Maulin P. Shah *Editor*

Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment

Springer

Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment

Maulin P. Shah
Editor

Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment

Springer

Editor
Maulin P. Shah
Divison of Applied and Environmental
Micro Lab
Industrial Waste Water Research Lab
Ankleshwar, Gujarat, India

ISBN 978-3-031-44617-7 ISBN 978-3-031-44618-4 (eBook)
https://doi.org/10.1007/978-3-031-44618-4

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Paper in this product is recyclable.

Preface

Day by day wastewater treatment is becoming a challenging task for environmentalist. If they do not maintain the discharge norms of their treatment plant, they will be in big trouble by the governing authorities. To keep safer side, wastewater treatment is enormously required. Study of antibiotic-resistant genes is among one of the most important tasks in industrial wastewater treatment. The cleaning of toxic contaminants or refractory pollutants from industrial wastewater treatment processes is an essential step for the protection of public health and of course for the environment. Biological wastewater treatment plants that use sludge represent one of the most used biotechnological processes. Elimination of organic carbon and other nutrients mainly is nitrogen (N) and phosphorus (P), which make sludge microbes essential to prevent eutrophication and deterioration of receiving surface waters. The complexity of wastewater microbial communities, based on 16S rRNA sequence analysis, is known enormous. Biological wastewater treatment is among the most important environmental biotechnological applications, and, as drivers of key processes, microorganisms are critical to its success. Therefore, the genomic study of wastewater microorganisms has an obvious applied significance; however, the importance of wastewater treatment reactors as model systems for microbial ecology is often overlooked. Modern molecular techniques, including environmental genomics, have identified key microbial actors unexpected for nutrient removal and/or sludge foaming and have provided many interesting insights into niche diversity, functions, and differentiations. Now is the time for wastewater microbiology to be recognized as a mature and dynamic discipline, offering much towards a deeper understanding of life in complex microbial communities. Comparative genomics of antibiotic-resistant genes isolated from conventional activated sludge and biological aerated filter wastewater treatment plants will be discussed.

Ankleshwar, India — Maulin P. Shah

Contents

Characterization Methods for Microbial Communities Present in Contaminated Soils

Sonia Sethi and Aakanksha Kalra

Abstract Microorganisms present in soil ecosystems contaminated with hydrocarbons and heavy metals are one of the most complex and diverse assemblages. The influence of long-term soil contamination on the activities and roles of microorganisms, community structure and genetic diversity are the focus of much interest. With the advent of recombinant DNA technology, microbial community have developed as a potential resource for various biotechnological products, bioenergy production and novel biotransformations. Long-term contamination of soil with high amounts of heavy metals and other organic compounds is expected to induce gradual changes in the composition of microbial communities. This is turn leads to a rise in the tolerance of strains and also spread of these metal resistance genes in the environment and other organisms via horizontal transfer. Identification and characterization of such microbial communities has been limited to those which are culturable so far but with the advancements in the approaches such as genomics, transcriptomics, proteomics, metabolomics and lipidomics has led to the discovery of previously unrecognized microorganisms and complex microbial diversity which shows an exciting opportunity for bioremediation strategies. In the view of this, the chapter examines the current applications of molecular techniques for the characterization of microbial communities in soil contaminated with heavy metals and petroleum hydrocarbon. Various "stress-on-stress" studies including metagenomics and meta-transcriptomics investigate microbial communities under environmentally stressful conditions which provides a deeper insight on predictable species assemblages.

Keywords Microbial community · Heavy metals · Organic compounds · Bioremediation

S. Sethi (✉) · A. Kalra
Dr. B. Lal Institute of Biotechnology, Malviya Nagar, Jaipur, Rajasthan, India
e-mail: soniakaura@blalbiotech.com

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_1

1 Introduction

The environment is referred to as place where humans, plants, animals and microorganisms survive. It is made up of land, atmosphere, water, microorganisms, plant and animal life, of amalgamation or correlation among and between them. Also, anthropogenic activities including various conditions that affect health of human beings. An environment can be polluted or contaminated. Contamination is different from pollution; however, contaminants can be pollutants, and possess deleterious effect on the environment. Pollution is anything that is introduced by humans either directly or indirectly, any substances or energy causing harmful effects to living resources, human health and reduction of amenities. On the other hand, contamination is the appearance of high concentrations of substances in the environment above the standard level (Wong, 2012).

2 Types of Pollutants

Pollutants causing environmental issues are causing a great challenge toward global society. Various types of pollutants are known in nature, namely inorganic, organic and biological. All of them have been given significant attention because of the consequences they are causing to the environment. Issue related to the amount of toxic substances in the environment is at alarming condition among the population.

2.1 Inorganic Pollutants

Pollutants from agriculture, industry and domestic wastes causing adverse effects to human and animals are considered as inorganic pollutants. It includes metals, salts and minerals (Wong, 2012). They occur naturally, but human interference has altered their amount in the environment. Anthropogenic activities including metallurgical, chemical and smelting processes lead to accumulation of these pollutants in food chains.

2.2 Organic Pollutants

Organic pollutants are considered as contaminants of environment which are easily degradable. They are also naturally occurring, but human activities have contributed an intensive amount to environment to meet their needs. Organic pollutants include polycyclic aromatic hydrocarbons (PAHs), human and food waste, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), pesticides, petroleum

and organochlorine pesticides (OCPs) (Lepp, 2012). Nowadays, organic pollutants are gaining more attention because of their characteristics and persistent nature causing toxicological effects.

2.3 Biological Pollutants

Pollutants' resulting due to human's actions and their impact on the environment quality are biological pollutants. It includes microorganisms, animal dander, cat saliva, dust, cockroaches and pollen. These pollutants are from various sources including plants (Pollens), humans, animals, (microorganisms), etc.

2.4 Heavy Metals

Heavy metals (HM) are metals of high density. Soil contamination by heavy metals is a censorious worldwide environmental issue. Metals are crucial constituents of the environment, and its accessible concentrations depend mainly on various processes (Appenroth, 2010). There are two types of metals one that is necessary for living beings called as microelements and other elements with unrevealed biological character and "inert" with regard to organisms. The metals acting microelements in living organisms are present in minute quantities, and their inadequacy and overabundance poorly influence living organisms (Szyczewski et al., 2009).

Heavy metals are known to be toxic in nature, but this is not true every time because its effect on living organisms depends upon the concentration available. Some heavy metal ions are significant for various metabolic pathways at low concentrations but are toxic at high concentrations. Metals such as arsenic, lead, cadmium, nickel, mercury, chromium, cobalt, zinc and selenium are highly toxic even in minor quantity. Increased discharge of amount of these heavy metals through various anthropogenic processes leads to remarkable ecological and health complications. Due to persistence of most of the heavy metals in terrestrial ecosystem, toxicological and deleterious consequence on the environment and humans (Järup, 2003; Shah, 2020, 2021a, 2021b) is considered a predominant ecological concern across world.

Heavy metals originate naturally and anthropogenic activities of humans and land up in various parts of environment. Various natural sources of heavy metals include volcanic eruptions, sea-salt sprays, forest fires, rock weathering, biogenic sources and wind-borne soil particles. Natural weathering processes also release metals to different environment compartments. Heavy metals can be found in the form of hydroxides, oxides, sulfides, sulfates, phosphates, silicates and organic compounds. The most common heavy metals are lead (Pb), nickel (Ni), chromium (Cr), cadmium (Cd), arsenic (As), mercury (Hg), zinc (Zn) and copper (Cu) (Van der Ent., 2013).

Various anthropogenic activities such as industries, agriculture, wastewater, mining and metallurgical processes, and runoffs causes release of pollutants to

different environmental compartments. Other processes include automobile exhaust which releases lead; smelting which releases arsenic, copper and zinc; insecticides which release arsenic and burning of fossil fuels which release nickel, vanadium, mercury, selenium and tin (Ebbs & Kochian, 1997; Shah, 2021).

Nickel (Ni) causes losses in the yield of crop and also results in harmful health effects when it enters the food chain. At very high concentrations, it stands in the way of various processes of plant body (Gajewska et al., 2006), leading to the growth retardation and loss of chlorophyll, wilting, etc. Also, it interferes with various enzyme activity and oxidative stress. Arsenic is more hazardous as compared to chromium and copper resulting in bonding with phosphorous and sulfur thereby uncoupling oxidative phosphorylation and blocking protein synthesis (Tamaki & Frankenberger, 1992). Chromium (VI) diffuses across the membrane and results in toxicity due to interaction with radicals.

Though soil is considered as a native source of heavy metals, changes influenced by people ameliorate their magnitude which is detrimental (Chibuike & Obiora, 2014). Heavy metals are transported off shore in gaseous and coarse aspect leading to aggregation in soil, water and living systems rapidly. Anthropogenic actions like unusual and everlasting applications of agrochemicals, sewage sludge, industrial waste disposal, waste incineration, and vehicle exhausts are major resources in soils.

Microorganisms present in soil including free living or in association with the plants can ameliorate the biomass productivity of plant and phytoremediation activity. Although microbial population size, growth, structure, metabolism, degradation of protein or cell membrane, and overall activities are influenced by presence of heavy metals in soil, these are necessary for degradation of soil organic matter; absorption of nutrient from soil by the plant is adversely affected by reduction in microbial diversity.

Microorganisms are responsible for reducing the magnitude of contaminants and biological activities in native surroundings (Díaz, 2010). Microbial communities are regarded as biological indicators because they are highly sensitive to changes occurring in the environment (Keshri et al., 2015). Indigenous microbial communities have adapted long-term remediation of heavy metal contamination and their demolition through pollution results in lowering or diminishing of the plant or animal populations.

Among different types of heavy metals, cadmium (Cd), arsenic (As), lead (Pb), mercury (Hg) and chromium (Cr) are the most toxic substances (Alloway, 2013) and potentially damage the ecosystems. For example, Cd inhibit calcium metabolism resulting in kidney and skeletal damage (Johri et al., 2010). Arsenic intake results in gastrointestinal symptoms, heart and nervous system. Pb poisoning leads to headache, abdominal pain, neurological disease and blood–brain barrier of children. Acute Hg poisoning causes lung damage. Heavy metals are absorbed by food chain and water (Wongsasuluk et al., 2014), and therefore, accurate concentrations of heavy metal and steps to remove them should be taken.

In terrestrial ecosystems, soils are the major sink for metal contamination. Soil microbes especially the rhizospheric population play important role in HM detoxification in contaminated soils. This input of the rhizomicrobial population

is also referred to as rhizoremediation. This involves higher metabolic activity of microbes including prokaryotes and eukaryotes near the vicinity of plants' root. In contaminated soils with heavy metals, predominant population of bacteria includes Firmicutes, Proteobacteria, Actinobacteria, Bacillus, Pseudomonas and Arthrobacter (Pires et al., 2017). Rhizobia is an important plant growth promoting (PGP) microbes occurring in the rhizosphere and is responsible for nodulation and nitrogenase activities which are found to be very sensitive to heavy metal stress. There are some HMT rhizobial strains which have also been reported effectively carrying out symbiotic nitrogen fixation in contaminated sites. Symbiosis between Legume–rhizobia can detoxify HM and ameliorate the characteristics of contaminated soils (Checcucci et al., 2017).

Among fungi, Ascomycota and Basidiomycota and soil with fewer nutrients are frequently colonized by arbuscular mycorrhizal (AM) fungi in polluted soils with heavy metals (Narendrula-Kotha & Nkongolo, 2017). As a matter of fact, binding of metal ions on the surface of cells drives the intracellular activities of AM fungi and other rhizosphere microbes like transformation, transport, toxicity, dissolution and deterioration. This interaction depends upon physico-chemical nature of soil, type and concentration of metal species, metabolic activity and diversity of microbes (Kong & Glick, 2017).

2.5 Petroleum Hydrocarbons

One of the major energy resource and substance for various industries is hydrocarbons which is a serious environmental problem caused by oil fields and stay for long term (Varjani & Upasani, 2016). Hydrocarbons are produced by large-scale production, transport, oil refining, shipping and accidental spilling. Polycyclic aromatic hydrocarbons or petroleum hydrocarbons are released in the environment due to various anthropogenic activities like partial ignition of organic matter, for instance in coking plants or gas factories. Also, commercial activities leave large amount of PAH resulting in soil contamination, frightening ecosystems outcome and health of human beings (Sajna et al., 2015).

Under such conditions, unique bacterial communities adapted to the contamination conditions develop in the soil. Long-term pollution due to petroleum products in soil results in aggregation of both polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. Deterioration of hydrocarbon required genes and pathways. Aliphatic hydrocarbons are chains which can easily undergo biodegradation while large branched chains and aromatic hydrocarbons remain in environment for long term (Hasanuzzaman et al., 2007).

Therefore, polycyclic aromatic hydrocarbons (PAHs) are considered to be carcinogenic, cytotoxic, genotoxic and environmentally toxic. PAHs are fused aromatic ring compounds found in the atmosphere and relatively resistant to the biodegradation; as such, they accumulate to significant levels into the environment. PAH presence in environment affects microbial biodiversity and functions of soil in a negative way,

where the accumulation has been going on for many years. Therefore, such areas which are polluted on the regular basis have no chance for efficient remediation, but there are some groups of microorganisms which are active in degradation of PAH. Degradation of mixtures can be achieved by consortium of microbes with different activities and potential to utilize them as carbon and energy resource (Galazka & Galazka, 2015).

Decomposition is brought about by a process of co-metabolism with their decomposition occurring step by step by contribution of variety of microbes. Therefore, co-metabolism involves transformation of PAHs in soil, e.g., *Pseudomonas stutzeri* co-metabolize 4 nitrophenol and diethylphosphate and phenol. Sometimes to activate the activity of enzyme responsible for degradation from one species, introduction of bacteria is required inactive for decomposition (Dellangnezze et al., 2016). Enzymes can carry out hydrolysis, denitrification, hydroxylation, deamination, etc., during mineralization of PAHs by co-metabolism process (Chen et al., 2017).

Examples of bacteria responsible for degradation PAHS belong to Actinobacteria and Proteobacteria (Yergeau et al., 2015). Various studies have reported that transcripts related to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Acidobacteria* are capable of degradation of PAHs. Also, other prevalent bacteria are *Actinomycetales*, *Rhodospirillales*, *Burkholderiales*, *Alteromonadales*, *Solirubrobacterales*, *Caulobacterales* and *Rhizobiales* (Pagé et al., 2015). Decomposition of phenanthrene is favored by the activities of bacterial degraders belonging to *Pseudomonadales, Actinobacteria, Caulobacterales, Rhizobiales and Xhantomonadales* (Thomas & Cébron, 2016).

Fungi such as *Trichoderma harzianum*, *Cunninghamella elegans,* Aspergillus, Penicillium, Fusarium, Amorthopeca, Neosartorya, Paecilomyces, Talaromyces and *Trametes versicolor* Graphium are microorganisms which can degrade persistent pollutants.

2.5.1 Microbial Degradation of PAH

Degradation of hydrocarbon requires tremendous treatment and is affected by amount and nature of hydrocarbon present. The restricted or poor microbial diversity in environment limits the degradation of hydrocarbon or oil contaminants. Hydrocarbons with high molecular weight cannot be degraded. There are few bacteria and filamentous fungi which play a role in degradation of hydrocarbons. Ligninolytic fungi producing lignin peroxidases, phenol oxidases, laccases, tyrosinases and H_2O_2-producing enzymes degrade hydrocarbons (Lee et al., 2015).

2.5.2 Hydrocarbon Degradation Mechanism

Mechanism behind the fast and complete degradation of hydrocarbon requires aerobic conditions. Steps involved in degradation involves oxidation and activation via oxygenates and peroxidases. For hydrocarbon decomposition, specific enzyme

system is required and also occurs by attachment of microbes onto cell surfaces and production of biosurfactant. Degradation of hydrocarbon can be carried out by selective metabolism of individual strain of microorganism or microbial consortium of same or different genera showing more possibility of degradation (Varjani & Upasani, 2016).

2.5.3 Role of Enzyme in Hydrocarbon Degradation

Degradation of hydrocarbons by microbes involves the role of Cytochrome P450 hydroxylases including *Candida apicola*, *C. maltose* and *C. tropicalis*. In prokaryotes, alkane oxygenases, di-iron alkane hydroxylases, copper-containing methane monooxygenases and soluble di-iron methane monooxygenases actively participate in the hydrocarbon degradation. Fungi also produce extracellular enzymes, which can degrade contaminants like hydrocarbon, and their efficiency level depends on various factors including nutrient availability, aerobic conditions, pH, temperature, etc. Fungi produces lignin, extracellular peroxidases, lignin peroxidases, manganese peroxidases, LiP, MnP and laccase enzymes for the degradation of hydrocarbons like anthracene and pyrene. The degradation of pyrene and anthracene by Trametes versicolor, Pleurotus ostreatus and Phanerochaete chrysosporium depends on levels of MnP and laccase secreted in the soil (Zhang et al., 2015).

Although fungal species are not as effective as bacterial species for PAH degradation, they are non-specific and able to hydroxylate various xenobiotics. The system of extracellular enzymes from six *Aspergillus* species, isolated from crude oil-polluted soil, efficiently degrades crude oil and shows potential for crude oil recovery. Jové et al. (2016) evaluated the anthracene degradation efficiency of three non-ligninolytic and ligninolytic fungi, observed that the anthracene degradation efficiency of Phanerochaete chrysosporium is higher than those of Pleurotus ostreatus and Irpex lacteus. Balaji et al. (2014) analyzed the ability of various fungal species to produce extracellular enzymes, like laccase, lipase, protease and peroxidase.

Traditional methods of microbial community analysis.

Hydrocarbon and heavy metals can be degraded in culture by different microbes isolated from soil including bacteria and fungi. This is bioremediation in which living organisms are used to remove pollutants from sites, causing stimulation of indigenous degraders by applying or improving conditions of contaminated soil. Therefore, culturing degraders and applying it to polluted soils enhances the degradation, resulting in bioaugmentation. To describe microbial community from polluted environments, two methods—culture-dependent and culture-independent methods—have been used predominantly.

Although culture-dependent methods basically retrieve negligible handout diversity from environment of soil, the potential of cultured isolates in situ or ex situ allows in vitro evaluation of microbial physiology and degradation capability creating a metagenomic database for gene identification useful for land restoration. Therefore, polluted soils are more amenable for culture-dependent sampling than other because

contamination leads to decline in diversity due to which sampling effort lowers the proportion of active community (Bell et al., 2014).

Microbes are sensitive index of soil health because it represents a transparent understanding between diversity and ecosystem sustainability and this contributes to analysis and devising practical measures. Studies related to process including biomass, respiration and enzymatic activities but these measurements are limited to explain specific microbial ecosystem. Therefore, analyses based on community-level interactions are effective methods because it helps in determining microbial diversity and function.

3 Culture-Dependent Methods of Community Analysis

3.1 Dilution Plating and Culturing Methods

Community analysis of soil microbes can be done by culturing techniques using different culture media for retrieval of various microbes. Although, to accelerate the retrieval of diverse microbial groups, attempts to devise new culture media have been done, because only 0.1% of microbes can be cultured using these media formulations. Culturing techniques involve the identification process which is carried out based on morphological, microscopically, and biochemical techniques.

3.2 Community-Level Physiological Profiles

Community-level physiological profiles are common method of scrutinizing soil microbial communities. In this, species of bacteria are identified on the basis of their carbon source utilization. For analysis, BIOLOG® system is available for community-level physiological based on consumption of carbon sources which is determined by the reduction of a tetrazolium dye, resulting in change of color estimated spectrophotometrically. Different communities present different fashion of oxidizing substrates which can be compared (Hackett & Griffiths, 1997).

3.3 Culture-Independent Methods of Community Analysis

Now due to some restrictions of culture-based methods, environmentalists are turning toward culture-independent methods of microbial community analysis. The community can be analyzed by specific molecules extraction and quantification or advanced fluorescence microscopic techniques. Specific molecules include phospholipid fatty acids and nucleic acids (Morgan & Winstanley, 1997), and microscopic techniques

involve hybridization of fluorescent-labeled nucleic acid probes with total RNA extracted from soils or hybridizations with cells in situ.

3.4 Phospholipid Fatty Acid Analysis

Phospholipid fatty acid (PLFA) analysis used for assessing the structure and soil disturbances of soil microbial communities, pollution, fumigation, and changes in soil quality (Petersen et al., 1998). Phospholipids are present in cell membrane, and on degradation, it is metabolized rapidly causing death of cell, and therefore, it acts as principal indicator of microbial biomass.

The presence and abundance of these signature fatty acids in soil reveals the presence and abundance of particular organisms or groups of organisms in which those signatures can be found. These studies clearly demonstrate the utility of this method in determining gross community changes associated with soil management practices. These are further described in detail in the later sections.

3.5 Nucleic Acid Techniques

Microbial community structure is easily understandable by nucleic acids found as cell component, which represent diversity. Analysis can be carried out by DNA reassociation/reannealing time, greater the sequence diversity of the DNA, the greater the DNA reannealing time. Among all nucleic acid techniques, the most useful is 16S ribosomal RNA (rRNA) genes (i.e., encoded by rDNA) in prokaryotes and 5S or 18S rRNA genes in eukaryotes. These small subunit (SSU) rDNA molecules are found universally in all three forms of life: the domains Bacteria, Archaea, and Eucarya, composed of highly conserved regions and its relatively large size (e.g., ∼1.5 kb for the 16S rDNA molecule) and the presence of many secondary structural domains. These are discussed in detail in the later sections.

3.6 Phylogenetic Analysis

Different methods are included in phylogenetic analysis like rDNA and rRNA and ranging from molecular to morphological traits (Olsen & Woese, 1993). Phylogenetic analysis provides the data required for identification of similarities among organisms, leading to know about the physiology and ecology of microbes.

3.7 Fluorescent in Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) helps in identification and quantification of microbial communities and across all levels of phylogeny (Amann et al., 1995). It is described in detail in the later sections.

4 Molecular Techniques for Microbiome Analysis

The characterization of soil microbes holds special importance for the fact that they constitute 2–3 times biomass than that of total plants and animals on earth (Ciesielski et al., 2013; Paul et al., 2018). Besides, they play a crucial role in the behavior and fertility of the soil and in turn are majorly affected by the anthropogenic activities and pollution. Thus, soil microbe analysis comprises of structure and composition of microbial community, functions associated with the genes present in these microbes and role of these functions in the major biogeochemical cycles of the ecosystem. The age-old tradition of identifying microbes via culture-based methods and biochemical characterization has been long replaced with molecular approaches. These methods have enhanced the characterization of the microbes at the level of strains as well as isolates thereby providing insights in the polymorphisms. Besides that, another major advantage of molecular methods is characterization of uncultivable microorganisms. This holds special importance in case of soil microbe analysis since about 20% soil microbes belong to phylum Acidobacteria, majority genera of which are uncultivable.

Tremendous technological advances in molecular biology have been made over the years with numerous methods available for the same. This chapter constitutes come of the most widely used methods which have been categorized in five types on the basis of the principle involved. These include library-based methods, amplification and digestion-based methods, electrophoresis-based methods, sequencing-based methods and fluorescence-based methods. The specific techniques in each of these categories have been described in detail in the upcoming section along with their advantages and limitations. Also, since non-nucleic acid approaches have also been developed for this analysis, lipid profile analysis and its importance in determination of soil microbiome have been discussed briefly toward the end of the chapter.

4.1 Library-Based Methods

This is one of the oldest approaches for the identification and characterization since analysis of the total genes helps in accurate identification. This method gained much more importance after the development of recombinant DNA technology since it paved the methods for library generation. The basic idea is to create a library of all the genes present in the soil sample and then align those for the identification of

existing microbes. The technique involved is clone library method which involves synthesis of clones of the genome present in the sample via amplification and its further analysis. The method has been discussed in detail in the upcoming section along with the steps involved as well as role of bioinformatics in the process. The section also describes the importance of selection of target genes in the process.

4.1.1 Clone Library Method

One of the most primitive and most widely used molecular biology methods for determination of soil microbes is the construction of clones of corresponding genes followed by their taxonomic assignment, referred to as clone library method. In this technique, the diverse environment samples are analyzed at a time wherein individual gene segments are amplified by polymerase chain reaction (PCR) in different soil samples. These amplified products are then used for the construction of DNA library commonly called as a clone library using recombinant DNA technology. Briefly, the amplified PCR products are ligated in an appropriate vector followed by transformation of the host cell with the recombinant vector. The transformed host cells are then separated from the non-transformed cells or non-recombinant transformed cells (cells comprising of self-ligated vectors).

The transformed clones are then selected for good-quality sequence in terms of size, specificity and purity. The selected clones are compared with multiple databases for the taxonomic identification. 16S and 23S rRNA are the most commonly used molecules for clone library method of taxonomic classification and phylogenetic analysis. The most common databases used for taxonomic classification include SILVA, Ribosomal Database Project (RDP), GreenGenes, National Center for Biotechnological Information (NCBI) and Open Tree of life Taxonomy (OTT). Owing to the size and complexities of these databases, it has also been observed that SILVA, RDP and GreenGenes can be mapped in NCBI, and these four can further be mapped in OTT but the vice versa is not true (Dorn-In et al., 2013) (Balvočiute & Huson, 2017). One of the major advantages of this technique is the analysis of vast number of genes of microbes since all the amplified genes are analyzed. Also, it helps to assign the cloned sequences at lower levels including genus as well as species. However, high time consumption as well as labor-intensive nature of this technique limits its use for microbe identification.

Owing to the large number of microbes present in the soil, about 40,000 clones need to be evaluated for the determination of only 50% of the soil microflora while only a thousand clones can be evaluated in one go, thereby increasing the time duration of the complete analysis. In spite of the limitations of this method, it has been used in the recent past for the identification of bacterial communities associated with rhizosphere of wild plant species in natural settings (Pascual et al., 2016) and also for hydrocarbon containing soil samples in USA (Beaver et al., 2016).

4.2 Amplification and Digestion-Based Methods

One of the most common limitation of nucleic acid analysis is the scarce amount present in the sample thereby limiting its detection. However, this limitation has been overcome since the discovery of polymerase chain reaction by Kary Mullis in 1983. This helps to amplify the present DNA in the sample in order to obtain the same in the detectable limits. Depending on the primers used for this amplification, they are categorized as non-specific and specific amplifications. With minor variations in the reaction mixture and conditions, more than 30 types of PCR exist. However, this section includes the PCR types which are specifically useful for the analysis of soil microbiome. The methods described here include Random Amplification of Polymorphic DNA (RAPD), quantitative PCR (q-PCR) and Ribosomal Intergenic Spacer Analysis (RISA). Apart from amplification-based strategies, use of restriction endonucleases for the identification of specific DNA sequences has also been widely exploited after the discovery of these enzymes by Arber, Smith and Nathans. Since these endonucleases particularly class II are specific in terms of DNA recognition and restriction, they are used for identification purposes. The method that exploits these restriction endonucleases discussed here is Restriction Fragment Length Polymorphism (RFLP) or Terminal-RFLP (T-RFLP). These two approaches, viz amplification as well as digestion, have also been combined for better specificity as well as sensitivity; amplified ribosomal DNA restriction analysis (ARDRA) is one such example especially used for the analysis of soil microbiome.

4.2.1 RAPD

Random amplification of polymorphic DNA (RAPD) is one of the most widely used PCR based technique for the identification and characterization of microbiome. The technique involves the use of short random primer sequences of about 6 to 10 bp long, mostly repetitive, for the amplification of regions in the genome to determine the polymorphisms between isolates. The use of random primer is the biggest advantage of the technique since no prior information of specific genes or even the microbes is needed, thereby facilitating the microbiome analysis consisting of varied types of microbes. It has widely been used for genetic fingerprinting of the microbial communities present in an ecological niche and also to differentiate between the strains and isolates of a particular species (Adzitey et al., 2012). However, low reproducibility of results obtained from RAPD is one major drawback besides others which include limitations associated with DNA isolation and characterization. Since the genotyping is done on the basis of size of amplicons observed without considering the sequences, the technique has a limited use (Kumari & Thakur, 2014).

4.2.2 Quantitative PCR

As against conventional PCR strategies which provide qualitative analysis of amplification and thus presence of corresponding DNA fragment, q-PCR or real-time PCR is a measure of the actual number of copies of the gene per unit volume of the sample. It involves the use of either the intercalating dyes or fluorescent probes for the detection of amplification in the real time. The most commonly used intercalating dyes include SYBR green or propidium monoazide while fluorescent probes include TaqMan probes, molecular beacons, scorpion probes, etc. The genes targeted for this amplification are usually 16S or 23S rRNA or other bacterial or fungal specific genes such as methane or ammonia oxidizers. Though the technique provides real-time information of the genes in a quantitative manner, it is still not relatable to the cell count of a particular species in the given sample since a number of target genes are present in more than one copy in the species.

4.2.3 Ribosomal Intergenic Spacer Analysis (RISA)

In addition to specific genes, intergenic regions have also been exploited for identification and characterization of microbes. One of such regions include the intergenic spacer region (ISR) which is present between the large 23S and small 16S subunit of rRNA operon. These fragments are generated by amplification of the DNA using primers complementary to 16S and 23S rRNA genes. This will help in the identification of the dominant members of the community, and its automated version (ARISA) has also been used for the determination of bacterial community of freshwater systems. Besides, this technique has been successfully used for the identification of microbial community in soils and also in anaerobic treatment plants or bioreactors (Ciesielski et al., 2013). In spite of its usual limitations such as qualitative DNA isolation, primer mismatch and identification of appropriate annealing temperature, it is still widely used for determination of composition of microbial communities in environmental samples.

4.2.4 RFLP/T-RFLP

Restriction fragment length polymorphism or RFLP is another molecular method for the identification of microbes on the basis of presence of recognition sequences corresponding to restriction endonucleases. It is very well known that restriction endonucleases (RE) particularly class II RE recognize a specific DNA sequence usually a palindrome and restrict it between the recognition sequences. Also, the recognition and restriction are specific to even a single bp change in the sequence which is exploited in RFLP technique to identify polymorphisms in the strains and isolates. A modified version of RFLP known as terminal-RFLP (T-RFLP) was invented in 1997 by Liu et al., wherein the 16S rRNA sequences were specifically amplified using primers with 5'end labeled with a fluorochrome. These amplified fragments

were then digested using restriction endonucleases, and the digested fragments are separated by capillary or polyacrylamide gel electrophoresis before detection of their fluorescence. Although this technique is quite similar to ARDRA, presence of a fluorochrome as well as separation of digested fragments in T-RFLP improves the efficacy of identification. The peaks obtained in electropherogram, which is the graphical representation of T-RFLP results, are indicative of the relative abundance in a particular community.

4.2.5 ARDRA

Amplified ribosomal DNA restriction analysis, commonly known as ARDRA, originally identified in 1993 is another molecular method for microbiome identification which is based on the amplification as well as restriction digestion techniques. As the name suggests, the technique is involved in the amplification of conserved regions of 16S rRNA followed by their digestion with restriction endonuclease at specific sites. The enzymes used for digestion are usually tetra-cutters owing to the size of the amplified products and are therefore the most crucial step of the technique. The digested fragments are analyzed on gel electrophoresis (agarose or polyacrylamide) for both genotyping and strain typing. Also, it is used for the analysis of genetic changes occurring in the isolates under different conditions. The major limitations of this technique include high time consumption, labor intensive and complex profiles obtained when studying the entire community.

4.3 Electrophoresis-Based Methods

The DNA fragments can be separated on the basis of their molecular weight or their secondary structural conformations. The analysis of separated fragments helps to reduce the background by other sequences and provide comparatively accurate results. Certain modifications in the electrophoretic measures which have normally been used to visualize or separate DNA have helped in the identification and characterization of soil microbiome. The methods involved in this category include denaturing gradient gel electrophoresis (DGGE) or temperature Gradient gel electrophoresis (TGGE) and single-stranded conformation polymorphisms (SSCPs) which are described in great detail in the next section.

4.3.1 DGGE/TGGE

The application of electrophoresis for visualization and separation of biological macromolecules particularly DNA, RNA and proteins has been exploited since a very long duration. The use of polyacrylamide gel electrophoresis (PAGE) for the

separation of small DNA fragments has been exploited in the identification of bacterial strains. Invented in 1980 by Fischer & Lerman (Keinänen et al., 2004), denaturing gradient gel electrophoresis (DGGE) is a technique in which a gradient PAGE gel is prepared comprising of increasing concentrations of a denaturing agent in which amplified DNA fragments are passed. The increasing denaturant present in the gel leads to the denaturation of the DNA fragments depending on the size as well as sequence of the fragment, thereby slowing the migration process and thus separating the fragments. Needless to say, smaller fragments with AT rich regions melt easily when compared to larger GC-rich fragments. A similar variant of the technique is temperature gradient gel electrophoresis (TGGE), wherein the denaturation of amplified fragments is facilitated by high temperatures.

A major advancement in the technique is the introduction of GC clamp which is a 20 to 60 nucleotide long GC stretch at the 5' terminal of the forward primer which improves the resolution to a great extent as a double-stranded fragment is maintained at very high temperature or high denaturant concentrations. The technique initially involved 16S rRNA primers, but the use of specific markers as reference has further revolutionized the process. The low comprehensiveness of the results obtained by this technique when compared to sequencing can be compensated by the low input cost and time required for the former. This technique offers great advantages for presumptive identification of microorganisms as well as for analyzing the microbial community changes under various conditions and/or parameters. On the other hand, similar melting points of various fragments, too small fragment size leading to improper identification and sequence heterogeneity in operons, are major limitations of this technique.

4.3.2 Single-Strand Conformation Polymorphism (SSCP)

As against all the other techniques which characterize the primary sequence of the DNA, SSCP is focused on the separation of DNA molecules on the basis of their secondary structures. Identified by Masato Orita et al. in 1989, the technique involves amplification of the DNA present in the environmental samples followed by their denaturation and then separation on polyacrylamide gel electrophoresis. Since the denaturing conditions leads to the formation of secondary structures of DNA fragments which is sequence dependent, various secondary structures are formed which migrate at different positions in the gel. The sensitivity of the technique is pretty high as even a single base pair substitution can be detected owing to variation in the secondary structure. Other advantages of the technique include the time required for processing and relatively low cost. It has been used for the identification of fungi among multiple bacterial species in a sample (Dorn-In et al., 2013) and also for successful differentiation of pure cultures.

4.4 Sequencing-Based Methods

The sequencing of DNA fragments was initially described by Sanger in 1977 by chain termination method using the dideoxynucleotides followed by Maxam Gilbert's "chemical degradation method" employing certain chemicals with specific cleaving capacities. These two methods formed the basis of DNA sequencing which was followed by further advanced approaches such as pyrosequencing, massive parallel sequencing as well as mapping strategies including clone contig and shot gun approaches. With the advancements in the sequencing approaches, they have been widely exploited for the soil microbiome analysis. The most common sequencing methods used in this analysis include 16S, 23S rRNA sequencing as well as high throughput or next generation sequencing which are discussed in detail.

4.4.1 16S rRNA Sequencing

Since the identification of this technique in 1970s, it has been used widely for microbial characterization of environmental samples particularly soil microbes. This sequencing is aimed at the identification, classification as well as quantification of the microbes. 16S rRNA is an essential and highly conserved molecule present in the transcriptional machinery of all the microbes which have DNA as their genetic material. As a result, universal primers targeting this RNA have been designed for almost all the species, thereby facilitating amplification of multiple microbes from a single complex sample such as soil. Here, it is important to note that these RNA molecules also possess some variable regions in addition to the conserved regions. Thus, this combination of conserved and variable regions helps in the amplification and distinguishing respectively of different species.

The universal primers are used for the amplification of these regions in the soil samples followed by sequencing and then comparing the sequences with the sequences present in the curated databases such as SILVA, RDP or GreenGenes. Though this method is one of the best as 16S rRNA are highly conserved, it still has two major limitations. One of the limitations is its inaccuracy at species-level classification particularly when analyzing very closely related species while the other being its biasness toward bacterial species disregarding all other microbes present in the soil microflora. One of the examples of biasness is its inability to identification of viruses due to absence of these regions in the viruses. Besides, this conservatory nature of 16S rRNA leading to its low evolutionary rate also limits the identification of certain distinguished bacteria. Apart from 16S rRNA sequencing for taxonomic identification directly, these RNA molecules have also been exploited as probes in other advanced techniques such as FISH microscopy, ARDRA, DGGE/TGGE, T-RFLP, and RISA, which have been discussed separately in detail in the further sections.

4.4.2 23S rRNA Sequencing

It is another conserved molecule present in all the bacterial species which is exploited for the species identification similar to 16S rRNA sequences. Similar to 16S rRNA molecules, 23S rRNA sequences are universally distributed, have conserved functions and possess both invariant as well as variable regions. In addition, they possess additional diagnostic sequences which are larger than 16S rRNA and also characteristic insertions and/or deletions, thereby making them better phylogenetic markers due to greater sequence variations (Hunt et al., 2006). Thus, it can be stated to consist of almost twice the information present in 16S rRNA and is thus used an additional marker for phylogenetic analysis of bacteria. However, lack of broad-range bacterial PCR amplification and sequencing primers has limited its use in phylogenetic analysis. To combat this limitation, an ARB software package was developed which analyzed the specificity of 23S rRNA primers. On the other hand, similar to 16SrRNA sequences, 23S rRNA is also used as probes in various advanced techniques which are described in the later sections. These have gained more importance as probes due to reduction in the sequencing cost as well as development of advanced techniques such as microarrays, RISA, etc.

4.4.3 High-Throughput Sequencing/Next-Generation Sequencing

With the advancements in the sequencing strategies during the human genome project, they have become one of the most widely used and most accurate strategies for microbiome identification. They offer added advantages over 16S or 23S rRNA sequencing since the analysis now is based on a number of genes as against only rRNA genes. The whole genome or metagenomics analysis is robust, rapid and provide extensive data for analysis. However, these techniques are labor intensive and require skilled professionals for accurate results. The major sequencing strategies developed so far include Roche 454, Illumina Solexa and SOLiD, all three of which employs fluorescent molecules for detection. The basic principle behind Roche 454 and Illumina Solexa is pyrosequencing; however, the latter offer advantages of formation of clone contigs wherein both the forward and reverse strands are read providing more accuracy. On the other hand, SOLiD sequencing employs the activity of DNA ligase instead of DNA polymerase and the use of heptanucleotide sequences for sequencing. All the three techniques have been described elsewhere in detail; however, the major differences lie in the read length, accuracy, cost and time requirement and the choice of the sequencer depend on the starting material as well as the precision of the result required.

