since, day by day, an increasing amount of data is being generated, analysis of these data hampers the bioremediation process.
(b) Owing to the lack of standard referenced chemicals, especially in metabolomics, there is a need for databases for the unknown metabolites.
(c) Varying environmental conditions are another challenge because the bioremediation efficiency rates of some microbes might change according to varying environmental factors, especially regarding data generation and overload of information.
(d) Contaminants can also hinder the hybridization method in transcriptomics and hence can lead to poor isolation of mRNA from samples. Moreover, DNA, mRNA, proteins, etc. of numerous microbes from contaminated sites were unable to be properly cultured in the laboratory.
(e) Omics tools and techniques are still in their developing phase. Thus, a lot of dedication, time, and research works need to be put in. Moreover, there is a requirement for research studies to generalize the omics approach to make it more affordable and convenient.
(f) Development of biomarkers is important as it will enhance the analysis and identification of the microbiomes in contaminated sites. These biomarkers could be enzymes, metabolites, or any gene in function.
(g) New addition of gene database across the nations not only increase feasibility of sharing, distribution and comparison but also for the quick utlization.

(h) A holistic approach to develop culture media for microbial culture using omics will support the knowledge of potency of microbes in bioremediation.

## 20.8 Conclusions

When it comes to examining microbial communities in detail, there are several advantages to using high-throughput metagenomics and metatranscriptomics rather than traditional sequence-based methods. In order to get the most out of today's low-cost and widely available omics technologies (such as metagenomics and metatranscriptomics), researchers should regularly employ fully integrated omics studies when studying unculturable microbial consortia. However, despite the abundance of data, contemporary metagenomic assemblies and analytic approaches do not allow for the comprehensive resolution of microdiversity like genetic heterogeneity among or within microbes. Metaproteomics is rapidly advancing, but community-wide metabolomic research is still hindered by "poor detection/sensitivity of high-throughput metabolomic equipment and the significant dependence on a limited knowledge base as reflected in existing metabolite databases". Long-read sequencing, chromatography, and mass spectrometry are all expected to witness considerable technological advancements in the near future. There will be no shortage of cutting-edge data processing and analysis tools to accompany these technological improvements, allowing for complete "multi-level snapshots of microbial population structures and activities to be obtained in situ".

To maximize the "biotechnological potential of microbial consortia, a knowledge of gene function, regulation, and physiological potential using integrated omics data across many spatial and temporal dimensions is necessary". As a result of the recent advances in integrated genomics and hypothesis testing, new methods for optimizing biotechnologically important compounds under varied environmental conditions may be developed. As a consequence of this knowledge basis, the fast-increasing toolset of synthetic biology may be used to fine-tune metabolic pathways at the transcription, translation, and post-translational levels.

Integrated omics has the potential to be a crucial tool in future bioprospecting since it allows direct linkages to be made between the genetic potential and final phenotype. Integrative omics is expected to become a standard practice in microbial consortia analysis in coming years, making it possible for meta-omics to fulfil its promise of discovering all biotechnology-relevant traits in a natural consortium.

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# Microbial Biodegradation and Metagenomics in Remediation of Environmental Pollutants: Enzymes and Mechanisms

21

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**Abstract**

Nowadays, pollution and the number of contaminated sites are increasing due to widespread industrialization around the world, which threatens the lives of humans and every living organism on Earth. Biological methods such as biodegradation, bioremediation, phytoremediation, and vermiremediation are effective ways to reduce pollution in contaminated sites. Microorganisms play an imperative role in the biodegradation and bioremediation of pollutants by catalyzing chemical and biochemical reactions via their enzymes. At present, exceptional novel enzymes with exceptional functions is in progress around the world. To date, many researchers have taken considerable efforts to improve the properties of the available enzymes by various methods such as protein engineering. However, there is still a great demand for enzymes with extraordinary properties that make them resistant yet still active under harsh conditions and extreme

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environments. The most important source of current enzymes is culturable microorganisms (nearly 0.1% of these microorganisms), whereas unculturable microorganisms have a great potential to be the source of novel enzymes. In this context, metagenomics, a culture-independent approach, enables us to excavate this diverse source.

In this chapter, the metagenomic principle, along with recent advances, has been reviewed. Thereafter, contaminated sites and microbial biodegradation pathways are introduced. The main focus of this chapter is on exploring degrading enzymes by metagenomics and their applications in microbial biodegradation.

**Keywords**

Metagenomics · Degrading enzymes · Degradative genes · Biodegradation · Bioremediation · Pollutants

## 21.1 Introduction

The quality of our environment significantly affects the quality of our life. The ever-increasing growth in technology as well as industry has deterred the environment from recovering. Disposal of recalcitrant and hazardous industrial, domestic, agricultural, and municipal wastes into terrestrial and aquatic environments causes severe problems that threaten life on Earth. In this regard, waste disposal technologies including physical and chemical methods, such as adsorption, electrochemical treatments, filtration, ion exchange, etc. (Robinson et al. 2001), have been developed to reduce the dangerous effects of these wastes on the environment; however, there are some drawbacks such as complexity, formation of unwanted products, and the high costs of processing that make cleaning up the environment a more challenging issue. Despite the appropriate efficiency of traditional methods, the number of contaminated areas is not decreasing. Hence, researchers have focused on biological approaches, such as bioremediation and biodegradation, which are carried out by different organisms and microorganisms. Microbial bioremediation and biodegradation principally depend on the enzymatic capacity of microorganisms. Microbial enzymes can catalyze biochemical reactions, resulting in the conversion of hazardous pollutants to nonhazardous products (Ufarté et al. 2015a).

Among microorganisms, bacteria and fungi can broadly contribute to the degradation of various organic compounds such as drugs, antibiotics, azo dyes, polymeric substances, plastics, herbicides, pesticides, or even oils that are disposed of into the environment (Rieger et al. 2002). Based on their metabolic capacity, bacteria and fungi can break down natural and synthetic compounds that are also critical for the global carbon cycle. Microbial degradation of these compounds can occur aerobically or anaerobically (Suenaga et al. 2007; Zan et al. 2022). However, it is estimated that nearly 99% of microorganisms have not yet been identified because most of them are unculturable under laboratory conditions. Therefore, culture-independent

methods are preferred by researchers for discovering microbial diversity, metabolic capacity, and novel genes among microbial communities existing in different environments. These methods provide proper possibilities to understand how different microbial communities interact to degrade pollutants. Moreover, discovery of novel genes and proteins including enzymes can result in developments in synthetic biology and metabolic engineering. One of the culture-independent methods is referred to as metagenomic approaches that make it possible to nonselectively clone all of the environmental genomes into a library. This library can be easily screened for various goals, such as novel genes involved in the biodegradation pathways of nitriles, toluene, naphthalene, organophosphorus compounds, or even plastics. For instance, metagenomic libraries prepared from activated sludge can aid researchers in studying the microbial communities of a complex system and their biodegradation capacity through specific genes (Suenaga et al. 2007; Ufarté et al. 2015b).

In this chapter, we first seek to provide an appropriate context on pollutants, microbial biodegradation processes, and metagenomic approaches. Then, we summarize how these approaches can be accommodated to determine the role of the microorganisms involved in the biodegradation of pollutants in contaminated sites. Furthermore, we have discussed the recent applications of metagenomic libraries in exploring enzymes participating in microbial biodegradation.

## 21.2 Pollutants and Biodegradation

### 21.2.1 Pollutants

The rapid industrialization of human societies and succeeding anthropogenic activities have led to an increasing trend of various pollutants and xenobiotics in the environment, which pose significant risks to human life (Chandra and Kumar 2017a, b; Rodríguez et al. 2020). Sources of environmental contamination include various industries (plastics, wood, textile, microelectronics, refineries, etc.), mining activities (smelting, river dredging, mine spoils and tailing, metal industries, etc.), agrochemicals (excessive use of fertilizers and pesticides), and waste disposal (sewage, sludge, leachate from landfills, fly ash disposal, etc.). Pollutants include organic and inorganic compounds from toxic wastes as well as toxic xenobiotic compounds that are abundantly produced for industrial, agricultural, and domestic applications (Chandra and Kumar 2015). These compounds include insecticides; herbicides; chlorinated solvents; petroleum hydrocarbons; polycyclic aromatic compounds such as benzene, toluene, ethylbenzene, or xylene (BTEX); phthalates (plasticizers); heavy metals such as iron, lead, chromium, cadmium, nickel, zinc, copper, and mercury; personal care products; pharmaceutically active micropollutants such as antibiotics and other high-consumption drugs; and other industrial chemicals (Sravya and Sangeetha 2022; Steliga et al. 2020). For instance, among pollutants, polychlorinated biphenyls (PCBs) are classified as dangerous chlorinated hydrocarbons that are human-created to be employed in many industrial

and domestic applications. Although manufacturing this persistent pollutant is banned, many PCBs are present in all ecosystems, such as soils and sediments. PCBs can accumulate in soil organic carbon and lipid tissues of animals and humans. PCBs consist of one to ten chlorine atoms that are attached to a biphenyl ring but at different positions. Due to the persistence of PCBs in environmental remediation, solutions are needed for their removal (Steliga et al. 2020). In this regard, traditional technologies such as phytoremediation, microbial remediation (Jing et al. 2018) by pure or mixed culture (Steliga et al. 2020), chemical reagents, and activated carbon are used for PCB removal. However, many innovative technologies have been developed for this purpose including supercritical water oxidation, ultraviolet radiation, bimetallic systems, and nano iron. Moreover, multi-technologies such as biofilm-coated activated carbon and nano iron with other metals have been used for PCB removal (Jing et al. 2018)..

Another threatening pollutant that is broadly widespread in the environment is pesticides. Compounds such as chlorpyrifos, cypermethrin, ethalfluralin, lindane, phosmet, and prometryn have adverse effects on various organisms and on human health (Tsaboula et al. 2016). Consequently, after the pollutants enter into the environment, human health is threatened in various ways, resulting in severe diseases and cytotoxic and genotoxic effects (Tsaboula et al. 2016; Dhakal et al. 2018; Mishra et al. 2020). Hence, it is important not to produce hazardous compounds and instead take efforts to remediate the present contaminated sites using modern, eco-friendly, and affordable technologies like biological processes.

### 21.2.2 Microbial Biodegradation Processes

Biodegradation is a biological mechanism in which toxic chemicals are degraded by microorganisms (bacteria and fungi), resulting in the bioremediation of polluted environments, solid wastes, and wastewater (Kumar et al. 2018, 2022). Degraded chemicals can be used and reused by other organisms in nutrient cycling that reduces the concentration of pollutants. Overall, bioremediation can be employed both in situ and ex situ, with the former including biostimulation, bioattenuation, bioaugmentation, and bioventing. Ex situ bioremediation that includes biopiles and landfarming techniques is the most applicable method for contaminated soils. In this method, contaminated areas are excavated and transported to be treated at another place (Adams et al. 2015; Sravya and Sangeetha 2022).

#### 21.2.2.1 Biostimulation

In this kind of bioremediation, accelerating degradation rates in the indigenous microbial consortia of contaminated sites, e.g., soil, are stimulated by adding specific nutrients (nitrogen, phosphorous, or even carbon sources, fertilizers, growth supplements, or trace minerals). Similarly, this method is the preferable choice when natural biodegradation has not occurred. Environmental factors such as temperature, oxygen, and pH may be effective in the increasing rate of metabolic degradation pathways (Kumar et al. 2011; Adams et al. 2015).

#### 21.2.2.2 Bioattenuation

Natural attenuation is referred to as the reduction of contaminants via biological processes (aerobic and anaerobic biodegradation or uptake by plants and animals), physical reactions (dilution, dispersion, diffusion, volatilization, etc.), and chemical reactions (abiotic transformation, ion exchange, or complexation) (Mulligan and Yong 2004). During microbial degradation of pollutants found in soils and groundwater, especially petroleum contamination, different microbes can use these compounds as their carbon, nitrogen, or energy sources and, consequently, water and related gases are produced. This mechanism mitigates pollutant content. Moreover, pollutants can also be immobilized in soil, which prevents groundwater pollution. On the other hand, these compounds can be diluted during mobilization within the groundwater and soil. However, some of these may evaporate into the atmosphere (Abatenh et al. 2017).

#### 21.2.2.3 Bioaugmentation

In this type of bioremediation, the biodegradation capacity of microbial communities present in contaminated sites is improved by adding natural, exotic, or genetically engineered degrading microorganisms. During bioaugmentation, the biodegradation rate of pollutants, especially recalcitrant chemicals that are not remediated by bioattenuation or biostimulation, can be enhanced. However, this method may have unidentified impacts on ecosystems; therefore, it is essential to conduct it under severe control and clarify each probable effect (Niu et al. 2009).

#### 21.2.2.4 Bioventing

This process is an in situ remediation method in which oxygen and nutrients are provided by injection via wells that stimulate aerobic, microbial degradation of pollutants, particularly volatile materials in unsaturated contaminated soils. In addition to this technique, biosparging and bioslurping that are similar to bioventing are frequently used in contaminated soils as the former is suitable for saturated areas. The latter technique is a multistage extraction method, which is applicable for a depth of 25 ft. from the surface (Sravya and Sangeetha 2022).

#### 21.2.2.5 Biopiles

This technique is a method for bioremediation of hydrocarbon-contaminated soils by supplying air to the biopile system (such as compost piles), which increases microbial respiration and causes high degradation of petroleum pollutants (Abatenh et al. 2017)..

### 21.2.3 Biodegradation of Hydrocarbons

Environmental pollutions involving aliphatic and aromatic petroleum hydrocarbons have increased around the world due to population growth and society renovation (Brzeszcz et al. 2020; Agrawal et al. 2021). Oil is a strategically important energy source for the anthropogenic activities of all countries. However, the use of oil leads

to environmental deterioration. Incidents such as explosions during oil field development, leaks from oil pipelines and storage tanks, leaks in tankers and oil wells that occur during oil production, storage and transportation, refining, and processing tend to release hydrocarbons into the ecosystem (Wang et al. 2018). Due to incomplete recovery of leaked oil in the environment by physicochemical methods, oil compounds remain in the polluted area and cause permanent hazards to the environment. Remediation of oil pollutions can be carried out using degrading bacteria (Xu et al. 2018). By developments in microbial biotechnology and high-throughput sequencing technology, hydrocarbon-degrading microorganisms have been screened and identified.

Many degradation pathways have been identified for petroleum hydrocarbons, each of which employs specific bacterial oxygenase for degrading alkanes and aromatic hydrocarbons. Tremblay et al. (2017) reconstructed bacterial genomes by metagenomics and transcriptomics and estimated petroleum-degrading gene abundance and activity in marine oil spills in the presence or absence of chemical dispersants. Metagenomic bins (a draft version of a microbe's entire genome) of *Pseudoalteromonas* were found as dominant bacteria in the microbial profile of the samples with an enhanced alkane degradation rate by dispersants at 7 days, whereas *Alcanivorax* bins dominated at 42 days in oil with dispersants in winter. In contrast, a high abundance of *Thalassolituus* bins was seen in oil with dispersants at all sites in summer.

In general, members of the genera *Bacillus*, *Tissierellia*, and *Clostridium* are involved in the hydrocarbon degradation, which strict anaerobic *Clostridium* is capable grow at dumpsites with low oxygen. Candidatus Entotheonella palauensis Metagenomic studies in petroleum hydrocarbon- and oil-polluted hypersaline environments found 15 genera, namely *Candidatus* Entotheonella palauensis, *Desulfotignum*, *Draconibacterium*, *Enhygromyxa*, *Gemmatirosa*, *Gimesia*, *Haliangium*, *Meiothermus*, *Melioribacter*, *Nitrococcus*, *Nitrolancea*, *Salinibacter*, *Schlesneria*, *Sphaerobacter*, and *Thioalkalivibrio* (Verma and Sharma 2020; Lackner et al. 2017; Martins and Peixoto 2012). For aromatic pollutant degradation, halophilic bacteria such as *Enhygromyxa*, *Haliangium*, *Salinibacter*, *Schlesneria*, *Sphaerobacter*, and *Thioalkalivibrio* were found (Martins and Peixoto 2012; Berben et al. 2017; Verma and Sharma 2020). Moreover, Verma and Sharma (2020) showed the microbial diversity of solid tannery wastes and pollutant-degrading bacteria such as *Pseudomonas*, *Halanaerobium*, *Bacillus*, and *Clostridium*.

The microbial communities of crude- and refined petroleum-contaminated urban and agricultural soils were investigated through a metagenomic approach like next-generation sequencing (NGS). The ability of soil microbial communities in hydrocarbon degradation and plant growth promotion was studied through in silico analysis. The sequencing of 16S rRNA amplicons indicated a petroleum hydrocarbon-degrading microbial community. In all soil samples, *Proteobacteria* was the most dominant phylum, followed by the *Actinobacteria* phylum. The phylum *Firmicutes* was dominant in crude petroleum-contaminated soils and was the least in refined petroleum-contaminated soils. In addition, this study revealed the presence of 61 hydrocarbon-degrading, enzyme-encoding genes such as alkane

monooxygenase, alcohol dehydrogenase, and aldehyde dehydrogenase genes (Auti et al. 2019).

A recent study has revealed that a bacterial consortium degrading 70% diesel oil hydrocarbons was isolated by enrichment of a microbial population from oil-contaminated soils. The microbial population's role in the degradation of BTEX (benzene, toluene, ethylbenzene and xylene) and its metabolic pathways were investigated by metagenomic analysis. The dominant class of bacteria in the enriched consortium included *Alphaproteobacteria* (87%) and *Actinobacteria* (13%). The results showed 1005 putative coding DNA sequences for activation of BTEX and degradation of catechol and alkyl catechols. Most of the DNA coding sequences belonged to the genus *Acidocella*, which was detected as the dominant bacterium in the consortium. Other represented genera in the consortium included *Acidiphilium*, *Acidobacterium*, and *Aquabacter*. The enzyme-encoding genes involved in the ortho-cleavage of catechol include catechol 1,2-dioxygenase (*catA*), muconate cycloisomerase (*catB*), muconolactone D-isomerase (*catC*), and 3-oxoadipate enol-lactonases (*pcaD* and *pcaL*), which mostly belonged to two genera, namely *Acidocella* and *Aquabacter*, followed by *Acidobacterium*, *Bradyrhizobium*, *Alsobacter*, *Pelagibaca*, and *Chitiniphilus*. The genes for meta-cleavage of catechol and its intermediates included *dmpBCDH*, *praC*, *mhpDEF*, and *todEF*; more than 60% of them belonged to *Acidocella* and *Aquabacter*. Other coding DNA regions belonged to *Pannonibacter*, *Acidobacterium*, and *Sulfitobacter* (Eze 2021).

### 21.2.4 Hydrocarbons and Heavy Metal Co-contamination

Due to the synergic toxic effects of hydrocarbons and heavy metals, the bioremediation of contaminated sites by these compounds is a challenging issue. The study of the metapopulation structure of a natural microbial consortium to detect orthologous gene copies of exopolysaccharide (EPS) synthesis and hydrocarbon biodegradation showed a decrease in its metabolic activity and biodiversity in the presence of heavy metals (Staninska-Pięta et al. 2020). The mechanisms of metals' toxic effects on microbial cells include intracellular free radical formation, enzyme inactivation, degeneration of the cell membrane via reaction with thiol groups, competition by other ions or cell compounds, and chelation by cell metabolites (Lenart-Boroń and Boroń 2014). On the other hand, biodegradation of aromatic hydrocarbons was increased because EPSs secreted by microbes in the presence of metals affected the interaction between hydrocarbons and microbial cells. These results confirmed the correlation between EPS synthesis pathway activation and the biodegradation pathway of aromatic hydrocarbons (Staninska-Pięta et al. 2020).

### 21.2.5 Pesticides

Pesticides are chemicals widely used to kill or control arthropods, flies, pests, and nematodes in agricultural practices and cause environmental pollution and toxicity

that threaten human health. A high percentage of applied pesticides that remain in the environment exert harmful effects on other organisms and ecosystems. Bioremediation of these pollutants by microorganisms is considered because it is a less expensive and eco-friendly technology compared to chemical methods. The efficient degradation of pyrethroid pesticides such as cypermethrin via pyrethroid hydrolases was observed in the bacterial genera *Pseudomonas*, *Arthrobacter*, *Bacillus*, *Cunninghamella*, and *Raoultella* and in the fungal genus *Trichoderma* (Lin et al. 2020).

A metagenomic study on pond sediments contaminated by lindane (a synthetic organochlorine insecticide) was performed by Negi and Lal (2017) via comparative genomics. The results showed degradative enzymes, such as isomerases, lyases, hydrolases, and oxidoreductases, with genetic variations in the pond sediments involved in the biodegradation of hexachlorocyclohexane, chlorobenzene, and some other xenobiotic compounds. Moreover, the metabolism of lindane includes the *linA* and *linB* genes found in the pond sediments. Dunon et al. (2018) discovered the presence of IS1071 as a carrier of catabolic genes in xenobiotic-contaminated environments through metagenomic analysis. The results indicated that IS1071 was involved in the adaptation of bacterial communities to pesticide biodegradation.

### 21.2.6 Pharmaceuticals

Pharmaceuticals such as drugs and personal care products that are readily available in hospitals, pharmacies, and stores are a group of micropollutants that are often identified in aquatic environments. Some of these drugs are available without a prescription (e.g., acetaminophen, ibuprofen, naproxen, and aspirin). Although these drugs are made for human consumption and animal care, they are not fully metabolized in the body. Residues and metabolites from drugs are excreted in sewage by humans and animals. Wastes from the pharmaceutical industry as well as expired drugs are also a source for drugs to enter an aquatic environment. Untreated effluents discharge these drugs directly into rivers, lakes, and reservoirs, which can be deposited naturally on sediments or transported elsewhere. These compounds can also be simultaneously converted into by-products by chemical and biological decomposition in surface waters (Zhou et al. 2009; Kim et al. 2011; Deziel 2014). A few pharmaceutical micropollutants include diclofenac, erythromycin, clarithromycin, amoxicillin, ciprofloxacin, azithromycin, and some hormones needed to measure for setting their European environmental quality standards (Silva et al. 2019).

Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) that affects ecosystems because it cannot be entirely metabolized in the human body. There are no degrading microorganisms in wastewater treatment plants that could metabolize this drug at a high level. On the other hand, the photolysis of naproxen produces more toxic compounds in water and wastewater (Isidori et al. 2005). Dzionek et al. (2020) introduced a loofah sponge that immobilized *Bacillus thuringiensis* into trickling filters containing wastewater treatment plant microflora for naproxen

removal. The microbial community structure and species diversity changed, and pollutant removal was increased in this system. The community structure was found by the PCR of the V3–V5 region of 16S rRNA (for bacteria) and ITS1F-ITS4 (for fungi) and denaturing gradient gel electrophoresis (DGGE) analysis. *B. thuringiensis* B1 (2015b) is capable of degrading naproxen into intermediates of the tricarboxylic acid cycle. The degradation pathway of naproxen comprises demethylation by tetrahydrofolate-dependent *O*-demethylase, then probably hydroxylation of *O*-desmethylnaproxen to 7-hydroxy-*O*-desmethylnaproxen and degradation to 2-formyl-5-hydroxyphenylacetic acid and salicylic acid, which is transformed into maleyl pyruvate, 2-oxo-3,5-heptadienedioic acid, or cis,cis-muconic acid.

### 21.2.7 Phthalates

Phthalates are from plasticizers that are used in polyvinyl chloride, paints, glues, cosmetics, lotions, and perfumes. Phthalate compounds have toxic and mutagenic effects on humans and animals, with their endocrine-disrupting behavior causing damages to reproduction and development (Chang et al. 2016). The aerobic biodegradation of various phthalate compounds has been widely studied. Wright et al. (2020) surveyed the biodegradation pathways for three plasticizers, including dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), and an eco-friendly alternative, acetyl tributyl citrate (ATBC), by proteogenomic and metabolomic strategies in a marine environment. Their study showed the removal of the ester side chain from these plasticizers by esterase and beta-oxidation pathway enzymes. Cutinase and hydrolase for hydrolysis of DBP and ATBC in *Mycobacterium* sp. and esterase in *Halomonas* sp. were identified by a proteogenomic approach. The removal of the ester-bound side chains in DBP and ATBC occurred sequentially and produced phthalate, citrate, and butanol, which was observed by culture supernatant metabolomics through liquid chromatography-mass spectrometry (LC-MS). Furthermore, the enzymes of the beta-oxidation pathway are involved in the sequential shortening of DEHB side chains. Interestingly, in *Mycobacterium* sp., phthalate dioxygenase and dihydroxy phthalate decarboxylase genes clustered with the degradation pathway genes of protocatechuate and benzoate. Besides, *Halomonas*, a phylogenetically distinct bacterial genus from *Mycobacterium*, can catabolize DBP through benzoate and related genes. Thoroughly, degradation genes of aromatic xenobiotics are mostly expressed constitutively and condensed into a genomic cluster. The metagenomic and metabolomic study of DEHP degradation in aerobic and anaerobic soils conducted by Zhu et al. (2020) revealed functional genes and a bio-degradative microbiome. The initial hydrolysis of DEHB to mono (2-ethylhexyl) phthalate and, in the next step, hydrolysis to phthalic acid and benzoic acid, as further intermediates, occurred in both aerobic dryland and anaerobic flooded soil samples. *Actinomycetales* members (*Nocardioides*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, and *Pimelobacter*) as dominant aerobic degrading bacteria and *Acidobacteria*, *Bacteroidetes*, *Proteobacteria* (*Ramlibacter* and *Burkholderia*) and *Gemmatimonadetes* as anaerobic degrading bacteria were

indicated. Under aerobic conditions, enriched genes by DEHB, 50% of esterase, lipase, and cytochrome P450 belonged to *Nocardioides*, a novel degrader bacterial genus in the soil environment..

## 21.3 Metagenomic Approaches

It has been a long time since scientists have focused on culturable microorganisms as culturing techniques have been the main platform for developing microbiological knowledge. However, further studies showed a discrepancy between the size of a population detected in the culture-based and microscopic studies. Novel findings revealed that a high percentage of microorganisms are unculturable. For example, only 0.1–1% of soil bacteria can be cultured in standard common media. Because of these observations, scientists used analysis of *rRNA* genes to describe the diversity of microorganisms in an environment and tried to develop PCR technology and primer designing to provide a better view of the microbial population without the bias of culture methods. However, the primers are also limited, so, metagenomics, the genomic analysis of a microbial population using high-throughput sequencing, has emerged as a powerful method to gain access to the diversity, physiology, and genetics of uncultured microorganisms (Handelsman 2004). Metagenomics is the direct study of microbial communities in an environment by genomic tools without any prior cultivation (Kumar et al. 2021). The first step of metagenomics is extracting total DNA from the environment, and, then, genomic DNA is sequenced by high-throughput sequencing platforms, and, finally, the obtained data are analyzed using bioinformatic tools to study the microbial diversity and/or functional potential of the environment (Thippeswamy et al. 2021). In Fig. 21.1, a schematic representation of this approach for biodegradative enzymes is shown.

### 21.3.1 Targeted Sampling

There are numerous microbial communities with the ability to degrade pollutants even in unpolluted ecosystems since there is sufficient taxonomical and functional diversity and similarity between targeted pollutants and one of the natural substrates in such natural environments. Despite unpolluted environments being rich in microbial communities with microbes being able to thrive by degrading pollutants for investigation of the microbial communities with the potential to degrade and metabolize the pollutants, many metagenomic studies have been conducted in polluted areas (Ufarté et al. 2015a). Hence, sampling areas should be accurately selected to access novel degrading genes existing in the microbial communities.

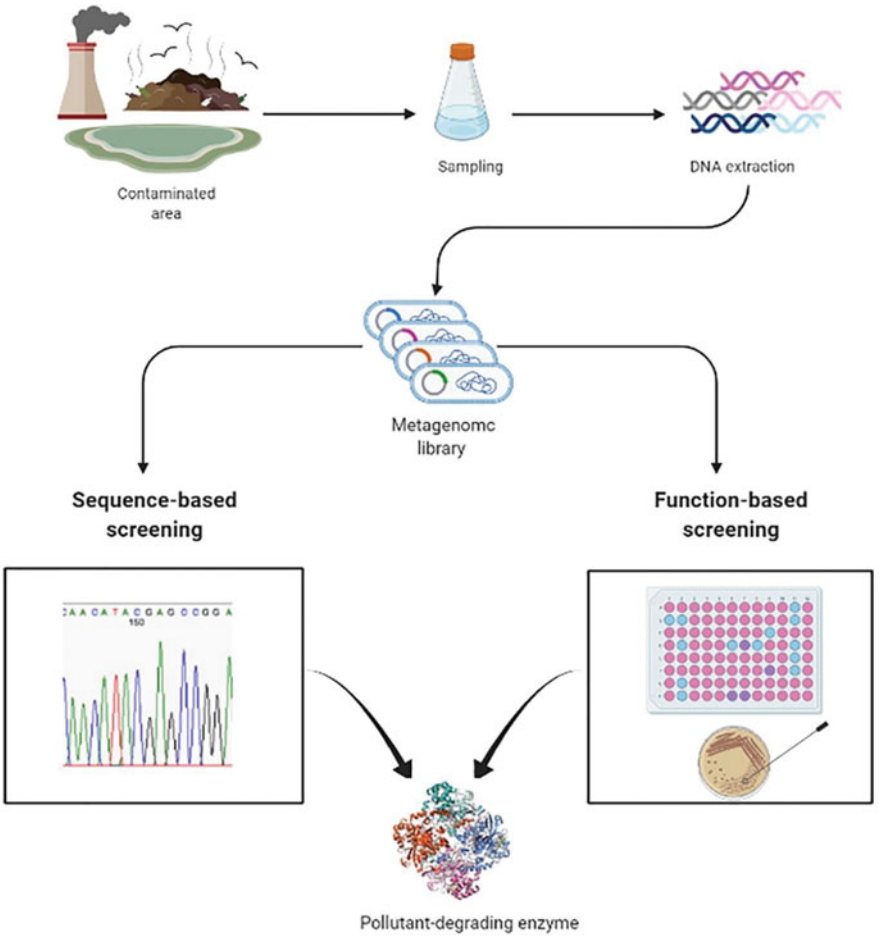


**Fig. 21.1** Schematic representation for discovery of pollutant-degrading enzymes through the metagenomic approach

### 21.3.2 DNA Extraction

DNA extraction is considered the most imperative step in the metagenomic approach. Obtaining pure and undegraded DNA that shows the diversity in an environment is a crucial step. There are various physical, chemical, enzymatic, and mechanical methods for metagenomic DNA extraction (Datta et al. 2020). Physical methods such as freeze-thawing, ultrasonication, and bead mill homogenization have been used for cell lysis to achieve equal proportions of all groups of microorganisms. In comparison to chemical methods, mechanical bead beating has been shown to recover more diversity; however, small-sized DNA fragments (5–10 kb or less) after physical treatments increase the risk of chimera formation.

Studies have shown chemical methods using SDS, leading to higher DNA yields in comparison to freeze-thawing. Moreover, during sediment analysis, using further SDS results in more cell lysis compared to bead milling. So, based on the source and microbial diversity, a combination of methods has been reported (Singh et al. 2009). Similarly, there are commercial kits for the extraction and purification of DNA that help researchers extract DNA more easily with considerable reproducibility (Pushpanathan et al. 2014).

### 21.3.3 Construction of a Metagenomic Library

Metagenomic libraries form the basis of genomic studies. Small-insert libraries are constructed by plasmid cloning vectors, and, for large-insert libraries, cloning vectors such as cosmids, fosmids, or BACs are used. So, the size of the genes that have to be screened affects the choice of vectors and metagenomic DNA libraries. *Escherichia coli* are typically used as the heterologous host. Subsequent screening of the library involves employing sequence- or function-based approaches (Pushpanathan et al. 2014; Kennedy et al. 2011). Other techniques for DNA library screening are DNA stable-isotope probing (DNA-SIP) and fluorescence in situ hybridization (Zhang et al. 2021).

### 21.3.4 Screening of a Metagenome Library

#### 21.3.4.1 Sequence-Based Analysis

A sequence-based metagenomic analysis is based on the screening of the reads obtained from sequencing methods. The two main steps of a sequence-based screening are gene prediction by identification of the reads with a desired sequence and gene annotation by linking the desired sequence to a database (Sharma et al. 2020). In a sequence analysis, oligonucleotide primers or probes are usually used to screen the target genes, and molecular methods such as PCR and gene hybridization are employed in this method. This method is suitable for gene screening of highly conserved sequence genes and also small DNA fragments. Not needing a host cell is the advantage of this method, but the disadvantage is missing the unknown genes with completely different sequences from the existing genes (Zhang et al. 2020).

#### 21.3.4.2 Function-Based Analysis

Phenotype-based screening is a function-based approach. This method depends on the expression profile of the library clones that are assayed by substrate-induced gene expression (SIGEX) and metabolite-regulated expression (METREX) profiles. Function-based methods help find novel enzymes that are not predicted via the sequence-based approach. The size of the gene inserts is important for successful screening during the function-based analysis. There are some specific challenges such as the quality and length of the gene insert, the number of clones selected to identify the whole microbial community, choosing an appropriate expression host, a

weak expression, and low product yields. In addition, low sensitivity and low-to-medium throughput are the limitations of the conventional agar plate screening. Methods like fluorescence-activated cell sorting (FACS)-driven and microfluidics-based screening can help overcome the limitations of agar plate screening (Sharma et al. 2020). Functional screening can be divided into two approaches. In the first one, screening of clones is performed in selective media and remarkable characterizations such as color and plaque indicate the existence of exogenous genes in the host cell. The second approach is referred to as the screening of the host and its mutants by studying their complementary growth under selective conditions (Zhang et al. 2021).

## 21.4 Application of Metagenomics in Contaminated Sites

Environmental pollution is increasing every year and can be a critical concern that threatens public health. The World Health Organization (WHO) has reported that about nine out of ten people are in danger of the harmful effects of polluted air. Moreover, soil and water contaminations all over the world have led to adverse impacts on the environment and human health. Microbial communities and specific genes can be biomarkers for monitoring air, soil, and water pollution (Datta et al. 2020). Metagenomics can be an adequate approach for analysis of microbial communities and functional diversity, e.g., the microbial population involved in the biodegradation and detoxification of organic and inorganic pollutants (Bharagava et al. 2019; Kumar and Chandra 2020).

### 21.4.1 Soil

#### 21.4.1.1 Microbial Community Profiling in Contaminated Soils

Conceivably, the highest diversity of microbial populations in the environment is related to soils as in each gram of a soil sample, ten billion microorganisms consist of thousands of different species. Recent advances in DNA sequencing have resulted in knowledge about microbial communities by generating millions of sequence reads (Myrold et al. 2014; Kirubakaran et al. 2020). The first study of microbial diversity in the soil by metagenomics was conducted by Tringe et al. (2005), who constructed a phage library with random lengths of DNA that was developed by the Sanger sequencing method. Because of the insufficient depth of the sequencing, researchers tried to use the shotgun method that directly sequences the extracted DNA without creating clone libraries (Myrold et al. 2014). Shah et al. (2013) worked on the taxonomic profile of contaminated soil collected from a canal in an industrial area and reported that the most abundant phylum and genus were *Proteobacteria* and *Pseudomonas*, respectively. Mapping the reads on sequenced microbial genomes showed that the highest numbers of reads were assigned to *Pseudomonas stutzeri*.

#### 21.4.1.1.1 Diesel and Hydrocarbon Contamination

The metagenomic study of diesel-contaminated soil by Yergeau et al. (2012) showed the increasing abundance of *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* and decrease of *Acidobacteria*, *Bacteroidetes*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, and *Deltaproteobacteria* in contaminated soils. This study confirmed that the most abundant organisms in contaminated soils were the *Pseudomonas* species that RT-qPCR assays approved; *Pseudomonas* and *Rhodococcus* species actively expressed hydrocarbon degradation genes. Similarly, Gao et al. (2021) studied diesel-contaminated soil by a metagenomic approach and investigated the changes in the microbial community, soil enzyme genes, and chemical analysis throughout a 30-day incubation period. They also focused on the effects of nitrogen sources on the bioremediation process. This study revealed that the two dominant phyla in diesel remediation in contaminated soil are *Proteobacteria* and *Actinobacteria* and that the addition of nitrogen enhances the bioremediation of diesel-contaminated soil. They showed that ammonium is the preferred nitrogen source and that glutamine synthetase and glutamate synthetase are the key enzymes in the nitrogen metabolic pathway.

Metagenomic analysis of hydrocarbon-contaminated soils and microbial diversity by Jurelevicius et al. (2022) revealed the positive correlation between the abundance of *Cytophaga*, *Methyloversatilis*, *Polaromonas*, and *Williamsia* and the concentration of total petroleum hydrocarbons (TPHs) and/or polycyclic aromatic hydrocarbons (PAHs). Another study of hydrocarbon-contaminated soils and metagenomic analysis was conducted by Bao et al. (2017). In their research, *Gammaproteobacteria* and *Alphaproteobacteria* were reported to be the key players in petroleum hydrocarbon degradation.

Many researchers have worked on the isolation and investigation of the consortia capable of xenobiotic degradation through the metagenomic approach. For instance, Eze (2021) reported the metagenomic analysis of a bacterial consortium with the ability to degrade diesel fuel. The metagenomic analysis demonstrated the role of microbial populations in BTEX degradation and the pathways involved. According to coding DNA sequences analysis, *Acidocella* was reported to be the dominant bacterial genus.

#### 21.4.1.1.2 Heavy Metal Contamination

Mining sites, leatherworking, electronic manufacturing, and metallurgical industries are the main sources of soil pollution due to their release of toxic wastes containing heavy metals (Amith et al. 2021; Singh et al. 2021). Heavy metals such as lead (Pb), cadmium (Cd), antimony (Sb), arsenic (As), zinc (Zn), etc. are a serious problem, particularly in agricultural soils. These metals, even in low concentrations, can accumulate in crops and animal tissues, which affect human health. The toxic effects of heavy metals gradually increase, and, therefore, it is essential to remediate contaminated sites via cost-effective and eco-friendly methods like bioremediation (Kumar 2018; Khashei et al. 2019). In this context, novel molecular approaches such as metagenomics can improve heavy metal bioremediation through discovering microorganisms that harbor heavy metal resistance genes. Luo et al. (2014) studied

the effects of As and Sb contamination on microbial diversity by a metagenomic approach along with physicochemical characterization. This research elucidated that *Actinobacteria*, *Firmicutes*, *Nitrospirae*, *Tenericutes*, and *Gemmatimonadetes*, among the 18 phyla detected in soil samples, were positively correlated with As and Sb concentration. Moreover, they showed that functional genes (including *arsC*, *arrC*, *aioA*, *arsB*, and *ACR3*) were much higher in the soils contaminated by As and Sb. Another metagenomic study of heavy metal-contaminated soils was conducted by Hemmat-Jou et al. (2018) who studied the microbial diversity of soils in three sites contaminated with Pb and Zn from slightly low to slightly high concentrations. They showed that most bacteria in the samples belong to *Actinobacteria* (*Solirubrobacter* and *Edaphobacter*), *Proteobacteria* (*Pseudomonas*, *Geobacter*, *Nitrosomonas*, *Xanthobacter*, and *Sphingomonas*), *Gemmatimonadetes* (*Gemmatimonas*), *Bacteroidetes* (*Pedobacter*), and *Chloroflexi* (*Ktedonobacter*). Among archaea, *Nitrososphaerales*, which is important in the nitrogen cycle, had the highest abundance in the samples. This study revealed that although Pb and Zn had negative effects on alpha and beta diversity, microbial diversity in contaminated soils did not show any significant change. A metagenomic study of the soil samples contaminated by toxic trace elements (TTEs) from an Iranian mine and in comparison with unpolluted control soils revealed that *Actinobacteria* and *Acidobacteria* in the polluted soils were lower than in controls. An increase in the ratio of archaea to bacteria and a decrease in the alpha diversity of polluted soils compared to control samples were reported in this research. Heavy metal resistance investigation of contaminated soils uncovered the major functional pathways that included carbohydrate metabolism, stress response, amino acid and derivative metabolism, clustering-based subsystems, iron acquisition, iron metabolism, cell wall synthesis, capsulation, and membrane transportation (Hemmat-Jou et al. 2021).

Additionally, Zhang et al. (2020) examined nitrate-dependent arsenite (As (III))-oxidizing bacteria (NDAB) with the combination of DNA stable-isotope probing (SIP), amplicon sequencing, and shotgun metagenomics. *Azoarcus*, *Rhodanobacter*, *Pseudomonas*, and *Burkholderiales* and related bacteria were identified in the As-contaminated soils, and *aioA* was identified as an important gene in As (III) oxidation.

#### 21.4.1.1.3 Radioactive Contamination

Yan et al. (2016) worked on the effects of uranium contamination on microbial communities by a metagenomic approach. They studied microbial communities in six soil samples from uranium-contaminated and uncontaminated areas. The obtained results showed that *Actinobacteria* and *Proteobacteria* were the dominant phyla in both contaminated and uncontaminated soils. At the genus level, *Robiginitalea*, *Microlunatus*, *Alicyclobacillus*, and *Azorhizobium* were significantly higher in radioactive soil. They showed that a change in the microbial community resulted in a change in the metabolism of amino acids (e.g., beta-alanine, taurine, and hypotaurine), carbohydrate metabolism, signal transduction, and membrane transport. They offered various uranium-degrading bacteria that may be useful for bioremediation of uranium-contaminated soil.

#### 21.4.1.1.4 Other Xenobiotics

Salam and Varma (2019) investigated the effects of electronic waste pollution on bacterial diversity in contaminated soils by a metagenomic study. *Proteobacteria* and *Firmicutes* were the most abundant phyla, and the most common classes under *Proteobacteria* were *Deltaproteobacteria* and *Betaproteobacteria*. They also studied alteration of the soil bacteria diversity by DGGE analysis and demonstrated a decrease in the number of *Proteobacteria* and *Firmicutes* and the emergence of *Actinobacteria* in the polluted soil samples. Cai et al. (2020) have worked on perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in perfluorinated compounds (PFCs) due to their increasing concerns in recent years. They studied the effects of these contaminants on microorganisms in the soil by a metagenomic approach. Results indicated that the dominant bacterial phyla were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Gemmatimonadetes*. *Bacillus* and *Sphingomonas* were the two genera that showed various degrees of suppression by PFC contamination.

### 21.4.2 Water

#### 21.4.2.1 Microbial Community Profiling in Contaminated Waters

Water bodies contain microorganisms that play a crucial role in ecosystem maintenance, water quality, biogeochemical cycles, and dissolved chemical structure. They can act in the decontamination of xenobiotics in contaminated water. Metagenomic approaches can be efficient in assessing microbial populations in fresh or marine water. Moreover, researchers can evaluate the microbes in contaminated ecosystems using these approaches. After collection, the water samples should be concentrated and subjected to filtration before DNA extraction for metagenomic analysis (Kirubakaran et al. 2020).

Abbai and Pillay (2013) studied the metagenomics of hydrocarbon-contaminated groundwater and reported an abundance of *Proteobacteria* (mainly *Betaproteobacteria*) as the dominant phylum, whereas the predominant orders were *Flavobacteriales*, *Sphingobacteria*, *Burkholderiales*, and *Rhodocyclales*. Among these orders, *Flavobacteria*, *Dechloromonas aromatica* RCB, and *Azoarcus* were involved in the degradation of aromatic compounds. Das et al. (2017) considered the structural and functional diversity in arsenic-contaminated groundwater via metagenomics. This research revealed that *Proteobacteria* with 62.6% was the dominant phylum and that *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, Actinobacteria, and *Firmicutes* with 11.7%, 7.7%, 5.6%, 3.7%, and 1.9% abundance, respectively, were the other bacterial phyla in the sample. They also worked on arsenic-resistant mechanisms in the sample and reported the dominant enzyme in the removal mechanisms to be the arsenate reductase enzyme.

For evaluation of microbial populations in water bodies, there are some reports on studying the river sediments. Chen et al. (2021) researched antimony (Sb) contamination by a metagenomic approach in a river sediment sample. They revealed that in all of the sediment samples, *Proteobacteria* and *Actinobacteria*

showed a high relative abundance and resistance to Sb contamination. *Deinococcus*, *Sphingopyxis*, and *Paracoccus* were reported as potential Sb-tolerant genera in sediment samples.

Somee et al. (2021) studied water and sediment samples from Persian Gulf's pollution continuum by a metagenomic approach and reported the role of *Oceanospirillales*, *Flavobacteriales*, *Alteromonadales*, and *Rhodobacterales* phyla in oil pollution and that of *Oceanospirillales*, *Alteromonadales*, and *Pseudomonadales* in high aliphatic pollution and showed the domination of *Alteromonadales*, *Cellvibrionales*, *Flavobacteriales*, and *Rhodobacterales* in polycyclic aromatic polluted samples.

Some reports investigated the effects of antibiotic contamination on the diversity of microorganisms and studied the antibiotic resistance genes in water samples in wastewater treatment plants. One of these research studies was conducted by Wang et al. (2013). The obtained results of the metagenomic analysis of a tannery wastewater treatment plant showed that *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* are the dominant bacteria.

## 21.5 Exploring Enzymes Involved in Bioremediation by Metagenomic Strategies

Microorganisms are unique sources of biocatalysts and enzymes that make them significant to be explored by scientists and researchers. However, most microorganisms are not cultured using conventional methods employed in laboratories, and this challenge affects the full access of exploring microbial enzymes. Therefore, non-culture-based methods such as metagenomics are the most suitable and popular possibilities for this purpose, especially for novel microbial enzymes that may affect the biodegradation of pollutants.

### 21.5.1 Oxidoreductases

Oxidoreductases include enzymes that facilitate electron transfer from an oxidant to an electron acceptor molecule. Oxidases, oxygenases/hydroxylases, peroxidases, and dehydrogenases/reductases are the four important groups of this diverse enzyme class. These groups of oxidoreductases play a significant role in the biodegradation of pollutants that converts them into less toxic and less hazardous compounds. The use of chromogenic and fluorogenic substrates is an appropriate and amenable screening method for the exploration of metagenomic libraries for these enzymes, particularly marine metagenomic libraries (Kennedy et al. 2011; Karigar and Rao 2011).

#### 21.5.1.1 Alkane Hydroxylases

Alkane hydroxylases from oxygenases/hydroxylases are the key enzymes in the aerobic degradation of alkanes. A few research studies have been conducted to target these enzymes among metagenomic libraries. For example, Xu et al. (2008) recognized two novel alkane hydroxylases in a metagenomic library of the east Pacific deep-sea sediments, which phylogenetically belonged to a special branch of integral membrane alkane hydroxylases. In another study, a metagenomic library constructed from aerobic and anaerobic microbial communities grown on n-hexadecane or an oil sample was screened for alkane-degrading enzymes. The obtained results showed that 72 clones grew in the minimum solid medium enriched with hexadecane as a carbon source and, among them, two clones revealed a remarkable ability for the biodegradation of branched and linear n-alkanes (De Vasconcellos et al. 2010).

#### 21.5.1.2 Laccases

Another important group of oxidoreductases is classified as benzenediol/oxygen oxidoreductases or laccases, which catalyze the oxidation of phenolic, chlorinated phenolic, polycyclic aromatic hydrocarbons (PAHs), and non-phenolic compounds along with the reduction of molecular oxygen to water. They can be employed in contaminated environments for bioremediation purposes (Ufarté et al. 2015a). Despite the diverse applications of laccases, these enzymes are sensitive to alkaline conditions and lose their activity in the presence of chlorine. Therefore, researchers are focusing on fungal laccases, which are resistant to alkaline pH and are less sensitive to chlorine; however, genes encoding laccases have been found in bacterial genomes and metagenomes (Ausec et al. 2011; Fang et al. 2011). For example, a South China Sea marine microbial metagenomic library was checked through a sequence-based approach, and the obtained results reported a bacterial laccase that showed considerable tolerance to chlorine while it decolorized azo dyes under high pH values (Fang et al. 2011). In a study led by Ye et al. (2010), a metagenomic library constructed using environmental DNA collected from mangrove soils was considered for laccase activity in a liquid medium supplemented with guaiacol. Clones of brownish-red color were selected as laccase-positive and checked on media with α-naphthol and catechol as specific substrates. The main property of the explored laccases was related to their highly alkaline optimal pH, which makes them compatible to be used for various purposes in industry, biorefinery, and bioremediation in alkaline environments. Along with laccases, peroxidases can participate in the oxidation of phenolic compounds such as lignin in the presence of hydrogen peroxide. Peroxidases belong to a diverse group of oxidoreductases, which are found in plants, fungi, bacteria, mammals, etc. (Karigar and Rao 2011).

#### 21.5.1.3 Oxygenases

In addition to hydroxylases and laccases, microbial oxygenases can participate in the oxidation of reduced organic pollutants, particularly halogenated and aromatic compounds that are used in a wide range of pesticides, herbicides, fungicides, and plasticizers (Ufarté et al. 2015a). Flavin adenine dinucleotide (FAD), nicotinamide

adenine dinucleotide hydrogen (NADH), and nicotinamide adenine dinucleotide phosphate (NADPH) are the main coenzymes of oxygenases that facilitate oxygenase-mediated reactions. Two types of oxygenases are identified: monooxygenases and dioxygenases that increase the solubility and reactivity of aromatic and aliphatic compounds or even break down the aromatic ring (Karigar and Rao 2011). The function of some dioxygenases such as extradiol dioxygenases (EDOs) can determine the catabolic pathway specificity. These enzymes play an essential role in the biodegradation of aromatic pollutants in the environments. Catechol can be used as a specific substrate to screen constructed metagenomic libraries for EDO-positive clones (Suenaga et al. 2007). The screening of clones for mono- and dioxygenases can be performed in solid or liquid media. In solid media, desired substrates such as naphthalene are used as the carbon source to select clones able to degrade the substrates. However, these specific substrates should not be toxic to host cells; otherwise, it limits the application of this screening strategy. Liquid media have been used in most studies to estimate the activities of selected clones in the presence of the same substrates or their structural analogues employed during preliminary screening in solid media (Ono et al. 2007). Environmental DNA samples from bacteria have been used for the construction of libraries that are searched for oxygenases. Most of these libraries employ *E. coli* strains as hosts, and some of them have been expressed in *Pseudomonas putida* strains degrading naphthalene (Ufarté et al. 2015a). Furthermore, artificial microcosms that are enriched with pollutants have been used for these libraries' construction (Silva et al. 2013). Finally, after preliminary screening and activity estimation, the target gene is sequenced via direct sequencing or PCR, and, then, by subcloning, novel gene and enzyme characteristics can be evaluated (Ufarté et al. 2015a). Interestingly, novel oxygenases have been discovered from libraries constructed from highly contaminated sites such as petroleum and pharmaceutical wastewaters (Sharma et al. 2012; Silva et al. 2013).

### 21.5.2 Hydrolases

In general, hydrolases belong to a class of enzymes that break down chemical bonds using water molecules and classically destroy larger molecules into small molecules. This function has a significant effect on the large organic polymers that cannot pass through cell membranes and pores. Moreover, hydrolases may reduce the toxicity of oil spills and organophosphate, organochlorine, or carbamate insecticides (Karigar and Rao 2011). Some common members of this class are glycosidases, aminohydrolases, amidolydrolases, peptidases, nucleosidases, and esterases (Alcántara et al. 2011).

#### 21.5.2.1 Amidases

The enzymatic breakdown of various chemical bonds in pollutants is an essential step in the microbial biodegradation of these compounds. Amidases or amidohydrolases can attack amide bonds in organophosphorus insecticides such as chlorpyrifos. In a study, *E. coli* clones from a cow rumen metagenomic library were

grown on 3,5,6-trichloro-2-pyridinol (TCP), and only one clone could attack the terminal amide bond in TCP. It was assumed that this enzyme has homology with ureases and amidases (Math et al. 2010).

#### 21.5.2.2 Nitrilases

Nitrilases or aminohydrlases are enzymes responsible for hydrolyzing nitriles to ammonia and their related carboxylic acids. Nitriles are frequently used in herbicides, chemical polymers, and plastics. Due to the importance of nitrilase activity in the microbial biodegradation of xenobiotic compounds, some efforts were taken to explore metagenomic libraries for nitrilase activity. Most of these libraries were constructed from marine and terrestrial samples. Library screening was performed by employing a liquid medium supplemented with different nitriles as a nitrogen source that resulted in the screening of 140 nitrilases showing enantio- and regioselective properties (Bayer et al. 2011; Robertson et al. 2004).

#### 21.5.2.3 Cellulases

Among glycosidases, cellulases, which degrade lignocellulosic materials, are the most important examples. Mostly, lignocellulosic materials are recalcitrant to cellulase functions. On the other hand, current cellulases show quite low activities. Hence, identification and functional screening of novel microbial cellulases with high activities is necessary. In this regard, screening of metagenomic clones of cellulolytic microorganisms can be performed by growing an *E. coli* library in media containing cellulosic materials such as carboxymethyl cellulose (CMC). Clones with cellulase activity can be detected through the Congo red staining method. The better the enrichment step during metagenomic library construction, the more novel cellulase-encoding genes are acquired (Kennedy et al. 2011). In general, cellulases are a cocktail of different enzymes including endoglucanase, cellobiohydrolase, and β-glucosidase that hydrolyze crystalline cellulose fibers to glucose units (Karigar and Rao 2011).

#### 21.5.2.4 Esterases

Esterases belong to hydrolases and play an essential role in the bioremediation of oily soils and in the biodegradation of pesticides and herbicides. These enzymes may reduce the total hydrocarbon content in soils through hydrolysis (ester bond cleavage), esterification, alcoholysis, interesterification, or aminolysis. Lipolytic activity in contaminated soils is an indicator to track oil spill biodegradation (Karigar and Rao 2011). Due to the importance of esterases in the biodegradation process, finding effective enzymes that tolerate harsh environmental conditions can be performed via metagenomic approaches (Table 21.1). Metagenomic libraries can be screened using solid media containing routine esterase substrates such as tributyrin or X-caprylate. Afterward, hit clone activity against contaminants should be checked to select those with the highest activity for further characterization (Ufarté et al. 2015a).

Many works have focused on discovering a new esterase family or highly resistant esterases. For example, one of the toxic compounds that are commonly used in the plastic manufacturing process is phthalate esters (PEs). These

**Table 21.1** Esterases discovered through screening of various metagenomic libraries

| Sample source | Library host | Pollutant category | Library size | Number of hit clones | References |
|---|---|---|---|---|---|
| Soil | *E. coli* | Insecticides | 390 Mb | 1 | Li et al. (2008) |
| | | | 100 Mb | | Fan et al. (2012) |
| Cow rumen | | | Unspecified size | | Kambiranda et al. (2009) |
| Soil | | Plastics | Unspecified size | 10 | Kang et al. (2011) |
| Compost | | | 100 Mb | 7 | Mayumi et al. (2008) |
| Oil-contaminated water | | | 1.43 Gb | 95 | Tchigvintsev et al. (2015) |
| Wastewater treatment plant biofilms | | Ectoparasiticides | 400 Mb | 3 | Jiao et al. (2013) |
| Compost | | Antibiotics | Unspecified size | 1 | Park et al. (2020) |
| Paper mill sludge | | Oils | Unspecified size | 1 | Jia et al. (2019) |

compounds act as plasticizers that can be easily washed from the main products and discharged into numerous environments, even the atmosphere. Short-chain types of PEs are more toxic due to their high solubility and are suspected to be carcinogenic; therefore, they have been classified as serious environmental pollutants. During the last few decades, biodegradation of PEs by microbial enzymes, especially dialkyl PE hydrolases, have been considered and well-studied in *Acinetobacter*, *Micrococcus*, *Gordonia*, and *Rhodococcus* spp. For instance, Jiao et al. (2013) constructed a metagenomic library from biofilms of a wastewater treatment plant in China that screened for a cold-active PE hydrolase. The obtained sequencing results showed no homology of the *dphB* gene with other reported genes that indicated that DphB is a novel enzyme in the family of PE hydrolases. In addition to these studies, screening of metagenomic libraries was performed to discover new esterases, which were effective in monoester and polyester compounds (Jiao et al. 2013; Mayumi et al. 2008; Tchigvintsev et al. 2015).

## 21.6 Conclusions and Future Perspectives

Despite the increase of pollutants in the environment, scientists and researchers are strongly attempting to mitigate pollutant content and even their hazardous effects. The omics approaches developed during the last few decades provide a novel context for the discovery of microbial potential in the biodegradation and bioremediation of

various pollutants. It is of considerable significance to employ omics technologies for unculturable microorganisms and to indirectly unlock their capabilities. Metagenomic approaches are one of the most beneficial technologies that explore microbial metagenomes for applicable data such as novel enzyme families, gene discovery, biodiversity, etc. In this regard, consideration of microbial metagenomes, originating from highly contaminated sites or natural environments with harsh conditions, aim at discovering enzymes that can be adapted to desired goals. Nevertheless, some bottlenecks may restrict researchers from achieving the total capacity of metagenomics. One of these limitations is referred to as the screening of numerous clones (sometimes thousands of clones) for a particular function or a novel reaction. Another issue is the handling of too many different hazardous organic compounds. However, these issues can be overcome by developing new screening techniques and employing safe, structural organic analogues rather than toxic compounds. In addition to these bottlenecks, most of the metagenomic literature focuses on the narrow range of enzymes (predominantly esterases or oxidases), while other enzymes such as oxygenases, proteases, laccases, etc. play an essential role in the biodegradation of xenobiotics and other hazardous pollutants and have to be studied in detail. For instance, by identifying the precise role of these enzymes in contaminated sites, biomarkers can be developed to track the variations in these sites. Currently, the rapid growth of developing novel high-throughput techniques increases the detection of new enzymes from metagenomic libraries and reduces the total cost. Therefore, the employment of explored enzymes for the biodegradation processes will be affordable and with enhance the process effectiveness.

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# Omics in Biofuel Production: A Sustainable Approach

22

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**Abstract**

Multiple environmental issues are associated with the use of fossil fuels, such as the emission of polluting gases that can generate acid rain and contribute intensely to the greenhouse effect. Aiming to alleviate these problems, biofuels—mainly biodiesel, bioethanol, and biogas—are presented as a sustainable alternative because they are renewable and less polluting, in addition to having many other advantages. These biofuels can be produced using microorganisms (bacteria and fungi) and algae, making the process even more sustainable. However, yields from this production are often relatively moderate. Aiming at more efficient production and, consequently, higher yields, several techniques related to genomics (proteomics, metagenomics, transcriptomics, metabolomics, and lipidomics, known as omics) have been used for various purposes such as identification, genetic improvement, and enzyme isolation, among others. Therefore, in this chapter, we will discuss about biofuels, their

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generation, and how they are normally produced. We will also explore the applications of microorganisms and algae in the production of biofuels and their advantages and how omics techniques can be used, helping in the choice of microorganisms or plants or modifying them to obtain higher yields in the production of renewable fuels.

**Keywords**

Sustainability · Renewable energy · Biofuels · Microorganism · Algae · Omics

## 22.1 Introduction

Obtaining energy through oil and natural gas sources still holds the place for the largest means of energy generation in the world, causing a high degree of dependence on the productive sector and, consequently, on the economy (Dey et al. 2021). The big question is what will happen when the sources of oil and natural gas become scarce in a few years, which could lead to a possible energy crisis, given that the world economy depends mainly on obtaining energy through these means (Mahapatra et al. 2021).

Although energy sources come from various products and by-products in nature, what is referred to as primary energy sources include oil, hydro, coal, solar, wind, and biomass. Among them, oil, natural gas, and coal—that are non-renewable sources—continue to be used as the principal methods of generating energy (Dey et al. 2021). Based on this scenario, there is an enormous need to explore new sources of renewable energy, since non-renewable sources are limited and there is a forecast of exhaustion in the future (Bonneuil et al. 2021).

The energy problem of the modern globalized world is based on an economic–ecological–social tripod. The world energy matrix is based on fossil energy, which, in addition to being non-renewable, will soon become scarce, causing a possible and imminent energy crisis. It is combined with the fact that it generates several environmental problems that are much discussed today, such as the greenhouse effect and climate change. The greenhouse effect, despite being a naturally occurring phenomenon, results in changes in the climate due to the increase in carbon dioxide and methane emissions. The release of these gases derives mainly from anthropogenic processes, such as transport, deforestation, agriculture, livestock, and the generation and consumption of energy (Pilecco et al. 2020). Another important factor to be mentioned is the concern with the environment that has increased in the last couple of years. Issues related to climate change and the greenhouse effect, which have been getting progressively worse, have given rise to the Kyoto protocol (Bonneuil et al. 2021).

Thus, given the factors presented, there is a need to look for other sources of energy. In this context, using biomass to generate biofuels may be a solution. Biofuels are derived from biomass (a renewable source) and can be used in internal

combustion engines or for other types of energy generation, partially or totally replacing fossil fuels (Kumar and Thakur 2020; Bashir et al. 2022).

We can define renewable energy as any energy produced from a fuel derived from biological materials—metabolic residues of living beings and non-fossilized organic matter (Bart et al. 2010a). Some better-known examples are biofuels produced from sugarcane, corn, soy, sunflower seeds, wood, and cellulose (Bart et al. 2010b). This concept includes solid-, liquid-, and gaseous-phase fuels, and it can be encountered in the forms of biogas, bioethanol, bioether, biodiesel, and ethanol, with the latter two being the most common.

The production of biogas takes place through the decomposition of organic waste, releasing a sticky and dark liquid (leachate), which, in turn, produces methane ($CH_4$). Biogas is the result of the combustion of gases formed by decomposing organic matter and the anaerobic fermentation of bacteria present in the biomass (Lee et al. 2022). The gases contained in biogas in smaller quantities are nitrogen ($N_2$), oxygen ($O_2$), hydrogen ($H_2$), and hydrogen sulfide ($H_2S$), and, in larger amounts besides methane, carbon dioxide ($CO_2$) is also present. These gases are captured by employing equipment responsible for the combustion of the released gases, called an anaerobic biomanager, and the product of this combustion generates biogas (Govarthanan et al. 2022). Therefore, the use and production of biogas present several advantages that are related to sustainability, since this is a renewable energy source, produced through waste, and is therefore infinite, being cleaner than natural gas (Lee et al. 2022).

Biodiesel is a biofuel derived from vegetable oils (soybean, corn, palm, castor, palm, etc.) and animal fats (beef tallow, chicken fat) (Bart et al. 2010b; Kumar and Thakur 2020). Given this, the energy generated by biodiesel is considered "renewable energy." This biofuel is highly similar to petroleum diesel in practically all properties. However, biodiesel has some additional advantages when compared to fossil fuel, such as it has reduced emissions of particulate matter, contains compounds with sulfur and carbon dioxide, can be obtained from renewable raw materials, is biodegradable because it has a higher flash point, has subsidies for safely handling and storing biodiesel, and has superior lubricity (Silva Junior et al. 2021; Takeno et al. 2021).

Triacylglycerols (TAGs) or triglycerides are compounds most present in vegetable oils and animal fats. Triglycerides have great potential to replace petroleum diesel and are the best option as a renewable energy source. TAGs are fatty acid esters with glycerol, and, for biodiesel to be produced, these TAGs undergo a process called transesterification (Gómez-Trejo-López et al. 2022). In this process, TAGs react when a catalyst is present, normally base, but acid catalysts can be used with short-chain alcohol (methanol, ethanol, or butanol), and transesterification consists of breaking down oil molecules in order to produce alkyl esters (Mendonça et al. 2019a, b; Kumar et al. 2021a). Transesterification, also known as alcoholysis, causes a decrease in the viscosity of vegetable oils as well as improves fuel performance in diesel engines (Geris et al. 2007).

Currently, biodiesel is industrially obtained through transesterification reactions between vegetable oils and short-chain alcohols (ethanol is the most suitable) when a

catalyst is present. The chemical catalysts used in the transesterification process can be acidic or alkaline (Barros et al. 2020). Sulfuric hydrochloric, organic sulfonic acids, and, including here, Lewis acids, such as aluminum chloride, are the most frequently used acid catalysts (Mukhtar et al. 2022). Acid-catalyzed transesterification is extremely slower than the alkaline one, reaching up to 4000 times slower for the same amount of catalyst. However, it is indicated in cases in which the moisture and concentration of free fatty acids found in the oil are high.

The most widely used alkali catalysts are hydroxides, carbonates, methoxides, sodium ethoxide, propoxide, or butoxide (Mukhtar et al. 2022). Due to their greater efficiency, base catalysts are more commonly used commercially. Despite a catalyst's efficiency, when it is in a medium with high humidity, above 1%, it causes saponification reactions that consume the catalyst, thus reducing its efficiency, increasing viscosity, favoring the formation of gels, and making it difficult to separate glycerol (Mariano et al. 2019; Mendonça et al. 2019b). When it has a fatty acid content above 0.5%, the amount of catalyst must be increased to compensate for the formation of soaps (Chouhan and Sarma 2011). Sodium hydroxide (NaOH) and potassium hydroxide (KOH) catalysts produce water when they react with alcohol, which can reverse the reaction (i.e., transform the ester obtained into a fatty acid). Thus, sodium methoxide and the like are more suitable to be used. Regarding the molar ratio, studies indicate that the ideal ratio for base catalysts is 6 moles of alcohol per 1 mole of triglyceride, that is, 100% excess when compared to the stoichiometric amount (3:1) (Demirbas 2005). The standard process commercially used to obtain biodiesel uses alkaline catalysts for the transesterification of oil or fat, in the presence of alcohol, producing methyl esters (if the alcohol is methanol) or ethyl esters (if ethanol) of fatty acids and glycerol (Mendonça et al. 2019b). However, this procedure has some factors that interfere in the process, for example, the difficulty in recovering glycerol, the use of an alkaline catalyst, which is retained in the reaction medium, subsequent treatment of alkaline effluents, highly energetic process problems caused in the reaction yield by the presence of free fatty acids, and moisture (Demirbas 2005). Because of this, several works seek other means to circumvent these disadvantages.

Enzymatic catalysis has emerged as an alternative to producing biofuels since it is possible to specifically synthesize alkyl esters and recover glycerol (Cavalcante et al. 2021). Another great advantage of enzymatic catalysis is the lower production of waste (Ching-Velasquez et al. 2020).

In addition to biodiesel, the first generation (1G) of biofuels also uses agricultural and food processing derivatives such as sugar, starches, vegetable oils, and animal fats to produce bioethanol and biogas. The second generation (2G) uses lignocellulosic raw materials, such as agricultural and forestry residues, grasses, and trees. In the third generation (3G), biofuels are produced from algae, in addition to using omics (various techniques related to genomics) to enhance the process (Wang et al. 2021).

Microorganisms may be able to produce molecules with characteristics suitable to produce biofuels. The genetic modifications in these microbes make them capable of synthesizing molecules that are not normally synthesized. These genetic

modifications consist of introducing genes so that the microorganisms start to express specific characteristics that they were not able to before, in order to increase the productivity of such a compound of interest (Levin et al. 2015). Thus, they can be applied in the conversion of simple sugars (e.g., glucose or sucrose) as well as sugars from lignocellulosic biomass into compounds of interest (Freitas et al. 2016). In this context, microorganisms can help in the production of different biofuels, where this type of energy production is directly linked to environmental sustainability since it does not cause negative environmental impacts and does not produce substances that cause damage to health, and, therefore, this form of producing energy is a clean process (Patade et al. 2018).

Bacteria, yeasts, fungi, and algae have been applied in several studies for the production of oil, where they store lipids in more than 20% of their biomass and are classified as oleaginous microorganisms (Majidian et al. 2018). Lipids of microbial origin are similar to those obtained by plants (Chintagunta et al. 2021). Due to these similar properties in both the composition and structure of fatty acids, the oil derived from microorganisms has a high potential to produce biodiesel. Wastes that usually are erroneously discarded, causing increased pollution and affecting public health, can also be used as nutrients in this process, providing both economic and environmental benefits (Zhang et al. 2022).

Biomass produced by microalgae can also be used in the production of third-generation biofuels, together with industrial waste and lower greenhouse gas emissions due to its high availability in nature, high oil production, and rapid and easy reproduction rate, besides being able to be produced without enrichment in marine, freshwater, and even sewage systems (Nanda et al. 2021).

Several compounds found in microalgae (i.e., carbohydrates, antioxidants, lipids, proteins, and even pigments) can be used to effectively produce carbon-neutral biofuels, without competing with food sources, and encourage the use of land plants as an alternative raw material for the production of biofuels for the next generation (Moshood et al. 2021).

In this context, we will address in more detail the main biofuels and their generation; we will discuss the main microorganisms used both in the production of oil and biofuels, besides exploring omics and how these techniques have been enhancing the application of microbes and their enzymes in biofuel synthesis.

## 22.2 Biofuels and Their Generation

The growth of industrialization and the constant use of fossil fuels have resulted in increases in greenhouse gas emissions and in an increase in the prices of their derivatives (Saravanan et al. 2021). The use of fossil fuels for energy generation was 83.1% in the year 2020, producing about 32 billion tons of carbon dioxide ($CO_2$), in addition to other harmful gases to the environment (Wang et al. 2022). Thus, we are heading toward the depletion of fossil fuels, causing an imminent energy crisis, as it is estimated that by 2040 the demand for fuel will be 17.39 billion L/day (Aziz et al. 2020).

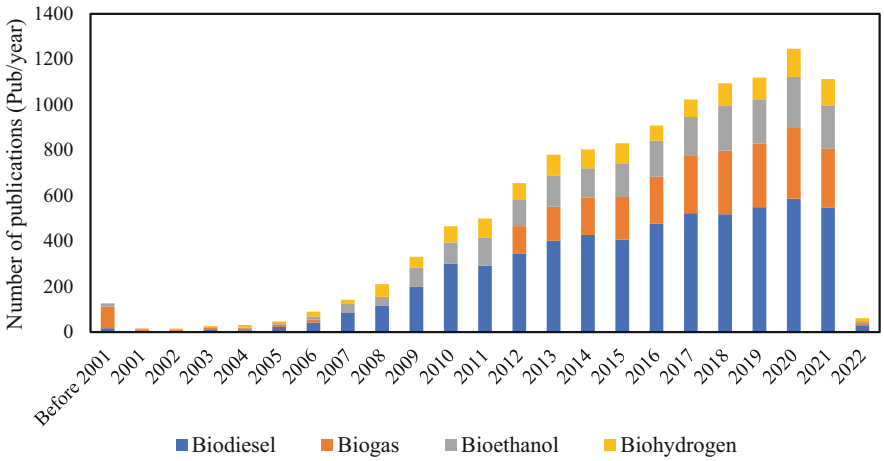


**Fig. 22.1** Number of publications found in the Web of Science. Search keys: "*biofuel name* production" or "*biofuel name* synthesis" only in the titles

On the other hand, several studies are seeking alternative sources of cheap and renewable energy, such as biomass residues that are abundant sources, including residues from wood derivatives, cattle and poultry manure, food residues, and algae and bacteria, which are sources of energy and do not emit carbon (Wang et al. 2022). Under the biomass concept, three major trends tend to dominate the energy agriculture market: those derived from products that are intensive in carbohydrates or starch, such as ethanol; lipid derivatives, such as biodiesel; and wood derivatives and other forms of biomass, such as briquettes or charcoal.

In this context, the number of publications about biofuels (bioalcohols, biogas, biogasoline, biohydrogen, and biodiesel) has increased significantly (Fig. 22.1) since they have properties with the same characteristics as fuels produced from petroleum, are considered a good renewable source of energy, and can be produced from various agro-industrial residues (Godbole et al. 2021; Moshood et al. 2021). The production of biofuels such as biodiesel, biogas, and bioethanol is around 50 billion L/year (Alalwan et al. 2019), which shows that these technologies are highly profitable.

Biofuels are classified according to their generations—i.e., first (1G), second (2G), third (3G), or fourth generation (4G) (Aziz et al. 2020). 1G biofuels are produced from food crops such as sugarcane, corn, wheat, and other foods that contain starch and oil. However, it is known that the use of these products raises concerns about the impacts on sustainability, food safety, and prices of these products (Aamer Mehmood et al. 2021; Shokravi et al. 2021). The disadvantage of using this technology for the production of biofuel is that it can cause impacts on food supply, in addition to the use of fertilizers, water consumption, and the need for large areas for crops (Alalwan et al. 2019). 2G biofuels are those derived from non-food sources, mainly lignocellulosic biomasses, which are mainly composed of cellulose, lignin, and hemicellulose (Barros et al. 2021a, b). These raw materials are

highly attractive due to their abundance, sustainability, economy, and non-carbon emission (Wang et al. 2022). 3G biofuels are linked to the use of micro- and macroalgae and cyanobacteria for the production of fuels (Abbasi et al. 2021). Micro- and macroalgae are promising sources to produce biodiesel. The technology involving algae presents itself as advantageous, given its easy adaptation to small environments, growth in natural and artificial environments, a high oil content, and greater photosynthesis efficiency compared to other plants, in addition to reducing the level of $CO_2$ in the environment (Alalwan et al. 2019). Lipids extracted from algae can be transesterified with ethanol or methanol to produce biodiesel, but other parts of the algae that have lignocellulosic material can be thermochemically or biochemically converted, producing bioalcohol, biogasoline, or other biofuels (Lin and Lu 2021). Its disadvantages are financial costs, as it requires more advanced technologies (Lin and Lu 2021).

Modified genetic engineering involves molecular biology and physicochemical analysis on microalgae, yeast, fungi, cyanobacteria, and genetically modified microbes, being these techniques applied in the production of fourth generation biofuels. In this context, genetic engineering is used to improve the biofuel production process derived from genetically modified algae (Alalwan et al. 2019). Generally, computational methods and metabolic engineering are used to aid processing in research on manipulating the genetics of photosynthetic organisms. This generation is related to the development of genetically modified strains of algae for the production of lipids and thus the production of biofuels (Shokravi et al. 2021).

The most used genetically modified algae are *Chlamydomonas reinhardtii*, *Synechocystis* sp., *Phaeodactylum tricornutum*, and various *Chlorella* species (Godbole et al. 2021). The advantages of using these species for the fourth generation (4G) of biofuels are their accelerated growth, high oil content, and their structure, presenting numerous possibilities for commercial applications (Alalwan et al. 2019).

Describing the generations of fuels, we will discuss the main biofuels and their characteristics.

### 22.2.1 Bioethanol

It is known that this biofuel is generally produced via fermentation of sugarcane or other lignocellulosic materials, such as rice straw, sugar beet, and grass. However, its main disadvantage is that it competes with the application as food, which leads to increased pressures on these foods (Arias et al. 2021).

In recent decades, bioethanol has received increasing attention as a more renewable biofuel alternative, as it has advantages, such as decreasing $CO_2$ concentrations in the atmosphere and decreasing dependence on fossil fuels, reducing environmental impacts (Alalwan et al. 2019).

On the other hand, bioethanol manufactured using metabolic microorganisms (yeasts—*Saccharomyces cerevisiae*—or bacteria—*Escherichia coli*) does not compete with food sources (Arias et al. 2021), given the possibility of applying

lignocellulosic biomasses. In this context, cyanobacterial biomass can also be applied in the production of alcohols (bioethanol and biobutanol), in addition to the production of hydrogen gas (Maia et al. 2018, 2019).

### 22.2.2 Biodiesel

For the production of biodiesel, more than 350 edible or inedible oil crops were suggested, where about 75% of the production cost is due to the raw material (Alalwan et al. 2019).

The conversion of triglycerides into biofuel usually occurs through transesterification, thermal cracking, and hydrogenation, using basic, acidic, enzymatic, heterogeneous, or photocatalytic catalysts (Lin and Lu 2021; Long et al. 2021).

When using the transesterification reaction, triglycerides and various chain alcohols undergo action under a catalyst to obtain esters of fatty acids and glycerol. Unlike petroleum, esters produced by transesterification have advantages, including non-toxicity, biodegradability, and low sulfur levels and nitrogen, reducing environmental pollution (Long et al. 2021).

The raw materials for the production of biodiesel are usually derived from agro-industrial plantations, such as jojoba oil, salmon oil, rubber tree, tobacco seed, cooking oil, tallow, and animal fats (Alalwan et al. 2019). The disadvantage of biodiesel is the low safety presented by raw materials derived from animals, which can be contaminated, in addition to competition with edible oils (Alalwan et al. 2019), which makes this biofuel a little more expensive.

This biofuel can also be produced by the action of microorganisms, where they produce enzymes capable of catalyzing the reaction. However, the price of these enzymes is a key factor in the application of this technology.

### 22.2.3 Biogas

Biogas is a biofuel mainly composed of methane ($CH_4$) and carbon dioxide ($CO_2$), and it is a source for generating energy and heat (Iweka et al. 2021). As a bioproduct, biogas has great potential to replace petroleum derivatives—mainly natural gas—and become an important part of the energy system (Zhang et al. 2021). Its production is derived from the anaerobic processing of manure and other sources of biomass, such as plant and animal residues (Alalwan et al. 2019).

The anaerobic digestion methodology is a promising process that consists of converting organic materials into carbon dioxide and methane; its advantages are low energy consumption, high organic load rate, and low sludge production, in addition to having economic benefits. In the environmental area, the benefits include efficient management of waste, in addition to the reduction of water and air pollution (Zhang et al. 2021).

It is known that to produce biogas, manure is usually the most used. However, it is not economically viable as gas production is low. Agro-industrial residues such as potato skins, corn, cereal, bananas, cassava, and pineapple, among other organic residues, can be applied for the production of gas (Hashemi et al. 2021; Iweka et al. 2021). The advantage of applying lignocellulosic materials is that they do not compete with food production and are abundant and available, having a large amount of carbon (Hashemi et al. 2021). Although the structure and composition of lignocellulosic materials can limit the efficiency of digestion, needing to be pretreated before being inserted into a digester for hydrolysis, its use is still viable, as it is given an application to the residues (Iweka et al. 2021).

For the production of biogas, there are several factors to be observed, since they can affect the anaerobic process and, consequently, the amount of biogas produced: digester temperature, retention time, digester pressure, volatile fatty acid, and fermentation pH (Iweka et al. 2021; Mahanta et al. 2005; Noorain et al. 2019); other factors such as agitation, toxicity, additives, loading, and dilution can also affect the quality and quantity of the biogas produced (Mahanta et al. 2005).

## 22.3 Microorganisms Applied in the Synthesis of Biofuels

Since ancient times, fermentation techniques using microorganisms have been used in the food industry, mainly in the production of beverages (Dashko et al. 2014). However, the application of microorganisms in the production of biofuels has gained relevance over the decades, as the energy industry has been looking for alternative energies with low environmental impact for its development, which can compete with fossil fuels (Majidian et al. 2018). Within this perspective, some microorganisms have gained attention due to their several advantages: they are organisms of simple cellular machinery, have versatile properties and highly accelerated development and reproduction cycles, require little space for culture, and can be found in different environments (Hahn-Hägerdal et al. 2007; Miao and Wu 2006).

Microorganisms, by definition, are beings that can only be observed with the use of a microscope and can be classified, mostly, into protozoa, fungi, algae, and bacteria (Behera et al. 2019). According to their application in biofuels, microorganisms can be used either as lipid feedstock (Siwina and Leesing 2021), replacing the use of vegetable oils, or in the production of enzymes for catalysis of esterification and transesterification reactions (John et al. 2017; Lee et al. 2010), for example. Microalgae are also considered photosynthetic microorganisms that, in addition to producing lipids, contribute to the reduction of atmospheric $CO_2$ (Atsumi et al. 2009). Thus, they contribute to the reduction of gases released by the burning of fossil fuels.

In order to improve the production of lipids and enzymes by microorganisms, several studies have been carrying out genetic modifications, aiming to increase the viability of microorganisms for the production of biofuels. This technique is based

on the manipulation of the microorganism's DNA, developing new characteristics after being genetically altered (Liu et al. 2011).

### 22.3.1 Oleaginous Microorganisms

Oleaginous microorganisms are those that can produce and store lipids inside their cells by metabolizing different substrates. A wide variety of substrates can be used for the development of these microbes. Waste from the agricultural industry can also be applied, being even more economically advantageous.

To produce oil, cells rich in lipids must undergo extraction processes, where the cell walls must be broken. According to Khot et al. (2020), different extraction methods can be used: ultrasound, osmotic shock, microwave, and autolysis, among others (Chintagunta et al. 2021). After cell disruption, the lipid portion can be obtained and the levels quantified. Table 22.1 summarizes some studies carried out with species of microbes, molds, yeast, and microalgae as well as the relationship of substrates and lipid levels produced by each of them.

### 22.3.2 Lipolytic Enzyme-Producing Microorganisms (Catalysts)

Some microorganisms, when metabolizing substrates, produce lipolytic enzymes that break down lipid molecules, thus acting as catalysts in reactions for the production of biofuels (Lee et al. 2010).

Enzymatic catalysis using lipases has interesting advantages, such as easier separation of post-reaction phases, lower cost of wastewater treatment, easier glycerol recovery, and absence of undesirable side reactions, when compared to transesterification catalyzed by homogenous or heterogeneous catalysts.

Among the lipase-producing microorganisms are *Candida rugosa* (Lee et al. 2010) and *Candida antarctica* (Ko et al. 2012) from the yeast group; *Burkholderia cepacia* (Sasso et al. 2016) and *Pseudomonas fluorescens* (Ferrero et al. 2016) from bacteria group; and *Rhizopus oryzae* (Luna et al. 2014) representing the fungi.

However, there are some disadvantages to the use of enzymes (i.e., lipases) in the production of biofuels, such as product contamination with undesirable residual enzyme activity and the high cost of commercial enzymes (Mishra et al. 2021). To overcome these problems, several studies have been looking for ways to immobilize enzymes on a solid support, or in a transport matrix, allowing their reuse for several cycles of reactions (Mishra et al. 2021).

**Table 22.1** Lipid production by oleaginous microorganisms and substrates [adapted from Chintagunta et al. (2021)]

| Source | Oleaginous species | Lipid production | References |
|---|---|---|---|
| Microbes | *Gordonia* sp. DG | 71%[a] | Gouda et al. (2008) |
| | *Rhodococcus opacus* | 14.2%[a] | Wei et al. (2015) |
| | *R. opacus* | 26.9%[a] | Goswami et al. (2017) |
| | *R. opacus* PD630 | 83%[a] | Voss and Steinbüchel (2001) |
| | *R. opacus* Xsp8 | 45.8%[a] | Kurosawa et al. (2013) |
| Molds | *Alternaria* sp. | 60.3 mg/gds[b]<br>NA* | Dey et al. (2011) |
| | *Aspergillus oryzae* | 62.9 mg/gds[b]<br>NA%[a] | Hui et al. (2010) |
| | *Colletotrichum* sp. | 68.2 mg/gds[b]<br>NA%[a] | Dey et al. (2011) |
| | *Microsphaeropsis* sp. | 80 mg/gds[b]<br>10.2%[a] | Peng and Chen (2008) |
| | *Mortierella isabelina* | 0.016–0.11 mg/gds[b]<br>29.47–38.36%[a] | Economou et al. (2011) |
| | *Mucor circinelloides* Q531 | 42.43 ± 4.01 mg/gds[b]<br>28.8 ± 2.85%[a] | Qiao et al. (2018) |
| | *M. circinelloides* | 32%[a] | Chan et al. (2020) |
| | *Phanerochaete chrysosporium* ATCC 24725 | >40%[a] | Liu et al. (2019) |
| Yeast | *Candida phangngensis* PT1–17 | 9.8 g/L[c] | Quarterman et al. (2018) |
| | *Cryptococcus curvatus* | 10.83 g/L[c]<br>61–73.26%[a] | Liang et al. (2012), Chang et al. (2015) |
| | *Cryptococcus vishniaccii* MTCC232 | 7.8 g/L[c]<br>53.4%[a] | Deeba et al. (2016) |
| | *Cutaneotrichosporon cutaneum* | 4–5 g/L[c] | Wang et al. (2016) |
| | *Cutaneotrichosporon dermatis* | 20.36 g/L[c]<br>56%[a] | Yu et al. (2020) |
| | *Lipomyces starkeyi* | 8.1 g/L[c]<br>26.9–55%[a] | Huang et al. (2014), Xavier et al. (2017) |
| | *Meyerozyma guilliermondii* | 37.99 ± 0.003%[a] | Ananthi et al. (2019) |
| | *Naganishia álbida* | 13.5 g/L[c]<br>20%[a] | Sathiyamoorthi et al. (2019) |
| | *Pichia kudriavzevii* | 28.57 ± 0.009%[a] | Ananthi et al. (2019) |
| | *Rhodotorula glutinis* | 1.4–5.5 g/L[c]<br>11.86–36.4%[a] | Liu et al. (2015) |
| | *R. glutinis* | 10.42 g/L[c]<br>51%[a] | Liu et al. (2018) |

(continued)

**Table 22.1** (continued)

| Source | Oleaginous species | Lipid production | References |
|---|---|---|---|
| | *Rhodotorula paludigenum* | 3.29 g/L[c]<br>58%[a] | Chaiyaso et al. (2019) |
| | *Rhodosporidium toruloides* | 14–39.6 g/L[c]<br>43.3–63.4%[a] | Zhao et al. (2010), Wang et al. (2012) |
| | *Trichosporon cutaneum* | 9.8–12.3 g/L[c]<br>32.1–40%[a] | Liu et al. (2012b), Gao et al. (2014) |
| | *Vishniacozyma psychrotolerans* | 46%[a] | Deeba et al. (2017) |
| | *Yarrowia lipolytica* | 5.2–6.68 g/L[c]<br>48–58.5%[a] | Tsigie et al. (2012) |
| Microalgae | *Auxenochlorella protothecoides* | 5.7 g/L[c]<br>66%b[a] | Patel et al. (2018) |
| | *A. protothecoides* | 5.3 g/L[c]<br>63%[a] | Patel et al. (2018) |
| | *Chlorella protothecoides* | 2.14 g/L[c]<br>22–55.2%[a] | Xu et al. (2006), Wei et al. (2009), Lu et al. (2010) |
| | *C. protothecoides* | 58 g/L[c]<br>34.0%[a] | Mu et al. (2015) |
| | *Chlorella pyrenoidosa* | 1.55 g/L[c]<br>53.6% [a] | Li et al. (2011) |
| | *Nannochloropsis* sp. | 3.2 g/L[c]<br>11.0%[a] | Cheirsilp et al. (2017) |
| | *Schizochytrium limacinum* | 2.15–4.95 g/L[c]<br>55.3–70.5%[a] | Liang et al. (2010) |
| | *Schizochytrium mangrovei* | 3.52 g/L[c]<br>16.4%[a] | Pleissner et al. (2013) |
| | *Schizochytrium* sp. | NA g/L[c]<br>45.15%[a] | Nguyen et al. (2018) |

a - lipid yield (mg/g dry substrate); b - lipid content (% w/w); c - lipid content

## 22.4 The Role of Omics in Biofuel Production

### 22.4.1 Omics Approaches for Biofuel Production

Omics approaches assist the energy agriculture market by associating technologies that aim to improve the efficiency of biofuels profitably with cost reduction in production and maintenance processes, besides favoring the environment. In a post-fossil fuel era, its contributions, through the modernization of technologies, accompany, refer, and adapt according to the proposed models of biofuels, be they first-, second-, third-, or fourth-generation (Moravvej et al. 2019). In this context, each generation has certain prerogatives, challenges, and even embargoes, where the omics studies have brought, at each stage, important contributions to energy solutions, which can be readapted or even redesigned according to the progress toward a more "green technology" in future perspectives (Yadav et al. 2018). Thus,

knowing some omics approaches and their associations becomes essential for the next steps in the path of bioenergetic evolution, since they allow elucidating more viable metabolic pathways for the production of biological molecules of commercial value, taking into account the abiotic effects and the biological events in the layers of genetic code and protein function (Patade et al. 2018). In this sense, omics studies will be clarified and demonstrated as one of the tools that have added to the economic and productive improvement in each generation of biofuels. In addition, their emphases will be discussed for the next generation of biofuels.

#### 22.4.1.1 What Are Omics Studies?

Omics studies are a legacy that had as its beginning the practices of manipulation of DNA molecules (Giani et al. 2020). According to the growing domain of sequencing and genetic modification techniques, added to the techniques related to biochemical functionality, they aim, in an integrated manner, to evaluate molecular biology factors and their processes observed in biological species: from genotypes to their respective phenotypes (Benfey and Mitchell-Olds 2008). From biochemistry, studies that have an extremely close relationship are derived because the functionality of a cell, whether at the intra- or intercellular level, follows the genetic code contained in it, and the variations of external factors are in line with these limits (Morange 2009).

As a starting point, genomics is a branch of biochemistry that has the function of global analysis of DNA or a smaller genetic scale. It studies DNA structure, function, evolution, and genome mapping, in addition to characterizing and quantifying those genes that lead to the production of proteins with the help of other biological molecules (Vailati-Riboni et al. 2017). In this axis, genomics is linked to a set of complementary sciences that aim to structure themselves in the collective of functional biological information, still often generalized as "functional genomics" (Hieter and Boguski 1997). In a few years, its integration gained the neologism called "omics." In this context, transcriptomics is the study of the set of all messenger RNA molecules of a cell, tissue, or organism and quantifies and characterizes their identities (Lowe et al. 2017). Proteomics addresses the studies of a global or specific set of proteins of a cell, tissue, or organism, investigates their biochemical properties and functionality, and takes into account post-translational modifications—fundamental to the functional and evolutionary characterization of living species (Vailati-Riboni et al. 2017). Metabolomics deals with the studies of the set of metabolites of a cell, tissue, or organism, evaluates their biochemical metabolic pathways, and often supports the joint elucidation with the other omics sciences (Vailati-Riboni et al. 2017). Other sciences were derived from these sciences: lipidomics, which evaluates the lipid composition distributed in cellular compartments and supports elucidation in conjunction with the other omics sciences (Han 2016), and metagenomics, also known as "environmental and community genomics," which is based on the genomic analysis of microorganisms in a community, where genomes are usually isolated from the environment, fragmented, and then cloned by microorganisms with the ability to replicate to create metagenomic libraries (Nazir 2016). There are currently other omics approaches that have gained

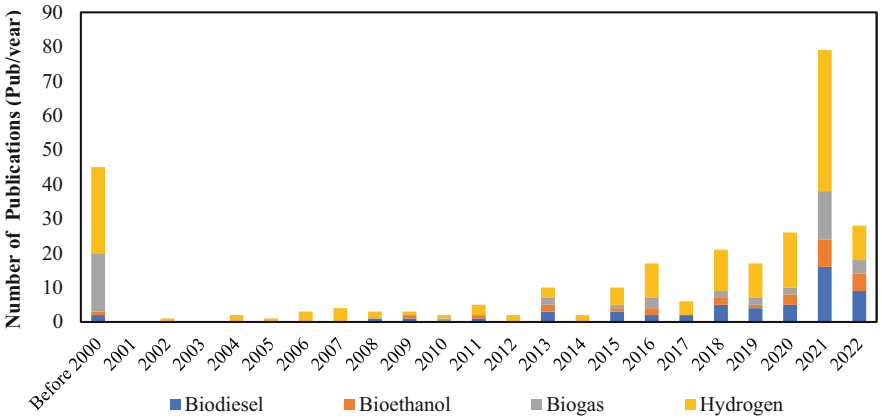


**Fig. 22.2** Number of publications found in *ScienceDirect*. Search keys: "*biofuel name* AND production" + "OMICS AND techniques"

space as technological proportions allow the modernization of techniques, instrumentation, and bioinformatics, serving as a tool for the various areas of genetic or metabolic engineering in the search for solutions that enable the efficient and economic exploration of living organisms of commercial importance, in this particular case, energy agriculture. In this context, several works are using omics techniques to improve the production of biofuels, growing considerably in the last year (Fig. 22.2).

### 22.4.2 Contributions of Omics and Their Perspectives on Biofuel Generation

The advances of omics techniques in organisms applied to the production of biofuels became more modernized over their generation, since the main species of commercial importance had the complete sequencing, molecular mapping, and bioinformatics of their genomes, providing a powerful strategy for the discovery of genes and their biological functionalities, as exemplified in Table 22.2.

Oil plants are the main source of lipids used in both food and biofuels. Therefore, the growing demand for vegetable oils has focused on research to increase the amount of its content. In this context, soybean can be highlighted, which is one of the plant organisms that has a great commercial value due to its high production of both oil and protein for food consumption, where a part of its production is converted into biodiesel. Omics studies have enabled soybeans to edit their genomes, genomic selection, and characterization of genetic loci, obtain transgenic lines, and develop nutritional quality and seed composition (Kumar et al. 2021b). Soybean breeding efforts have shown that multiple loci (the position that a gene occupies on the chromosome) contribute to the final oil and protein content stored in seeds (Lardizabal et al. 2008). Using the genomic approach, the levels of these two

**Table 22.2** Sequencing the complete genome of some cultures or important organisms to produce biofuels [adapted from Yadav et al. (2018)]

| Biofuel crop/organism | Genome size (Mbp) | Biofuel generation | Web link for whole-genome sequencing information |
|---|---|---|---|
| *Saccharum officinarum* (sugarcane) | ~1000 (monoploid) | 1st | http://sugarcanegenome.org/ |
| *Jatropha curcas* (physic nut) | 416 | 1st | http://www.kazusa.or.jp/jatropha/ |
| *Glycine max* (soybean) | 1150 | 1st | http://www.phytozome.net/soybean |
| *Brassica rapa* (canola) | 530 | 1st | http://www.brassica.info/resource/sequencing.php |
| *Helianthus annuus* (sunflower) | 3600 | 1st | http://www.sunflowergenome.org/ |
| *Sorghum bicolor* (sorghum) | 730 | 2nd | http://www.plantgdb.org/SbGDB/ |
| *Zea mays* (maize) | 2500 | 2nd | http://www.maizegdb.org/ |
| *Panicum virgatum* (switchgrass) | 1500 (haploid) | 2nd | http://switchgrassgenomics.org/ |
| *Triticum aestivum* (wheat) | 17,000 | 2nd | http://www.wheatgenome.org/ |
| *Botryococcus braunii* (green microalga) | 166 | 3rd | http://jgi.doe.gov/why-sequence-botryococcus-braunii/ |

products show an inverse correlation, where the accumulation of one affects the reduction of the other: a strategy to improve production was the insertion of a transgene, through a fungus, with the function of specifically modulating a metabolic pathway to improve oil production without greatly impacting the protein content or yield (Lardizabal et al. 2008). Similarly, other oilseed plants of commercial value gain a perspective with these studies, such as canola and *Jatropha* (Table 22.2).

As for some plants used for bioethanol production, sugarcane has the particularity of high ethanol productivity due to the cost and ease of breaking down its sugars in the fermentation process (Canilha et al. 2012). Proteomics in sugarcane is in progress, where its first studies made it possible to analyze sugarcane stalks (Amalraj et al. 2010), in which it was possible to identify the proteins involved in sugar metabolism. In this manner, they can be targets for additional manipulation to increase or modify sugar content with potential benefits for bioethanol production (Wu and Birch 2007). Other approaches aim at the incorporation of enzymes capable of hydrolyzing lignocellulosic materials, transforming them into sugars with potential applications in the production of bioethanol or modification of lignin biosynthesis pathways to reduce biomass resistance (Harrison et al. 2011; Jung et al. 2013). Further omics advances are being made to understand the metabolism of sugars and also other cellular processes involved in favoring bioethanol production (Ali et al. 2019). Corn also takes advantage of genetic engineering approaches applied to sugarcane, as it contains a large amount of soluble sugars and biomass, which can improve bioethanol production (Shen et al. 2012). Similarly, other plants with the potential to produce bioethanol are under development for proteomic studies, such as

the case of sugar beet and sorghum, and microorganisms applied to the improvement of enzymatic catalysis of biomass are evaluated by other omics approaches for the selection of more tolerant crop stresses involved in the processes (Table 22.2).

For 3G biofuels, the most economically viable sources are photosynthetic unicellular microalgae cultures. With the modernization of omics tools, a safe perspective is generated for microalgae to be used and leveraged as a source of biofuel production on an industrial scale (Salama et al. 2019). However, the challenge for some omics approaches lies in sample extraction, as some species have a high content of polysaccharides that contaminate observations, for example, in electrophoretic instrumentations – often used in the proteomic analysis (Ndimba et al. 2013). For this, several strategies have been developed to obtain a cleaner analysis. Despite this, high-throughput proteomics provides a deeper interpretation of the microalgae proteome and metabolomic techniques have enabled the remodeling of metabolic pathways, improving the production of lipids and dyes. In addition, genetic engineering tools have contributed to the improvement of strains (Chakdar et al. 2021).

The investigations aimed at microalgae are often linked not only to the accumulation of lipids but also to the characterization of their species to assess their quality because microalgae produce high amounts of both polar and non-polar lipids (Shanmugam et al. 2020). Triacylglycerol (TAG) is a non-polar lipid convertible to biodiesel; on the other hand, glycolipids and phospholipids are polar and have unsaturations that do not allow conversion to biodiesel, which makes the process more expensive (Arif et al. 2020). Typing lipids, in addition to quantifying them, has been done through studies in lipidomics, in which several species of microalgae are evaluated as potential candidates to produce biodiesel. An extra in these studies is the evaluation of the produced biomass and the property of taking advantage of the crop to remove nutrients from wastewater (Arif et al. 2020). In this context, lipidomics together with transcriptomics is applied for manipulation of the gene associated with the synthesis of acetyl-CoA in order to improve the production of lipids accumulated by microalgae (Arif et al. 2020). As the cultivation of microalgae is relatively easy to handle and maintain compared to conventional agriculture, several studies are carried out aiming at the yield of products. In this context, the omics studies explore metabolic pathways that can be altered to improve the production of lipids and carbohydrates (Brar et al. 2021). One of the emphases subjects microalgae to stress conditions to evaluate their productive properties (Salama et al. 2019). The investigation of nitrogen deprivation combined with heterotrophism in a microalgae species is also promising to produce biodiesel since it stimulates the accumulation of lipids in algae. Through the proteomic analysis of this study, it was observed that the significantly altered proteins, with some of them being related to carbohydrate metabolism and gene regulation of fat oxidation, lead to the prospect of future studies of additional functional analysis of these altered proteins to elucidate cell growth and the accumulation of lipids in microalgae (Li et al. 2013).

Bacteria are also organisms that have interesting properties to contribute to the production of biofuels as "micro-factories." In this context, the biosynthesis of alkanes by cyanobacteria are the oldest studies, and some species are evaluated for

the tolerance of some products since certain compounds such as hexane are considered toxic to the biological environment (Liu et al. 2012a). A proteomic study that evaluated a species of cyanobacteria in response to the stress of the presence of hexane shows that the altered proteins are related to transporters, membranes, oxidative stress, and photosynthesis. These results lead us to believe that hexane defense mechanisms may be associated with the metabolic pathway of these altered proteins (Ndimba et al. 2013). Other studies (Table 22.3) that aim at selection criteria for cyanobacterial species for bioprospecting in the production of biodiesel and other biologically based chemical compounds use lipid profile and metabolomic engineering as a means of evaluation (Kato et al. 2022; Shanmugam et al. 2020).

In addition to cyanobacteria as one of the candidate organisms for the production of biodiesel, there are other bacteria with the same potential, as shown by a study carried out with chemolithotrophic bacteria, which use inorganic compounds as their energy source, in the special case in which $CO_2$ present in the atmosphere (Kumar et al. 2017). In this emphasis, other studies evaluate several known routes for $CO_2$ fixation with interest in biofuel production and other valuable chemical compounds, where the fixation means presented are both natural and synthetic at in vivo, in vitro, and in silico levels. The in silico level relies on multi-omics tools to be designed before being tested in vitro and transplanted in vivo. In this manner, it is possible to see that the tools of the omics sciences can accompany the next advances in metabolic engineering (Nisar et al. 2021). Autotrophic organisms, by their very nature, are equipped to convert $CO_2$. Therefore, integrated technologies that solve its high-yield fixation for biomass can cooperate in the policies of the next generation of biofuels (Dutta et al. 2014).