Variants of sequencers which exploit the H^+ released during the incorporation of the base in the growing DNA chain instead of fluorescent molecules are also synthesized which are collectively known as bench top sequencers. They majorly exploit the pH changes that occur owing to H^+ ion release during the DNA synthesis, and this variation is directly converted to digital information in an Ion Torrent PGM machine. However, a library flanked by Ion Torrent adapters need to be generated

first in order to analyze the sequences which are usually amplified by emulsion-based PCR in ion sphere particles.

4.5 Fluorescence-Based Methods

The application of fluorescence molecules for tagging specific DNA molecules and thus characterization is also widely exploited. Several fluorescence molecules have been discovered and exploited for the generation of labeled probes which are then hybridized to target sequences to be analyzed by microscopy, hybridization or flow cytometry methods. The methods described here include FISH microscopy, flow cytometry and microarray technology. Besides that, the section also describes the quantitative analysis of these fluorescence molecules and also sheds light on the design and characteristics of a probe molecule, which is the center of all these techniques.

4.5.1 FISH Microscopy

Fluorescence in situ hybridization microscopy or commonly known as FISH microscopy is one of the advanced techniques for microbiome classification. The basic principle behind this technique is the identification of phylogenetic group-specific sequences particularly rRNA sequences via fluorescent labeled probes (labeled at 5'end) complementary to the target sequences. The probes hybridize to the complementary sequences which are then imaged by fluorescence microscope. The procedure includes fixing of the cells obtained from environmental samples followed by hybridization with oligonucleotide probes (labeled) which are specific to taxa. The hybridized cells are then visualized by scanning confocal laser microscopy (SCLA). Due to large number of ribosomes in all types of cells and thus high amount of rRNA molecules, these are selected as target molecules thereby increasing the detection probability. In addition, the length of the probe, GC content as well as targeted region of the gene affect the selection of probe for hybridization. It is very clear that probe designing is the key to the successful characterization of this technique for which an online database, probeBase, can be used which provides optimal hybridization conditions as well as specificity (http://www.microbial-ecology.de/prob). Other such software include PRIMROSE (http://www.cf.ac.uk/biosi/research/biosoft) and Probe Design Tool of ARB software package (available at http://arb-home.de/).

The steps involved in probe design include identification of specific short sequences in the target group, generation of oligonucleotide probes, their modification to optimize hybridization conditions and finally in silico validation of these probes. The major advantages of this technique include no requirement for cultivation, DNA extraction, PCR amplification or cloning thereby avoiding the limitations associated with each of these processes. Having said that, this technique is very time

consuming and has very low detection limit which is majorly affected by microscope magnification, fixation dilution and number of microscopic fields studied. The number of microscopic fields analyzed is a major cause of biased results by this technique. However, it still has been used for analysis of composition of microbial communities of various environmental conditions particularly soil samples (Müller et al., 2016). Another modification in this technique wherein coupling it with mass spectrometry helps to identify spatial location as well as metabolic status of the bacteria at the analyzed time.

4.5.2 Flow Cytometry

It is another fluorescent-based technique for the identification of soil microbiome. The basic principle behind this is the size, granularity of the cells as well fluorescence emission at different wavelengths. The size and granularity of the cell is measured by forward and side scatter of the flow cytometer, respectively. The application of flow cytometry initially was confined only for the quantification of cells (Czechowska et al., 2008) since inorganic particle interfere with the bacterial cells. However, with the advent of the methods to get rid of inorganic particles from the soil or sediment samples, the role of flow cytometry in microbiome analysis has increased. These methods include chemicals such as ionic and non-ionic detergents, mechanical such as ultrasonication or enzymatic treatments (Falcioni et al., 2006). As against FISH microscopy, it is a rapid technique and also has better detection limit. It has therefore been used for determining the difference in the microbiome present in soils, sediments and sludges, all comprising of large number of microbes (Frossard et al., 2016).

4.5.3 Microarray Technology

The techniques discussed so far have focused on the analysis of DNA fragments present in the genome of the microbe. However, microarray technology determines the expression levels of the genes, thereby connecting the genome to the functional aspects. The technique basically analyzes the gene expression of the same cell under two different conditions. Briefly, the mRNA of the cells under different conditions are isolated and converted to cDNA via reverse transcriptase enzyme. The cDNA of different pools is labeled with two different colored fluorescent markers; Cy5 and Cy3 are the most commonly used markers. Another variation of this technology is the use of total DNA of the cell, wherein the DNA is isolated, fragmented, selectively amplified and then labeled as discussed. However, in this case, in place of the expression levels, the presence of gene is identified. Nevertheless, the two different pools of cDNA/DNA are then mixed and hybridized on a single chip consisting of DNA probes attached to it. The hybridization is then detected either by high resolution microscopy or by scanning and depending on the color detected; the regulation status of the genes under the studied conditions is evaluated. This technology has been used for the analysis of environmental samples using GeoChips or PhyloChips

(Asuming-Brempong, 2012). One of the most critical factors for microarray technology is its high sensitivity even from unpurified soils. Having said that, when DNA samples are considered for the study, the amplification step creates biasness in the results.

The mRNA levels in a cell can also be analyzed by in situ monitoring of gene expression via RT-PCR which involves an additional step of amplifying the mRNA within the cell. This method involves a labeled probe as primer for the amplification of mRNA which is then detected either by reporter genes or by microbial activity. The major advantage offered by this advancement is improving the detection limit to great extent especially for genes with low expression. However, appropriate permeabilization of the cells for components of PCR reaction mixture is of utmost importance as too little permeabilization will hinder component entry while too much permeabilization will cause cell lysis.

The quantitative analysis of rRNA molecules for the determination of microbial diversity by quantitative DOT BLOT is another hybridization strategy. The rRNA isolated from soil samples are isolated and transferred on a stable membrane following which they are detected by hybridization with either radioactive or fluorescent probes. An advantage of this technology over other hybridization methods is the ability to measure the intensity of the spot, thus quantifying the RNA levels. This is also exploited to determine the specificity of the newly designed probes along with the hybridization conditions, so that these probes can further be used for analysis.

4.6 Non-nucleic Acid-Based Approaches

All the methods discussed for far have used nucleic acids (both DNA and RNA) as the target macromolecules for microbiome identification. However, recent years have witnessed the emergence of "omics" approaches with the developments in the bioinformatics approaches as well in biophysical techniques such as nuclear magnetic resonance (NMR), and mass spectrometry. These omics include lipidomics, proteomics, metabolomics, etc., involving the study of lipids, proteomes or metabolomes, respectively, of the cells/samples. Of these approaches, lipid analysis has been widely exploited for the characterization of soil microbiomes specifically depending on the phospholipid contents of the microbial cells. Thus, a brief introduction of the same has been included in the chapter.

4.6.1 Microbial Lipid Analysis

Though nucleic acids have been extensively exploited for the identification and characterization of microbiome present in both medical and environmental samples, lipids particularly phospholipids (PFLA) can also be used for the purpose (Goupil et al., 2015). Similar to nucleic acids, lipid analysis also does not require cultivation of microbes, thereby helping in the identification of non-culturable microbes as well.

The phospholipids present in the membrane of microbes contain specific fatty acid molecules and thus can be treated as signature molecules for identification. The isolation of these molecules from environmental samples particularly soil samples is quite easy, and their methyl esters have been accepted as markers for species identification during taxonomic analysis. Also, since these molecules degrade rapidly after cell death, they help to determine the active population of microbes in the studied sample. Not only characterization of microbiome, PFLA analysis has also been employed to determine pollution levels, variation in quality of soil, association of soil microbiome with type of crops or role of fertilizers and pesticides on the soil microbiome (Bossio et al., 1998).

References

Adzitey, F., Rusul, G., Huda, N., Cogan, T., & Corry, J. (2012). Prevalence, antibiotic resistance and RAPD typing of campylobacter species isolated from ducks, their rearing and processing environments in Penang, Malaysia. *International Journal of Food Microbiology*, *154*(3), 197–205. https://doi.org/10.1016/j.ijfoodmicro.2012.01.006

Alloway, B. J. (2013). Introduction. In *Heavy metals in soils* (pp. 3–9). Springer.

Amann, R., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews, 59*, 143–169.

Appenroth, K. J. (2010). Definition of "heavy metals" and their role in biological systems. In I. Sherameti, & A. Varma (Eds.), *Soil heavy metals, soil biology* (p. 19: 19–29). Springer.

Asuming-Brempong, S. (2012). Microarray technology and its applicability in soil science—A short review. *Open Journal of Soil Science, 02*(03), 333–340. https://doi.org/10.4236/ojss.2012.23039

Balaji, V., Arulazhagan, P., & Ebenezer, P. (2014). Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. *Journal Environment Biology*, *35*(3), 521.

Balvočiute, M., & Huson, D. H. (2017). SILVA, RDP, Greengenes, NCBI and OTT—how do these taxonomies compare? *BMC Genomics*, 18. https://doi.org/10.1186/s12864-017-3501-4

Beaver, C. L., Williams, A. E., Atekwana, E. A., Mewafy, F. M., Abdel Aal, G., Slater, L. D., & Rossbach, S. (2016). Microbial Communities Associated with Zones of Elevated Magnetic Susceptibility in Hydrocarbon-Contaminated Sediments. *Geomicrobiology Journal, 33*(5), 441–452. https://doi.org/10.1080/01490451.2015.1049676

Bell, T. H., El-Din Hassan, S., Lauron-Moreau, A., Al-Otaibi, F., Hijri, M., & Yergeau, E., et al. (2014). Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-contaminated soils is related to plant phylogeny. *The ISME Journal*, *8*, 331–343. pmid:23985744.

Bossio, D. A., Scow, K. M., Gunapala, N., Graham, K. J., & Ecol, M. (1998). *Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles MICROBIAL ECOLOGY* (Vol. 36).

Checcucci, A., Bazzicalupo, M., & Mengoni, A. (2017). Exploiting nitrogenfixing rhizobial symbionts genetic resources for improving phytoremediation of contaminated soils. In A. Naser, A. Anjum, S. S. Gill, & N. Tuteja (Eds.), *Enhancing cleanup of environmental pollutants: Biological approaches*, (Vol. 1, pp. 275–288). Springer International Publishing. https://doi.org/10.1007/978-3-319-55426-6_13

Chen, Y., Kuang, J., Jia, P., Cadotte, M. W., Huang, L., Li, J., et al. (2017). Effect of environmental variation on estimating the bacterial species richness. *Frontiers in Microbiology, 8*, 690. https://doi.org/10.3389/fmicb.2017.00690

Chibuike, G. U., & Obiora, S. C. (2014). Heavy metal polluted soils: Effect on plants and bioremediation methods. *Applied and Environmental Soil Science, 2014*, 752708. https://doi.org/10.1155/2014/752708

Ciesielski, S., Bułkowska, K., Dabrowska, D., Kaczmarczyk, D., Kowal, P., & Mozejko, J. (2013). Ribosomal intergenic spacer analysis as a tool for monitoring methanogenic archaea changes in an anaerobic digester. *Current Microbiology, 67*(2), 240–248. https://doi.org/10.1007/s00284-013-0353-2

Czechowska, K., Johnson, D. R., & van der Meer, J. R. (2008). Use of flow cytometric methods for single-cell analysis in environmental microbiology. In *Current opinion in Microbiology* (Vol. 11, Issue 3, pp. 205–212). https://doi.org/10.1016/j.mib.2008.04.006

Dellagnezze, B. M., de Vasconcellos, S. P., de Melo, I. S., Santos Neto, E. V., & de Oliveira, V. M. (2016). Evaluation of bacterial diversity recovered from petroleum samples using different physical matrices. *Brazilian Journal of Microbiology, 47*, 712–723. https://doi.org/10.1016/j.bjm.2016.04.004

Díaz, E. (2010). Bacterial degradation of aromatic pollutants: A paradigm of metabolic versatility. *International Microbiology, 7*, 173–180.

Dorn-In, S., Hölzel, C. S., Janke, T., Schwaiger, K., Balsliemke, J., & Bauer, J. (2013). PCR-SSCP-based reconstruction of the original fungal flora of heat-processed meat products. *International Journal of Food Microbiology, 162*(1), 71–81. https://doi.org/10.1016/j.ijfoodmicro.2012.12.022

Ebbs, S. D., & Kochian, L. V. (1997). Toxicity of zinc and copper to Brassica species: implications for phytoremediation. *Journal of Environmental Quality, 26*, 776–781.

Falcioni, T., Manti, A., Boi, P., Canonico, B., Balsamo, M., & Papa, S. (2006). Comparison of disruption procedures for enumeration of activated sludge floc bacteria by flow cytometry. *Cytometry Part B - Clinical Cytometry, 70*(3), 149–153. https://doi.org/10.1002/cyto.b.20097

Frossard, A., Hammes, F., & Gessner, M. O. (2016). Flow cytometric assessment of bacterial abundance in soils, sediments and sludge. *Frontiers in Microbiology*, *7*(JUN). https://doi.org/10.3389/fmicb.2016.00903

Gajeswska, E., Slaba, M., Andrzejewska, R., & Sklodowska, M. (2006). Nickel-induced inhibition of wheat root growth is related to H_2O_2 production, but not to lipid peroxidation. *Plant Growth Regulation, 49*, 95–103.

Gałązka, A., & Gałązka, R. (2015). Phytoremediation of polycyclic aromatic hydrocarbons in soils artificially polluted using plant-associated-endophytic bacteria and Dactylis glomerata as the bioremediation plant. *Polish Journal of Microbiology, 64*, 241–252. https://doi.org/10.5604/01.3001.0009.2119

Goupil, K., Nkongolo, K. K., & Nasserulla, S. (2015). Characterization of fungal communities in limed and unlimed lands contaminated with metals: Phospholipid fatty acid (PLFA) analysis and soil respiration. *American Journal of Biochemistry and Biotechnology, 11*(2), 45–56. https://doi.org/10.3844/ajbbsp.2015.45.56

Hackett, C. A., & Griffiths, B. S. (1997). Statistical analysis of the time course of Biolog substrate utilization. *Journal Microbiology Methods, 30*, 63–69.

Hasanuzzaman, M., Ueno, A., Ito, H., Ito, Y., Yamamoto, Y., Yumoto, I., & Okuyama. (2007). Degradation of long-chain n-alkanes (C 36 and C 40) by *Pseudomonas aeruginosa* strain *WatG International Biodeterioration & Biodegradation*, *59*(1), 40–43.

Hugenholtz, P., Tyson, G. W., & Blackall, L. L. (n.d.). Design and evaluation of 16S rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization. In *From: Methods in molecular biology* (Vol. 179). Retrieved from http://www.williamstone

Hunt, D. E., Klepac-Ceraj, V., Acinas, S. G., Gautier, C., Bertilsson, S., & Polz, M. F. (2006). Evaluation of 23S rRNA PCR primers for use in phylogenetic studies of bacterial diversity. *Applied and Environmental Microbiology, 72*(3), 2221–2225. https://doi.org/10.1128/AEM.72.3.2221-2225.2006

Järup, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin, 68*, 167–182.

Johri, N., Jacquillet, G., & Unwin, R. (2010). Heavy metal poisoning: The effects of cadmium on the kidney. *BioMetals, 23*, 783–792.

Jové, P., Olivella, M. À., Camarero, S., Caixach, J., Planas, C., Cano, L., & De Las Heras, F. S. (2016). Fungal biodegradation of anthracene-polluted cork: a comparative study. *Journal of Environmental Science and Health*, Part A, *51*(1), 70–77.

Keinänen, M. M., Martikainen, P. J., & Kontro, M. H. (2004). Microbial community structure and biomass in developing drinking water biofilms. *Canadian Journal of Microbiology, 50*(3), 183–191. https://doi.org/10.1139/w04-005

Keshri, J., Mankazana, B. B., & Momba, M. N. (2015). Profile of bacterial communities in South African mine-water samples using Illumina next-generation sequencing platform. *Applied Microbiology and Biotechnology, 99*, 3233–3242.

Kong, Z., & Glick, B. R. (2017). The role of plant growth-promoting bacteria in metal phytoremediation. *Advances in Microbial Physiology, 71*, 97–132. https://doi.org/10.1016/bs.ampbs.2017.04.001

Kumari, N., & Thakur, S. K. (2014). Randomly amplified polymorphic DNA-A brief review. *American Journal of Animal and Veterinary Sciences, 9*(1), 6–13. https://doi.org/10.3844/ajavssp.2014.6.13

Lee, H., Yun, S. Y., Jang, S., Kim, G. H., & Kim, J. J. (2015). Bioremediation of polycyclic aromatic hydrocarbons in creosote-contaminated soil by *Peniophora incarnat*a KUC8836. *Bioremediation Journal*, *19*(1), 1–8.

Lepp, N. W. (2012). Effect of heavy metals pollution on plants. In *Metals in the environment, Pollution Monitoring series., Applied Science Publishers. Department of Biology*. Liverpool, Liverpool Polytechnic 2.

Morgan, J. A. W., & Winstanley, C. (1997). Microbial biomarkers. In J. D. van Elsas, J. T. Trevors, & E. M. H. Wellington (Eds.), *Modern soil microbiology* (pp. 331–352). Marcel Dekker.

Müller, H., Bosch, J., Griebler, C., Damgaard, L. R., Nielsen, L. P., Lueders, T., & Meckenstock, R. U. (2016). Long-distance electron transfer by cable bacteria in aquifer sediments. *ISME Journal, 10*(8), 2010–2019. https://doi.org/10.1038/ismej.2015.250

Narendrula-Kotha, R., & Nkongolo, K. K. (2017). Microbial response to soil liming of damaged ecosystems revealed by pyrosequencing and phospholipid fatty acid analyses. *PLoS ONE, 12*, e0168497. https://doi.org/10.1371/journal.pone.0168497

Olsen, G. J., & Woese, C. R. (1993). Ribosomal RNA: A key to phylogeny. *The FASEB Journal, 7*, 113–123.

Page, A. P., Yergeau, E., & Greer, C. W. (2015). Salix purpurea stimulates the expression of specific bacterial xenobiotic degradation in a soil contaminated with hydrocarbons. *Plus ONE, 10*, e0132062.

Pascual, J., Blanco, S., García-López, M., García-Salamanca, A., Bursakov, S. A., Genilloud, O., Bills, G. F., Ramos, J. L., & van Dillewijn, P. (2016). Assessing bacterial diversity in the rhizosphere of Thymus zygis growing in the Sierra Nevada National Park (Spain) through culture-dependent and independent approaches. *PLoS ONE*, *11*(1). https://doi.org/10.1371/journal.pone.0146558

Paul, D., Kumar, S., Mishra, M., Parab, S., Banskar, S., & Shouche, Y. S. (2018). *Molecular genomic techniques for identification of soil microbial community structure and dynamics* (pp. 9–33). https://doi.org/10.1007/978-981-10-6178-3_2

Petersen, S. O., Debosz, K., Schjonning, P., Christensen, B. T., & Elmholt, S. (1998). Phospholipid fatty acid profiles and C availability in wet-stable macro-aggregates from conventionally and organically farmed soils. *Geoderma, 78*, 181–196.

Pires, C., Franco, A. R., Pereira, S. I. A., Henriques, I., Correia, A., Magan, N., et al. (2017). Metal(loid)-contaminated soils as a source of culturable heterotrophic aerobic bacteria for remediation applications. *Geomicrobiology Journal*, 1–9. https://doi.org/10.1080/01490451.2016.1261968

Sajna, K. V., Sukumaran, R. K., Gottumukkala, L. D., & Pandey, A. (2015). Crude oil biodegradation aided by biosurfactants from Pseudozyma sp. NII 08165 or its culture broth. *Bioresource Technology, 191*, 133–139.

Shah, M. P. (2020). *Microbial bioremediation and biodegradation.* Springer.

Shah, M. P. (2021a). *Removal of refractory pollutants from wastewater treatment plants.* CRC Press.

Shah, M. P. (2021b). *Removal of emerging contaminants through microbial processes.* Springer.

Szyczewski, P., Siepak, J., Niedzielski, P., & Sobczyński, T. (2009). Research on heavy metals in Poland. *Polish Journal of Environmental Studies, 18*, 755–768.

Tamaki, S., & Frankenberger Jr., W. T. (1992). Environmental biochemistry of arsenic. *Reviews of Environmental Contamination and Toxicology, 124*, 79–110.

Thomas, F., & Cebron, A. (2016). Short term rhizosphere effect on available carbon sources, phenanthrene degradation and active microbiome in an aged contaminated industrial soil. *Frontiers in Microbiology, 7*, 92.

Van der Ent, A., Baker, A. J. M., Reeves, R. D., Pollard, A. J., & Schat, H. (2013). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil, 362*, 319–334.

Varjani, S. J., & Upasani, V. N. (2016). Carbon spectrum utilization by an indigenous strain of *Pseudomonas aeruginosa* NCIM 5514: Production, characterization and surface active properties of biosurfactant. *Bioresource Technology, 221*, 510–516.

Wong, M. H. (2012). *Environmental Contamination: Health risks and ecological restoration.* United states of America: Taylor & Francis Group.

Wongsasuluk, P., Chotpantarat, S., Siriwong, W., & Robson, M. (2014). Heavy metal contamination and human health risk assessment in drinking water from shallow groundwater wells in an agricultural area in Ubon Ratchathani province, Thailand. *Environmental Geochemistry and Health, 36*, 169–182.

Yergeau, E., Maynard, C., Sanschagrin, S., Chanpagne, J., Juck, D., lee, K., & Greer, C. W. (2015b). Microbial community composition, functions and activities in the gulf of mexico 1 year after the deepwater horizon accident. *Applied and Environmental Microbiology*, *81*, 5855–5866.

Zhang, S., Ning, Y., Zhang, X., Zhao, Y., Yang, X., Wu, K., Yang, S., La, G., Sun, X., & Li, X. (2015). Contrasting characteristics of anthracene and pyrene degradation by wood rot fungus *Pycnoporus sanguineus* H1. *International Biodeterioration and Biodegradation, 105*, 228–232.

RETRACTED CHAPTER: Antibiotic Resistance Genes as Contaminants in Industrial Wastewater Treatment

Raunak Dhanker, Merwin Mammen, Anjali Singh, Shubham Goyal, Touseef Hussain, and Priyanka Tyagi

The Volume Editor retracted this chapter because its authors withdrew their consent prior to publication. The book has been updated accordingly.

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_2

Bacteriophages: A Strategy to Combat Antibiotic Resistance in Wastewater Treatment Plants

Kanika Bhargava, G. K. Aseri, Gopal Nath, and Neelam Jain

Abstract The widespread proliferation of antibiotic resistant bacteria (ARB) carrying antibiotic resistance genes (ARGs) in wastewater confirms their prevalence. Antibiotic resistance is an emerging, silent worldwide epidemic, caused primarily due to antibiotic overuse and misuse. The emergence and accelerated spread of drug resistance determinants has raised concerns about the imminent threat to the world and human health. To design preventive strategies, health risks affiliated with the burgeoning of ARBs and their respective genes must be acknowledged and taken seriously. Wastewater and wastewater treatment plants (WWTPs) may serve as reservoirs and manufacturers of antibiotic resistance due to continuous introduction of antibacterial drugs, which are then distributed to various ecosystem components. According to the World Health Organization (WHO), we could be thrown back into the pre-antibiotic period, with people suffering from minor injuries that result in infection and complications. To improve human health, a long-term ecological balance of all in our climate, including infectious organisms, should be one of the priorities where phage therapy can be employed. Anaerobic–aerobic treatment reactors, wetlands, and disinfection processing plants have been reported to have shown significant efficiencies for removal of such resistant bacteria. However, microbial purification by using natural cleaners such as phages is yet to be explored as it has shown promising results when used clinically in the form of phage therapy. This manuscript's main objective is to consider bacteriophages as one of the treatment methods and mechanisms in the wastewater system for the battle against ARB carrying ARGs.

K. Bhargava · G. K. Aseri
Amity Institute of Microbial Technology, Amity University Rajasthan, Jaipur 303 002, India

N. Jain (✉)
Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur 303 002, India
e-mail: njain1@jpr.amity.edu

K. Bhargava · G. Nath
Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_3

Keywords Antibiotic resistant bacteria (ARB) · Multidrug resistant (MDR) · Antibiotic resistance genes (ARG) · Wastewater treatment plant (WWTP) · Antimicrobial resistance (AMR)

1 Introduction

Antibiotic resistance is without a doubt one of the most serious issues confronting public health in modern medicine. Antibiotics have been extensively employed in disease management and animal husbandry in recent decades. Antimicrobial use has resulted in an increase in ARB in the public health, livestock, and agriculture sectors (Pazda et al., 2020). ARB and MDR carrying ARGs may undergo mutations as a result of antibiotic abuse and improper disposal (Barancheshme & Munir, 2019). Resistant bacteria and their genes are evolving contaminants that pose a danger to food quality and public safety in the twenty-first century (Xu et al., 2017). According to published data, ARB caused 671,689 infections and more than 33,000 fatalities in the European Union/European Economic Activity (EU/EEA) countries in 2015 (Cassini et al., 2019). Also, more than two million people in the United States are diagnosed with antibiotic resistant infections per year, resulting in at least 23,000 deaths (CDC, 2013; Dadgostar, 2019). According to European ICU physician third-generation cephalosporin-resistant Enterobacteriaceae, meticillin-resistant *S. aureus* (MRSA), carbapenem-resistant Enterobacteriaceae (CRE), carbapenem-resistant *P. aeruginosa*, and vancomycin-resistant *enterococci* (VRE) were the most commonly encountered MDR bacteria (Lepape et al., 2020). The estimated basic economic impediment in Europe AMR is projected to be at least EUR 1.5 billion, with hospital costs exceeding EUR 900 million (Pazda et al., 2020) while in the United States, according to CDC (Center for disease control and prevention), AMR has a surplus of $20 billion in direct healthcare amenities (CDC, 2013; Dadgostar, 2019).

Antibiotics are the most commonly used agents in the treatment of infectious diseases; however, antibacterial metals, on the other hand, are used in hospitals and food processing facilities to prevent adhesion of bacteria and biofilm formation. Antibiotics have been identified in soil and surface water, sewage sludge and manure, in addition to wastewater (Barancheshme & Munir, 2019). The existence of pharmaceuticals in the aquatic ecosystem has eco toxicological consequences, altering the composition of the algal population and influencing the composition of the food web of streams (Brodin et al., 2014). Human actions such as improper waste disposal contribute to more environmental contamination. Since municipal wastewater is a breeding ground for emerging pollutants such as antibiotics, it has the ability to trigger antibiotic resistance in bacteria and their associated genes. According to the UNESCO World Water Development Report for 2020, irrigation uses up to 69% of global freshwater withdrawals from natural waterbodies (UNESCO, 2020). As the global population is projected to grow to 9.7 billion by 2050, there will be an increase in demand for water, so treated wastewater is a realistic solution to water shortages (Hong et al., 2018). Organic materials such as antibiotics and inorganic matter such as

pathogens (ARBs containing ARGs) are found in treated urban wastewater (2020b; Wang et al., 2020a) and their reuse can lead to further environmental pollution and ARB spread, causing public health concerns.

Humans have disposed of waste in rivers and wastelands throughout history, but since the industrialization of the late eighteenth and early nineteenth centuries, waste disposal has increased enormously (Mohammadali & Davies, 2017). In recent years, some European countries have developed WWTPs for hospital wastewater treatment such as using membrane bioreactors as a pretreatment, ozonation, powdered and granulated activated carbon for micropollutant removal (Baresel et al., 2019). However, less effort has been made to eliminate ARB from WWTPs, which can be solved by the use of bacteriophages. To prevent a return to the dark ages of drugs, a number of initiatives to encourage non-drug alternatives, such as bacteriophages/phages and others, are recommended. Bacteriophages are bacterial predators, the ubiquitous organisms of our ecosystem. Phages can be found in different locations where their hosts (bacteria) thrive, including sewers, rivers, soil, and patients' urine and stools (Batinovic et al., 2019). Unlike traditional antibiotics, phages are biological entities with unprecedented diversity and adaptability, and many surprises may be in store (Nikolich & Filippov, 2020). Researchers are reconsidering phages due to the difficulties in treating many life-threatening bacteria as their products are non-self-antigens, which means the immune system can recognize and respond to them but they do not cause infection (Principi et al., 2019). As a result, antibiotic resistant bacteria should be treated at the source instead of just being disposed of in the environment. The purpose of this report is to present a brief overview of antibiotic resistance in wastewater as well as alternative (bacteriophage) for combating it.

2 Occurrence of AMR in WWTPs

AMR is a global concern and, despite the clinical consequences, the problem is not confined to hospital facilities and laboratories. The majority of antibiotics consumed by humans at home or in hospital facility end up in the sewage. As a consequence, one of the most common sources of ARBs that contain ARGs is urban wastewater treatment plants (Fouz et al., 2020). WWTPs serve as a repository, receiving sewage from different sources and bacteria from various habitats allowing bacteria to communicate and exchange genes horizontally; allowing ARGs to spread even further (Hultman et al., 2018). Given the high concentration and variety of microorganisms in a nutritionally rich area, and selective pressure induced by contaminants like antibiotic metabolites, disinfectants, drug products, and heavy metals, even in trace quantities, facilitate horizontal gene transfer (HGT) and increases the probability of ARB proliferation with ARGs (Pazda et al., 2020). WWTPs are a peculiar link between humans and the environment, since waste from residences and hospitals contain antibiotics and bacteria of human origin, potentially creating selective pressure for ARB before they are introduced into the environment (Fouz et al., 2020).

In WWTPs and their effluent, resistant bacteria have been discovered, suggesting that such facilities are failing to remove these pathogens as the overall load of bacteria is reduced 10–100 fold thereby reducing the level of resistance but are not eliminated completely (Bengtsson-Palme et al., 2016). Hospitals are ARB reservoirs because their resistance profile includes resistance to major antibiotics (Kraemer et al., 2019), and they are considered ARB dissemination hotspots (Amarasiri et al., 2020). *E. coli* and *enterococci* are the most common bacterial species found in WWTPs, along with other clinically significant antibiotic resistant bacteria such as MRSA, VRE, CRE, and Gram-negative bacterial species like *Enterobacteria, Pseudomonads, and Acinetobacter* (Savin et al., 2020). All of these bacteria were found to be resistant to fluoroquinolones and carbapenems and produce extended-spectrum β-lactamases (Kraemer et al., 2019; Smyth et al., 2020). The reuse of sewage, which serves as a source of essential nutrients as water demand rises, increases the likelihood of antibiotic resistance dissemination globally.

3 Antibiotics Leading to Resistance Among Bacteria

Once ARB has acquired access to WWTPs, they can spread their resistant genes to other bacterial species in the ecological microbial environment (Kraemer et al., 2019). The presence of various antibiotic compounds (tetracyclines, sulfonamides, and fluoroquinolones) in rhizosphere soil irrigated with treated water was investigated, and substantial concentrations of drugs were detected in the specimens (Wang et al., 2014). As a result, antibiotics accumulate in recycled wastewater-irrigated soils at levels several times greater than those found in wastewater (Barancheshme & Munir, 2019). Another research examined into the role of wastewater treatment in the dissemination of resistance in *Acinetobacter* spp. found in various habitats where multiple isolates were isolated from five different locations, and susceptibility to eight different antibiotics was assessed using a disk diffusion method (Zhang et al., 2009). According to this research, the use of traditional biological treatment in WWTPs results in an increment of ARBs population (Barancheshme & Munir, 2019; Zhang et al., 2009). Macrolides are a category of natural and semisynthetic antibiotic compounds widely used in human and veterinary medicine (Fernandes et al., 2017). According to a WHO study on antibiotic use in Europe from 2016 to 2018, macrolides and cephalosporin were the two most frequently used antibiotics and have recently designated macrolides, as a top priority in order to improve their usage and mitigate their resistance (WHO, 2018). Klein et al., 2018 conducted a study on antibiotic usage in 76 countries and predicted total global consumption of antibiotic through 2030 where defined daily dose increase up to 65% and the consumption rate increase to 39%. All this unhindered use of drugs ultimately is unloaded in the wastewater treatment plant and the atmosphere and leads to AMR. Biological treatment of wastewater may result in the accumulation of ARB and its associated mobile elements, resulting in the modification of sludge microbiota due

to the selection pressure of antibiotic residues (Kraemer et al., 2019). Thus, industrial WWTPs are "worst case" situations for the pool of ARB, where they should be closely studied and examined (Bengtsson-Palme et al., 2019). Understanding habitats with high antibiotic selection pressures is critical not only for avoiding resistance to antibiotics, but also for understanding the negative effects of antibiotic exposure on microbial flora.

4 Dissemination of Antibiotic Resistance Among Bacteria

Long-term use of antibiotics has been shown to cause MDR, a phenomenon generally seen in intestinal bacteria and other niches in both clinical and veterinary sectors (Pacios et al., 2020). Disposal of antibiotic residues, combined with long-term drug use, increases the risk of MDR in environmental bacteria (Kraemer et al., 2019). Li et al. (2010) performed resistance profiles of bacterial isolates against ten antibiotics from seven different groups to back up the claim. Tetracyclines have long been used in veterinary and human medicine, and some have also been used as livestock and aquaculture growth promoters; after widespread use, resistance was soon encountered in a number of commensal and pathogenic bacteria. One of the key environmental problems of ARB containing ARG is the transfer of genes between organisms through horizontal gene transfer (Amabile-Cuevas, 2021). Horizontal gene transfer can take place in four ways: A. Conjugation-It is a method of transferring DNA from one cell to another through sexual pilus (cell-to-cell contact) where the recipient cell that was previously susceptible becomes resistant as a result of newly acquired resistant genes; B. Transformation-It is a process where absorption, incorporation, and foreign genetic material (naked DNA) of environmental bacteria; C. Transduction-It is a method of genetic recombination by which genes from one bacteria (host cell) are inserted into the genome of another host cell (bacteriophage) which is then transferred to another host cell; D. Gene transfer agents (GTA)-They are DNA segments that are found in the host (bacterial cell) and are transferred to the recipient cell (Von Wintersdorff et al., 2016). The marine environment is continuously polluted with antimicrobial compounds, making them ideal for the emergence and dissemination of ARBs and ARGs (Kraemer et al., 2019). These ARB and their genes are aggregated in the environment and transmitted via HGT to other pathogens will result in a future failure of antibiotic treatment (Lazar et al., 2021; Shah, 2020, 2021a, 2021b). However, there are some breaches in our understanding, such as how HGT occurs in wastewater and how the WWTP influences the microbial resistome habituating in the environment.

5 Sources of Antibiotic Resistant Bacteria (ARB)

Antimicrobial resistance, particularly multidrug resistance (MDR), has spread widely across pathogenic bacteria and has become one of the most significant challenges in medical practice (WHO, 2020). Researchers have used a variety of microbial techniques to study the emergence and dissemination of antibiotic resistance in the environment in order to avoid a return to pre-antibiotic times. The significant concentration of antibiotics when released facilitates the selection of ARB and their genes that navigate their escape into their surrounding habitats. Owing to the possible health threats, a variety of experiments have been conducted to investigate ARB recovered from different environments (Ventola, 2015). Once upon a time, hospital wastewater was expected to be the main source of antibiotics in marine ecosystems, however industrial, agricultural, and aquatic wastewater also have now been recognized as a major source of these metabolites, ARBs, and ARG (Kraemer et al., 2019). A document also stated that treated wastewater contains significantly higher levels of antibiotic metabolites than other aquatic habitats, making it a possible reservoir for ARB (Kraemer et al., 2019). Several studies have shown that the use of even a single antibiotic during human or veterinary clinical therapy will result in MDR strain selection. A few other reports on AMR and ARB are addressed in the following sections below (Table 1). To find solutions for managing antibiotic resistance dissemination (1) it is necessary to explore creative techniques on a large scale and over

Table 1 Summarized studies for AMR and ARB in the environment

S. No	Source	Sample type	Findings/organism	References
1	Sewage and water bodies	Sewage, river, pond, and swimming pool water	AMR bacteria (*Escherichia coli* and *Salmonella spp.*)	Nahar et al. (2019)
2	Sewage and environmental waters	Sewage water, WWTP	ESBL producing *E. coli*	Haberecht et al. (2019)
3	Sewage and environmental waters	Hospital and municipal wastewater	Carbapenemase producing Enterobacteriaceae	Cahill et al. (2019)
4	Sewage	Hospital and municipal wastewater	Vancomycin and ampicillin resistant *Enterococcus faecium*	Gouliouris et al. (2019)
5	Sewage	Hospital wastewater	MDR bacteria (*E. coli, K. pneumonia, A. baumannii, A. hydrophila*)	Yousfi et al. (2019)
6	Sewage	WWTP	Carbapenem producing *K. pneumoniae*	Sekizuka et al. (2018)
7	Sewage	Sludge	ESBL enterobacteriaceae, MRSA, VRE	Galler et al. (2018)

a long period of time (2) conduct risk assessment research to adequately comprehend the frequency and quantity of ARB/ARGs for the evaluation of possible public health risks (3) consider the environmental and organizational factors that affect each treatment procedure's efficacy.