## 22.5 Conclusions

Biotechnology developed until the present day has enabled solutions for the bioenergy market. So far, four types of biofuel generation have been introduced, which are classified according to their biomass sources, their limitations as a renewable energy source, and their technological progress. 1G and 2G biofuels still compete with food production for the use of conventional land, and 3G biofuels have the differential of being developed in non-competitive spaces that have more developed engineering for the growth of the culture of microorganisms, whether they are producers of oils or enzymes (catalysts). Not only plants with high commercial production but also several classes of microorganisms have been widely explored to improve productivity and reduce costs in biofuel crops directly or indirectly. Microalgae have been largely elucidated through modern omics approaches and have great potential for the development of 4G biofuels because, in addition to their high productivity in a short time, they can solve certain environmental problems concerning wastewater treatment and capture of $CO_2$. However, they still face limitations regarding the most efficient methods for extracting their raw material and are in the study phase for selection, characterization, and

**Table 22.3** Some contributions from the omics studies for each generation of biofuels and their generated perspectives, based on some organisms or cultures

| Biofuel generation | Organism/crops | Omics approaches | Purpose and perspective | References |
|---|---|---|---|---|
| 1st | Soybean | Genomics, transcriptomics, proteomics | Species selection based on genomic databases and stress responses, genetic modification for increased oil production, genome editing opportunities | Lardizabal et al. (2008), Kumar et al. (2021a) |
| 1st | Canola | Genomics, transcriptomics | Genetic modification through gene transfer from yeast to canola results in increased oil production by the plant | Vigeolas et al. (2007) |
| 1st | Sugarcane | Genomics, transcriptomics, proteomics, metabolomics | Search for biomarkers that are reflected in the development of high-throughput omics technologies, proteins associated with sugar metabolism, genetic modification to increase sugar production, lignin biosynthesis pathway | Ndimba et al. (2013), Ali et al. (2019) |
| 1st | *Jatropha*, sugar beet | Proteomics | Search for biomarkers that are reflected in the development of high-throughput omics technologies | Ndimba et al. (2013) |
| 2nd | Sorghum, maize | Proteomics | Search for biomarkers that are reflected in the development of high-throughput omics technologies | Ndimba et al. (2013) |
| 2nd | Microorganisms | Metagenomics, proteomics, metabolomics | Increase production for biofuels by knowing metabolic pathways and further | Martien and Amador-Noguez (2017), Xing et al. (2012), Poudel |

(continued)

**Table 22.3** (continued)

| Biofuel generation | Organism/crops | Omics approaches | Purpose and perspective | References |
|---|---|---|---|---|
| | | | characterizing the pathways for new products, proteomic studies to increase enzyme efficiency, enzyme mining of microbial communities | et al. (2017), Guo et al. (2021) |
| 3rd | Microalgae | Genomics, proteomics, metabolomics, transcriptomics, lipidomics | Detection of proteins related to lipid accumulation through N deprivation, carbohydrate accumulation, driving genetic alterations, and metabolic engineering based on stress responses and biomass production along with lipid and carbohydrate content, reengineering to program production through understanding in proteomic studies and responses in metabolic pathways, identification of gene expression linked to lipid production | Li et al. (2013), Nagappan et al. (2020), Brar et al. (2021), Shahid et al. (2020), Arif et al. (2020), Chakdar et al. (2021), Kato et al. (2022), Shanmugam et al. (2020) |
| 3rd | Cyanobacteria | Metabolomics, proteomics, lipidomics | Understanding through proteomics to pave the way for other omics approaches; lipid profile assessment as a selection criterion in bioprospecting | Kato et al. (2022), Shanmugam et al. (2020), Ndimba et al. (2013) |

bioenergetic evaluation. In parallel, cyanobacteria also cooperate to produce 3G biofuels, as they have properties highly similar to microalgae, being frequently explored by omics tools. Other classes of bacteria have been studied in a way that generates perspectives for the increase of biomass in cultures since it is a problem faced by cultures of autotrophic organisms. In this manner, we can list in the solutions for the development of the next generation of biofuels, the policy of negative carbon in the atmosphere—these were previously caused by human activities since the advent of the Industrial Revolution to the present day, mainly arising from the burning of fossil fuels. The amount of omics studies linked to each generation has brought about highly promising perspectives for genetic and metabolic engineering and has opened doors to synthetic biology, that is, the possibility of engendering artificial biological combinations for highly efficient energy applications.

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# Part IV

# Recent Trends and Development in Omics Technologies

# High-Throughput Sequencing Technologies in Metagenomics: Advanced Approaches for Algal Research

23

Neha Saini, Sumit Kumar, Bansal Deepak, and Sharma Mona

**Abstract**

The fields of biology and environmental research have been shaped by high-throughput sequencing (HTS) technologies in the past few years. The study of microorganisms that are found everywhere on Earth has faced many challenges in terms of their discovery and their interactions with the environment. The thriving area of metagenomics is likely to solve these problems. The discovery of new microorganisms has faced a transition from culture-based techniques to non-culture-based techniques and is discussed in this chapter. Emphasis is placed on different types of high-throughput sequencing technologies to understand the fundamentals of DNA sequencing and their analysis with the help of bioinformatic tools. Advancements in algal research as a result of metagenomics in the quantification, diversity study, molecular detection, and functional assessment of microalgae are discussed in this chapter.

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Metagenomics · Microalgae · Diversity · DNA sequencing · Microbial diversity

## 23.1 Introduction

Microorganisms are found everywhere, i.e., in habitable places on Earth and inside and on the surface of living organisms (Jo et al. 2020; Kumar and Chandra 2020). Microbial communities are instrumental in almost all biological processes, from natural ecosystems to animal bodies. A total of $10^{30}$ microbes are estimated to be on Earth with 100 trillion microbes in the human body alone. These microbial communities remain undiscovered and are not cultured in the laboratory and need to be revealed to humankind (Di Bella et al. 2013). The term "metagenomics" emerged more than 20 years ago, exploring the microbial universe. It has become a driving force for discoveries in microbial ecology and biotechnology (Taş et al. 2021). The use of high-throughput sequencing (HTS) protocols in metagenomics provides a faster, easier way of evaluating microbial diversity and ecological interactions between all life forms in a cost-effective manner. These technologies can identify novel genes and novel sources of drugs and energy (Wang et al. 2013). HTS technologies have revolutionized genome research due to their potential to produce large data in relatively shorter time. These help determine bacterial community composition, which helps keep a check on environmental biodegradation. Environmental metagenomics helps explore the diversity of organism-specific DNA in environmental samples without culturing them, which enhances our knowledge to improve drinking water quality (Kumar et al. 2020, 2021; Wani et al. 2021). DNA extraction, library preparation, and sequencing through Illumina, Roche 454, Ion Torrent (short read) or PacBio, and Oxford Nanopore (long read) techniques are all straightforward processes. Every metagenome analysis starts with quality control (QC) by minimizing sequence bias and removing adapter sequences and contaminant sequences. Next-generation sequencing (NGS) technologies, particularly second-generation sequencing (SGS) and third-generation sequencing (TGS) platforms, have evolved a lot in the past few years. Some of the second-generation platforms include Roche GS FLX by 454 Life Sciences, Illumina HiSeq and Illumina NextSeq genome analyzer by Illumina, Inc., SOLiD by ABI, Illumina MiSeq, and Ion Torrent by Life Technologies. Third-generation sequencing platforms include LLC, SeqLL Helicos™ Genetic Analysis System, Pacific Biosciences single-molecule real-time (SMRT) Sequencing, GnuBIO by BioRad, Oxford Nanopore's Nanopore sequencing, and Complete Genomics by the Beijing Genomics Institute (Ambardar et al. 2016). Life scientists need to have correct and accurate knowledge of bioinformatic experimental designs to extract information from the data obtained after sequencing. New computational methods are constantly evolving to obtain useful information from sequencing datasets (Escobar-Zepeda et al. 2015). HTS has gradually dominated the algal research. This has helped in new algal species discovery, nomenclatural decisions, clarifying higher-level

classification, and species boundaries. This has paved the way for the analysis of non-model microalgal strains for biofuel production. Success in obtaining axenic cultures of microalgae has always been problematic due to the presence of culturable and non-culturable bacterial contaminations. HTS has helped scientists evaluate bacterial presence and obtain axenic algal cultures (Heck et al. 2016; Oliveira et al. 2018).

This chapter provides an overview of the brief history of sequencing technologies that have emerged in recent times by shedding light on different platforms of sequencing available. It also deals with bioinformatic analysis and tools available for the interpretation of the sequencing databases. Application of HTS platforms in algal research is also covered in this chapter.

## 23.2 Historical Background of Sequencing Technologies

Earlier, microcopy was the only medium available to study microorganisms and their interactions. However, to scientists, the catch was that the number of microorganisms observed and the number of microorganisms cultured in the laboratory was unequal. A brief overview of the history of emergence of different molecular technologies is shown in Fig. 23.1. Morphology, biochemical profiles, and growth patterns have been the basis for the study of microorganisms for almost 300 years. In the late 1970s, Carl Woese suggested the application of ribosomal RNA genes as molecular markers for classifying microbes. To study and classify microorganisms, a revolution was brought about through the Sanger automated sequencing method (Escobar-Zepeda et al. 2015). Since then, scientists have been increasingly interested in overcoming the barriers of microscopy. In 1977, the first DNA sequencing technologies were developed by Sanger (Sanger dideoxy synthesis) and Maxam–Gilbert (chemical cleavage). These are "first-generation sequencing" (FGS) methods. Sanger's method was more suitable than Gilbert's method and was well-accepted due to the development of a thermal cycler, use of non-hazardous chemicals, automation, and commercialization (Ambardar et al. 2016; Kchouk et al. 2017). Staden in 1979 proposed the idea of "shotgun sequencing." In this method,

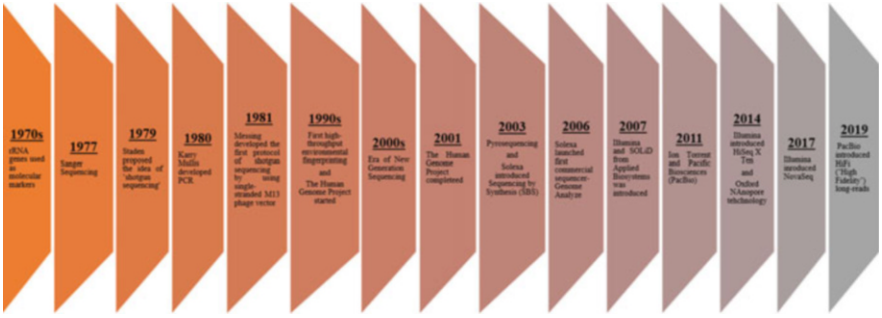


**Fig. 23.1** Historical overview of sequencing technologies

the DNA is cloned through bacterial vectors, which are then sequenced and assembled using overlaps, thus helping in sequencing larger genomes in a shorter time. In 1985, Norman Pace and his colleagues used 5S and 16S rRNA sequences to detect microbial communities. In 1981, Messing used a single-stranded M13 phage vector to develop the first protocol of shotgun sequencing. The 48,502 bp-long genome of the λ-phage was sequenced by Sanger in 1982. The US National Institutes of Health (NIH) sequence database known as GenBank was also established in the year 1982. This database contained more than 40 million bases at the end of the decade. Since then, it is adding almost tenfold sequences every 5 years, and the growth rate has not ceased. In 1988, Sanger published an article titled "Sequences, Sequences, and Sequences," expressing general excitement. Technological breakthroughs occurred in the 1980s and 1990s due to the selection of industrial operations to increase output and reduce sequencing errors (Giani et al. 2020). During the 1990s, the first high-throughput environmental fingerprinting techniques were developed. In these, the size difference of amplicons of polymerase chain reaction (PCR) was used and the banding pattern obtained was used as the "fingerprint" of individual taxa. Some of the representative techniques are temperature gradient gel electrophoresis (TGGE), terminal restriction fragment-length polymorphism (TRFLP), and denaturing gradient gel electrophoresis (DGGE). High-throughput environmental fingerprinting is limited to detecting only the most abundant or dominant community. Next, DNA microarray technology was proposed. This technology uses short sequences (called probes) from a specific genome locus for phylogenetic classification. Gene chip microarray analysis was developed to study biogeochemical processes and the activities of microbial communities. Moreover, this method has successfully provided details about the mechanisms involved and about the dynamics of metal-reducing bacteria for detecting the bioremediation of uranium (Jo et al. 2020). Until the advent of NGS technologies, the most frequently used sequencing technologies by scientists throughout the world were Sanger and Maxam–Gilbert sequencing technologies (Kchouk et al. 2017). The Human Genome Project (HGP) was performed in labs filled with ABI3730 Sanger sequencing instruments. These aimed to sequence complete human chromosomes and produce genetic and physical maps of the same. This labor-intensive approach cost $3 billion US and took 13 years to complete right from sample collection to publication of the completed human genome. On the other hand, NGS technologies can complete the sequencing of human genomes de novo for $one million US in 2 months, i.e., they reduce the cost by 10% and 0.9% of the time of Sanger sequencing (Jones 2010).

In the 2000s, "high-throughput sequencing (HTS)" technologies or "next-generation sequencing (NGS)" technologies were introduced, which were cost-effective technologies with progressively higher sequencing output. They contained procedures of data acquisition and analysis, which makes them non-labor-intensive and highly efficient than Sanger sequencing technologies (Mardis 2017). High-throughput and single-molecule DNA sequencing are the hallmarks of NGS, irrespective of the sequencing platform. The first NGS technology was "pyrosequencing," which was commercialized and introduced by 454 Life Sciences in 2003 as an NGS DNA sequencer GS20 (Giani et al. 2020). The literature divides

NGS technologies into two groups: (1) second-generation sequencing (SGS) and (2) third-generation sequencing (TGS). SGS was the newest technology developed after FGS as it was distinguished to prepare sequence banks prior to the sequencing of amplified DNA fragments. TGS is capable of producing long reads in a shorter time and at lower costs and sequence a single molecule without creating amplification libraries and is, therefore, also called single-molecule sequencing technology. SGS technologies include Illumina, Ion Torrent platforms, 454, Solexa, Agencourt, and Applied Biosystems, whereas TGS technologies include Pacific Biosciences or Oxford Nanopore technology platforms (Kchouk et al. 2017). In 2003, Solexa came up with a new technology known as sequencing by synthesis (SBS). In 2006, Solexa launched the first commercial sequencer—Genome Analyzer. In 2007, Illumina acquired Solexa, and SOLiD from Applied Biosystems was also introduced in the same year. A new approach based on proton detection in semiconductors called Ion Torrent came about in 2011 (Ansorge 2009). The company reduced the cost of sequencing human genomes to $1000 in a single day. In 2012, a more dynamic machine called Ion Proton was released. However, it was abandoned as it also mismeasured the length of homopolymers unlike SOLiD. Illumina commercialized the SBS approach, and, by 2014, the company took over 70% of the DNA sequencing market and 90% of all the DNA produced. They also introduced HiSeq X Ten in the same year and NovaSeq in 2017. TGS sequences single DNA molecules without the need for amplification and produces longer reads than does SGS. From 2010 onward, the new technologies ushered in an era of TGS. The idea of Nanopore sequencing technology was initially proposed in the 1990s, but it was brought to the market in 2014 by Oxford Nanopore Technologies (ONT). Craighead, Korlach, Turner, and Webb developed single-molecule real-time (SMRT) sequencing, and this has been commercialized by Pacific Biosciences (PacBio) since 2011. In 2019, HiFi ("high-fidelity") long reads were developed by PacBio, which are as accurate as Illumina short reads and can generate 10–20 kbp long circular consensus sequences (CCSs). In the same year, they released Sequel II (Giani et al. 2020). The only direct competitor of SMRT sequencing, Nanopore, has released a portable sensing device called MinION in 2014, which became available in May 2015, and R10 with improved base calling in 2019 (Ambardar et al. 2016). Since the introduction of the first method of sequencing, these technologies have evolved every day and are still evolving.

## 23.3 High-Throughput Sequencing Platforms

As discussed above, the three generations of sequencing platforms and their general mechanisms and their characterization is briefly described in Table 23.1.

### 23.3.1 First-Generation Sequencing (FGS) Platforms

The first genomes sequenced by Sanger sequencing were phiX174 (in 1980) and the bacteriophage λ genome having a size of 5374 and 48,501 bp, respectively. It is also

**Table 23.1** Characterization of different NGS technologies

| Sequencing technology | Read length (bp) | Run time (h) | Error rate (%/bp) | Output (Gb/run) | Accuracy | Chemistry | Amplification for library construction | References |
|---|---|---|---|---|---|---|---|---|
| *First-generation sequencing (FGS)* | | | | | | | | |
| Sanger | 400<br>900 | – | 0.3 | 0.00069<br>0.0021 | – | Sequencing by synthesis | Yes | Kchouk et al. (2017) |
| *Second-generation sequencing (SGS)* | | | | | | | | |
| Roche 454 GS FLX+ | 700 | 18<br>20 | 1 | 1 | – | Pyrosequencing | Yes | Scholz et al. (2012) |
| Roche 454 GS junior | 400 | 10 | 1 | – | – | Pyrosequencing | Yes | Scholz et al. (2012) |
| Illumina Hiseq 2500 | 2 × 150 | 11 days | 0.26 | 720–800 | – | Reversible terminator | Yes | Ambardar et al. (2016) |
| Illumina Hiseq 2500 Rapid Run | 2 × 150 | ~27 | 0.26 | 150–180 | – | Reversible terminator | Yes | Ambardar et al. (2016) |
| Illumina NextSeq | 2 × 150 | – | 0.8 | 100–120 | – | Reversible terminator | Yes | Ambardar et al. (2016) |
| Illumina NextSeq 550 | 2 × 150 | 11<br>29 | – | 120 | Q30 ≥ 75% | Sequencing by synthesis | Yes | Hu et al. (2021) |
| Illumina NextSeq 1000 and 2000 | 2 × 150 | 11<br>48 | – | 330 | Q30 ≥ 75% | Sequencing by synthesis | Yes | Hu et al. (2021) |
| Illumina MiSeq | 2 × 300 | 27 | 0.8 | 8.35 | Q30 ≥ 75% | Reversible terminator | Yes | Di Bella et al. (2013) |
| Illumina MiSeqDx | 2 × 300 | 24 | 0.1 | ≥5 | – | Sequencing by synthesis | Yes | Hu et al. (2021) |
| Illumina MiniSeq | 2 × 150 | 5–24 | 0.8 | 6.5–7.5 | Q30 ≥ 80% | Sequencing by synthesis | Yes | Kchouk et al. (2017) |
| Illumina iSeq | 2 × 150 | 9.5<br>19 | – | 1.2 | Q30 ≥ 80% | Sequencing by synthesis | Yes | Hu et al. (2021) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Illumina NovaSeq 6000 | 2 × 250 | 13<br>44 | – | 3000 | – | Sequencing by synthesis | Yes | Hu et al. (2021) |
| SOLiD 5500 W | 75 | 8 h | 0.1 | 160 | – | Sequencing by ligation | Yes | Kchouk et al. (2017) |
| 5500xl W SOLiD | 75 | – | 0.1 | 320 | – | Sequencing by ligation | Yes | Kchouk et al. (2017) |
| Ion PGM | 100–200 | 7.3 | ~1 | 2 | – | Sequencing by capturing hydrogen ion | Yes | Escobar-Zepeda et al. (2015) |
| Ion PGM-dx | 200 | 4.4 | – | 1 | – | Sequencing by capturing hydrogen ion | Yes | Hu et al. (2021) |
| Ion PGM 314 chip v2 | 400 | 3 | 1 | 0.06–0.1 | – | Sequencing by capturing hydrogen ion | Yes | Scholz et al. (2012) |
| Ion PGM 316 chip v2 | 200 | 3 | 1 | 0.6–1 | – | Sequencing by capturing hydrogen ion | Yes | Scholz et al. (2012) |
| Ion 318 chip v2 | 400 | 3 | 1 | 1.2–2 | – | Sequencing by capturing hydrogen ion | Yes | Scholz et al. (2012) |
| Ion proton | 200 | – | 1 | 10 | – | Sequencing by capturing hydrogen ion | Yes | Kchouk et al. (2017) |
| *Third-generation sequencing (TGS)* | | | | | | | | |
| PacBio | 50,000 | 2 | 13 | 0.1 | – | Sequencing through single molecule | No | Ambardar et al. (2016) |
| PacBio RS C1 | 432 | 2 | 15 | 0.54 | – | Sequencing through single molecule | No | Kchouk et al. (2017) |

(continued)

**Table 23.1** (continued)

| Sequencing technology | Read length (bp) | Run time (h) | Error rate (%/bp) | Output (Gb/run) | Accuracy | Chemistry | Amplification for library construction | References |
|---|---|---|---|---|---|---|---|---|
| PacBio RS II P6 | 600 | 2 | 12 | 0.5–1 | – | Sequencing through single molecule | No | Kchouk et al. (2017) |
| C4PacBio RS C2 XL | 432 | 2 | 15 | 0.5–1 | – | Sequencing through single molecule | No | Kchouk et al. (2017) |
| Oxford Nanopore MinION | – | 18 | 38.2 | 1 | – | Sequencing through single molecule | No | Ambardar et al. (2016) |
| Oxford Nanopore MinION Mk | 100 | – | 12 | 1.5 | – | Sequencing through single molecule | No | Kchouk et al. (2017) |
| Oxford Nanopore PromethION | – | – | – | 2–4 Tb | – | Sequencing through single molecule | No | Kchouk et al. (2017) |
| Heliscope™ | 2 × 35 | – | 0.5 | 35 | – | Sequencing through single molecule | Yes | Ambardar et al. (2016) |

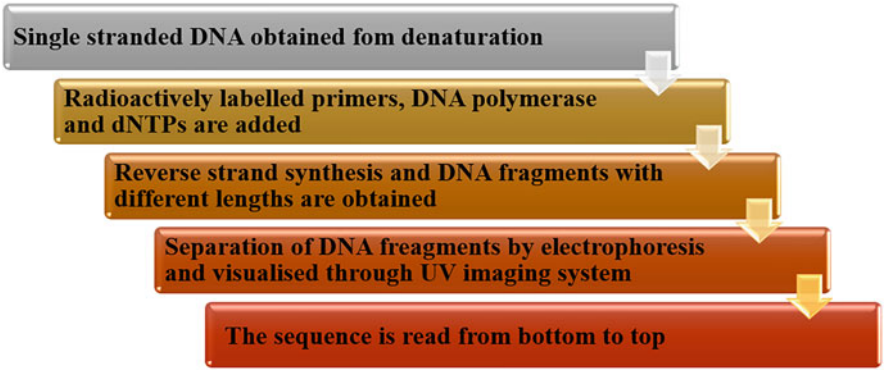


**Fig. 23.2** Overview of the Sanger sequencing technology

called sequencing by the synthesis method or the dideoxynucleotide method or the chain termination method. An overview of the technology is presented in Fig. 23.2. This method uses a single strand of double-stranded DNA as a template, dNTPs, radioactively labeled primers, and DNA polymerases. Four dNTPs are run in a separate reaction to terminate the polymerization randomly when dNTPs are incorporated into the DNA strand. Reverse strand synthesis occurs on the polymerized DNA strands using a known priming sequence present upstream of the sequence. At the end, different DNA fragments with different lengths are obtained, which are separated according to their size difference using a gel slab or by capillary electrophoresis. The corresponding DNA fragments are visualized by an X-ray or an ultraviolet (UV) light imaging system. The final sequence of the DNA is known by reading the complement of the original template from bottom to top (Kircher and Kelso 2010; Kchouk et al. 2017). Sanger sequencing is slow when compared to current NGS technologies, but, with time, innovations, such as the use of thermal cyclers and fluorescent dyes, software developments to interpret results, etc., have helped the Sanger process in enhancing its speed and ease. Several sequencing machines based on the Sanger sequencing technology are present like PerkinElmer, Licor, Dupont, and MilliGen. The only leader in automated Sanger sequencing is Applied Biosystems (ABI), which is now part of Thermo Fisher Scientific (Slatko et al. 2018). It is still a useful technique in which a high throughput is not required.

### 23.3.2 Second-Generation Sequencing (SGS) Platforms

From the start of the 2000s, NGS has marked its emergence to break the limitations faced in FGS technologies. SGS platforms can generate millions of copies of short reads of DNA in parallel, have enhanced speed and low cost, and have no need of electrophoresis. These are divided into two categories: (1) sequencing by hybridization and ligation (SBL) and (2) sequencing by synthesis (SBS). The

major platforms are Roche/454, Illumina/Solexa, ABI/SOLid, and Ion Torrent (Ambardar et al. 2016).

#### 23.3.2.1 Roche/454

Pal Nyren and Mostafa Ronaghi developed the Roche/454 sequencing platform at the Royal Institute of Technology, Stockholm, in 1966. This is based on the principle of the pyrosequencing technique and came to the market in 2005. Roche/454 was the first of the NGS sequencing platforms (Kircher and Kelso 2010). The approach is sequencing by synthesis or sequencing during extension. Each random fragment of DNA is attached to a single bead. The beads have primers carrying oligonucleotides, which are complementary to the DNA fragments. Each bead is isolated and produces millions of copies of each DNA fragment during amplification in the PCR emulsion. The pyrosequencing technique is applied on the beads, which are transferred to a picotiter plate (PTP). Each of the two million wells in the plate can accommodate exactly one 28-μm diameter bead (Kchouk et al. 2017). During the process of incorporation of nucleotides in the pyrosequencing technique, ATP is formed by ATP sulfurylase because of the release of pyrophosphate per nucleotide. After the incorporation of the complementary nucleotides into the template DNA, one nucleotide is washed over by polymerases. A high-resolution charge-coupled device (CCD) camera is used to capture the light signal produced by luciferase during the production of ATP. This is performed for all wells in parallel, and the final sequence of the DNA is deduced (Ansorge 2009). Deoxyadenosine-50-(a-thio)-triphosphate (dATPaS) is used for the base incorporation reaction to prevent the dATP from being used in the light reaction. The remaining nucleotides are the standard deoxyribose nucleotides. The unincorporated nucleotides are removed, and the next nucleotides are added after capturing the light signal (Escobar-Zepeda et al. 2015; Ambardar et al. 2016). The number of flow cycles, the order of the bases in the sequence to be determined, and the base composition determine the length of the reads. The Roche/454 GS FLX Titanium platform can determine the length between 300 and 500 nt and sequence 1.5 million beads. The current instrument launched, GS FLX+, produces ~one million reads per run with 1000 bp length. The error rates are due to the insertions and deletions caused by the occurrence of homopolymer regions. This can be corrected by higher coverage. The substitution error rate, excluding insertion/deletion, is in the range of $10^{-3}$ to $10^{-4}$ (Kircher and Kelso 2010). In bead preparation, some of the beads carry copies of multiple different sequences, which are automatically removed during software post-processing. The cost of this technology is high in comparison to those of other NGS technologies, i.e., about 20 $/Mb, and an output of 750 Mb of DNA sequence per day can be achieved (Ambardar et al. 2016; Kchouk et al. 2017).

#### 23.3.2.2 Illumina/Solexa

Illumina uses fluorescently labeled reversible terminator technology based on the SBS approach. It is currently the most used technology in the NGS market. Figure 23.3 shows the steps involved in Illumina technology briefly. A special sequencing library is prepared by randomly fragmenting the DNA samples, which are then

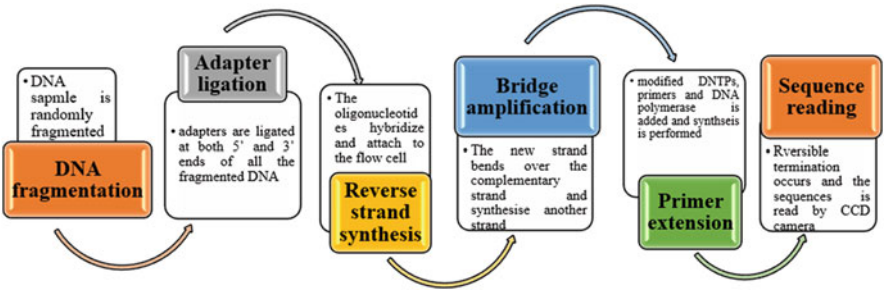


**Fig. 23.3** Illumina sequencing technology

amplified and immobilized for sequencing (Goodwin et al. 2016). They are ligated to the adapters at both 5′- and 3′-ends of all the fragmented DNA molecules, which are attached to the complementary adapters. A solid plate (flow cell) consists of many immobilized complementary oligonucleotide variants of adapters. Reverse strand synthesis occurs from the double-stranded DNA formed due to the hybridization of the oligonucleotides to the single-stranded library molecules and thus a new strand is created (Kchouk et al. 2017). After this, the process of bridge amplification occurs. In this, the new strand formed during reverse synthesis attaches to the other complementary adapter sequence by bending on the free end of the strand and by synthesizing a second covalently bound reverse strand. This produces millions of identical copies of each sequence known as clusters, containing both the forward and reverse strands of the original sequences. The extension reaction by complementary base pairing is hindered by the oligonucleotides, and it is checked by cleaving one of the two strands selectively at base modifications of oligonucleotides (Bentley et al. 2008). At the end, each cluster on the flow cell consists of identical copies of the same sequence. For sequencing of these copies, sequencing primers (hybridized to the sequences), DNA polymerases, and four modified nucleotides are added as a mix. DNA polymerases perform a reversible termination reaction, and the incorporated fluorescently labeled nucleotides are optical readouts. The fluorescently labeled nucleotides, having an inactive 3′-hydroxyl group, are used by DNA polymerases to extend the primers. Nucleotide sequences are translated by light signals emitted by each nucleotide of a cluster and are detected by a coupled-charge device (CCD) camera. At the 3′-end of the base, the terminator group and the fluorescent dye are removed and the synthesis cycle is continued (Reuter et al. 2015; Kchouk et al. 2017). The paired-end sequencing of Illumina NGS platforms generates more data with in-depth coverage along with high-quality sequences and high numbers of reads as compared to single-end sequencing. It has an error rate of 0.1% and is the most accurate base-by-base sequencing technology (Hu et al. 2021). The first sequencer with Illumina technology called Illumina/Solexa GA was able to produce extremely short reads of only ~35 bp. The top five sequencers are NextSeq 550, NextSeq 550Dx, NextSeq 1000, NextSeq 2000, MiSeq, MiniSeq, iSeq and MiSeqDx with differing sequencing outputs and total reads per run. The patterned flow cells has billions of nano wells at fixed locations which results in providing high

level of throughput and maximum data output as compared to normal flow cell. The limitation of Illumina is high requirement for sample loading control and high run time have reduces with the advancements in more Illumina models (Thermes 2014).

#### 23.3.2.3 Applied Biosystems/SOLiD

In 2005, the Harvard Medical School and the Howard Hughes Medical Institute developed Supported Oligonucleotide Ligation and Detection (SOLiD). In 2007, Applied Biosystems (ABI) developed a sequencing by ligation (SBL)-based approach called ABI/SOLID sequencing technology by acquiring SOLiD (Shendure and Ji 2008). This is different from the SBS approach as it uses DNA ligases to carry out the extension reaction instead of DNA polymerases. The ABI/SOLiD technique consists of multiple sequencing rounds. Initially, DNA is fragmented and known adapters are attached to it, which are further attached to the beads. Only one fragment is bound per bead (Shao et al. 2011). These are amplified by emulsion PCR (emPCR) and are deposited on the glass surface. Eight/nine base-long probes are hybridized or annealed to the complementary sequence of the target DNA. The probes contain three degenerate bases and three universal bases attached to one of the four fluorescently labeled probes. DNA ligases add short primers, and non-ligated probes are removed. The fluorescently labeled probes are detected by fluorescence imaging. After detection, the system is prepared for another round of ligation by cleaving the probes and the sequence of the complete target DNA is known. The cycle is repeated, and the next cycle is started at the $n - 1$ position of the template. Each base is sequenced twice in a cycle. The output format is a color space where the fluorescent recovered data are translated to DNA letters and the sequence of the DNA fragment is known. This requires the last base of the used library adapter sequence known as the first base (Breu 2010). This leads to a reduction in the average error rate by detecting machine errors. One of the limitations is short reads and less output, which has improved from 35 bp to 85 bp and 3 Gb/run to 30 Gb/run, respectively (Goodwin et al. 2016). Moreover, higher background noise due to amplification in ligation cycles poses another limitation. This technique is comparable to Illumina in terms of throughput and price, i.e., 5000 Mb/day and 0.50 $/Mb, respectively (Kircher and Kelso 2010).

#### 23.3.2.4 Ion Torrent

In 2010, the Ion Torrent semiconductor sequencing technology was commercialized in the form of a benchtop Ion PGM sequencer. It is also called pH-mediated sequencing, semiconductor sequencing, or silicon sequencing (Mascher et al. 2013). This technology is based on the Roche/454 pyrosequencing platform through the detection of hydrogen ions released during the sequencing process not on enzymes, altered bases, or optical detection. This technique occurs in millions of wells with a semiconductor chip and a bead. The semiconductor chip consists of a complementary metal-oxide semiconductor (CMOS) pH sensor and a flow chamber. The nucleotide sequence is directly converted into digital information on a semiconductor chip. DNA fragments are present in a water-in-oil emulsion droplet (micelle) and are ligated to the adapter sequences. The complementary adapters are put on the

beads along with deoxynucleotides (dNTPs), primers, and DNA polymerases. PCR amplification of adaptor-ligated DNA fragments occurs in each micelle, which acts like a micro-PCR reactor (Goodwin et al. 2016). In this process, millions of beads contain multiple copies of one DNA sequence. Only one bead can enter an individual well while flowing across the chip. Moreover, the appropriate nucleotide is incorporated, a hydrogen ion is released, and the change in pH is induced when flowing across the chip. The pH change is detected by a sensor, which is placed at the bottom of the microwell, and a voltage signal is produced. The voltage signal is proportional to the number of bases incorporated (Slatko et al. 2018). This technique saves time, having run times between 2.5 and 4 h, and cost and generates read lengths of 200–600 bp (Reuter et al. 2015). The substitution error rate is low for this technique, i.e., per base rate of $<0.1\%$. The limitation of this technique is that it is error-prone due to its homopolymer sequencing. This leads to the production of higher electronic signals by the release of hydrogen ion repeats incorporated into every cycle (Ambardar et al. 2016). Some of the Ion Torrent platforms are Ion PGM Dx, Ion Proton™ System, Ion GeneStudio S5, ION S5 XL system, Genexus Instruments, Ion S5 System, and Ion Hi-Q sequencing, which are extremely useful for whole-genome and transcriptome sequencing (Hu et al. 2021).

### 23.3.3 Third-Generation Sequencing (TGS) Platforms

The third-generation high-throughput NGS technology is also called single-molecule real-time (SMRT) sequencing technology. The quality of genome assemblies has greatly been improved by TGS. This overcomes short read lengths and PCR bias limitations of the SGS platforms. Very long read lengths and low sequencing context bias help find many missing regions of NGS-based data to provide more coverage and accuracy of the resulting genome assemblies (Giani et al. 2020). In TGS platforms, a single DNA template is sequenced instead of sequencing a clonally amplified DNA template. Moreover, biochemical usage is minimal. This can generate sequences of more than 10 kb. Initially, there were inaccuracies in the technologies, but, with recent modifications and improvements, this has provided exciting possibilities for sequencing large DNA fragments. The more advantages of TGS are that it gives the maternal and paternal contributions of the diploid or polyploid genomes separately and characterization of the epigenetic marks along with DNA sequencing (Merker et al. 2018). There are five types of TGS sequencing platforms, i.e., Pacific Biosciences, GnuBio by BioRad, Helicos' Genetic Analysis System, nanopore sequencing by Oxford Nanopore, and Complete Genomics by the Beijing Genomics Institute. In 2010, Pacific Biosciences commercialized the single-molecule real-time (SMRT) sequencing technology. It is the most widely used third-generation sequencing technology (Rusk 2009). In this, a SMRT bell template (a single strand of a circular DNA template to be sequenced) and the high-fidelity DNA polymerases derived through $\phi 29$ are bound at the bottom of the zero-mode waveguide (ZMW). The SMRT bell template is produced by the ligation of hairpin adaptors called SMRT bell adapters to both ends of the double-

stranded DNA (dsDNA) template molecule. ZMWs are microfabricated wells in an aluminum cladding film of 70 nm diameter and 100 nm depth deposited on a silica glass. It guides the light into an area of small dimensions where it decays inside the chamber as it cannot propagate if the diameter of the aperture is less than its wavelength. This property helps in developing images only at the bottom of the ZMW. The DNA polymerase incorporates the dye-labeled nucleotides in a growing chain. The DNA polymerase travels the SMRT bell in the 5′ to 3′ direction on the priming sites present on the adapters attached at both ends (Ambardar et al. 2016; Slatko et al. 2018). The light signal is detected as nucleotide sequences by monitoring the active site of the enzymes and is called a continuous long read (CLR). Pacific Biosciences sequencing platforms show a high error rate of 13–15% due to insertion and deletion errors (Kchouk et al. 2017). Oxford Nanopore Technology (ONT), a sequencing flow cell, consists of hundreds of microwells, each having an artificial lipid bilayer perforated by genetically modified staphylococcal a-hemolysin (aHL) protein nanopores. The single-stranded nucleic acid passes through nanopores. The adaptors are ligated to double-stranded DNA along with the preloaded motor enzymes at the 5′-end. The single DNA strand passes through the nanopores and disrupts the current in the pores, which is detected by a semiconductor sensor, and the current changes are recorded and translated into DNA sequences (Clarke et al. 2009). The first nanopore sequencing device is the USB-powered portable sequencer MinION, which was released in 2014, and can produce >90 Mb data in 18 h. In spite of producing high error rates of 5.1% for substitution, 4.9% for insertion, and 7.8% for deletion, this technology is of low cost (Reuter et al. 2015). Helicos was the first company to sell a single-molecule sequencer instead of molecule assemblage requiring PCR amplification. SeqLL, LLC commercialized the HeliScope Genetic Analysis System platform. In this platform, a poly-A tail is attached to the sheared DNA and blocked at 3′-OH using terminal transferase and a dideoxy nucleotide. The poly-A fragments are hybridized and sequenced in the flow cell containing oligo-dT. It uses fluorescently tagged nucleotides attached to the flow cell through poly-T tails. The next base incorporated into the synthesized strand is determined by an analysis of the emitted light signal. The read length is between 24 and 70 nucleotides and is shorter than those of other platforms. TGS platforms with single-molecule real-time sequencing have error reduction in progress and are evolving.

The main questions while studying microorganisms is "what we are looking for?" and "what is their role in the environment?" HTS or metagenomics answer these questions in two different ways, i.e., (1) amplicon sequencing (2) shotgun metagenomics. Specific regions of DNA are amplified using known primers in amplicon sequencing. The most common primer used should have more phylogenetic coverage in databases. So, for prokaryotes, the most used gene is *16S rRNA*, and, for eukaryotes, it can be the large subunit (LSU) gene or internal transcribed spacers (ITSs). In amplicon sequencing, the analysis involves just one gene present in all the organisms of the community, and this is called "meta-profiling." Although this is a cost-effective approach, it has low resolution at the species level, bias generated due the use of a single gene, horizontal transfer of the *16S rRNA* gene, and low abundance of the marker gene in the sample. Moreover, the effect of primer

mismatch leads to error in detection of taxa within a community (Wang et al. 2013). On the other hand, the whole shotgun sequencing approach uses total DNA to prepare libraries and random primers to sequence the overlapping regions of a genome. This approach is more accurate to identify the taxa at the species level and novel genes in organisms. Due to the high cost associated and requirement of extensive data analysis, this approach has been less used to study microbial profiling than 16S rDNA amplicon profiling. The choice of sequencing platforms will dependent upon the requirements of the researcher and the availability of the computational resources and the software to process the sequencing output (Escobar-Zepeda et al. 2015).

## 23.4 Bioinformatic Tools for Metagenome Analysis

Researchers need to have good knowledge of computational tools to design experiments correctly and to extract useful information as high-throughput sequencing techniques generate huge numbers of datasets consisting of text files in the FASTA or FASTQ format with millions of reads. The generation of bigger datasets requires large data storage and advanced computational resources and more complex bioinformatic analyses. Programming and scripting knowledge along with UNIX operative system skills are desirable with high-end servers to install and run metagenome software for the interpretation of the results (Ladoukakis et al. 2014). Table 23.2 shows the different computational tools for bioinformatic analysis. The assessment of the output quality of the sequencing technologies is an important step before bioinformatic analysis. The characterization of a microbial community is affected by the bias and error rate of each sequencing platform. Different programs for quality control (QC) or Phred score analyze the sequencing output, i.e., information about the guanine–cytosine (GC) content, the number of reads, and the length of the sequence obtained. Tools are also available to modify the reads, i.e., quality filtering and adapter removal. QC analysis involves removal of low-quality sequences or parts of sequences based on the type of downstream analysis and the algorithm used. In 16S rRNA amplicon sequencing, high levels of intra- and inter-sequencing center technical variations are reported. This can alter the estimation of β-diversity and can be removed by removing ambiguous bases from the reads, trimming low-quality sequences, and using denoising and less computationally intensive algorithms like SeqNoise, PyroNoise, DeNoiser, and single linkage clustering (Wang et al. 2013). After denoising, chimeric sequences need to be removed, which arise due to reannealing of prematurely terminated fragments to another template DNA. Some of the tools are UCHIME, Perseus, ChimeraSlayer, and Decipher (Kim et al. 2013). Operational taxonomic units (OTUs) are greatly influenced by chimeras, sequencing errors, and clustering algorithms. There are two types of clustering methods, i.e., alignment-based and alignment-free clustering methods. An OTU represents a clustered-together sequence with a particular percentage of divergence. The number of OTUs decreases as the divergence and the number of reads increase. A species-level resolution is considered for 97% identity

**Table 23.2** Bioinformatic software and their applications used to study databases produced by sequencing technologies

| S. no. | Bioinformatic tool | Application | References |
|---|---|---|---|
| 1 | PyroNoise | Denoising | Wang et al. (2013) |
| 2 | DeNoiser | Denoising | Wang et al. (2013) |
| 3 | Single linkage clustering | Denoising | Wang et al. (2013) |
| 4 | SeqNoise | Denoising | Wang et al. (2013) |
| 5 | DADA | Denoising | Kim et al. (2013) |
| 6 | Acacia | Denoising | Kim et al. (2013) |
| 7 | AmpliconNoise | Denoising | Di Bella et al. (2013) |
| 8 | UCHIME | Chimeric sequence removal | Kim et al. (2013) |
| 9 | ChimeraSlayer | Removal of chimeric sequences | Kim et al. (2013) |
| 10 | Perseus | Chimeric sequence removal | Kim et al. (2013) |
| 11 | Decipher | Removal of chimeric sequences | Kim et al. (2013) |
| 12 | UCLUST | OTU picking | Di Bella et al. (2013) |
| 13 | BeBAC | OTU picking | Di Bella et al. (2013) |
| 14 | CROP | OTU picking | Di Bella et al. (2013) |
| 15 | ESPRIT-tree | OTU picking | Di Bella et al. (2013) |
| 16 | M-pick | OTU picking | Di Bella et al. (2013) |
| 17 | BLAST | Mapping and local alignment of translated sequences | Escobar-Zepeda et al. (2015) |
| 18 | MEGAN | Uses data derived from BLASTX or BLASTP for gene annotation, | Escobar-Zepeda et al. (2015) |
| 19 | QIIME | Analysis of raw reads, taxonomic annotation, and comparison of meta-profiling or metagenomic data | Escobar-Zepeda et al. (2015) |
| 20 | MG-RAST | Annotation and analysis of taxonomy, comparative metagenomics | Escobar-Zepeda et al. (2015) |

(continued)

**Table 23.2** (continued)

| S. no. | Bioinformatic tool | Application | References |
|---|---|---|---|
| 21 | Meta velvet | de Bruijn assembler | Escobar-Zepeda et al. (2015) |
| 22 | IDBA-UD | de Bruijn assembler | Escobar-Zepeda et al. (2015) |
| 23 | MetaSPAdes | de Bruijn assembler | Jo et al. (2020) |
| 24 | Megahit | de Bruijn assembler | Jo et al. (2020) |
| 25 | Ray meta | de Bruijn assembler | Jo et al. (2020) |
| 26 | Anvi'O | de Bruijn assembler | Jo et al. (2020) |
| 27 | MetAMOS | OLC assembler | Jo et al. (2020) |
| 28 | Genovo | OLC assembler | Jo et al. (2020) |
| 29 | BBAP | OLC assembler | Jo et al. (2020) |
| 30 | Map | OLC assembler | Jo et al. (2020) |
| 31 | Omega | OLC assembler | Jo et al. (2020) |
| 32 | SOAPdenovo | De novo assembler | Kim et al. (2013) |
| 33 | Meta-IDBA | de Bruijn assembler | Kim et al. (2013) |
| 34 | ALLPATHS | de Bruijn assembler | Scholz et al. (2012) |
| 35 | ABySS | de Bruijn assembler | Scholz et al. (2012) |
| 36 | MetaGeneAnnotator | Prediction of genes | Di Bella et al. (2013) |
| 37 | MetaGeneMark | Prediction of genes | Di Bella et al. (2013) |
| 38 | Orphelia | Prediction of genes | Di Bella et al. (2013) |
| 39 | Metagenomic gene caller (MGC) | Prediction of genes | Di Bella et al. (2013) |
| 40 | HUMAnN2 | Functional annotation | Jo et al. (2020) |
| 41 | Prokka | Bacterial genome annotation | Roumpeka et al. (2017) |
| 42 | GenScan | Eukaryotic gene annotation | Roumpeka et al. (2017) |

(continued)

**Table 23.2** (continued)

| S. no. | Bioinformatic tool | Application | References |
|---|---|---|---|
| 43 | CAMERA | Gene annotation | Di Bella et al. (2013) |
| 44 | MetaPath | Analyze pathways in metagenomes | Escobar-Zepeda et al. (2015) |
| 45 | ProViDE | Viral diversity estimation | Escobar-Zepeda et al. (2015) |

with the other sequences (Di Bella et al. 2013). OTU picking is assigned to a taxonomic identity if the reference sequence belongs to the cluster. A taxonomy-dependent approach provides a direct taxonomic assignment to each query sequence and helps perform a more standardized community analysis (Kim et al. 2013). There are three approaches for taxonomy assignment, i.e., (1) phylogenetic tree-based methods, which are based on the evolutionary models of reconstruction of targeted molecular markers, (2) similarity search methods, and (3) composition-based methods. Similarity search methods are of two types, i.e., comparison-based methods and homology- or alignment-based methods. The homologies and metagenome sequences have been successfully identified by the Basic Local Alignment Search Tools (BLASTs) and profile Hidden Markov Models (pHMMs). To interpret the BLAST output, there are computational requirements for programs like MG-RAST, which trusts the best hits with low e-values and discards the less significant hits. BLAST is the most widely used tool and the most approved method to match query databases. The lowest common ancestor (LCA) algorithm implemented in a MEtaGenome Analyzer (MEGAN) is the alternative approach. If a minimum assignment dose not match the statistical significance, then LCA helps assign sequences to higher taxonomic levels. It considers all available reference sequences. Moreover, while interpreting the results, missing data in the reference databases and uneven representation of taxa need to be considered. Another integrated environment approach to analyze 16S rRNA amplicon sequences is the Python-based software pipeline Quantitative Insights Into Microbial Ecology (QIIME). This software is publicly available and ready to use once installed (Wang et al. 2013).

The assembly of reads into contigs and providing taxonomic classification can help assemble sequence fragments into larger coherent sequence constructs or contigs or scaffolds, which contain authentic connections between them. Furthermore, functional assignments or read-based reconstruction of the metagenome components are also performed (Scholz et al. 2012). Each of these constructs can be analyzed for the detection of open reading frames (ORFs), which belong to a part of the genome or the genome itself. This poses an important limitation for numerous algorithms such as short read assemblies require a large amount of memory to reconstruct (Ladoukakis et al. 2014). The wide range of genomes also creates complications in the assembly. Furthermore, the widely abundant levels of genomes

in a metagenomic sample result in highly non-uniform coverage, and low-abundance genomes might not assemble at all (Lapidus and Korobeynikov 2021). There are two assembly computational approaches, i.e., mapping reads to a template genome (reference-based) or assembly-free and *de novo* assembly, where the latter falls under one of the two types, i.e., overlap–layout–consensus (OLC) or the de Bruijn graph approach. The assembly-free approach uses a reference genome and provides authentic results as it can avoid issues due to short read lengths, sequence repeats, low coverage, and unbiased estimation of low abundance. The *de novo* assembly approach is the most intensive and complex in terms of computational work as it uses a reference-free strategy for constructing contigs. It performs a method known as overlap–layout–consensus (OLC), which can search for all possible overlaps between the reads (Ladoukakis et al. 2014). In the OLC approach, OLC assemblers are highly accurate but do not work well for short reads. Longer contigs can be obtained by comparing all sequence reads pairwise to find the overlaps where these are combined in the graph. The *de novo* assembly approach relies upon mathematical models like de Bruijn graphs, which work by reading the consecutive k-mers that are defined as the sequences of k-base long where k represents the length of the nucleotides, which in turn, constructs longer contiguous sequences. Due to the use of k-mers, this is more sensitive to sequence errors (Roumpeka et al. 2017). Some of the OLC assemblers are MetAMOS, Genovo, BBAP, Map, and Omega, whereas de Bruijn assemblers are MetaSPAdes, Megahit, Ray Meta, Anvi'O, and IDBA-UD. Ray Meta, MetaVelvet, and Meta-IDBA perform well on short reads like Illumina reads, whereas MAP and Genovo perform well on longer reads like 454 sequencing reads (Jo et al. 2020).

After metagenome assembly, the next step is gene annotation and finding out "what are they doing?" The abundance of information contained in a metagenomic population cannot be used without proper interpretation. The final steps of metagenomics are the functional assignments and pathway reconstructions that help know the potential of uncultivated microbes. It is suggested to use algorithms that consider preferential bias in codon usage, di-codon frequency, start and stop codon patterns, species-specific ribosome-binding site patterns, GC content of the coding sequence, and open reading frame (ORF). Mostly, gene annotation is performed by measuring the homology with respect to the already known genes from public databases. The web-based workflows provided by some portals along with high-performance computers are useful in assessing the annotation task. This takes place in two steps: one is finding genes in the sequence and the second is the prediction of their function. Some of the de novo gene annotation tools are Orphelia, MetaGeneAnnotator, MetaGeneMark, FragGeneScan, Glimmer-MG, and metagenomic gene caller (MGC). MetaGeneMark and Glimmer-MG identify genes using heuristic models and second-order Markov chains (Escobar-Zepeda et al. 2015). MetaGeneAnnotator and Orphelia utilize di-codon usage information and machine learning algorithms and Hidden Markov Models (HMMs). Glimmer-MG identifies insertions/deletions. Longer sequences give more functional annotations of the genes. It is generally based on a homology-based approach and involves a BLAST search against databases such as the Clusters of Orthologous

Groups of Proteins (COGs) system, Kyoto Encyclopedia of Genes and Genomes (KEGG), SEED, the Conserved Domains Database (CDD), eggNOG, and TIGRFAM (Di Bella et al. 2013). COGs determine well-known genes and show less attention toward recently discovered genes. The Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) and the Human Microbiome Project Unified Metabolic Analysis Network (HUMAnN) are other tools useful for metagenomic projects. The HUMAnN2 annotation pipeline is fast and easy to use. It constructs a sample-specific database and nucleotide-level mapping along with realigning the unmapped sequence reads (Jo et al. 2020). The Pfam and TIGRfam databases are used for protein family analysis. Information on putative protein–protein interactions is provided by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) tool along with MG-RAST. PRIAM is used for enzyme detection, which uses the already available ENZYME database and provides KEGG graphs for imaging (Kim et al. 2013). Prokka is used for annotation of bacterial genomes. It uses the already published open-source software tools for the prediction of protein-coding and *tRNA/rRNA* genes. GenScan and GeneMark are used for the analysis of single eukaryotic genomes and for the prediction of exons, introns, and intergenic sequences (Roumpeka et al. 2017). Computational tools are becoming faster and more specialized and are able to investigate high-throughput sequences.

## 23.5 Application of High-Throughput Sequencing Technologies in Algal Research

Algal studies, through the use of molecular data, have increased in recent years. They help study algal systematics (Oliveira et al. 2018), algal cultures (Heck et al. 2016), cyanobacterial blooms (Huang et al. 2017), cyanotoxins (Kurobe et al. 2018), algal cell identification and phylogenetics (Ebenezer et al. 2011), transcriptional expression in microalgae mutants and microalgae cultures (Yao et al. 2017), algal-based wastewater treatment (A-WWT) systems (Cheng et al. 2021), genomes of industrially useful algae (Graham et al. 2015) and novel proteins (Schultz-Johansen et al. 2018), etc. Table 23.3 summarizes the high-throughput sequencing techniques and their applications in algal research. The study of microalgae depends upon culturing and isolation, which become difficult for unculturable and rare species. HTS has helped study these species. Approximately 110,000 operational taxonomic units (OTUs) were found by The Tara Oceans research using the *18S rRNA* gene for marine eukaryotic plankton. Metabarcoding surveys revealed most of the species of unculturable Chlamydomonadaceae in snow. Algal classification has always been challenging, but molecular data have helped set algal species boundaries. Sanger-sequenced single-locus datasets and multi-locus methods are dominated by algal species delimitation studies in sensitivity and accuracy, respectively. RADseq has been used to identify speciation in *Durvillaea*, and HTS of mitochondrial and plastid genomes helped delineate the *Sargassum* (Oliveira et al. 2018). Heck et al. (2016) used Illumina sequencing in order to study uncultured bacteria in the axenic cultures

**Table 23.3** High-throughput technologies and their applications in algal research

| S. no | Algae | Technique used | Application | References |
|---|---|---|---|---|
| 1. | *Fischerella* sp. strain CENA161 | Illumina MiSeq platform | Non-cyanobacterial contaminants in cyanobacterial cultures | Heck et al. (2016) |
| 2. | *Microcystis* and the associated bacteria | Visualization-enhanced binning method | Metabolic pathways of the microbial community and cooperative interaction | Huang et al. (2017) |
| 3. | *Microcystis*, *Aphanizomenon*, *Cylindrospermum*, *Leptolyngbya*, *Moorea*, *Hassallia*, *Nannochloropsis*, *Chlamydomonas*, *Volvox*, *Planktothrix*, and bacteria | Shotgun metagenomic analysis | Study of the community structure | Kurobe et al. (2018) |
| 4. | *Dunaliella tertiolecta* | Illumina MISEQ and HISEQ 4000 | Found 17,845 protein-coding transcripts | Yao et al. (2017) |
| 5. | *Galdieria sulphuraria* | Illumina HiSeq platform | Check wastewater treatment system for antibiotic resistance genes (ARGs) and virulence genes (VGs) | Cheng et al. (2021) |
| 6. | Durvillaea | RADseq | Speciation | Oliveira et al. (2018) |
| 7. | *Ectocarpus* | RADseq | Speciation | Oliveira et al. (2018) |
| 8. | *Nostoc commune* | Metagenomics | Finding >30 types of nifH gene sequences | Graham et al. (2015) |

of *Fischerella* sp. CENA161 akinetes before and after treatment of Extran detergents and sodium hypochlorite. The results showed that the uncultured bacteria were still there but the number of bacteria reduced before and after the treatment. A metagenomic approach was used to study the relationship between *Microcystis*-dominated blooms and the associated bacteria in the Lake Taihu, China. It revealed that heterotrophic bacteria depend upon the microalgae for carbon and energy (Huang et al. 2017). Shotgun metagenomic analysis was used to study cyanobacterial blooms and bacteria in the brackish and freshwater region in the waterways of the San Francisco Estuary (SFE). At the six sampling stations, the most abundant genus with 94.4% of the cyanobacterial population was *Microcystis*. Other cyanotoxin(s)-producing algae were also found, i.e., *Planktothrix*, *Leptolyngbya*,

*Aphanizomenon*, *Moorea*, and *Cylindrospermum*. The toxins produced by *Microcystis* were abundant than other cyanobacterial toxins (Kurobe et al. 2018). RNA-Seq studies of *Dunaliella tertiolecta* were conducted by Illumina MISEQ and HISEQ 4000. *De novo* assembly and mpiBLASTX search were performed and generated 17,845 protein-coding transcripts. This has improved microalgal transcriptomic database and explained triacylglycerol (TAG) production induced as a regulatory response of nitrogen deprivation (ND) for promoting biofuel production (Yao et al. 2017). Antibiotic resistance genes (ARGs) and virulence genes (VGs) in an algal-based wastewater treatment (A-WWT) system were studied. The A-WWT had the thermoacidophilic algal strain *Galdieria sulphuraria*. Using the shotgun metagenomic approach, 111 ARG and 93 VG subtypes were detected with the help of the Illumina HiSeq platform. The results showed that only four subtypes survived, only one new subtype was generated in the algal-based treatment, whereas 24 ARG subtypes were found and 37 new ARG subtypes were generated in the traditional treatment. Only macrolide, aminoglycoside, and beta-lactam resistance lasted in the algal effluents and seven of them were eliminated from the A-WWT system (Cheng et al. 2021). The diversity of algae and macrophyte species was studied in three wastewater stabilization ponds (WSPs) at a wastewater treatment plant in Ontario, Canada. The bioinformatic analysis was performed by a metagenomic analysis pipeline mainly based on the QIIME package. A mixture of green algae and macrophytes was observed in WSP#1, and, in WSP#2, the Chlorophyceae were dominant and macrophytes and milfoil plants dominated WSP#4 (Wallace et al. 2015). From an enrichment culture of brown algae *Fucus vesiculosus* and *Fucus serratus*, three new GH107 fucanases were discovered. Whole-metagenome sequencing was performed using paired-end sequencing on an Illumina HiSeq platform. The three encoded proteins, Fp273, Fp277, and Fp279, were likely to be responsible for the breakdown of sulfated fucan (Schultz-Johansen et al. 2018). High-throughput sequencing studies will enable us to add more algal genomes, which will help in the advancement in the diverse areas of basic and applied studies.

## 23.6 Conclusions

Sequencing technologies have been constantly evolving since the first-generation sequencing that came about half a century ago, producing massive amounts of data with improved efficiencies. The objective of evolving scientific progress is to help researchers get specific, sensitive results faster at a reliable cost. These high-throughput technologies produce millions of reads inexpensively. Furthermore, the development of new computational technologies has enabled to identify and characterize new genes and proteins and microbial communities. A good and careful experimental design is required to effectively explore the complete population. Metagenomics has been significant in algal research by providing insights into community structure. Each technology has its pros and cons when applied to the detection of algae and differs from species to species. NGS is remarkably helpful in

studying algae, but the insufficient database leads to unassigned OTUs. More research and efforts are required in the future to get a comprehensive understanding of the algal diversity and potential novel genes.

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# Metagenomic Approaches for the Discovery of Pollutant-Remediating Enzymes: Recent Trends and Challenges

24

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**Abstract**

Metagenomic studies in diverse environments have generated petabytes of sequencing data, allowing biologists to peer into the uncultivated microbial majority with unprecedented clarity. Such advances have heralded a new era of enzyme discovery where proteins of interest can be directly extracted from metagenomic sequencing data, although this remains a challenging task. Traditionally, metagenomic enzyme discovery, including that involved in xenobiotic degradation, has largely relied on functional insights derived from activity-guided or PCR-based metagenomics. Due to its untargeted and holistic nature, metagenomics allows us to probe the unknown and underexplored microbial diversity, which represents a key resource for novel biocatalyst discovery. Metagenomic shotgun sequencing-based enzyme discovery, additionally, avoids common biases introduced through PCR-based or activity-guided functional genomic methods. In this chapter, we have provided an overview of metagenomics in novel enzyme discovery with discussions on both the experimental and computational aspects of the same. We discuss in detail computational strategies for identifying possible enzyme candidates from shotgun sequencing data and experimental strategies for characterizing candidate enzymes once they have been identified. Finally, we review emerging methods of metagenomic

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enzyme discovery as well as future goals and challenges with an emphasis on metagenomic-based approaches.

**Keywords**

Metagenomics · Xenobiotics · Pollution · Enzyme discovery · Shotgun sequencing · Protein function

## 24.1 Introduction

Increasing industrial, agricultural, and domestic activities coupled with economic globalization have led to large amounts of diverse pollutants being released into the environment. These include diverse pollutants such as pesticides, herbicides, dyes, petroleum hydrocarbons, and plastics, among others. Most pollutants are polymeric aromatic molecules with cyclic or planar units, which contribute to their stability and persistence in the environment. Indeed, the most frequently persistent and recalcitrant pollutants in natural habitats include polycyclic aromatic hydrocarbons (PAHs), steroids, and dyes. Other persistent pollutants, which are not necessarily polymeric aromatic compounds, include long-chain aliphatic hydrocarbons, organocyanides, organophosphates, polyurethanes, and pyrethroid herbicides and pesticides. Their recalcitrance to natural degradation, a direct result of their highly stable structures, contributes to the toxicity of these compounds in the environment and in animals. These compounds are often categorized as persistent organic pollutants (POPs), with some of them being potent carcinogens, mutagens, and/or capable of endocrine disruption (Ballschmite et al. 2002).

Restoration of polluted soil, lacustrine, and marine and groundwater environments can be a major challenge. Different methods to clean up such contaminated habitats include physical and chemical methods such as pollutant adsorption, electrokinetic coagulation, ion exchange, electrochemical treatments, and biological techniques. Most physical and chemical methods for remediation of polluted sites have major drawbacks including the generation of undesirable by-products, economic infeasibility, and high sludge production (Robinson et al. 2001). Biological remediation methods, in contrast, employ bacteria, fungi, algae, or enzymes derived from such microbes for degradation and mineralization of pollutants in the environment into stable, innocuous end products (Cerniglia 1997; Sutherland et al. 2004). Additionally, as bioremediation can attain complete removal of contaminants from the environment, such methods are considered more effective and environment-friendly compared to the more intrusive physical and/or chemical methods (Colleran 1997; Kumar et al. 2018). Bioremediation exploits the metabolic versatility of microorganisms to degrade a wide variety of organic pollutants in the environment as opposed to directly applied physical or chemical methods. Indeed, since most organic pollutants resemble certain naturally occurring compounds, most contaminated habitats would have certain endemic microorganisms that may be capable of metabolizing these pollutants as their primary carbon source.

The microbial degradation of pollutants is mediated by specific microbial enzymes that are the key components of bioremediation strategies. In most cases, such enzymes have a broad specificity for a range of compounds having similar structures, thereby contributing to the metabolic plasticity of microbes in the environment. Upon detection and isolation, directed evolution-based strategies can be employed to improve the stability and catalytic efficiency of such enzymes (Theerachat et al. 2012). Most major advances in recent years in such works have, however, been derived from 'omics'-based techniques, more specifically, next-generation sequencing (NGS)-based methods. Indeed, NGS-based methods have provided several insights into the catabolism of contaminants in the environment by bacteria and fungi through elucidation of relevant genes, operons, and metabolic pathways. This has helped us gain a better understanding of the genetic and molecular determinants involved in microbial degradative processes in the environment and, in turn, has aided in the development of better bioremediation strategies. Most of such knowledge has been gathered from functional genomic studies, in which functional characterization of microbial genomes after genome sequencing and/or activity-based screening of genomic libraries has led to identification of genes directly involved in the degradation of pollutants of interest (Pieper et al. 2004). However, given that a majority of microbial species in environmental microbiomes are not detectable through culture-dependent methods (Bakken 1997; Pham and Kim 2012), it can be reasonably speculated that most microorganisms capable of xenobiotic degradation remain uncharacterized.

Functional metagenomics, which endeavours to assign a function to proteins in all genomes in a microbial community, in contrast to functional genomics that focuses on a single microbial genome, is a highly efficient method to access the uncharacterized microbiome. Since NGS-based functional metagenomic studies involve no isolation or cultivation steps, they represent a high-throughput method for accelerated discovery of novel biocatalysts from the plethora of uncharacterized and uncultivated microbes in the environment. In this chapter, we discuss the various considerations, tools, and strategies for functional metagenomic-based enzyme discovery, including those that involve metagenomic cloning and heterologous expression. Additionally, we discuss emerging techniques, methods, and approaches in functional metagenomics with special emphasis on sequencing-based ones. Finally, we review in brief the upcoming goals and future challenges in the fast-changing discipline of enzyme discovery. We have endeavoured to focus on those aspects of functional metagenomics that represent the frontiers of the discipline and limit discussions of classical approaches, which are elaborated elsewhere in this book. Ultimately, we present a largely generalized approach to metagenomic enzyme discovery that can be applied in most investigations of nature, with discussions specific to pollutant-degrading enzymes wherever appropriate.

## 24.2 Metagenomics and Functional Characterization of Microbiomes

The term 'metagenomics' was first coined by Handelsman et al. (1998), and it involves the study of environmental nucleic acids, specifically DNA. The first direct sequencing-based study of microbial communities in the environment can, however, be attributed to phylogenetic studies carried out by Pace et al. (1985). Metagenomic studies not only include natural environments but also investigation of microbial communities in the human and animal guts (Almeida et al. 2019; Xia et al. 2017), built-up spaces (Danko et al. 2021), and, indeed, the International Space Station (Mora et al. 2019), among others. Although early metagenomic studies followed a low-throughput, cloning-based strategy, the advent of next-generation sequencing (NGS) technologies has transformed metagenomics into a high-throughput, holistic method to investigate microbiomes (Kumar et al. 2020, 2021). Combined with continually decreasing sequencing costs, this has contributed to an exponential growth of sequencing repositories and has propelled metagenomics into becoming a scientific discipline in its own right. Indeed, sequencing repositories are estimated to accommodate approximately $10^{18}$ bp of nucleotide data within the next 5 years (Stevens et al. 2015).

Towards the end of the twentieth century, it became increasingly obvious that culture-dependent techniques were inadequate for recovering the majority of microbial diversity from environmental microbiomes. Most microorganisms in the environment do not survive in isolation and often exhibit a fastidious dependence on specific nutrients or metabolic fluxes to thrive. Indeed, only 1–5% of microbes in the highly studied rhizosphere could be recovered in the laboratory using culture-based methods (Bakken 1997). The inability of culture-based methods in the laboratory to replicate such environmental conditions therefore meant that the majority of genetic information in environmental microbiomes remained elusive. Due to its culture-independent nature, metagenomics, more specifically NGS-based metagenomics, can provide researchers access to the genetic information of a greater proportion of microbiomes, and, therefore, it holds a major advantage over culture-based methods. Indeed, the 'rare biosphere', i.e. microbes occupying specific habitat niches and present in low abundance, can only be accessed through culture-independent, metagenomic techniques. The capability of metagenomic approaches to elucidate the functional characteristics of a largely uncultivated microbial majority has been demonstrated in the identification of metabolically diverse lineages including candidate phyla radiation and DPANN archaea (Lomakina et al. 2018; Nicolas et al. 2020). Other examples include the elaboration of the biosynthetic potential of several uncultivated microbial phyla such as *Eelbacter*, *Candidatus tectomicrobia*, and *Angelobacter*, among others (Wilson et al. 2014; Crits-Christoph et al. 2018). Indeed, the heterologous expression of genes of interest from *Candidatus tectomicrobia* has allowed the characterization of novel biosynthetic products and metabolic pathways (Wilson et al. 2014; Crits-Christoph et al. 2018; Mori et al. 2018). Although promising, the discovery of novel enzymes or products from

metagenomes presents unique challenges that can primarily be attributed to the uncultivable nature of microbes/DNA through metagenomic approaches.

## 24.3 Targeted Metagenomic Sampling

Several microbial communities have been demonstrated to have xenobiotic degradation potential. However, the ability to degrade and mineralize xenobiotic compounds is not exclusive to microbes in contaminated ecosystems. Sufficient taxonomic and functional diversity along with the ability to metabolize compounds similar to pollutants of interest as the primary carbon source can allow microbial communities from non-polluted environments to successfully negotiate contamination events. In recent years, several such natural habitats, which have never or rarely been exposed to pollution events, have been investigated for xenobiotic-degrading enzymes. Indeed, such studies have revealed the presence of genes approximating to nearly 15% of the entire annotated human gut microbiome catalogue to be involved in the degradation and mineralization of xenobiotic compounds (Qin et al. 2010). However, due to the inherently contaminated nature of polluted environments, such ecosystems represent genetic sinks enriched with microbes able to degrade and thrive on xenobiotic compounds. Understandably, most metagenomic studies aimed at xenobiotic-degrading enzyme discovery have been carried out in polluted habitats including contaminated soils and groundwater, activated sludge, and wastewater, among others. Several such studies have employed stable-isotope probing (SIP) to deconvolute the degradation of a pollutant of interest by microbial communities, where the contaminant has been radiolabelled. For example, to understand petroleum hydrocarbon degradation by microbial communities in contaminated groundwater, Wang et al. (2012) supplemented contaminated groundwater microcosms with $^{13}$C-labelled naphthalene. The heavy, radiolabelled DNA was subsequently separated from the unlabelled genetic material, sequenced, and cloned to screen for desirable degradative properties. Such an enrichment-based approach is adequate for investigating the active degraders of primarily simpler xenobiotic compounds. However, more complex pollutants, such as PAHs and complex aliphatics, often require the participation of multiple microbial species to accomplish complete degradation into simple products. Indeed, such approaches may overlook or miss those microbes involved in the degradation of the most recalcitrant pollutant molecules and only detect microbes catalysing the metabolism of simpler products in the final steps of degradation.

## 24.4 Experimental Designs for Metagenomic Enzyme Discovery

In this section, we briefly discuss the various approaches, both in silico and experimental, to metagenomic enzyme discovery. Although our primary emphasis will be on enzyme discovery from shotgun sequencing data, we will briefly outline both

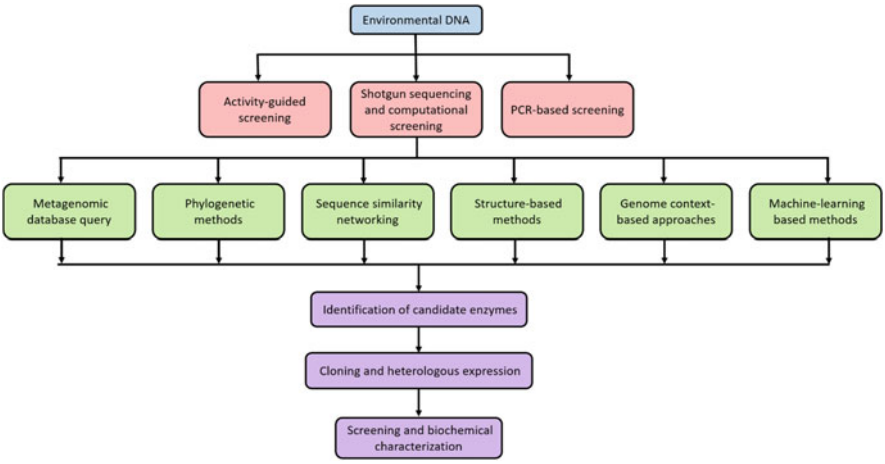


**Fig. 24.1** Flowchart of experimental and computational approaches in metagenomic enzyme discovery

activity-guided and PCR-based functional metagenomic approaches (Fig. 24.1). Comprehensive reviews on the latter are available elsewhere (Katz et al. 2016).

### 24.4.1 Activity-Guided Functional Metagenomics

Functional metagenomic library screening, through identification of desirable activities, was one of the earliest methods developed for metagenomic enzyme discovery. In contrast to other methods, activity-based metagenomic methods are capable of simultaneously screening for novel enzymes as well as obtaining biochemical data. The approach relies on the observation of desirable phenotypes, which, in this case, is the degradation of pollutants of interest by metagenomic clones. There are three main steps in such an approach: (1) cloning of environmental DNA or complementary DNA (cDNA) ranging between 2 and 200 kb into appropriate expression vectors such as fosmids, cosmids, or bacterial artificial chromosomes to create metagenomic or metatranscriptomic clone libraries; (2) heterologous expression of the cloned fragments in an appropriate microbial host; and (3) implementation of appropriately sensitive phenotypic screening strategies to identify 'hits', i.e. clones of interest exhibiting desirable properties. Metagenomic clones identified as 'hits' are then subjected to sequencing to reveal the genes or coding regions in the environmental DNA fragment and to further investigate the protein of interest. Since such functional screening does not depend on any prior information, such as sequence homology with known genes, activity-guided metagenomics is particularly effective in identifying novel protein families, i.e. de novo enzyme discovery, as well as in assigning new functions to previously identified protein families. Indeed, such screening has been widely used to identify

additional properties of known enzymes, especially those considered commercially relevant, such as lipases, amylases, and cellulases, among others (Berini et al. 2017). Activity-based metagenomic studies have been instrumental in providing proof of function for xenobiotic-degrading enzymes as well. The two protein families involved in pollutant degradation and identified through activity-guided screening are oxidoreductases and hydrolases; these have been reviewed in detail elsewhere (Ufarté et al. 2015). Activity-based metagenomic screening, however, suffers from a number of disadvantages. First, many enzymes require specific substrates or cofactors to function and so may evade detection through general screening assays developed for primary metabolic enzymes. Additionally, incompatibility in codon usage, low levels of expression, and differential metabolic requirements, among others, in metagenomic library hosts may lead to detection of a lower number of 'hits'. Activity-based metagenomic studies have been more productive compared to sequence-based ones in providing evidence for enzyme function with respect to xenobiotic-degrading enzymes. The two primary families of enzymes involved in pollutant degradation that have been unravelled through the use of activity-guided screening are oxidoreductases and hydrolases. *Escherichia coli* was the most frequently used host for enzyme validation, although non-conventional hosts such as *Bacillus*, *Thermus*, *Pseudomonas*, and *Sphingomonas*, among others, were also used in a limited capacity (Ufarté et al. 2015). The transformation efficiency of *E. coli* in various plasmid types was, indeed, significantly higher than that of other hosts, with the ability to appropriately express genes from taxonomically distant species as well (Tasse et al. 2010).

### 24.4.2 PCR- and Microarray-Based Functional Metagenomics

Microbiomes can be directly explored for known enzyme families using polymerase chain reaction (PCR)-based methods or through DNA/DNA and DNA/cDNA hybridization methods. This can be achieved through the design of degenerate primers or probes from conserved sequences in genes of interest. PCR-based screening strategies are highly specific and sensitive as such characteristics are inherent to PCR itself, with upscaling and throughput possible through pooling and other deconvolution methods (Ferrer et al. 2016). PCR-based screening for pollutant-degrading enzymes is fairly common, and numerous examples of the same are available. For instance, ammonia monooxygenases, which are the key enzymes involved in denitrification, were amplified and identified in soil, marine, and laboratory microcosm samples contaminated with nitrates (Nogales et al. 2002; Treusch et al. 2005) using PCR-based methods. Similarly, PCR-based screening techniques were also employed to understand the diversity and abundance of 2,4-D/α-ketoglutarate and $Fe^{2+}$-dependent dioxygenases that are involved in the degradation of phenoxyalkanoic acid herbicides (Zaprasis et al. 2010). Interestingly, in most PCR-based screening studies, the emphasis is on understanding the genetic diversity of the proteins of interest and the taxa contributing to the same in a given habitat. Consequently, reports of identification of full-length gene sequences and

subsequent characterization of the enzyme products aimed at the capture of novel catalysts are rare. One such example is the identification and validation of polychlorinated biphenyl-degrading enzymes by Sul et al. (2009). In this study, microcosms of river sediments contaminated with polychlorinated biphenyls were amended with $^{13}$C-biphenyl and the labelled DNA was later extracted and used to construct a cosmid library with 30–40 kb inserts. These inserts were subsequently screened using primers designed against aromatic dioxygenases, leading to the identification of a biphenyl dioxygenase-encoding gene, which was subcloned, expressed, and validated for its biphenyl degradation activity. Another such example is the identification of novel bacterial laccases through PCR-based screening of a marine metagenomic library and subsequent experimental validation of their activity against azo dyes, catechol, syringaldazine, and 2,6-dimethoxyphenol, among others, by Fang et al. (2012). Probe-based metagenomic enzyme discoveries involve high-density arrays designed to detect sequences specific to certain genes of interest and allow investigators to gain a detailed understanding of the diversity and abundance of such sequences in the habitat under study (Eyers et al. 2004). For instance, GeoChip 4.0, which accommodates 83,992 50-mer oligonucleotide probes targeting 152,414 genes, was used by Lu et al. to elucidate the differences in hydrocarbon degradation processes when comparing deep-sea water samples and non-contaminated ones (Lu et al. 2012). This microarray-based method successfully highlighted the functional differences between the two microbial communities as well as quantified the hydrocarbonoclastic genes involved in aerobic degradation of petroleum hydrocarbons.

In the last few decades, metagenomic investigations have established that the actual enzyme diversity in the environment is much higher than that predicted from functional genomic data. To access the enzyme diversity unavailable using conventional primers, certain innovative strategies have been designed in recent years. For instance, Iwai et al. developed a hybrid approach in which PCR-based screening is combined with next-generation sequencing of generated amplicons to iteratively construct new primers from other conserved regions in the gene of interest (Iwai et al. 2010). Such an approach allows the detection of structurally diverse and divergent proteins belonging to the same functional group. Indeed, such approaches have allowed the identification of thousands of full-length dioxygenases from novel, unknown clusters of sequences recovered from metagenomes of polychlorinated biphenyls and 3-chlorobenzoate-contaminated soils (Iwai et al. 2010; Morimoto and Fujii 2009). In a latter study by Morimoto and Fujii (2009), genes that became dominant after addition of pollutants of interest were identified through PCR denaturing gradient gel electrophoresis (DGGE)-based methods. These dominant DNA sequences were subsequently used as anchor fragments to facilitate PCR metagenome walking that led to the retrieval of full-length genes as well as to the elucidation of upstream and downstream genes/sequences.

The major drawback of PCR-based screening approaches is, understandably, their dependence on sequence homology, i.e. prior information on the gene or protein family of interest. This severely limits the usefulness of PCR-based methods in probing the unknown enzyme diversity. Additionally, being based on PCR, such

methods have inherent amplification biases against guanine-cytosine (GC)-rich sequences and the less abundant members of microbiomes. Furthermore, short amplicons from genes do not provide reliable and/or complete information about the taxonomy of the source organisms or the co-occurrence information regarding other neighbouring genes. Although this can be somewhat overcome through PCR metagenome walking as mentioned above, the process is tedious and low-throughput. CONKAT-Seq, an innovative method recently developed by Libis et al. (2019), has used a co-occurrence network analysis-based approach to identify rare gene clusters. It employs position-barcoded domain amplification in an array of highly partitioned metagenome libraries, following which co-occurrence networks are computed for various protein domains and statistical analysis is carried out to elucidate highly linked but rare gene clusters. Amplicon sequencing remains a favoured choice among researchers due to its low cost, but, with decreasing sequencing prices, it is anticipated to be superseded by metagenomic shotgun sequencing in functional metagenomic studies aimed at enzyme discovery.

### 24.4.3 Metagenomic Shotgun Sequencing-Based Approaches

As mentioned above, metagenomic shotgun sequencing refers to the direct, untargeted sequencing of environmental DNA. Methods for library preparation in shotgun sequencing and bioinformatic analysis have been discussed elsewhere (Ayling et al. 2019; Almeida and De Martinis 2019). Metagenomic shotgun sequencing-based enzymatic discovery has several advantages over traditional functional metagenomic-based approaches. For example, shotgun sequencing-guided methods typically introduce less bias compared to functional metagenomics as PCR amplification and microbial hosts such as *E. coli* are not required. Additionally, shotgun sequencing is a high-throughput, highly scalable method that is less labour-intensive compared to fosmid/cosmid library-based functional metagenomics that takes much longer to produce sequencing/gene-level data. Furthermore, since sequencing data are now typically deposited in public repositories, analysis of these metagenomes can be carried out by different research groups around the world to provide further insights into genes and gene clusters of interest, their regulation, and other relevant aspects; this could never be possible with large-insert, library-based functional metagenomics. However, while shotgun sequencing is capable of providing information at an accelerated rate compared to functional metagenomics, downstream cloning and heterologous expression of the genes of interest are still necessary for biochemical validation of enzymes identified through both methods.

One of the major challenges in shotgun sequencing-guided enzyme discovery is the retrieval of environmental DNA of sufficient quality and quantity from complex habitats/microbiomes and the achievement of adequate sequencing depth that may allow error correction. This can be partly resolved through targeted enrichment methods in sequencing, which are particularly effective in extracting longer reads from specific taxa or gene clusters of interest from low amounts of environmental

DNA. For instance, recent advances made by Samplix Technologies (Kvist et al. 2014) have relied on microdroplet multiple displacement amplification of unknown sequences, flanking short, desired sequences to enable an indirect capture and sequence enrichment of the former. Other disadvantages of shotgun sequencing-based approaches include computational costs attached to such projects, limitations of bioinformatic approaches, and the inherent biases and inaccuracies of metagenomic assembly, annotation, and binning. Shotgun sequencing projects frequently employ the Illumina sequencing platform, which produces short reads that can be an impediment to the recovery of full-length genes/gene clusters, even with the significant advances made in metagenomic assemblies in recent years. Short-read sequencing can, however, be complemented with other sequencing methods to improve metagenomic assemblies. For instance, techniques such as Hi-C chromosome capture for a proximity-guided assembly of short reads have successfully demonstrated improvements in the genome-level resolution of the human gut and cow rumen microbiomes (Yaffe and Relman 2020; Stewart et al. 2018). Additionally, long-read sequencing technologies such as Oxford Nanopore (Jain et al. 2016) or PacBio HiFi (Hon et al. 2020) can be combined with short-read sequencing to dramatically improve metagenomic assemblies. This is particularly important for the retrieval of full-length genes/operons of microbial enzymes involved in secondary metabolism, which are often clustered together in microbial genomes.

In recent years, several studies have investigated heavily polluted sites using metagenomic methods to understand microbial community structures, functional characteristics, species interactions, and genetic determinants necessary for survival in such habitats. However, discovery of enzymes from direct shotgun sequencing as compared to functional metagenomic approaches remains rare. Indeed, a recent estimate has reported that only seven studies have identified novel enzymes using shotgun sequencing methods compared to more than 300 such instances using functional metagenomic methods (Berini et al. 2017). A metagenomic analysis of activated biomass from an effluent plant carried out by Jadeja et al. has allowed the identification and retrieval of novel oxygenase sequences (Jadeja et al. 2014). Additionally, samples of the activated biomass were demonstrated to degrade a range of aromatic hydrocarbons, but the degradation activity was not attributed to a specific taxon or enzymatic component. A study by Wang et al. (2012) discovered a key operon involved in naphthalene degradation by combining stable-isotope probing and shotgun sequencing. Shotgun sequencing of $^{13}$C-labelled DNA was followed by cloning of the identified operon and experimental validation of naphthalene degradation. This remains one of the few examples in which massively parallel sequencing has been successfully employed to identify and characterize new pollutant-degrading enzymes. With increasing focus on shotgun sequencing studies, a considerable reservoir of metagenomes and annotated genes is now available, which can be analysed in silico for identification of novel catalysts in bioremediation. Indeed, 1200 bacterial laccases have been identified in genomic and metagenomic datasets, which remain to be experimentally validated (Ausec et al. 2011).

## 24.5 Computational Methods for Metagenomic Enzyme Discovery

### 24.5.1 Searching Metagenomic Databases

Due to the petabytes of sequencing data being deposited in public repositories, a wealth of information is available for researchers to mine and identify novel enzymes and predict new enzyme functions in protein families, including those involved in xenobiotic degradation. After sampling, DNA extraction, and sequencing, shotgun sequencing-based metagenomic studies deposit their data in public repositories such as the Sequence Read Archive (SRA) (Leinonen et al. 2011), Joint Genome Institute Integrated Microbial Genomes and Microbiomes Resource (JGI IMG/M) (Chen et al. 2019), or MGnify (Mitchell et al. 2020), among others. MGnify is unique compared to other metagenome resources as it allows researchers to probe metagenomes using Hidden Markov Models (HMMs) compared to more commonly implemented sequence alignment-based methods such as BLAST (McGinnis and Madden 2004) or DIAMOND (Buchfink et al. 2015). This is an important distinction as compared to alignment-based methods, as HMM-guided methods are more effective in identification of remote homologues and, as an extension, novel enzymes. Sets of aligned sequences are used to build probabilistic models, i.e. HMM profiles, which can sensitively detect distantly related enzymes at the boundaries of protein families. Custom HMM profiles can be built for defined clades of closely related sequences to investigate metagenomes for protein subfamilies as desired by the user. A complementary approach to HMM-based searches involves the reconstruction of metagenome-assembled genomes (MAGs) from metagenomic shotgun sequencing data. Assembly and binning of metagenomic contigs into MAGs allow researchers to extract full-length genes at a genome-level resolution, which, in turn, enables recovery of full-length genes/operons involved in secondary metabolism from the unknown microbial biodiversity. Indeed, a recent study on ~10,000 diverse environmental metagenomes by Nayfach et al. (2021) using MAG-based approaches has led to the identification of >100,000 biosynthetic gene clusters, thereby underlining both the challenges and opportunities in metagenomic enzyme discovery from metagenomic shotgun sequencing data. Given the exponential rise in shotgun sequencing data being generated, there is a distinct need for improved tools and platforms, HMM-based, MAG-based, or otherwise, to facilitate and accelerate metagenomic novel enzyme discovery.

### 24.5.2 Phylogenetic Methods

Phylogenetics, which aims to understand the links between shared functional traits including proteins with similar functions, can be a particularly useful tool for enzyme discovery. Most enzymes tend to group on the basis of their shared preference of substrates or similar functionality and not their taxonomic origin, unlike other genes such as the *16S rRNA*. This makes phylogenetics a powerful tool for

reference-based enzyme discovery, where sequences of genes of interest can be aligned with uncharacterized, metagenomic sequences to identify distantly related enzymes. Reference sequences of characterized enzymes of interest can be obtained from Swiss-Prot (Bairoch and Apweiler 1996), the Protein Data Bank (Berman et al. 2000), or through literature search. In such an approach, reference sequences act as anchors or seeds around which the phylogenetic tree is expanded, with distantly related, uncharacterized sequences forming distinct clades with no seed sequences. This provides easy, visual inspection of distantly related, possibly novel sequences and is often a good place to start investigations for enzymes preferring different substrates or with novel functionalities. A key drawback of phylogenetic trees is that their accuracy is dependent on the quality of the multiple sequence alignments that they are built on. Although there are several multiple sequence alignment tools available, such as MAFFT (Katoh et al. 2002), MUSCLE (Edgar 2004), and Clustal Omega (Sievers and Higgins 2018), manual inspection of the alignment to trim regions outside the core alignment as well as large gaps is essential to ensure high-quality trees downstream. Tools such as Gblocks (Castresana 2000) and trimAl (Capella-Gutiérrez et al. 2009) are well-suited to carry out such intermediate steps before treeing. An additional limitation of phylogenetic methods is the computational cost of creating trees from large multiple sequence alignments. While rigorous maximum-likelihood-based treeing tools such as RaxML (Stamatakis 2014) or PhyML (Guindon et al. 2005) make few assumptions and require significant investment in time and resources, newer algorithms such as FastTree (Price et al. 2010) and RaxML-NG (Kozlov et al. 2019) are faster, less resource-hungry, and nearly as accurate. FastTree employs heuristic methods to shrink the tree search space and make approximations of maximum-likelihood probabilities, thus, in turn, achieving drastically reduced treeing times. RaxML-NG, in contrast, allows computational scalability for large metagenomic datasets while retaining the improved accuracy of RaxML. IQ-Tree (Nguyen et al. 2015) is yet another popular treeing tool that includes automated model selection as well as ultrafast bootstrapping. The ETE3 toolkit (Huerta-Cepas et al. 2016) or ggtree (Yu et al. 2018) implemented in R can be used for advanced annotation and visualization of phylogenetic trees.

Ancestral state reconstruction (Hochberg and Thornton 2017) is a related method that analyses contemporary protein sequences to infer their evolutionary history and may help identify evolutionarily distant relatives of reference genes of interest. FastML (Ashkenazy et al. 2012) is a web-based tool that allows ancestral state reconstruction to be explored by non-experts. Indeed, ancestral state reconstruction of diterpene cyclases by Hendrikse et al. and subsequent experimental validation of the predicted sequences revealed that ancestral forms of the enzymes had improved thermostability and broader substrate specificity (Hendrikse et al. 2018). Tools like EvoMining (Sélem-Mojica et al. 2019) and CORASON (Navarro-Muñoz et al. 2020) can be implemented in phylogeny-based genome mining to identify new enzymes. CORASON, or CORe Analysis of Syntenic Orthologs to prioritize Natural Product Biosynthetic Gene Clusters, takes a query gene and genome database to identify gene clusters that share the same genomic core and reconstructs multi-locus phylogenies to explore evolutionary relationships. Although originally created to

explore biosynthetic gene clusters, the tool can be used for any gene cluster. EvoMining is based on the assumption that primary metabolic enzymes often undergo duplication or horizontal gene transfer and that both events can lead to the rise of secondary metabolic enzymes with novel functions. It has been recently implemented in the discovery of novel enzymes involved in the biosynthesis of arseno-organic molecules (Cruz-Morales et al. 2016). Overall, phylogenetics is a key first step in enzyme bioprospecting from metagenomes and can be highly effective when used in combination with some of the other relevant methods described in this section.

### 24.5.3 Genome Context-Based Approaches

Gene neighbourhoods, i.e. genes flanking the gene of interest, can often provide important information regarding the substrates, cofactors, or partner proteins required for enzyme activity. Indeed, gene context is a critical but frequently overlooked aspect of metagenomic enzyme discovery. Gene neighbourhood analysis can be automated using the Genome Neighbourhood Tool (EFI-GNT) in addition to the Enzyme Function Initiative Enzyme Similarity Tool (EFI-EST) (Gerlt et al. 2015; Zallot et al. 2019). The EFI-GNT provides statistical analysis on gene co-occurrence to facilitate identification of possible functional linkages as well as generates genome neighbourhood networks that allow visual inspection of the genome context. Other tools such as BiG-SLICE (Kautsar et al. 2021) and BiG-SCAPE (Navarro-Muñoz et al. 2020) are designed to identify and group biosynthetic gene clusters. The latter is integrated with CORASON, thereby allowing simultaneous analysis using neighbourhood clustering as well as phylogenetics. BiG-SLICE dramatically reduces computational resource requirement and run time by clustering gene clusters in Euclidean space rather than by pairwise comparison. Indeed, BiG-SLICE is well-suited for genome context-guided metagenomic enzyme discovery due to its ability to cluster millions of gene clusters from metagenome-assembled genomes within a reasonable run time. The STRING web resource is another platform that is helpful in the identification of gene functions through investigations into the gene context (Szklarczyk et al. 2019). STRING employs text mining of the scientific literature and gene associations inferred from diverse data, including co-expression data and gene orthology, to model organisms to facilitate functional analysis of proteins including prediction of protein–protein interactions. Although STRING is not specifically designed for metagenomic investigations, it can nevertheless be used to confidently infer protein–protein interactions. CO-ED is a more specific tool, which can implement network analysis to identify co-occurring domains in multi-domain proteins (De Rond et al. 2020). CO-ED relies on PFAM information as input, which can be extracted from metagenomes using PfamScan (Madeira et al. 2019). Upon completion of analysis, CO-ED highlights the domains in the input data that are already present in public repositories (e.g. UniPROT (The UniProt 2019), MIBiG (Kautsar et al. 2020)) and other domains that have not yet been characterized. CO-ED, therefore, provides

insights into unusual domains or combination of protein domains (in multi-domain proteins) in metagenomes and, in turn, facilitates identification of possible points of initiation for enzyme discovery investigations.

### 24.5.4 Sequence Similarity Networking

Sequence similarity networks (SSNs) are graphs that display the relationships between protein families and were first published in 2009 for the purpose of investigations into protein superfamilies (Atkinson et al. 2009). SSNs use scores from an all-against-all BLAST search for a user-defined custom dataset to generate network graphs in which each protein sequence is represented by a node and pairwise sequence similarities define the edges between the nodes. Smaller clusters of protein families of interest can be revealed by pruning the network graph through manipulation of the sequence similarity threshold. Clusters of metagenomic sequences of interest can therefore be analysed relatively quickly to identify protein subfamilies that may yield novel biocatalysts. As mentioned above for phylogenetics, it is a useful and, indeed, common practice to include representative sequences as seed or anchor points, which, in turn, helps draw robust inferences for relationships between protein families and subfamilies. A major advantage of SSNs is their ability to visually inspect thousands of sequences and interpret the relationship between proteins. Additionally, SSNs can be computed much faster than bootstrapped maximum-likelihood phylogenetic trees and are computationally less intensive as well. Visual inspection of network graphs can be carried out interactively using network visualization tools such as Cytoscape (Shannon et al. 2003). However, while the graphical user interface in Cytoscape makes network inspection more accessible to researchers, it may be difficult to reproduce such graphs/networks due to the point-and-click system. Python, R, or C/C++ can be used to create reproducible SSN workflows for CyREST API or igraph (Csardi and Nepusz 2006; Ono et al. 2015); users without coding or programming experience can alternatively use the EFI-EST platform for automated sequence similarity network construction online (Gerlt et al. 2015). A key drawback of SSN analysis is the bias introduced during pruning of the network graph using sequence similarity threshold scores. BLAST e-values form an important parameter in such decisions, which can often be misleading since BLAST e-values are derivatives of database size that in turn make comparisons between SSNs infeasible as the size of the sequence databases may vary. Furthermore, a variety of graph layouts may lead to different interpretations of the same data. As a compromise, sequences, codes, network layouts, similarity scores, and BLAST e-values can be made publicly available, which will allow third-party examination of SSN workflows and limit bias introduced through preferences of the involved researchers.

### 24.5.5 Structure-Based Methods

Traditionally, the lack of structural information for most proteins has been a roadblock for inferring enzyme functions from metagenomes. Although in recent years, efforts have been made to characterize more proteins at the structural level, these have disproportionately focused on culturable organisms. Indeed, only a small proportion of PDB entries originate from metagenomes (Robinson et al. 2001). Surprisingly, large-scale efforts to structurally characterize proteins, as mentioned above, have yielded much fewer novel protein folds than expected (Jaroszewski et al. 2009). While it is expected that the repertoire of protein folds will be expanded as we further interrogate the unknown microbial majority, it must be noted that given the enormous possibilities of amino acid combinations, only a small fraction of protein conformations has been represented in biological macromolecules studied to date. Observations that even the most conserved protein folds can catalyse a variety of reactions underline our nascent understanding of the effects of the multidimensional enzyme structure on catalytic diversity. Nevertheless, protein structures can provide important insights into functionality beyond the primary amino acid sequence. Powerful structural prediction tools such as AlphaFold2 (Jumper et al. 2021) can significantly advance our understanding of uncharacterized proteins. However, AlphaFold2 is currently not available publicly, and web-based homology modelling tools such as I-TASSER (Roy et al. 2010), Phyre2 (Kelley et al. 2015), and SWISS-MODEL (Schwede et al. 2003) can be alternatively used to predict the protein structure. Importantly, structural prediction is often the first step towards detection of active sites as well structurally vital motifs, both of which play critical roles in determining enzymatic/protein function.

Enzyme-active sites represent a minute proportion of the full-length, folded protein volume in three-dimensional space. Catalytic residues in enzyme-active sites as well as the architecture of the site itself are highly conserved compared to the rest of the enzyme. However, the same catalytic residues can accommodate a wide range of substrates as well as catalyse a diversity of reactions, most famously in case of the Ser-His-Asp catalytic triad (Rauwerdink and Kazlauskas 2015), thereby indicating the multifunctional capabilities of even highly conserved residues in the enzyme-active site. The prediction of enzyme function from examination of the active site alone can therefore be challenging, if not impossible. An alteration of a single residue in the active site can drastically change the substrate specificity or enantioselectivity of an enzyme; protein engineering for enzyme discovery can therefore lead to several dead ends (May et al. 2000). In this respect, identification of naturally occurring active-site variants across genomes/metagenomes can provide an alternative and complementary route in enzyme discovery besides protein engineering. Studies on active-site variants targeted at enzyme discovery from metagenomic shotgun sequencing are rare, with unusual active sites only reported upon enzyme characterization. Tools such as CASTp are useful for the de novo, automated detection of active sites/active-site variants (Tian et al. 2018). Databases such as the Mechanism and Catalytic Site Atlas (M-CSA), which catalogues active-site architecture and chemistry, can also be useful in this regard (Ribeiro et al. 2018).

Although M-CSA currently contains more than 1000 curated entries representing approximately 73k Swiss-Prot entries and 15k PDB entries, it still represents less than 10% of proteins with solved structures (Robinson et al. 2001). Additionally, predicted active-site information is also available in UniProt, which can be useful in identifying variant active sites in structurally similar metagenomic sequences. Other conserved motifs beyond active sites, such as cofactor binding sites that can be detected using tools such as ScanProSite (De Castro et al. 2006), are also generally conserved and can be useful in enzyme discovery. Overall, strategies such as motif searching combined with sequence homology modelling or de novo identification of conserved/variant structural features and cofactor binding sites, can be particularly useful in structure-based enzyme discovery from metagenomic sequences.

### 24.5.6 Machine Learning-Based Methods

Machine learning (ML) has made great strides in the past few decades with the continuous advancements in computing power and algorithms, particularly in the life sciences and chemistry. Indeed, more than 35 machine learning-based methods for protein prediction have been published in the past decade; these approaches provide researchers with the opportunity to apply methods beyond homology modelling to understand unknown links between protein sequence, structure, and function. One of the key drawbacks of machine learning-based methods is their requirement for large amounts data to train. The quality and quantity of data directly influence the accuracy and sensitivity of the resulting machine learning model, with more complex models requiring greater amounts of training data. Machine learning models, particularly deep neural networks, frequently suffer from overfitting, where they become highly specific for a given dataset and cannot be implemented in a generalized manner for similar studies or datasets. Availability of high-quality, curated data in public databases such as MiBIG (Kautsar et al. 2020) and Swiss-Prot (Bairoch and Apweiler 1996) is therefore essential for adequate training of machine learning algorithms and for enabling enzyme discovery. Transfer learning is an emerging method that is designed to handle the scarcity of experimentally verified data, where machine learning models are pre-trained on large quantities of unlabelled data such as metagenomic sequences in order to learn the general characteristics of the data and, in turn, to improve performance on separate, related tasks such as protein function prediction. Further advances in transfer learning methods as well as semi-supervised machine learning will allow researchers to investigate large metagenomic datasets with machine learning models trained on relatively smaller amounts of labelled data.

Machine learning-based methods can incorporate a wide variety of features to produce better models. These include protein sequences, structural information, protein–protein interaction data, and physicochemical properties for amino acids, among others (Robinson et al. 2001). Certain ML methods have also employed text mining to extract feature information available in text format, particularly in journal articles (You et al. 2018). Autoencoders, which are artificial neural networks that can

automate feature extraction, thereby removing human biases, have been recently employed in unsupervised encoding of protein features. Although such automation is attractive, autoencoders require even larger datasets and longer compute times, which, in turn, may limit their usability. A diverse array of machine learning models including simple logistic regression, random forest, and multi-layer neural networks, among others, have found use in protein function prediction (Bonetta and Valentino 2020). However, benchmarking on different machine learning models can be difficult due to the inconsistent classification systems used for prediction of protein functionality. Most machine learning models use hierarchically structured protein classification systems such as enzyme classification (EC) (Webb et al. 1992), gene ontology (GO) (Ashburner et al. 2000), or functional catalogue (Ruepp et al. 2004), among others, as objectives. Understandably, this creates difficulties in comparing various protein function prediction models, but recent initiatives such as the Critical Assessment of Functional Annotation Challenge have made important efforts to standardize the diverse approaches undertaken (Zhou et al. 2019). Importantly, deep neural networks have been outperformed by simple homology modelling and logistic regression models in certain instances of protein function prediction and underlines the nascent and developing nature of ML in this field (Bonetta and Valentino 2020). A primary drawback of the currently available ML methods is their limitation of predicting truly novel protein functions. Although ML models can be trained on a variety of objectives (i.e. GO or EC), they are unable to predict entirely new enzyme classes. Negative selection algorithms can be employed as an alternative to multi-objective training, where enzymes can be classified based on their ability to perform a particular function (Youngs et al. 2014). In this method, proteins are not forced into previously defined enzyme classes but can be predicted to not fit into any known classes or fit into multiple classes, indicating potential substrate promiscuity (Copley 2003).