6 Bacteriophage

Because human beings are greedy by nature, we create or find things that are easier and more compact in our lives. Bacteriophages are essential because pharmaceutical industries pipelines are completely dry, and there is no other way to treat antibiotic resistant bacteria. Antibiotic resistant bacteria have emerged, not only in humans but also in animals and plants, necessitating careful monitoring of their use in both animals and plants. The widespread prevalence of ESKAPE bacteria (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and *Enterobacter spp*) in the environment, which are extremely difficult to treat due to their high resistance potential, has put a lot of stress on the private and public health systems (Mulani et al., 2019). Bacteriophage is a type of virus that survives in different types of bacterial hosts (Stone et al., 2019). Due to the proficient properties of phages, they are constantly being armed with human research to target various species that cause complex diseases, allowing them to be used to treat WWTPs (Fig. 1). Phages are not self-propelled; they must physically come into contact with bacteria in order to infect

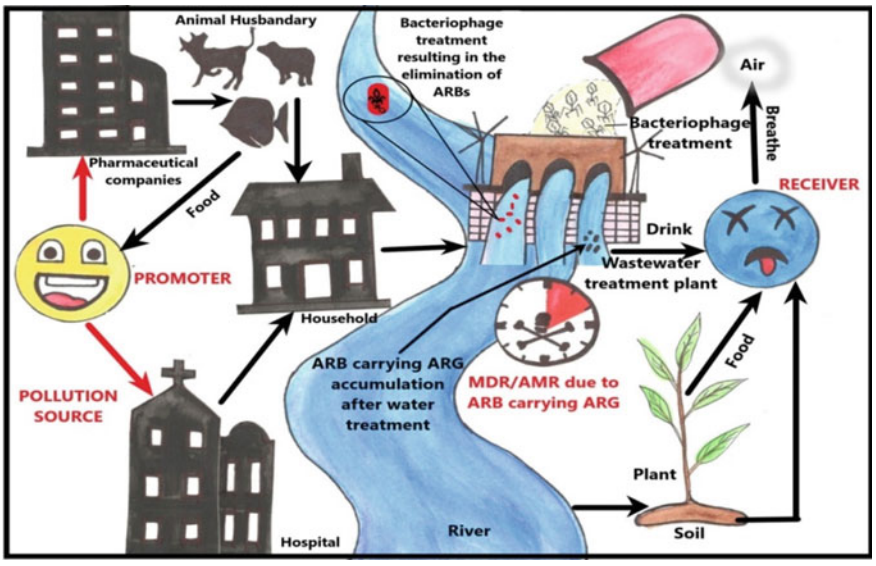

Fig. 1 Bacteriophage treatment: A solution to antibiotic resistant bacteria (ARBs) in wastewater treatment plants

Table 2 Properties of bacteriophages

Bacteriophage attributes	Comments	Reference
Highly specific	Bacteriophages are bacteria-specific, which ensures that they will not destroy any non-specific bacteria in the microbial environment	Brives and Pourraz (2020)
Self-limiting	Bacteriophages thrive alongside their hosts, and when the host is destroyed, the phage particles are dispersed harmlessly	Prabhurajeshwar et al. (2020)
Self-replicating	Bacteriophages have a distinctive advantage in terms of local applications because they can replicate as long as their host is present	Stone et al. (2019)
Naturally abundant	Phage therapy is effective in treatment of all bacterial infections since every bacteria found in nature has at least one specific bacteriophage that explicitly infects its complementary species	Lin et al. (2017)
Low production cost	Bacteriophages are environmentally friendly, production is easy, relatively inexpensive, and because it is ubiquitous, isolation and detection are relatively easier as compared to antibiotics which can take years, cost millions of dollars in clinical trials	El-Shibiny and El-Sahhar (2017)
Evolve naturally	Phage selection is a natural phenomenon, and eventually evolves with its host, thereby supporting the principle of evolutionary arguments	Fortuna et al. (2019)
Infinitely present	Bacteria may also develop resistance to phage and as they are abundant, it is much easier to identify and isolate new ones as compared to antibiotic resistance which is worrisome	Rohde et al. (2018)

them. Bacteriophages are highly specific, with each phage targeting and infecting only one bacterial species or, in some cases, one bacterial strain with numerous other properties mentioned below (Table 2) (Prabhurajeshwar et al., 2020).

7 Bacteriophage Life Cycle

The utilization of phages dates back over a century, leading scientists to rethink their use due to AMR. Bacteriophages, like all viruses, are extremely species-specific in terms of their hosts, infecting either a single bacterial species or specific strains within a species. If the bacteriophage attaches to its host, one of the two cycles (lytic or lysogenic) is used for propagation. During the lytic cycle, the bacteriophage binds to the receptors on the surface of the bacterium, delivers its genomic material to its host, executes viral replication in the cytosol via bacterial transcription, translation, and replication, and then escapes through the cytoplasm through cell lysis, resulting in the

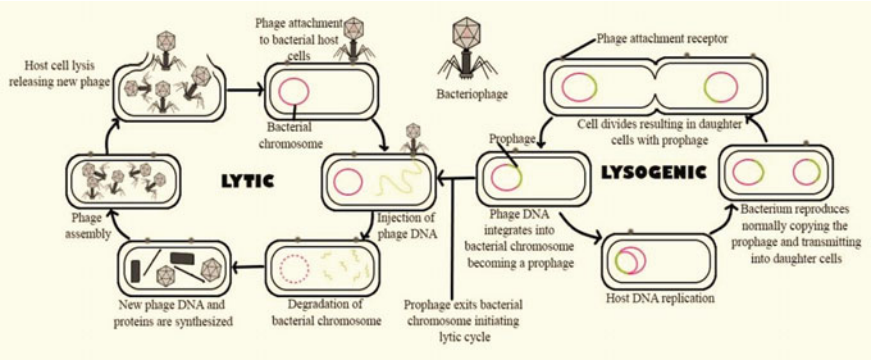

Fig. 2 Bacteriophage Life cycle

formation of new prophages (Fig. 2) (Kortright et al., 2019). This whole procedure is repeated when phages infect other susceptible bacteria (host). This exemplifies phage therapy's long-standing strength, as phages are a self-amplifying organism; a natural antimicrobial entity that destroys its target and is then eliminated (Lin et al., 2017). The top candidates for phage therapy are obligate lytic or virulent phages against ARB bacteria that are very difficult to eliminate (Fernandes et al., 2017). During the lysogenic cycle, the phage genome integrates with its bacterial host, which is ultimately passed down to daughter cells via binary fission. However, lysogenic phage genome excises from the bacterial genome and enters a lytic infection cycle when exposed to environmental or physiological stress (Kortright et al., 2019).

8 Studies of Phages Against ARBs

Foaming in treatment plants, sludge dewaterability and digestibility, infectious pathogens, and conflict among harmful microbes and essential bacterial species, all these problems can be regulated via bacteriophage treatments (Withey et al., 2005). As a result, they can be used not only for medicinal purposes but also for wastewater treatment. ESKAPE pathogens are MDR isolates and the most common source of hospital-acquired infections worldwide (Mulani et al., 2019). The majority of them are multidrug-resistant isolates, which is one of the most difficult issues to deal with in clinical practice as they easily evade commonly used antibiotics, and their prevalence in the environment makes them difficult to handle. Immunocompromised individuals, infants, and/or critically ill people are at risk from such ESKAPE pathogens sourced from the environment (Schultz et al., 2020). As a result, when antibiotics are no longer effective in treating drug-resistant infections, an alternative therapeutic technique such as bacteriophage therapy may be used. Few clinical phage therapies conducted are described below (Table 3) on resistant organism isolated from patients, which further gets discharged into the environment causing other multiple infections.

Table 3 Clinical phage therapy studies

Organism	Phages used	References
Enterococcus faecalis	EF-P29	Cheng et al. (2017)
Staphylococcus aureus	SA phage	Hamza et al. (2016)
Escherichia coli *Proteus mirabilus* *Pseudomonas aeruginosa* *Enterococcus spp* *Staphylococcus spp*	Pyophage solution	Leitner et al. (2017)
ESBL Klebsiella pneumoniae	VTCCBPA43	Anand et al. (2020)
Acinetobacter baumannii	IME285	Wang et al., (2020a, 2020b)
Pseudomonas aeruginosa	ΦPan70	Holguin et al. (2015)

Mulani et al. (2015) harnessed bacteriophages against pathogens isolated from water sample taken from Poona hospital, Pune for reduction of pathogen in wastewater. Ghorpade et al. (2019) also discussed in a review the effect of *Pseudomonads* on human health and the methods (filtration devices, continuous dosing via chlorine dioxide, UV sterilization, ozone installation with copper-silver ionization, phage treatment) to control them in water system. Reyneke et al. (2020) isolated and characterized bacteriophages (PAW33 and PFW25) against *Pseudomonas spp.* for biological regulation of rainwater samples, both pre-treated and non-pre-treated. Another study was conducted where the effect of MS2 phage was evaluated for inactivation of *E. coli* host to comprehend the population dynamic of bacteriophages and its host interaction (Voumard et al., 2019). A model of biofilm contained in ecosystem was analyzed and discussed for phage interaction with its host pathogen (Abedon, 2015).

Bacteriophages have been used as markers or smoke grenades for the presence of bacteria in WWTPs in a number of reports (Jassim et al., 2016; Withey et al., 2005). The main feature of phage-based detection of its host cells is due to bacteriophage affinity for particular host cells where phage's ability to identify specific bacterial surface receptors (pili, flagella, external polysaccharides, or a variety of membrane-bond proteins) can restrict or extend the host range (Bayat et al., 2021). To target bacterial pathogens, phages have been effectively used as a specific identification vector in foods, environmental conditions, and human infections (Principi et al., 2019), hence, these techniques can help predict bacterial contamination in wastewater, allowing for the use of additional disinfection strategies for infectious agent management. Withey et al. (2005), Goldman et al. (2009) all have suggested utilization of bacteriophages directly in wastewater treatment as they are an eco-friendly technique for management of biological foam and treatment of infectious pathogens (Jassim et al., 2016; Khairnar et al., 2014). Table 4 summarizes phage studies against pathogens isolated from wastewater system (Bayat et al., 2021).

Table 4 A study of phage-based research against pathogens isolated from wastewater systems

Host	Bacterial source	Phage source	Reference
H. hydrossis	Biomass bulking in the activated sludge	HHY phage from Local wastewater treatment plant	Kotay et al. (2010)
Gordonia and *Nocardia* sps	Foams on the surfaces of aerobic reactors of activated sludge	GTE7 phage from activated sludge mixed liquor of wastewater treatment plant	Petrovski et al. (2011)
Salmonella serovars	Wastewater	Sww65, sww275, and sww297 phages from wastewater	Turki et al. (2012)
E. coli, Pseudomonas sp. Streptococcus sp and *Bacillus spp*	Hospital wastewater	Hospital sewage	Periasamy and Sundaram (2013)
Nocardioforms	Activated sludge plants	NOC1, NOC2, and NOC3 phage from wastewater	Khairnar et al. (2014)
Gordonia spp	Activated sludge foam	Wastewater and natural water bodies of environment	Dyson et al. (2015)
S. flexneri and *S. sonnei*	Lab culture	Bacteriophage, pSs-1 from environmental water	Jun et al. (2016)
Escherichia coli (NDM-1)	Activated sludge from aeration tank	PER01 and PER02 from Sludge	Yu et al. (2017)
NDM-positive *E. coli* PI-7	Municipal wastewater	Wastewater influent	Al-Jassim et al. (2018)
Nocardia transvalensis, Nocardia brasiliensis and *Nocardia farcinica*	Lab culture	NTR1 phage from activated sludge	Taylor et al. (2019)
Escherichia coli, Pseudomonas aeruginosa and *Salmonella Typhi*	Sewage, agricultural and industrial waste	Wastewater	El-Dougdoug et al. (2020)

9 Conclusion

The revival of bacteriophage research as a way of managing diseases caused by bacteria has dispersed from the clinical industry to agriculture, aquaculture, and the food sector. Phage therapy diversified non-clinical application has sparked interest for wastewater treatment. One of the major obstacles in controlling WWTPs using bacteriophages is a lack of understanding of microbiota dynamics and interactions during wastewater treatment, which needs to be addressed and studied properly. The use of bacteriophages in wastewater treatment systems would almost certainly necessitate a

better understanding of wastewater microbiology and marine ecosystems in general. Failure to conduct phage therapy research would not only thwart phage application development, but it would also restrict our understanding of modern microbiology, which is crucial for future medicine due to the dangling sword of antimicrobial resistance. With a greater understanding of the microbial ecology of WWTPs, bacteriophage therapies will become viable alternatives and feasible solution to wastewater treatment challenges and optimization.

References

Abedon, S. T. (2015). Ecology of anti-biofilm agents II: Bacteriophage exploitation and biocontrol of biofilm bacteria. *Pharmaceuticals, 8*(3), 559–589.

Al-Jassim, N., Mantilla-Calderon, D., Scarascia, G., & Hong, P. Y. (2018). Bacteriophages to sensitize a pathogenic New Delhi metallo β-lactamase-positive Escherichia coli to solar disinfection. *Environmental Science & Technology, 52*(24), 14331–14341.

Amabile-Cuevas, C. F. (2021). Antibiotic resistance from, and to the environment. *AIMS Environmental Science, 8*(1), 18–35.

Amarasiri, M., Sano, D., & Suzuki, S. (2020). Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. *Critical Reviews in Environmental Science and Technology, 50*(19), 2016–2059.

Anand, T., Virmani, N., Kumar, S., Mohanty, A. K., Pavulraj, S., Bera, B. C., Vaid, R. K., Ahlawat, U., & Tripathi, B. N. (2020). Phage therapy for treatment of virulent Klebsiella pneumoniae infection in a mouse model. *Journal of Global Antimicrobial Resistance, 21*, 34–41.

Barancheshme, F., & Munir, M. (2019). Development of antibiotic resistance in wastewater treatment plants. In *Antimicrobial resistance-a global threat*. IntechOpen.

Baresel, C., Harding, M., & Fang, J. (2019). Ultrafiltration/granulated active carbon-biofilter: Efficient removal of a broad range of micropollutants. *Applied Sciences, 9*(4), 710.

Batinovic, S., Wassef, F., Knowler, S. A., Rice, D. T., Stanton, C. R., Rose, J., Tucci, J., Nittami, T., Vinh, A., Drummond, G. R., Sobey, C. G., Chan, H. T., Seviour, R. J., Petrovski, S., & Franks, A. E. (2019). Bacteriophages in natural and artificial environments. *Pathogens, 8*(3), 100.

Bayat, F., Didar, T. F., & Hosseinidoust, Z. (2021). Emerging investigator series: bacteriophages as nano engineering tools for quality monitoring and pathogen detection in water and wastewater. *Environmental Science: Nano.*

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C. F., Fick, J., Kristiansson, E., Tysklind, M., & Larsson, D. J. (2016). Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Science of the Total Environment, 572*, 697–712.

Bengtsson-Palme, J., Milakovic, M., Švecová, H., Ganjto, M., Jonsson, V., Grabic, R., & Udikovic-Kolic, N. (2019). Industrial wastewater treatment plant enriches antibiotic resistance genes and alters the structure of microbial communities. *Water Research, 162*, 437–445.

Brives, C., & Pourraz, J. (2020). Phage therapy as a potential solution in the fight against AMR: Obstacles and possible futures. *Palgrave Communications, 6*(1), 1–11.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., & Jonsson, M. (2014). Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 369*(1656), 20130580.

Cahill, N., O'Connor, L., Mahon, B., Varley, Á., McGrath, E., Ryan, P., et al. (2019). Hospital effluent: A reservoir for carbapenemase-producing Enterobacterales? *Science of the Total Environment, 672*, 618–624.

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., & Burden of AMR Collaborative Group. (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect Diseases*, *19*(1), 56–66.

CDC. (2013). Antibiotic resistance threats in the United States. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf. Accessed March 15, 2021.

Cheng, M., Liang, J., Zhang, Y., Hu, L., Gong, P., Cai, R., Zhang, L., Zhang, H., Ge, J., Ji, Y., Guo, Z., Feng, X., Sun, C., Yang, Y., Lei, L., Han, W., & Gu, J. (2017). The bacteriophage EF-P29 efficiently protects against lethal vancomycin-resistant Enterococcus faecalis and alleviates gut microbiota imbalance in a murine bacteremia model. *Frontiers in Microbiology, 8*, 837.

Di Cerbo, A., Pezzuto, F., Guidetti, G., Canello, S., & Corsi, L. (2019). Tetracyclines: insights and updates of their use in human and animal pathology and their potential toxicity. *The Open Biochemistry Journal*, *13*(1).

Dadgostar, P. (2019). Antimicrobial resistance: Implications and costs. *Infect Drug Resist, 12*, 3903–3910.

Dyson, Z. A., Tucci, J., Seviour, R. J., & Petrovski, S. (2015). Lysis to kill: evaluation of the lytic abilities, and genomics of nine bacteriophages infective for Gordonia spp. and their potential use in activated sludge foam biocontrol. *PLoS One*, *10*(8), e0134512.

El-Dougdoug, N., Nasr-Eldin, M., Azzam, M., Mohamed, A., & Hazaa, M. (2020). Improving wastewater treatment using dried banana leaves and bacteriophage cocktail. *Egyptian Journal of Botany, 60*(1), 199–212.

El-Shibiny, A., & El-Sahhar, S. (2017). Bacteriophages: The possible solution to treat infections caused by pathogenic bacteria. *Canadian Journal of Microbiology, 63*(11), 865–879.

Fernandes, P., Martens, E., & Pereira, D. (2017). Nature nurtures the design of new semi-synthetic macrolide antibiotics. *Journal of Antibiotics, 70*(5), 527–533.

Fortuna, M. A., Barbour, M. A., Zaman, L., Hall, A. R., Buckling, A., & Bascompte, J. (2019). Coevolutionary dynamics shape the structure of bacteria-phage infection networks. *Evolution, 73*(5), 1001–1011.

Fouz, N., Pangesti, K. N., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Ghany, M. A. E. (2020). The contribution of wastewater to the transmission of antimicrobial resistance in the environment: Implications of mass gathering settings. *Tropical Medicine and Infectious Diseases, 5*(1), 33.

Galler, H., Feierl, G., Petternel, C., Reinthaler, F. F., Haas, D., Habib, J., Kittinger, C., Luxner, J., & Zarfel, G. (2018). Multiresistant bacteria isolated from activated sludge in Austria. *International Journal of Environmental Research and Public Health, 15*(3), 479.

Ghorpade, K. B., Suryawanshi, M., & Shinde, S. M. (2019). Elimination of pseudomonas aeruginosa from water systems: A review. *Journal of Biomedical and Pharmaceutical Research, 8*(5), 124–127.

Goldman, G., Starosvetsky, J., & Armon, R. (2009). Inhibition of biofilm formation on UF membrane by use of specific bacteriophages. *Journal of Membrane Science, 342*(1–2), 145–152.

Gouliouris, T., Raven, K. E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., et al. (2019). Detection of vancomycin-resistant Enterococcus faecium hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. *Genome Research, 29*(4), 626–634.

Haberecht, H. B., Nealon, N. J., Gilliland, J. R., Holder, A. V., Runyan, C., & Oppel, R. C., et al., (2019). Antimicrobial-resistant Escherichia coli from environmental waters in northern Colorado. *Journal of Environmental and Public Health.*

Hamza, A., Perveen, S., Abbas, Z., & Rehman, S. U. (2016). The lytic SA phage demonstrate bactericidal activity against mastitis causing Staphylococcus aureus. *Open Life Sciences, 11*(1), 39–45.

Holguín, A. V., Rangel, G., Clavijo, V., Prada, C., Mantilla, M., Gomez, M. C., Kutter, E., Taylor, C., Fineran, P. C., Barrios, A. F., & Vives, M. J. (2015). Phage ΦPan70, a putative temperate phage, controls Pseudomonas aeruginosa in planktonic, biofilm and burn mouse model assays. *Viruses, 7*(8), 4602–4623.

Hong, P. Y., Julian, T. R., Pype, M. L., Jiang, S. C., Nelson, K. L., Graham, D., Pruden, A., & Manaia, C. M. (2018). Reusing treated wastewater: Consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes. *Water, 10*(3), 244.

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., & Virta, M. (2018). Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS Microbiology Ecology*, *94*(4), fiy038.

Jassim, S. A., Limoges, R. G., & El-Cheikh, H. (2016). Bacteriophage biocontrol in wastewater treatment. *World Journal of Microbiology and Biotechnology, 32*(4), 70.

Jun, J. W., Giri, S. S., Kim, H. J., Yun, S. K., Chi, C., Chai, J. Y., et al. (2016). Bacteriophage application to control the contaminated water with Shigella. *Scientific Reports, 6*(1), 1–7.

Khairnar, K., Pal, P., Chandekar, R. H., & Paunikar, W. N. (2014). Isolation and characterization of bacteriophages infecting nocardioforms in wastewater treatment plant. *Biotechnology Research International*.

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences of the United States of America, 115*(15), E3463–E3470.

Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019). Phage therapy: A renewed approach to combat antibiotic-resistant bacteria. *Cell Host & Microbe, 25*(2), 219–232.

Kotay, S. M., Datta, T., Choi, J., & Goel, R. (2010). Biocontrol of biomass bulking caused by Haliscomenobacter hydrossis using a newly isolated lytic bacteriophage. *Water Research, 45*(2), 694–704.

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic pollution in the environment: From microbial ecology to public policy. *Microorganisms, 7*(6), 180.

Lazar, V., Gheorghe, I., Curutiu, C., Savin, I., Marinescu, F., Cristea, V. C., Dobre, D., Popa, G. L., Chifiriuc, M. C., & Popa, M. I. (2021). Antibiotic resistance profiles in cultivable microbiota isolated from some romanian natural fishery lakes included in Natura 2000 network. *BMC Veterinary Research, 17*(1), 1–11.

Leitner, L., Sybesma, W., Chanishvili, N., Goderdzishvili, M., Chkhotua, A., Ujmajuridze, A., Ujmajuridze, A., Schneider, M. P., Sartori, A., Mehnert, U., Bachmann, L. M., & Kessler, T. M. (2017). Bacteriophages for treating urinary tract infections in patients undergoing transurethral resection of the prostate: A randomized, placebo-controlled, double-blind clinical trial. *BMC Urology, 17*(1), 1–6.

Lepape, A., Jean, A., De Waele, J., Friggeri, A., Savey, A., Vanhems, P., Gustin, M. P., Monnet, D. L., Garnacho-Montero, J., & Kohlenberg, A. (2020). European intensive care physicians' experience of infections due to antibiotic-resistant bacteria. *Antimicrobial Resistance & Infection Control, 9*(1), 1–11.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., & Qi, R. (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Applied and Environmental Microbiology, 76*(11), 3444–3451.

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics, 8*(3), 162–173.

Mohammadali, M., & Davies, J. (2017). Antimicrobial resistance genes and wastewater treatment. *Antimicrobial Resistance in Wastewater Treatment Processes*, 1–13.

Mulani, M. S., Azhar, S., Azharuddin, S., & Tambe, S. (2015). Harnessing the power of bacteriophage for pathogen reduction in wastewater. *International Journal of Current Microbiology and Applied Sciences, 2*, 152–161.

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Frontiers in Microbiology, 10*, 539.

Nahar, A., Islam, M. A., Sobur, M. A., Hossain, M. J., Zaman, S. B., Rahman, M. B., et al. (2019). Detection of tetracycline resistant E. coli and Salmonella spp. in sewage, river, pond and swimming pool in Mymensingh, Bangladesh. *African Journal of Microbiology Research*, *13*(25), 382–387.

Nikolich, M. P., & Filippov, A. A. (2020). Bacteriophage therapy: Developments and directions. *Antibiotics, 9*(3), 135.

Pacios, O., Blasco, L., Bleriot, I., Fernandez-Garcia, L., González Bardanca, M., Ambroa, A., Lopez, M., Bou, G., & Tomás, M. (2020). Strategies to combat multidrug-resistant and persistent infectious diseases. *Antibiotics, 9*(2), 65.

Pazda, M., Rybicka, M., Stolte, S., Piotr Bielawski, K., Stepnowski, P., Kumirska, J., Wolecki, D., & Mulkiewicz, E. (2020). Identification of selected antibiotic resistance genes in two different wastewater treatment plant systems in Poland: A preliminary study. *Molecules, 25*(12), 2851.

Periasamy, D., & Sundaram, A. (2013). A novel approach for pathogen reduction in wastewater treatment. *Journal of Environmental Health Science and Engineering, 11*(1), 1–9.

Petrovski, S., Seviour, R. J., & Tillett, D. (2011). Prevention of Gordonia and Nocardia stabilized foam formation by using bacteriophage GTE7. *Applied and Environmental Microbiology, 77*(21), 7864–7867.

Prabhurajeshwar, C., Desai, P. P., Waghmare, T., & Rashmi, S. B. (2020). An overview of bacteriophage therapy over antibiotics; as an alternative for controlling bacterial infections. *International Journal of Pharmaceutical Sciences and Research, 11*(3), 993–1006.

Principi, N., Silvestri, E., & Esposito, S. (2019). Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Frontiers in Pharmacology, 10*, 513.

Reyneke, B., Khan, S., Fernández-Ibáñez, P., & Khan, W. (2020). Podoviridae bacteriophage for the biocontrol of Pseudomonas aeruginosa in rainwater. *Environmental Science: Water Research & Technology, 6*(1), 87–102.

Rohde, C., Wittmann, J., & Kutter, E. (2018). Bacteriophages: A therapy concept against multidrug–resistant bacteria. *Surgical Infections, 19*(8), 737–744.

Savin, M., Bierbaum, G., Hammerl, J. A., Heinemann, C., Parcina, M., Sib, E., Voigt, A., & Kreyenschmidt, J. (2020). ESKAPE bacteria and extended-spectrum-β-lactamase-producing Escherichia coli isolated from wastewater and process water from German poultry slaughterhouses. *Applied and Environmental Microbiology*, *86*(8).

Schultz, F., Anywar, G., Tang, H., Chassagne, F., Lyles, J. T., Garbe, L. A., & Quave, C. L. (2020). Targeting ESKAPE pathogens with anti-infective medicinal plants from the greater Mpigi region in Uganda. *Scientific Reports, 10*(1), 1–19.

Sekizuka, T., Yatsu, K., Inamine, Y., Segawa, T., Nishio, M., Kishi, N., & Kuroda, M. (2018). Complete genome sequence of a blaKPC-2-positive Klebsiella pneumoniae strain isolated from the effluent of an urban sewage treatment plant in Japan. *Msphere*, *3*(5).

Shah, M. P. (2020). *Microbial Bioremediation & Biodegradation.* Springer.

Shah, M. P. (2021a). *Removal of refractory pollutants from wastewater treatment plants.* CRC Press.

Shah, M. P. (2021b). *Removal of emerging contaminants through microbial processes.* Springer.

Smyth, C., O'Flaherty, A., Walsh, F., & Do, T. T. (2020). Antibiotic resistant and extended-spectrum β-lactamase producing faecal coliforms in wastewater treatment plant effluent. *Environmental Pollution, 262*, 114244.

Stone, E., Campbell, K., Grant, I., & McAuliffe, O. (2019). Understanding and exploiting phage–host interactions. *Viruses, 11*(6), 567.

Taylor, S., Brown, T. L., Tucci, J., Lock, P., Seviour, R. J., & Petrovski, S. (2019). Isolation and characterization of bacteriophage NTR1 infectious for Nocardia transvalensis and other Nocardia species. *Virus Genes, 55*(2), 257–265.

Turki, Y., Ouzari, H., Mehri, I., Ammar, A. B., & Hassen, A. (2012). Evaluation of a cocktail of three bacteriophages for the biocontrol of Salmonella of wastewater. *Food Research International, 45*(2), 1099–1105.

UN-Water, U. N. E. S. C. O. (2020). *United Nations world water development report 2020: Water and climate change*.

Ventola, C. L. (2015). The antibiotic resistance crisis: Part 1: Causes and threats. *P T, 40*(4), 277.

Von Wintersdorff, C. J., Penders, J., Van Niekerk, J. M., Mills, N. D., Majumder, S., Van Alphen, L. B., Savelkoul, P. H., & Wolffs, P. F. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology, 7*, 173.

Voumard, M., Giannakis, S., Carratalà, A., & Pulgarin, C. (2019). E. coli–MS2 bacteriophage interactions during solar disinfection of wastewater and the subsequent post-irradiation period. *Chemical Engineering Journal, 359*, 1224–1233.

Wang, F. H., Qiao, M., Lv, Z. E., Guo, G. X., Jia, Y., Su, Y. H., & Zhu, Y. G. (2014). Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China. *Environmental Pollution, 184*, 247–253.

Wang, C., Li, P., Zhu, Y., Huang, Y., Gao, M., Yuan, X., Niu, W., Liu, H., Fan, H., Qin, Y., Tong, Y., Mi, Z., & Bai, C. (2020a). Identification of a novel Acinetobacter baumannii phage-derived depolymerase and its therapeutic application in mice. *Frontiers in Microbiology, 11*, 1407.

Wang, J., Chu, L., Wojnárovits, L., & Takács, E. (2020b). Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview. *Science of the Total Environment, 744*, 140997.

WHO. (2020). Antimicrobial resistance. https://www.who.int/health-topics/antimicrobial-resistance

Withey, S., Cartmell, E., Avery, L. M., Stephenson, T. (2005). Bacteriophages–potential for application in wastewater treatment processes. ***Science of the Total Environment***, *1, 339*(1–3), 1–18

World Health Organization. (2018). *WHO report on surveillance of antibiotic consumption: 2016–2018 early implementation.* World Health Organization.

Xu, Y. B., Hou, M. Y., Li, Y. F., Huang, L., Ruan, J. J., Zheng, L., Qiao, Q. X., & Du, Q. P. (2017). Distribution of tetracycline resistance genes and AmpC β-lactamase genes in representative non-urban sewage plants and correlations with treatment processes and heavy metals. *Chemosphere, 170*, 274–281.

Yousfi, K., Touati, A., Lefebvre, B., Garneau, P., Brahmi, S., Gharout-Sait, A., et al. (2019). Characterization of multidrug-resistant Gram-negative bacilli isolated from hospitals effluents: First report of a bla OXA-48-like in Klebsiella oxytoca Algeria. *Brazilian Journal of Microbiology, 50*(1), 175–183.

Yu, P., Mathieu, J., Lu, G. W., Gabiatti, N., & Alvarez, P. J. (2017). Control of antibiotic-resistant bacteria in activated sludge using polyvalent phages in conjunction with a production host. *Environmental Science & Technology Letters, 4*(4), 137–142.

Zhang, Y., Marrs, C. F., Simon, C., & Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. *Science of the Total Environment, 407*(12), 3702–3706.

The Emergence of Wastewater Treatment Plant as a Leading Source for Dissemination of Antibiotic-Resistant Gene

Bidisha Ganguly and Subhasish Dutta

Abstract Antibiotic-resistant genes have been evolving as one of the leading contaminants threatening human health. The emission of novel microbial pollutants and chemicals in the aquatic environment released from the wastewater treatment plant (WWTP) affects the environment. World Health Organization has predicted that if this continues, the future will look mostly like the past, where people were dying due to trivial injuries. Thus, it is necessary to balance everything in the environment, including bacteria, for our well-being. The book chapter deals with different reasons for developing and disseminating antibiotic-resistant genes (ARG) in the environment. The wastewater treatment plant's role in the spread of antibiotic-resistant genes and antimicrobial resistance (AMR) will also be discussed. The book chapter also highlights a few techniques by which ARG's increasing effect can be eliminated or reduced to a considerable extent by different methods, including WWTP. Finally, the chapter ends with a discussion of the prospects and limitations of the world of ARG and AMR.

Keywords Antimicrobial resistance · Gene · Wastewater treatment plant · Microbial pollutants · Aquatic environment

1 Introduction

The evolution of antibiotic-resistant pathogen is a very recent process as antibiotics have massively increased in few years. In hospitals and veterinary medicine, agriculture the large-scale synthesis of antibiotics has led to its adverse effect on the environment. Earlier, scientists thought that only through mutation bacterial genetic variability can be changed. Consequently, only target modification could affect the antibiotic resistance, so it remains clonal. This mechanism is applicable still now for

B. Ganguly · S. Dutta (✉)
Biosafety Support Unit (BSU), Regional Centre for Biotechnology (RCB), 3rd Milestone, Faridabad-Gurgaon Expressway, Faridabad Rd, Faridabad 121001, Haryana, India
e-mail: subhasish@bsu.rcb.res.in

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_4

quinolones, rifampin, fosfomycin, etc. It promotes the horizontal gene transfer evolution like extended-spectrum beta-lactamases or ESBLs. But this antibiotic resistance which can be driven only through mutation occurs during in-host evolution only. Chronic infection is an example of such mutation-driven in-host evolution (Maciá et al., 2005). Present-day antibiotic resistance is proof that ARGs have a long history of evolution. By many means of selection, diversification of the present antibiotic era has come (Aminov & Mackie, 2007). Beta-lactamases are the best examples by which the evolution of ancient antibiotic resistance and its present structure, its phylogeny can be illustrated. These beta-lactams are widely used antibiotic, the resistance to which can be a serious problem for society. Because the beta-lactams resistance is of low contamination, its excessive use to treat infections can hurt the ecosystem and human population. The primary mechanism for antibiotic resistance holds inactivation of the enzyme through beta-lactam ring separation by beta-lactamases. The beta-lactam ring has a serine active site and two classes of Metallo-β-lactamases (Bush, 1998). The structure-based phylogeny has been able to construct both of the β-lactamase's group for evolution. This phylogeny had proved that it was almost two billion years ago when these enzymes originated on the Earth. The β-lactamases were found to reside on plasmid almost a million years ago before its present-day use (Hall & Barlow, 2004). A study on β-lactamase genes has shown that it has been residing on the plasmid for a hundred million years. The enzyme did not develop any antimicrobial resistance while it was residing on a plasmid (Fevre et al., 2005). The metagenomic study on β-lactamase was analyzed using cold-seep sediments (Song et al., 2005). It is interesting to know that those evolutionary forces that are unknown to us also contribute to the creation of diverse antibiotic-resistant genes (Allen et al., 2009). Various ARGs grant resistance to the antibiotics, which are unrelated to the structure of those ARGs. These antibiotic-resistant genes exhibit different action mechanism and phylogenetic analysis to the structurally different antibiotics (Aminov & Mackie, 2007). There are two stages for the evolution of antibiotic-resistant genes. The macro evolutionary or pre-antibiotic era is defined by diversification of the environmental ecosystem through mutation and duplication. On the other hand, the micro-evolutionary or antibiotic era is characterized by large-scale antibiotic production. In this evolutionary period, there is strong pressure exertion toward the bacteria of various ecosystems. The macro evolutionary period has a very less amount of horizontal gene transfer contributing to antibiotic production's evolutionary process. But one thing that is still unknown to us is if the antibiotic resistance mechanism is involved in any other metabolic function or not. The micro-evolutionary or antibiotic era has been occurring for seventy years. Thus, it is a very recent period involving antibiotic production. There are many rare genes relative to other genes that grant the antibiotic-resistant genes. These genes, once used, participate in other cellular activities. But they opted for resistance characteristics, and they moved from the ecological genomic pools into taxonomically varying pathogenic bacteria. It is a fast process where the mobile genetic elements drove the horizontal gene transfer. The contribution of mobile genetic elements (MGE) in the evolutionary process of antibiotics was significant. If ARGs' solitary functional

contribution is to protect the lethal concentration of antibiotics, then ARGs' selection and spread are connected to those anthropogenic components. Its reason is that the antibiotic concentration in the harmless areas is below the detection boundary and does not exist in the minimum inhibitory concentration or MIC (Chee-Sanford et al., 2009). Another study showed that pristine freshwater of an area where the antibiotic-resistant genes did not affect contains almost sixty percent of *Enterobacteriaceae* with multidrug-resistant genes (Lima-Bittencourt et al., 2007). Thus, it has now been generalized that there is a huge diversity of antibiotics and antibiotic-resistant genes that occur naturally in nature. Many soil bacteria reside on antibiotics in nature for their survival as they get carbon from antibiotics. Carbon is their sole source of survival (D'Costa et al., 2007). The ultimate goal of these genes is to clean up the infection. In this book chapter, the antibiotic-resistant genes have been displayed to harm the environment and human beings. The antibiotic-resistant genes existing in the wastewater treatment plants are sources for the contamination of freshwater, drinking, and usable waters. For the past few years, the excessive utilization of antibiotics has led to the generation of such antibiotic resistance whose cure is unknown to scientists. Thus, we must remove these ARGs as soon as possible. This book chapter includes different ARGs that are mainly obtained in WWTPs and the sources of different ARGs. Various complex treatment methods that are effective for the removal of ARGs and ARBs have also been discussed. Finally, it has discussed how to deal with these ARGs in WWTPs in future.

2 Antibiotic-Resistant Genes and Its Dissemination in Wastewater Treatment Plant

According to the healthcare system, the growth of antibiotic-resistant bacteria and the abundance of infections caused by it have been considered one of the most significant issues. As per the report, about one million people died in the time period between the year 2014 and 2016. The reason behind this is none other than the infections caused by antibiotic-resistant bacteria. There was no treatment method to cure those infections as the scientists were unable to develop any antibiotics. The situation is becoming more severe if we consider the future antibiotic drug development under consideration. Scientists have predicted that if this rapid growth of antibiotic-resistant bacteria continues, then there will be millions of death happening in the upcoming two decades. Indeed, the evolutionary process of antibiotic resistance and its gene transfer is complex. It is proved that one of the principal reasons for the development of antibiotic-resistant genes and ARB is the excessive use and misuse of antibiotics in human and veterinary medicines (Holmes et al., 2016).

Additionally, the mobilization or rally of the antibiotic-resistant genes has made it difficult to remove the ARB systematically once the transmission starts to broaden. This process gets enhanced when the density of bacterial cells is high with nutrient-rich habitat (Baquero et al., 2008). It has been seen that the activated sludge process

of the wastewater treatment plant is the perfect system for the transfer of ARGs. As the WWTP cannot remove antibiotic resistance from water, the ARGs and ARBs get into the aquatic environment after being discharged by WWTPs in water (Hembach et al., 2017).

Almost one thousand different β-lactamases variants that fall under ARGs have been found and analyzed. This discovery proves that the diversity of antibiotic resistance has been increased abruptly and added to the ARG pool. As a result, people are getting affected by so many different ARBs, the treatment of which is still not found yet, proving the fact that those antibiotic-resistant bacteria have acquired the resistance from environmental source through horizontal gene transfer. The number of multi-resistant pathogens is also increasing as the ARGs are being accumulated. This made the treatment of antibiotics difficult.

The development of multi-resistant genes has resulted in insufficient hygiene in the clinical system, thereby increasing the growth of ARBs. *Staphylococcus aureus*, extended-spectrum β-lactamase synthesizing *Enterobacteriaceae*, vancomycin-resistant *Enterococci*, etc. These all are examples of the multi-resistant gene for which many infections are becoming very difficult to treat. These organisms which reside in healthy people can become pathogenic when possible.

The aquatic environment consists of millions of resistance gene, which are entirely unknown to us. It serves as a suitable platform for the antimicrobial resistance and antibiotic-resistant gene to develop and disseminate. Apart from this, there is a considerable diversity of ARGs released from hospitals and communal wastewater into the aquatic surroundings on an everyday basis. This incidence creates known and unknown kinds of ARGs in a more significant number, which has become a real threat to the world (Bengtsson-Palme et al., 2018). Industrial pollutions, heavy metals, detergents, antimicrobial agents, and disinfectants serve as the activators for developing and disseminating resistant genes in the aquatic environment. Millions of environmental bacteria out there serve as the source of resistant genes when entering into pathogenic organisms. The point to be noted here is that many of such genes do not exist as resistant genes, but tend to convert in ARGs. These are called resistome (D'Costa et al., 2006). This book chapter has jotted down the sources of antibiotic-resistant genes, the reason for the abrupt growth of ARGs. Also, what role wastewater treatment plant plays in the spread of ARGs has been discussed later. And lastly, the various techniques for reducing ARGs will be covered.

3 Different ARGs Available in Wastewater Treatment Plants

Extensive research showed various antibiotic-resistant genes are available in WWTPs. There are two principal approaches by which problems regarding antibiotic resistance in wastewater can be analyzed, i.e., culture-based and molecular-based approaches. Culture-based approach is used to detect the phenotypic characteristics

of the bacteria isolated from the environment. One disadvantage of this method is that it is limited to 1% of total environmental bacteria only as only this amount of bacteria has been isolated so far. On the other hand, the molecular-based approach deals with the isolated DNA from the samples analyzed and detected sequences of nucleotides of those specific DNAs which code for ARGs under polymerase chain reactor. In other words, the quantitative polymerase chain reaction identifies those target DNAs used to detect nucleotides that cannot be cultured in the laboratory (Dumas et al., 2006). These DNAs, although they grow very steadily, donate substantially to the antibiotic resistance problems. A known fact is that in PCR detection, the quality of the DNA isolated, as well as the extraction method of the nucleic acid, are fundamental conditions. The detection of microbial or bacterial species becomes difficult in wastewater treatment plant as it contains organic and inorganic matter, inhibitors, detergents, etc. The molecular high-throughput sequencing technologies are essential tools to analyze natural microbial groups, their various genomic ranges, and gene expression activities. The WWTPs can affect the providence of antibiotic-resistant genes and antibiotic-resistant bacteria in multiple ways. The initial step involves oil, grease withdrawal, and deposition of large particles. The next step consists of the reduction of organic matter in wastewater by using microbial organisms. In the ultimate step, tertiary wastewater treatment is waged to improve its quality further (Quach-Cu et al., 2018). Many other processes like membrane bioreactor, moving bed biofilm reactor, and fixed bed bioreactor are used in municipal wastewater treatment plants. Below are some of the names of antibiotic-resistant genes found in the wastewater treatment plant.

3.1 Beta-Lactam Resistant Genes

There is a beta-lactam ring in the molecular constitution of such antibiotics, which serves a vital role or, more precisely, the principal role in the antibacterial activity. The branches of the ring possess a diversity of pharmacological characteristics. These antibiotics obstruct the biosynthesis of the bacterial cell wall. This PDB is the enzyme required for the peptidoglycan layer preparation and is used for medicinal and veterinary purpose. Penicillins and cephalosporins are some of the most used antibiotics. As per the European hospital sector, the defined daily dose per one thousand inhabitants per day for the consumption of systemic use is 11.4. But for beta-lactamase consumption, it is just 0.92 DDD per 1000 inhabitants per day. They have taken over half of the available antibiotics commercially. Although beta-lactams and penicillin are the most common antibiotics available in the market, they are relatively lesser in the wastewater treatment plant. The resistance to beta-lactams occurs by reducing the number of pours in the channels. It results in a change in the target protein, i.e., penicillin-binding protein and alters the absorptive power of the bacterial outer membrane. And hence, the drug cannot enter the cell wall as the previous way. Beta-lactamase enzyme mechanism of resistance is widespread in gram-negative bacteria

(Zapun et al., 2008). There are specific resistance genes for specific genetic elements, for example, ampC for the chromosome, ampR, bla_{TEM}, bla_{CIT}, bla_{FOX} for plasmid, etc.

3.2 Macrolide Resistance Genes

Macrolides are a community of inherent admixture which have a lactone ring. This lactone ring is replaced by ketone, hydroxyl (−OH), and alkyl groups. Their mode of action includes inactivation of the protein synthesis at an early stage by tying up with 50S ribosomes of bacteria. As macrolides cannot be metabolized or, more precisely, are not metabolized in body, so they are ejected from the body through bile and feces. These ejected macrolides are then thrown into the wastewater, and there they stay as the unchanged parent compound. Their daily consumption in hospitals is 0.15 DDD per one thousand inhabitants per day, while in the community, this number is about 3.0. These are good examples of the substitute for penicillin. These macrolides are the principal metabolite of erythromycin and erythromycin-H_2O. The transformation of the erythromycin occurs under high instability of this depressant and high acidic condition. At the sample preparation stage, it directly changes erythromycin to macrolides and consequently increases its concentration in the experimental samples (Göbel et al., 2007). Synthesis of methylase is the basis of the pathway for the transfer of bacterial resistances. Synthesis of methylase involves methylation of 23S rRNA where the antibiotics act. Moreover, division of lactone ring cleavage and its mechanism, inhibition of antibiotics by modifying enzymes or macrolide phosphotransferases, the active pumping of drug from the cell, etc. are some of the examples of the mechanisms of bacterial resistance to macrolides.

3.3 Quinolone Resistance Genes

Quinolones and its derivatives form a group of chemotherapeutic substance which has an antibacterial pursuit. These families have four different generations. The derivatives of quinolones are fluoroquinolones. These derivatives can comprise a subset along with a central ring which can be substituted with fluorine. These compounds inactivate DNA gyrase and topoisomerase IV, which are two crucial enzymes for DNA's replication. Quinolones are essential substances that are primarily used in hospitals for treating various infections and prophylactic purposes. Moreover, it has a wide range of applications for veterinary purposes as growth promoters and medicinal purposes. As per the global marketing, they stood out to be the third-largest chemotherapeutic groups. It holds almost seventeen percent of world trading. In a community, its consumption is 1.7 DDD per 1000 inhabitants in a day, whereas for hospital purposes, its daily utilization is 0.23 DDD per 1000 inhabitants per day. Quinolones, like macrolides, are disseminated into municipal sewages by urine and

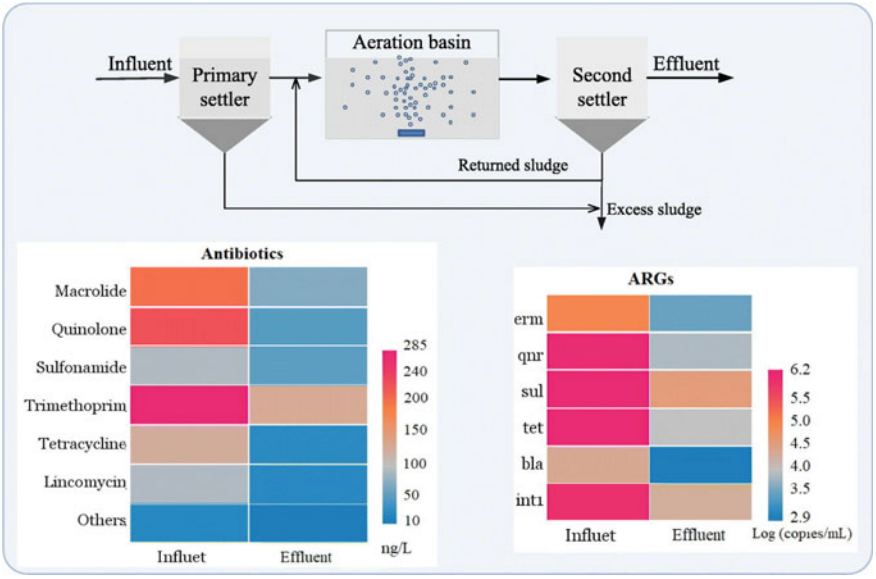

Fig. 1 Filtration of ARGs in Wastewater Treatment Plant (Wang et al., 2020)

feces. The percentage of urine is 45–62%, and that of wastes is fifteen to twenty-five percent in quinolones excretion. There is a substantial number of quinolones detected in the wastewater treatment plant. There are two effective mechanisms for chromosomal quinolones that can be acted. One is by modifying the target enzyme, and the other is by decreasing permeability of the outer membrane through porin swapping and upregulation of the efflux pump. The qnr genes can arbitrate quinolone resistance. It protects DNA gyrase and topoisomerase IV against the quinolones. Qnr genes code for protein and are situated on MGEs (mobile genetic elements), e.g., plasmids. These plasmids can actively transfer genes in HGT (horizontal gene transfer) (Fig. 1).

3.4 Sulfonamide and Trimethoprim Resistance Genes

The resistance genes mentioned above are categorized under a group of synthetic antimicrobial agents. It is specific genes that act on particular bacteria as a competitive inhibitor of dihydropteroate synthase enzyme. DHPS is involved in the synthesis of folic acid. These resistance genes are used for treating bacterial and protozoal infection and as medicated feed additives in veterinary fields. In the case of the treatment for human-related diseases, it is used for respiratory, urinary tract, and chlamydia infections. Its combination with pyrimethamine is used for treating malaria and toxoplasmosis. Daily consumption of sulphonamides and trimethoprim

in hospital purposes is 0.06 DDD per one thousand inhabitants. On the other hand, its intake in community area is about 0.6 DDD per thousand inhabitants per day. These compounds are also excreted as the other two resistance genes in wastewater or municipal water as metabolites. The report says that the purification mechanism for sulphonamides is about seventy-five to eighty-five percent, and they are slightly insoluble in water. The use of these sulphonamides has reduced significantly after the end of nineteenth century, but these genes are still available in wastewater treatment plants. The diversity of the mechanism of the antibiotic resistance to sulphonamides is more than that of other antibiotics. The mechanism includes various points like—alteration of metabolism; target enzyme is mutated. Consequently, this target enzyme reduces the affinity toward the inhibitor, cell permeability changes, and it influences the absorption of drug, etc. (Then & Angehrn, 1982). It is to be notified that those resistances picked up for a particular substance can affect the resistance to other sulphonamide compounds. For example, sulI, sulII, and sulIII genes code for DHPS are the main reasons for which resistance to sulphonamides happens. These DHPS or dihydropteroate synthases have a lower affinity toward the sulphonamides. These sulfonamide genes are found both on transposons and plasmids in mobile genetic elements. On the other hand, if the dihydrofolate reductase synthesis increases, then bacterial resistance causes to trimethoprim.

4 Sources of Antibiotic-Resistant Genes

Antibiotic-resistant genes can occur in various ways. A low concentration of ARGs occurs in the environment through natural selection. But in the case of high-level ARBs or ARGs in the environment, human activities occur. There are two primary sources through which antibiotic-resistant bacteria enter the environment—human sources and animal sources. Wastewater treatment plants are one of the principal sources for the development and dissemination of ARG and ARB. On the other hand, the land usability of dung is animal sources (Rizzo et al., 2013). The discharges in wastewater treatment plant are received from different origins. The WWTPs are the hotspot for the resistant genes, and the existence of these ARGs is linked with clinical pathogens (Devarajan et al., 2015; Rizzo et al., 2013). Research has shown that aquatic systems are the ideal sites for developing and disseminating ARGs as the antimicrobial compounds keep polluting those areas. These antimicrobial compounds come from anthropogenic activities. The genetic evolution for antibiotic resistance takes place frequently in four places; human and animal microbiota, hospitals and well-established care facilities, biological residues, and wastewater and environments including soil, surface, and groundwater (Baquero et al., 2008). As urban wastewater doesn't always undergo suitable treatment, thus the trash excreted from the wastewater may impact the receiving environments. The antimicrobial substances can be present there, i.e., in wastewater at a concentration that can be detected effortlessly. Many examples of rivers initially didn't have any antibiotic-resistant genes or antibiotic-resistant bacteria in there. There is a high concentration of those genes

in those rivers in the sediment sample experiments (Pruden et al., 2006). Animal cultivation related field is a vital place for ARG detection as these resistant genes can be found there substantially. Even the pig dung consists of the source of diverse ARGs. Thus it is also an example of a major source of antibiotic-resistant genes. For example, the fields that deal with agriculture, such as feedlots and fishponds, are crucial sources of ARGs in the habitat. The researchers have detected antibiotic resistance in massive amounts in shrimp, salmon fish, catfish, trout, tilapia, and swai. Eleven different countries do use ARGs in these animals. By studying these animals, it has been observed that forty-seven various ARGs are there, and only five of them have been traced so far. Another essential source of ARGs to be noted is constructed wetlands as the microbes keep releasing sediment to water in there. Constructed wetlands affect the pattern variation and the concentration of ARGs based on operational and environmental factors. And these wetlands which affect those factors are more important than that to the polluted source. The constructed wetlands are found in a larger number of domestic sewages. A study was done on where scientists constructed wetlands, and sul(1), sul(3), tet(A), tet€, tet(C) were analyzed. The result showed that the microbes, ARB and antibiotics, metals are there in those wetlands samples. Moreover, another study where six mesocosm scale constructed along with three flow types was made showed the presence of sul(1), sul(2), tet(o), tet(X), erm(B), etc. in there. As mentioned earlier, the hospital and healthcare sectors are the major facilities for the intake of ARGs. These places are key sources for the existence and dissemination of antibiotic-resistant genes (Devarajan et al., 2015). As the human consume antibiotics in a more significant proportion, the presence of ARGs in these hospital and healthcare sectors have been considered to hold tet(M), tet(O), tet(S), tet(Q), etc. antibiotic resistance (Zhang et al., 2009a). Various studies have proved that the high concentration of ARGs in hospital sectors is due to antibiotics like penicillin and aminoglycosides. A research from Oslo city has shown that there was a presence of antibiotic resistances like tetracycline, ciprofloxacin, and fluoroquinolones in every waste sample. The necessary and preliminary steps to disinfect the wastewater releasing from hospitals can reduce the spread of ARGs significantly in the environment. The detection, analysis, and quantification of ARGs were always toward the soil water, surface water, WWTPs, and constructed wetlands. But more concentrations are given to leachates of landfills holding municipal solid wastes because these areas have a high chance to save antibiotic-resistant genes. Moreover, these landfills may also consist of antibiotics, metals, mainly organic pollutants, etc. Such elements in solid waste landfills and landfill leachates can increase the resolution of ARGs in a considerable proportion.

5 Role of Wastewater Treatment Plant in the Dissemination of ARGs

Wastewater treatment plant plays a crucial role or more precisely one of the principal roles in the spread of antibiotic-resistant genes. The urban freshwater, nowadays, is lacking in its proportion. As a result, the total amount of freshwater has become limited (Zhang et al., 2009a). According to UNESCO's report in 2015, people need seventy percent of groundwater and river water for fertilization purposes. Thus, it is proved that clearing the wastewater from all kinds of wastes, including ARGs and filtering up the freshwater, is essential for the human population to exist. As the amount of water existing in nature cannot be altered, reusing the treated wastewater is the only option we have left with. The people need to use it wisely, and consequently, the problem regarding water shortage can be overcome.

On the other hand, the waste and discharges of effluents in the wastewater can be solved by this recycling process. The WWTPs may also be free from eutrophication and algal production. The used urban wastewater contains various organic and inorganic substances apart from ARG and ARBs. Thus, the reuse of the treated wastewater may result in toxicity in the environment and increase ARG and ARBs in the aquatic environment. Due to the shortage of freshwater in irrigation, wastewater is used and supported by governments and official organizations. Another reason for using wastewater in fertilization is increasing poverty in urban areas that lack enough water (Zhang et al., 2009b). A study on the *Acinetobacter* spp. for the contribution of WWTPs in antibiotic resistance development has been studied, and it has been found from water, sewage, soil, and food environments. Raw influent, second effluent, final effluent of WWTPs, upstream, downstream: these five points have contributed to the isolation of this *Acinetobacter* spp (Figs. 2 and 3).

Another study on detecting ARBs in WWTPs has been jotted down here, and the process is polymerase chain reaction or PCR. In this study, one hundred and ninety-two PCR primer pairs with resistance genes have been isolated and analyzed. One hundred forty plasmid-borne ARGs of the WWTPs have been detected (Szczepanowski et al., 2009). The samples were taken from the effluents of wastewater treatment plants and activated sludge as well. And the result of this study has

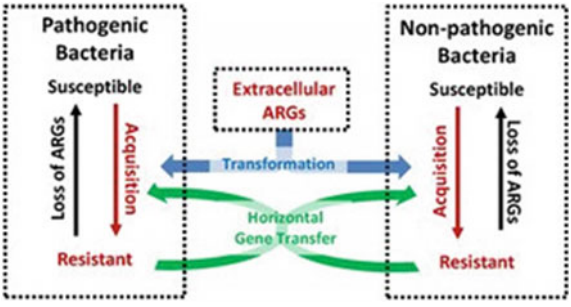

Fig. 2 An overview of how classical quantitative microbial risk assessment framework can be applied to understand the risk from ARGs and ARBs (Hong et al., 2018)

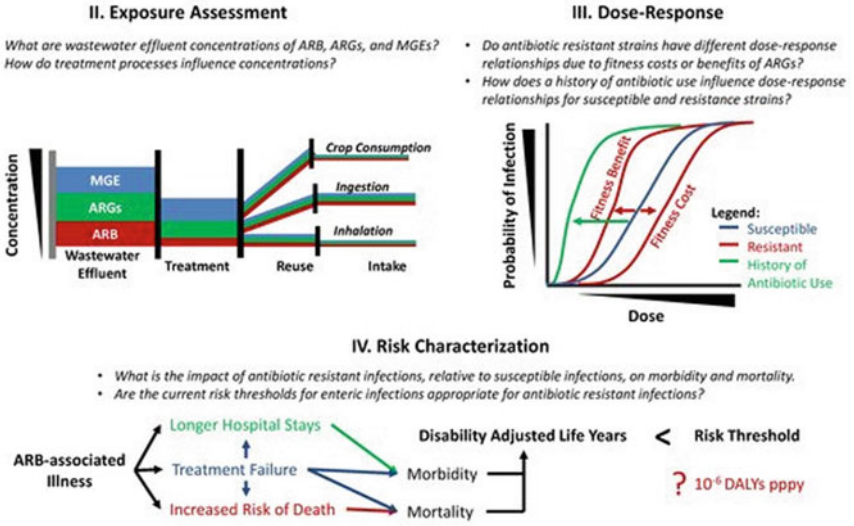

Fig. 3 An overview of how classical quantitative microbial risk assessment framework can be applied to understand the risk from ARGs and ARBs (Continuation) (Hong et al., 2018)

shown plenty of antibiotic-resistant genes in the wastewater. These ARGs can consequently participate in the gene transfer and gene exchange between clinical and bacteria in the wastewater treatment plant. The ultimate product, i.e., the effluent of the WWTP, also has been seen to contain a pool of ARGs that proves that wastewater helps to spread antibiotic resistance genes and antibiotic-resistant bacteria in the environment (Szczepanowski et al., 2009).

Scientists recently discovered that cell-free DNA and cell-associated DNA are critical sources of ARG obtained from wastewater treatment plants. Extracellular DNA can transfer to other cells. On the other hand, the cell-associated DNA is intercellular DNA through which 0.22 μm filters intercept. Sul(2), tet(C), blaPSE-1, erm(B): these four ARGs were taken as cell-free and cell-associated DNA, and they have been quantified by polymerase chain reaction method. The result has shown that the number of antibiotic resistance was more prominent in the wastewater than in ARG fractions. However, after going through sludge settling, membrane filtration, disinfection, etc. processes, the cell-associated ARGs were successfully removed. Although the ARG which is cell-free and was present in wastewater, the number was significantly less. This study has proved that cell-free to cell-associated ARGs ratio has been increased (Zhang et al., 2018). D'Angelo observed the number of antibiotics present in biosolid amendment and sorption and desorption of tetracycline. There were different types of amendments of which the research was done. Some of them are biosolids, poultry manure, wood chip litter, rice hull litter, etc., at various temperatures. The equilibrium constants of sorption and desorption were seen to have twenty times higher than that of other amendments as these contain aluminum and ferric ion in their biosolid materials. The results have shown an increase in the sorption

of tetracycline. The diffusion rates of antibiotics were decreased significantly after treating with amendments. The irrigation of wastewater of the urban areas and its effect on the fungal and microbial world was studied. Both of them were in a larger amount of urban sewage. One important thing to notice in the result was that the amount of microbial persuasion of soil inundated by the wastewater was much more in amount than microbial activities in freshwater. Thus, this is proof of the fact that wastewater increased soil fertility. Soil microbial biomass in wastewater may alter the microbiological components. These microbial components are biotic factors of soil.

The research was done to detect ten tetracycline resistance genes in freshwater and activated sludge WWTPs. The result shows various types of tetracycline resistance genes than the natural lake water specimen (Auerbach et al., 2007). It was also revealed that in WWTPs tetQ and tetG were reduced, but the amount of ARGs was not decreased even after UV disinfection (Auerbach et al., 2007).

6 Different Strategies for Treating the ARGs by WWTPs

The effluents releasing from WWTPs are known as one of the principal sources of pollution to water resources. About four million people of America have fallen ill after using those water resources that they considered safe. WWTPs, as mentioned earlier, have been considered a hotspot for ARG, ARB, and metals (Ben et al., 2017). There has been an uncountable amount of ARGs in different fields of the environment like in landfills, solid leachates, dairy farm soil, surface water, etc. To combat these ARGs, we need suitable treatment strategies that can destroy the ARG and harm the animals and human beings (McKinney & Pruden, 2012).

6.1 Anaerobic and Aerobic Treatment Reactors

The aerobic and anaerobic treatment reactors are helpful process to remove ARGs from water. These methods are generally used to treat the chemical oxygen demand or COD, but these are recognized as one of the best methods to remove ARGs and ARBs (Christgen et al., 2015). Aerobic and anaerobic processes are harmless and eco-friendly. It uses the air or oxygen and microorganism to transform the contaminants into harmless carbon dioxide, water, and other biomass. On the other side, the anaerobic treatment processes are used to convert organic pollutants to methane, biomass, and carbon dioxide, but in the absence of air, microorganisms (Grady et al., 2011). A study for observing variation among different ARGs like tet(G), tet(W), sul(1), etc., was done. And the processes used in the WWTPs were aerobic, anaerobic, anoxic, and MBR process. The result concluded that under anaerobic and anoxic treatment, the ARGs were removed considerably from the aerobic process. It is likely due to

because the microorganisms under anaerobic condition cannot exhibit bioactivity (Du et al., 2015). The MBR discharge process also helped in the removal of ARGs.

The anaerobic–aerobic sequence bioreactor or AAS is a low energy strategy to treat the ARGs. Anaerobic treatment is done first to remove the carbon, and then the aerobic treatment process is done. AAS has removed almost eighty-five percent of ARGs from wastewater, whereas its individual treatment has resulted in only sixty-two percent of ARGs. Another study concluded that removal efficiency of sulfamethazine, sulfamethoxazole, trimethoprim, and lincomycin were 13.1%, 51.9%, 69%, and −11% (Behera et al., 2011). Thus, the removal efficiency of antibiotics was less than that of pharmaceutical compounds. The negative removal efficiency of lincomycin is due to its high load (Behera et al., 2011). Thus, in a gist, it can be said that the combination of anaerobic and aerobic treatment is enough to combat the ARGs and ARBs in wastewater. But aerobic reactors alone are not that sufficient to remove ARGs. Membrane-based technology (MBR) combined with aerobic reactors effectively results in ARG removal from wastewater.

6.2 Constructed Wetlands

Constructed wetlands consist of various types of microbes and microbial community that propagates in there. In constructed wetlands, different physical and chemical reactions take place. It is a kind of portable semi-aquatic ecosystem. Constructed wetlands, as it is understood from its name, are human-made. It is another practical approach to remove ARGs (Fang et al., 2017). It is also cost-effective, has fetching municipal, industrial, and agricultural WWT techniques, and is simple. There are many processes by which nutrients, antibiotics, and other pollutants, including ARGs can be removed efficiently. Some of the functions are—flow configuration, biodegradation, substrate absorption, plant uptake, etc. These all processes play a vital role in the effective removal of pollutants from wastewater. A study by Fang et al. (2017) showed that almost 77% and sixty percent removal of ARGs happened in summer and winter (Fang et al., 2017). Total ARGs used were fourteen. This result also firmly pointed out that mobile genetic elements or MGEs have a lasting effect on disseminating ARGs in these wetlands (Fang et al., 2017). By observing various studies, it has been seen that constructed wetlands can remove about seventy-five percent to ninety-eight percent of antibiotics and eighty-four percent of ARGs. Also plants, subsurface flow constructed wetlands also have an impact on the removal of pollutants (Chen et al., 2016).

6.3 Disinfection

Disinfecting water and wastewater is another effective process for killing a considerable amount of pathogenic organisms. These pathogenic organisms can cause bacterial and parasitic diseases. Disinfection consists of chlorination, ozone, ultraviolet radiation, etc. Among these, chlorination is the most effective disinfection process for removing ARGs and ARBs from WWTPs as WWTPs are a hotspot for antibiotic resistance. Also, in WWTPs there is a presence of many heterotrophic bacteria that exhibit resistance to the antibiotic (Pang et al., 2016). Effective chlorination on various antibiotics, e.g., cephalexin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, and sulfadiazine was analyzed. Also, its effect on inactivation on ARG and ARBs has been monitored. Moreover, ozone, ultraviolet radiation, and Fenton reagent have been applied to the disinfection process. Yusan et al. (2015) showed that the chlorination process had removed 60% of nine different ARGs as per the real-time PCR examination (Yuan et al., 2015). The ARGs used in that study were ere(A), ere(B), erm(A), erm(B), tet(A), tet(B), etc. Similar research was carried out to look into the inactivaon of sul(1), tet(G), intl(1) by chlorination, UV radiation, and Ozonation. The samples were taken from municipal effluents of the WWTPs. The result showed that chlorination was the most fruitful method among all the three processes, which was succeeded in removing ARGs from WWTPs (Zhang et al., 2015).

6.4 Nanomaterial

Antimicrobial nanotechnology has stood out to be one of the effective ways to remove antibiotic-resistant genes and ARBs. Different combinations of nanomaterial can treat pollutants of wastewater treatment plants. There are two effective nanomechanisms by which ARGs are removed or can be removed from WWTPs. First one is the entrance of nanomaterials inside antibiotic-resistant bacteria and cleans out all the toxic ion existing inside ARBs. Another mechanism is the synergistic effect, resulting from the combination of nanomaterials and antibiotics, combating the antibiotic-resistant genes individually (Aruguete et al., 2013). Research carried out by Aruguete et al. (2013) showed antibiotic-resistant pathogens by nanomaterial to analyze the potential of these nanomaterials to restrict the multiplication of the ARGs (Aruguete et al., 2013). Liposomes, dendrimers, some antibiotics existing inside polymers nanomaterials, antibiotics inside inorganic nanoparticles, etc. were used to combine the nanoparticles and the antibiotics. This study proved that the nanomaterials or nanoparticles are eligible to combat nanomaterial resistant organisms and can resist the ARGs from propagating. In this experiment, the polyvinyl pyrrolidone was used to remove the nanomaterial resistant genes or organisms. The nanomaterials were also successful in removing *Pseudomonas aeruginosa* bacteria which are harmful to WWTPs.

Another vital characteristic of nanomaterial is that it can combat multiple drug resistance due to its antimicrobial activity (Yu et al., 2017). Their activity mainly depends on two factors—nanoparticle's physicochemical characteristics and types of target bacteria. Although this is a very convenient way of treating ARGs, scientists prefer to perform experiments under the nanomaterials accessible to them (Hajipour et al., 2012). Nanoparticles are not an excellent example for the use in full-scale production in combination with physicochemical properties. Nowadays, the adverse effect of nanomaterial is coming to the surface. It has shown toxicity toward fauna, flora, and humans as well. Several infections like tissue ulceration, cell capability reduction, and infections caused by silver nanoparticles have become a matter of concern.

Hence, it can be stated that though it has some significant characteristics which helps to remove the ARGs and ARBs from WWTPs, but the adverse effect of it cannot be unseen either. Thus more information about how nanomaterials can achieve the goal without harming the society should be a matter to deal with. Consequently, it can be an effective process for developing resistance against microbial resistance.

6.5 Biochar

By pyrolyzing carbon-rich biomass, biochar can be obtained. Biochar has porous and a large surface area in it. It can absorb the contaminants into its pour, thus reducing the amount of pollutants of WWTPs. According to the size of the pores in the surface area of biochars, it can be classified into micropores, mesopores, and macropores. Sorption is the main mechanism of biochar. Based on the type and size of contaminants, the pores are micro, meso, or macropores biochar treats in WWTPs. Another mechanism by which biochar acts is electric repulsion (Zheng et al., 2013). Various studies have been done on biochar to analyze its effect on ARGs in WWTPs. And it has been seen that there has happened significant changes in microbial communities. If the phylogenetic composition of bacteria is changed, it can affect the composition of ARGs. Thus, biochar can also be categorized under a practical strategy for treating ARGs and ARBs existing in soil and WWTPs.

7 Human Health Risk Due to Antibiotic-Resistant Genes

When enters into the human body, the water of WWTP, the antibiotic residues, or the antibiotic-resistant genes interconnect with the humans microbiome. The human microbiome contains various microorganisms that reside on or inside the human body (National Academies of Sciences, Engineering, and Medicine, 2018). If antibiotic residues are taken daily inside the human body, then the human gastrointestinal tract is surrounded by approximately thousands of bacterial species. Also, more than seven thousand various strains colonize in the gastrointestinal tract. Among

these bacterial species, ninety-five percent of bacteria are harmless to health. But the remaining five percent consists of harmful and pathogenic bacteria (National Academies of Sciences, Engineering, and Medicine, 2018). A balance between the human body and bacteria is continuously residing in the ecosystem. Some of the micro-ecological bacteria are dominant and some are minor. The predominant taxa are the Bacteroidetes, and Firmicutes and the minor bacteria are *Actinobacteria, Proteobacteria, and Verrucomicrobia.* Apart from these, many bacteria phyla have been not isolated and analyzed yet. The composition of intestinal microbiom directly affects the subjection of the antibiotics. This is because the microbiome community has a broad-spectrum influence instead of one target bacteria at a time (Blaser, 2016; Shah Maulin, 2020, 2021a, 2021b). By antibiotic treatment, these intestinal microbiota's organization can be altered. The increase in Firmicutes and reduction in Bacteroidetes can help to change this composition (Pérez-Cobas et al., 2013). But this therapy leads to the generation of antibiotic-resistant bacteria or ARG, which can survive inside the human body for much time. If the balance in the intestinal bacteria changes, then the growth of harmful and bacteria occurs, leading to the generation of various diseases like Pseudomembranous colitis, colorectal cancer, etc. Also, the intestinal tract does not act accordingly, which is called intestinal disorders. If somehow, antibiotic resistance develops inside intestinal bacteria, it propagates rapidly inside the intestine and gives birth to super-bugs. This super-bug and the multiplication of intestinal bacteria can cause death as it cannot be treated. A hypothesis explains that if human beings undergo antibiotic treatment, the harmful taxa that are low in amount can be removed. But the antibiotic-resistant genes would be generated and propagate inside the human intestine. The malfunction of taxa can damage the security of body and result in anxiety disorder, immunologic development, and bone growth (Cho et al., 2012). The staple food contains a considerable amount of antibiotic residues. The antibiotic residues, hence the ARBs generating inside human beings, are cumulative and thus transferred to the next generation. Although the relationship between antibiotic residues and human microbiome is critical to understanding and needs further research, it is very well understood that ARG and ARB have a substantial impact on human health. The ARG and ARBs also affect the gastrointestinal microbiota balance. The veterinary antibiotics, ARGs and *Acinetobacter* do not impact human health as per various reports. It is still to be found that if WWTPs and groundwater have such significant impact on human health or not. Antibiotic-resistant genes and antibiotic-resistant bacteria may result in pathogenicity, various diseases, disease transmission, extended morbidity and mortality (Shah Maulin, 2021b). More risk assessment methods addressing the interaction between ARG and other ecological stressors are needed.

8 Future Recommendation

The ARGs and ARBs have not been tackled yet effectively. Various things are unknown to us, and more research is needed to explore the world of antibiotic resistance. Until then, the elimination of wastewater treatment plants ultimately is not required. Various treatment processes like size fixation in nanofiltration, coagulation, flocculation, sedimentation, etc., have underlined that there are many deficiencies in the wastewater treatment plant methods; positive outcomes have also been spotlighted. AOP or advanced oxidation process has been regarded as a critical treatment process to combat ARG and ARBs (Zhang et al., 2016). In the pre and post-treatment treatment process of it is undoubtedly necessary to be clean and careful. Because any malfunction could result in toxicity of AOP. However, if treated carefully, the ARGs can be removed in the pre-treatment process, leading to the destruction of the cell's ability to regenerate ARGs.

On the other hand, the post-treatment method should be stored for the future to manage the escaped ARGs and ARBs very well. Moreover, the carbon residue association with repair and regeneration of cell ought to be explored further. The primary motive behind this is to assure the cell regeneration process is caused by a deficiency in mineralization. It is seen that hydrogen peroxide, even at low concentration, can hinder cell reactivation and has a tendency to improve the advanced oxidation process. As oxidation of antibiotic resistance by specific reactive oxygen species is not enough, scientists have suggested hydrogen peroxide to be applied to ARB and ARGs. Photoreactors are also recommended to improve the in-situ generation of H_2O_2. It could be a lifeline for reducing ARGs. Future research can also focus on composite photocatalyst as it might be an example for combating ARGs. It is said to have improved surface area, compactness, accessible, and available charge carriers. Thus, a combination of pre, post treatment, and composite photocatalyst can be an important alternative to access AOP to reduce ARG effectively.

References

Allen, H. K., Moe, L. A., Rodbumrer, J., Gaarder, A., & Handelsman, J. (2009). Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. *The ISME Journal, 3*(2), 243–251.

Aminov, R. I., & Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiology Letters, 271*(2), 147–161.

Aruguete, D. M., Kim, B., Hochella, M. F., Ma, Y., Cheng, Y., Hoegh, A., Liu, J., & Pruden, A. (2013). Antimicrobial nanotechnology: Its potential for the effective management of microbial drug resistance and implications for research needs in microbial nanotoxicology. *Environmental Science: Processes & Impacts, 15*(1), 93–102.

Auerbach, E. A., Seyfried, E. E., & McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research, 41*(5), 1143–1151.

Baquero, F., Martínez, J. L., & Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology, 19*(3), 260–265.

Behera, S. K., Kim, H. W., Oh, J. E., & Park, H. S. (2011). Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Science of the Total Environment, 409*(20), 4351–4360.

Ben, W., Wang, J., Cao, R., Yang, M., Zhang, Y., & Qiang, Z. (2017). Distribution of antibiotic resistance in the effluents of ten municipal wastewater treatment plants in China and the effect of treatment processes. *Chemosphere, 172*, 392–398.

Bengtsson-Palme, J., Kristiansson, E., & Larsson, D. J. (2018). Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews*, *42*(1), p.fux053.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M. N., & Kreuzinger, N. (2015). Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology, 13*(5), 310–317.

Blaser, M. J. (2016). Antibiotic use and its consequences for the normal microbiome. *Science, 352*(6285), 544–545.

Bush, K. (1998). Metallo-β-lactamases: a class apart. *Clinical infectious diseases*, *27*(Supplement_1), S48–S53.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., Maxwell, S., & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality, 38*(3), 1086–1108.

Chen, J., Wei, X. D., Liu, Y. S., Ying, G. G., Liu, S. S., He, L. Y., Su, H. C., Hu, L. X., Chen, F. R., & Yang, Y. Q. (2016). Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: Optimization of wetland substrates and hydraulic loading. *Science of the Total Environment, 565*, 240–248.

Cho, I., Yamanishi, S., Cox, L., Methé, B. A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., & Li, H. (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature, 488*(7413), 621–626.

Christgen, B., Yang, Y., Ahammad, S. Z., Li, B., Rodriquez, D. C., Zhang, T., & Graham, D. W. (2015). Metagenomics shows that low-energy anaerobic−aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environmental Science & Technology, 49*(4), 2577–2584.

D'Costa, V. M., Griffiths, E., & Wright, G. D. (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Current Opinion in Microbiology, 10*(5), 481–489.

D'Costa, V. M., McGrann, K. M., Hughes, D. W., & Wright, G. D. (2006). Sampling the antibiotic resistome. *Science, 311*(5759), 374–377.

Devarajan, N., Laffite, A., Graham, N. D., Meijer, M., Prabakar, K., Mubedi, J. I., Elongo, V., Mpiana, P. T., Ibelings, B. W., Wildi, W., & Poté, J. (2015). Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in Central Europe. *Environmental Science & Technology, 49*(11), 6528–6537.

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., & Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A 2 O-MBR system. *Environmental Science and Pollution Research, 22*(5), 3715–3726.

Dumas, J. L., Van Delden, C., Perron, K., & Köhler, T. (2006). Analysis of antibiotic resistance gene expression in Pseudomonas aeruginosa by quantitative real-time-PCR. *FEMS Microbiology Letters, 254*(2), 217–225.

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., Liao, W., & Liang, X. (2017). Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. *Chemosphere, 173*, 99–106.