## 24.6 Characterization of New Enzymes

### 24.6.1 Primary Selection and Cloning

Until this point in this chapter, we have discussed various computational methods for prediction of enzyme function and identification of potential candidate enzymes. While such in silico techniques might be useful for prioritization of newer areas of the protein space to be explored, they remain predictions at best and require functional validation through rigorous experimentation. In the following sections, we will review and discuss some of the newer methods and approaches to successfully navigate functional validation of novel enzymes, primarily selection, cloning, expression, and screening. We will not discuss the classical methods for heterologous expression and screening here; they have been reviewed in detail previously (Katz et al. 2016). An important initial step when selecting proteins for characterization in the lab is to ensure removal of truncated or chimeric sequences that in turn may give rise to dysfunctional proteins. Additional clues for mispredicted start or

stop codons may be obtained from visual inspection of multiple sequence alignments, in which erroneous sequences will show up as outliers in terms of sequence length. Several tools are currently available to facilitate the selection of candidate sequences from hundreds of thousands of metagenomic sequences for enzyme discovery. Clustering tools such as CD-HIT, UCLUST, and Linclust (available through the MMSeq2 software suite) (Li and Godzik 2006; Edgar 2010; Steinegger and Söding 2017) can cluster metagenomic sequences by similarity to produce automatically selected representative sequences. Representative sequences for protein subfamilies of interest can be subsequently selected through SSN analysis and visualization in Cytoscape (Shannon et al. 2003) or igraph (Csardi and Nepusz 2006). The strategy here, irrespective of the clustering and selection pipeline followed, is that highly similar proteins will almost always perform similar functions. Although exceptions to this rule have been reported (North et al. 2020), clustering remains a useful tool in the initial stages for selection of representative candidate sequences from metagenomes.

Further filtering of representative sequences may be necessary depending on the dataset size. In many cases, the absence of high-throughput enzyme activity assays can preclude further investigation of several metagenomic sequences. Most frequently, however, metagenomic sequences found in culturable organisms are readily selected since functional characterization in this case would be possible in the native host. Yet another popular strategy is to select proteins from thermophilic organisms that tend to have increased thermostability. It must be noted that this is also a generalization, since proteomes of diverse organisms across the tree of life have exhibited highly variable melting temperatures, even for organisms adapted to extreme temperatures (Jarzab et al. 2020). Alternatively, proteins with higher GC content, transmembrane regions, or extended disordered regions may be filtered out to obtain candidate proteins that are more likely to be stable. Various tools such as XANNPred (Overton et al. 2011), CrystalP2 (Kurgan et al. 2009), XtalPred (Slabinski et al. 2007), and ParCrys (Overton et al. 2008), among others, have been developed to predict crystallization proclivities and can be used to predict protein stability. Multiple tools can be used to assess protein stability and results that are aggregated and ranked in order to prioritize representative sequences for experimental validation. Such an approach ensures that biases and weaknesses in individual tools are offset through an ensemble-based analytical approach and, in turn, enables robust identification of the most promising candidates for enzyme discovery. Removal of signal peptides, i.e. 16–30 amino acids at the end of the N-terminus of many prokaryotic and eukaryotic proteins, is an approach that increases the likelihood of obtaining soluble proteins. Signal proteins are short sequences that direct the export of proteins from the cytosol and can therefore influence the solubility of proteins during heterologous expression experiments. SignalP (Teufel et al. 2022) has been the most commonly used tool for detection and removal of N-terminal signal peptides in recent years, but more advanced, machine learning-based tools for signal peptide detection are now available (Wu et al. 2021). With a better understanding of the links between signal peptides and protein function, these can be used as attributes to filter representative sequences in enzyme discovery.

### 24.6.2 Heterologous Expression

Once genes or enzymes of interest have been identified, constructs for heterologous expression of the same must be created. Most vectors used in the construction of metagenomic libraries in functional metagenomics, however, are typically ill-suited for heterologous expression. Additionally, certain secondary metabolism gene clusters, which can be quite extensive in length, may be captured in a truncated form in fosmid or cosmid vectors that have maximum insert sizes of approximately 4.5 kb. New methods, besides classical restriction-based cloning and Gibson assembly methods, have been developed to facilitate efficient cloning of genes/gene clusters of interest in heterologous hosts (Zhang et al. 2019; Bordat et al. 2015). Among these new techniques, transformation-associated cloning relies on natural homologous recombination in yeasts to aggregate the overlapping cosmid/fosmid clones, whereas genetic recombineering employs diverse bacteriophage proteins to enable homologous recombination in *E. coli* (Zhang et al. 1998). The latter also allows rapid and efficient cloning of large gene clusters using RecET direct cloning in combination with Redαβ recombination (Wang et al. 2016). The availability of sufficient genetic material for cloning can be an issue in heterologous expression, and PCR amplification remains one of the most reliable methods to produce the same if the original environmental sample or the culturable organism is available. In the event of the absence of the genetic material for amplification, gene synthesis can be carried out for the enzyme/gene of interest. Advantages of gene synthesis include codon optimization for the heterologous host of choice, which can be particularly useful for the expression of metagenomic sequences in non-conventional, distantly related hosts. Heterologous expressions of metagenomic sequences often fail even when constructs are properly prepared. One of the reasons behind this may be that some enzymes exhibit activity only when co-expressed with surrounding genes, i.e. the entire gene cluster. Additionally, other factors such as suitable coenzymes, cofactors, or post-translational modification machineries may be absent in the host, leading to a failure of heterologous expression or production of dysfunctional proteins.

Non-conventional hosts can be used as an alternative to conventional hosts such as *E. coli* for heterologous expression when the latter fails. To do this, homologous sequences for metagenomic sequences of interest can be mined from genomes and such sequences, in turn, can be cloned and expressed in *E. coli* or other conventional hosts. In this manner, researchers can obtain information on the sequence/gene/domains of interest by studying similar proteins from related organisms that can be expressed in conventional hosts. Examples include the investigation of FR900359, a Gq protein inhibitor identified from a leaf metagenome but later characterized using a homologous protein from *Chromobacterium vaccinii* expressed in *E. coli* (Hermes et al. 2021). Yet another strategy for heterologous expression can be the selection of the closest culturable taxonomic relatives if genetic engineering tools are available for the same. Indeed, such an approach has been frequently implemented in the expressions of various biosynthetic gene clusters from *Streptomyces* spp. in the model host *Streptomyces coelicolor* A3(2) (Zhang et al. 2016). Protein expression

in *E. coli* for sequences from evolutionarily distant organisms, however, can be successful in certain cases. Overall, the choice of a host for heterologous expression of metagenomic sequences of interest is still based on a trial-and-error process. Design-build-test-learn pipelines in synthetic biology may be able to produce optimized hosts for heterologous expression in the future, thereby reducing the trial-and-error process (Carbonell et al. 2018).

### 24.6.3 Enzyme Activity Screening

The final challenge in the characterization of enzymes is carrying out assays, in vitro or in vivo, to assess enzyme activity. Generally, a compromise is required at this stage between throughput and specificity for enzyme screening methods. Typically, activity screening assays involve visual confirmation for zones of inhibition around cultured bacterial colonies or production of colour or fluorescence due to enzymatic action on chromophores or fluorophores, respectively. However, suitable substrate analogues may not be available for numerous, especially novel, uncharacterized enzymes; the presence of large chromophores or fluorophores may also interfere with the activity of candidate enzymes. Mass spectrometry (MS) can be used as an alternative here as it is sensitive and allows generalized application without the need for substrate analogues. Although this allows accurate monitoring of enzyme activity, MS suffers from low throughput due to the requirement for chromatographic separation. Alternative workflows are now available that bypass the chromatographic step to allow MS-based enzyme activity screening with higher throughput. For instance, nanostructure-initiator mass spectrometry (NIMS) employs a nanostructured surface to trap compounds that can be subsequently released by laser irradiation for MS (Northen et al. 2008). A washing step promotes non-covalent fluorous interactions on the said surface that, in turn, enables compound separation. An improvement on NIMS, PECAN (probing enzymes with click-assisted NIMS), relies on click chemistry, thereby allowing screening of enzymes, which are not able to accommodate bulky perfluoroalkylated tails in their active site. As it uses click chemistry, substrate analogues with 'clickable' alkynes or azides are still required in PECAN, but, being much less bulkier than the perfluoroalkylated tails used in NIMS, a wider range of enzyme classes can be assayed by this method (De Rond et al. 2019). Another alternative to NIMS is SAMDI-MS (self-assembled monolayers for matrix-assisted desorption/ionization-MS), in which proteins or metabolites are immobilized on self-assembled monolayers of alkanethiolates on gold. This allows significantly higher throughput compared to MS-based methods while conserving the specificity and generalizability of the latter (Mrksich 2008). Additionally, SAMDI-MS does not require labelling, which makes it ideal for enzymes without established screening assays. Although these methods have only been employed in a limited capacity for metagenomic enzyme discovery, they are well-suited for the same, with specialized equipment and expertise needed for these methods being the primary barriers to their successful implementation in the discipline. Besides mass spectrometry-based methods, biosensors may also be used for

metagenomic enzyme discovery. Indeed, biosensors have been employed in identifying a novel enzyme capable of cyclizing ω-amino fatty acids from a marine sediment metagenomic library (Yeom et al. 2018). Methods such as mRNA displays have been recently used to screen a naturally occurring RiPP maturase (Fleming et al. 2020). It must be noted that in contrast to MS-based methods, biosensor and mRNA displays are generally tailored to enzyme classes and are therefore high throughput but less generalizable. Overall, several emerging techniques have been discussed in this section, which have been used in a variety of applications and can be adapted and implemented in metagenomic enzyme discovery from diverse environments.

## 24.7 Metagenomic Enzyme Discovery: Outlook and Emerging Techniques

In this section, we discuss the upcoming methods and techniques that can be used in association with metagenomics to accelerate enzyme discovery. These include meta-omics methods such as metabolomics and metatranscriptomics as well as single-cell genomics, cell-free techniques, and sequence-independent methods, among others.

### 24.7.1 Cell-Free Methods

Cell-free systems such as filtered lysates from *E. coli* or other expression hosts of choice can be a viable alternative to heterologous expression and purification of proteins of interest. All cellular organelles required for successful expression remain in the lysates, and additional components such as amino acids, cofactors, and DNA can be exogenously added to induce the expression of proteins or metabolic pathways of interest (Bogart et al. 2021). In contrast to whole-cell hosts, cell-free platforms allow rapid transcription and translation of the sequences of interest unbound to cellular growth. Additionally, cell-free platforms are also more tolerant to toxic metabolite production that may kill whole-cell hosts in heterologous expression. Throughput in such cell-free systems can be further increased using integrated screening methods such as in-droplet reaction microfluidics and mRNA displays, among others (Bogart et al. 2021). Low yield, however, remains a frequently encountered issue for cell-free platforms, particularly when involving exogenous DNA distantly related to *E. coli*. Other commonly encountered issues include rapid degradation of mRNA templates and other critical cellular machinery. Despite these issues, cell-free systems are rapidly growing in popularity and it is anticipated that they will be widely implemented in metagenomic enzyme discovery in future.

### 24.7.2 Meta-Omics Approaches

Although we have discussed enzyme discovery in this chapter primarily in terms of metagenomic shotgun sequencing, other meta-omics applications, both sequencing-dependent and sequencing-independent, can make important, complementary contributions to enzymes. Indeed, integration of omics methods such as transcriptomics/metatranscriptomics, metabolomics, and metaproteomics, among others, can provide a powerful framework to link genetic information to phenotypic data, thereby facilitating hypothesis generation. For instance, transcriptomic studies provide insights into the transcription profile of an organism of interest at any given time and can elucidate differentially expressed genes (of both known and unknown functions) under conditions of interest. Instances of RNA-Seq involvement in enzyme discovery and expansion include the identification of biosynthetic pathways for domoic acid and the expansion of the enzymatic repertoire of DadH-related enzymes in the gut (Rekdal et al. 2020; Chekan et al. 2020). Metatranscriptomic-based approaches can similarly be used for enzyme discovery from microbiomes. Although metatranscriptomic-based studies in enzyme discovery are still sparse, ease of sequencing and increasingly accessible bioinformatic tools are driving a steady increase in these numbers. For instance, BiG-MAP is a recently released tool that has enabled differential expression analysis of biosynthetic gene clusters in transcriptomic/metatranscriptomic datasets (Pascal Andreu et al. 2020). While similar tools are not yet available specifically for pollutant-degrading enzymes, most of the tools described in this chapter, frequently developed for biosynthetic gene clusters, can be used for xenobiotic-degrading enzyme discovery. Integration of metatranscriptomic and increasingly available metabolomic data, along with analytical tools such as BiG-MAP, will contribute to an accelerated metagenomic mining for relevant enzymes of interest. Metaproteomics, compared to other omics methods, remains an under-utilized technique. Recently, a functional metaproteomic workflow has been proposed by Sukul et al., in which proteins are directly isolated from soil samples, separated using two-dimensional (2D)-polyacrylamide gel electrophoresis, and are refolded to assay, screen, and identify novel lipolytic enzymes using a fluorogenic lipase substrate (Sukul et al. 2017). Proteins exhibiting desired activities are then subjected to mass spectrometric analysis. Simultaneous metagenomic shotgun sequencing of the same soil sample allowed searching of the lipolytic enzymes identified using mass spectrometry against a custom environmental database to recover full-length sequences and taxonomic origins for the proteins of interest (Sukul et al. 2017). This study employed in-gel assays, which may be challenging to execute for screening enzymes without well-established colorimetric or fluorometric substrates. Thus, low yields of proteins from environmental samples, refolding of proteins in-gel, and limitations regarding available assays may be some issues that need to be resolved before metaproteomic methods can be better integrated into meta-omics workflows. Nevertheless, integration of meta-omics methods, as discussed, provides a promising avenue towards metagenomic enzyme discovery.

### 24.7.3 Single-Cell Genomics

Increasingly popular single-cell genomics can be implemented as both an alternative to shotgun sequencing and/or as a complimentary application. In single-cell genomics, microbial cells are first sorted, usually using FACS or a microfluidics-based method, with subsequent lysis and whole-genome multiple displacement amplification using high-fidelity polymerases (Woyke et al. 2017). One of the major drawbacks of single-cell genomics is the low yield of DNA material from a single cell even after a billion-fold amplification of single-cell genetic material, which, in turn, results in poor quality of genomes. Additionally, minute amounts of contaminating DNA can result in contributing a significant proportion of the DNA material due to the initial amplification step and in turn destabilize the entire pipeline. Recent advances have contributed to the development of optimized protocols implementing high-fidelity polymerases to improve such issues along with amplification bias from GC-rich genetic material, commonly encountered in secondary metabolism-related gene clusters from Actinobacteria and other organisms with high GC content (Stepanauskas et al. 2017). A clear advantage of single-cell genomics over shotgun sequencing-based metagenomics is the ability of the former to directly link taxonomic information to genomic and phenotypic data without the requirement for binning and annotation. Ideally, best results would be obtained through simultaneous examination of the samples of interest using both single-cell genomics and shotgun sequencing, in which limitations of either can be offset to maximize result quality. Reference genomes from cultivated isolates have been found to rarely overlap with MAGs or single-amplified genomes (SAGs; genomes amplified using single-cell genomic methods) and this indicates that diverse sequencing applications may contribute to greater genome-level resolution of microbial communities (Paoli et al. 2021). Importantly, since single-cell genomic methods sequence every cell individually, it has contributed to a better understanding of the within-population genomic variability and evolution. Here, it is notable that gene clusters involved in secondary metabolism are frequently strain-specific, and single-cell sequencing therefore provides a high-throughput, reliable, and sensitive method to screen for the same in microbiomes. Single-cell RNA sequencing has been used to show spatial transcription heterogeneity in microbial populations that are genetically identical (Imdahl et al. 2020). Since spatial heterogeneity has been reported in secondary metabolite production, single-cell transcriptomics may be a useful method to probe microbial communities for unknown genes with functions of interest (Tobias and Bode 2019). Overall, single-cell genomics remains a nascent field of research with wide applications, including metagenomic enzyme discovery.

### 24.7.4 Sequence-Independent Methods

Most techniques to infer protein function described in this chapter have either been sequence-based on structural homology-based. However, such approaches may be insufficient for identifying and characterizing enzymes that do not have any

structural or sequence similarity with known protein families. Although limited to certain protein families, few tools are available now to investigate and aid in such de novo enzyme discovery. decRiPPter (Data-driven Exploratory Class-independent RiPP TrackER) has been recently developed to identify new RiPP classes without consulting homology to known RiPP classes or enzymes (Ziemert et al. 2016). Briefly, decRiPPter employs a filtering step where it implements pangenomic analyses to infer sparsely distributed operons within taxonomic clades, thereby identifying genes/gene clusters that may be involved in secondary metabolism. Although this approach has been previously used to identify a new family of RiPP maturases, a major drawback of the process remains the increased possibility of false positives compared to homology-based methods when searching for novelty. Due to their limited implementation in current research, sequence- and structure-independent methods remain an open and emerging field for metagenomic enzyme discovery.

## 24.8 Conclusions and Future Challenges

Several challenges remain before the rate of enzyme discovery, particularly from the unexplored microbial space, can see significant acceleration. First, studies on metagenomic enzyme discoveries are heavily skewed towards prokaryotes and eukaryotes in the environment and thus are rarely investigated for enzymatic properties. Eukaryotes, however, play an important role in several ecosystems with some cultivable fungi being frequently used in bioremediation. Moving forward, eukaryotic populations in the environment need to be screened more diligently, with possible expression in new screening hosts, to facilitate discovery of novel biocatalysts from the unknown eukaryotic biodiversity. A similar bias can be observed in the existing literature for studies involving esterases and oxidases with diverse substrate specificities. Although such enzyme discovery studies are common, investigations into proteases, which are likely to be effective in the hydrolysis of amide bonds in pollutants, are sparse. Additionally, uncharacterized protein families enriched in polluted habitats are seldom further investigated. Future efforts in metagenomic enzyme discovery may require certain changes in established approaches. For example, a recent meta-analysis has revealed that among metagenomic enzymes discovered between January 2014 and March 2017, >84% were esterases or cellulases and >82% were discovered through activity-guided screening (Berini et al. 2017). This indicates a bias towards industrially relevant enzymes that can be detected using colorimetric assays. Additionally, hosts besides *E. coli* need to be considered for heterologous expression of candidate enzymes/genes in metagenomic enzyme discovery studies. Indeed, a controlled study reported the successful expression of ~30–40% of environmental bacterial genes and only 7% of high GC-content DNA in the *E. coli* model system (Gabor et al. 2004). Given that many organisms classified as high-GC content are important producers of secondary metabolites and represent interesting reservoirs of novel enzymes, alternative and non-conventional hosts should be assessed for heterologous expression. Additional

complications, leading to failed heterologous expression in *E. coli* hosts, may involve a lack of cofactors, coenzymes, post-translational modification systems, and protein folding factors, among others. Proteins with reduced sequence identity with reference proteins with known functions are more likely to catalyse novel reactions and utilize a broader range of substrates, thereby making them interesting targets for enzyme discovery. In order to detect more distantly related sequences, iterative HMM-based strategies or PSI-BLAST may be used. For the former, an initial HMM can be supplemented with BLAST searches to identify distant neighbours of the gene, to rebuild the model newly identified sequences, and to iteratively carry out a search until no new homologues are identified. Ultimately, enzymes/genes of interest need to be studied and understood as clusters of genes that generally do not exist in isolation. Since bacterial genes involved in secondary metabolism are highly linked, identification of coexpression modules from metatranscriptomic datasets can help predict the functions of unknown genes through coexpression with genes with known functions. It can be anticipated that advances in long-read sequencing methods will allow examination of genetic loci directly from environmental DNA compared to requirements for assembly and binning for short-read sequencing datasets. Overall, the milieu of genetic clusters, transcriptomes, and protein interactomes represents the underexplored routes in enzyme discovery.

The emergence of meta-omics methodologies in the last few decades has enabled researchers to access the functional potential of the hitherto unknown microbial populations in environmental microbiomes. Indeed, activity-guided screening of metagenomes has led to the discovery of numerous enzymes, primarily bacterial in origin, frequently from highly contaminated environments or habitats with other extreme characteristics. Notably, such de novo enzyme discovery using shotgun sequencing-based metagenomics is still rare. Thus, even with the continually accelerating accrual of meta-omics data in public repositories, the ease of next-generation sequencing has not necessarily translated to increased functional characterization of novel protein families. Functional metagenomic approaches are currently limited in several ways. These include the logistical impossibility of screening hundreds of thousands of metagenomic clones for desired properties, the requirement to design novel screening assays for new enzymatic reactions, and the need to handle hazardous, toxic materials when genes involved in xenobiotic degradation are sought, among others. Some of these limits are being overcome by the development of multi-step, activity-based screening strategies as well as the use of non-toxic structural analogues of pollutants of interest in primary screening. Although continual advances in bioinformatic algorithms and next-generation sequencing methodologies show great promise in providing new avenues for enzyme discovery, functional metagenomic techniques will remain important pillars for de novo enzyme identification and expansion. Importantly, we anticipate that shotgun sequencing-based methods in combination with mass spectrometry-based molecular networking methods will facilitate rapid de-replication of candidate enzymes/genes and limit rediscovery, thereby providing proper direction and indirectly expediting truly novel enzyme discovery.

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# Recent Trends in Metagenomic Approaches in Environmental Cleanup

25

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**Abstract**

Metagenomic approaches are being widely used to detect all culturable and unculturable microbes present in environmental samples in a time-saving and efficient manner. It is applied to perceive the composition of microbes and their activity during the remediation of polluted environments. Metagenomic tools such as next-generation sequencing technologies provide authentic information regarding the major genes and enzymes associated with the degradation and detoxification of contaminants present in soil and water. This chapter highlights the various metagenomic approaches, such as metatranscriptomics, metaproteomics, metabolomics, and fluxomics, used in environmental analysis. This chapter also elaborates on high-throughput next-generation sequencing technologies and the procedures associated with library preparation. The workflow of metagenomics, which is used as targeted and shotgun metagenomics, is discussed. Various examples of microbes in environmental cleanup and the bioinformatic tools utilized in metagenomic data analysis are also elaborated. Finally, the challenges associated with metagenomic approaches are discussed.

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Metagenomics · Bioremediation · Targeted sequencing · Shotgun sequencing

## 25.1 Introduction

Expeditious urbanization and industrialization have prompted the cognizance and deeper comprehension to link environmental damage to human health. Of the several sources of pollution, effluents released by various industries are probably the most prevalent ones. Industries employ a range of chemicals to process raw materials to produce high-quality products in a short duration of time in a frugal manner. Often, non-biodegradable and cheap chemicals are utilized and their perniciousness is usually overlooked. Various studies are publicly available, which demonstrate the presence of several noxious compounds in industrial effluents (Bharagava et al. 2018a).

Industrial wastewaters contain a wide range of inorganic and organic pollutants, posing major environmental and health risks to living organisms (Goutam et al. 2018). High total dissolved solids, chemical oxygen demand, total suspended solids, biological oxygen demand, and many other recalcitrant inorganic and organic contaminants all typify the wastewater discharged by industries (Bharagava et al. 2018a; Kumar et al. 2021). Organic contaminants encompass endocrine-disrupting chemicals, polychlorinated biphenyls, phenols, azo dyes, pesticides, chlorinated phenols, polyaromatic hydrocarbons, etc., and, on the other hand, inorganic contaminants comprise toxic heavy metals such as lead (Pb), chromium (Cr), mercury (Hg), arsenic (As), cadmium (Cd), etc. The non-biodegradability and enormously higher concentration of recalcitrant organic contaminants and inorganic metal pollutants in effluents pose pronounced threats to human health and environmental safety, therefore necessitating the adequate treatment of effluents before discharging them into the environment (Saxena et al. 2020; Kumar et al. 2022).

Bioremediation is an environmentally safe management process that utilizes the natural ability of microbes like bacteria, algae, and fungi to eliminate inorganic and organic contaminants from industrial wastewaters; on the other hand, biodegradation is the process of transformation of toxic organic contaminants into less toxic forms (Bharagava et al. 2018b; Kumar et al. 2018). If the contaminants are highly persistent in the environment, then biodegradation may take several steps involving several enzymes and microorganisms. The capacity of bacteria to break down, detoxify, or transform contaminants is reliant on their metabolic potential, which is also governed by the pollutants' accessibility and bioavailability (Megharaj et al. 2011). Microbes are indispensable to the degradation of organic and inorganic contaminants in industrial wastewaters (Maszenan et al. 2011). Nonetheless, due to the incompetence of the native microbiota to degrade noxious contaminants, bioremediation of contaminants takes a longer time. Exogenous microbes and their respective enzymes could be used to remediate the environment to ameliorate the degradation process. However, introducing an exogenous organism into a

contaminated environment can be intrusive because the exogenous organism may exist for a long time even after removal of the contaminant, hence modifying the ecology of the system. Therefore, it is pivotal to consider the importance of microbes in the bioremediation of an environment, as being the fundamental operators of the bioremediation their performance can impact the presence of pollutants in the system (Techtmann and Hazen 2016). Metagenomics is employed to perceive the composition of microbes and their activity during the remediation of polluted environments. Metagenomic tools such as next-generation sequencing technologies provide authentic information regarding the major genes and enzymes associated with the breakdown and detoxification of contaminants in the environment. Therefore, the goal of this chapter is to provide a foundational understanding of metagenomic tools and their applications to better understand the activities and structure of the microbiota during bioremediation of contaminants in a polluted matrix.

## 25.2 Definition and History of Metagenomics

Metagenomics is elucidated as the study of the entire genetic material (metagenome) available in an environment. It is an acclivitous field of genomics that involves a variety of genomic tools to characterize the communities of microbes in environmental samples and to unravel the genetic material of the unculturable microbiota, revealing a wide range of phylogenetically and taxonomically relevant genes, whole operons, and catabolic genes (Riesenfeld et al. 2004; Schmeisser et al. 2007; Uhlik et al. 2013; Kumar et al. 2020). In 1998, Handelsman and colleagues coined the term "metagenome" to describe "the combined genetic material of the whole microbial community residing in nature." Lane, Pace, Olsen, and Stahl were the first to effectively utilize the direct cloning of DNA from the environment (1986) (Pace et al. 1986). Nevertheless, the first successful metagenomic libraries were developed by Pace, DeLong, and Schmidt in 1991 using DNA recovered from marine picoplankton (Schmidt et al. 1991). At the outset, the total genomic DNA in metagenomic experiments was obtained from the environment and then arbitrarily fragmented and sequenced.

Nevertheless, the breakthrough in high-throughput next-generation sequencing (NGS) has revolutionized shotgun metagenomics, and this method no longer requires the construction of a cloned library (Handelsman 2005; Schmidt et al. 1991). These days, metagenomic approaches are thriving, due to the expeditious refinement and cost-effectiveness of high-throughput next-generation sequencing technologies. Metagenomic approaches are making remarkable progress on a massive scale in genomic applications and aid in understanding the functional and taxonomic makeup of the microbiota (Schloss and Handelsman 2005). Various aquatic environments, including sewage, thermal, marine, and freshwater, have also been investigated to determine the variety of microbial populations. Furthermore, metagenomics plays a major part in recognizing various unknown antibiotic resistance genes (ARGs), incorporating those for aminoglycosides, bleomycin, tetracycline, and lactams (Schmieder and Edwards 2011).

## 25.3 Metagenomic Approaches

Several metagenomic methodologies are now available to examine the profiling of microbial populations to showcase the potentials of distinct metagenomic approaches categorized as whole-genome and amplicon-based sequencing, as shown in Fig. 25.1. These are based on the sequencing of target sequences known to be capable of differentiating microorganisms and allowing the sequencing of the whole pool of DNA isolated from a specific sample (Wani et al. 2022).

### 25.3.1 Metatranscriptomics

Metatranscriptomics is carried out with genes that respond to a specific environment in the time it takes to transcribe and possess other functions (Zhang et al. 2009). Metatranscriptomics is a robust approach in which is depicted a set of genes immediately expressed by various eukaryotic microbiota from the environment. Metatranscriptomics has a lot of potentiality in biotechnology for exploring new genes of interest for bioremediation and biomarkers. When metatranscriptomics is employed in a soil system, it entails the isolation and analysis of mRNA, which provides data on the regulation and expression of microbial communities. It is based on the isolation of RNA rather than DNA from environmental samples and, thereafter, the purification of eukaryotic polyadenylated mRNA in the entire environmental

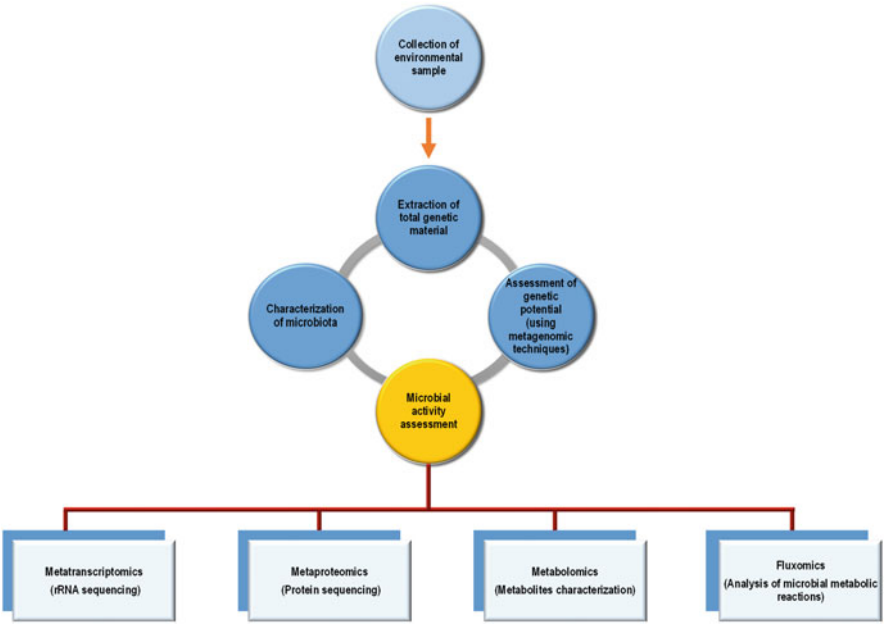


**Fig. 25.1** Typical flowchart of metagenomic approaches followed in an environmental analysis

RNA mix using affinity. The polyadenylated mRNAs obtained could be converted to complementary DNA (cDNA) and are amplified using expression vectors like plasmids, allowing the cloned genes to be expressed in eukaryotic hosts (Yadav et al. 2014).

### 25.3.2 Metaproteomics

Wilmes and Bond coined the term "metaproteomics" in 2004 to define the "massive-scale analysis of the whole protein component of the microbial community present at a time in the environment" (Wilmes and Bond 2004). Today, metaproteomics can determine a plethora of proteins from the environment and provides details on the proteins expressed by microbes as well as their abundances and sources (Hagen et al. 2017). However, in contrast to the anticipated amount of proteins in environmental samples and the rates of protein determined in the proteomics of a single organism, metaproteomics yet faces hurdles in protein determination. Various obstacles include protein extraction from complex systems, sequence unavailability, huge sequence databases, and search engine sensitivity (Kunath et al. 2019). The major impediments to metaproteomic studies include uneven species distribution, wide genetic heterogeneity within microorganisms, and a wide range of protein expression levels among microbial communities (Bharagava et al. 2018a).

### 25.3.3 Metabolomics

A metabolomic analysis involves the identification and quantification of small chemical molecules, which are released by organisms (metabolites) into their immediate surroundings. It is the immediate measure of the status of environmental health or any changes in the homeostasis of an environment as alterations in the synthesis of signature metabolites are linked to the variation in the activity of metabolic pathways (Bernini et al. 2009). Thus, for this reason, metabolomics is a useful tool in pathway analysis. A metabolomic outline of the microbiome is generally highly dependent on environmental factors (such as environmental stressors and xenobiotic exposure diet), providing important information regarding the characteristics of the microbiota and their interactions with the host environment (Krumsiek et al. 2015; Manor et al. 2014). As a result, metabolomics strives to ameliorate our knowledge regarding the contribution of the microbiota to the degradation of toxins and other abiotic factors that could disrupt the equilibrium of the environment. The microbiome has a significant impact on biogeochemical cycles, thus analyzing their metabolome aids in the development of environmental stress biomarkers (Lankadurai et al. 2013). Microbes are considered as reactors that could transform hazardous substances and resources into either valuable or deleterious products in their environment, depending on the genetic pool. Identification and quantification of metabolites generally involve consolidation of chromatography techniques (such as gas and liquid chromatography) and some methods of detection including mass spectrometry

and nuclear magnetic resonance, which create spectra comprising patterns of peaks (Bharagava et al. 2018a).

### 25.3.4 Fluxomics

Metabolomics involves the detection of all metabolic substances present in a biological sample at any particular time. As part of the set of metabolic processes that occur in cells, metabolites that make up the metabolome are constantly transformed. These activities are coupled to form a convoluted set of metabolic pathways that work together to form the metabolic network, which is directly dependent on both the metabolome and fluxome. Comprehensive information about various cellular processes is provided by the fluxome, or a complete set of fluxes of the metabolic system of a cell, and, hence, each cell has distinct phenotypic characteristics. Flux analysis focuses on understanding the functional interactions of the metabolome with the environment and the genome, providing a complete dynamic picture of the phenotype. Fluxomics has many advantages over proteomics and genomics, as it is based on data from metabolites, which are significantly less than those from proteins and genes (Cascante and Marin 2008).

## 25.4 High-Throughput Next-Generation Sequencing (NGS) Technologies

Next-generation sequencing techniques are the most robust methods for the sequencing of genes, and they offer a comprehensive understanding of microbial ecology-aided processes including environmental pollutant breakdown, wastewater quality, and pathogen transmission (Bharagava et al. 2018a; Kumar and Chandra 2020). The evolving procedures of the technology are effectively integrated with data procurement and analysis, thus relieving the society from work-intensive and inefficient Sanger sequencing methods and enabling a massive rise in data output. DNA fragmentation, DNA end repair, adaptor ligation, surface attachment, and in situ amplification are all common steps in second-generation techniques, such as in the case of Ion Torrent and Illumina systems. These "short-read" sequencing tools include millions of individual sequencing processes taking place simultaneously. These types of technologies require reassembling of data over long sections of DNA, which might pose challenges with low complexity regions and structural variances. Third-generation sequencing approaches, such as Oxford Nanopore or Pacific Biosciences, can achieve read lengths of up to 10 kb, more than that of short-read or Sanger sequencing technologies. In contrast to second-generation approaches, process improvements consist of fewer steps for the preparation of a library and real-time targeting of unfragmented DNA molecules. The accuracy of the reads is an early shortcoming of these third-generation approaches in contrast to second-generation approaches, although this has improved with time, especially with software analysis breakthroughs (Hu et al. 2021).

### 25.4.1 Short-Read Next-Generation Sequencing

Short-read sequencing is the further step in the development of sequencing technology next to first-generation Sanger sequencing. Short (250–800 bp) and amplified molecules of DNA are concurrently sequenced in short-read techniques (Tucker et al. 2009). Library preparation, sequencing, and data analysis are all constituents of NGS (Hu et al. 2021).

#### 25.4.1.1 Preparation of Libraries

A high-quality library is required for NGS. For sequencing, procurement of templates proportional to molecules of interest is the first step for preparing a library, followed by processing fragments, to make them suitable to the sequencing platform (Head et al. 2014). RNA sequencing (RNA-Seq) and DNA sequencing (DNA-Seq) are two types of NGS (RNA-Seq) techniques (Lightbody et al. 2019).

##### 25.4.1.1.1 Preparation of a DNA-Seq Library

A DNA sequence can comprise whole-exome sequencing (WES), whole-genome sequencing (WGS), targeted sequencing, or epigenome sequencing, depending on the sequencing template (Rizzo and Buck 2012). Hybridization capture-based technologies and polymerase chain reaction (PCR)-based technologies are two methods for preparing templates. To create a template for targeted sequencing, PCR-based procedures are often used (Head et al. 2014; Lightbody et al. 2019; Rizzo and Buck 2012). Initially, amplicon sequencing was confined to a small number of genes (short-range PCR). With the introduction of long-range PCR (LR-PCR), shotgun sequencing became the standard method, allowing whole genes to be sequenced, including the downstream, upstream, untranslated, and intronic regions. As a result, LR-PCR was able to resolve sequence ambiguities that were present in short amplicon sequencing (Hu et al. 2021). Template preparation in targeted sequencing and WES is carried out using a hybridized capture-based approach, in which biotinylated complementary probes get hybridized with domains of interest and are further separated using magnetic beads of streptavidin coating (Liang et al. 2018). This target-oriented strategy offers a low-cost methodology for targeted sequencing of massive genomic areas and a considerable set of genes than do PCR-based approaches, with lower allele dropout rates. These two approaches, however, are not mutually exclusive. The genomic regions of the major histocompatibility complex (MHC) (Dapprich et al. 2016) and other genomic regions (Pinney et al. 2013) have been captured using a combination strategy known as regional-specific extraction (RSE). The RSE technique encompasses an enzymatic extension of an oligonucleotide (non-biotinylated) hybridized to a certain sequence, integrating biotinylated nucleotides as it moves further (Hu et al. 2021).

Afterward, streptavidin-coated magnetic beads are used to capture the extended DNA, which is then enriched using whole-genome amplification procedures and processed for further sequencing. Whole-genome bisulfite sequencing (WGBS) and chromatin immunoprecipitation (ChIP) are the most prevalent methods for examining epigenetic changes and their effects on gene regulation, followed by

NGS (ChIP-Seq). WGBS provides genome-wide DNA methylation study at a resolution of base pair. WBGS samples for NGS are typically prepared following the post-bisulfite treatment of DNA and de-tagging prior to index adaptor binding. ChIP-Seq enables modifications of histone at the base-pair resolution and the base-pair resolution mapping of DNA-binding proteins across the genome. In ChIP-Seq, natural or formaldehyde-fixed chromatin is broken down either by sonication or micrococcal nuclease (MNase) and, thereafter, immunoprecipitated with magnetic beads attached to a target-specific antibody. Libraries are constructed from DNA extracted from protein-precipitated DNA complexes (Head et al. 2014). The major steps of DNA-Seq library building include fragmentation, end repair, adaptor ligation, and size selection (Podnar et al. 2014). Fragmentation snips the DNA into a certain size range for the optimal platform. Enzymatic (such as fragmentase or transposase tagmentation), chemical (heating with a divalent metal cation), and physical (acoustic shearing or sonication) methods are all used to fragment DNA (Head et al. 2014). Longer pieces provide proximal phase information, whereas small fragments provide sequencing data of higher quality (Gandhi et al. 2017). End repair prepares libraries by making certain that there are no overhangs that possess 30 hydroxyl and 50 phosphate groups or by dA tailing and blunt endings of the DNA fragment for blunt ending Ion Torrent or Illumina (Hu et al. 2021).

Adaptors are sequences that are restricted to a specific platform for the detection of a fragment on a sequencing apparatus, for example, a fragment of DNA binding to the Illumina flow cells. Individual samples are identified by a unique, short sequence known as an index or barcode, which allows multiple samples to be gathered and further sequenced concomitantly in a single go (multiplex sequencing) (Hu et al. 2021).

After the process of adapter ligation, DNA fragments are enriched by size selection defined by a certain size range, and contaminants are removed to increase the efficiency of sequencing. An appropriate selection of size maximizes phasing, produces higher-quality data, improves the runs of sequencing, and increases the number of samples sequenced. The library could be PCR-amplified before being sequenced or could be sequenced directly (Gandhi et al. 2017).

#### 25.4.1.1.2 RNA-Seq Library Preparation

RNA-Seq is an efficacious tool for the functional analysis of a genome including alternative splicing, variant detection, and differential gene expression (Mutz et al. 2013; Love et al. 2015). Small RNA sequencing (smRNA-Seq), mRNA sequencing (mRNA-Seq), and whole-transcriptome sequencing (WTS) are the three different types of RNA-Seq techniques. The three main steps for the preparation of samples comprise RNA isolation, target RNA enrichment, and reverse transcription of RNA into cDNA. For the construction of WTS libraries, an Illumina Stranded Total RNA Prep with Ribo-Zero kit is used, which involves rRNA elimination from total RNA, chemical fragmentation of residual RNA, and, thereafter, random priming for the purpose of reverse transcription (Hu et al. 2021).

For the sample preparation of mRNA-Seq, mRNA is procured with the help of oligo-dT-enriched magnetic beads and is isolated from the complete RNA present.

The Illumina TruSeq Stranded mRNA kit is used to prepare mRNA libraries, corresponding to the way that WTS libraries are prepared (Hu et al. 2021). Small RNAs are non-coding RNAs with a total length of less than 200 nucleotides, but the most scrutinized species are microRNAs (miRNAs), which are crucial in gene regulation. However, the construction of a smRNA-Seq library is easy because miRNA has a 5′-terminating phosphate in its initial state (Head et al. 2014). The construction of the library begins with a two-step ligation. A 3′-end adenylated, enriched DNA adaptor is ligated with RNAs with the help of T4 RNA ligase 2, ensued by a second 50 RNA adapter ligation using RNA ligase 1. Thenceforth, reverse transcription-PCR is used to convert ligated miRNAs into cDNAs, and thereafter, amplification results are processed for sequencing after gel size selection (Hu et al. 2021).

#### 25.4.1.2 Sequencing Platforms

Clonal amplification and sequencing are sequential steps in the short-read sequencing process. In clonal amplification, DNA fragments are amplified in the solid phase to produce detectable and strong signals at the time of sequencing. Beads (Ion Torrent) and flow cell surfaces (Illumina) can be used as the solid phase in which single DNA fragments are attached. For the amplification of attached fragments of DNA into a number of separated template fragments, "bridging" PCR (Illumina) or emulsion PCR (Ion Torrent) is deployed, based on the platform for sequencing. Both Ion Torrent and Illumina technologies are constructed on the concept of sequencing by synthesis (SBS) in which, on the extended DNA chain, DNA polymerase-dependent nuclease is incorporated (Goodwin et al. 2016).

##### 25.4.1.2.1 Ion Torrent Sequencing

Clonal amplification in the case of Ion Torrent is accomplished via emulsion PCR using a bead technique on Ion Sphere particles in a microwell. The DNA fragments are ligated to adapter sequences and then collected with a bead containing DNA polymerase, complementary adapters, primers, and deoxynucleotide triphosphates (dNTPs) in a water-in-oil emulsion droplet (micelle). Individual micelles serve as a micro-PCR reactor, enabling separate PCR amplification. Micelles are put on a semiconductor chip into microwells, and A, T, G, or C nucleotides are progressively flooded into the device. It is the foremost technique to conduct semiconductor sequencing without optical sensing (Levy and Myers 2016). It could perform fast sequencing (in 2.5–4 h) with reads between 200 and 600 bp. The intricacy of sequencing across homopolymer sections is an integral shortcoming of the Ion Torrent chemistry. With the consolidation of various identical bases, there is indeed a possibility of losing the linearity of response due to erroneous voltage pulse magnitude measurements, which might manifest as insertion/deletion errors in a single read. The technology, on the other hand, has a low substitution error rate (less than 0.1% per base rate) (Merriman et al. 2012). The Ion Genestidio S5, Genuxus, and PGM Dx instruments are all part of the Ion Torrent platform (Hu et al. 2021).

#### 25.4.1.2.2 Illumina Sequencing

The Illumina technology is based on the principle of SBS in which a fluorescently labeled reversible terminator is used (Koboldt et al. 2013). On the platform (flow cell), DNA libraries of cluster amplification (cluster generation) are executed before preceding sequencing using "bridging amplification" PCR, which is governed by the system. For the purpose of sequencing, optical readout of fluorescent nucleotides is used, which are further linked by a DNA polymerase to a reversible terminator. During each sequencing cycle, a fluorescently tagged, reversible, and terminator-bound dNTP is integrated into the chain of nucleic acid, and the ensuing fluorescent signal is visualized. To enable the preceding labeled dNTP to be inserted, the fluorescent dye and terminator are sniped from the inserted dNTP. Furthermore, Illumina NGS platforms can adequately perform paired-end sequencing, which involves sequencing a DNA fragment from both ends, resulting in sequencing data of high quality with extensive handling and a large number of reads. The Illumina MiSeq instrument, which is widely utilized for clinical purposes in HLA typing, prefers 350–500 bases of fragment size; however, fragments of 600–900 bases or larger are best for phasing distal polymorphisms, countenanced by paired-end sequencing (Gandhi et al. 2017). Reversible Illumina sequencing is coupled with end-pair sequencing, which makes it a reliable base-by-base sequencing technique (with an error of 0.1%, mainly due to substitution or extremely hard due to insertions/deletions). Illumina offers six major sequencers (NextSeq 550, iSeq, NextSeq 1000, MiniSeq, NextSeq 2000, and MiSeq) with varying outputs and total reads per run as well as two in vitro diagnostic tools (NextSeq 550Dx and MiSeqDx). More recent versions, such as the NovaSeq 6000, MiniSeq, and NextSeq 550, could perform all four base calls using a two-channel SBS approach with two images in each cycle, thus minimizing the data processing and sequencing time and ensuring greater accuracy and quality (Hu et al. 2021).

### 25.4.2 Long-Read Sequencing

Long-read sequencing methods, often known as third-generation sequencing approaches, can yield sequences >10 kb directly from native DNA, as compared to short-read sequencing technologies. Prior implementations of these techniques had errors, whereas more recent adjustments and enhancements have allowed the sequencing of large DNA molecules and have significantly improved accuracy. Oxford Nanopore Technology (ONT) and Pacific Biosciences (PacBio) are two key long-read technologies based on different principles (Logsdon et al. 2020).

#### 25.4.2.1 Pacific Biosciences (PacBio)

Single-molecule real-time (SMRT) sequencing is a third-generation sequencing technology produced by Pacific Biosciences (PacBio). The DNA to be sequenced is a single-stranded circular DNA known as the SMRTbell template in this approach. SMRTbell adapters (hairpin adaptors) are ligated to both the terminals of the double-stranded DNA (dsDNA) molecule to construct the SMRTbell template. The process

takes place in an SMRT cell chip with several small pores known as zero-mode waveguides (ZMWs), which are roughly around 100 nm in depth and 70 nm in diameter. The earlier PacBio RS II system had 150,000 ZMWs per SMRT cell, and the advanced system has a million ZMWs per SMRT cell, which are two PacBio sequencing platforms. A single SMRTbell template can be sequenced using a single molecule of DNA polymerase bound to every ZMW (Rhoads and Au 2015).

#### 25.4.2.2 Oxford Nanopore Technology

Sequencing with Oxford Nanopore Technology (ONT) could provide reads larger than 1 Mb; additionally (Miga et al. 2020), all together, it can computationally assemble larger than 2 Mb (Payne et al. 2019). The movement of single-stranded nucleic acid (DNA or RNA) along a protein pore named staphylococcal α-hemolysin (αHL) provides a framework for ONT sequencing (Clarke et al. 2009). The ligation of an adaptor to double-stranded DNA makes it easier for the protein pore to capture it. The libraries are fed into a flow cell that has a membrane comprised of hundreds of thousands of nanopores implanted in it. An ion current is applied, as a result of which the single strand is transported along the pore by a preloaded motor enzyme on the adaptor at the 5′-ends. Each nucleotide passes through the pore, causing a peculiar discontinuity in ion current, which is detected by sensors and recorded (Hu et al. 2021).

## 25.5 Types of Metagenomic Approaches

Mullany (2014) outlined three distinct metagenomic strategies for analyzing the microbiota such as sequence-based, targeted (microarray or PCR-based), and functional metagenomics. Functional metagenomics is a promising method for detecting the expression, isolation, and identification of new antibiotic resistance genes (ARGs) from the unculturable component of the microbiome. Both sequence-based and functional metagenomics could provide information about the detection of mobile genetic elements harboring antibiotic resistance genes as well as the association of resistance genes with the structure of a community. Targeted (PCR-based) metagenomics could be utilized to assess the occurrence of certain gene families and resistance genes within or between ecosystems. To detect the occurrence of antimicrobial resistance (AMR) genes, PCRs such as multiplex, conventional, and real-time PCRs are commonly used. The amplification of resistance genes of interest within metagenomes is accomplished using PCR primers. Real-time PCR could yield semi-quantitative data that can be used to assess the relative abundance of various genes in the microbiota. All known resistance genes could be identified using PCR-based metagenomics. In sequence-based metagenomics, DNA is extracted from samples and, further, next-generation sequencing methods are employed to obtain entire sequences. The generated contiguous sequence read lengths could further be compared to public databases to find resistance genes. This methodology may be used to identify all known and unknown

resistance genes in a metagenome. Unfortunately, no details on the expression of resistance genes are provided in this study (Nowrotek et al. 2019).

## 25.6 Metagenomics in Environmental Cleanup

Metagenomic techniques aid in determining the dynamics and composition of a microbial community in an ecological system. They also contribute to our knowledge of microbial degradation and the removal of inorganic and organic contaminants from polluted sites. These techniques are effective in identifying a potential microbial degrader or catabolic gene accountable for the breakdown and detoxification of a certain contaminant for bioremediation. It is also applied to evaluate the functionality of microbiome diversity at various polluted sites. Furthermore, advanced sequencing technologies like NGS aid in delving deeper into microbial communities and provide an unbiased understanding of the functional diversity and phylogenetic composition of the environmental microbiome (Zwolinski 2007). The accessibility of whole-genome sequences from a variety of microbes is relevant to bioremediation aids in comprehending the gene pool of enzymes engaged in the breakdown of anthropogenic contaminants (Galvão et al. 2005). Metagenomics overcomes the hitch of cultivation-based studies by directly extracting the genetic material from environmental samples, which could theoretically represent the whole collection of genomes residing in the microbial community of an ecosystem (Desai et al. 2010). Metagenomics has been proved advantageous in identifying novel gene families as well as microorganisms involved in xenobiotic bioremediation in recent years. DNA microarrays have recently been used in microbial ecological studies to monitor microbial communities and the performance of various bioremediation procedures (Bae and Park 2006). In bioremediation processes, these "omics" tools have enormously been utilized for determining new biodegradation routes and monitoring or characterizing contaminant-biodegrading microbial populations (Bharagava et al. 2018a).