Fevre, C., Jbel, M., Passet, V., Weill, F. X., Grimont, P. A., & Brisse, S. (2005). Six groups of the OXY β-lactamase evolved over millions of years in Klebsiellaoxytoca. *Antimicrobial Agents and Chemotherapy, 49*(8), 3453–3462.

Göbel, A., McArdell, C. S., Joss, A., Siegrist, H., & Giger, W. (2007). Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Science of the Total Environment, 372*(2–3), 361–371.

Grady, C. L., Jr., Daigger, G. T., Love, N. G., & Filipe, C. D. (2011). *Biological wastewater treatment*. CRC Press.

Hajipour, M. J., Fromm, K. M., Ashkarran, A. A., de Aberasturi, D. J., de Larramendi, I. R., Rojo, T., Serpooshan, V., Parak, W. J., & Mahmoudi, M. (2012). Antibacterial properties of nanoparticles. *Trends in Biotechnology, 30*(10), 499–511.

Hall, B. G., & Barlow, M. (2004). Evolution of the serine β-lactamases: Past, present and future. *Drug Resistance Updates, 7*(2), 111–123.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., & Schwartz, T. (2017). Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology, 8*, 1282.

Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet, 387*(10014), 176–187.

Hong, P. Y., Julian, T. R., Pype, M. L., Jiang, S. C., Nelson, K. L., Graham, D., Pruden, A., & Manaia, C. M. (2018). Reusing treated wastewater: Consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes. *Water, 10*(3), 244.

Lima-Bittencourt, C. I., Cursino, L., Gonçalves-Dornelas, H., Pontes, D. S., Nardi, R. M. D., Callisto, M., Chartone-Souza, E., & Nascimento, A. M. A. (2007). Multiple antimicrobial resistance in Enterobacteriaceae isolates from pristine freshwater. *Genetics and Molecular Research, 6*(3), 510–521.

Maciá, M. D., Blanquer, D., Togores, B., Sauleda, J., Pérez, J. L., & Oliver, A. (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in Pseudomonas aeruginosa strains causing chronic lung infections. *Antimicrobial Agents and Chemotherapy, 49*(8), 3382–3386.

McKinney, C. W., & Pruden, A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environmental Science & Technology, 46*(24), 13393–13400.

National Academies of Sciences, Engineering, and Medicine. (2018). *Environmental chemicals, the human microbiome, and health risk: a research strategy*. National Academies Press.

Pang, Y., Huang, J., Xi, J., Hu, H., & Zhu, Y. (2016). Effect of ultraviolet irradiation and chlorination on ampicillin-resistant Escherichia coli and its ampicillin resistance gene. *Frontiers of Environmental Science & Engineering, 10*(3), 522–530.

Pérez-Cobas, A. E., Gosalbes, M. J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K., Otto, W., Rojo, D., Bargiela, R., von Bergen, M., & Neulinger, S. C. (2013). Gut microbiota disturbance during antibiotic therapy: A multi-omic approach. *Gut, 62*(11), 1591–1601.

Pruden, A., Pei, R., Storteboom, H., & Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environmental Science & Technology, 40*(23), 7445–7450.

Quach-Cu, J., Herrera-Lynch, B., Marciniak, C., Adams, S., Simmerman, A., & Reinke, R. A. (2018). The effect of primary, secondary, and tertiary wastewater treatment processes on antibiotic resistance gene (ARG) concentrations in solid and dissolved wastewater fractions. *Water, 10*(1), 37.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., & Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment, 447*, 345–360.

Shah, M. P. (2020). *Microbial Bioremediation & Biodegradation.* Springer.

Shah Maulin, P. (2021a). *Removal of refractory pollutants from wastewater treatment plants*. CRC Press

Shah Maulin, P. (2021b). *Removal of emerging contaminants through microbial processes*, Springer.

Song, J. S., Jeon, J. H., Lee, J. H., Jeong, S. H., Jeong, B. C., Kim, S. J., Lee, J. H., & Lee, S. H. (2005). Molecular characterization of TEM-type $\beta $-lactamases identified in cold-seep sediments of Edison seamount (south of Lihir Island, Papua New Guinea). *Journal of Microbiology, 43*(2), 172–178.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Guetzkow, T., Eichler, W., Pühler, A., & Schlueter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology, 155*(7), 2306–2319.

Then, R. L., & Angehrn, P. E. T. E. R. (1982). Trapping of nonhydrolyzablecephalosporins by cephalosporinases in Enterobacter cloacae and Pseudomonas aeruginosa as a possible resistance mechanism. *Antimicrobial Agents and Chemotherapy, 21*(5), 711–717.

Wang, J., Chu, L., Wojnárovits, L., & Takács, E. (2020). Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview. *Science of The Total Environment*, 140997.

Yu, W., Zhan, S., Shen, Z., Zhou, Q., & Yang, D. (2017). Efficient removal mechanism for antibiotic resistance genes from aquatic environments by graphene oxide nanosheet. *Chemical Engineering Journal, 313*, 836–846.

Yuan, Q. B., Guo, M. T., & Yang, J. (2015). Fate of antibiotic resistant bacteria and genes during wastewater chlorination: Implication for antibiotic resistance control. *PLoS ONE, 10*(3), e0119403.

Zapun, A., Contreras-Martel, C., & Vernet, T. (2008). Penicillin-binding proteins and β-lactam resistance. *FEMS Microbiology Reviews, 32*(2), 361–385.

Zhang, X. X., Zhang, T., & Fang, H. H. (2009a). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology, 82*(3), 397–414.

Zhang, Y., Marrs, C. F., Simon, C., & Xi, C. (2009b). Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. *Science of the Total Environment, 407*(12), 3702–3706.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., & Xu, K. (2015). Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Science of the Total Environment, 512*, 125–132.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Xu, K., & Ding, L. (2016). Reduction of antibiotic resistance genes in municipal wastewater effluent by advanced oxidation processes. *Science of the Total Environment, 550*, 184–191.

Zhang, Y., Li, A., Dai, T., Li, F., Xie, H., Chen, L., & Wen, D. (2018). Cell-free DNA: A neglected source for antibiotic resistance genes spreading from WWTPs. *Environmental Science & Technology, 52*(1), 248–257.

Zheng, H., Wang, Z., Zhao, J., Herbert, S., & Xing, B. (2013). Sorption of antibiotic sulfamethoxazole varies with biochars produced at different temperatures. *Environmental Pollution, 181*, 60–67.

Increasing Prevalence of Antibiotic-Resistant Genes in Wastewater: Impact on Public Health

Navneet Kour, Jigyasa Singh, and Harvinder Kour Khera

Abstract As emerging contaminants, both antibiotic-resistant bacteria and antibiotic resistance genes have captured global attention, imposing adverse effects on the surrounding environment. Effluents released into the surface water are the primary sources of emerging contaminants that contribute to soil and groundwater pollution. Wastewater treatment plants are the hotspots for the spread of antibiotic resistance through horizontal gene transfer. Given the prevalence of genetic elements and resistance genes detected in waste water samples, a concerning scenario emerges, exacerbating the challenges associated with antibiotic treatment in the healthcare system. In this chapter, we focus on the methods employed for the detection of antibiotic resistance genes in wastewater and the latest research in this area. By delving into public health implications, we analyze the potential routes of exposure to antibiotic genes, the increasing threat of antibiotic resistance in healthcare settings, and the broader environmental and societal consequences.

Keywords Antibiotic-resistant genes · Industrial wastewater · Public health · Gene transfer · Environmental risk

N. Kour
Sher-E-Kashmir University of Agricultural Sciences, Jammu, Jammu and Kashmir, India

J. Singh
Banasthali Vidyapith Jaipur, Jaipur, Rajasthan, India

H. K. Khera (✉)
Tata Institute for Genetics and Society, Bangalore Life Science Cluster (BLiSC), inStem Building, NCBS Campus, GKVK Post, Bellary Road, Bengaluru, Karnataka, India
e-mail: harvinder.khera@tigs.res.in

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_5

1 Introduction

Worldwide for decades, antibiotics have been predominantly used for the efficient management of infectious diseases. The spread of antibiotic resistance stands as a paramount global concern, presenting a formidable and complex challenge to public health. Antibiotic-resistant diseases significantly heighten the risk to public health. They are anticipated to become even more prevalent in the future, putting individuals in jeopardy, even during routine infections and minor surgical procedures. Common infections caused by AMR microbes result in high mortality (Laxminarayan et al., 2013). Recently, the Global Antimicrobial Surveillance System (GLASS) of the World Health Organization (WHO) reported that many regions of the world are grappling with increased levels of resistance in numerous serious and common bacterial infections (WHO, 2019). According to reports from the US Center for Disease Control and Prevention (CDC), 35000 deaths occur every year due to antimicrobial resistance (AMR) in the United States (CDC report, 2019). Antibiotic resistant bacteria (ARB) led to approximately 671,689 infections that caused overall 33,000 deaths in European Economic activity countries in 2015 (Cassini et al., 2019). The situation is worsening in developing countries where the demand for antibiotics is driven by the high incidence of infectious diseases (Lirermore, 1995). Despite extensive measures taken by certain nations to curtail antibiotic usage, there have been noticeable increases in antibiotic resistance rates. This underscores the intricate nature of the public and environmental concerns associated with this issue (O'neill, 2014). Antibiotic resistance is developing at a faster rate than the production of new antibiotics suggesting a challenging situation arising day by day for identifying new antibiotics (Ling et al., 2015). As most antibiotics are not completely metabolized within humans and animals, about 30–90% of these drugs are expelled via urine and feces into water bodies and soil. Industrial and municipal wastewater entering wastewater treatment plants (WWTPs), along with sewage sludge, can contain a diverse range of contaminants, including heavy metals, antibiotics, various chemicals, organic matter, nutrients, bacteria, viruses, and more. These contaminants, originating from domestic, industrial, and commercial sources, contribute to the potential reservoir of antibiotic resistance genes (ARGs), which can further exacerbate the issue of antibiotic resistance. The specific composition and prevalence of ARGs in wastewater is influenced by local factors, such as industrial practices, healthcare protocols, and regional regulations. Effective monitoring and management of ARGs in wastewater are crucial components of addressing the broader challenge of antibiotic resistance in the environment and its implications for public health. The main drivers of antibiotic-resistant bacteria (ARB) and multidrug-resistant (MDR) bacteria are the improper use and dispersal of antibiotics. These bacteria carry an evolved set of antibiotic-resistant genes (ARGs). Both ARGs and ARB are present in air (aerosols) and water wastewater treatment plants (WWTPs) and may pose a

risk of dispersion to nearby workers. Moreover, these WWTPs are not designed to eliminate ARB or ARGs, but contain antibiotics that contribute to the development of antibiotic resistance. The contribution of residual antibiotics toward the development of antibiotic-resistant bacteria and their impact on the ecological system are the two serious concerns. WWTPs are considered as the hotspot for ARGs and play a major role in horizontal gene transfer further leading to the dispersal of ARGs in the broader sense. In WWTPs, the evolution of resistance and dissemination of ARGs is not evidenced clearly. In sludge of various WWTPs, many of the antibiotics like sulfamethoxazole, norfloxacin, tetracycline, ofloxacin, and ciprofloxacin are present in higher amounts. Antibiotics from the sewage of households and hospitals create selective pressure on both ARB and ARGs preceding their dispersal into the surroundings (Martínez, 2009).

The effluents (antibiotics, ARB, ARGs) present in WWTPs get released into surface water then enter the environment. Antibiotic resistance is developed by a different mechanisms in bacteria that makes antibiotic ineffective. The genes responsible are situated either on a bacterial chromosome or extrachromosomal region and they can be transmitted via horizontal or vertical gene transfer. Horizontal gene transfer through conjugation is more common in high-density bacteria which may also form biofilms.The spread and proliferation of ARGs are complex and can vary widely in different contexts and environments. Efforts to mitigate the spread of ARGs are a crucial part of combating antibiotic resistance and preserving the effectiveness of antibiotics, as, ARB remian the prime concern in circulating antibiotic resistance in the microbial system (Larson, 2007). Due to the improper use of antibiotics, excretion via humans and animals, the compounds, their metabolites, and transformed products get deposited in hospital and industrial wastewater. The observed concentration of tetracycline has been as high as 1300 ng/L and 1420 ng/L in WWTPs and effluents respectively (Minh et al., 2009). One of the sulfonamides i.e., sulfamethoxazole was identified with a concentration of 5597 ng/L (Peng et al., 2008) in effluent and 6000 ng/L (Batt et al., 2006) in a wastewater plant. Both the resistant genes of tetracycline (tet A, B, C, G, L, M) and sulfonamide (sull) have been investigated in WWTPs (Szczepanowski et al., 2009). The overall findings confirmed the effluents responsible for anthropogenic sources in an environment that further outspread the resistance to non-resistant bacteria (LaPara et al., 2011; Pruden et al., 2012).

A study reported the detection of NSAID (Non-steroidal anti-inflammatory drugs) in municipal WWTPs with the concentration of 0.42–367 mg/kg whereas in livestock WWTPs antibiotics have been shown to be present in the range of 43.6–142 mg/kg (Ekpeghere et al., 2017). Dispersal of ARGs in the environment is highlighted as a global concern as the adulterated water is supplied for various agricultural practices.

Sewage received by WWTPs, thus, contains residual antibiotics from different sources along with the corresponding ARB, including human pathogens (Borjesson et al., 2009). The designing of WWTPs aimed for the removal of solids, organic matter, and nutrients but not for the removal of antibiotics and ARGs. The presence and persistence of antibiotics and ARGs in WWTPs are posing a serious hazard to the environment and public health (LaPara et al., 2012). The existence of antibiotics even in trace amounts in WWTPs allows frequent horizontal gene transfer (HGT) between different bacterial species, and thus, several pathogens are developing resistance particularly against broad-spectrum antibiotics (Rahal et al., 1997). In developing countries of the world, the situation is even more difficult to control because of a breakdown in health services and sanitation which leads to the rapid dissemination of resistant pathogens (Dodge, 1990).

WWTPs significantly contribute toward the problem of antibiotic resistance due to issues associated with their treatment processes. The activated sludge process is the most used wastewater treatment process in the world intended to reduce and eliminate some antibiotics and other pharmaceutical compounds from wastewater. The biodegradation/sorption process is commonly used for the removal of non-volatile compounds such as pharmaceuticals. The sorption process cannot be used for the removal of all types of antibiotics because of the selective specificity of antibiotics toward biosolids (Chander et al., 2005). Chlorination and ultraviolet (UV) treatment are the other methods used for the removal of pathogens and also contribute to the elimination of some pharmaceuticals in wastewater. However, the findings of several studies suggested the inefficiency of disinfection processes in the removal of antibiotics (Batt et al., 2007; Jones et al., 2005). Volatilization (resulting from aeration) or photodegradation (triggered by sunlight) are recognized as effective removal mechanisms; however, they have limited or no impact on antibiotics due to their negligible or non-existent efficacy in breaking down these compounds. In WWTPs the transformation of antibiotics into compounds with increased hydrophilicity or hydrophobicity, as well as their conversion into carbon dioxide that adheres to the activated sludge, poses significant worldwide challenges. These processes make it more challenging to effectively degrade antibiotics in wastewater (Halling-Sørensen et al., 1998; Kümmerer, 2003). Further, finding the absence of the parent pharmaceutical from the WWTP effluent can mislead treatment strategies, the compound can be transformed and persist in the effluent. Identification of the antibacterial potential of the transformed compounds is another issue that needs consideration and immediate scientific attention. It is not enough to have an idea about the number of antibiotics discharged in the wastewater however, it is also important to identify the nature and quantities of degradation products because the transformation of a biologically active compound does not necessarily equate to detoxification. Owing to the above context it is challenging to identify the realistic load of antibiotics in WWTPs. Overall, the methods for ARB and ARGs removal in wastewater and sludge often fall short of complete elimination due to various factors. These include the resilience of certain ARB and ARG, variations in treatment conditions, potential shielding of contaminants, and the specificity of removal techniques. Biological treatment may not entirely eradicate these elements, and chemical treatments may

have varying effectiveness. Adsorption methods may not be universally suitable, and membrane filtration might not fully remove smaller molecules. UV disinfection may face limitations, while land application of biosolids carries environmental exposure risks. Comprehensive ARB and ARG removal typically necessitates a multifaceted approach, coupled with responsible antibiotic use to manage antibiotic resistance in the environment. In a study, metagenomic sequencing was used to assess microbial community diversity and the fate ARGs, antibiotic biosynthesis genes (ABSGs), and virulence factor genes (VFGs) in sewage samples from four major WWTPs in the United States. The study found that the diversity and composition of these genes were linked to the treatment processes. While overall ARG and VFG abundances decreased by over 20% after treatment, the activated sludge process (ASP) selectively enriched multidrug resistance ARGs and certain VFGs. Sub-groups of ABSGs also saw substantial increases during ASP. These insights shed light on how conventional wastewater treatment processes impact ARGs and VFGs, aiding in understanding their dissemination through sewage and suggesting strategies to mitigate antibiotic resistance spread via sewage discharge (Le et al., 2022).

2 Antibiotic-Resistant Genes

Maximum antibiotics, ARGs and ARB enter the environment via WWTPs into irrigation, rivers, and biosolids. As a result of anthropogenic activities, aquatic ecosystems contaminated by antibiotic compounds are the chief sites for the occurrence of ARGs. These are transferred to other microbial pathogens via horizontal gene transfer, rendering antibiotics ineffective for the treatment later (Zhou et al., 2016). Hospital wastewater has been shown to host a multitude of Antibiotic Resistance Genes (ARGs). Early studies, dating back to the 1970s, indicated a higher prevalence of transferable resistance in coliform bacteria from hospital wastewater compared to municipal wastewater (Grabow and Prozesky, 1973). Recent research in Romania identified ARGs for tetracyclines, aminoglycosides, chloramphenicol, β-lactams, sulphonamides, quaternary ammonium, and macrolide-lincosamide-streptogramin B antibiotics in hospital wastewater (Szekeres et al., 2017). A review of 37 studies found that hospital wastewater consistently carries more ARGs than community wastewater and serves as a significant source of antibiotic resistance in the environment (Hassoun-Kheir et al., 2020). A group of researchers evaluated the ARGs like *tet* (*X, W, G*), *sul-1*, and *intI-1* present in municipal WWTP that is comprised of anaerobic/aerobic/anoxic bioreactors. In anaerobic and anoxic units, a decrease in ARGs succeeding with an increase in ARGs of aerobic units was observed. The overall result demonstrated the effectiveness of anaerobic and anoxic treatments was more superior to aerobic ones (Du et al., 2015). Antimicrobial compounds act by targeting various bacterial cell sites, exhibiting bactericidal or bacteriostatic effects. Bacteria, in response to environmental stress, employ defense mechanisms to counteract these antimicrobials. Common resistance mechanisms involve altering antibiotic target sites, reducing drug affinity, decreasing drug accumulation, employing

inactivating enzymes, and acquiring alternative metabolic pathways. Genes encoding the antibiotics are usually located on mobile genetic elements like plasmids, transposons as well as integrons that take part in the dissemination of ARGs and emergence. More specifically integrons include resistance cassettes that code for different ARGs (aminoglycoside, sulfonamide) and efflux pump (qacEΔ1) resistance genes (Xu et al., 2017). In certain conditions with a shared promoter, multigene cassettes may contain various genes, including antibiotic resistance genes (ARGs). These cassettes can exhibit different ARGs' expression in response to selection pressure from specific antibiotics. For example, in the case of MRSA (methicillin-resistant *Staphylococcus aureus*), a cassette may carry the mecA gene, which imparts resistance to methicillin and related antibiotics (Di Cesare et al., 2016; Sharma et al., 2016).

Wetland sediments are a crucial source of aquatic ARGs because of the regular release of microbe into water. The level of ARGs mainly depends on the environmental factors of the wetland with domestic sewage as the major source of ARGs. A group of researchers demonstrated the presence of ARB, *sul* (1&3), *tet (A, B, C, E),* and *qnr (S)* genes in surface flow wetland (Fang et al., 2017). In wastewater associated with the microbial community, a large number of ARGs has been demonstrated and by the release of wastewater into the aquatic environment these can move along the water cycle. Notably, the prime sites for occurrence and dispersal of ARGS are the aquatic systems that get polluted with antibiotics mainly from various human activities (Rodriguez-Mozaz et al., 2015). Various qnr genes on plasmids encode proteins that reduce the effectiveness of DNA gyrase and topoisomerase IV, resulting in low-level fluoroquinolone resistance. qnrB genes, originating from different Citrobacter species, and qnrA genes from Shewanella algae are prevalent. These genes are often found on class 1 integrons, co-carried with other resistance determinants. qnr genes are frequently detected in aqueous environments, including WWTPs, their effluents, soil irrigated with wastewater, and wetlands along urban coasts, indicating potential environmental dissemination of antibiotic resistance determinants (Mutuku et al., 2022).

3 Methods for Analyzing Antibiotic Resistance Genes

As research on antibiotics is mainly focused on microbes for the past 70 years, wherein the foremost method employed is pure culture isolation. Testing of antibiotic susceptibility is reasonably affordable and provides fundamental data required for the treatment of patients related to resistance. Worldwide, the clinical breakpoints committees such as Clinical and Laboratory Standards Institute (CLSI) and EUCAST, www.eucast.org help in interpreting antibiotic resistance. But these clinical breakpoints are not applicable for wastewater bacteria. In this case, epidemiological cut-off values i.e., ECOFFs have been introduced for determining the wastewater. The term ECOFF is used to define the Minimum Inhibitory Concentration (MIC) threshold at which bacterial isolates exhibit phenotypically detectable

acquired resistance mechanisms (Kahlmeter and Turnidge, 2022). Pure culture isolation is a daunting task and may not work for organisms difficult to culture and therefore, for determining antibiotic-resistant genes, various molecular biology tools have been used like polymerase chain reaction (PCR), quantitative PCR, digital PCR and genome sequencing (Martínez et al., 2014).

3.1 PCR and its Variants

PCR is a basic genetic method and powerful detection tool with significant diagnostic values employed in numerous scientific areas. This method is used in the identification of resistance, virulence factors, and other properties of microorganisms. Based on amplification of specific target genes or application of probes, detection of antibiotic-resistant genes along with their expression was observed. Real-time PCR as compared to conventional PCR requires less duration of time and therefore yields products in a very short period (March Rosselló & Bratos Pérez, 2016). Various real-time PCR is known that can detect the pathogens and the genes responsible for antibiotic resistance of different specimens. Such kind of PCR is automated and can amplify the target within few minutes. Verigenesystem developed by nanosphere is used for identification of antimicrobial resistance marker. It has detected 9 species 3 genera of bacteria and 2 resistance genes (*mecA* & *vanA/B*) with high specificity (100%) and sensitivity in gram-positive bacteria. In the case of gram-negative bacteria, 5 species and 4 genera of bacteria and resistance genes (*CTX-M*, *ESBLs*, *KPC*) were detected within 3 h of time exhibiting 93% specificity and sensitivity (Ledeboer et al., 2015).

The Xpert Carba-Rcatridge from GeneXpert can detect genes for *KPC*, *NDM*, *VIM*, *IMP*, and *OXA-48* within an hour using PCR amplification. Eazyplex system carries out DNA amplification via LAMP (loop-mediated isothermal amplification). Amplification in this technique is done through chain displacement without a change in temperature and the amplicon formed is detected in real-time. Numerous such kits in different formats and different amplifuication methods that can determine the genes responsible for antibiotic resistance are in market (Hinic et al., 2015; Bloemberg et al., 2014).

Analysis of antibiotics resistant genes from environmental DNA can be achieved by PCR and quantitative PCR techniques. The kits available for clinical samples can be repurposed to detect the ARB or ARGs in WWTPs. The initial processing of the samples and nucleic acid isolation needs optimization. To achieve optimization, spiking and marker use are considered. For instance, for SARS-CoV2 process optimization, Phi6 bacteriophage was employed as a surrogate for enveloped viruses. In the analysis of Antibiotic Resistance (AR), sample processing is essential. This involves actions like concentrating Antibiotic Resistance Genes (ARGs), eliminating impurities, and ensuring sample uniformity. Common processing methods involve filtration and/or centrifugation. Filtration is typically employed for liquid samples, while centrifugation is preferred for samples with a significant solid content, such as

activated sludge. Hundreds of ARGs as well as other genes can be detected simultaneously in one run by qPCR array (Zhu et al., 2013). Using qPCR, a comparative analysis of 10 genes related to tetracycline resistance and three sulfonamide resistance genes was conducted in two Polish WWTPs. The study revealed an augmentation of selected ARGs following wastewater treatment processes highlighting the pivotal role of WWTPs in amplifying the distribution of antibiotic resistance determinants in the environment (Pazda et al., 2020). Droplet Digital PCR (ddPCR™) has been used to analyze Antibiotic Resistance Genes (ARGs). ddPCR offers precise quantification by counting nucleic acid molecules enclosed in defined water-in-oil droplets. The study assessed one mobile element integrase gene (intI1) and seven ARGs, including four tet genes (tetA, tetC, tetQ, tetW), one macrolide resistance gene (ermB), and one sulphonamide resistance gene (sul2). The impact of ultrasonic treatment on these ARGs was examined in different types of sludge from Wastewater Treatment Plants (WWTPs). The results indicate that ultrasonic pre-treatment had limited effects on the absolute concentrations of total ARGs, with some fluctuations observed in specific ARGs and intI1 abundance (Rumky et al. 2022). Thus, PCR based techniques provide a better opportunity for the detection of ARGs, sequences of mobile genetic elements, and target genes of bacterial species present in WWTPs or in the environement.

3.2 Microarrays

This method is known for the detection of target molecules hybridized to a specific probe on a solid base using image analysis. It can analyze vast number of resistance genes in a single assay. Many of the microarrays like Check MDR (*CT102*, *CT103*, *CT103XL*) can detect several genes that code for different types of β-lactamases (*ESBLs*, *AmpCs*, *carbapenemases*) have been marketed (Bogaerts et al., 2016; Cuzon et al., 2012; Stuart et al., 2012). Pathogen identification and microbial source tracking (MST) enhance water quality evaluation, health risk assessment, and pollution source remediation. A microarray, used with dead-end ultrafiltration and whole-genome amplification, detected various pathogens, viruses, and antibiotic resistance genes in different water types, including sewage-contaminated samples. Sensitivity for sewage-related gene targets was around 51–57%, lower than specificity (79–81%) (Li et al., 2016).

3.3 Metagenomics

With some limitations of qPCR targeted analysis and failure to design primers for new or unknown genes, the most suitable approach is Metagenomics. It is based on the sequencing of the whole metagenome present in the environmental sample. It is becoming a widely used and affordable approach for analyzing the ARB

and ARGs present in the waste water treatment plants (Thirunavukarasou et al., 2022). It is not only restricted to the detection of species already known genes but also has the potential to determine the new ARGs. A shotgun metagenomic approach was used to analyze ARGs and mobile genetic elements (MGEs) in activated sludge samples from two hospital wastewater treatment plants in Daegu, South Korea. Microbial community diversity was assessed through 16S rRNA metagenome sequencing. Cloacibacterium caeni and Lewinella nigricans dominated in domestic sewage wastewater (SWW) effluents, while Bacillus subtilis and Staphylococcus epidermidis were prevalent in hospital wastewater (HWW) effluents. Notably, HWW had higher ARG abundance, including multidrug resistance, macrolide-lincosamide-streptogramin, beta-lactam, bacitracin, and tetracycline, indicating antibiotic use in human medicine. Higher levels of MGEs in HWW raised concerns about horizontal gene transfer (Manoharan et al., 2021).

A group of researchers determined the ARGs present in WWTP of 8 activated sludges collected twice in both seasons. As a result, a wide spectrum of ARGs was detected with approximately 200 subtypes, many of which were reported for the first time. The ARGs identified were mainly aminoglycoside, tetracycline, sulfonamide, and chloramphenicol. It is quite evident from the above study that wastewater influent possessed the highest ARGs subsequently followed by affluent, anaerobic digestion and activated sludge. This approach has also been implicated for the detection of ARGs in different environmental samples (Yang et al., 2014). A study performed by Nesme et al. revealed the different ARGs present in 71 environment specimens. The other study also investigated the occurrence patterns of ARGs from 10 different samples including WWTPs. A total of 260 subtypes of ARGs were determined within a range of 5.4×10^{-6} to 2.2×10^{-1} copy of ARG/16s rRNA. The total ARG in a different environment was the same as the level of anthropogenic impact. It was clear from the results WWTPs contribute to the major hotspot of abundant ARG dissemination (Li et al., 2015). To detect low-abundance ARGs, a high-throughput amplicon sequencing method was developed, targeting 251 ARGs, 8 mobile genetic element genes, and 19 metal resistance genes. The new method outperformed traditional shotgun sequencing, offering significantly improved sensitivity, diversity, and cost-effectiveness. The approach was applied to environmental and clinical samples, demonstrating its efficiency in ARG surveillance and evolution assessment. By enhancing our understanding of resistome dynamics, this method provides valuable insights into the global challenge of antibiotic resistance (Li et al., 2022). Amplicon sequencing panels are available commercially as well such as The AmpliSeq for Illumina Antimicrobial Resistance Research Panel. The panel offers a fast, precise, and economical solution for the detection of 28 distinct antibiotic classes. It consists of two pools comprising 815 amplicons designed to evaluate the presence of 478 antimicrobial resistance (AMR) genes. This collaborative effort involved experts from Lawrence Livermore National Laboratory, University of Chicago, Argonne National Laboratory, Los Alamos National Laboratory, and Naval Research Laboratory.

4 Impact of Antibiotic Resistance on Public Health

The rapid emergence of antibiotic resistance development through wastewater and WWTPs is a serious concern and negatively affects the environment and public health. Heavily populated cities drinking-water aquifers are usually polluted with wastewater effluent (Sedlak et al., 2000). Through such practices, antibiotics and resistant bacteria are introduced into the drinking-water systems and increasing the potential risk of human exposure. There are different possible routes by which antibiotics and ARGs from WWTPs and agricultural runoffs can reach the consumers of treated water. The presence of discharge points of municipal WWTPs upstream of a pumping station, seepage and runoff of lagoon wastewater from animal feeding operations, and farm application of the lagoon sediments are few potentially risky sources that allow the introduction of antibiotics and antibiotic-resistant bacteria in the water supplies. The amount of antibiotics and ARB in the water supplies is highly influenced by the disinfection process employed and its frequency of use. Chlorine is a generally effective disinfection process, a significant increase in ARB was observed in chlorinated swine lagoons. The plausible reason behind that is the presence of chlorine-resistant bacteria may reduce bacterial removal efficiency during chlorination due to the development of resistance against disinfectants (Macauley et al., 2006). WHO has categorized 12 bacterial species and their accompanying AMR profiles that are found highly hazardous to human health (WHO, 2019). Majorly the Gram-negative bacteria are included in the list, the most common etiologic agents and mainly found in hospital- and/or community-acquired infections. Table 1 shows the categorization of these bacterial species and drugs for which they possess resistance.

The presence of even traces of antibiotics in WWTPs and the availability of high bacterial species make the environment highly favorable for the development of resistant strains. The presence of antibiotics works as a selection pressure and this stress induces mutations in bacteria and thus they acquire resistance not only against a single drug but also against multiple antibiotics. Further, these resistance strains rapidly transfer their ARGs to the available high bacterial population, and thus, resistance spread exponentially through HGT methods (Korzeniewska et al., 2013). Waste generated by hospitals is considered a major source of ARB which is further transferred to WWTPs and contaminates surface water/groundwater and agricultural soil. Proper safety measures should be acquired before the discharge of hospital waste and reliable testing of discharge samples must be employed to analyze a load of resistant bacterial strains and the number of antimicrobial residue/agents in the hospital waste to adopt necessary control measures for the same. To prevent the dissemination of ARB, the use of effective disinfection processes with their correct doses is essentially required (Exner et al., 2017). The use of sub-lethal doses can trigger resistance. Identification of effective disinfectants and measurement of their appropriate doses are very crucial and essential steps used to eliminate ARB from waste discharge. The discharge of antibiotics by living beings into the environment through their feces and urine is a serious matter of concern. The humans and animals generated waste contains a mixture of partially metabolized bioactive compounds

Table 1 Categorization of bacterial species according to their impact on human health and their potential to acquire resistance, along with their antimicrobial resistance profile

Category	Name of the bacterial species	Resistance against drug
Critical priority	*Acinetobacter baumannii*	Carbapenem
	Pseudomonas aeruginosa	
	Klebsiella spp.,	Carbapenem and extended-spectrum ß-lactamase
	Escherichia coli,	
	Serratia spp.,	
	Proteus spp	
High priority	*Enterococcus faecium*	Vancomycin
	Staphylococcus aureus	Methicillin, Vancomycin
	Helicobacter pylori	Clarithromycin
	Campylobacter spp.	Fluoroquinolone
	Salmonella spp.	Cephalosporin, Fluoroquinolone-
	Neisseria gonorrhoeae	
Medium priority	*Streptococcus pneumoniae*	Penicillin
	Haemophilus influenzae	Ampicillin
	Shigella spp.	Fluoroquinolone

Source WHO report (2019)

and xenobiotic compounds. The discharged antibiotics by this waste are further incorporated into the municipal sewers, sewage sludge, and the soil by different routes. As municipal wastewater contains high levels of inorganic and organic matter that accelerate the growth of microorganisms present in the waste including commensal, pathogenic, and non-pathogenic bacteria, further emergence of antibiotics in this waste causes the rapid development of resistance through HGT (Silva et al., 2006). Thus, the WWTPs are known as potential hot spots for promoting the spread of antibiotic resistance and overall increase ARB and ARGs in the environment (Moura et al., 2011). Research findings showed the presence of higher percentages of MDR bacteria in the effluent of treated wastewater than in the affluent, confirming the increased impact of remnants of antibiotics in the aquatic environment. Thus, there is an emerging need for the development of effective strategies of antibiotics removal/degradation from WWTPs as the persistence of microbial contaminants is a major threat to public health (Harnisz, 2013). Different factors like the design and operation of WWTPs affect the dynamics of ARB and ARGs in wastewater. Even after treatment of water, complete removal of microbes cannot be ensured, and their potential risks cannot be overlooked. In distribution system pipe proliferation of bacteria in drinking has been observed even after chlorination (Marathe et al., 2017).

Different factors like an increase of antibiotic-resistant bacteria, decrease in the count of antibiotic-sensitive bacteria, type of antibiotic resistance acquired, and dose of disinfectant, all affect the increase in the ARB content in the effluent of WWTPs. Pathogenic bacterial strains which acquire resistance against one or more antibiotics

might be transferred from the environment to humans and pose a significant threat (Blasco et al., 2008). The chances of acquiring resistance by human pathogens are also increasing and affecting their population dynamics. The presence of multi-resistance bacteria and genes in drinking water has been reported. Multi-resistant *Salmonella* is identified in water used for spraying vegetables (Parvathi et al., 2011). The development of multidrug resistance phenotypes imposes further limitations on the available therapeutic options. Thus, the selection of an effective process for wastewater treatment can play an important role in the reduction of ARB and ARG contaminants from the effluent which will further reduce their possible risks to the environment and public health. Different factors can control the choice of wastewater treatment method to be used. Improvement in treatment technologies can reduce the chances of ARB infections.

Transfer of ARGs from non-pathogenic strains to pathogenic strains that may infect humans and cause severe health risks is a serious issue. Pomati et al. (2006) reported inhibition in the proliferation of human embryonic cells by a complex mixture of therapeutic drugs (including four antibiotics) and these pharmaceuticals might potentially affect aquatic life as well. Inhibitory effects of low levels of pharmaceuticals were observed on the catalytic activities of different xenobiotic-metabolizing enzymes present in carp liver (Thibaut et al., 2006; Shah Maulin, 2020, 2021a, 2021b). In a study conducted by Wilson et al. (2003) eco-toxicological effects of ciprofloxacin antibiotic along with triclosan (antiseptic) and tergito NP10 (surfactant) were reported in the aquatic environment. The mixture of pharmaceutical products significantly affected the rate of algal biomass production and algal community structure. The authors used different concentrations of ciprofloxacin antibiotics and studied its impact on the algal community structure in the upstream and downstream samples of a WWTP. Findings suggested that that continuous exposure of ciprofloxacin influenced the algal community structure and later it shifts the food web structure of streams. Halling-Sørensen (2001) evaluated the EC50 value of certain antibiotics to activated sludge bacteria and *Nitrosomonas europaea* and found maximum toxic effects were exerted by chlortetracycline and oxolinic acid. The assessment of human health risks associated with ARB and ARGs in aquatic environments remains a challenge due to the lack of specific data, such as dose-response relationships and exposure assessments. Amarasiri et al. outlines recent studies on human health risk evaluations related to ARB and ARGs, emphasizing the need for additional data to conduct a refined quantitative microbial risk assessment (QMRA) for various scenarios. QMRA, which combines information on occurrence, exposure, and dose-response, has been applied to assess health risks related to pathogens like E. coli, Campylobacter, and Legionella. While QMRA is considered a suitable method for assessing the additional human health risks posed by ARB, more research is needed. Recent studies have evaluated exposure to antibiotic-resistant E. coli through drinking water and irrigation, indicating potential risks. The transfer of ARGs varies based on bacterial counts, and even a low dose of ARB can pose health risks, particularly for individuals with compromised immune systems. However,

limited data on exposure and dose-response for ARB and ARGs hinder the quantitative assessment of these risks, necessitating further research to better understand their implications in various scenarios (Amarasiri et al., 2020).

The antibiotic resistance ultimately decreases societal productivity and increases the number of side effects (WHO report, 2019). The major risk groups who are highly susceptible and can be devastatingly affected by antibiotic resistance are.

- Infants, especially premature babies, due to their less developed and weak immune systems.
- Seniors, who are living in long-term care facilities or seniors' residences for a long period. As they have more chances of infection exposure, they are in close contact with many other people and have weakened immune systems due to prolonged illness or extended use of medicines.
- Homeless people or those who are living in crowded or unhygienic conditions. They have a higher risk of infection exposure.
- People with weak immune systems due to prolonged illness or injury.
- People are associated with healthcare facilities and working in daycare centers or other settings where chances of spreading infections are very high, especially when proper infection prevention and control measures are not followed.

Antibiotic resistance is an emerging threat for society. To avoid the environmental spreading of ARB and ARGs, specific assessment tools and methods are required to identify the antibiotic residue and the antibiotic-resistant determinants present in the wastewater. Intensive and result-oriented research is required for developing effective treatment and disinfection methods for the complete elimination or degradation of ARB in WWTPs as their implications on public health and the environment are challenging and difficult to handle. The WWTPs are best suited for the removal of solids, organic matter, and nutrients, but unfortunately, their design and operation do not support the removal of antibiotics and ARB. However, advanced technologies are being used in WWTPs for water treatment but identification of effective treatment technology and complete removal of ARB and ARG from the effluent of WWTPs is still very challenging. Mathematical modeling appropaches can also help us understand how bacteria and antibiotics interact in water treatment plants. These models have been useful for wastewater treatment but haven't focused much on antibiotic resistance. Some models have explored resistance in specific environments. Baker et al., developed a mathematical model to quantify the spread of antimicrobial resistance in stored agricultural waste (Baker et al., 2016). More data is required to fine-tune such models and include resistance in everyday wastewater treatment plant operations. Combining different modelling approaches, from population-level models to individual-based models, could give a clearer picture of how antibiotic resistance spreads. The main challenge is the lack of data to validate and improve these models. Developing models that consider resistance, bacteria, gene transfer, and antibiotic levels could help us understand and control antibiotic resistance better.

5 Conclusion and Future Perspectives

Different studies suggest the presence of high microbial densities, residual antibiotics, and high microbial, growth rates are fundamental issues of conventional WWTP design that may represent the perfect niche for promoting rapid transfer of ARGs and spread of multi antibiotic resistance among residents bacteria. Wastewater treatment plants are well designed for the treatment of wastewater but to improve their antibiotic removal efficiencies their designs and operation should be optimized. The advanced treatment technologies used in drinking-water production such as activated carbon, ozonation, and membrane technologies can efficiently reduce the concentration of micropollutants, however; these are not affordable for many municipal WWTP facilities. Using the combined physical and biological treatment process and optimizing the operational condition of WWTPs can help in the effective removal of pharmaceuticals. It is difficult to identify the actual load of antibiotics in WWTPs as many of them transformed into another form and reactivate. Furthermore, in the complex environment of WWTPs, the assessment of effective doses of antibiotics which can raise risks of resistance development is also very difficult. For evaluation of the complete risk of pharmaceuticals in the environment, it is important to identify the types, abundance of transformed products, and their biological activities that contribute to adverse ecological and health effects. The advance and sensitive analytical instrumentation can be used for the identification of traces of metabolites present in the WWTPs. Toxicity assessment of metabolites and mixtures of microcontaminants is essentially required for a complete and reliable assessment of their adverse ecological and human health consequences. There is an emerging need to update the current water quality standards to identify the quality of water in terms of the acceptable levels of micropollutants present in it before its discharge into the environment. Critical quality assessment of wastewater, recycled into WWTPs, is important to reduce the significant hazards posed on the environment and public health.

The absence of standardized regulations for monitoring microcontaminants has led to a rise in environmental antibiotic resistance, endangering public health and ecological stability. Wastewater treatment plant effluents, containing ARGs, significantly contribute to the spread of resistance among various bacteria through horizontal gene transfer. This poses a risk to humans and animals, challenging the "One Health" initiative endorsed by the World Health Organization and hindering the achievement of the United Nations Sustainable Development Goals. To mitigate this, advanced technologies are essential for removing antibiotics and ARGs from wastewater. Setting stringent limits on antibiotic release from sources like hospitals and agriculture, while also controlling other drugs and biocides driving resistance, is crucial.

The effluent of WWTPs is used for many purposes without knowing the potential hazards accompanied by it. Critical assessment of actual microbial load, amount of residual antibiotics or their transformed compounds, and their biological activities must be checked before the recyled water is used. Antibiotic resistance and water

sustainability are global grand challenges that need immediate attention and must be addressed. There is a need for continuous investment and innovation in research, technology, and policy development, particularly where the two challenges intersect. Very few epidemiological studies have been conducted to evaluate evidence of recycled water as a source of microbial illness. Next-generation sequencing, metagenomics, and other new molecular epidemiological approaches are providing insights into the relative contributions of various sources to antibiotic-resistant diseases in humans. Assessment of their impact on the environment and public health is required. However, quantification of risks with the effect of other related sources is quite challenging. Identification of low-cost mitigation strategies that work with existing infrastructure or upgrade plans, and can also provide additional benefits of water treatment, nutrient recycling, and watershed protection is the need of the hour. Further research on environmental antibiotic contamination and its link to resistance development is needed to enhance intervention measures. Although the occurrence of antibiotic ARGs in the One-Health cycle may vary, it is believed that some ARGs create connections between different niches and environmental areas. Integrated monitoring programs are necessary to track the spread of ARB and ARGs across the One-Health framework. Challenges include aligning methods and sites, deciding what biological entities to target (bacteria, mobile genetic elements, or ARGs), and ensuring method sensitivity and accuracy. The ultimate goal is to provide data for authorities and policymakers to develop guidelines for preventing resistance spread, but translating scientific data into practical information remains a significant challenge.

The above-mentioned areas need susbtancial scientific inputs to address the issue of the rapid emergence of antibiotic resistance and its potential hazards to the environment and public health. To conclude, addressing the issue of ARGs and ARBs and their potential impact on public health, several strategies should be considered. These strategies involve implementing more robust surveillance and monitoring systems to track the trends and presence of ARGs and ARBs in WWTPs, researching and developing advanced wastewater treatment technologies that specifically target and reduce these contaminants, and establishing regulatory measures and guidelines to control their release into the environment. Promoting public awareness, fostering collaboration among research institutions, wastewater authorities, and healthcare organizations, and adopting a holistic “One Health” approach that recognizes the interconnectedness of human, animal, and environmental health are all pivotal. Furthermore, encouraging antibiotic stewardship within healthcare settings, promoting international cooperation, and continuing to research innovative technologies for ARG and ARB removal are essential components. Long-term health impact studies are also necessary to gain insights into the consequences of the increased presence of ARGs and ARBs in the environment. These comprehensive strategies are designed to mitigate the risks associated with ARGs and ARBs in wastewater and protect public health from the growing menace of antibiotic resistance.

References

Amarasiri, M., Sano, D., & Suzuki, S. (2020). Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. *Critical Reviews in Environmental Science and Technology, 50*(19), 2016–2059.

Baker, M., Hobman, J. L., Dodd, C. E., Ramsden, S. J., & Stekel, D.J. (2016). Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. *FEMS Microbiology Ecology*, *92*(4), fiw040.

Batt, A. L., Bruce, I. B., & Aga, D. S. (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environmental Pollution, 142*, 295–302.

Batt, A., Kim, S., & Aga, D. (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere, 68*, 428–435.

Blasco, M. D., Esteve, C., & Alcaide, E. (2008). Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. *Journal of Applied Microbiology, 105*, 469–475.

Bloemberg, G. V., Polsfuss, S., Meyer, V., Bottger, E. C., & Hombach, M. (2014). Evaluation ofthe AID ESBL line probe assay for rapid detection of extended-spectrumbeta-lactamase (ESBL) and KPC carbapenemase genes in Enterobacteriaceae. *Jantimicrob Chemotherapy, 69*, 85–90.

Bogaerts, P., Cuzon, G., Evrard, S., Hoebeke, M., Naas, T., & Glupczynski, Y. (2016). Evaluation of a DNA microarray for rapid detection of the most prevalent extended-spectrumbeta-lactamases, plasmid-mediated cephalosporinases and carbapenemases inEnterobacteriaceae, Pseudomonas and Acinetobacter. *International Journal of Antimicrobial Agents*, *48*, 189–193.

Borjesson, S., Melin, S., Matussek, A., & Lindgren, P. E. (2009). A seasonal study of the mecA gene and Staphylococcus aureus including methicillinresistant S. aureus in a municipal wastewater treatment plant. *Water Research*, *43*, 925−932.

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M.E ., Devleesschauwer, B., & Cecchini, M. et al. (2015). Faculty opinions recommendation of attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: A population-level modeling analysis. *Fac Opin Post-Publ Peer Rev Biomed Lit*, 2019(19), 56–66.

CDC report. (2019). The biggest antibiotic-resistant threats in the U.S. Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/biggest-threats.html.

Chander, Y., Kumar, K., Goyal, S. M., & Gupta, S. C. (2005). Antibacterial activity of soil-bound antibiotics. *Journal of Environmental Quality, 34*, 1952–1957.

Cuzon, G., Naas, T., Bogaerts, P., Glupczynski, Y., & Nordmann, P. (2012). Evaluation of a DNAmicroarray for the rapid detection of extended-spectrum beta-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). *Journal of Antimicrobial Chemotherapy, 67*, 1865–1869.

Di Cesare, A., Eckert, E. M., D'Urso, S., Bertoni, R., Gillan, D. C., Wattiez, R., et al. (2016). Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Research, 94*, 208–214. https://doi.org/10.1016/j.watres.2016.02.049.

Dodge, C. P. (1990). Health implications of war in Uganda and Sudan. *Social Science & Medicine, 3*(1), 691–698.

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., & Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A2O-MBR system. *Environmental Science and Pollution Research., 22*(5), 3715–3726.

Ekpeghere, K. I., Lee, J. W., Kim, H. Y., Shin, S. K., & Oh, J. E. (2017). Determination and characterization of pharmaceuticals in sludge from municipal and livestock wastewater treatment plants. *Chemosphere, 168*, 1211–1221.

Exner, M. et al. (2017). Antibiotic resistance: What is so special about multidrug- resistant gram-negative bacteria? *GMS Hygiene and Infection Control*, *12,* Doc05. *PMC*. Web. October 8, 2017.

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., et al. (2017). Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. *Chemosphere, 173*(99–10), 6. https://doi.org/10.1016/j.chemosphere.2017.01.027.

Grabow, W. O., & Prozesky, O. W. (1973). Drug resistance of coliform bacteria in hospital and city sewage. *Antimicrobial Agents Chemotherapy*, *3*, 175–180. https://doi.org/10.1128/AAC.3.2.175.

Halling-Sørensen, B. (2001). Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. *Archives of Environmental Contamination and Toxicology, 40*, 451–460.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhøft, H. C., & Jorgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment—A review. *Chemosphere, 36*, 357–393.

Harnisz, M. (2013). Total resistance of native bacteria as an indicator of changes in the water environment. *Environmental Pollution, 174*, 85–92.

Hassoun-Kheir, N., Stabholtz, Y., Kreft, J.-U., de la Cruz, R., Romalde, J. L., Nesme, J., et al. (2020). Comparison of antibiotic-resistant bacteria and antibiotic resistance genes abundance in hospital and community wastewater: a systematic review. *Science of the Total Environment*, *5*, 140804. https://doi.org/10.1016/j.scitotenv.2020.140804.

Hinic, V., Ziegler, J., Straub, C., Goldenberger, D., & Frei, R. (2015). Extended-spectrumbeta-lactamase (ESBL) detection directly from urine samples with the rapidisothermal amplification-based eazyplex(R) SuperBug CRE assay: proof of concept. *Journal of Microbiology Methods*, *119,* 203–205.

Jones, O. A., Lester, J. N., & Voulvoulis, N. (2005). Pharmaceuticals: A threat to drinking water? *Trends in Biotechnology, 23*, 163–167.

Kahlmeter, G., & Turnidge, J. (2022). How to: eCOFFs—the why, the how, and the don'ts of EUCAST epidemiological cutoff values. *Clinical Microbiology and Infection*, *28*(7), 952–954.

Korzeniewska, E., Korzeniewska, A., & Harnisz, M. (2013). Antibiotic resistant Escherichia coli in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and environmental Safety, 91*, 96–102.

Kümmerer, K. (2003). Significance of antibiotics in the environment. *Journal of Antimicrobial Chemotherapy, 52*, 5–7.

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M., & Eichmiller, J. J. (2011). Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into duluth-superior harbor. *Environmental Science and Technology, 45*, 9543–9549.

LaPara, T. M., & Burch, T. R. (2012). Municipal wastewater as a reservoir of antibiotic resistance? In P. Keen, & M. Monforts (Eds.), *Antimicrobial Resistance in the Environment*. Wiley, ISBN 978-0-470-90542-5.

Larson, E. (2007). Community factors in the development of antibiotic resistance. *Annual Review of Public Health, 28*(1), 435–447.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., et al. (2013). Antibiotic resistance-the need for global solutions. *The Lancet Infectious Diseases, 13*, 1057–1098.

Le, L. T., Huang, Z., Whiteson, K., & Jiang, S. (2022) The occurrence and diversity of antibiotic resistance and virulence factor genes in wastewater from four North American treatment plants. *Environmental Science: Water Research & Technology*, *8*(8), 1650–1664.

Ledeboer, N. A., Lopansri, B. K., Dhiman, N., Cavagnolo, R., Carroll, K. C., Granato, P., et al. (2015). Identification of gram-negative bacteria and genetic resistance determi-nants from positive blood culture broths by use of the Verigene Gram-NegativeBlood culture multiplex microarray-based molecular assay. *Journal of Clinical Microbiology, 53*, 2460–2472.

Li, X., Harwood, V. J., Nayak, B., & Weidhaas, J. L. (2016). Ultrafiltration and microarray for detection of microbial source tracking marker and pathogen genes in riverine and marine systems. *Applied and Environmental Microbiology*, *82*(5), 1625–1635.

Li, Y., Shi, X., Zuo, Y., Li, T., Liu, L., Shen, Z., Shen, J., Zhang, R., & Wang, S. (2022). Multiplexed target enrichment enables efficient and in-depth analysis of antimicrobial resistome in metagenomes. Microbiology Spectrum, *10*(6), e02297–22.

Li, B., Yang, Y., Ma, L. P., Ju, F., Guo, F., Tiedje, J. M., & Zhang, T. (2015). Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME Journal, 9*(11), 2490–2502.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., … Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature, 517*, 455.

Lirermore, D. M. (1995). _lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*, *8*(5), 57–84.

Macauley, J. J., Qiang, Z., Adams, C., D., Surampalli, R., & Mormile, M. R. (2006). Disinfection of swine wastewater using chlorine, ultraviolet light and ozone. *Water Research*, *40*, 2017–2026.

Manoharan, R. K., Srinivasan, S., Shanmugam, G., & Ahn, Y. H. (2021). Shotgun metagenomic analysis reveals the prevalence of antibiotic resistance genes and mobile genetic elements in full scale hospital wastewater treatment plants. *Journal of Environmental Management*, *296*, 113270.

Marathe, N. P., Pal, C., Gaikwad, S. S., Jonsson, V., Kristiansson, E., & Larsson, D. G. J. (2017). Untreated urban waste contaminates Indian river sediments with resistance genes to last resort antibiotics. *Water Research, 124*, 388–397.

March Rosselló, G. A., & Bratos Pérez, M. A. (2016). Antibiograma rápido en MicrobiologíaClínica. *Enfermedades Infecciosas y Microbiología Clínica*, *34*, 61–68.

Martínez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution, 157*, 2893–2902.

Martínez, J. L., et al. (2014). What is a resistance gene? Ranking risk in resistomes. *Nature Reviews Microbiology, 13*, 116–123.

Minh, T. B., Leung, H. W., Loi, I. H., Chan, W. H., So, M. K., Mao, J., Choi, D., Lam, J. C. W., Zheng, G., Martin, M., et al. (2009). Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour. *Marine Pollution Bulletin, 58*, 1052–1062.

Moura, A., Carolina Pereira, C., Henriques, I., & Correia, A. (2011). Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters. *Research Microbiology*, 163(2011), 92–100.

Mutuku, C., Gazdag, Z., & Melegh, S. (2022). Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology*, *38*(9), 152.

O'neill, J. (2014). *Review on antimicrobial resistance: Tackling a crisis for the health and wealth of nations*. HM Government.

Parvathi, P., Vijayan, J., Murali, G., & Chandran, P. (2011). Comparative virulence genotyping and antimicrobial susceptibility profiling of environmental and clinical Salmonella enterica from Cochin, India. *Current Microbiology, 62*, 21–26.

Pazda, M., Rybicka, M., Stolte, S., Piotr Bielawski, K., Stepnowski, P., Kumirska, J., Wolecki, D., & Mulkiewicz, E. (2020). Identification of selected antibiotic resistance genes in two different wastewater treatment plant systems in Poland: A preliminary study. *Molecules*, *25*(12), 2851.

Peng, X., Tan, J., Tang, C., Yu, Y., & Wang, Z. (2008). Multiresidue determination of fluoroquinolone, sulfonamide, trimethoprim, and chloramphenicol antibiotics in urban waters in China. *Environmental Toxicology and Chemistry, 27*, 73.

Pomati, F., Castiglioni, S., Zuccato, E., Fanelli, R., Vigetti, D., Rossetti, C., & Calamari, D. (2006). Effects of a complex mixture of therapeutic drugs at environmental levels on human embryotic cells. *Environmental Science and Technology, 40*, 2442–2447.

Pruden, A., Arabi, M., & Storteboom, H. N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. *Environmental Science and Technology, 46*, 11541–11549.

Rahal, K., Wang, F., & Schindler, J. et al. (1997). Reports on surveillance of and microbial resistance on individual countries. *Clinical Infectious Diseases*, *24*(Suppl 1), S169-75.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez- Melsió, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Research, 69*, 234–242. https://doi.org/10.1016/j.watres.2014.11.021.

Rumky, J., Kruglova, A., & Repo, E. (2022). Fate of antibiotic resistance genes (ARGs) in wastewater treatment plant: Preliminary study on identification before and after ultrasonication. *Environmental Research*, *215*, 114281.

Sedlak, D. L., Gray, J. L., & Pinkston, K. E. (2000). Understanding microcontaminants in recycled water. *Environmental Science and Technology, 34*, 508A-515A.

Shah, M. P. (2020). *Microbial bioremediation & biodegradation.* Springer.

Shah, M. P. (2021a). *Removal of refractory pollutants from wastewater treatment plants.* CRC Press.

Shah, M. P. (2021b). *Removal of emerging contaminants through microbial processes.* Springer.

Sharma, V. K., Johnson, N., Cizmas, L., McDonald, T. J., & Kim, H. (2016). A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. *Chemosphere, 150*, 702–714. https://doi.org/10.1016/j.chemosphere.2015.12.084.

Silva, J., Castillo, G., Callejas, L., Lopez, H., & Olmos, J. (2006). Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile, Elect. *Journal of Biotechnology, 9*, 533–540.

Stuart, J. C., Voets, G., Scharringa, J., Fluit, A. C., & Leverstein-van Hall, M. A. (2012). Detec-tion of carbapenemase-producing Enterobacteriaceae with a commercial DNAmicroarray. *Journal of Medical Microbiology, 61*, 809–812.

Szekeres, E., Baricz, A., Chiriac, C. M., Farkas, A., Opris, O., Soran, M. L., et al. (2017). Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals. *Environment and Pollution*, *225*, 304–315. https://doi.org/10.1016/j.envpol.2017.01.054.

Szczepanowski, R., Link e, B., Krahn, I., Gartemann, K.-H., Gützkow, T., Eichler, W., Pühler, A., & Schlüter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, *2009*(155), 2306–2319.

Thibaut, R., Schnell, S., & Porte, C. (2006). The interference of pharmaceuticals with endogenous and xenobiotic metabolizing enzymes in cap liver: An in-vitro study. *Environmental Science & Technology*.

Thirunavukarasou, A., Kaur, S., & Khera, H. K. (2022). Metagenomics for studying microbes in wastewater treatment plants. In *Microbial Community Studies in Industrial Wastewater Treatment*, (pp. 171–183). CRC Press.

WHO. (2017). *Antibacterial agents in clinical development: An analysis of the antibacterial clinical development pipeline, including tuberculosis.* World Health Organization. WHO/EMP/IAU/2017.11.

Wilson, B. A., Smith, V. H., deNoyelles, F., Jr., & Larive, C. K. (2003). Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environmental Science Technology*, *37*, 1713–1719.

World Health Organization. (2019). *Report on global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics.*

Xu, Y. B., Hou, M. Y., Li, Y. F., Huang, L., Ruan, J. J., Zheng, L., et al. (2017). Distribution of tetracycline resistance genes and AmpC b- lactamase genes in representative non-urban sewage plants and correlations with treatment processes and heavy metals. *Chemosphere, 170*, 274–281. https://doi.org/10.1016/j.chemosphere.2016.12.027.

Yang, Y., Li, B., Zou, S. C., Fang, H. H. P., & Zhang, T. (2014). Fate of antibioticresistance genes in sewage treatment plant revealed bymetagenomic approach. *Water Research, 62*, 97–106.
Zhou, B., Wang, C., Zhao, Q., Wang, Y., Huo, M., Wang, J., et al. (2016). Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy farms. *Journal of Hazardous Materials, 320*, 10–17. https://doi.org/10.1016/j.jhazmat.2016.08.007.
Zhu, Y.-G., et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 3435–3440.

Antibiotic Resistance Genes as Emerging Contaminants in Industrial Wastewater Treatment

Gayatri Suresh, Agnieszka Cuprys, and Satinder Kaur Brar

Abstract About 70% of antibiotics used in human and veterinary medicine are excreted unmetabolized and subsequently end up in sewage. The presence of antibiotic residues in the environment has led to the selection of antibiotic resistance genes (ARGs), which have been classified as emerging environmental pollutants. Additionally, ARGs can also be transferred to non-resistant bacteria by horizontal gene transfer (HGT), thus further disseminating resistance. As wastewater treatment plants (WWTPs) are the point of confluence for sewage and bacteria from various sources, they are believed to be "hotspots" for antibiotic resistance and can act as possible reservoirs for the selection of ARGs. Hence, it is essential to identify the ARGs present in WWTPs. Molecular biology tools such as high-throughput sequencing and metagenomics have been utilized to analyze the resistome in the environmental DNA obtained from the WWTPs, to understand the risk of the spread of antimicrobial resistance. This chapter discusses the prevalence, identification and fate of not only ARGs but also mobile genetic elements (MGEs) present in WWTPs that are responsible for the dissemination of antibiotic resistance. Since current WWTPs are not equipped to remove ARGs completely, the effluent from these can subsequently disseminate antibiotic resistance into water or soil resources. Therefore, upcoming techniques for the reduction and/or inactivation of ARGs in WWTPs have also been reviewed in this chapter. A detailed understanding of the dynamics of antibiotic resistance in WWTPs would guide the development of novel technologies to increase

G. Suresh
Ocular Pharmacology and Pharmacy Division, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India
e-mail: Gayatri.Suresh@ete.inrs.ca

A. Cuprys
Norwegian University of Life Sciences, Elizabeth Stephansens Vei 15, 1430 Ås, Norway
e-mail: agnieszka.cuprys@nmbu.no

S. K. Brar (✉)
INRS-ETE, UniversitéduQuébec, 490 Rue de La Couronne, Québec, QC G1K 9A9, Canada
e-mail: Satinder.Brar@ete.inrs.ca

Department of Civil Engineering, Lassonde School of Engineering, Toronto, Canada

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_6

the efficiency of WWTPs, as well as policy implementation for appropriate waste management to control the spread of drug resistance from WWTPs.

Keywords Wastewater treatment plants · Antibiotic resistance · Resistome sequencing · Reduction · Inactivation

1 Introduction

The serendipitous discovery of penicillin by Sir Alexander Fleming in 1928 spearheaded the advent of the "Golden Era" of antibiotics. During this period, several antibiotics were discovered and developed, which have been used for human, agricultural and veterinary purposes. However, the long-term use and abuse of antibiotics in medicine, agriculture and animal husbandry has resulted in the accelerated emergence and dissemination of antimicrobial resistance (AMR), thus severely restricting their potential to treat infections. AMR leads to increased mortality, higher medical costs as well as economic losses and thus poses a serious challenge to public health. It has been estimated that if unchecked, by 2050, AMR would cause 10 million deaths annually, along with a reduction of gross domestic product amounting to 100 trillion USD (O'Neill, 2016). Hence, to counter this global health crisis, it is vital to understand the role of the environment in AMR.

2 AMR and ARGs in Wastewater

Though the primary cause of AMR is the indiscriminate use of antibiotics, in recent times it has been demonstrated that selective pressure due to the presence of environmental reservoirs of antibiotics has a critical role not only in the transmission of resistant bacteria but also the emergence of novel resistant bacterial strains (Bengtsson-Palme et al., 2019; Berendonk et al., 2015; Larsson et al., 2018). Antibiotic residues, along with antibiotic resistance genes (ARGs), have been classified as environmental pollutants, as in addition to modification of the microbial community, these can also affect the overall health of the ecosystem (Keen & Patrick, 2013). Wastewater treatment plants (WWTPs) have been identified as a hotspot for the selection of antibiotic resistance as well as a reservoir of ARGs. About 70–90% of the antibiotics consumed by humans and animals remain unmetabolized and are excreted into the urine or feces. These antibiotics, therefore, find their way into the sewage system and eventually into the WWTPs (Kümmerer, 2009, Limb, 2017). Additionally, the improper disposal of unused antibiotics as well as effluents from hospitals and pharmaceutical industries can also serve as sources of antibiotics in the sewage (Kumar & Pal, 2018). The presence of antibiotics, pharmaceuticals, heavy metals and disinfectants in wastewaters even in low concentrations provides selection pressure for AMR. WWTPs receive influents from various sources and therefore have a high bacterial density, which

Table 1 Wastewaters as a reservoir of ARGs

S. No.	Antibiotic-resistant genes in wastewater	References
1	*vanA, ampC, mecA,*	Volkmann et al. (2004)
2	*bla*OXA, *bla*TEM, *bla*CTX-M, *bla*EBC, *bla*FOX, *bla*CIT	Amador et al. (2015)
3	*tetC, tetE, tetG, tetM, tetO, tetQ, tetT, tetW, sul1, sul2, sul3, oqxB, qnrS, qnrD, ermB, ermC, aph, aadD, aac, acrA, acrB*	Chen et al. (2015a)
4	*tetA, tetB, tetC, tetG, tetL, tetM, tetO, tetQ, tetW, tetX, sul1, sul2*	Li et al. (2015)
5	*bla*KPC, *bla*CTX-M, *bla*SHV, *bla*TEM, *qnrS1, qnrS2*	Ng et al. (2017)
6	*mef, msr, mph erm,*	González-Plaza et al. (2018)
7	*etM, int1, qacEΔ1, blaOXA-58*	Hultman et al. (2018)
8	*nrA*, *tetC*	Huang et al. (2019)
9	*mcr*-3, *mcr*-4, *mcr*-5, *mcr*-7, *mcr*-1	Kneis et al. (2019)
10	*bla*CTX-M, *bla*TEM, *bla*SHV, *mcr-1*	Hassen et al. (2020)
11	*bla*SHV, *bla*CTX, *bla*NDM *sul1*, *sul2, vanA*, *ermB, qnrS, qnrA, tetO, tetW*	Zhang et al. (2020)

provides a conducive environment for HGT of ARGs, thus facilitating the spread of AMR to novel phenotypes (Karkman et al., 2018). Several studies have previously detected the presence of ARGs in wastewaters (Table 1).

3 Dissemination of ARGs in WWTPs

The resistance to antibiotics can be either due to random mutations in the bacterial chromosome (intrinsic) or can be via horizontal gene transfer (HGT) mechanisms (extrinsic), where antibiotic resistance genes (ARGs) and transferred between bacterial species (Suresh et al., 2018). HGT can occur by one of the three mechanisms: (i) transformation, where extracellular DNA is taken up by the bacteria from the environment, (ii) transduction, where genes are transferred between bacteria by bacteriophages and (iii) conjugation, where genes are directly transferred from a donor to a recipient cell (Burmeister, 2015). HGT mechanisms are primarily responsible for the widespread dissemination of ARGs from environmental to pathogenic species mobile genetic elements (MGEs) such as plasmids, transposons and integrons have been extensively studied and linked to the transfer of ARGs between different environmental and clinical reservoirs (Von Wintersdorff et al., 2016).

3.1 Plasmids

Plasmids are extrachromosomal DNA molecules that can replicate autonomously, independent of the host cell. Plasmids have been extensively studied for their role in the dissemination of drug resistance as they can either inherently carry ARGs or acquire ARGs from bacterial chromosomes by recombination. Additionally, multiple ARGs can be clustered on the same plasmid, conferring multidrug resistance in a single "horizontal transfer event" (Barlow, 2009). The dissemination of resistance to multiple antibiotics such as quinolones, tetracyclines, β-lactams, aminoglycosides, sulfonamides, carbapenem and colistin via plasmids has been reported in the literature (Carattoli, 2013; Huddleston, 2014; Liu et al., 2016).

3.2 Transposons

Transposons or transposable elements (TE) are MGEs that can move from one DNA molecule to another or within a DNA molecule. Composite transposons (Tn) flanked by insertion sequences (IS) are the carriers of ARGs. have been implicated in the transfer of ARGs between bacterial cells Tn903, Tn9, Tn10, Tn3 Tn21, Tn1331, Tn1721, Tn4430 and Tn5403 are some of the transposons that have been reported to be major carriers of ARGs against multiple antibiotics (Babakhani & Oloomi, 2018).

3.3 Integrons

Integrons are conserved double-stranded DNA sequences that can capture ARG cassettes by site-specific recombination and thus facilitate the spread of resistance via plasmids or transposons (Akrami et al., 2019). Of the four classes of integrons, it is the class 1 integrons that are associated with ARGs mobilization. Though class 1 integrons may be associated with several ARG cassettes, the most frequently detected ARGs are for streptomycin-spectinomycin and trimethoprim resistance (Sultan et al., 2018).

The presence of ARGs on the above-mentioned MGEs facilitates the easy transfer of AMR between related as well as unrelated bacterial species. Table 2 attempts to summarize the previously published literature on the presence of several MGEs coding for resistance against the major classes of antibiotics in wastewaters (Table 2).

Table 2 Resistance against major classes of antibiotics mediated by MGEs in wastewater

Antibiotic class	ARGs present on plasmids	Transposons	Integrons	References
Macrolides	*ereA*, *ereB*, *ermC*, *mphA*	*ermA*, *ermB*, *ermF*	*ereA2*	Zhang et al. (2009), Pazda et al. (2019)
Quinolones	*qnr*			
Tetracyclines	*tetA*, *tetA*, *tetC*, *tetD*, *tetE*, *tetK*, *tetY*	*tetB*, *tetH*, *tetM*	*tetG*	
Sulfonamides	*sul1*, *sul2*, *sul3,*	*sul1*, *sul2*, *sul3*	*sul1, dhfrA1*	
β-Lactams	*ampR, bla*TEM*, bla*CIT*, bla*FOX*, blaSH* *V bla*CTX-*M, bla*OXA-2			
Aminoglycosides	*aadA2 aadA13 strA*			

4 Enrichment of ARGs in Industrial Wastewater Treatment

WWTPs function as the site of confluence for wastewaters from several sources, consequently, have a diverse bacterial population that may carry ARGs. In low-income countries, influent wastewaters—particularly from hospitals and pharmaceutical industries—typically have high levels of resistant bacteria as well as ARGs (Cahill et al., 2019; Gouliouris et al., 2019; Khan et al., 2019). However, studies have also reported that even after treatment, effluents from hospital and pharmaceutical WWTPs contain a higher abundance of antibiotics, resistant bacteria as well as ARGs (Chagas et al., 2020; Hembach et al., 2017; Seki et al., 2011). Wang et al. reported that in pharmaceutical WWTPs high levels of ARGs associated with clinically relevant antibiotics were found in the effluent (Wang et al., 2015). The presence of high levels of integrons associated with antibiotic resistance in wastewater samples was also reported in several studies (Gaze et al., 2011; Ma et al., 2011; Mokracka et al., 2012; Tchuinte et al., 2016).

While conflicting studies have previously also reported the removal of ARGs in the liquid effluent, it is estimated that up to 90% of ARGs in WWTPs are enriched in the sludge (Fig. 1) (Hayward et al., 2019; Korzeniewska & Harnisz, 2018; Redhead et al., 2020; Yang et al., 2014). Activated sludge treatment is the commonly used technique for biological treatment of industrial wastewaters; however, several factors such as the high microbial density and diversity, sub-inhibitory concentrations of antibiotics in flocs, the extracellular polymeric substance matrix and proper aeration facilitate the enrichment of ARGs in the sludge. The application of sludge as manure in agriculture can thus introduce these ARGs into the soil reservoir. Resistant bacteria from the aeration tank can also be discharged with the effluent, thus leading to contamination of water resources with ARGs (Zhang, 2016). Some studies have even reported the selection of ARGs in the last step of disinfection in WWTPs, while removing residual bacteria (Fiorentino et al., 2019).

Since WWTPs are not equipped for the complete removal of antibiotics, resistant bacteria and ARGs, these can enter the environment through WWTP effluent.

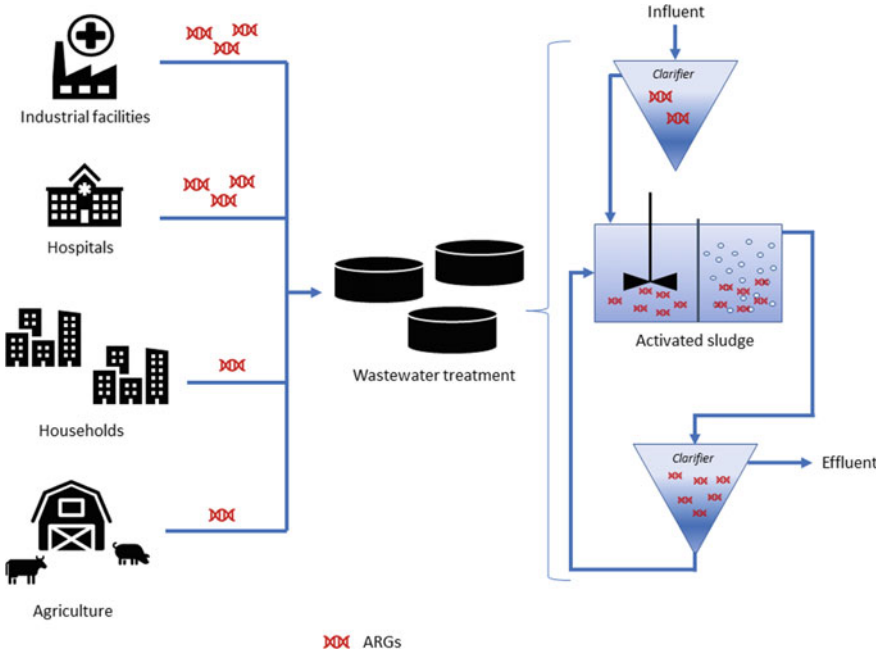

Fig. 1 ARG transfer in wastewater treatment plant

The treated effluent is often used for irrigation for the production of drinking water, which provides even more avenues for ARGs and resistant bacteria to enter the human ecosystem. Previously, several studies have reported a higher abundance of ARGs, such as those coding for resistance against β-lactams (*ermB*), fluoroquinolones (*qnrS*), tetracyclines (*tetW, tetC, tetB, tetM, tetO, tetG, tetA, tetO, tetC, tetX, tetM, tetQ* and *tetW*) and sulfonamides (*sul1* and *sul2*) in rivers that receive WWTP effluents (Sabri et al., 2020a). Therefore, to understand the eventual effect of discharge of treated WWTP effluents into the environment, it is crucial to detect and quantify the ARGs present in WWTP.

5 Identification of ARGs

Several methods are available for the detection of ARGs in wastewater, which can be either culture-dependent or culture-independent.

5.1 Culture-Dependent Method

As the name suggests, this method is based on the isolation of cultivable bacteria from wastewater. Once pure cultures of the bacterial strains are obtained on specific culture media, their resistance to different antibiotics can be measured by standard techniques such as disc diffusion method or broth microdilution method (McLain et al., 2016). Culture-based methods offer the advantages of determination of minimum inhibitory concentration (MIC) of antibiotics, monitoring the resistance in clinically relevant bacterial strains, characterization of phenotypes of resistant bacteria, as well as assessing the bacterial capacity to participate in HGT mechanisms However, as a majority of the microorganisms cannot be cultured, this method does not give the true picture of the prevalence of ARGs in wastewater (Manaia et al., 2018).

5.2 Culture-Independent Methods

These methods are based on the direct analysis of DNA or RNA in the wastewater.

Two main techniques used for this are as follows.

5.2.1 Quantitative Polymerase Chain Reaction (qPCR)

This method is used for the genotypic identification of ARGs in cultivable as well as non-viable microorganisms. It utilizes specifically designed primers for the real-time amplification of the targeted genefragment. The amplicon produced can be quantified by correlation with the emission signal produced by fluorescent-tagged dyes or probes. It is a highly specific targeted analysis method and can also be used to detect a specific gene or gene mutation. Additionally, high-throughput qPCR arrays can quantify hundreds of ARGs as well as MGEs associated with ARGs simultaneously (Volkmann et al., 2004). However, this technique can be used only for the detection of those ARGs for which primer designing is possible, thus restricting the discovery of novel ARGs. Additionally, environmental samples may contain inhibitor molecules that can affect the accuracy of the technique (Karkman et al., 2018; Manaia et al., 2018).

5.2.2 Metagenomic Analysis

Metagenomic analysis refers to the analysis of the collective genomes extracted directly from environmental samples and when combined with high-throughput sequencing technique can be used to not only determine the abundance and diversity of ARGs in wastewater, but also to study the microbiological profile in wastewater samples. In this technique, the complete community DNA is sequenced to capture

the total resistome in the sample, and hence, prior knowledge of ARGs is not needed. Metagenomics has aided the discovery of novel ARGs against aminoglycosides, β-lactams, tetracycline and bleomycin (Schmieder & Edwards, 2012; Manaia et al., 2018).