A study of fungi from sediment samples in the Zuari and Mandovi inlets, Goa, India, was conducted using a metagenomic molecular method (Haldar and Nazareth 2019). *Eurotiomycetes*, *Agaricomycetes*, *Dothideomycetes*, *Sordariomycetes*, and *Saccharomycetes* are some of the fungi that were detected. The researchers suggested that the diverse species of fungi were accountable for bioremediation, which could further be used for remediation of other sites. Similarly, two separate sediment samples from mangrove forests, which are found along the subtropical and tropical coasts, were used in a metagenomic investigation. The analysis revealed that the microbiota of these areas possess microbial activity, which is associated with methanogenesis and nitrogen fixation (Li et al. 2019). Ammonia-oxidizing archaea (AOA) are a promising prospect for the bioremediation of nitrogen in wastewater (Yin et al. 2018). The seven AOA genomes from the Pearl River estuary were compared to the *Nitrosopumilus maritimus* SCM1 strain using a metagenomic approach. Surprisingly, a relative genomic analysis revealed that this type of strain with essential genes for contaminant degradation can be used in eutrophic

environments (Zou et al. 2019). Furthermore, the microbial population involved in the breakdown of bisphenol A (BPA) compounds was studied using a combination of approaches that included metabolomics, metatranscriptomics, and metagenomics. In contrast to the *Sphingomonas* species alone, a coculture of the *Pseudomonas* and *Sphingomonas* species showed enhanced BPA degradation (Yu et al. 2019).

Microbial interactions are essential to the biodegradation of contaminants, and comprehending this type of interaction might help clean up the environment. The insertion sequence ISI1071, which is covered by a xenobiotic gene, was found to aid in the breakdown of pesticides in a metagenomic analysis (Dunon et al. 2018). Furthermore, the screening of strains using a metagenomic approach could be used to degrade xenobiotic pollutants found in specific habitats (Mani 2020). Naphthalene is a naturally occurring pollutant that is a primary cause of contamination in anaerobic environments. Metagenomics and metabolomics play a major role in knowing the degradation mechanism of naphthalene-degrading bacteria, as they are hard to cultivate.

In a study, it was found that *Desulfuromonadales* plays a role in the degradation of naphthalene (Toth et al. 2018). Vinyl chloride (VC) is a strenuous substance to degrade because of its carcinogenic properties. In total petroleum hydrocarbon (TPH)-polluted groundwater, a bioaugmentation-based analysis was proposed. TPH was decreased from 1564 to 89 mg/L after 32 days due to the adoption of the bioaugmentation approach. In addition, a metagenomic study revealed that there was an alteration in the microbial community after the initial treatment period (Poi et al. 2018). The microbiota linked to oil sand process-affected water were also analyzed using metagenomics. In a study, it was found that the *Rhodococcus* bacteria were present in abundance in sand samples. Biofilter systems were shown to be effective in reducing naphthenic acids by 21.8% in this investigation (Zhang et al. 2018). Metagenomic molecular techniques were also used to assess the diversity of microbes in a study of microorganisms in diesel bioremediation. Several species of bacteria, notably *Chryseobacterium*, *Pseudomonas*, *Sphingomonadaceae*, and *Aquabacterium*, have been identified in a mixture of hydrocarbons (aliphatic and aromatic nature) residing in diesel (Garrido-Sanz et al. 2019). These bacteria can also be used to break down hydrocarbons. The global ecosystem has been extensively damaged by the liberation of thiocyanate ($SCN^-$) from gold and coal mining. Microbiomes are employed in bioreactors to study the breakdown of $SCN^-$, and, in a metagenomic study, it was observed that $SCN^-$ was degraded by the $SCN^-$ hydrolase-coding *Gammaproteobacteria* (Watts et al. 2019).

Metagenomic approaches have also been used to study o-xylene-degrading genes from the methanogenic group of bacteria, and it was found that the fumarate-adding enzyme, napthylmethylsuccinate synthase (nmsA)-, alkylsuccinate synthase-, and benzylsuccinate synthase-coding genes were associated with o-xylene molecule degradation (Rossmassler et al. 2019). This concept could be implemented to mineralize o-xylene in various polluted locations. To better comprehend the role of bioaugmentation in the elimination of trichlorinated ethylene/cis-dichloroethylene (TCE/cDCE) from polluted locations, researchers used a combination of metagenomic and metatranscriptomic approaches. The *Rhodococcus* genus,

including strain RHA1, rose from 0.1% to 76.6% of the total microbiota, according to the study. Other TCE/cDCE-degrading bacteria, on the other hand, have been found to diminish with time. In addition, a metatranscriptomic analysis revealed that during the experimentation, a high level of expression of aromatic compound-degrading genes (bphA1–A4) was observed (Watahiki et al. 2019). HGT of antibiotic resistance genes (ARGs) from one bacterium to another is a worldwide issue that society and clinicians must deal with. Moreover, landfills promote these issues by serving as a repository for these antibiotics and genes. A metagenomic analysis was conducted to better understand the dynamics of ARGs in a bioreactor, in which an increased level of ARGs was revealed (Liu et al. 2018). Furthermore, a metagenome-based investigation of bioreactor samples was conducted, demonstrating that the presence of solids resulted in a reduction in microbial diversity. Thiobacilli, which are responsible for the breakdown of thiocyanate ($SCN^{-}$), were also repressed in the bioreactor (Rahman et al. 2017). To comprehend the bacteria and genes associated with the metabolic pathways of nitrifying sludge, a combined approach of metatranscriptomics and metagenomics was employed and bacterial communities such as *Betaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidetes* were detected in the nitrifying sludge. Furthermore, metatranscriptomics has demonstrated a substantial expression of genes, which degrades aromatic compounds (Sun et al. 2019). In a study, metagenomics was employed to assess the polyaromatic hydrocarbon ring hydroxylating dioxygenase (PAH-RHDα) gene diversity of bacterial species in mangrove sediments and oilfield soils. Various bacteria such as *Polymorphum gilvum*, *Mycobacterium*, *Pseudomonas*, *Burkholderia*, *Sciscionella marina*, and *Rhodococcus* have expressed PAH-RHDα gene diversity (Liang et al. 2019). In a metagenomic sequence assembly-based genome of bacteria, it was found that the *Methylocystis* species (HL 18 strain) has robust mercuric reductase genes that are associated with the bioremediation of Hg (II) and As (V) in polluted aquatic habitats. After conducting a sequence analysis, it was analyzed that the mercuric reductase genes are more similar to Hg (II)-reducing bacteria such as *Paracoccus halophilus* and *Bradyrhizobium* species strain CCH5-F6. Microorganisms of this kind can be utilized in contaminated places for heavy metal bioremediation (Shi et al. 2019).

## 25.7 Tools for Metagenomic Data Analysis

To evaluate molecular data, a variety of web resources, pipelines, algorithms, and in silico software are used (Desai et al. 2010). Table 25.1 summarizes some of the most common computational and bioinformatic software used in the study of metagenomic data.

**Table 25.1** Different bioinformatic software used in the metagenomic analysis of data (Arya and Ravindra 2020; Bharagava et al. 2018a)

| Name of the software | Application |
|---|---|
| Metagenome Seq | Evaluation of the abundance of *16S rRNA* genes in meta-profiling |
| UCLUST | A clustering tool, which utilizes USEARCH to allocate sequences to clusters |
| Mothur | Used in the quality analysis of reads for taxonomic classification |
| NGSQC toolkit | Method of performing quality control analysis in a direct environment |
| RDP (Ribosomal Database Project) | Biodiversity analysis, sequence arrangement, alignment, trimming, and taxonomic classification of sequences |
| Pfam | A large collection of families and domains expressed by profile HMMs and multiple sequence alignments |
| Prodigal | Identification of translation initiation sites in prokaryotic genes |
| CAMERA | A server for a metagenomic database containing sequences from environmental samples collected during the GOS |
| envDB | Prokaryotic taxa environmental distribution database and tool server |
| myPhyloDB | A tool used for the purpose of storage and metagenomic analysis |
| FUNGI Path | A database used for metagenomic and orthological studies of fungi |
| PyNAST | Aligned sequences of representative OTUs |
| Meta MIS | Analysis of microbial interaction |
| FOAM | Created to screen environmental metagenomic sequencing datasets and to offer a novel functional ontology specialized in categorizing gene functions pertinent to environmental microbes using HMMs |

## 25.8 Challenges in Metagenomics

Notwithstanding the fact that metagenomics is a robust tool for uncovering varied microbial communities and their significance in the environment, it also exhibits a number of pitfalls. It encompasses a diverse set of assembly techniques, each with its own set of benefits and drawbacks and especially lacking an optimal guideline for every specific metagenomic community. The quality of metagenomics is significantly affected by the employment of numerous technologies with longer read lengths, a large volume of data, and various error models. For instance, amplicon-based sequencing suffers from biased amplification due to a variety of factors, including specific primer sets for known eukaryotic (18S, ITS1), archaeal (16S), and bacterial sequences, and is unable to amplify unknown organisms with sufficiently diverged sequences from the primers. A further major difficulty is that distinct sequencing technologies interpret the same datasets differently. As a result, metagenomic data processing varies widely based on the researcher's goals and the types of data provided (Bharagava et al. 2018a).

## 25.9 Conclusions

Industries are the key source of pollutants, releasing waste materials into the environment, which are either untreated or partially treated. These contaminants might reach the soil and aquatic environments and, as a result, could enter the bodies of humans and animals via food chains. These pollutants could be harmful, lethal, and cause changes in the genetic makeup of organisms. Natural microbial communities are accountable for the elimination of organic and inorganic contaminants from the environment. However, it is unclear how pollutants are degraded by microbes; because of the accessibility of metagenomic approaches, we can easily comprehend the metabolic pathways adopted by microbial communities in the environment. Additionally, we can evaluate viable microbial strains, potential metabolite enzymes, and genes to biotransform these environmental pollutants using metaproteomic, metagenomic, metabolomic, and metatranscriptomic approaches. Due to the advancements in metagenomic technology, it is now possible to conduct culture-independent analysis of microbes using either sequence-based or biotechnologically important genes. The development of a metagenomic library is critical for comprehending the numerous enzymes, proteins, and mechanisms involved in the growth and metabolism of a microbial community during the process of bioremediation.

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# Applications of Metagenomics in Microbial Diversity and Function Analysis: Recent Trends and Advances

26

Harshitkumar J. Savalia and Anupama Shrivastav

**Abstract**

Every year, two billion tonnes of waste is generated globally, most of which is kept in landfills and then disposed into oceans. This is a potential threat to the environment as it contaminates the land. Due to industrial wastewater, the atmosphere is also contaminated. Many microorganisms can be found in polluted areas. A municipal dump is an example of a man-made ecosystem that supports a diverse community of microorganisms. Microorganisms can degrade waste by secreting enzymes. Because of their excellent specificity and cost-effectiveness, enzymes or microbial cells are used as biological catalysts. Organic and inorganic wastes are degraded and detoxified by microorganisms. In the biomethanation process, methane gas is produced, which is used as a biofuel, and compost is produced from the composting process, which is used in agricultural processing; many microorganisms living in waste are useful in these processes. Metagenomics is an advanced molecular technique to identify the microbial diversity present in different types of wastes and is used for environmental cleanup. Metagenomics describes the genomic analysis of microbial DNA extracted directly from a sample, and it is a high-throughput gene-level study of mixed microbial communities. During bioremediation in a contaminated environment, metagenomic approaches are currently being used to better understand the makeup and study of microbial communities. The major enzymes and genes involved in the breakdown and detoxification of environmental contaminants can be accurately identified using the next-generation sequencing (NGS) technology. The purpose of this chapter is to provide a fundamental understanding of the metagenomic methodology and applications in order to better understand microbial diversity, functions, and structures in contaminated environments.

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**Keywords**

Metagenomics · Microbial community · Environment · Contamination

## 26.1 Introduction

Developed countries produce 521.95–759.2 kg of waste per person per year (kpc), whereas underdeveloped countries produce 109.5–525.6 kpc. The global municipal solid waste (MSW) production has reached two billion tonnes per year, according to the latest estimates, posing a threat to the environment. As a result, MSW (municipal solid waste) management seems to be one of the most pressing challenges for current and future environmental preservation (Karak et al. 2012).

With an increase in population, waste generation is definitely going to increase and an increasing amount of land would be required for waste disposal. According to CPCB Annual Report (2013), the state of Gujarat is facing a shortage of landfill sites and has decided to have one landfill site between three to four villages. The matter has also been discussed in the State Assembly, where the minister reported that even if the local administration identifies a site, the local residents near the site oppose the allotment. This indicates public awareness about the health and environmental implications associated with MSW disposal without any treatment.

Different countries across the world employ different strategies to treat MSW. For instance, thermochemical technologies like combustion, gasification, or pyrolysis are used for non-biodegradable waste, whereas biogas or compost technologies are used for biodegradable waste. During incineration or combustion, MSWs are burned at high temperatures so as to convert them into residue and gaseous products, while the heat is harnessed in the process. However, with an increase in environmental awareness, the public outcry hampers the establishment of such facilities. Conventional and plasma-assisted gasification is also employed for waste treatment; however, so far, the latter has not been a financially viable technology. In gasification, syngas or producer gas produced at the end of the process can be purified and used either for heating purposes or fed to gas generators to produce power or can be converted to fuel like jet fuel, gasoline, or diesel through the Fischer–Tropsch reaction or catalytic reaction.

Composting is the best process of organic waste disposal as it can convert organic products into compost. Few biomethanation-based plants are also installed at certain locations in India, where the organic portion of MSW, normally from hotels and restaurants, are fed and biogas along with biodigested slurry is generated. The biogas can be purified and used for cooking/heating purposes or fed to a gas generator to generate power, whereas the slurry is used as a soil conditioner in farming and gardening.

## 26.2 Waste Production

With an increase in population, waste generation is definitely going to increase and waste would require an increasing amount of land for its disposal. This indicates public awareness about the health and environmental implications associated with MSW disposal without any treatment. The number of new landfill sites is declining due to the lack of available land and public objection to the production of methane and other toxic gases, as well as particulate matter, which cause pollution in the surrounding area. The leachate from landfills also contaminates groundwater aquifers as it seeps. The land where a landfill is established loses its fertility (Atalia et al. 2015; Nandan et al. 2017) (Fig. 26.1).

## 26.3 Waste Management

Waste treatment and waste sorting involve various activities associated with waste treatment until the waste is collected in waste storage containers that also need proper handling. Classification of the different types of wastes is the main step in solid waste management and storage. Solid waste management is not scientific in India. A large amount of solid waste is dumped without any primary process of segregation. Because of this, groundwater contamination and air pollution are increasing. However, due to the rising demand of these commodities, plastic and paper recycling has seen a significant increase.

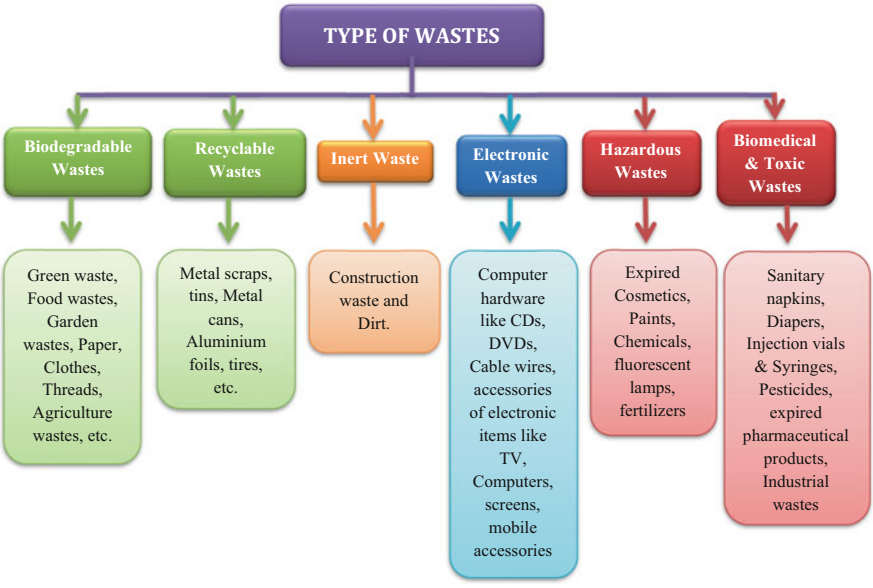


**Fig. 26.1** Different categories of wastes and waste materials

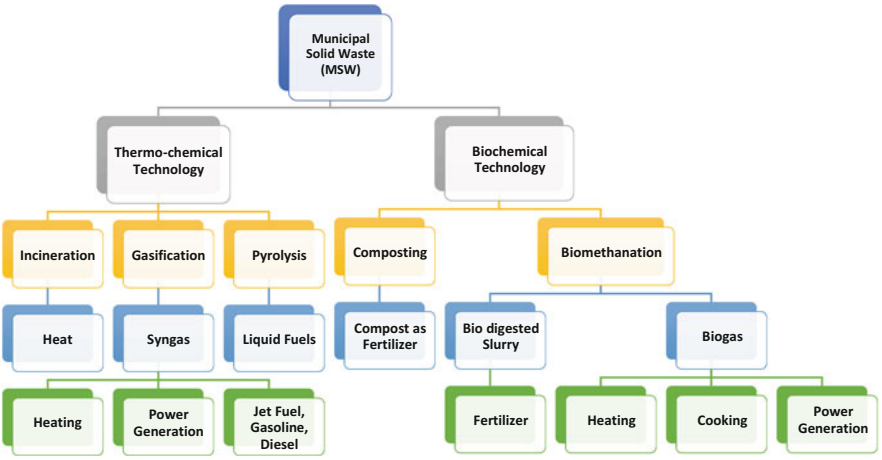


**Fig. 26.2** Application of wastes

In a systems analysis, various garbage disposal techniques created for municipal solid waste were examined. Municipal solid waste can be processed to reduce the total quantity and weight of materials required for the final disposal. The treatment changes the form of the waste and makes it easier to handle. It can also serve as a material for recycling or reuse as well as for recovering thermal energy. Incineration, recycling of separated plastic and containers, and biological treatment of biodegradable trash such as anaerobic digestion and composting were all investigated and compared to landfilling (Eriksson et al. 2005).

Municipal solid waste management (MSWM) is one of India's most serious environmental issues. The inappropriate management of municipal solid waste puts residents in danger. According to numerous studies, almost 90% of MSW is dumped in landfills and open dumps without being professionally analyzed, posing a threat to public health and the environment. To promote extensive input and review of the existing MSW, generation, treatment, and disposal technologies as well as collection and transportation (Sharholy et al. 2005) are required (Fig. 26.2).

## 26.4 Microbial Diversity in Waste Sites

Microbial activity in discarded garbage plays an important role in the decomposition of organic waste and in the release of greenhouse gases. Because of the abundance of microorganisms and the complexity of the substrates in landfills, they have been referred to as microbial pools. At the bottom of landfills, methanogens and acid-producing bacteria degrade organic materials and produce methane. *Syntrophus*, *Clostridium*, and some others produce methane with the monooxygenase enzyme in aerobic conditions in landfills by hastening the breakdown of hydrocarbons (Wang et al. 2017; Gieg et al. 2014).

The study of microbial diversity for understanding the microbial ecology in an ecosystem is not easy (Kumar et al. 2021). Because of the enormous diversity of phenotypes as well as genotypes, the characterization of the microbial community remains one of the most challenging aspects. Diversity refers to the variety of species in ecosystems as well as the genetic variability within each species, and it is thus the range of considerably different kinds of organisms and their relative abundance in natural assemblages and habitats. The amount and distribution of individual species information in a natural community are defined as biodiversity; therefore, a fair estimate of microbial biodiversity is also critical for understanding the functional activity of microorganisms in ecosystems.

There is currently a special interest in the relationship between biodiversity, which is defined as the quality and quantity of microbial species present in a particular ecosystem, and their function there.

Agenda 21, produced at the United Nations Conference on Environment and Development held in Rio de Janeiro in 1992, emphasized the relevance of biodiversity in ecosystem functioning. The document aided in scientific and international collaboration in order to gain knowledge of the biodiversity and the role of biodiversity in ecosystems. Most organisms are functionally unnecessary, according to experimental findings, and functional attributes of component species are at least as important as the total number of species in maintaining crucial processes. Microbial enzymes can also be used to convert harmful and hazardous waste into more environmentally acceptable forms (Paul et al. 2005).

Solid waste management reduces the negative impact of wastes on the environment and human health while simultaneously improving the economic growth and quality of life. There are many processes to manage solid waste after proper segregation processes like recycling, composting, dumping, harmonizing, etc.

## 26.5 Metagenomics

Metagenomics is an advanced molecular technique to identify the microbial diversity present in different types of wastes and is used for environmental cleanup (Kumar et al. 2020; Kumar and Chandra 2020). Metagenomic approaches are required to retrieve massive amounts of data from microbial DNA isolated from waste samples. Last year, soil metagenome sequencing revealed a new ecology of soil microbes as well as a good and powerful method for recovering novel genes and biomolecules (Wani et al. 2022).

Metagenomics is a culture-independent method. Microbial DNA, which is directly isolated from environmental samples, is cloned and analyzed. The following are the main steps:

1. DNA isolation
2. Purification and fragmentation of DNA
3. Insertion of the DNA into suitable vectors
4. DNA cloning and transformation of host cells

5. Delivery into a metagenomic library
6. Screening and analysis

Metagenomics describes the genomic analysis of microbial DNA directly from the communities present in samples, and it is a high-throughput gene-level study of mixed microbial communities. The metagenomic objective is to better study the microbial diversity, their composition, and activity of bioremediation in a polluted environment. Metagenomic methods, such as next-generation sequencing (NGS) technology, can provide information on enzymes and genes, which are involved in the degradation and detoxification of biodegradable wastes (Feng et al. 2018; Panigrahi et al. 2018; Mocali and Benedetti 2010) (Fig. 26.3).

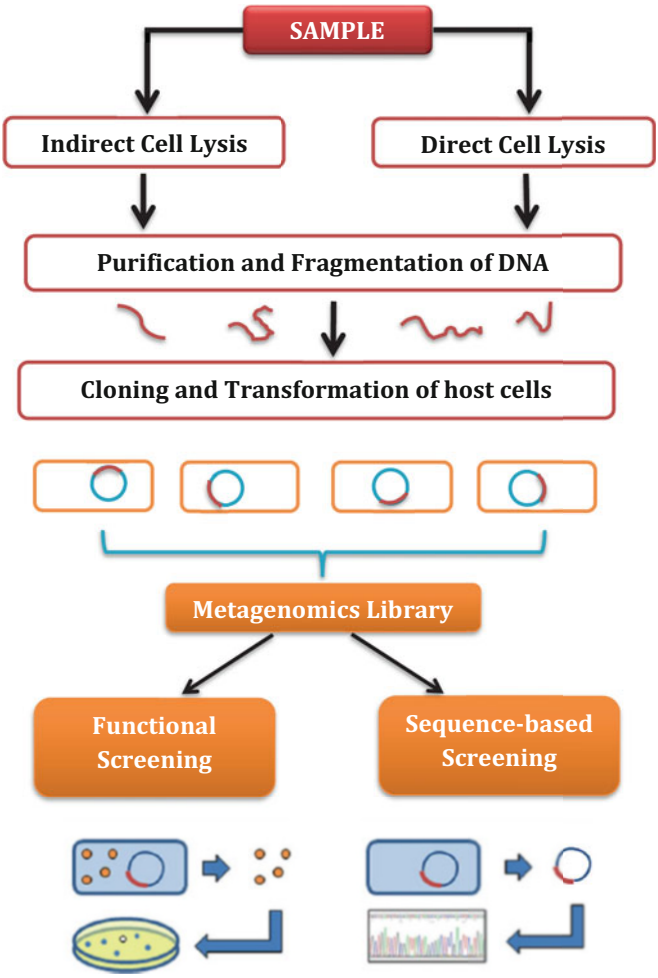


**Fig. 26.3** General work flow of metagenomics

To begin, constructing a sample-based library necessitates a substantial amount of DNA that represents the microbial population in a sample. Appropriate DNA extraction methods and cloning methodologies are required to clone whole gene clusters encoding metabolic pathways for secondary metabolites. PCR amplification, restriction enzyme digestion, and efficiency of cloning and transformation can be affected due to the contaminants present in the sample (Ghosh et al. 2018; Riesenfeld et al. 2004).

The second step is cloning DNA into suitable host cells with the use of different vectors for the preparation of sample libraries. Cloning strategies and vector selection are heavily influenced by the experiment's eventual goal. Plasmids (<15 kb), fosmids and cosmids (<40 kb), and BAC vectors (>15 kb) are examples of cloned vectors.

Functional screening and sequence-based screening are two metagenomic techniques. The first is based on clone metabolism, whereas the second is based on nucleotide sequencing. For genes encoding novel enzymes or drugs in general, a functional screening technique is performed instead of a sequence analysis. For the examination of metagenomic libraries, microarray technology is used (Verma and Sharma 2020).

## 26.6 Future Prospects

In recent years, the whole world has been facing the major problem of different types of wastes. Data show that developed countries generate 521.95–759.2 kpc waste and 109.5–525.6 kpc wastes are generated by developing countries. There are many microbial communities present in waste sites, which degrade and detoxify organic and inorganic wastes. There are many types of microorganisms like methanogens and acid-producing bacteria present at the bottom of landfills, which degrade organic materials and produce methane. Metagenomics and genomics are powerful tools for exploring microbial diversity, and, with the help of these techniques, we also examine uncultivated microorganisms. Function-based metagenomic studies will help discover many new enzymatic activities. These techniques also help optimize the different types of degrading and detoxifying enzymes, which are present in microorganisms. These molecular-based microbial profiling techniques can better understand the community structure and functions of microbial diversity available in environmental samples.

## 26.7 Conclusions

The aim of this chapter is to use DNA sequencing and analysis to better understand the composition and operation of complex microbial diversity found in environmental samples. In waste sites and contaminated places, there are numerous microorganisms. A municipal landfill is a man-made ecosystem that supports a diverse range of microbial cultures. Organic and inorganic contaminants are broken

down and detoxified by microorganisms. Metagenomics is an advanced molecular technology for identifying the microbial variety contained in various sorts of wastes, and it is used to clean up the environment. Metagenomics is a high-throughput gene-level investigation of mixed microbial communities as well as the genomic study of microbial DNA directly from the communities present in samples.

## 26.8 Summary

Waste disposal is the key element of environmental protection. Waste management provides the development of recycling programs. Waste can be used in two ways by reusing and recycling through thermochemical and biochemical technology. Poor waste management is linked to a rise in health concerns, ranging from vector-borne disease epidemics to the negative effects of groundwater contamination. The final action is disposal in landfills through incineration by gaining residue products like fertilizers, power generation, liquid fuel, heat, etc. Moreover, use of microorganisms provides major benefits like decomposition of dead organic wastes of plants and animals and converting them into simple substances and also promoting methane production by accelerating hydrocarbon degradation. Species diversity allows a more varied and flexible response to environment changes. It is essential to the ecosystem's functioning since it is necessary to maintain ecological processes such as organic matter decomposition, nutrient cycling, soil aggregation, and pathogen control. To identity microbial diversity, metagenomics is an advanced molecular technique. It analyzes genomic data, providing knowledge of the species. The basic aspect of metagenomics is to provide genomic analysis of microorganisms by direct extraction and cloning of DNA from their natural environment.

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# CRISPR/Cas-Mediated Functional Gene Editing for Improvement in Bioremediation: An Emerging Strategy

27

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**Abstract**

The recent surge in high-throughput genomic profiling has resulted in the identification of an extensive range of genes that respond to various functions in biological systems, including advancements in bioremediation. Pollution has been steadily increasing over the years, with the major sources being in the soil, water, and air with serious consequences for human health and well-being. Human activities like mining, utilization of industrial waste, fossil fuels, and the widespread use of agrochemicals are among the most significant sources of pollution today. Researchers can change the regulation of gene expression at specific places via genome editing, thus providing new insights into the advancements of three major areas of bioremediation: microbial bioremediation, phytoremediation, and mycoremediation. Recent advancements in this technology have resulted in a breakthrough using clustered regularly interspaced short palindromic repeats (CRISPR)-assisted type of remediation. CRISPR is a game-changing genomic tool that allows plants, bacteria, and fungi to improve specific

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characteristics. We summarize the current progresses in our understanding of CRISPR/Cas-based functional gene editing for improvements in microbial bioremediation, phytoremediation, and mycoremediation in this chapter. Furthermore, we have discussed recent strategies involving CRISPR/Cas in the improvement in bioremediation for successful waste management.

**Keywords**

CRISPR/Cas · Bioremediation · Phytoremediation · Mycoremediation · Waste management

## Abbreviations

| | |
|---|---|
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| gRNA | Guide ribonucleic acid |
| MTBE | Methyl tert-butyl ether |
| PAHs | Polycyclic aromatic hydrocarbons |
| PCBs | Polychlorinated biphenyls |
| PCR | Polymerase chain reaction |
| rRNA | Ribosomal RNA |
| RT-qPCR | Real-time quantitative PCR |
| TALENs | Transcription activator-like effector nucleases |
| TCE | Trichloroethylene |
| T-DNA | Transfer deoxyribonucleic acid |
| ZFNs | Zinc-finger nucleases |

## 27.1 Introduction

Today, pollution has become a serious issue, and bioremediation can help clean up pollution sources. Because of the ongoing development of new contaminants as a result of anthropogenic activities, remediation approaches, for example, chemical and physical approaches, are insufficient to address pollution issues. Traditional remediation methods are being replaced with bioremediation using bacteria, plants, and fungi, which is a more ecologically friendly and socially acceptable approach. Agriculture and the expansion of industry are the key components of modern human civilization. Over the past few decades, extreme use of pesticides as well as chemical fertilizers in agricultural techniques has damaged the environment (water, land, and air). The accidental discharge of harmful organic and inorganic compounds, as well as heavy metals, is one of the adverse side effects of industrialization (Larsson 2014; Cheng 2016).

Anthropogenic waste compounds are the chief source of environmental contamination, particularly in developing nations like India (Nigam and Shukla 2015;

Francová et al. 2017; Chandra and Kumar 2017a, b; Kumar et al. 2021a, 2022a). The principal notable pollutants released are industrial by-products, paints, chemical solvents, polycyclic aromatic hydrocarbons (PAHs), etc., which are harmful to the environment (Perelo 2010; Deblonde et al. 2011; Xu et al. 2016). Besides, excessive production and use of nanomaterials endanger human health and ecosystems, thus resulting in biodiversity loss (Chen et al. 2017a, b). These substances can be carried into the marine environment via rivers, mainly the coastal marine ecology, once they have been released. The continual cycling of these compounds via volatilization and condensation has resulted in their presence in rain, fog, and snow, with deleterious impacts on the biogeochemical cycle and harmful consequences to all aspects of the surrounding environment (Boehler et al. 2017; Chakraborty et al. 2017). This issue is a global problem with millions of polluted sites estimated. Direct or indirect contact with these chemicals causes undesirable health consequences and harms all kinds of lives on Earth.

Water scarcity is expected to affect 80% of the world's total population, with pollution and water resource engineering developments being the leading culprits in the most affected areas (Vörösmarty et al. 2010). To protect a healthy atmosphere and identify the existing extremely polluted places, these pollutants must be removed from waste streams to prevent them from spreading in the environment. In certain situations, pollutant remediation is carried out using long-established physicochemical approaches, for instance, evaporation, filtration, combustion, solidification, oxidation and reduction, electrochemical treatment, reverse osmosis, ion-exchange methods, and chemical precipitation (Porcelli and Judd 2010; Erdem and Özverdi 2011; Aksoy et al. 2014; Shi et al. 2014; Vasudevan and Oturan 2014; Kanadasan and Abdul Razak 2015). However, these traditional techniques are restricted by high reagent necessities, high handing out costs, enormous volumes, and the release of inferior environmental contaminants. When dealing with mine effluents, contaminated groundwater, and other industrial wastewater, the limits of these approaches become obvious (Bhalara et al. 2014; Dasgupta et al. 2015). Bioremediation, on the contrary, is a more efficient and environmentally friendly strategy that depends on bacteria's and plants' inherent ability to remove or neutralize contaminants found in the environment (Eevers et al. 2017; Singh et al. 2017; Kumar and Chandra 2020; Kumar et al. 2022b). The pros and cons of bioremediation are explained in Table 27.1. Plants and an extensive range of microbes, including fungi, algae, and bacteria, have been revealed to have a high level of neutralization ability (Bell et al. 2014; Shukla et al. 2014). Because it mostly relies on enzymes generated by bacteria and plants and participates in metabolic pathways, bioremediation with microbes and plants is more acceptable. These bacteria target contaminants, either totally degrading them or converting them into less hazardous compounds (Nikolaivits et al. 2017; Sivaperumal et al. 2017).

Researchers can utilize genome editing to modify gene expression regulation at targeted places, allowing them to get novel insights into functional genomics more quickly (Wolt et al. 2016). The incredible progression in these new techniques has surely accomplished a breakthrough by efficiently using CRISPR-assisted phytoremediation (Kaur et al. 2018). CRISPR is a game-changing genome editing

**Table 27.1** Pros and cons of bioremediation

| Technology | Pros | Cons | Examples |
|---|---|---|---|
| In-situ | Cost-efficient in nature<br>Noninvasive and comparatively passive<br>Natural reduction process | Environmental restrictions<br>Unlimited treatment time (not fixed)<br>Difficulties in monitoring the system | Bioaugmentation<br>In situ bioremediation process<br>Biosparging<br>Bioventing |
| Ex situ | Cost-proficient<br>Lower cost<br>Site-specific in nature | Needs space<br>Lengthy treatment period abiotic loss must be controlled and mass transfer issues must be addressed | Biopiles<br>Landfarming<br>Composting |
| Bioreactors | Rapid degradation<br>Kinetic<br>Optimized environmental parameters | Here, the soil needs excavation<br>Comparatively high cost | Aqueous reactors slurry reactors |

tool that allows for the development of specific plant features. It has become one of the most promising functional genomic tools (Piatek et al. 2015). The CRISPR/Cas method is a combination of CRISPR along with Cas proteins (Bortesi and Fischer 2015). This strategy is a practical method for improving phenotypes; additionally, it functions flawlessly on plant-based genomes. Furthermore, it is reported to be extra-predisposed toward monocotyledon agricultural plants due to their elevated guanine–cytosine (GC) content (Miao et al. 2013). A number of the most challenging plant alterations, which were previously difficult to modify, are stated to be simple using this tool (Bolotin et al. 2005). The CRISPR technology assists in altering the DNA that can then be employed to alter plant genetic links in order to reduce the negative effects of toxicants as well as other pollutants in the soil. Through plant–microbe–microbe interactions, this technique also improves nutrient uptake and metal bioavailability in plants (Abhilash et al. 2012).

Furthermore, CRISPR-based bioremediation could be utilized to efficiently purify the soil profile, eliminate hazardous elements from the rhizosphere, and reuse them for agricultural purposes and other beneficial initiatives such as agroforestry as well as social forestry (Thijs et al. 2016). An effective bioremediation necessitates the finding of main genes connected to the plant–microbe signaling network as well as an understanding of gene functions. Gene editing technologies such as CRISPR could be used to modify target genes. As a result, the phytoremediation properties are enhanced, and it could be useful in a variety of atmospheres subjected to its extremes (Basu et al. 2018). Taking these concepts into account, our aim here is to represent the work done so far in CRISPR/Cas-mediated functional gene editing for improvements in microbial bioremediation, phytoremediation, and mycoremediation. Furthermore, we have discussed the recent

strategies involving CRISPR/Cas in the improvement in bioremediation for successful waste management.

## 27.2 What Is Known About Bioremediation?

Microorganisms may eliminate many pollutants from the environment through a variety of enzymatic activities. We found a huge number of bacteria, which carry out bioremediation reactions (Wackett 1997; Anderson and Lovley 1999; Kumar et al. 2018; Kumar and Chandra 2018). An incessant kind of bioremediation includes the oxidation of unsafe, natural toxins to nonpoisonous mixtures, most frequently carbon dioxide. The vigorous breakdown of a wide range of natural foreign substances has been broadly explored, from aromatic hydrocarbons like benzene to xenobiotics like insect sprays (Wackett 1997), regardless of the way in which a wide assortment of microorganisms is equipped for high-impact toxic degradation (Wackett 1997; Anderson and Lovley 1999). The *Pseudomonas* species and firmly related living beings stand out enough to be noticed because of their capability to break down a broad scope of contaminants.

Many polluted circumstances, like sea-going residues, aquifers, and lowered soils, are regularly anoxic, and, certainly, microorganisms can anaerobically oxidize numerous pollutants utilizing optional electron acceptors, like sulfate, nitrate, and Fe (III) oxides (Anderson and Lovley 1999; Lovley 2001) and also potentially electrodes (Bond et al. 2002). Depending on the electron acceptor availability and the competition for electron donors among different respiratory infection-causing bacteria, the utilization of these abovementioned electron acceptors is usually assigned to a separate category. In underground environments, Fe(III) is habitually the most latent electron acceptor for oxidation of natural materials (Lovley et al. 2004), and expanding the accessibility of Fe(III) for microorganism decrease can enormously increase the anaerobic breakdown of natural pollutants (Lovley et al. 1994, 1996). The *Geobacter* species that can absolutely oxidize organic substances with Fe(III) reduction (Lovley 2000) are substantially enriched in subsurface climates where organic pollutants are oxidized with Fe(III) reduction. Sulfate is a significant electron acceptor for the anaerobic breakdown of toxins in oceans because of its enormous concentration in saltwater (Coates et al. 1997), and the extra sulfate in groundwater can extraordinarily speed up degradation in aquifers (Anderson and Lovley 2000). Sulfate-decreasing microorganisms, for example, the *Desulfobacula* and *Desulfobacterium* species, can oxidize hydrocarbons (Spormann and Widdel 2000).

In bioremediation, some contaminants accept electrons rather than electron transfer. Reductive dechlorination is indeed a type of bioremediation in which microbes remove chlorines from toxins, including chlorinated solvents as well as polychlorinated biphenyls, and use these substances as electrophiles in respiration (Mohn and Tiedje 1992; Chandra and Kumar 2015). Numerous organic entities are recognized to be fit for dehalogenation (Holliger et al. 1998); however, as clarified further below, the *Dehalococcoides* species seem to assume an especially significant

part in catalyzing this interaction in tainted subsurface settings. A few microscopic organisms are equipped to corrupt inorganic contaminations, for example, nitrate and perchlorate, transforming them into innocuous side effects (Anderson and Lovley 1999; Coates and Anderson 2000). Metals are one more kind of poison that can go about as an electron acceptor in microbial metabolism (Anderson and Lovley 1999). Metal decrease does not annihilate them; however, it normally alters their dissolvability. The *Geobacter* species could use uranium (U) as just an electron acceptor, converting the soluble, oxidized type of uranium, U(VI), to an undissolved structure, U(IV) (Lovley et al. 1991), hence promoting *Geobacter* growth in uranium-contaminated subterranean habitats. This causes the uranium in polluted groundwater to precipitate, thus stopping it from spreading further (Anderson et al. 2003).

Without the need for human intervention, microbial cleansing of the environment occurs frequently. "Intrinsic bioremediation" is the term for this process (Maitra 2018). Fundamental bioremediation is becoming the strategy of choice if it could be illustrated that pollution does not really pose an instant health hazard and remains localized as well. Assuming that natural bioremediation is an extremely slow process, ecological circumstances can be adjusted to speed up the action of microorganisms capable of degrading or immobilizing pollutants of concern. Designed bioremediation methodologies incorporate the expansion of electron contributors that enable the development or digestion of microbes involved in bioremediation, the expansion of supplements that restrict the development or action of the microbes, and the change of microbes with desired properties.

## 27.3 Pre-genomic Approaches Toward Bioremediation

The 'bioremediation research,' wherein pollution in air tests are incubated in a research facility and the rates of contaminant immobilization are reported, is currently used in most practical microbiological analyses of bioremediation (Kumar et al. 2021b; Agrawal et al. 2021). This type of examination assesses the likely metabolic action in a population of microbes but provides little information about the microbes accountable for bioremediation or why certain changes that can be examined for planned bioremediation presentations generate action while certain others do not . While focusing on bioremediation processes in greater depth, efforts are generally taken to limit capable life forms (Allard and Neilson 1997). Pure culture isolation and characterization have been and continue to be vital for the creation and evaluation of molecular biology investigations.

Researchers can examine not just the biodegradation responses of these isolates but also other features of their metabolism, which are believed to influence their proliferation as well as action in contaminated environments, as detailed below.

Prior to using molecular methods in bioremediation, it was unclear whether the microorganisms isolated during the process were relevant to in situ bioremediation or whether they were rapidly generated and were thus not the primary organisms accountable for the environmental processes.

## 27.4 What Microorganisms Are Present Based on the 16S rRNA Approach?

The 16S rRNA approach is a well-known method for reporting microorganisms that comprise the microbial system, and it represents a vital progress in the area of microbial systems (Amann et al. 1995; Watanabe and Baker 2000). This has been beneficial to the field of bioremediation since it means that researchers could conclusively determine the phylogenetic order of microorganisms linked to bioremediation by studying the 16S rRNA sequences in pollutant environments (Allard and Neilson 1997; Watanabe and Baker 2000). One of the conclusions drawn from the 16S ribosomal RNA method for bioremediation is that bacteria that thrive throughout bioremediation are inextricably linked to living forms that could be refined from natural environments (Lovley 2001).

This contradicts the universal challenge in life sciences, namely, the difficulty in recovering the utmost environmentally related species (Amann et al. 1995). In impure aquifers where microbes oxidize pollutants by the reduction of Fe(III) oxides, for example, a considerable concentration in bacteria with 16S ribosomal RNA sequences is closely linked to those of the formerly cultured *Geobacter* species (Röling et al. 2001; Lovley et al. 2004; Bandopadhyay et al. 2020). The *Geobacter* species in an unadulterated culture are fit for oxidizing natural toxins through Fe (III)oxide reduction (Lovley et al. 1989), and it is reported that the *Geobacter* species play a fundamental role in impurity debasement in situ. The *Geobacter* species can likewise change solvent U(VI) to insoluble U(IV) and, in this manner, eliminate uranium from contaminated water (Lovley et al. 1991). At a point when uranium-tainted groundwater was treated with acetic acid derivation to empower the microbial decrease of U(VI), the quantity of the *Geobacter* species increased to a significant degree, representing up to 85% of the groundwater microbial community (Holmes et al. 2002; Anderson et al. 2003). In the month of springs where the native microbial populace was debasing the dissolvable trichloroethene (TCE), 16S rRNA successions that are almost like the 16S ribosomal RNA grouping of an unadulterated culture of the TCE-degrader *Dehalococcoides methanogens* were distinguished (Fennell et al. 2001; Hendrickson et al. 2002; Richardson et al. 2002). Microorganisms having 16S rRNA arrangements that are firmly connected with NaphS2, an unadulterated culture-accessible anaerobic naphthalene degrader, were viewed as particularly moved in marine dregs with high anaerobic naphthalene breakdown rates (Hayes and Lovley 2002). The power of groundwater to vigorously corrupt the fuel oxygenated methyl tert-butyl ether (MTBE) was strongly associated with the amount of organisms with 16S rRNA successions that were about 100% connected to the MTBE-debasing bacterium, strain PM-1, which is open in a pure culture method (Hristova et al. 2003).

The fundamental drawback of the 16S ribosomal RNA approach is understanding the phylogeny of bioremediation organisms, which does not essentially predict the main elements of its physiology (Pace 1997; Clarridge 2004; Kumar et al. 2020). Microbes with 16S ribosomal RNA sequences strongly linked to the TCE degrader *D. ethanogenes* can vary in the amount of chlorine that they can deteriorate, making

it difficult to predict which of these compounds an uncultured organism will degrade based on its 16S rRNA sequence alone (Bunge et al. 2003; He et al. 2003; Hendrickson et al. 2002). When there are not many similar organisms available in bacterial isolates, differentiating their physiology from their phylogeny becomes much more difficult..

## 27.5 Recent Genomic Tools and Techniques Used to Improve Bioremediation

The lack of knowledge regarding cellular processes, enzymes, and their encoded genes limits their utilization during the mineralization process in countless microbial communities (Zhao and Poh 2008). Genomics can help address this constraint by providing a global picture of the genetic material expressed in microorganisms during pollution exposure, such as DNA and RNA. This method mostly comprises genome sequencing and bioinformatic study with diverse tools and algorithms. A total of 270,567 genomic sequences of organisms have been sequenced recently, with more than 46,000 genome sequencing studies now underway worldwide. The genomes of certain microbes are sequenced, which is used in the bioremediation process (Lasa and Romalde 2017; Yang et al. 2017). *Pseudomonas* sp. KT2440's genome sequences (6.2 Mb) revealed the presence of numerous qualities encoding compounds or proteins, for example, cytochromes, dehydrogenases, sulfur-metabolizing proteins, ferredoxins, glutathione-*S*-transferases, oxidoreductases, and oxygenase efflux pumps, among others, which undertake an important role in the corruption of a few synthetic substances set free from modern effluents. Using the total genome arrangement of *Pseudomonas putida* CSV86, the chromosomal area of qualities liable for naphthalene debasement was explored (Belda et al. 2016). Moreover, *Arthrobacter* sp. strain's whole-genome sequencing of LS16 and YC-RL1 uncovered metabolic organizations engaged with the bioremediation of fragrant synthetic substances such as naphthalene, 4-nitrophenol, fluorene, bisphenol A, 1,2,3,4-tetrachlorobenzene, biphenyl, and p-xylene (Paliwal et al. 2014; Hassan et al. 2016; Ren et al. 2016).

Another investigation discovered multiple *bph* gene clusters in the genomic sequence of *Pseudomonas alcaliphila*, JAB1, encoding for biphenyl and its derivative degradation pathways (Ridl et al. 2018). Furthermore, based on whole-genome sequencing data, various scientific papers have proposed the role of other microbes in bioremediation of heavy metals, dyes, etc. Metagenomics is another genomics-based method that includes collecting genomic DNA directly from an ambient microbial sample and thereafter sequencing and analyzing it in a laboratory capable of producing bacterial pure cultures (Garza and Dutilh 2015; Baweja et al. 2016). This is the most utilized process for bacteria that cannot thrive in a laboratory setting. As a result, the metagenomic method can aid in the investigation of microbial diversity's diverse degradation paths, which are currently understudied and undiscovered.

The knowledge gained from these techniques can be used to produce added, capable, and customized microbes for precise bioremediation applications. Moreover, a better understanding of the many ways in which microbial populations interact with environmental contamination is necessary for determining the pollution recovery capabilities of polluted sites, which improves the bioremediation process' success.

The bioremediation capability of *Pseudomonas* in the case of diesel-tainted Canadian extreme frosted soils was investigated using a metagenomic method, and various hydrocarbon-debasing properties were discovered using real-time polymerase chain reaction (RT-PCR) (Yergeau et al. 2012). This approach has also been used to evaluate the PAH breakdown capacity of aerobic microbial populaces in soil (Duarte et al. 2017). A metagenomic examination of soil and water samples from beaches in Mexico affected by the deep water oil catastrophe revealed a strange spreading in *Vibrio cholerae*, a dramatic spreading in *Rickettsia* sp., and a drop in *Synechococcus* sp. (Kimes et al. 2013).

According to one study, the Ion Torrent technology was used to sequence directly isolated DNA. In the same study, a taxonomic analysis revealed the presence of *Proteobacteria* inside the metagenome along with *Firmicutes* in consortia specimens. In addition, the Biosurfactants but also Biodegradation Databases can be used (BioSurfDB), functional analysis of the samples revealed a continuous trend. The pathways involved in fatty acid and chloroalkane degradation were also observed to be enhanced in consortia samples. These data indicate that these consortia's principal application will be in driving residue bioremediation (Guerra et al. 2018).

rDNA technology provides a wide range of methods for tackling environmental challenges. Two recent applications of rDNA technology are bioremediation and waste treatment. In environmental biotechnology, natural microorganisms are used in several waste management applications. Bioremediation, which uses bacteria, is currently used to clean up contaminated streams, land, air, and lakes (Vashishth and Tehri 2015). Similarly, bacteria are utilized in the treatment of wastewater, industrial waste, sewage, and other types of wastes (Wagner et al. 2002). To protect the environment, microorganisms are used in bioremediation (Omokhagbor Adams et al. 2020). In bioremediation, *Nitrosomonas europaea* and *P. putida* are utilized (Chapman et al. 2006; Zuo et al. 2015). The easiest and safest approach to producing genetically engineered antibiotic-resistant bacteria is the incorporation of biomarkers. This may distress normal ecosystems, e.g., microorganisms with petroleum-debasing abilities destroy imported petroleum products (Robinson 2016). Bioindicators are also created using rDNA technology. These are bacteria that were genetically altered to be 'bioluminescent,' meaning that they may generate light in response to a range of chemical pollutants (Vashishth and Tehri 2015). These microorganisms have been utilized as biological markers to evaluate harmful chemical pollution in the environment. Biofuels are fuels that are good for the environment because they are made from biomass. They are both cheap and renewable. For large and useful biofuel production such as bioethanol, biodiesel, and biohydrogen, the application of rDNA technology is critical. Many microbes, particularly

cyanobacteria, are involved in the creation of hydrogen. Its creation, however, demands the employment of particular enzymes. By strengthening microorganisms, recombinant DNA technology has improved product tolerance and yield (Lü et al. 2011). These microbes are produced utilizing cutting-edge rDNA technology, resulting in high bioenergy output (Ganesh et al. 2012). Unlike traditional energy sources, bioenergy production does not emit $CO_2$ or other toxic substances. As a result, it contributes to environmental safety and cleanliness (Tiwari and Pandey 2012). This technique has been established to be effective in an extensive range of commercial compounds, especially energy transporters, including medium- and short-chain alcohols (Savakis and Hellingwerf 2015). Because they make better use of solar energy, some other crop plants are also used to generate biomass. Fermentation microorganisms are also employed in the production of biogas. All of this is only feasible because of the advancements in rDNA technology.

## 27.6 CRISPR/Cas Approaches in Bioremediation

Over the last 20 years, genome editing has gained popularity as a technique for creating advanced phenotypes to fulfill global demands. From sequence variations to mega-nitrogenous base cutting, everything was handled. Approaches based on combinatorial omics will be essential for revolutionizing genetic engineering tools (Fig. 27.1). Access to the CRISPR/Cas9 technology has the potential to significantly improve ambitious assigned tasks in plant biology. By faster gene identification and associated trait improvement among plant species, CRISPR/Cas9 techniques will help subjugate the expanding functional genomics and systems biological sciences data. Mammalian experimentation has been used in the majority of CRISPR/Cas9-based studies. The findings were assumed to apply to other model organisms, but this is not the case. Future CRISPR system research will demand the optimization of the sgRNA scaffold, which has strong binding affinity for the Cas9 protein. Aside from the known studies, additional composite genome plants such as wheat, sugarcane, and others should be further investigated in the field of bioremediation employing this method.

## 27.7 Phytoremediation

The CRISPR technology is utilized for the phytoremediation of plants, allowing for genome editing. Model phytoremediators like *Brassica juncea*, *Arabidopsis halleri*, *Noccaea caerulescens*, *Pteris vittata*, and *Hirschfeldia incana* among others have had their genomes completely or partially sequenced. The research phase of the phytoremediation procedure is substantially aided by this process of altering the plants' genetic sequence (Xie et al. 2009; Mandáková et al. 2015; Auguy et al. 2016; Yang et al. 2016; Briskine et al. 2017). The editing technique makes it easier to characterize and identify the genetic determinants involved in phytoremediation processes like phytoextraction, phytostabilization, phytodegradation, and

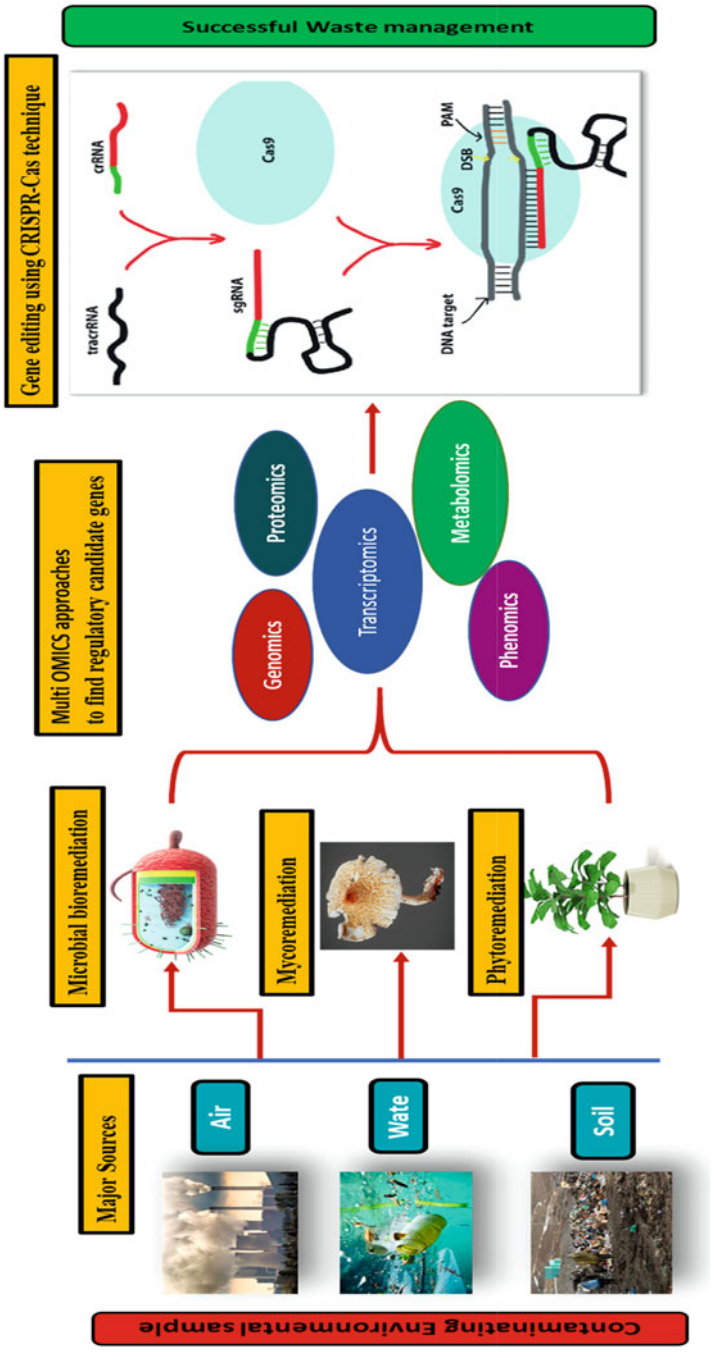


**Fig. 27.1** Multi-omics-based functional gene editing using the CRISPR/Cas technique for successful waste management

phytodesalination, among others. CRISPR systems are designed to target the pathways involved in pollution accumulation, volatilization, and decomposition, exposing them to phytoremediation with the process of targeted engineering (Estrela and Cate 2016). Plant differentiation from single cells, T-DNA-delivered gRNA–Cas9, modular cloning systems like GoldenBraid, and even the cloning-free technique can all be examined for their ability to promote phytoremediation. T-DNA-transported gRNA–Cas9 has also been explored (through *Agrobacterium*-mediated T-DNA transformation), but due to the transient effect of T-DNA in callus initiation, action in somatic tissues has been detected via genome integration (Bortesi and Fischer 2015; Mikami et al. 2015).

This strategy may require the combination of many gRNAs and Cas9 in a particular T-DNA, all in a plasmid process that would surely advance editing. Cloning methods such as GoldenBraid make it simpler to link pre-made DNA pieces to multigene complexes. Multiplex editing regulatory attempts offer the cloning-free technique to assure cell integration of a gRNA but at a lower throughput. However, the use of gRNA-guided dCas9 to control gene expression is a subgroup of CRISPR research that has the potential to be more useful for phytoremediation (Miglani 2017). Transcription factors could be useful in dCas9 to suppress or improve RNA polymerase transcription, which results in up- or downregulation of one gene or genes of interest. Plants have been successfully used to control genetic expression across a 1000-fold variety using CRISPRi and CRISPRa techniques (Gilbert et al. 2014; Piatek et al. 2015; Lowder et al. 2018). Tang et al. (2017) exhibited CRISPR's capacity to lessen Cd aggregation in rice by thumping down the OsNramp5 metal carrier quality. The last option is assuredly the main accomplishment of CRISPR in phytoremediation to date, exhibiting the innovation's true capacity in quality record guidelines. CRISPRi and CRISPRa may before long be utilized to control the outflow of qualities in plants and microorganisms that produce metal carriers, development factors, metal-solubilizing exudates, or oxidative pressure metabolites for phytoremediation.

CRISPR/Cas9 has been previously used for successfully editing the genomes of a variety of plant species (Estrela and Cate 2016; Song et al. 2016). Conventional breeding procedures require longer turnaround times for each experiment due to the complexities of plant genomes, such as high ploidy. Furthermore, site-specific mutagenesis is challenging due to the less frequency of homologous recombination (Estrela and Cate 2016). As a result of gRNA–Cas9 allowing for targeting multiple sequences of a wide range of phenotypes, there may be an increasing demand for improved plant biomass, growth rate, disease and climate resistance, metal tolerance, and metal accumulation. CRISPR/Cas9 systems have already been used to modify the genomic information of poplar as well as maize species known for their phytoremediation uses (Fan et al. 2015). Poplar is a popular option for phytoremediation owing to its increased biomass production, fast development, deep and wide root system, distinct ability to adapt to diverse soils and climates, marked tolerance to organic and inorganic pollutants, and exceptional ability for vegetative propagation, which aids in spreading (Baldantoni et al. 2014). In contrast, maize is a fast-growing, high-yielding species with a high metal accumulation

capacity that has shown promising results in phytoremediation and phytomining (Anderson et al. 2005; Ali et al. 2013). Agarwal et al. (2018) summarized the significant advances in maize genetic modification using the CRISPR/Cas9 system to date. More importantly, both poplar and maize have been increasingly used to combine their phytoremediation capabilities with bioenergy generation (Meers et al. 2010; Pandey et al. 2016), thus demonstrating that CRISPR can be used not only to increase process efficiency but also to generate bioenergy on a larger scale.

The CRISPR/Cas9 system, as available now, has had a massive effect on expanding the number of plants that can be subjected to genetic modifications (Seth and Harish 2016). Several factors have contributed to this, the most important of which was the accessibility of plant exome sequencing, which was aided more by the presence of development tools and other computational biology techniques. The limitation modeling approach is used in mathematical modeling to explain physiology (Zhu et al. 2016). Similarly, flux balance analysis for evaluating metabolic pathways (Maurice Cheung et al. 2015) and omics methods for evaluating genome integration are available (do Amaral and Souza 2017; Flexas and Gago 2018). Cas9 variants that are codon-optimized for both dicots and monocots benefit from this process, allowing genome editing to be performed on a diverse range of plants. Phytoremediation recycling entirely centers around the expression levels via clustered regularly interspaced short palindromic expressions to improve the synthesis of metal ligands (e.g., metallothioneins and phytochelatins) as well as the formulation of metal transport proteins (i.e., from the CDF, HMA, and ZIP families), plant growth hormones, other plant growth factors (CKs and GAs), and root exudates (particularly siderophores) (Prasad and Aranda 2018). Several genetic transfers have been shown to increase phytoremediation, as proven by several studies conducted since 2000, which included the transmission of specific plants and bacterial genes to target plants and demonstrated good results for the phytoremediation (Pandey and Singh 2018). NAS1-enhanced Arabidopsis and tobacco crops had significantly increased tolerance to metals like Cd, Cu, Fe, Ni, and Zn as well as increased uptake of metals like Mn and Ni. When metallothionein-encoding genes (such as *MTA1*, *MT1*, and *MT2*) were overexpressed, tobacco and Arabidopsis plants demonstrated an increase in their ability to withstand and accumulate metals such as Cd, Cu, and Zn (Sebastian et al. 2018). When the metallothionein gene *MT2b* is expressed, *H. incana* increases its tolerance and, as a result, accumulates Pb. When the genes *APS* and *SMT* were introduced into *B. juncea* plants, they demonstrated greater tolerance to Se. These two genes are responsible for the synthesis of ATP sulfurylase and selenocysteine methyltransferase, respectively (Lv et al. 2013). CRISPR systems have the potential to greatly boost these genes to significantly newer and higher levels, and Fasani et al. (2018) have recently evaluated all of these genes, as well as numerous more, which can be enhanced in this manner. They analyzed all of these genes with the potential for augmentation, utilizing CRISPR technology in a publication regarding the reclamation of metal-polluted soils by transgenically changed plants that have been transgenically modified. Their investigation, which is rather lengthy in paper, contains a detailed explanation of the individual genes that were transferred, their unique sources, and their impacts on the target plant. The

results ranged from increased tolerance to toxic levels of metals present to increased metal absorption capacity, which, in some cases, led to metal hyperaccumulation (Huang et al. 2019). These effects may be advantageous to phytoremediation. It should be noted, however, that allowing the accumulation of a specific metal in the target plant to occur through the introduction of any specific gene can result in the development of hypersensitivity to that specific element in the plant in some cases, invariably with the possibility of causing plant decay. When the plasma membrane protein (NtCBP4) was overexpressed in tobacco plants, it boosted the plant's ability for Pb accumulation while also increasing its sensitivity to Pb. Similarly, in Arabidopsis and tobacco plants, the *Mer C* gene increased Hg metal accumulation while simultaneously making the plants hypersensitive to Hg (Fasani et al. 2018). Additional pollutants including not only polycyclic aromatic hydrocarbons but also polychlorinated biphenyls are also targeted by this method (Banerjee and Roychoudhury 2018). This method also employs explosives including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). Genes associated with plant organic xenobiotic detoxifying have been found in several research studies (Jaiswal et al. 2019). The findings of these studies can be utilized to seed future experiments employing CRISPR-based augmentation of the systems in plant enzymes responsible for the removal and subsequent detoxification of organic pollutants. These techniques could also be utilized to address pollutants like polychlorinated biphenyl and polycyclic aromatic hydrocarbons. CRISPR-mediated development of plant enzyme systems that remove contaminants should be pursued further (Pandey and Singh 2018). Recently, genes expressing tolerance to naphthalene and phenanthrene have been generated in rice and Arabidopsis by altering the genes responsible for naphthalene dioxygenase synthesis (Peng et al. 2014). Tolerance to polychlorinated biphenyls (PCBs) and 2,4-dichlorophenol rose considerably after altering the *bph* gene expression in alfalfa plants (Wang et al. 2015). Another study with Arabidopsis plants discovered that the cytochrome P450 reductase complex effectively cleaned up RDX pollutants. The *XplA* and *XplB* genes of the bacterial genome were found to be transferred, whereas the cytochrome genetic makeup was altered (Rylott et al. 2015). CRISPR has also been recognized for its potential to engineer more growth-promoting rhizobacteria. The plant–microbe interaction, also known as the microbiome, plays an important role in conferring tolerance on the plant in terms of rhizobacteria, phytohormone levels, and nitrogen fixation capacity (Mosa et al. 2016). Controlling phosphate ion solubility and other direct and indirect methods could improve the plant's capacity for pollutant reclamation (Chinnaswamy et al. 2018; Thode et al. 2018). Even though CRISPR/Cas9 is an excellent tool for genome editing in plants, the results differ. Many factors, including target regions inside plants, delivery mechanisms, and plants with variable genetic makeup, play a role in determining the success response rate. Although they have shown great promise in terms of enhancing the cleanup process within facilities, all of these findings are still being kept in labs for the time being. The field success ratio must still be documented.

## 27.8 Microbe-Based Bioremediation

To deal with all these toxic metals, microbes have developed a resistance system, which is based on the stability of take-up and efflux procedures (Pedone et al. 2004). Many of these processes are also discovered in mesophiles, and they can display distinctive properties in thermophilic bacteria/archaea (Ranawat and Rawat 2018). The understanding of heavy metal resistance methods in thermophiles has been aided by the gene expression system, which allows for such proof of identity of presumed molecular factors (Panyushkina et al. 2019; Aulitto et al. 2021). A minimum of four heavy metal resistance mechanisms have been proposed to date: periplasmic barrier, effective metal cation transport (efflux), enzyme-mediated metal–ion minimization, and intracellular sequestering (Cha and Cooksey 1991; Cervantes and Gutierrez-Corona 1994; Bruins et al. 2000; Cervantes et al. 2001; Cazorla et al. 2002; Cánovas et al. 2003; Lin et al. 2006). Some bacteria are capable of constructing complexes or chelating agents with cell surface polymers, thereby limiting metal absorption (El-Helow et al. 2000). Heavy metals, on the other hand, can bypass this framework and go into the cell via uptake systems, such as arsenic, which goes into the bloodstream through the glucose or phosphate transporter system (Yang and Rosen 2016). Heavy metal resistance is generally caused by the synchronized function of subcellular enzymatic oxide reduction as well as heavy metal efflux pumps, both of which take in ATP and push the toxic metal outside the cell (Schelert et al. 2004; Smith et al. 2014). Operons are generally used to start organizing resistant genes, which also ensure the expression of transcriptional activation that regulates the overall structure. While defining intracellular sequestration of heavy metals, the basic mechanism predicts that some cysteine-rich proteins will generate metal–ion or thiol–metal complexes (Ranawat and Rawat 2018). *Escherichia coli* possesses a copper proactive transportation system (Cop A) and a multicopper oxidase-based system (Cue O) for periplasmic copper detoxification as well as the CusCFBA transport system (Bondarczuk and Piotrowska-Seget 2013). The abovementioned mechanisms of resistance are frequently stimulated as a response to stress (Bartolucci et al. 2013; Ranawat and Rawat 2017), and recognizing their molecular basis is critical for implementation in metal contamination monitoring systems (biosensing) and/or for establishing bioremediation processes (Gallo et al. 2018; Vasudevan and Jayshree 2020). Biometallurgy is a biotechnology subfield that analyzes the interrelations of microbes with metals or metal-bearing minerals (Zhuang et al. 2015). It includes microbial activities such as heavy metal adsorption, bioaccumulation, and biomining; such procedures are vital to the source of critical materials, since they have the ability to provide eco-efficient alternatives to conventional pyro- or hydrometallurgical measures (Hennebel et al. 2015; Garole et al. 2020), and thus play a role in the development of metal biomonitoring and bioremediation approaches. The utilization of thermophiles in this context has many benefits due to their sustainability in extreme states and deteriorate refractory mineral organisms. Moreover, they have the capacity for metal bioremediation and/or in situ recovery in any environment (Castro et al. 2019).

Zinc-finger nucleases, activation effector nucleases, and endogenous homologous recombination systems were the dominant genome engineering methods until 2013 (Kim et al. 1996; Cava et al. 2007; Li et al. 2012). These techniques employed designed fusion proteins that combined a DNA-binding domain with the nonspecific nuclease domain of the restriction enzyme Fok1. These processes were widely used to modify eukaryotic microbe genomes. A new genome editing technology based on RNA-guided engineered nucleases (the CRISPR/Cas9 system) has recently emerged. Despite being unearthed in the late 1980s (Ishino et al. 1987), the function of a CRISPR array was unknown until 2005 (Pourcel et al. 2005). It was discovered to be a bacterial innate defense system only in 2007 (Barrangou et al. 2007; Mohanraju et al. 2016).

When it was proven that target genomic DNA could be reconfigured by removing 20 nucleotides inside the CRISPR RNA (crRNA) and that the crRNAs trying to target specificity could be combined with both the structural features of the tracrRNA inside a chimeric single-guide RNA, the CRISPR/Cas platform was transformed from just a biological condition to a platform for genetic manipulation (sgRNA). In addition, evidence that sgRNAs with other specificities might be produced, which are allowed for the concurrent modification of more loci, lends validity to CRISPR mania (Jinek et al. 2012; Pennisi 2013). Innumerable gene editing applications have been developed for animal and human cells and have lately been adapted for use in bacteria for genome editing, transcriptional control, and extensive genome screening. In actuality, using the CRISPR/Cas system, some genetic modification prokaryotes were acquired. When trying to apply it to thermophiles, the fact that this genetic editing tool is premised on a mesophilic system poses a problem. In current history, a heat-stable gene editing tool based on the thermophilic Cas9, which can be used at temperatures up to 55 °C and contains everything needed for genome editing inside a single plasmid, has been developed; with the emergence of the ThermoCas9, genome modification in moderate thermophilic bacteria has now become possible, attempting to make production much more pleasant and time saving as well (Mougiakos et al. 2017).

A genome-based editing tool for reasonable thermophiles was developed by the use of Cas12a from the genome of *Francisella novicida* (Mohanraju et al. 2021); this methodology allows knockout mutants to be generated in much less time than 7 days with high editing effectiveness. The FnCas12a has the right to modify the genomes of several archaea and thermophilic bacteria. The proteins Cas9 and 12a are the clustered regularly interspaced short palindromic multi-domain nucleases that break DNA strand targets by the use of guide RNA. The protein Cas9 originally belonged to the type II-a family, whereas the protein Cas12a corresponds to the type V family. The initial enzyme works extremely well, and it edits the genome with the nuclease. Cas12a has emerged as a promising option in the past few decades. These isoenzymes have diverse origins, evolution, and structural configurations, resulting in various molecular pathways; in fact, Cas9's as well as Cas12's nuclease activities, as well as the subsequent DNA repair results, are influenced by cell type, target sequence, and genetic contexts (Swarts and Jinek 2018). Their genetic variations influence them being used as genome editing tools. In some organisms, Cas9 activity

is the greatest option, whereas in others, Cas12a is the best option. The features of Cas9 and Cas12a, as well as those of their developed variations, are complementary, resulting in a powerful and versatile toolset.

## 27.9 Mycoremediation

Mycoremediation holds great promise for cleaning up hostile environments contaminated by polyaromatic organic contaminants. Recognizing the genetic as well as the molecular foundation of a recovery method is necessary to tie together the natural mechanism for sensible uses. However, when attempting to compare with specific bacterial pathways, the fungal deterioration system has attracted scant attention. Moreover, our understanding of the genetic grounds for metabolic activity remains extremely limited. Genomic approaches, coupled with advances in next-generation sequencing methods, have sparked a new degree of comprehension in the mycoremediation method, which is expected to result in a revolution in the field (Malla et al. 2018).

Over the previous decade, a large amount of fungi have already sequenced their genomes to explore their possibility for bioremediation from entire genomes (Min et al. 2015; Blasi et al. 2017; Morales et al. 2017; Singh et al. 2019). From various fungal genomes, a large set of gene-encoding biocatalysts like proteases, lipases, cellulases, and glycosidases have indeed been recognized. Each genome's many prevalent metabolic pathways have indeed been anticipated and reproduced. Comparative genomics could indeed disclose the major differences in metabolic processes between genomes and also their physiological abilities for mycoremediation (Tkavc et al. 2018). Gene cluster prediction has also aided in the rebuilding of complex metabolic processes and the proof of identity of the novel as well as unusual genes involved in the process (Blasi et al. 2017). These approaches, even so, are far off from understanding the complete mycoremediation procedure in the absence of experimental verification. The most popular technique in the bioremediation process is the oxidation of lethal organic pollutants to produce harmless substances (Tuomela and Hatakka 2019). Oxygen is perhaps the most prevalent acceptor for microbial respiration and the agent responsible for aerobic decomposition of a wide range of pollutants throughout bioremediation. Although both bacteria and filamentous fungi have the possibility for use in bioremediation, fungi have just a few benefits over bacteria. Mycelial systems could indeed enhance chemical bioactivity, and their catabolic enzymes are compatible with a wide variety of metabolic functions (Deshmukh et al. 2016). Furthermore, because lignin-degrading enzymes are also extracellular, ligninolytic fungi can transform toxic chemicals without having to transport pollutants all over the cell membranes to fulfill cytosolic enzymes (Novotný et al. 2004). Oxidoreductases, ligninolytic enzymes, cytochrome P450, as well as dioxygenase, a specialized enzyme in mycoremediation, are some of the enzymes involved in mycoremediation. The procedures of gene tagging in these enzyme-coded genes have not yet been studied.

## 27.10 Contributions of the CRISPR/Cas System to Bioremediation and Future Strategies

Traditional metabolomics using the recombinant DNA technology is singularly suitable for trying to introduce a small number of genes, so renovating a whole metabolic process necessitates the use of many genes involved (Gaj et al. 2016). It demands advanced technology, constantly adding an entire set of genes for a selected process to silence multiple genes, thus editing to redirect a presented pathway in a certain direction (Xia et al. 2019). For metabolic engineering, nuclease-mediated genome editing can be a viable option. This premise is rapidly changing following a breakthrough in the field of nucleases that are used in genetic engineering (Choi et al. 2019). The principal genome editing tools are ZFNs (zinc-finger nucleases), TALENs (transcription activator-like effector nucleases), and the CRISPR/Cas (clustered regularly interspaced short palindromic repeats) system (Gupta and Shukla 2017; Liu et al. 2019). A comparison of the CRISPR/Cas system, TALENs, and ZFNs for genome editing is provided in Table 27.2.

However, given the fact that this innovation is still in its initial stages, it is not widely used for bioremediation. There are some limitations to microbial genome editing with modern nucleases, which limit its applications in the areas of bioremediation. Some of the constraints of microbial genome editing are that is expensive, time-consuming, has the same large size of nucleases, which hinders genome editing

**Table 27.2** A comparison of the CRISPR/Cas system, ZFNs, and TALENs for gene editing systems

| | CRISPR/Cas | Zinc-finger nucleases | TALENs |
|---|---|---|---|
| Recognition sites | RNA–DNA | Protein–DNA | RNA–DNA |
| Vital elements | Cas 9 protein and guide RNA | Zinc–Fok fusion protein | TALE–Fok1 fusion protein |
| Function | Here, the guide RNA recognizes the target DNA sequence, which is next to an NGG motif; Cas9 encourages DSBs of DNA DSBs that are necessitated by NHEJ/HDR | Here, zinc-finger proteins recognize the target DNA sequence dimerization of Fok1; nuclease DSBs of DNA DSBs are necessitated by NHEJ/HRD | Here, the TALE proteins recognize the target DNA sequences, which help in dimerization of Fok1; nuclease DSBs of DNA DSBs are necessitated by NHEJ/HRD |
| Pros | • Easy to conduct<br>• Highly efficient in nature<br>• Capable of editing multiple sites at a time | • Highly efficient<br>• More specific in nature | • Extremely efficient<br>• More and more specific in nature |
| Cons | • Requires the PAM motif besides the target sequence | • Requires large-scale screening process<br>• Time-consuming<br>• Expensive during construction | • Additionally tedious<br>• More time-consuming for construction |

effectiveness, includes addition of spontaneous and nonspecific mutations, etc. (Li et al. 2019). Because of their large genome size, the implementations of gene editing in plant engineering are well-recognized in bioremediation. Moreover, the use of tissue culture-free genome engineering processes has the ability to improve productivity by minimizing the need for *Agrobacterium*-mediated transformation, which generates a genetically mutated plant and is much more costly, time-consuming, and asset (Manghwar et al. 2019).

This technique has been increasingly implemented in plant genome editing to confer resistance on viruses, environmental conditions, and other purposes (van Eck 2018). As a result, it can be used to edit or try and influence the microbial genome in bioremediation. Scientists are continuously working to use gene editing tools to modify the genomes of microorganisms to produce desired enzymes by adjusting metabolic pathways (Bi and Yang 2017). The production of genetically stable organisms, as well as employing a diverse variety of DNA sequences in a range of microorganisms, is a prerequisite condition for genome editing. These implements have not been extensively used in metabolic engineering so far; however, they do have the ability to improve the bioremediation of many pollutants in the future (Kim et al. 2017). There is an urgent need to overcome these gene editing tools' limitations. Off-target impacts, unusual gene mutations in the host DNA, ineffectual selection, and so on are also examples (Zhang et al. 2014). Furthermore, engineering of many protein techniques has been used to continue improving the action and selectivity of ZFN and TALEN domains (Schmidt and Pei 2015).

Omics-based techniques such as genomics, proteomics, metagenomics, and transcriptomics as well as computational techniques that were developed to analyze the statistics generated by these techniques offer vital confirmation for understanding the intricate actions of microbes that play a key role in bioremediation. ZFNs, TALENs, and CRISPR/Cas are the three gene editing tools that intend to restore the purpose of an expressive gene in a microbe with precise enzymes involved for bioremediation.

Biotechnological investigations into microbes that can substitute chemical reactions are fueled by rising awareness in the age of multi-omics and the growing need to deal with ecological pollution in a progressive, eco-friendly manner. The currently added genome editing tools derived from the CRISPR/Cas approach pave the way for these goals to be fully realized in a variety of fields, including bioremediation.

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# Metabarcoding Approach in Identifying Potential Pollutant Degraders

28

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**Abstract**

Different groups of microorganisms are considered notable degraders of pollutants. This microbial richness remains little explored; however, better knowledge about the biodiversity and ecology of microbial communities that inhabit contaminated environments is essential for the conduction of strategies to optimize the bioremediation potential of these organisms. In contaminated environments, resident microorganisms play a crucial role; although due to the limitations imposed by the cultivation-dependent methods, relevant information about the composition and functions of microbial communities remains unknown. Comprehending the composition of microbial communities, and their role in the bioconversion of organic contaminants in their natural environment, depends on the knowledge of microbial dynamics and the responses of these microorganisms to environmental changes, including the noncultivable ones. Environmental DNA (eDNA) investigation is a powerful technology for monitoring biodiversity in an ecosystem, and DNA metabarcoding, which unites the fundamentals of environmental and next-generation sequence barcoding, generates large amounts of reads/information for this monitoring. In contaminated environments, monitoring biodiversity makes it possible to understand the natural process of biodegradation, and, with the help of metabarcoding, microorganisms, consortia, and associations that participate in the degradation of pollutants can be identified. In this sense, this chapter will address the importance of studies carried out with DNA metabarcoding technology in environmental

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samples, aiming at the identification of microbial communities that possibly act in the processes of removal of polluting compounds present in the environment.

**Keywords**

Pesticides · Hydrocarbons · Heavy metals · Urban waste · Metabarcoding

## 28.1 Introduction

The rapid population and economic growth observed in recent decades have resulted in an increased global demand for resources to combat environmental degradation (Nasrollahi et al. 2020). Quality of life is closely associated with environmental health, and, when the environment is constantly deteriorated by intense industrial, agricultural, and human activities, the results of these actions are perceived when they exert deleterious effects on health and the environment (Singh et al. 2021). The large and increasing amount of pollutants in the ecosystem has raised concerns, and, for this reason, efforts to remediate and restore the health of the environment are intensifying (Luka et al. 2018). Pollution affects human health directly or indirectly, and, xenobiotic compounds can be classified into biodegradable pollutants, which comprise sewage effluents and organic matter, and nonbiodegradable substances, which comprise compounds that are not naturally degraded, such as heavy metals, plastics, and detergents (Wasi et al. 2013).

Microorganisms have a vast metabolic arsenal and perform essential functions for the dynamics and functioning of ecosystems. This metabolic capacity can be directed toward the purpose of bioremediation of contaminated areas, making microorganisms potential removers of environmental pollutants, thus representing an ecologically friendly and economical alternative for the treatment of contaminated areas (Borchert et al. 2021). Traditionally, the microbial degradation of pollutants uses microorganisms previously isolated; however, as less than 1% of microorganisms are cultivable, the use of microbial isolates for this purpose is limited, and nonculturable microbial groups that may have the ability to remove contaminants from the environment remain underexplored. In this scenario, a more comprehensive investigation into the composition and functions of the noncultivable microbial community residing in the contaminated environment may reveal promising and yet unknown candidates capable of assisting the bioremediation of contaminated sites (Pushpanathan et al. 2014).

In this sense, DNA barcoding and metabarcoding analyses of different environments show that the current knowledge about biodiversity does not reflect the actual composition present in ecosystems and that most species remain unknown and sibylline. New sequencing technologies support the identification and monitoring of species and communities, and, in addition, these technologies make it possible to relate communities to their respective functional and genetic characteristics (Weigand et al. 2017). This knowledge may be the key to improving mechanisms for the treatment of environmental pollutants.

## 28.2 Metabarcoding

A barcode comprises a short variable region, flanked by highly conserved gene regions that allow taxonomic affiliation (Hebert et al. 2003), whereas metabarcoding consists of an approach that, through the amplification of specific regions present in environmental DNA, simultaneously identifies different taxonomic groups present in a sample. In metabarcoding, the purpose is not to identify a single organism but a group of organisms with the DNA barcode in common (Fig. 28.1).

In 2012, Pierre Taberlet and colleagues introduced this term to "designate high-throughput multispecies (or higher-level taxon) identification using the total, and typically degraded, DNA extracted from an environmental sample (i.e. soil, water, feces, etc.)" (Taberlet et al. (2012); Comtet et al. 2015). Although known as a variation of the metagenomic technique, the metabarcoding approach does not involve genome-level functional analysis and focuses primarily on taxonomy (Comtet et al. 2015).

The adoption of DNA metabarcoding analyses has been gradually growing, due to this approach's diversified applicability and use as a tool for environmental assessment and monitoring. This technology, combined with state-of-the-art sequencing and in silico analysis, enables taxonomic affiliation of the set of amplified sequences of all species contained in a sample (Compson et al. 2020).

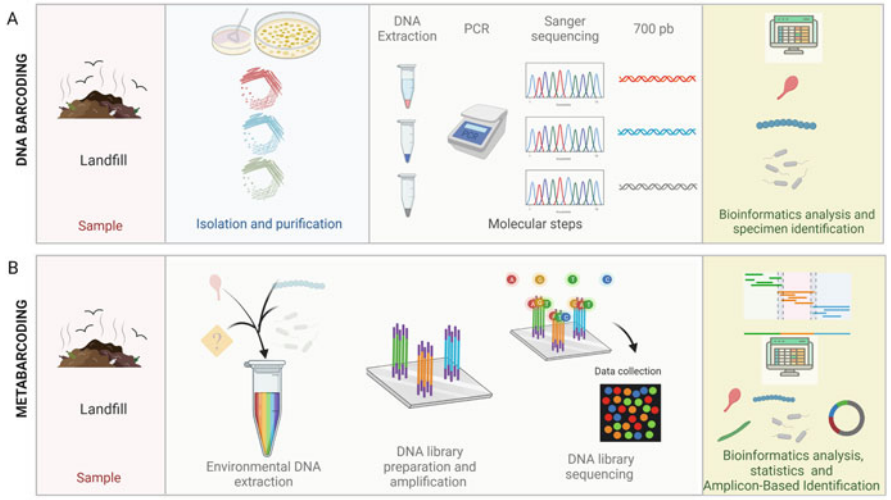


**Fig. 28.1** DNA barcoding and metabarcoding of contaminated sample (e.g., landfill) pipelines. (**a**) In DNA barcoding, each individual has its DNA extracted independently and submitted to polymerase chain reaction (PCR) for amplification of a specific fragment (target sequence) for subsequent sequencing. (**b**) DNA metabarcoding approach, without previous cultivation and isolation of microorganisms. Entire environmental samples are processed, and the total DNA contained in the samples is extracted. The extracted DNA is subjected to PCR with universal primers, and the amplicons are compared to sequences contained in databases for taxonomic affiliations. (Source: the authors)

For metabarcoding DNA sequencing, different platforms can be used, but some are more efficient for this purpose, such as those provided by Illumina® and Thermo Fisher Scientific®. Currently, these platforms sequence longer DNA fragments (above 600 bp), which is a relevant advance to produce more robust data and, consequently, more reliable taxonomic associations. It is important to note that accurate taxonomic affiliations not only depend on good sequencing but also on computational and statistical analysis performed on platforms and software appropriate for this type of study (Abdelfattah et al. 2018).

Success in opting for DNA metabarcoding analyses depends on selecting the appropriate marker gene. For taxonomic description of prokaryotic groups, the most used gene is the *16S rRNA*, whereas to identify eukaryotic communities, the internal transcribed spacer (ITS) region is generally the most applied. The ITS region has the ITS1 and ITS2 subloci separated by the *5.8S rRNA* gene and is located between the 18S (SSU) and 28S (LSU) rRNA genes in eukaryotic rRNA (Francioli et al. 2021).

Historically, metabarcoding has been primarily applied to water and soil samples. In 2015, studies began with airborne sampling (Johnson et al. 2021). In plants and animals, metabarcoding analyses can be used to evaluate the microbiome. The microbiome can contain an immense group of organisms, both prokaryotic and eukaryotic, including bacteria, archaea, viruses, fungi, and protozoans (Christian et al. 2015).

Presently, DNA metabarcoding is not only applied to the knowledge of the biodiversity of a given environment but also mainly to monitor this biodiversity and the ecosystem. An efficient strategy to accompany environments is to use metabarcoding analyses together with traditional monitoring methods, thus deepening the knowledge about the target area. In addition, the metabarcoding approach allows retrieving genetic information from rare or totally unknown species, monitoring the responses of the microbial community due to disturbances in the environment (Ruppert et al. 2019). Other practical applications of environmental DNA metabarcoding are the discovery of species present in the environments, ecosystem reconstruction, and identification of biological invasion, among others (Ladin et al. 2021).

The insertion and persistence of polluting substances in environments cause changes in ecosystems and affect microbial communities. The responses of resident microorganisms to these disturbances can be tracked with the aid of metabarcoding, which reveals those species that are more resistant and those that are more susceptible to the presence of a particular contaminant. In environments where the presence of pollutants is known, it is possible to monitor the behavior of microbial communities (Ruppert et al. 2019) as well as to identify microorganisms resistant to pollutants and with the potential to degrade these compounds, which allows improving the techniques of bioremediation and removal of environmental pollutants currently used.

## 28.3 Challenges in Pollution Bioremediation and the Potential of Microorganisms

Environmental pollution affects the air, water, soil, and sediments and impacts the health of the environment and those that inhabit it (Landrigan and Fuller 2015). Polluting compounds can be recalcitrant and remain in the ecosystems for long periods of time, thus prolonging their harmful action. In addition, besides the known prevalent pollutants, such as industrial effluents containing dyes and heavy metals, hydrocarbons, and urban wastes, it is currently necessary to deal with the so-called emerging pollutants, which comprise natural or anthropogenic trace organic contaminants, such as personal care products, plasticizers, pharmaceuticals, pesticides, surfactants, etc., that are also difficult to remove from the environment (Wasi et al. 2013; Taheran et al. 2018).

Biodiversity plays a key role in the stability and functionality of ecosystems, and disturbances in the environment directly influence the diversity of species and the composition of the environment. Thus, understanding how the ecosystem responds to variations, such as those caused by the presence of pollutants, helps in the development of efficient intervention strategies (Mancuso et al. 2021). The vast microbial diversity is responsible for the functioning of habitats in which microorganisms live, and they are able to quickly respond to changes that may occur in the environment. These organisms are constantly exposed to changes, and their ability to adapt makes them excellent candidates for application in bioremediation (Das and Dash 2014). As environmental metabarcoding has been increasingly used to study biomes, with multiple markers for the assessment of different organisms (Brandt et al. 2021), the use of this approach for the study of microbial communities present in environments contaminated with toxic and recalcitrant pollutants can be an important tool in the discovery of potential microorganisms for bioremediation.

### 28.3.1 The Use of Metabarcoding to Support Heavy Metal Removal

Some heavy metals, such as copper and chromium, in trace amounts are essential for cellular metabolism; however, at high concentrations, both essential and nonessential heavy metals are toxic to all living organisms due to the formation of complex compounds within cells (Mohammed et al. 2011; Vareda et al. 2019). As they are not biodegradable, these elements are highly persistent and pollute the air, water, and soil (Mohammed et al. 2011). Heavy metals are released into the environment by anthropogenic activities such as mining, industry, combustion of fossil fuels, and pesticides (Vareda et al. 2019). Controlling pollution by these elements includes reducing the bioavailability, mobility, and toxicity of metals (Mohammed et al. 2011), and two widely studied routes of decontamination of polluted areas include phytoremediation and microbial activity (Vareda et al. 2019). To decontaminate polluted areas, microorganisms have developed tolerance mechanisms to interact and survive in the presence of inorganic metals, such as adsorption, complexation,

biotransformation, extrusion, use of enzymes, exopolysaccharide production, metallothionein synthesis, or use of heavy metals as electron acceptors (Mohammed et al. 2011; Igiri et al. 2018). The presence of a stressor like heavy metals causes imbalances in the environment, and more tolerant organisms can replace more sensitive taxa (Yang et al. 2018).

Some naturally or genetically modified microorganisms are able to chelate or decompose hazardous materials, such as heavy metals, representing an important bioremediation strategy. The role of microorganisms in decontamination directly depends on their resistance to heavy metals, and the remediation of these compounds occurs through biosorption, bioaccumulation, and biosurfactant production (Wu et al. 2017). Thus, the analysis of genetic sequences obtained with the aid of the metabarcoding approach for the search for heavy metal resistance strains is the first step to finding potential microorganisms for heavy metal bioremediation.

The factors responsible for the responses of microbial communities to changes that occur in the environment due to anthropogenic activities remain poorly understood; however, genomic studies suggest that horizontal gene transfer plays a fundamental role in the adaptation of these microorganisms (Hemme et al. 2016). In a study on the microbial response to heavy metal contamination in groundwater, Hemme et al. (2016) compared the reference genomes of *Rhodanobacter*, a dominant genus found in contaminated groundwater, with amplicon sequences based on the 16S rRNA gene, and observed that in contaminated wells containing high levels of nitrate and heavy metals and with low pH values, there was an abundance of populations of *Rhodanobacter*, which did not occur in uncontaminated wells, where sequences of this genus were rarely found. The authors, through analysis of the *Rhodanobacter* pangenome, observed that $Co^{2+}/Zn^{2+}/Cd^{2+}$ efflux and mercury resistance operon genes appeared to be highly mobile within populations of this genus and, in addition, found evidence of multiple duplications of a mercury-resistant operon normally found in most *Rhodanobacter* strains. Such characteristics are probably present within *Rhodanobacter* found in heavy metal sites with the aid of metabarcoding, allowing them to thrive in that environment.

Sequence analysis obtained via metabarcoding can also be used to access information on resistance to heavy metals by the fungal community present in the soil. Pathak et al. (2020), studying soil samples collected from two locations of the US Department of Energy (DOE) with a history of contamination with heavy metals, including mercury, and analyzing ITS-based amplicon sequences, observed that the fungal genera *Penicillium*, *Thielavia*, *Trichoderma*, and *Aspergillus* were significantly abundant in most contaminated soils. The authors also observed the correlation between *Penicillium* spp. and total mercury (THg), whereas *Trichoderma* spp. and *Aspergillus* spp. were correlated with methylmercury (MeHg), thus concluding that these genera selected suitable traits to ensure their survival in contaminated soils.

The metabarcoding approach was used in a study by Yang et al. (2018), with wetland sediments (Warrandyte, Melbourne, Australia) using a microcosm to assess the effects of copper on the biodiversity of microbial communities. The authors used bacterial 16S rRNA and 18S eukaryotic rRNA amplicons and found that some

species replaced others as more abundant in treatments with higher copper concentrations, especially in prokaryotes. This study highlights the potential of the metabarcoding approach to access information about potential organisms for heavy metal bioremediation. The effects of mercury on the composition and structure of a soil microbial community were also studied by Fatimawali et al. (2020), who analyzed 16S rRNA sequences obtained from total DNA extracted from gold mining waste disposal site soil (containing mercury used in mining) and soil from a rice field located 100 m from the mine waste area in Indonesia. By analyzing the sequences, it was possible to identify the dominant prokaryotic groups in the presence of higher concentrations of mercury (waste disposal area), with emphasis on the genus *Bacillus*, indicating that this genus can be further explored for mercury decontamination. Passarini et al. (2021), through the analysis of 16S rRNA sequences obtained by metabarcoding, observed that in a Brazilian leachate containing heavy metals, mostly cadmium and copper, the most abundant phylum was *Firmicutes*, elucidating the importance of the members of this phylum in removing heavy metals from the environment.

Thus, it is evident that the metabarcoding approach represents an important tool in the identification of microorganisms with the potential to remove heavy metals, guiding efforts to the most promising groups.

### 28.3.2 The Use of Metabarcoding to Support Pesticide Bioremediation

The relevant increase in agricultural productivity that occurred in the past 60 years had, as one of the main culprits, the use of synthetic pesticides. However, if, on the one hand, pesticides brought benefits to crops, on the other hand, the intense use of these substances entailed the degradation of the physical–chemical and biological health of the soil, subsequently negatively impacting productivity (Basu et al. 2021).

Sustainable agriculture production must consider the conservation of biodiversity, and, in the opposite way, the use of chemical fertilizers and pesticides exerts undesirable effects on the environment, for the health and for the nontarget soil communities and organisms (Prashar and Shah 2016). Despite being considered recalcitrant and potentially toxic substances, pesticides are susceptible to decomposition by biotic and photolytic factors; however, the intense use of these chemical substances has led to a persistent contamination of the environment (Chowdhury et al. 2008; Vryzas 2018).

The most efficient and accepted mechanism for pesticide degradation is microbial metabolism, which is considered an effective, safe, and ecologically correct alternative for this purpose (Kumar et al. 2021a). Pest management alters microbial diversity and community composition, and technologies such as metabarcoding represent a powerful tool for accessing information that allows understanding the responses and adaptation mechanisms of microorganisms under stress caused by the presence of pesticides (Fournier et al. 2020).

By sequencing the *16S rRNA* gene, Bourhane et al. (2022) analyzed the composition of prokaryotic communities present in soils and sediments obtained from different locations of Ichkeul Lake (northern Tunisia), contaminated by different pollutants. In a sample collected at the site with the highest use of pesticides, the most abundant genera related to the presence of organic compounds (organochlorines) were *Rubrobacter*, *Gaiella*, *Gemmata,* and *Microvirga* together with *Skermanella* and *Blastococcus*. The authors highlighted that these same genera were found in soils contaminated with pesticides in other previous reports and stated that members of these groups have functionally important roles related to the biogeochemical nitrogen cycle.

Serbent et al. (2021) analyzed the prokaryotic and microeukaryotic communities of an irrigated rice field with a history of pesticide use since 1980. The authors analyzed the sequences of 16S rRNA and 18S rRNA from the influents, the rice rhizosphere soil, the effluent storage tank, and the sediments of the storage tank and initially found that the soil and sediment samples showed greater diversity. The authors also observed that the *Proteobacteria* phylum was the most abundant in all locations, but, interestingly, the *Actinobacteria* phylum was much more abundant in aquatic samples, with emphasis on the effluents, which had a higher concentration of pesticides, indicating the selective pressure of this phylum in the presence of these compounds. On the other hand, in soil and sediment samples, the phyla *Thaumarchaeota* and *Nitrospirae* were more abundant compared to those in aquatic samples. Regarding microeukaryotes, specifically fungi, the authors found similar diversity indices in the influents, rhizosphere, and sediments, but they reduced considerably in the effluents. Some operational taxonomic units (OTUs) belonging to Chytridiomycota, Mucoromycota, Zoopagomycota, and Cryptomycota (genus *Paramicrosporidium*) were not found in the effluent sample, indicating that this is a selective environment for fungi.

Aiming to optimize the consortia of degrading bacteria from fluorinated pesticides, including Epoxiconazole, Alexandrino et al. (2021) used the dependent and independent methods of cultivation. In the analyses with isolates, the authors verified that the consortium composed of *Hydrogenophaga eletricum* 5AE and *Methylobacillus* sp. 8AE was able to defluorinate 80% of the pesticides. 16S rRNA sequence analysis was carried out in the microbial consortium originated from agricultural soil enriched with pesticides (among them Epoxiconazole), which gave rise to the tested isolates. The consortium was enriched with Epoxiconazole and incubated for 28 days, and the total DNA of the consortium was analyzed at the beginning and at the end of the incubation period. From the metabarcoding data, the authors verified that the most efficient strains in the tests with isolates were a minority in the consortium, showing that the degradation of Epoxiconazole can be driven by less abundant phylotypes in the community.

The impacts of alternative fumigation products on the soil microbial population structure were investigated by Wei et al. (2016), who used the metabarcoding approach to obtain 16S rRNA and 18S rRNA amplicons and analyzed the responses of soil communities to microencapsulated terpene, Brassica seed meal (BioFence™), and chloropicrin. The abundances of OTUs were followed for 16 weeks and

analyzed at three different times. The authors observed that chloropicrin dramatically altered bacterial and fungal populations after 4 weeks of application (time 2); however, after 16 weeks of application (time 3), little changes in the bacterial population structure were observed, whereas in the fungal population the impacts were more persistent. Terpene and BioFence™ did not significantly affect the soil microbiota. Among the interesting observations, it was verified that the phylum *Actinobacteria* was less affected by chloropicrin compared to the phylum *Firmicutes*; however, the presence of the pesticides increased the abundance of *Bacillales* (*Firmicutes*) and reduced the abundance of *Streptomycetes* (*Actinobacteria*), indicating greater tolerance of *Bacillales*, a group that may have individuals with the potential to metabolize chloropicrin.

The increasing adoption of the metabarcoding approach for analyzing environments contaminated with pesticides can be attributed to the richness of the data generated in these analyses, which provide important information about microbial communities tolerant to these compounds and help define the direction in which research efforts should focus.

### 28.3.3 The Use of Metabarcoding to Support Hydrocarbon Bioremediation

Considered important environmental contaminants, many hydrocarbons have harmful effects on human health, such as toxicity, mutagenicity, and carcinogenicity. Aromatic hydrocarbons comprise monoaromatic hydrocarbons, including benzene, toluene, ethylbenzene, xylenes (BTEX), and polycyclic aromatic hydrocarbons (PAHs). These aromatic compounds reach the environment from natural and anthropogenic sources, especially from industrial activities such as oil, textile, and coal processing, natural gas purification, and manufacture of paints and synthetic rubber, which are major generators of effluents containing aromatic hydrocarbons (Makós et al. 2018).

The accumulation of hydrocarbons in the environment occurs due to the high hydrophobicity and low solubility of these compounds, and their impacts are more deleterious in more sensitive environments, such as cold marine areas, permafrost, and deep waters, with consequent environmental problems (Tomasino et al. 2021).

Microorganisms are able to metabolize hydrocarbons and, for this reason, represent a sustainable and economical strategy to streamline the complete removal of pollutants present in the environment (Tomasino et al. 2021; Logeshwaran et al. 2018). These microorganisms are widely distributed in the environment and have the ability to use hydrocarbons as a carbon source for their development (Tomasino et al. 2021; Varjani and Upasani 2017). Cultivation-dependent methods are well-known and relevant for understanding the physiological potential of microbial isolates; however, this technique does not allow accessing information on the composition of microbial communities (Van Elsas et al. 1998; Varjani and Upasani 2017). To overcome this limitation, the use of molecular methods, including the analysis of gene sequences such as 16S rRNA, proved to be efficient in the characterization of

microbial communities in different environments, including oil reservoirs (Varjani and Upasani 2017), without the previous need for isolation of microorganisms. The analysis of the effects generated by the presence of hydrocarbons on the dynamics and diversity of microbial communities is a key element for the success of bioremediation, as it provides information on the degradation potential of these compounds by microorganisms as well as allows the identification and selection of the indigenous hydrocarbon-degrading microbial consortia present in the contaminated environment, enabling the improvement of their biodegradation potential (Tomasino et al. 2021; Logeshwaran et al. 2018).

The dynamics of prokaryotic communities in different soil microcosms before and after oil pollution was studied by Manucharova et al. (2020). The authors added 20% of soil mass of oil to three different soils (chernozem, gray forest, and chestnut) collected in Russia, and, after 1 month of assay, the total DNA of the soil microcosms was extracted for 16S rRNA gene sequencing. Little difference was observed between the microbial communities of the different soils; however, compared to the microbial communities present at the beginning of the trial, the authors verified changes in the metabolically dominant groups, in particular, an increase in the community of *Gammaproteobacteria* and *Actinobacteria* (among the bacterial groups) and in that of *Thaumarchaeota* and *Crenarchaeota* (the archaea group).