Metagenomic approaches for sequencing can be divided into two: Functional metagenomics and sequence-based metagenomics. In functional metagenomics, a metagenomic library is created by the expression of cloned environmental DNA fragments in a surrogate host (e.g., *Eschericiha coli*). Following this, the host is tested for susceptibility to different antibiotics, and clones showing phenotypic resistance are further screened for ARGs via mutagenesis, proteomics or *in-silico* analyses. Functional metagenomics has been used to identify previously unknown genes coding for antibiotic inactivation, and efflux pumps (Mullany, 2014). In sequence-based metagenomics, random DNA fragments are sequenced using next generation sequencing (NGS) techniques. These sequences are then mapped to known sequences deposited in ARGs databases such as MG-RAST Sequence Read Archive and CAMERA, to identify ARGs in the wastewater samples (Schmieder & Edwards, 2012).

While both these techniques have definitive advantages over culture-based techniques and qPCR, they are not without limitations. In the case of functional metagenomics, for identification of the gene, it has to be functional outside its natural host. Additionally, novel ARGs that have been detected by functional metagenomics need to be characterized biochemically as well as microbiologically as in the recombinant host, and the genes may exhibit a different phenotype. For sequence-based metagenomics, the biggest challenge remains the scarcity of reference sequences, which could lead to an underrepresentation of ARGs in the environmental sample (Boolchandani et al., 2019).

For metagenomic analyses, it may be difficult to identify those ARGs whose abundance decreases during wastewater treatment or to analyze polymorphic ARG variants of clinical relevance (Manaia et al., 2018). Finally, the insufficiency of standard techniques or universally accepted tools for metagenomic analyses could cause the lack of data reproducibility and comparability (Escobar-Zepeda et al., 2015).

In addition to the above-mentioned techniques, few emerging techniques for analysis of ARGs from environmental samples are also under review. Spencer et al. reported a novel technique for high-throughput screening of genes—emulsion, paired isolation and concatenation PCR (epicPCR), by linking functional genes with phylogenetic markers in single uncultured cells. This technique can be used to determine the host of a particular ARG, which is a limitation with all the culture-independent techniques (Spencer et al., 2016).

6 Removal of ARGs in WWTP

The contribution of WWTP to the selection of AMR and ARGs has been well established. The effluent from WWTPs will subsequently carry these ARGs into aquatic ecosystems, thus causing a large-scale dissemination of AMR into the environment.

Hence, treatment processes in WWTPs must also be removing resistant bacteria as well as ARGs. Conventional WWTPs are not designed for the removal of micropollutants such as ARGs. This could be attributed to several factors such as inefficient adsorption, sieve pore size, the negation of coagulation of ARGs by reconfiguring surface charge, cell reactivation and growth (Anthony et al., 2020). While some studies have previously reported a decrease in the ARG concentration after WWTP treatment, others have reported a significant enrichment of ARGs after conventional secondary wastewater treatment (Sabri et al., 2020b; Pärnänen et al., 2019).

Hence, it has been postulated that additional treatment technologies could be effective in the reduction or removal of ARGs in the WWTP effluent. Broadly, these treatment strategies can be classified into four categories:

6.1 Biological Treatment

While both aerobic and anaerobic strategies have been used for ARG removal, Christgen et al. reported that lower concentrations of ARGs were obtained in the final effluent with anaerobic treatment as compared to aerobic treatment. The same study also reported a combination of the two treatments, i.e., an anaerobic pre-treatment followed by aerobic treatment in anaerobic–aerobic sequence (AAS) bioreactors resulted in an 85% removal of ARGs in the effluent as compared to isolated aerobic and anaerobic treatments (Christgen et al., 2015).

6.2 Capturing ARGs

In this technique, ARGs are physically captured or trapped and removed from the wastewater (Fig. 2). These strategies include:

6.2.1 Coagulation

Coagulation is a commonly used tertiary treatment process in WWTPs for the removal of organic matter and suspended particles. In a recent study, Li et al. demonstrated the removal of tetracycline and sulphonamide-resistant genes as well as the integrase gene using FeCl3 and poly ferric chloride (PFC) as coagulating agents. WWTP effluents generally have a pH value of 7–8, due to which the ARGs gets negatively charged. The iron species in both FeCl3 and PFC are positively charged, and coagulation of ARGs is primarily attributed to charge neutralization (Li et al., 2017).

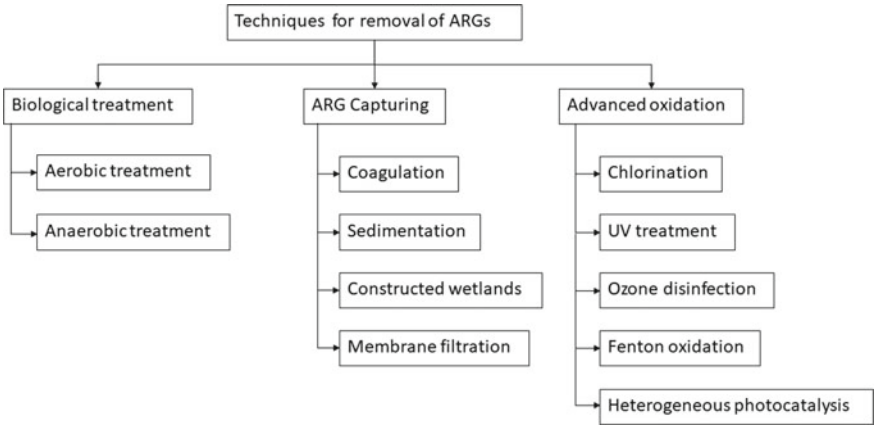

Fig. 2 Techniques for removal of ARGs

6.2.2 Sedimentation

Sludge sedimentation is an effective technology for ARG removal as a majority of the ARGs would be separated with the sludge. Previous studies have reported a decrease in the genes coding for resistance to tetracyclines, sulphonamides, fluoroquinolones and integron 2 (Anthony et al., 2020).

6.2.3 Constructed Wetlands

Constructive wetlands (CW) is an economically and environmentally feasible engineering system that utilizes soils, wetland vegetation and the associated microbial community in a controlled environment for the treatment of wastewater (Vymazal, 2010). It is proposed that physical sorption to organic matter or sediment and biological processes such as ARG uptake by plants via roots, increased ARG biodegradation facilitated by the root microbiome as well as bacterial cell death are the major mechanisms of removal of ARGs in CWs (Chen et al., 2015b; Sabri et al., 2021).

6.2.4 Membrane Filtration

Membrane filtration is based on the physical separation of the pollutant on a molecular sieve. Based on the size of the filter used, membrane filtration can be of the following types: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). It has been postulated that size exclusion by membrane filtration removes the antibiotic-resistant bacteria, thus decreases the load of ARGs in the effluent. The efficiency of the removal of ARGs depends on the pore size as well as the composition of the membrane. A study carried out by Breazeal et al. reported that

an alumina membrane of pore size 1kDa was quite effective for the removal of ARGs (Breazeal et al., 2013). Previous studies have reported a decrease in the abundance of ARGs in WWTP effluent by membrane filtration (Lan et al., 2019). With respect to wastewater treatment, NF is a particularly effective technique for the removal of ARGs due to the pore size of the membrane (100–500 Da). However, after membrane filtration, an additional step is required for the elimination of the resistant bacteria and ARGs that have accumulated on the membrane (Wang & Chen, 2020).

6.3 Advanced Oxidation

6.3.1 Chlorination

Chlorination has been used as a cheap and effective technique for the reduction of ARGs. However, it does not cause any direct damage to the DNA. Chlorination disinfects the resistant bacteria, thus causing a reduction in the proportion of ARGs. However, a major drawback associated with this technique is that during dell lysis,

ARGs can be released into the effluent, where they can be taken up by the environmental bacteria. In addition, chlorination does not affect the structure of plasmids which play a major role in the horizontal transmission of resistance (Hiller et al., 2019; Singh et al., 2019).

6.3.2 UV Disinfection

The disinfection mechanism of UV exposure by the disruption of the nitrogen bases in DNA has been well characterized. While previous studies have reported favorable results for disinfection of ARGs in WWTPs in comparison with biological methods (McKinney & Pruden, 2012; Zheng et al., 2017), Sharma et al. stated that the efficacy of UV treatment could be increased by combining it with other oxidants such chlorine, H2O2, peroxymonosulphate and photocatalysts (Sharma et al., 2019).

6.3.3 Ozone Disinfection

Ozone is a strong oxidizing agent, and it can oxidize either directly or via the production of free radicals. ARGs inactivation by O3 depends on the type of ARG, the bacterial community, genome compositions, O3 dosage as well as the hydraulic retention time in the WWTP (Wang & Chen, 2020). Alexander et al. reported that post O3 treatment, an enrichment of certain ARGs was observed, which could be attributed to a high guanosine content, which made these genes less susceptible to ozonation (Alexander et al., 2016).

6.3.4 Fenton Oxidation

In the Fenton reaction, H2O2 is catalyzed by ferrous ions to produce a series of free radicals, which cause oxidation. Fenton oxidation, in combination with UV, has been evaluated as a means to eliminate ARGs (Chen et al., 2015a), while other studies reported a higher ARG removal efficiency via solar-Fenton oxidation (Anthony et al., 2020).

6.3.5 Heterogeneous Photocatalysis (HPC)

HPC is an advanced oxidation technology that utilizes the capacity of semiconductor oxides to generate reactive oxygen species on interaction with a near-UV, UV or visible light source. These highly ROS (e.g., OH•, $O2^{-}$ and HO2•) can degrade organic pollutants, eventually mineralizing them into water and CO2 (Ahmed & Haider, 2018). Titanium dioxide (TiO2) has a wide degradation potential is cost-effective, chemically stable and non-toxic, and therefore has been widely studied as a photocatalyst in wastewater treatment (Al-Rasheed, 2005). Several studies have reported an increase in the photocatalytic activity of TiO2 by hybridization with other semiconductors, modification of the semiconductor surface via carbon material coating or anion adsorption and the addition of oxidant species such as O3 and H2O2 (Loddo et al., 2018). The inactivation of ARGs by HPC could be either due to the generation of ROS which can oxidize nucleic acids as well as proteins, leading to the deactivation of ARGs and ARBs, or, by physical cell damage caused by modified cell permeability due to adsorption of the photocatalyst by bacterial cells (Wang & Chen, 2020). Ren et al. observed that ARGs on the bacterial genome were more prone to degradation by ROS produced by TiO_2/polyvinylidene fluoride, as compared to those located on plasmids (Ren et al., 2018). The removal of ARGs and ARBs in wastewater was also reported to be enhanced by coupling the photocatalysts with external electron acceptors such as peroxydisulphate, H2O2 and O3 (Anthony et al., 2020). However, there is a lack of data regarding the decrease in the relative abundance of ARGs and ARBs following HPC, and further studies need to be carried out on this aspect (Iervolino et al., 2020).

7 Conclusion

With antibiotic resistance becoming a global threat to the human population, it is essential to have a deeper understanding of all factors that contribute to the emergence and dissemination of resistance in the environment. The microbial load, organic content and sub-inhibitory concentration of antibiotics present in the wastewater make WWTPs “hotspots” for ARG selection and transmission. While several techniques are being used to evaluate the ARGs in WWTPs, it is essential to have a

standard protocol for the quantitative determination of ARGs to compare efficiencies of different WWTPs. Additionally, as conventional WWTP treatment techniques do not eliminate ARGs in the effluent completely, it is also imperative to closely look at the different tertiary treatment techniques in WWTPs for the removal or reduction of ARGs in the effluent, to further the research in this area. This would be a crucial step in the effort against the spread of antibiotic resistance.

References

Ahmed, S. N., & Haider, W. (2018). Heterogeneous photocatalysis and its potential applications in water and wastewater treatment: A review. *Nanotechnology, 29*(34), 342001.

Akrami, F., Rajabnia, M., & Pournajaf, A. (2019). Resistance integrons; A mini review. *Caspian Journal of Internal Medicine, 10*(4), 370.

Al-Rasheed, R. A. (2005). Water treatment by heterogeneous photocatalysis an overview. In *Fourth SWCC acquired experience symposium.*

Alexander, J., Knopp, G., Dötsch, A., Wieland, A., & Schwartz, T. (2016). Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Science of the Total Environment, 559*, 103–112.

Amador, P. P., Fernandes, R. M., Prudêncio, M. C., Barreto, M. P., & Duarte, I. M. (2015). Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of Class A and Class C β-lactamases. *Journal of Environmental Science and Health, Part A, 50*(1), 26–39.

Anthony, E. T., Ojemaye, M. O., Okoh, O. O., & Okoh, A. I. (2020). A critical review on the occurrence of resistomes in the environment and their removal from wastewater using apposite treatment technologies: Limitations, successes and future improvement. *Environmental Pollution, 263*, 113791.

Babakhani, S., & Oloomi, M. (2018). Transposons: The agents of antibiotic resistance in bacteria. *Journal of Basic Microbiology, 58*(11), 905–917.

Barlow, M. (2009). What antimicrobial resistance has taught us about horizontal gene transfer. *Horizontal Gene Transfer*, 397–411.

Bengtsson-Palme, J., Milakovic, M., Švecová, H., Ganjto, M., Jonsson, V., Grabic, R., & Udikovic-Kolic, N. (2019). Industrial wastewater treatment plant enriches antibiotic resistance genes and alters the structure of microbial communities. *Water Research, 162*, 437–445.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., & Martinez, J. L. (2015). Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology, 13*(5), 310–317.

Boolchandani, M., D'Souza, A. W., & Dantas, G. (2019). Sequencing-based methods and resources to study antimicrobial resistance. *Nature Reviews Genetics, 20*(6), 356–370.

Breazeal, M. V. R., Novak, J. T., Vikesland, P. J., & Pruden, A. (2013). Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Research, 47*(1), 130–140.

Burmeister, A. R. (2015). Horizontal gene transfer. *Evolution, Medicine, and Public Health, 2015*(1), 193.

Cahill, N., O'Connor, L., Mahon, B., Varley, Á., McGrath, E., Ryan, P., Cormican, M., Brehony, C., Jolley, K. A., & Maiden, M. C. (2019). Hospital effluent: A reservoir for carbapenemase-producing Enterobacterales? *Science of the Total Environment, 672*, 618–624.

Carattoli, A. (2013). Plasmids and the spread of resistance. *International Journal of Medical Microbiology, 303*(6–7), 298–304.

Chagas, T. P. G., L. Seki, J. Cury, J., Oliveira, A., Dávila, D. S., & Asensi, M. (2011). Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *Journal of Applied Microbiology, 111*(3), 572–581.

Chen, B., Hao, L., Guo, X., Wang, N., & Ye, B. (2015a). Prevalence of antibiotic resistance genes of wastewater and surface water in livestock farms of Jiangsu Province, China. *Environmental Science and Pollution Research, 22*(18), 13950–13959.

Chen, J., Liu, Y.-S., Su, H.-C., Ying, G.-G., Liu, F., Liu, S.-S., He, L.-Y., Chen, Z.-F., Yang, Y.-Q., & Chen, F.-R. (2015b). Removal of antibiotics and antibiotic resistance genes in rural wastewater by an integrated constructed wetland. *Environmental Science and Pollution Research, 22*(3), 1794–1803.

Christgen, B., Yang, Y., Ahammad, S. Z., Li, B., Rodriquez, D. C., Zhang, T., & Graham, D. W. (2015). Metagenomics shows that low-energy anaerobic−aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environmental Science & Technology, 49*(4), 2577–2584.

Escobar-Zepeda, A., Vera-Ponce de León, A., & Sanchez-Flores, A. (2015). The road to metagenomics: From microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics, 6*, 348.

Fiorentino, A., Di Cesare, A., Eckert, E. M., Rizzo, L., Fontaneto, D., Yang, Y., & Corno, G. (2019). Impact of industrial wastewater on the dynamics of antibiotic resistance genes in a full-scale urban wastewater treatment plant. *Science of the Total Environment, 646*, 1204–1210.

Fouz, N., Pangesti, K. N., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Ghany, M. A. E. (2020). The contribution of wastewater to the transmission of antimicrobial resistance in the environment: Implications of mass gathering settings. *Tropical Medicine and Infectious Disease, 5*(1), 33.

Gaze, W. H., L. Zhang, N. A., Abdouslam, P. M., Hawkey, L., Calvo-Bado, J., Royle, H., Brown, S., Davis, P. K., & Boxall, A. B. (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *The ISME Journal, 5*(8), 1253–1261.

González-Plaza, J. J., Šimatović, A., Milaković, M., Bielen, A., Wichmann, F., & Udiković-Kolić, N. (2018). Functional repertoire of antibiotic resistance genes in antibiotic manufacturing effluents and receiving freshwater sediments. *Frontiers in Microbiology, 8*(2675).

Gouliouris, T., Raven, K. E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., Naydenova, P., Horner, C., Brown, N. M., & Corander, J. (2019). Detection of vancomycin-resistant *Enterococcus faecium* hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. *Genome Research, 29*(4), 626–634.

Hassen, B., Abbassi, M. S., Ruiz-Ripa, L., Mama, O. M., Ibrahim, C., Benlabidi, S., Hassen, A., Torres, C., & Hammami, S. (2020). Genetic characterization of extended-spectrum β-lactamase-producing Enterobacteriaceae from a biological industrial wastewater treatment plant in Tunisia with detection of the colistin-resistance mcr-1 gene. *FEMS Microbiology Ecology, 97*(3).

Hayward, J. L., Huang, Y., Yost, C. K., Hansen, L. T., Lake, C., Tong, A., & Jamieson, R. C. (2019). Lateral flow sand filters are effective for removal of antibiotic resistance genes from domestic wastewater. *Water Research, 162*, 482–491.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., & Schwartz, T. (2017). Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Frontiers in Microbiology, 8*, 1282.

Hiller, C., Hübner, U., Fajnorova, S., Schwartz, T., & Drewes, J. (2019). Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. *Science of the Total Environment, 685*, 596–608.

Huang, Y.-H., Liu, Y., Du, P.-P., Zeng, L.-J., Mo, C.-H., Li, Y.-W., Lü, H., & Cai, Q.-Y. (2019). Occurrence and distribution of antibiotics and antibiotic resistant genes in water and sediments of urban rivers with black-odor water in Guangzhou, South China. *Science of the Total Environment, 670*, 170–180.

Huddleston, J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes. *Infection and Drug Resistance, 7*, 167.

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., & Virta, M. (2018). Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS Microbiology Ecology, 94*(4).

Iervolino, G., Zammit, I., Vaiano, V., & Rizzo, L. (2020). Limitations and prospects for wastewater treatment by UV and visible-light-active heterogeneous photocatalysis: A critical review. *Heterogeneous Photocatalysis*, 225–264.

Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. (2018). Antibiotic-resistance genes in waste water. *Trends in Microbiology, 26*(3), 220–228.

Keen, P. L., & Patrick, D. M. (2013). Tracking change: A look at the ecological footprint of antibiotics and antimicrobial resistance. *Antibiotics, 2*(2), 191–205.

Khan, F. A., Söderquist, B., & Jass, J. (2019). Prevalence and diversity of antibiotic resistance genes in Swedish aquatic environments impacted by household and hospital wastewater. *Frontiers in Microbiology, 10*, 688.

Kneis, D., Berendonk, T. U., & Heß, S. (2019). High prevalence of colistin resistance genes in German municipal wastewater. *Science of the Total Environment, 694*, 133454.

Korzeniewska, E., & Harnisz, M. (2018). Relationship between modification of activated sludge wastewater treatment and changes in antibiotic resistance of bacteria. *Science of the Total Environment, 639*, 304–315.

Kumar, A., & Pal, D. (2018). Antibiotic resistance and wastewater: Correlation, impact and critical human health challenges. *Journal of Environmental Chemical Engineering, 6*(1), 52–58.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part I. *Chemosphere, 75*(4), 417–434.

Lan, L., Kong, X., Sun, H., Li, C., & Liu, D. (2019). High removal efficiency of antibiotic resistance genes in swine wastewater via nanofiltration and reverse osmosis processes. *Journal of Environmental Management, 231*, 439–445.

Larsson, D. J., Andremont, A., Bengtsson-Palme, J., Brandt, K. K., de Roda Husman, A. M., Fagerstedt, P., Fick, J., Flach, C.-F., Gaze, W. H., & Kuroda, M. (2018). Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. *Environment International, 117*, 132–138.

Li, J., Cheng, W., Xu, L., Strong, P., & Chen, H. (2015). Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environmental Science and Pollution Research, 22*(6), 4587–4596.

Li, N., Sheng, G.-P., Lu, Y.-Z., Zeng, R. J., & Yu, H.-Q. (2017). Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. *Water Research, 111*, 204–221.

Limb, M. (2017). *Environment's role in global antibiotic resistance 'overlooked' until now*. BMJ Publishing Group British Med Assoc House, Tavistock Square.

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., & Huang, X. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *The Lancet Infectious Diseases, 16*(2), 161–168.

Loddo, V., Bellardita, M., Camera-Roda, G., Parrino, F., & Palmisano, L. (2018). Chapter 1—Heterogeneous photocatalysis: A promising advanced oxidation process. In A. Basile, S. Mozia, & R. Molinari (Eds.), *Current trends and future developments on (bio-) membranes* (1–43). Elsevier.

Ma, L., Zhang, X.-X., Cheng, S., Zhang, Z., Shi, P., Liu, B., Wu, B., & Zhang, Y. (2011). Occurrence, abundance and elimination of class 1 integrons in one municipal sewage treatment plant. *Ecotoxicology, 20*(5), 968–973.

Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I. C., & Zammit, I. (2018). Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environment International, 115*, 312–324.

McKinney, C. W., & Pruden, A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environmental Science & Technology, 46*(24), 13393–13400.

McLain, J. E., Cytryn, E., Durso, L. M., & Young, S. (2016). Culture-based methods for detection of antibiotic resistance in agroecosystems: Advantages, challenges, and gaps in knowledge. *Journal of Environmental Quality, 45*(2), 432–440.

Mokracka, J., Koczura, R., & Kaznowski, A. (2012). Multiresistant Enterobacteriaceae with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water Research, 46*(10), 3353–3363.

Mullany, P. (2014). Functional metagenomics for the investigation of antibiotic resistance. *Virulence, 5*(3), 443–447.

Ng, C., Tay, M., Tan, B., Le, T.-H., Haller, L., Chen, H., Koh, T. H., Barkham, T. M. S., Thompson, J. R., & Gin, K. Y.-H. (2017). Characterization of metagenomes in urban aquatic compartments reveals high prevalence of clinically relevant antibiotic resistance genes in wastewaters. *Frontiers in Microbiology, 8*(2200).

O'Neill, J. (2016). Review on antimicrobial resistance: Tackling drug-resistant infections globally: final report and recommendations.

Pärnänen, K. M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T. U., Cacace, D., Do, T. T., Elpers, C., Fatta-Kassinos, D., Henriques, I., & Jaeger, T. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science Advances, 5*(3), eaau9124.

Pazda, M., Kumirska, J., Stepnowski, P., & Mulkiewicz, E. (2019). Antibiotic resistance genes identified in wastewater treatment plant systems–a review. *Science of the Total Environment, 697*, 134023.

Redhead, S., Nieuwland, J., Esteves, S., Lee, D.-H., Kim, D.-W., Mathias, J., Cha, C.-J., Toleman, M., Dinsdale, R., & Guwy, A. (2020). Fate of antibiotic resistant *E. coli* and antibiotic resistance genes during full scale conventional and advanced anaerobic digestion of sewage sludge. *PLoS ONE, 15*(12), e0237283.

Ren, S., Boo, C., Guo, N., Wang, S., Elimelech, M., & Wang, Y. (2018). Photocatalytic reactive ultrafiltration membrane for removal of antibiotic resistant bacteria and antibiotic resistance genes from wastewater effluent. *Environmental Science & Technology, 52*(15), 8666–8673.

Sabri, N., Schmitt, H., Van der Zaan, B., Gerritsen, H., Zuidema, T., Rijnaarts, H., & Langenhoff, A. (2020a). Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *Journal of Environmental Chemical Engineering, 8*(1), 102245.

Sabri, N. A., van Holst, S., Schmitt, H., van der Zaan, B. M., Gerritsen, H. W., Rijnaarts, H. H. M., & Langenhoff, A. A. M. (2020b). Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. *Science of the Total Environment, 741*, 140199.

Sabri, N. A., Schmitt, H., van der Zaan, B. M., Gerritsen, H. W., Rijnaarts, H. H. M., & Langenhoff, A. A. M. (2021). Performance of full scale constructed wetlands in removing antibiotics and antibiotic resistance genes. *Science of the Total Environment, 786*, 147368.

Schmieder, R., & Edwards, R. (2012). Insights into antibiotic resistance through metagenomic approaches. *Future Microbiology, 7*(1), 73–89.

Sharma, V. K., Yu, X., McDonald, T. J., Jinadatha, C., Dionysiou, D. D., & Feng, M. (2019). Elimination of antibiotic resistance genes and control of horizontal transfer risk by UV-based treatment of drinking water: A mini review. *Frontiers of Environmental Science & Engineering, 13*(3), 1–9.

Singh, R., Singh, A. P., Kumar, S., Giri, B. S., & Kim, K.-H. (2019). Antibiotic resistance in major rivers in the world: A systematic review on occurrence, emergence, and management strategies. *Journal of Cleaner Production, 234*, 1484–1505.

Spencer, S. J., Tamminen, M. V., Preheim, S. P., Guo, M. T., Briggs, A. W., Brito, I. L., Weitz, D. A., Pitkänen, L. K., Vigneault, F., Virta, M. P., & Alm, E. J. (2016). Massively parallel sequencing

of single cells by epicPCR links functional genes with phylogenetic markers. *The ISME Journal, 10*(2), 427–436.

Sultan, I., Rahman, S., Jan, A. T., Siddiqui, M. T., Mondal, A. H., & Haq, Q. M. R. (2018). Antibiotics, resistome and resistance mechanisms: A bacterial perspective. *Frontiers in Microbiology, 9*(2066).

Suresh, G., Das, R. K., Kaur Brar, S., Rouissi, T., Avalos Ramirez, A., Chorfi, Y., & Godbout, S. (2018). Alternatives to antibiotics in poultry feed: Molecular perspectives. *Critical Reviews in Microbiology, 44*(3), 318–335.

Tchuinte, P. L. S., Stalder, T., Venditti, S., Ngandjio, A., Dagot, C., Ploy, M.-C., & Barraud, O. (2016). Characterisation of class 3 integrons with oxacillinase gene cassettes in hospital sewage and sludge samples from France and Luxembourg. *International Journal of Antimicrobial Agents, 48*(4), 431–434.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., & Obst, U. (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *Journal of Microbiological Methods, 56*(2), 277–286.

Von Wintersdorff, C. J., Penders, J., Van Niekerk, J. M., Mills, N. D., Majumder, S., Van Alphen, L. B., Savelkoul, P. H., & Wolffs, P. F. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology, 7*, 173.

Vymazal, J. (2010). Constructed wetlands for wastewater treatment. *Water, 2*(3), 530–549.

Wang, J., & Chen, X. (2020). Removal of antibiotic resistance genes (ARGs) in various wastewater treatment processes: An overview. *Critical Reviews in Environmental Science and Technology*, 1–60.

Wang, J., Mao, D., Mu, Q., & Luo, Y. (2015). Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants. *Science of the Total Environment, 526*, 366–373.

Yang, Y., Li, B., Zou, S., Fang, H. H., & Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Research, 62*, 97–106.

Zhang, S., Huang, J., Zhao, Z., Cao, Y., & Li, B. (2020). Hospital wastewater as a reservoir for antibiotic resistance genes: A Meta-analysis. *Frontiers in Public Health, 8*, 574968–574968.

Zhang, T. (2016). Antibiotics and resistance genes in wastewater treatment plants. *AMR Control, 9*, 11–20.

Zhang, X. X., Zhang, T., & Fang, H. H. (2009). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology, 82*(3), 397–414.

Zheng, J., Su, C., Zhou, J., Xu, L., Qian, Y., & Chen, H. (2017). Effects and mechanisms of ultraviolet, chlorination, and ozone disinfection on antibiotic resistance genes in secondary effluents of municipal wastewater treatment plants. *Chemical Engineering Journal, 317*, 309–316.

Characterization and Dynamic Shift of Microbial Communities in Wastewater Treatment Plant

Agnieszka Cuprys, Joanna Lecka, and Satinder Kaur Brar

Abstract Apart from chemical pollutants and organic matter, wastewater treatment plants release microorganisms into the environment. This microbial community, released into the environment, may pose a biological risk due to present pathogens or multi-resistant bacteria. The latter is especially dangerous; antibiotic-resistant microorganisms may potentially transfer resistant genes to non-resistant microorganisms. Moreover, the various influent composition (e.g. micropollutants, total dissolved solids, chemical oxygen demand, nitrogen and phosphorous level), operating parameters of water treatment (e.g. pH, dissolved oxygen, temperature, hydraulic and sludge retention time) lead to a microbial shift. It may influence the wastewater treatment performance, especially biological treatment. Hence, it is essential to understand the relationship between microbial community and the water treatment process. It would help to recognize the crucial microorganisms to wastewater treatment performance as well as facilitate its optimization. Hence, this chapter aims to characterize the microbial community shift in wastewater treatment plants. The driving factors that affect the shift will also be revised. Moreover, the dynamics of microbial community shift will be discussed.

Keywords Microbial shift · Wastewater treatment · Dynamic shift · Pollution

A. Cuprys
Faculty of Science and Technology, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

J. Lecka
INRS-ETE, 490 Couronne St., Quebec, QC G1K 9A9, Canada

S. K. Brar (✉)
Department of Civil Engineering, Lassonde School of Engineering, York University, 204 Bergeron Centre, Toronto, ON M3J 2S5, Canada
e-mail: Satinder.Brar@lassonde.yorku.ca

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4_7

1 Introduction

Biological treatment requires functional stability of microbial community to properly biotransform pollutants present in wastewater. The microorganisms take part in the removal of organic matter, chemical oxygen demand (COD), suspended solids, nutrients (phosphorous and nitrogen) or odours. Moreover, some of the microbes have the potential to recover the high-value resources from wastewater, such as biogas, bioplastics or fertilizers (Sheik et al., 2014). However, the microbial community performance depends strictly on various aspects, which are summarized in Fig. 1. Each of them may affect the microbial ecosystem leading to the change of microbial consortium. Some of the microorganisms will not be able to survive in new environmental conditions, some will need time for acclimatization, and in the meantime, the new dominant species can emerge, which will result in competition between microbes over the nutrients. As an effect, the microbial shift can hinder or ameliorate the biological treatment performance.

Hence, the relation between functional stability, microbial community shift and the wastewater composition and treatment parameters has been widely investigated in recent years. The understanding of these correlations is essential to guarantee an efficient treatment process and ensure the functionality and stability of water treatment plants. It could be the basis of novel technical advances in wastewater treatment plants. This chapter aims to present the characterization of the microbial community in wastewater treatment plants, as well as to describe the most important aspects that may lead to dynamic microbial shift.

2 Characterization of the Microbial Community in Wastewater Treatment Plants

Woodcock et al. (2006) reported that the size of the microbial community in wastewater treatment plants may include up to 10^{18} individuals (Woodcock et al., 2006). This diverse group includes viruses, bacteria, archaea, fungi, algae or protozoa; hence, the identification of the species present in wastewater is a challenging task. Furthermore, the researchers have been trying to understand the biological treatment process and the interactions between the microbial community and pollutants removal potency.

2.1 Approaches to Characterize the Microbial Community

The extensive studies on microbial diversity and expansions of '-omics' have resulted in the development of molecular biology tools, such as sequencing or polymerase chain reaction, that help to characterize and understand the microbial community in various samples. The first approach is whole-genome sequencing, which allows

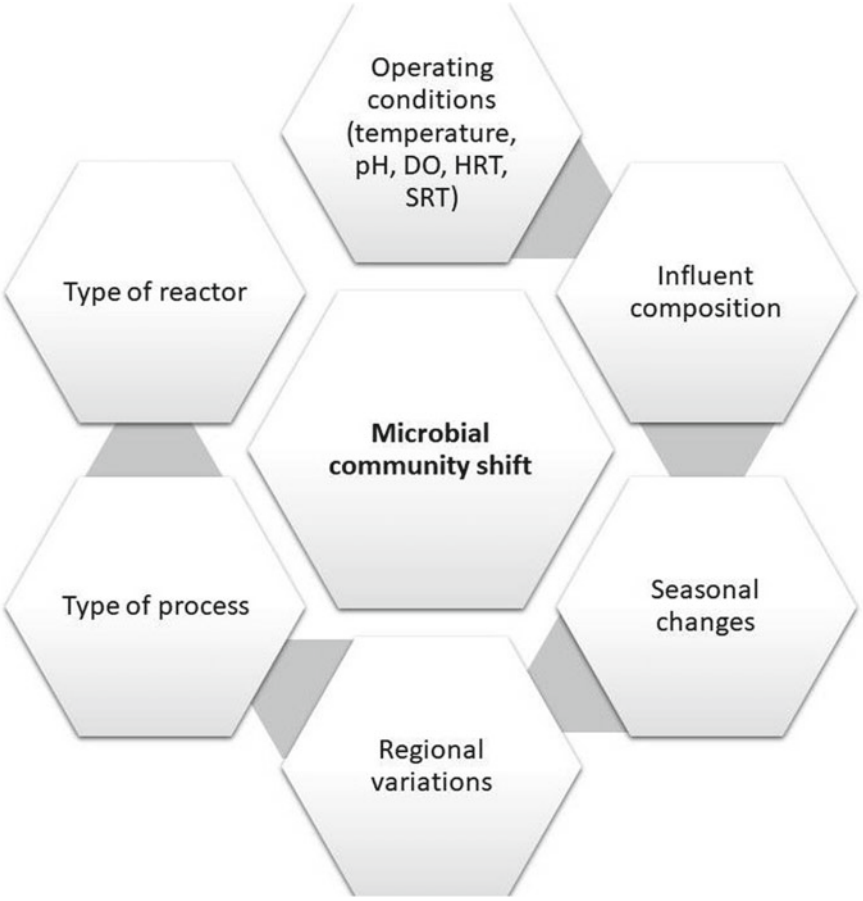

Fig. 1 Factors influencing the microbial community shift

to exposure of the relevant microbes present in wastewater. Further, the microbial community may undergo massive DNA sequencing (metagenomics) or expressed gene sequencing (metatranscriptomics). The metaproteomic studies may help to identify and/or discover the proteins that are involved in the treatment process. A similar function has the analyses of all the metabolites present in wastewater (metabolomics); however, due to the complexity of the matrix, this approach is the least used.

The general steps for microbial community characterization are presented in Fig. 2. Firstly, the culturing of individual species is the basic tool for understanding the physiology and behaviour of microbes. Moreover, it can be the basis for more advanced sequencing studies, for instance as a reference genome (Ferrera & Sánchez, 2016). The preliminary experiments consist of culture-independent techniques, i.e. denaturation gradient gel electrophoresis, terminal restriction fragment polymorphism or fluorescent in situ hybridization (Ferrera & Sánchez, 2016). The

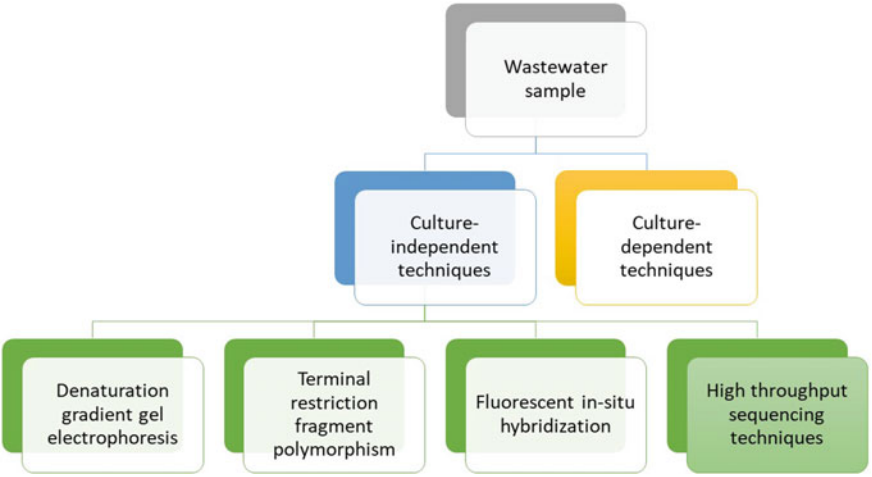

Fig. 2 Approaches of microbial community characterization

results obtained with these methods can be further confirmed with high throughput sequencing techniques (e.g. Illumina, 454-pyrosequencing). They can detect the groups that were not described before (Ferrera & Sánchez, 2016). To receive the complex characterization of the microbial community in wastewater, the combination of the above-described techniques is required.

2.2 Wastewater Microorganisms

The research connected to the investigation of microbial diversity in wastewater samples showed that Proteobacteria are the dominant group amongst detected microorganisms (Ferrera & Sánchez, 2016). The following groups, like Bacteroidetes, Actinobacteria or Firmicutes, as well as their proportion, are influenced by the treatment condition (Fig. 1). The composition of microbial consortia is also dependent on the type of treatment process. The microbial community in the nitrification process (Stadler & Love, 2016) will be distinct from the microorganisms present in anaerobic reactors (Si et al., 2018). The list of potential microorganisms present in different treatment processes is shown in Table 1.

Table 1 Examples of wastewater microorganism present in different processes

Function in the process	Organism	References
Ammonia oxidation bacteria	*Nitrosomonas europaea*	Liu et al. (2018)
Anammox bacteria	*Candidatus* Brocadia, *Candidatus* Jettenia, *Candidatus* Kuenenia, *Candidatus* Anammoxoglobus	Liu et al. (2018), de Almeida Fernandes et al. (2018), Hendrickx et al. (2014)
Toxic compounds removal	*Zoogloea ramigera*	Wang et al. (2008)
Methanogens	*Thermogymnomonas acidicola, Aciduliprofundum boonei, Methanosarcina acetivorans, Methanosarcina mazei*	Li et al. (2014), Zhou et al. (2016)
Denitrifying bacteria	*Acidovorax* sp., *Burkholderia* sp., *and Burkholderiales*	de Almeida Fernandes et al. (2018)
Syntrophic acetate oxidizing bacteria	*Clostridium ultunense*	Zhang et al. (2019a)
Phosphorous accumulating bacteria	*Pseudomonas* spp., *Dechloromonas, Aeromonoas*	Izadi et al. (2021)
Acidogenes	*Burkhoderia* sp., *Pantoea* sp., *Citrobacter* sp., *Pseudomonas* sp., *Alteromonas* sp	Win et al. (2016)
Hydrogen producers	*Clostridium* sp., *Klebsiella* sp.	Sivagurunathan and Lin (2016)

3 Factors Affecting the Microbial Shift

As mentioned in the previous section, the composition of the microbial community depends on the treatment conditions. Moreover, the disturbance during the treatment may result in change and shift of microbial community. The influence of most important factors, such as influent composition and process operating parameters, such as temperature, pH, dissolved oxygen, hydraulic retention time (HRT) or sludge retention time (SRT), are described in this section. Table 2 summarizes the dynamic shift of microbial community in studies that are presented below.