Spini et al. (2018), using 16S and ITS2 metabarcoding, analyzed the microbial communities present in oil-contaminated soils at different depths, in Fidenza, Italy. The polluted area has a history of contamination by BTEX, n-alkanes, and polycyclic aromatic hydrocarbons (PAHs), and the soil samples used in the study were applied in microcosms enriched with different pollutants, i.e., benzene, pyrene, phenanthrene, naphthalene, paraffin oil, and crude oil mixture from the contaminated Fidenza site. The authors found that the relative composition of bacterial genera was partially dependent on the pollutants used in the respective microcosm. In the presence of pyrene and phenanthrene, the dominant bacterial genera were *Azospirillum*, *Achromobacter*, and *Pseudomonas*; in enrichment with naphthalene, the most frequent genera were *Achromobacter* and *Pseudomonas*; in oil enrichment, *Pseudomonas* followed by *Achromobacter* predominated; and for enrichments with benzene and paraffin, a greater diversity of communities was observed, with a high relative presence of *Acinetobacter*, *Pseudoxanthomonas*, and *Pseudomonas*. Regarding fungi, no clustering of dominance associated with the added pollutants was observed, and all samples showed dominance of *Fusarium* (more than 80% on average), followed by *Aspergillus*, *Penicillium*, *Trichoderma*, and *Arthrinium*. No significant differences were observed in the composition of microbial communities at different depths.

Xie et al. (2018) used the metabarcoding approach to obtain information about the impacts of an oil spill that occurred in 2007 in South Korea on macrobial and microbial communities. Surface sediment samples were collected 1 month after the spill, and after 1, 2, 3, 4, 6, and 7 years after the spill, and 16S rRNA analysis of these samples provided the authors with an overview of the changes in the microbial structure over time. The bacterial communities found in the sediments containing residual oil contamination were significantly different from the

communities present in the samples collected at sites free from oil contamination (reference), indicating that changes in the structure of microbial communities occurred due to the presence of oil. The most abundant bacterial communities were *Proteobacteria* and *Firmicutes*, and bacterial communities differed between areas with different oil concentrations. The presence of bacterial families such as Aerococcaceae and Carnobacteriaceae in less contaminated sediments may indicate the sensitivity of these groups to oil pollution. On the other hand, families of bacteria known to degrade hydrocarbons such as Anaerolinaceae, Desulfobacteraceae, Helicobacteraceae, and Piscirickettsiaceae were resistant to the adverse effects of spilled oil. In addition, the analysis showed that the abundances of Desulfobacteraceae and Anaerolinaceae were correlated, indicating the cooperation between these groups in the biodegradation of petroleum hydrocarbons. The successional pattern of bacterial communities over time suggests the long-term impact of pollution caused by oil residues. Through eDNA metabarcoding, it was possible to understand the long-term effects of pollution caused by anthropogenic actions, such as oil spills, on sediment communities in a coastal marine environment.

Metabarcoding, used to monitor microbial communities in the presence of hydrocarbons, allows us to understand which are the tolerant groups and which are the most sensitive groups to the presence of these contaminants, helping future studies optimize potential hydrocarbon-degrading microorganisms.

### 28.3.4 The Use of Metabarcoding to Support Solid Waste Bioremediation

Organic waste can be defined as any useless and unwanted product in its solid state, which is derived from urban, industrial, commercial, mining, and agricultural activities and is discarded by society (Hoornwerg and Bhada-Tata 2012). Currently, one of the main concerns regarding the fate of organic waste is its destination in sanitary landfills (Abiriga et al. 2021). A major concern associated with municipal sanitary landfills is the generation of leachate and its treatment. Leachate is a dark liquid with a characteristic odor, constituted by the infiltration of rainwater into urban solid waste cells together with the degradation of organic matter existing in the domestic and hospital organic waste present there (Passarini et al. 2021). Leachate can contain several compounds, including dissolved organic matter, heavy metals such as copper, lead, and mercury, and other xenobiotic organic compounds, including aromatic hydrocarbons, pesticides, and plasticizers. The characteristics of this complex composition make the leachate highly toxic to the environment and human health (Kumar et al. 2021b; Passarini et al. 2021). Biological degradation of solid waste may involve different phases, including the hydrolytic, acidogenic, and methanogenic phases. During these phases, the formation of $CO_2$ and $H_2O$ occurs by the microbial metabolism of organic matter, which can form organic acids and ammoniacal nitrogen, leading to the formation of acetate and methane (Passarini et al. 2021).

The use of the 16S rRNA metabarcoding approach has been increasing continuously, aiming at the characterization of the microbiota associated with solid waste and allowing to improve the understanding of the microbial community responsible for the bioremediation process in organic waste including landfills (Abiriga et al. 2021; Álvarez-López et al. 2020; Srivastava et al. 2021; Passarini et al. 2021).

Recently, Abiriga et al. (2021) have conducted a study using an association of microbiological techniques, including the 16S rRNA metabarcoding approach, to analyze groundwater samples from an aquifer contaminated by a municipal landfill. The sequences obtained after sequencing were analyzed, and the authors observed that the vast majority of the microbial community was affiliated with eight major phyla, including *Proteobacteria* (the most abundant), followed by *Patescibacteria*, *Bacteroidetes*, *Actinobacteria*, *Cloroflexi*, *Acidobacteria*, *Verrucomicrobia*, and *Firmicutes*, out of a total of 57 other phyla found. The results showed that the microbiome of the aquifer impacted by leachate from landfills, compared to the microbiome of unimpacted site samples, was affected by the presence of leachate. Contaminated samples presented a greater diversity of microbial communities, and among them were groups capable of metabolizing hydrocarbons, sulfur, and iron.

In the work carried out by Passarini et al. (2021), the microbial community present in the leachate from a landfill was analyzed using the 16S rDNA metabarcoding methodology. The authors observed a domain of fermenting bacteria, representing the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Synergistetes*; however, about 60% of the amplicon sequence variants (ASVs) were not taxonomically classified. With the results of the study, it was possible to affirm that the use of metabarcoding technology allowed expanding the knowledge of bacterial diversity, including groups with a fundamental role in the bioremediation of toxic compounds from leachate, which may be associated with leachate detoxification processes. A metabarcoding analysis was performed by Srivastava et al. (2021), to characterize bacterial communities in urban solid waste vermicompost samples. The authors observed a successional displacement of the microbial community, initially dominated by groups belonging to the order Rhizobiales (the *Alphaproteobacteria* class), which were gradually replaced by the phylum *Firmicutes*, particularly, representatives from the Bacilli class. The authors concluded that metabarcoding technology can help manage emerging issues, such as dealing with organic waste.

Rossi et al. (2022) carried out a study using a plug flow reactor on a pilot scale to retrieve volatile fatty acids and biogas from the organic fraction of municipal solid waste, using the metabarcoding tool. *Defluviitoga* sp. was the most abundant bacterial genus, comprising 72.7% of the total bacterial population. The results allowed the authors to infer some functional associations of the bacterial community, such as the production of propionate by unidentified species belonging to the Lentimicrobiaceae and Proteiniphilum families and the hydrolysis of complex polysaccharides by members of the genus *Defluviitoga* sp.

Using the massive sequencing of metabarcoding, Stoeck et al. (2018) characterized bacterial and eukaryotic communities in activated sludge samples obtained from a wastewater treatment plant. *Proteobacteria*, *Verrucomicrobia*, and *Bacteroidetes* were the most abundant phyla in all samples and represented

approximately 95% of the diversity of OTUs. Regarding eukaryotes, most of the diversity was associated with protozoa. Other taxa including fungi and Metazoa were relatively undiversified among samples. The obtained results allowed the authors to suggest a strategy for the development of a bioindicator system based on metabarcoding, which can be implemented in future monitoring programs for wastewater treatment plant performance and effluent quality.

Thus, it is possible to say that the use of metabarcoding to characterize microbial communities in recycling and/or organic waste treatment processes can be a powerful tool for understanding the dynamics of communities and identifying taxonomic groups with the greatest potential to perform these processes.

## 28.4 Future Outlook

Using DNA metabarcoding, it is possible to access particular groups of organisms, through the use of specific primers, or broad fractions of biodiversity in different taxonomic sets (Compson et al. 2020). This tool also allows accessing information not directly linked to the taxonomy, through the amplification of specific genes for a given function, including genes that degrade a particular pollutant.

There are some alternatives when it comes to the treatment of environments contaminated with pollutants, including natural attenuation and bioremediation, which include biostimulation and bioaugmentation. In the case of bioaugmentation, exogenous microorganisms, known to be bioremediators of a particular pollutant, are inoculated into the contaminated environment in order to remove or remediate the contaminant. In bioinoculation, special attention must be given to the indigenous biota, which must not be harmed, and to the rapid proliferation of the inoculated organism. In this field of bioremediation, molecular tools, such as metabarcoding and metagenomics, have proved to be great allies, either by detecting degradative genes or by characterizing the biodiversity of a given environment by analyzing and monitoring indigenous and inoculated communities and, among other advantages, increasing the efficiency of bioremediation and allowing monitoring of the treatment (Speight 2018).

The use of microbial resources for the recovery of polluted environments is a promising alternative that has been extensively studied due to the economic and environmental advantages that they offer. There are many ways to prospect promising candidate microorganisms for this function, and the practical application of these resources can be quite challenging. Among the forms of prospecting, DNA metabarcoding represents a relevant molecular tool, which can greatly contribute to the search for microorganisms or groups of microorganisms capable of acting in the recovery of areas impacted by pollutants.

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# Artificial Intelligence in Bioremediation Modelling and Clean-Up of Contaminated Sites: Recent Advances, Challenges and Opportunities

29

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**Abstract**

The present environmental balance has been jeopardised by a scarcity of natural resources and sometimes harmful human efforts that try to impose authority on them. As a result, water sources are contaminated by various types of plastic debris and crude oil leakage from ships, and air pollution is caused by increased gas production. Polycyclic aromatic hydrocarbons, pyrethroids, insecticides, bisphenol A and dioxanes are also known to pollute groundwater, land and farmland. As a result, bioremediation may be a viable option for restoring a clean environment. However, most findings on bioremediation are now confined to the inherent capability of microbial enzymes. Biology with unwavering ethical standards can aid in circumventing natural engineering to boost $CO_2$ absorption. Furthermore, a combination of systems biology and the potential of algorithms to widen the scope of bioengineering is exciting. This chapter highlights the present level of knowledge on data-driven enzyme redesign in order to actively pursue new research using artificial intelligence.

**Keywords**

Artificial intelligence · Bioremediation · Contaminants · Environment

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## 29.1 Introduction

The term "bioremediation" refers to a specific approach to removing toxins from the environment. Several bioremediation systems based on artificial intelligence are well-recognised for removing environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), polyphenols, chlorobenzene compounds and endocrine-disrupting compounds (EDCs) (Arranz et al. 2008). Microbial-assisted bioremediation is the most widely utilised pollution removal technology. Several bioremediation technologies (phytoremediation, digesters, biological treatment, bioventing, landfarming, bioleaching and biostimulation) are all examples of biotechnologies, which are now in use. Bioremediation is a promising technology in which dangerous biocontaminants are removed using resources such as plants and bacteria. Microbe-aided toxin remediation entails the use of microorganisms to either totally decompose harmful substances into $H_2O$ and carbon dioxide or to expedite their transformation into less harmful forms (An et al. 2020). All of them, however, have unique capabilities, limits, benefits and prospects that are frequently related to the form of pollutants and the afflicted region. Over the last few decades, remarkable research has been conducted and published for bioremediation of diverse pollutants, including PAHs and a variety of other complicated toxins. Furthermore, typical bioremediation technologies cannot concurrently assess comparative toxicity, environmental effects and comprehensive information on total biologically altered substances, which is a major limitation of conventional bioremediation (Annadurai and Lee 2007). A few sophisticated bioremediation technologies frequently cause secondary (phytoremediation) contaminants to be released at the cleaning site. Biocontrol technologies are typically manufactured or modified techniques that are not synonymous with photocatalytic degradation. Biodegradation occurs in four distinct steps: (1) hydrolysis, (2) acidogenesis, (3) acetogenesis and (4) methanogenesis. The environmental advantages of anaerobic digestion systems have been recognised by the United Nations. Adopting biomethanation assists in decreasing emissions through a variety of means, including the replacement of fossil fuels, landfills, chemical fertiliser substitution (industrially created), manure management and organic diversion from landfills (Baker et al. 2004).

Toxic residues released into the atmosphere, as a result of rapid industrialisation, are the major cause of harmful environmental effects. Pollutants recognised to be harmful to people and the environment are released into the environment by the paper and pulp, tannery, textile and distillery industries. Phenolic chemicals have been discovered to be a potential carcinogen in the environment (Bao et al. 2019). It motivates the scientific community to discover and test rapid and effective responses to these threats. The type of pollutants and operating circumstances influence bioremediation technology, which consists of several approaches in terms of pollutants to be eliminated, depending on the unique environmental state. Aside from most chlorinated chemicals, EDCs and POPs are highly resistant to degradation in the environment (Barthlott et al. 2020). These are known to cause issues in male and female reproduction. To protect and maintain the environment's sustainability and to reduce the harmful effects of pollutants, a cleansing of all discharged

pollutants is urgently needed . Although bioremediation technologies are extensively used, they can be ineffective at times. They are unable to mitigate complex contaminants, and the process of restoring a balanced environment following application at polluted locations is lengthy (Baskaran et al. 2020). For cleaning and mitigation, a number of chlorinated phenolic compounds such as genetically engineered microbes (GEMs) have been widely used as part of different sophisticated bioremediation techniques. In order to fulfil the requirements of bioremediation, GEMs, unlike conventional organisms, have been created to fight against environmental toxins through distinct pathways, with more constant catabolic activity and a wider spectrum of substrates (Boethling et al. 1989). Other approaches, such as whole-genome sequencing, have also been shown to be effective in understanding enhanced microbial-mediated bioremediation.

The use of artificial intelligence in modelling the environmental and operational processes has gained traction as a means to better understand the dynamics that occur in water basins and to design feasible solutions for successful management. This study was conducted to better understand the applicability, research breakthroughs, limitations and significant obstacles of machine learning and artificial intelligence in studies for industrial wastewater treatment (Bueno et al. 2009). This review might assist environmental scientists, biogeochemists and industry practitioners in managing contaminated areas more effectively. This review also explains about the artificial intelligence system for stimulating, predicting and controlling therapeutic processes as well as for environmental cleaning.

## 29.2 Biosensors as Molecular Tools for Use in Bioremediation

Human activities such as industrialisation contribute to environmental contamination. Inadequate removal practises of sludge or spills can discharge inanimate chemicals into the atmosphere, such as heavy metals or harmful organic poisonous compounds. Heavy metals, for example, may be rather stable in nature. They build up in living things and the environment due to their low biodegradability; this scenario has a wide variety of consequences, including ecological toxicity and human disorders (Cakmakci 2007).

Traditionally, samples obtained from the atmosphere are subjected to chemical and physical investigations to precisely ascertain contamination. This strategy necessitates systematic equipment, making the entire process costly, time-consuming and slower (Chelani et al. 2002). This approach does not provide a complete picture of the chemical contaminants in the environment, as bioavailability and impacts on organisms are the two missing parts of the puzzle. As a result, tiny whole-cell biosensors are excellent equipment for use in environmental monitoring studies to reduce the genotoxicity of contaminants.

Cellular biosensors are frequently utilised to locate a wide range of heavy metal pollutants in the environment. Many ecological biosensors are focused on determining the lethality of a sample rather than identifying it. A transcription factor (TF) promoter pair and a reporter are used to create these biosensors. In *Bacillus*

*subtilis*, a cadmium sensor was created by combining the CadC (a regulatory protein) and the CadC promoters (a reporter gene) for firefly luciferase. This entire cell biosensor has nanomolar sensitivity to metals. A whole-cell biosensor in *Escherichia coli* responds to cadmium (Cd), zinc (Zn) and lead (Pb) via the ZntR-regulating protein and zatAp promoter. Other sensors are available to distinguish a broad array of ions (Coruh et al. 2014). Arsenic is extremely poisonous because of its incapacity to biodegrade; thrash metal gathers fast in organisms. Arsenic-detoxifying *E. coli* strains are used to identify arsenic. This sensor's operational algorithm is predicated by the adherence of the ArsR transcriptional proteins to their arsR promoter. Imagine arsenite is introduced into the reporter is transcribed by the algorithm, after ArsR decoupling as a result of its interaction with arsenite. Logic-gated cellular biosensors were created using an *E. coli*-based AND gate design to detect copper-detecting molecules in an aquatic medium, producing quantified fluorescence output. A triple-input logic gate was designed based on a prior system. The triple-input logic gate detects $As^{3+}$, $Hg^{2+}$ and $Cu^{2+}$ levels, which generate two populations that are connected by a synthetic cell–cell communication element (Darajeh et al. 2017). Later, a unique AND gate logic operation based on the hrp operon in *Pseudomonas syringae* was discovered. Two- and three-input AND gates were built utilising $As^{3+}$, $Hg^{2+}$ and *N*-acyl homoserine lactone (AHL) molecules as inputs in the researchers' study. HrpR and HrpS are manufactured in the two-input AND gate from a non-inverted $As^{3+}$ input controlled by the arsenic responsive promoter, whereas HrpV is produced from an inverted $Hg^{2+}$ input controlled by the mercury-responsive promoter. As a result, output will be enabled unless the systems are stimulated by arsenic, which engages HrpS–HspR. HrpS forms a compound with HrpV in the presence of both inducers, blocking the hrpL promoter, which encodes the output. The appearance of HrpS and HrpR was separated into two halves, and the two-input AND gate function was increased to three-input AND gate operations; HrpS is still controlled by the arsenic responsive promoter, and HspR is still controlled by the Quorum Sensing-responsive promoter (Devillers 1993).

## 29.3 Whole-Cell Biosensors for Monitoring Bioremediation

Whole-cell biomaterials are microorganism-based embedded systems to detect certain physical or chemical characteristics of an ambient sample. Microbial detection generates a signal, which is then translated into data that consumers may access. This information might be as basic as a sheet of coloured paper or as complicated as a quantitative computer display. A complete cell can be incorporated into the transducer or utilised as a multi-level attachment format separately (El-Naggar et al. 2021). The cells employed are unmodified genetically altered cells. Metabolism reporters are used to identify toxicity that impairs cell metabolism, whereas catabolic reporters can detect particular contaminants. Biosensors can offer data on pollutant bioavailability and are useful for monitoring bioremediation. Several commercially accessible sensors have been created, some of which have been thoroughly tested and proven to be useful in assessing contamination (Fawzy et al. 2018).

### 29.3.1 The Principle of Whole-Cell Biosensors

A biosensor is a unique expression of an analyte of interest by combining a sensitive natural element with a transducer. The natural element (e.g. microbes, enzymes, aptamers, antibodies and so on) interacts with the target by a simple list or recognition. This commerce generates a signal (optic, electrochemical) that is read by the transducer and is converted into a receiving signal. It also provides functional information related to processes such as protein conflation, apoptotic or necrotic cell death and the bioavailability of a target analyte, the quantum of the analyte that can cross the cell membrane and be taken up by the cell (Feng et al. 2019). The biocomponent of whole-cell biosensors can comprise prokaryotic or eukaryotic cells. Specific microbial biosensors can be acquired by conforming the microorganisms to a suitable substrate (target) of interest using picky culture conditions.

Optic discovery is also extensively used in microbial biosensors. The naturally bioluminescent bacterium *Vibrio fischeri* is extensively used as a bioreporter of ecotoxicity and forms the base of several marketable assays. Toxins that decrease metabolic exertion lead to dropped bioluminescence. A specific analyte induction of microbial fluorescence, luminescence or colour change is possible with genetically modified microorganisms having a journalist gene fused with a protagonist or inducible gene. Activation of the inducible gene by an analyte that suppresses the journalist gene leads to generation of an optic signal (Fernandes et al. 2020).

This review discusses four different biosensor strategies using unmodified whole-cell biosensors: (1) stimulation of microbial respiration by redundant nutrients; (2) inhibition of microbial metabolism due to environmental toxins; (3) specific pathways naturally decoded by microorganisms and (4) microbial declination of poisons to readily sensible products. The genetically modified metabolic catabolic and whole-cell bioindicators are also bandied.

### 29.3.2 Whole-Cell Immobilisation Strategies

Microbial immobilisation on the inquiry is a major step in the manufacture of microbial biosensors to maintain long-term cell viability, increase the reproducibility of responses and help in cell proliferation and spreading of transgenic cells in terrains, especially for field operations (Hattab et al. 2013). An immobiliser also provides a means of conserving microorganisms without the need for non-stop culture. Traditionally, whole cells were paralysed on the inquiry by physical methods (adsorption and encapsulation) or by chemical methods (covalent bond and cross-linking). More lately, natural biofilms have gained more interest.

#### 29.3.2.1 Artificial Films

Artificial films are used to incapacitate cell dormancies in an internal armature defined by encapsulation, adsorption or covalent bonding. Encapsulation involves enmeshing living cells in a matrix that provides a three-dimensional (3D) terrain

where cells remain feasible but stop growing. An ideal immobilisation matrix should be operable at a high temperature, ensure full cell retention, be non-toxic and be permeable to nutrients and oxygen (Hongwei et al. 2006). The matrix must guarantee viability of the cells over a long storehouse period. Organic (e.g. hydrogels such as agar) and inorganic (e.g. sol–gel) polymers have shown good performance in cell encapsulation, although the nebulosity of organic polymers frequently impairs optic signal discovery.

### 29.3.3 Unmodified Whole-Cell Biosensors

#### 29.3.3.1 Biological Oxygen Demand (BOD) Sensing

Removal of one of the oldest and most widespread environmental toxins is a goal rather than an asset of life. Redundant nutrients and organic carbon, whether applied to land and water for agrarian purposes or unintentionally emitted as a result of assiduity, husbandry and the general business of mortal life, eventually have the ability to destroy natural systems and tip them towards a state unfavourable to numerous multicellular beings (Huang et al. 2014). The stimulation of microbial growth is the cause of this environmental declination, which can affect blooms of poison-producing bacteria and/or oxygen privation, both of which are dangerous to numerous feathers of life that we value. One of the most common environmental hazards is organic pollution. Wastewater with a high organic lading is constantly treated before discharge by natural declination in erected-in systems or after discharge by natural refining processes.

### 29.3.4 Toxicity Measurement through Inhibition of Cellular Metabolism

BOD in terrains can be measured using whole-cell microbial biosensors for detecting and quantifying environmental toxins. Toxins can be assessed secondary to nutrient dimension using metabolic inhibition rather than metabolic exertion. The SciTOXTM fashion, for illustration, which is now being capitalized for quick Duck analysis, has also been successfully shown for use as a toxin assay. There are also some distinct metabolic pathways that have been used to quantify metabolic repression (Huuskonen 2001). This universal metabolic inhibition-based biosensor system detects general toxins but does not identify or quantify specific manures. Specific signalling pathways must be genetically integrated into the whole-cell element or microorganisms with unique and salutary metabolic pathways must be used to assess specific manures exercising a whole-cell biosensor approach. To descry specific environmental toxins, a number of microorganisms with new metabolic pathways are employed.

#### 29.3.4.1 Specific Metabolic Pathways

Certain substances could be detected using targeted metabolic repression. Dressings, for example, that inhibit photosynthesis can be detected utilising whole-cell biosensors with a photosynthetic organism as the detector biocomponent. The dressings atrazine and diuron were plant using a calcium alginate matrix fashion and an immobilized *Synechococcus* sp. Pesticide repression of photosystem II electron transport is used in the biosensor. Co-contamination of spots is more current than impurity with a single agent (Imran et al. 2017). As a result, analogous photosynthetic biosensors, including those for heavy and fungicides, have been retailed as general toxin detectors rather than pesticide-specific biosensors.

An electrode is utilised to quantify the oxygen reduction caused by substrate-specific bacterial declination for several surfactants. Surfactants with sweet rings have a high environmental stability and can be utilised to solubilise and concentrate other adulterants in the waterless phase. Nonylphenol is a wood pulp assiduity surfactant, and nonylphenol ethoxylate (NPEO) may be degraded by the bacteria *Comamonas testosterone* TI (Jaskulak et al. 2020).

This outfit had a discovery limit for NPEO of 0.25 mg/L that remained constant over a 10-day non-stop monitoring period. This approach has also been used to descry microbiological NPEO breakdown in wastewater, albeit using the NPEO-demeaning bacteria, *Alcaligenes*. Analogous whole-cell biosensors for surfactants have been demonstrated by *Pseudomonas* and *Achromobacter* strains, with target analytes including sodium dodecyl sulphate, volgnate, toluene sulphonate and alcylbenzene sulphonate (Karama et al. 2001).

#### 29.3.4.2 Substrate Degradation Products

Microorganisms degrade fungicides like parathion, releasing the electroactive emulsion p-nitrophenol, which can be detected using electrochemical methods that are quick, easy and affordable. Bacteria such as *Pseudomonas* and *Flavactenium* can naturally convert organophosphate fungicides to p-nitrophenol. Owing to inheritable manipulation, bacteria that overexpress enzymes that catalyse the conversion have been created and used as more sensitive biosensor strains (Karpinets et al. 2010). In recent times, the development of biosensors for the discovery of specific composites has evolved from employing whole cells that have not been altered in any manner. The current exploration seems to be concentrated on biosensors that use insulated factors of cells, such as enzymes, synthetic structures that bind specific substrates and whole-cell detectors that use genetically finagled metabolic pathways.

### 29.3.5 Genetically Engineered Whole-Cell Biosensors

#### 29.3.5.1 Metabolic Bioreporters

Genetically altered incentives, protozoa, algae, factory cells and, indeed, mortal cells have been used in environmental biosensor operations. Poison-demeaning bacteria put into the terrain can be detected and quantified using genetically finagled cells, and biosensors for general toxins can be used to reveal the presence and exertion of

certain poison-demeaning bacteria brought into the terrain. Depending on whether the journalist gene is expressed constitutively or inducibly, genetically finagled bioreporters are codified as catabolic or metabolic journalists (Karpinets et al. 2010).

Cell metabolism can also be re-engineered to ameliorate a cell's resistance or perceptivity to a substrate. This approach was used to develop an amperometric biosensor for detecting distinct toxins of organophosphate in a terrain, which improved the rate of respiration in genetically finagled *Moraxella.* Amino acid variations in the D1 subunit of photosystem II have also been used to develop finagled *Chlamydomonas* strains with increased perceptivity or resistance to a specific pesticide class (Khataee et al. 2012).

#### 29.3.5.2 Catabolic Bioreporters

Individual substances or groups of motes can be detected and quantified using catabolic bioreporters, whereas metabolic bioreporters can be used to cover cytotoxicity caused by the terrain. Catabolic bioreporters are formed by a journalist gene governed by an inhibitory protagonist, which activates when the target analyte is present. Owing to their extremely low detection limits, whole-cell catabolic bioreporters have been used to test particular adulterants in complex environments (Korvigo et al. 2018). Bioreporters' specificity can be altered by fusing the journalist gene with promoters that are convinced by a group of composites rather than a single emulsion or that are convinced in response to activation of a specific signal transduction pathway that responds to multiple inputs or further general conditions similar to a cellular redox state. A fluorescent journalist gene has been placed under the control of an incentive stress-seeing system to boost journalist gene conflation in response to environmental stress.

Journalist genes include the *E. coli lacZ* gene, which expresses galactosidase as a chromogenic marker, fluorescent protein genes like *GFP* and its wavelength- shifted performances, luciferase genes like the eukaryotic *lucF* gene and the bacterial *luxCDABE* gene cluster for bioluminescence. As it is a sensitive journalist gene that does not bear any external substrates, the *luxCDABE* gene mail from *Vibrio fischeri* has been constantly used.

## 29.4 Engineered Microorganisms as Sensing Machinery in Microbial Biosensors

Microbial sensors are valued devices used for routine heavy metal analysis in order to examine the landscape; however, they have several limitations, including poor sensitivity and selectivity, particularly in multiplex recognition, and intense information that is influenced by genetic and thus phenotypic heterogeneity and stochastic upregulation. However, these limitations may be overcome by combining micro-electromechanical systems (MEMs) with microbial biosensors (Kim et al. 2009). The prevalent commitment of whole-cell biomaterials is the incorporation of whole-cell biomaterials and micro/nanotechnologies into microfluidic systems. In collaboration with whole-cell biomaterials, a microfluidic device capable of sensing

metal ions was developed. The origin of this development is revealed as a mix of various aspects, including microfluidic chambers that provide a new growth medium and heavy metal ions as inducers while also eliminating trash created by the cells, despite several drawbacks, such as a restricted dynamic range and adaptability to high-throughput examination in numerous samples. A microfluidic device with cells was used to create a biodisplay platform. This biodisplay technique enables the screening of many analytes using individually programmable cell cultures. The reaction of modified bacterial cells grown on a biodisplay platform to various arsenite concentrations was examined, for example. There are claims that biodisplays exert dangerous effects. The biodisplay approach is also ecologically benign since it prevents genetic elements from escaping (Lee et al. 2016).

## 29.5 Heavy Metal and Organic Pollutant Sensing Using TCRS

A wide range of environmental signals, including illumination, respiration, acid levels and temperature, and perhaps a number of heavy metal and organic contaminants, may be detected by several regulators. Several reports have been published; however, few substantial metal- and organic pollutant-based sensors have been developed thus far. Bacteria detect heavy metals via a variety of TCRSs. In *E. coli*, a *HydHG* TCRS was discovered that recognises and regulates the expression of the *zraP* gene, which encodes the zinc efflux protein in the presence of high amounts of $Zn^{2+}$ and $Pb^{2+}$ in the aerobic state. The HydH polypeptide is closely connected to the cell membrane and is considered to detect excessive amounts of periplasmic $Zn^{2+}$ and $Pb^{2+}$. Following this, in the presence of a phosphoryl donor, HydG binds to the intergenic region inside zraP-hydHG, resulting in ZraP expression being upregulated (Lopez et al. 2017). Several TCRSs can control the expression of many genes in an operon or the whole operon. The SilRS TCRS improves *Salmonella enterica* resistance to silver cations by the coordinated sensing and activation expression of the periplasmic silver-specific binding protein. This is also true for the NrsSR TCRS discovered in *Synechocystis* sp. PCC6803. NrsSR detects $Ni^{2+}$ and $Co^{2+}$ ions and controls the production of the nrsBACD operon, which produces $Ni^{2+}$ resistance proteins (Ma et al. 2011).

The most biodegradable chemical carcinogens are aromatic molecules. However, the majority of bacteria are disrupted by the use of these chemicals, because of bacteria's genetic and metabolic flexibility. A multitude of TCRSs have been shown to be involved in aromatic chemical catabolisation from the beginning. *Pseudomonas putida* induces aromatic substrates such as toluene and ethylbenzene. TCRSs control the expression of tod genes, which code for enzymes involved in aromatic chemical breakdown. The StySR TCRS discovered in the *Pseudomonas* sp. strain Y2 induces the synthesis of the *styABCD* genes in response to fluctuations in styrene concentration in the environment (Masood et al. 2012). Another TCRS, BpdST, may be used to regulate heavy metal bioadsorption based on biphenyl or pTCRs in conjunction with a biosensor. One of the most significant advances in biosensor-based technology is the employment of a genetically engineered microorganism that

generates a clear signal when microorganisms come into contact with a target chemical. Several research groups have created a range of TCRS-based ecological contamination sensors based on the types of cells utilised. Heavy metal biosensors based on TCRSs have been developed for use in bioremediation applications, which are discussed below.

A zinc adsorption system was created using the ZraSR TCRS and the chimera zinc-binding OmpC. ZraSR recognises and activates the membrane protein ZraP, which is responsible for $Zn^{2+}$ ion efflux in a normal microbial environment. The designed zinc adsorption system is based on the standard ZraSR TCRS, in which ZraS detects $Zn^{2+}$ ions, but ZraR activates the ompC Zinc-binding amino acid chimera gene under the ZraP promoter rather than the resident ZraP (Moussa et al. 2021). Zinc-binding molecules on the cell surface can absorb exogenous zinc. Even in small amounts, this method is susceptible to zinc (0.001 mM). Similarly, the simultaneous detection and removal of copper ions from the bacterial surface was accomplished by combining the use of the CuSR TCRS with the cell surface exhibiting copper-binding peptides (CBPs) linked to the membrane protein OmpC. CuSR stimulates the expression of the chimera OmpCCBP when it recognises $Cu^{2+}$ ions in this approach. As a result, these chimeric proteins expressed on the bacterial cell surface can adsorb copper ions (Mullai et al. 2011).

The development of the chimeric OmpC with the metal-binding site is driven by metal ions, which is an intriguing characteristic of these adsorption systems. As a result, the development of a heavy metal biosensor in combination with a bioadsorption device would enhance analytical heavy metal detection methodologies, allowing for the rapid monitoring and removal of hazardous levels of bioavailable metal contaminants in industrial settings. Without the need for an induction device, the better biosensor paired with bioadsorption is capable of efficiently absorbing heavy metals. As a result, this artificial bacterial system is a great model for designing versatile synthesised systems capable of effectively removing and recovering the target molecule (Nourouzi et al. 2012).

## 29.6 Whole-Cell Bioreporters That Have Been Genetically Engineered for Environmental Monitoring

Bioreporters are real-time detectors to locate priority environmental pollutants and toxicologically significant substances. These devices are made up of bacterial or eukaryotic (fungi, algae and mammals) cells. These cells have evolved unique inheritable traits that allow them to acclimatise (e.g. metabolism) or show endurance (e.g. a bactericidal poison), allowing them to live and reproduce in nearly any ecological niche. A bioreporter emits light when exposed to a certain chemical or poison. This necessitates a thorough understanding of the inheritable medium underpinning the inheritable protagonist that has dominion over it. The protagonist in the cell governs the genes that behave like toxins (Nobrega et al. 2013). The protagonist's link to these genes is disassociated in the bioreporter cell, and the protagonist is replaced with a journalist gene, which is now transcribed and restated

into a journalist protein, which when actuated by the protagonist emits a bioluminescent. We will concentrate on bioreporter seeing bias that has been used in environmental conditions to establish their practical discovery and monitoring capabilities. As informed by the anthology, numerous further bioreporters than those listed are then still laboratory-bound and are capable of detecting a wide range of substances and chemical relations.

### 29.6.1 Common Reporter Elements

Bioluminescence is derived from bacteria, firefly (luc) genes, the green fluorescent protein (GFP) gene and its several coloured variations and colorimetric end points from the galactosidase (lacZ) gene.

#### 29.6.1.1 Bioluminescence

Bioluminescence—meaning in a living body, light is produced chemically—is used as a journalist element in ambient biosensing. An enzyme (luciferase) catalyses the chemical response that creates bioluminescence by responding with a substrate (luciferin) to form an agitated state patch that emits photons when it relaxes back to its ground state (Oguz and Ersoy 2014).

#### 29.6.1.2 Bacterial Luciferase (lux)

Bioluminescent response (*Photoradars, and Photobacterium rubrics*) is reduced riboflavin phosphate ($FMNH_2$), which is oxidised, the luciferase enzyme is activated. The genes *luxA*, *luxB*, *luxC*, *luxD* and *luxE* (denoted as *luxCDABE* to represent the order of the genes in the operon) control this system (Orellana et al. 2019). The *luxCDE* genes provide and replenish long-chain aldehydes and *luxA* and luxB (*luxAB*) gene products produce a heterodimeric luciferase.

Leftover oxygen and $FMNH_2$ reactants are salvaged by supplemental metabolic training within the cell. The end result is a 490-nm wavelength blue/green light signal. There are two orders of operation for lux-grounded bioreporters. The most basic method is just integrating the *luxAB* genes with bioreporters that only include the luciferase enzyme, which also demands the inclusion of an exogenous aldehyde, often n-decanal, to the process (Pinski et al. 2020).

#### 29.6.1.3 Firefly Luciferase (luc)

The *luc* gene, which is most typically taken from the firefly *Photinus pyralis*, a prominent journalist gene, produces powerful light products and has quick response kinetics. In the context of ATP–$Mg_2$ and oxygen, the Luc protein catalyses the oxidation of a reduced luciferin substrate to produce an unheroic/green 562-nm light, the highest yield of any bioluminescent system ever examined. As there are no post-translational modifications required for the Luc protein, it is ready for use as soon as it is produced (Rene et al. 2011). Luc-grounded bioreporters cannot reply autonomously or continually cover stoner-defined targets since luc journalist systems bear the exogenous input of the luciferin substrate. Despite this, its minimal light affair

equates to exceptionally sensitive assays for a wide range of chemical composites, heavy essence and estrogenic and endocrine disruptor chemicals that are applied to the terrain.

#### 29.6.1.4 Green Fluorescent Protein (GFP)

The Luc protein does not bear any post-translational changes; thus, it is ready for use right away. As luc journalist systems bear the external input of the luciferin substrate, luc-grounded bioreporters cannot reply autonomously or continuously cover stoner-defined targets. Despite this, their maximal light sensitivity corresponds to exceptionally sensitive tests for a range of chemical components, essence and chemicals found in the environment (Rustum et al. 2008).

#### 29.6.1.5 β-Galactosidase (lacZ)

The lacZ gene canons for a-galactosidase (- girl) enzyme can be able to catalyses the hydrolysis of-galactosidase disaccharides into monosaccharides. It was reproduced from *E. coli*. LacZ- grounded bioreporters handed a colorimetric signal when given the substrate o-nitrophenyl—ß-D-galactosidase (ONPG). LacZ- grounded mixtures to DNA-sensitive genes are utilised in commercially available accoutrements similar as the SOS Chromotest to screen environmental samples for mutagenic. Similarly, when a substrate is given to bioreporter cells, they must be permeabilised, resulting in inconsistent and frequently delayed data gathering (Sahinkaya 2009). To prevent permeabilisation, electrochemical and amperometric interfaces might be employed.

### 29.6.2 Ecological Evaluation Using Bacterial Bioreporters

Bacterial cells are used as host cells in the maturation of whole-cell bioreporters because they are genetically simple to alter and are tolerant of a wide range of conditions. By utilising natural cellular mechanisms such as stress response, poison defence and emulsion catabolism, bacterial bioreporters may be extremely well-developed and find conditions. Bioreporters for bacteria have been created and characterised in huge amounts. Still, because of restrictions that circumscribe or enjoin the release of recombinant DNA into the terrain, their practical operations are limited (Schryver et al. 2006). This section summarises the current bacterial bioreporter operations in environmental evaluation to help readers better understand what bacterial bioreporters may provide as environmental observers. Amongst the most important advantages of bioreporters is their ability to report on bioavailability. The relationship between bioreporter detection and bioavailability is impacted by a number of factors.

#### 29.6.2.1 Heavy Metal Detection and Monitoring Using Bioreporters

Owing to their widespread prevalence in the environment and intrinsic toxicity to humans and wildlife, heavy essence are essential inorganic pollutants for hazard assessment. A lengthy history of research into microbial essence resistance has resulted in a wealth of information on the inheritable rudiments and

non-supervisory mechanisms involved in essence resistance. Efflux transporters, which laboriously export dangerous and/or modified enzymes that transform the essence to lower poisonous forms, are generally used by bacteria to repel essence toxins. As the proteins involved in these defence mechanisms need energy for expression, they add to the body's metabolic cargo (Sudkamp and Haas 2000). When a specific transcriptional controller attaches to essence, it stimulates the recap of downstream defence-related genes, performing in the induction of a specific defence ministry. MerR and ArsR, for example, are transcriptional controllers that control Hg and As defences. To develop a non-generic essence bioreporter, a transcriptional controller with essence specificity, a protagonist/driver of defence-related transcription of related genes can be fused with a reporter gene with no promoter or with journalist genes. When the transcriptional controller is exposed to essence, it is activated and the transcriptional controller becomes active.

#### 29.6.2.2 Organic Pollution Detection and Monitoring Using Bioreporters

Human-induced conditioning has resulted in the release of a variety of colourful organic composites into the ecosystem. Despite their artificial utility, these composites are a significant source of environmental adulterants since they have a negative impact on human health. For decline in organic composites, microorganisms have established transcriptionally controlled catabolic processes. Similar to the case of essence resistance, an effector-activated non-supervisory protein promotes the expression of genes encoding declination enzymes in order to control catabolic processes at the site of recapitulation (Talwar et al. 2020). Bacterial bioreporters for organic substances are therefore based on non-supervisory proteins and their associated promoters. A plasmid containing a *luc* gene connected to the xylene-binding non-supervisory protein XylR and the XylR-responsive enzyme Pu, for example, was created and injected into *E. coli* DH5, resulting in a bioluminescent bioreporter that reacted to toluene and other related chemicals. Bioreporters have been developed for middle-chain alkanes, simple sweet hydrocarbons (similar to BTEX (benzene, toluene, ethylbenzene and xylene)), two-to-three ring polycyclic sweet hydrocarbons (PAHs) and phenolic composites (Tang et al. 2008).

### 29.6.3 Emerging Eukaryotic Whole-Cell Bioreporters

#### 29.6.3.1 Available Eukaryotic Bioreporter Protein Classes

Although bacterial bioreporters have been proven effective in the detection and monitoring of a wide variety of environmental factors, eukaryotic systems are increasingly being used in this field. One major problem is that the various ploidies of bacterial species might influence the mutagenic or carcinogenic behaviour of environmental toxins that arouse the journalist's interest or can lead to conditions in which inheritable revision cannot be discovered. Furthermore, like with estrogenic monitoring, these bacterial journalists may just be deserving of the necessary

attention (Terron-Camero et al. 2020). Factors for interacting with the target analyte Whole-cell eukaryotic bioreporters avoid these challenges by identifying specific composites.

#### 29.6.3.2 Lower Eukaryotic Hosts Are Used for Environmental Monitoring

GFP (green fluorescent protein) and its variations are utilised in eukaryotes to determine ambient adulterants, and when numerous fluorescent proteins with overlapping emigration wavelengths are used, they can enable the detection of several adulterants. Nonetheless, produced in bacteria, their activity in eukaryotic cells is hampered by the existence of fresh, naturally fluorescent mixtures inside the host. Given normal imaging conditions, this might result in substantial amounts of background fluorescence, decreasing the detectability of the intelligence signal, in situations of moderate induction (Titah et al. 2018). Bioluminescent intelligence systems, on the other hand, are not affected by such high background circumstances in eukaryotic hosts and are hence routinely preferred over their fluorescent counterparts for imaging smaller-cell populations or signal detection. The expression of bacterial lux genes has recently been optimised for non-supervisory eukaryotic regulation. They are the only intelligence systems that are suitable for recurring, real-time signalling since no substrate is required, even if their performing bioluminescence emigration is not as dazzling as luc.

### 29.6.4 An Evaluation of the Requirements for Whole-Cell Bioreporters in Environmental Applications

Whole-cell bioreporters have several advantages. Despite these advantages, bioreporters rarely, if ever, reach the commercialisation stage or attain the conventional train operation status due to a number of patent obstacles, the most significant of which, from a marketing standpoint, is the inability to patent most bioreporters because the intelligence gene technology they incorporate is well-established and lacks novelty (Vafaei et al. 2013). There is less profit to be made without a patent, and, as a result, there is less interest in the pursuit.

The fact that bioreporters are recombinant organisms has a significant influence on their capacity to perform in real-world settings, and the possibility of recombinant DNA dislocating from its initial host to other members of the microbial community is a difficult and poorly understood phenomena with potentially major environmental implications. Government-mandated restrictions and guidelines strictly limit the release of recombinant organisms into the environment, time-consuming & expensive thus it leaves no room for a marketable enterprise. As a result, real-world bioreporters have been developed. Operations will focus on biomaterials, wherein the bioreporter is incorporated into a monitoring device. As a result, it cannot be freely discharged into the environment (van der Werf and Zimmer 1998).

The ultimate benchmark of a bioreporter is its perceptivity, and because bioreporters have yet to reach the perceptivity (or specificity) of logical chemical

styles, several end-user actions remain out of bounds. In order to increase perceptivity, researchers have concentrated on enhancing promoter rudiments that have evolved low perceptivity or genetically modifying promoter rudiments to be more sensitive. Bioreporters also respond to more than one target, making it difficult to detect the location and amount of a given molecule within a complicated mixture. Variations in the transcriptional regulator engaged in the seeing pathway can provide an implicit mechanism for improving specificity (Wang et al. 2018). This shift away from visionless slicing decreases the exorbitant expenses associated with logical styles that consistently provide a high number of useless samples merely labelled as below-discovery limitations (Wolf et al. 2001).

## 29.7 The Use of Whole-Cell-Based Biosensors for Environmental Analysis

A quick identification of contaminants in an ecosystem and the evaluation of their influence on health is an important research subject. Despite the fact that standard physical and chemical analytical techniques can determine the exact composition and amount of toxins in samples with high precision and sensitivity, only a small number of toxins can be examined for bioavailability, toxicity and genotoxicity. In most circumstances, live cells can only be utilised to measure a few key properties. Whole-cell-based biosensors have two significant advantages: (1) they can be field-tested easily and (2) they can detect bioavailable pollutant components easily (Fig. 29.1).

BMB-PL is used to identify the presence of phenanthrene (PHE) in red soil samples (Yang et al. 2006). The starting concentrations of PHE ranging from 10 to 60 mg/kg were studied. HPLC was able to detect around 80% of the PHE. The sample extraction procedure utilised during the HPLC test was mostly responsible for this. In addition to organic detection, one experiment employed a comparable bioluminescent whole-cell biosensor to test the bioavailability of Pb and Cu concentrations in natural soil. This result was consistent with the findings of the PHE investigation; the whole-cell-based biosensor achieved significantly higher selectivity. Chemical analysis and the use of a specialised laboratory were really expensive. Finally, it has been established that whole-cell-based biosensors are capable of continually monitoring the bioavailability and concentration of dangerous chemicals (Zhang et al. 2018).

### 29.7.1 Reporter Genes

Reporter genes utilised to control transcriptional toxins and regulatory proteins coupled with these promoters frequently affect the performance of whole-cell biosensors for detecting environmental contaminants. In live cells used as sensors, a reporter gene can convert its biological reaction into a physicochemically visible signal. This method is critical for whole-cell biosensor sensitivity and selectivity.

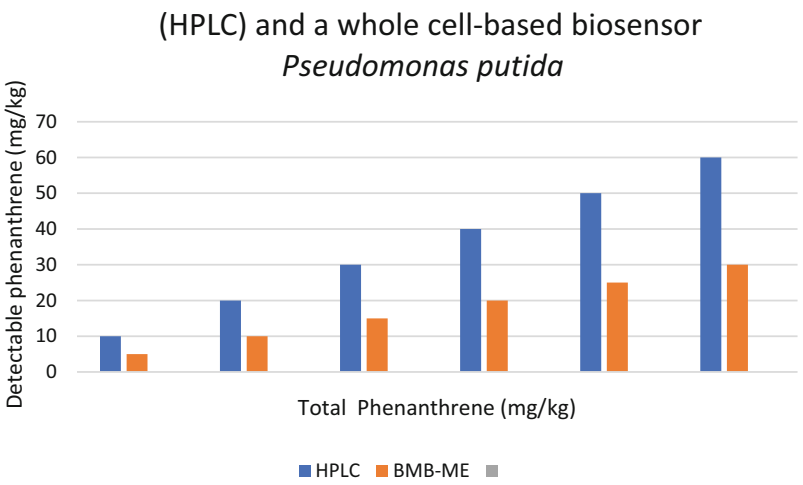


**Fig. 29.1** Comparison of the capabilities (HPLC) and whole-cell-based *Pseudomonas putida* biosensor

Some frequently used reporter genes have been shown to integrate well with whole-cell-based biosensors. Examples include luc (firefly luciferase) and lacZ (galactosidase). As there are so many reporter genes to choose from, choosing one to utilise may be tricky.

Table 29.1 summarises the advantages and disadvantages of frequently published reporter genes.

*Gfp* is a reporter gene. It causes signal interference. As a result, GFP-based biosensors are less sensitive than lux- and lacZ-based biosensors. Furthermore, GFP takes longer to produce a consistent fluorescence, reducing its maximal detection activity (Zhu et al. 2019). As a result, GFP-based whole-cell biosensors are frequently ineffective in rapidly identifying containments. Similarly, heat lability and dimeric protein interference limit the activity of bacterial luciferases (*lux*) as a reporter gene in human cells. The firefly luciferase (*luc*) reporter was extensively incorporated into mammalian cells to avoid these limits because of its high sensitivity and wide linear range (up to 7–8 orders of magnitude). Another often employed reporter in molecular biology is galactosidase (lacZ), a well-characterised bacterial enzyme that is an effective sensor of transfection efficacy. LacZ has distinct benefits for detection utilising colorimetric or fluorescence methods since its application to a sample is straightforward and quick. As lacZ chemiluminescent and electrochemical substrates are widely available, they provide ultra-high sensitivity, a low detection limit (as low as 2 fg) and a broad dynamic detection range. Fujimoto and co-workers developed crtA, a novel kind of reporter gene responsible for carotenoid synthesis in another reporter system (Zin et al. 2020). CrtA-based whole-cell biosensors, when applied to a sample, change the colour of the culture media from yellow to red substrate, making them an excellent choice for quick detection in emergency situations.

**Table 29.1** The list of frequent reporter genes identified in whole-cell sensors

| Gene | Detection method | Advantages | Disadvantages |
|---|---|---|---|
| *Lux* | Bioluminescence | Simple counting and quick answer | $O_2$ demand and thermal lability |
| *Luc* | Bioluminescence | High sensitivity and quick response time | $O_2$ and ATP needs |
| *Gfp* | Fluorescence | There is no need for a substrate and the product is quite stable | Autofluorescence |
| *lacZ* | Electrochemistry | Wide range of sensing techniques and detection with the naked eye | Low permeability due to substrate dependence |
| *crtA* | Colorimetry | Detection with the naked eye | Dependent on the substrate |

## 29.8 Conclusions

There is a vast array of different technologies that have been developed for environmental biosensing. Whole-cell, unmodified bioreporters for general toxin monitoring have been developed, and assay accoutrements based on this technology are extensively commercially available. Inheritable revision of whole cells has increased the mileage, perceptivity and particularity of these biosensors. Whole-cell biosensors allow provident quantification of biologically active pollutants and should be more extensively used in place of, or as a reciprocal addition to, more precious and complex chemical analyses. Published explorations into biosensors seem to show a relative increase in interest in biosensors that use genetically modified cells and also towards biosensors that incorporate biocomponents such as enzymes and DNA rather than unmodified whole cells. The unborn trend for all biosensors seems to be towards miniaturisation; high-output equipment and enhanced integration allow for the creation of a single bias with numerous channels, simpler support systems and faster fire reaction times at a reduced cost. The issue may thus not lie in the bioreporter's capacity to properly collect and send data.

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