3.1 Influent Composition

Wastewater contains a broad spectrum of organic and inorganic compounds that may influence the microbial community. In general, certain nutrients may affect the microorganisms directly, acting as the limiting substrate that may hinder the microbial activity, or indirectly via affecting and selecting the microorganisms that transform targeted substrate faster or slower.

Table 2 Examples of dynamics of microbial community shift

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
Influent composition: sucrose	Aerobic granular sludge sequencing batch reactor/swine wastewater	Decrease of *Acidovorax* and *Leadbetterella;* increase of *Aeromonas*, *Comamona* and *Chryseobacterium*; the dominant groups were *Flavobactrium*, *Stenotrophomonas* and *Comamonas*	COD removal 96.83%, NH_4^+–N removal 81.14%; total phosphorus removal 97.37%, tetracycline removal 81.40%, oxytetracycline removal 80.69%	Wang et al. (2020)
Influent composition: methanol	Aerobic granular sludge sequencing batch reactor/swine wastewater	Decrease of *Acidovorax Aeromonas*, *Comamona, Chryseobacterium* and *Leadbetterella;* the dominant groups were *Flavobactrium*, unclassified and *Hydrogenophaga*	COD removal > 90%, NH_4^+–N removal 62.67%; total phosphorus removal < 40%, tetracycline removal 74.52%, oxytetracycline removal 66.94%	Wang et al. (2020)
Influent composition: methanol	Lab-scale sequencing batch reactor/domestic wastewater	76% of OTUs closely related to *Betaproteobacteria*, 8% closely related *Hyphomicrobium* spp. of the *Alphaproteobacteria.* The remaining clones affiliated with *Bacteroides*	NH_4^+–N removal > 93%	Ginige et al. (2008)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
Influent composition: starch	Aerobic granular sludge sequencing batch reactor/swine wastewater	Decrease of *Acidovorax, Aeromonas*, *Comamona* and *Chryseobacterium;* dominant groups were *Leadbetterella* and *Methylobacillus*	COD removal > 90%, NH_4^+–N removal < 30%; total phosphorus removal 97.37%, tetracycline removal < 50%, oxytetracycline removal around 50%	Wang et al. (2020)
Influent composition: synthetic/real wastewater	Two-stage partial nitritation-anammox process/change from synthetic to real wastewater	Increase of heterotrophic bacteria; decrease of autotrophic species occurred in the partial nitritation reactor; slight decrease of almost all dominant bacteria in the anammox reactor; dominant species *Nitrosomonas europaea* and one species of *Candidatus Brocadia*	Morphological changes in sludge, but overall no obvious change was noticed in nitritation-anammox process	Liu et al. (2018)
Influent composition: antibiotics	Anoxic/aerobic membrane bioreactor/ wastewater	No impact on aerobic heterotrophic bacteria (40% abundance) and autotrophic nitrifying bacteria (7% abundance); decrease of representatives of denitrifying bacteria by 86% at high antibiotic concentration (2mg/L)	COD and ammonia removal efficiency remained stable; inhibition of denitrification at 2mg/L of antibiotics	Zhu et al. (2017)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
Influent composition: phenol	Anaerobic digestion/mashed biowaste, phenol, biochemical methane potential buffer	Up to 0.5g/L of phenol led to no changes in microbial shift; between 0.5 and 1.0g/L of phenol increase of *Methanoculleus* and *Synergistaceae* abundance; between 1.0 and 2.0g/L of phenol predominance of *Methanoculleus* and *Synergistaceae*, decrease of *Methanosarcina* and *Syntrophomonadaceae*	The inhibition of methagenesis was at 5g/L of phenol	Poirier et al. (2016)
Influent composition: pharmaceutically active compounds	Activated sludge process/municipal wastewater	Microbial shifts in the presence of ketoprofen and naproxen	Inhibition of removal ranging from 34 to 43% under the lowest organic loading for naproxen, ketoprofen and carbamazepine	Wang et al. (2008)
Influent composition: organic inhibitors	Anaerobic digestion/ hydrothermal liquefaction wastewater	Increase of abundance of detoxification bacteria, acetate- oxidizing bacteria in upflow anaerobic sludge bed reactor and acetogens in packed bed reactor and hydrogenotrophic methanogens (Archaea)	Decrease in methane production and COD removal	Si et al. (2018)
Temperature (decrease)	Two-stage partial nitritation-anammox process/change from synthetic to real wastewater	Limited impact on microbial shift	Morphological changes in sludge, but overall, no obvious change was noticed in nitritation-anammox process	Liu et al. (2018)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
Temperature (decrease from 35 to 20 °C)	Anammox reactor/ municipal wastewater	Shift to anammox bacteria (*Ca.* Brocadia and *Ca.* Anammoximicrobium) and denitrifiers (*Burkholderiales*, *Myxococcales*, *Rhodocyclales*, *Xanthomonadales*, and *Pseudomonadales*); decrease of *Anaerolineales* and *Clostridiales*	Decrease of nitrogen removal efficiency from 96 to 90%	de Almeida Fernandes et al. (2018)
Temperature (decrease from 20 to 10°C)	Submerged membrane bioreactor/hospital wastewater	Reduction in relative abundance of nitrifying bacteria (*Nitrosospira*, *Rhodanobacter*, and *Sphingobium*) and ciliate population at lower temperatures; dominance of the fungal group (e.g. Basidiomycota)	Decrease of suspended solid concentration with the temperature decrease (8.5/L at 20º to 5.2g/L at 15º)	Bhagyashree et al. (2021)
Temperature (decrease from to 10°C)	Anammox reactor/ domestic wastewater	Shift from non-purple phototropic bacteria to purple phototropic bacteria (> 50% abundance); the microbial community included *Rhodobacter*, *Rhodopseudomonas*, *Allochromatium* and *Chromatiaceae*	The treatment performance at 10°C and ambient temperature was comparable	Hülsen et al. (2016)
Temperature (decrease from to 10°C)	Anammox reactor/ synthetic wastewater	The dominant species was *Candidatus Brocadia fulgida*	Biomass yield (10°C) was 0.046g VS/g N converted, which is similar higher temperatures	Hendrickx et al. (2014)
Temperature (between 33 and 52°C)	Anaerobic digestion/synthetic PTA wastewater	The dominant species at 37°C was *Methanosaeta*, which was replaced by *Methanobrevibacter* and *Methanofollis* at lower (33°C) and higher temperatures (43–52°C)	The highest COD removal was at 37 °C (91.9%) and the lowest at 52°C (66.1%)	Li et al. (2014)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
pH (6.0–8.0)	Anaerobic digestion/pig manure	The dominant species at pH 7.0 was *Methanocorpusculum,* when the dominant species at pH 6.0 and 8.0 was *Methanosarcina*	Biogas production and organic matter degradation was the highest at pH 7.0	Zhou et al. (2016)
pH (5.0–7.0)	Anaerobic sludge blanket reactor/ sugar refinery wastewater	With the pH decrease, acidogenic bacteria *Prevotella, Streptococcus, Acidaminococcus* and *Megasphaera* were changed to *Butyricicoccus*, *Lactococcus*, *Brooklawnia*, *Armatimonadetes*_gp2 and *Megasphaera.* At pH 5.0 the hydrogenotrophic methanogens were not detected	Decrease of methane yield by 25.3% (pH 5.0)	Zhang et al. (2019a)
pH (6.5–7.0)	Enhanced biological phosphate removal process/acetate-rich wastewater	Almost 65% of clones detected in sludge at pH 7.0 were absent at pH 6.5, for instance *Proteobacteria*, *Actinobacteria*, *Bacteriodetes*/*Chlorobi* group, *Defluvicoccus* of the α-*Proteobacteria*	Decrease of pH resulted in inability to remove phosphate	Zhang et al. (2005)
pH (7.0–10.0)	Volatile fatty acids production/primary and activated wasted sludge	With the pH increase, the dominance of phyla *Actinobacteria* and *Proteobacteria* increased	Maximum production of volatile fatty acids was at pH 8.9	Chen et al. (2017a)
pH (5.0–10.0)	Volatile fatty acids production/primary and activated wasted sludge	With the increase of pH, there was higher relative abundance of *Clostridium.* The dominance of *Chloroflexi* negatively affected the volatile fatty acids production	Maximum production of volatile fatty acids was at pH 10.0	Atasoy et al. (2019)
DO	Phosphorous removal process/ synthetic wastewater	Decrease of DO resulted in higher abundance of phosphorous accumulating organisms and lower abundance of glycogen accumulating organisms	Higher phosphorus removal with the DO decrease	Izadi et al. (2021)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
DO	Nitrite accumulation/high ammonium wastewater	Increased DO resulted in the highest abundance of *Proteobacteria* (phylum) and *Nitrosomonas* (genus). Lower DO led to elimination of nitrite-oxidizing bacteria	High DO, along with high free ammonia concentration, favour nitrite accumulation	Sui et al. (2016)
DO	Partial nitrification process/wastewater	Ammonia-oxidizing organisms had the highest abundancy in both cases; the increase of DO concentration did not lead to recovery of nitrite-oxidizing organisms	Low DO concentration led to better efficiency of simultaneous nitrification and denitrification (45%) as compared to higher DO (3 mg/L, process efficiency 8%)	Guo et al. (2009)
DO	Nitrifying bioreactors/ synthetic wastewater	Long-term DO concentration resulted in enriched ammonia-oxidizing organisms, more diverse microbial community and higher biomass concentration	Long-term DO conditions resulted in faster pharmaceuticals biotranformation	Stadler and Love (2016)
HRT	Phosphorous removal process/ synthetic wastewater	Increase of aerobic HRT, results in a decrease in phosphorous accumulating organisms' population and increase in glycogen accumulating organisms' activity	Lower HRT resulted with higher phosphorus removal	Izadi et al. (2021)

(continued)

Table 2 (continued)

Factor	Process or reactor/ wastewater	Dynamics of microbial community shift	Water treatment performance	References
HRT	Anaerobic moving bed membrane bioreactor/synthetic domestic wastewater	Drastic change of HRT (8–4 h) resulted in boosted growth of homoacetogenic bacteria, *Thermoanaerobacteraceae.* After the HRT recovery, new microorganisms emerged: hydrogenotrophic archaea, *Methanocella* sp. and *Methanofollis* sp.	The methane production was hindered. After the HRT recovery, the methane production yield was higher than before the shock	Win et al. (2016)
HRT	Continuously stirred tank reactor, hydrogen production/ industrial beverage wastewater	The highest the abundance of *Clostridium* sp. and *Klebsiella*. At HRT 1.5 h *Ruminococcus albus, Clostridium pasteuarnium* and *Clostridium acetobutylicum* were not detected	The highest hydrogen production was achieved at HRT 1.5 h; the highest hydrogen yield was at HRT 6 h	Sivagurunathan and Lin (2016)
SRT	Activated sludge process/wastewater	The enrichment of phosphorous accumulating organisms is a result of increasing SRT	The COD and phosphorous removal increase, with the increase of SRT	Shao et al. (2020)
SRT	Anaerobic membrane bioreactor/kitchen food waste	Increased SRT resulted in higher relative abundance of *Aminomonas* and *Aminobacterium*	Increased SRT resulted in higher biodegradability potency	Durán et al. (2018)

For instance, the organic compounds, such as chitin, cellulose, sucrose or starch present in wastewater, may be used by microbes as a carbon source. The research shows that this aspect determines the diversity of microorganisms during biological treatment, their effectiveness in pollutants removal, but also the morphology and biomass of sludge (Ginige et al., 2008; Liu et al., 2018; Wang et al., 2020). For instance, Wang et al. (2020) compared the removal potential of aerobic granular sludge sequencing batch reactor while using three supplementary carbon sources: starch, methanol and sucrose (Wang et al., 2020). The results indicated that the additional supplementation resulted in higher bacterial diversity and their growth. There were 478, 358 and 555 unique operational taxonomic units (OTUs) in reactors supplemented with methanol, starch and sucrose, respectively, as compared to 186 unique operational taxonomic units in inoculated sludge samples (Wang et al., 2020). Hence, sucrose as an external carbon source resulted in the highest diversity of the microbial community.

Another study investigated the influence of influent composition change from synthetic wastewater to anaerobically pre-treated wastewater on microorganisms during a two-stage deammonification process (Liu et al., 2018). Even though there was no significant change in the water treatment process (based on nitrogen removal efficiency), there was a considerable shift in the microbial community. In the partial nitrification stage, only 21.9% of total OTUs were common before and after the change of influent (Liu et al., 2018). In the second stage, the anammox reactor, the number of shared unique OTUs was reduced even more (16.5%). It is associated with the overall number of unique OTUs, which was greater in reactors after the influent change; the increase was 191 OTUs to 586 OTUs and 397 OUTs to 1364 OUTs in partial nitrification and anammox stage, respectively (Liu et al., 2018). Thus, a more complex matrix with a high content of organic matter, like pre-treated sewage, resulted in the evolution of microbial community in the process.

The organic compounds that can be used as an additional carbon source may also exhibit some adverse effects on the microorganisms. For instance, methanol needs to be supplemented gradually, as the microbial community requires time to adapt to the new environment (Wang et al., 2020) and promote the growth of the methanol-utilizing microorganisms (Ginige et al., 2008). During the adaptation period, the removal efficiencies of COD, nitrogen or phosphorous were relatively lower than expected (Ginige et al., 2008; Wang et al., 2020).

Moreover, wastewater influent includes a load of compounds that may inactivate or destroy microorganisms. When the concentration of the inhibitor is high, it may lead to process blockage (Poirier et al., 2016). Moreover, the high organic load may result in hindering microbial growth, rather than the presence of inhibitors (Wang et al., 2008). However, the studies show that the microbial community exhibits a high level of adaptability. The presence of potential inhibitors, such as furfurals (Si et al., 2018),

N-heterocyclic compounds (Si et al., 2018), phenolics (Poirier et al., 2016; Si et al., 2018) or pharmaceuticals (Wang et al., 2008; Zhu et al., 2017), results in microbial community shift towards the organisms related to the detoxification (Table 2). For instance, the treatment of hydrothermal liquefaction, with the high content of organic

compounds, led to a higher abundance of known species, responsible for transformation of furfurals, aromatic and nitrogen compounds (shift from 15 to 21.5% relative abundance) (Si et al., 2018). Furthermore, the microbial community in the presence of potential inactivating compounds may maintain stability due to gradual acclimatization of microbes and/or develop resistance, such as antibiotic resistance species (Gao et al., 2015; Zhu et al., 2017).

3.2 Operating Parameters

3.2.1 Temperature

The temperature is a key parameter for microbial growth and metabolism. Each microorganism has its optimum growth temperature. This aspect has tremendous importance in countries with seasonal changes, such as European, Middle Eastern or North American countries. The tendency is that the higher temperature, the higher is the microbial growth rate (Rajeshwari et al., 2000). The regional variations also need to be considered during wastewater treatment plant management. Thus, the dynamics of microbial shift are associated with the changes in temperature. The wastewater treatment plants need to be designed in a way that would allow them to eliminate the high-temperature fluctuations.

Li et al. (2014) investigated the performance of anaerobic reactor at higher temperatures (Li et al., 2014). The water treatment at 37 °C had the best performance amongst all tested temperatures and had the greatest microbial diversity. The dominant species was *Methanosaeta*, which was replaced by *Methanobrevibacter* and *Methanofollis* at lower (33 °C) and higher temperatures (43–52 °C). The study of Tiwari et al. (2021) revealed that the decrease from 20 to 10 °C resulted in a relative abundance reduction of nitrifying bacteria and dominance of the fungal group (Bhagyashree et al., 2021). In both cases, the increase or decrease of temperature was followed by a worsening performance of water treatment (Bhagyashree et al., 2021; Li et al., 2014).

The anammox process, a cost-effective way to treat influents with high ammonia concentration, is one of the most challenging water treatment processes due to its sensitivity to temperature drop. The optimum temperature for anammox microorganisms is between 30 and 37 °C. For instance, the decrease of temperature from 35 to 25 °C resulted in an abundance increase of anammox and denitrifying bacteria, whereas the decrease of *Anaerolineales* and *Clostridiales* was observed (de Almeida Fernandes et al., 2018). The nitrogen removal decreased from 96 to 90%. Further decrease of temperature even further results in hindering the biomass-specific activity (Vázquez-Padín et al., 2011). The solution is to enrich the biomass with the cold-adapted species of anaerobic ammonia oxidation bacteria. For instance, Hülsen et al. (2016) used purple phototropic bacteria to treat domestic wastewater at 10 °C (Hülsen et al., 2016). The purple phototropic bacteria became the dominant group in the

reactor (> 50%), which was the result of competition between them and less compatible non-purple phototropic bacteria. Hendrickx et al. (2014) used the microorganisms that were isolated from sludge sampled in winter (Hendrickx et al., 2014). The enriched biomass, where the dominant species was *Candidatus Brocadia fulgida*, exhibited twofold higher specific activity compared to previous studies.

3.2.2 pH

Similarly to temperature, pH is an important parameter that influences microbial growth. The variations in reactors' operation parameters (i.e. temperature, organic load and dissolved oxygen) may result in pH variation. Usually, the microorganisms in wastewater treatment plants exhibit a good pollutant removal rate, when the pH is between 6.0 and 9.0. However, even the small change within the optimal pH range may result in microbial community shift, followed by removal aggravation (Table 2) (Zhang et al. 2005). The sudden pH shock may also lead to disturbance in the treatment performance, but in some cases, due to acclimatization and microbial shift, the ability to remove the pollutants may be regained (Zhou et al. 2018). Nevertheless, one should consider the stepwise pH change to avoid the wastewater treatment disturbance (Chen et al. 2017a).

The optimal pH depends on the targeted process. For instance, anaerobic digestion systems can produce methane between 6.0 and 8.0. However, even the variations within this range may lead to changes in the microbial community. For instance, Zhou et al. (2016) investigated the microbial shift during anaerobic digestion, when the pH varied between 6.0 and 8.0 (Zhou et al. 2016). There was the highest abundance of acetoclastic methanogens, but interestingly, the dominant species were different at pH 7.0 compared to pH 6.0 and 8.0 (Table 2). In another study, the acetoclastic methanogens were able to completely replace the hydrogenotrophic methanogens, when pH decreased to 5.0 (Zhang et al. 2019a).

In some cases, the increase of pH may lead to better performance of the process. The production of volatile fatty acids was more efficient when the pH increased to almost 9.0–10.0 (Chen et al. 2017a; Atasoy et al. 2019). It is associated with the microbial shift (Table 2). Moreover, the change of pH and following microbial shift influence the composition of volatile fatty acids (Atasoy et al. 2019).

3.2.3 Dissolved Oxygen (DO)

The concentration of DO in treated water is an important parameter for microbial growth, not only in anaerobic processes but also in anaerobic conditions (Garcia-Ochoa et al. 2010). Due to the low solubility of oxygen in water, DO can be considered as one of the limiting nutrients, responsible for microbial community shift. For instance, the diminution of DO in water results in increased dominance of phosphorous accumulating organisms, at the same time, decreasing the abundance of glycogen accumulating organisms (Izadi et al. 2021).

The different DO concentration also affects the nitrification and denitrification processes. Sui et al. (2016) showed that higher DO concentration (around 4 mg/L) led to increased abundance of *Nitrosomonas,* which was one of the dominant genera in the reactor, resulting in efficient nitrite accumulation (Sui et al., 2016). The decrease of DO was followed by the elimination of nitrite-oxidizing bacteria. In another study, low DO concentration (below 0.8 mg/L) led to better efficiency of simultaneous nitrification and denitrification (45%) as compared to higher DO (3 mg/L, process efficiency 8%) (Guo et al., 2009). Ammonia-oxidizing organisms had the highest abundance in both cases; the increase of DO concentration did not lead to the recovery of nitrite- oxidizing organisms.

Stadler et al. (2016) compared the transformation rate of pharmaceuticals and ammonia oxidation in high and low DO concentrations for a short and long period (Stadler & Love, 2016). High DO conditions (4 mg/L) resulted in faster biotransformation of pharmaceuticals as compared to low DO conditions (0.3 mg/L) in a short period. On the other hand, long-term low DO concentration resulted in a more diverse microbial community, greater mass concentration, lower specific ammonia oxidation rate, leading to a faster transformation of pharmaceuticals and nitrification rate (Stadler & Love, 2016). Hence, the benefits and drawbacks of both low/high DO lead to a conclusion that while planning the best strategy for efficient wastewater treatment plant operation, this parameter needs to be evaluated carefully. Some research suggests considering the application of different process cycles, such as low/high DO (Stadler & Love, 2016) or aerobic/anaerobic conditions (Izadi et al., 2021).

3.2.4 HRT/SRT

Alterations in HRT lead to volume loading and not all microorganisms will adapt to these changes. Hence, the sudden HRT change may influence not only microbial but also reactor performance. Win et al. (2016) studied the effect of shock change of HRT on methane production (Win et al., 2016). The sudden decrease of HRT from 8 to 4 h resulted in a reduction of methane production due to competition between methanogens and enhanced growth homoacetogenic bacteria, *Thermoanaerobacteraceae*, over hydrogen consumption. Interestingly, after the HRT recovery to 8 h, the methane production rate was higher than before shock, which may be associated with the activity of new hydrogenotrophic archaea, *Methanocella* sp. and *Methanofollis* sp. (Win et al., 2016).

The same process under different HRT can be characterized by various efficiency and microbial community. Short HRT may result in the wash-out of competitive and/or non-competitive microorganisms due to a high dilution rate. For instance, the highest hydrogen production was achieved at HRT 1.5 h due to the high organic load rate and the abundance of *Clostridium* sp., efficient hydrogen-producing bacteria (Sivagurunathan & Lin, 2016). On the other hand, the hydrogen yield at HRT 1.5 h was lower compared to HRT 6 h, probably due to the wash-out of *Ruminococcus albus*, *Clostridium pasteuarnium* and *Clostridium acetobutylicum.*

SRT also influences microbial community shift, however, in a different manner than HRT; its impact is focused more on microbial growth and proliferation (Chen et al., 2017b). For instance, long HRT resulted in a decrease in the abundance of phosphorous accumulating organisms and an increase in glycogen accumulation organisms' activity (Izadi et al., 2021). However, SRT exhibits the opposite phenomena; with the increase of SRT, there was an enhancement of phosphorous accumulating organisms (Shao et al., 2020). Durán et al. (2018) reported that with the increase of SRT, better biodegradability was observed (Durán et al., 2018). It was associated with a higher abundance of microorganisms with enhanced hydrolytic activity (Table 2).

4 Meteorological, Geographical and Different Process Parameters Influence Microbial Shift

4.1 Seasonal Changes and Regional Variations

It is very important to understand the relationships between microbial communities and their environment and how those relationships play out at local, regional and global scales. Using sequencing technologies Hollister et al. showed the diversity, complexity and dynamics of microbial systems in soils and sediments from La Sal del Rey, a hypersaline lake located in southern Texas, USA (Hollister et al., 2010). Also, Horton et al. reported significant differences in microbial community structure amongst coastal wetlands within the western basin of Lake Erie and all other wetlands (Saginaw Bay and Beaver Archipelago) (Horton et al., 2019).

Those studies have to be taken into account to create hypothetical networks of microbial shift that can be predicted by chemical structures involved in this process within a specific environment. Disturbance and change to carbon inputs through plant functions connected to climate and observed during seasonal changes. The composition of microbial communities strongly correlates with seasonal changes as it brings variations in nutrients. In the dry season, microeucaryotic communities' growth correlates significantly to dissolved organic carbon, total nitrogen, nitrate and soluble reactive phosphorus, while during the wet season such phenomena are observed as a response to nitrate and total phosphorus. The shift of microbial communities is not constant, but it is predictable as associated with nutrient variations (Liu et al., 2020). Seasonal alteration in resource availability, which is driven by plants via below ground carbon allocation, nutrient uptake and litter fall, also exerts effects on soil microbial community composition. Marianne Koranda et al. in their research showed that microbial communities in soils collected at different seasons and from experimentally changed nutrition load clearly altered as a response to chemical soil composition and this observation characterizes distinct physiological capacities of winter and summer microbial communities. The winter communities have a higher

capacity for degradation of cellulose and generally plant cell walls and lower utilization of glucose compare with the summer communities, which indicates a switch in the adaptation process of microbial communities (Tignat-Perrier et al., 2020).

As we can imagine those seasonal shifts are followed by geographical localization that is characterized by variable meteorological conditions. It was found by Tignat-Perrier et al. at the puy de Dôme's landscape with windy condition strongly affects the airborne microbial taxa (Tignat-Perrier et al., 2020). In the spring/summer season the presence of crop pathogens was observed while in autumn/winter time soil-associated microorganisms and dead material-associated microorganisms were found in higher relative abundance.

It seems that autumn has the biggest influence on the bacteria shift was particularly distinct from other seasons for bacteria connect with plant physiological changes, most probably with root exudates. Most climate and soil variables related to soil moisture as well as pH for bacteria temperature for fungi, and soil C:N for archaea explain significant variation for seasonal patterns in the different microbial communities (Stevenson et al., 2014).

Microbial diversity depends also on the localization in soil depths as it we can observe in the study of Luo et al. on subtropical orchard interaction with microbial communities localized at 0–5 and 5–20 cm soil depth during different seasons (i.e. spring, summer and autumn). The number of soil microorganism and the Simpson and Shannon–Wiener indexes were all highest in summer and were significantly negatively correlated with soil pH, total organic carbon, total nitrogen and the cation exchange capacity (Luo et al., 2019).

Interesting season depended phenomena were demonstrated in phytoplankton biomass growing during the winter season due to the significant export of organic carbon in the water column has a place. It can help to predict future effects of seasonal variations in temperature (and ice cover) that is connected with changes in carbon level (Wilhelm et al., 2014)

4.2 Type of Process and Type of Reactor

Microbial communities play an important role in water purification in drinking water treatment systems. Even though some bacteria present in the untreated water may help in its purification through biodegradation of the contaminants strict controls in the processes at wastewater factories are crucial as some bacteria may pose a threat to consumers as human pathogens. Liao et al. by clone library analysis showed the drastic decrease of the density of viable heterotrophic bacteria and bacterial populations through a pilot-scale drinking water treatment process using heterotrophic plate counts pre-ozonation, rapid mixing, flocculation, sedimentation, sand filtration post-ozonation and biological activated carbon filtration and clone library analysis (Liao et al., 2015).

The microbial community is significantly altered by chlorine disinfection, while other treatment processes were synergetic (Li et al., 2017). There is a strong distinction between bacterial communities (depends on pollutant presence that is degraded by particular organisms) in water and biofilms (this distinction is further observed between different biofilms), nevertheless, the functional composition of biofilms on different filters are similar. Multidimensional scaling analysis revealed tight clustering of biofilm samples collected from different treatment steps, with Nitrospira, the nitrite-oxidizing bacteria, noted at higher relative abundances in biofilm compared to water samples (Xu et al., 2017). The **microbial community** alterations are observed on of the oxic-settling-anaerobic/anoxic process treating real domestic **wastewater** by changing interchange ratios (Karlikanovaite-Balikci et al., 2019).

The potential shift of the bacterial community is observed during water source switching, especially in corrosion-related bacteria. Hu et al. showed that the bacteria community released from the pipeline reactor was significantly different under different finished water, and the variation was biggest at the genus level. The observed shift was explained by the lower carbon and nitrogen content of the new water source, nevertheless, corrosion-inhibiting bacteria, decreased after switching, still maintained dominance in three reactors (Hu et al., 2021). Similarly, it was observed significant shifts in planktonic and biofilm microbial communities that were receiving contaminated water (Wilpiszeski et al., 2020).

In the laboratory experiment of the bacterial and archaeal communities shift using anaerobic membrane bioreactor with a side stream tubular membrane dynamic during the start-up, steady-state, overloading and recovery periods of operation at mesophilic temperatures, a significant shift occurred in the recovery period, especially in the methanogen group, which shifted to acetoclastic methanogens. Interestingly, the impact of shock loading on the community caused that shifted bacteria did not recover their previous structure and population (Martin Vincent et al., 2018). It was also shown that to promote anammox bacteria immobilization leading to microbial community evolution an upflow porous-plate anaerobic reactor can be used. By this techniques exploration and promotion of biomass retention and growth was significant (Zhang et al., 2019b).

Study of bacterial and archaeal communities shift, investigated in an anaerobic batch reactor treating dairy-processing wastewater, associated with alterations in chemical profiles showed that its monitoring is also important as a diagnostic tool of anaerobic digestion (Lee et al., 2008).

5 Conclusion

Microbial communities' structure is very flexible. It is very important to understand the conditions that may influence microbial shift. As those organisms may play an important function in pollutants digestions, the shift in microbial communities may increase their value in process of water purification. On the other hand, some shifts may increase pathogen organisms that can be a danger to human health. The source

of the water–seasonal and geographical conditions as well as techniques used in wastewater factories had to be closely monitoring to reach the best pathways of bacterial community shifting towards improving the efficiency of wastewater treatment and finally the best quality of the water that will be used in households in fields and factories.

References

Atasoy, M., Eyice, O., Schnürer, A., & Cetecioglu, Z. (2019). Volatile fatty acids production via mixed culture fermentation: Revealing the link between pH, inoculum type and bacterial composition. *Bioresource Technology, 292*, 121889. https://doi.org/10.1016/j.biortech.2019.121889

Bhagyashree, T., Balasubramanian, S., & Sarah, P.-C., et al. (2021). Compositional microbial-community shift of submerged membrane bioreactor treating hospital wastewater at varying temperatures. *Journal of Environmental Engineering, 147*, 04020152. https://doi.org/10.1061/(ASCE)EE.1943-0001842

Chen, Y., Jiang, X., Xiao, K., et al. (2017a). Enhanced volatile fatty acids (VFAs) production in a thermophilic fermenter with stepwise pH increase—Investigation on dissolved organic matter transformation and microbial community shift. *Water Research, 112*, 261–268. https://doi.org/10.1016/j.watres.2017.01.067

Chen, Y., Lan, S., Wang, L., et al. (2017b). A review: Driving factors and regulation strategies of microbial community structure and dynamics in wastewater treatment systems. *Chemosphere, 174*, 173–182.

de Almeida, F. L., Pereira, A. D., Leal, C. D., et al. (2018). Effect of temperature on microbial diversity and nitrogen removal performance of an anammox reactor treating anaerobically pretreated municipal wastewater. *Bioresource Technology, 258*, 208–219. https://doi.org/10.1016/j.biortech.2018.02.083

Durán, F., Zamorano-López, N., Barat, R., et al. (2018). Understanding the performance of an AnMBR treating urban wastewater and food waste via model simulation and characterization of the microbial population dynamics. *Process Biochemistry, 67*, 139–146. https://doi.org/10.1016/j.procbio.2018.02.010

Ferrera, I., & Sánchez, O. (2016). Insights into microbial diversity in wastewater treatment systems: How far have we come? *Biotechnology Advances, 34*, 790–802.

Gao, P., He, S., Huang, S., et al. (2015). Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. *Applied Microbiology and Biotechnology, 99*, 3971–3980.

Garcia-Ochoa, F., Gomez, E., Santos, V. E., & Merchuk, J. C. (2010). Oxygen uptake rate in microbial processes: An overview. *Biochemical Engineering Journal, 49*, 289–307.

Ginige, M. P., Bowyer, J. C., Foley, L., et al. (2008). A comparative study of methanol as a supplementary carbon source for enhancing denitrification in primary and secondary anoxic zones. *Biodegradation, 20*, 221. https://doi.org/10.1007/s10532-008-9215-1

Guo, J., Peng, Y., Wang, S., et al. (2009). Long-term effect of dissolved oxygen on partial nitrification performance and microbial community structure. *Bioresource Technology, 100*, 2796–2802. https://doi.org/10.1016/j.biortech.2008.12.036

Hendrickx, T. L. G., Kampman, C., Zeeman, G., et al. (2014). High specific activity for anammox bacteria enriched from activated sludge at 10 °C. *Bioresource Technology, 163*, 214–221. https://doi.org/10.1016/j.biortech.2014.04.025

Hollister, E. B., Engledow, A. S., Hammett, A. J. M., et al. (2010). Shifts in microbial community structure along an ecological gradient of hypersaline soils and sediments. *The ISME Journal, 4*, 829–838. https://doi.org/10.1038/ismej.2010.3

Horton, D. J., Theis, K. R., Uzarski, D. G., & Learman, D. R. (2019). Microbial community structure and microbial networks correspond to nutrient gradients within coastal wetlands of the Laurentian Great Lakes. *FEMS Microbiology Ecology, 95*.https://doi.org/10.1093/femsec/fiz033

Hu, Y., Dong, D., & Wan, K., et al. (2021). Potential shift of bacterial community structure and corrosion-related bacteria in drinking water distribution pipeline driven by water source switching. *Frontiers of Environmental Science & Engineering, 15*.https://doi.org/10.1007/s11783-020-1320-3

Hülsen, T., Barry, E. M., Lu, Y., et al. (2016). Low temperature treatment of domestic wastewater by purple phototrophic bacteria: Performance, activity, and community. *Water Research, 100*, 537–545. https://doi.org/10.1016/j.watres.2016.05.054

Izadi, P., Izadi, P., & Eldyasti, A. (2021). Understanding microbial shift of Enhanced Biological Phosphorus Removal process (EBPR) under different Dissolved Oxygen (DO) concentrations and Hydraulic Retention Time (HRTs). *Biochemical Engineering Journal, 166*, 107833. https://doi.org/10.1016/j.bej.2020.107833

Karlikanovaite-Balikci, A., Ozbayram, E. G., Yagci, N., & Ince, O. (2019). Microbial community shifts in the oxic-settling-anoxic process in response to changes to sludge interchange ratio. *Heliyon, 5*.https://doi.org/10.1016/j.heliyon.2019.e01517

Lee, C., Kim, J., Shin, S. G., & Hwang, S. (2008). Monitoring bacterial and archaeal community shifts in a mesophilic anaerobic batch reactor treating a high- strength organic wastewater. *FEMS Microbiology Ecology, 65*https://doi.org/10.1111/j.1574-6941.2008.00530.x

Li, X., Ma, K., & Meng, L., et al. (2014). Performance and microbial community profiles in an anaerobic reactor treating with simulated PTA wastewater: From mesophilic to thermophilic temperature. *Water Research, 61*, 57–66.https://doi.org/10.1016/j.watres.2014.04.033

Li, Q., Yu, S., & Li, L., et al. (2017). Microbial communities shaped by treatment processes in a drinking water treatment plant and their contribution and threat to drinking water safety. *Frontiers in Microbiology, 8*.https://doi.org/10.3389/fmicb.2017.02465

Liao, X., Chen, C., & Wang, Z., et al. (2015). Bacterial community change through drinking water treatment processes. *International Journal of Environmental Science and Technology, 12*.https://doi.org/10.1007/s13762-014-0540-0

Liu, Y., Ren, Z., & Qu, X., et al. (2020). Seasonal water level fluctuation and concomitant change of nutrients shift Microeukaryotic communities in a shallow lake. *Water, 12*https://doi.org/10.3390/w12092317

Liu, W., Ji, X., Wang, J., et al. (2018). Microbial community response to influent shift and lowering temperature in a two-stage mainstream deammonification process. *Bioresource Technology, 262*, 132–140. https://doi.org/10.1016/j.biortech.2018.04.082

Luo, X., Wang, M.K., Hu, G., & Weng, B. (2019). Seasonal change in microbial diversity and its relationship with soil chemical properties in an orchard. *PLoS One, 14*.https://doi.org/10.1371/journal.pone.0215556

Martin Vincent, N., Wei, Y., & Zhang, J., et al. (2018). Characterization and dynamic shift of microbial communities during start-up, overloading and steady-state in an anaerobic membrane bioreactor. *International Journal of Environmental Research and Public Health, 15*https://doi.org/10.3390/ijerph15071399

Poirier, S., Bize, A., Bureau, C., et al. (2016). Community shifts within anaerobic digestion microbiota facing phenol inhibition: Towards early warning microbial indicators? *Water Research, 100*, 296–305. https://doi.org/10.1016/j.watres.2016.05.041

Rajeshwari, K. V., Balakrishnan, M., & Kansal, A., et al. (2000). State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. *Renewable & Sustainable Energy Reviews, 4*, 135–156.https://doi.org/10.1016/S1364-0321(99)00014-3

Shao, Y., Liu, G., & Wang, Y, et al. (2020). Sludge characteristics, system performance and microbial kinetics of ultra-short-SRT activated sludge processes. *Environment International, 143*, 105973. https://doi.org/10.1016/j.envint.2020.105973

Sheik, A., Muller, E., & Wilmes, P. (2014). A hundred years of activated sludge: Time for a rethink. *Frontiers in Microbiology, 5*, 47.

Si, B., Li, J., Zhu, Z., et al. (2018). Inhibitors degradation and microbial response during continuous anaerobic conversion of hydrothermal liquefaction wastewater. *Science of the Total Environment, 630*, 1124–1132. https://doi.org/10.1016/j.scitotenv.2018.02.310

Sivagurunathan, P., & Lin, C.-Y. (2016). Enhanced biohydrogen production from beverage wastewater: Process performance during various hydraulic retention times and their microbial insights. *RSC Advances, 6*, 4160–4169. https://doi.org/10.1039/C5RA18815F

Stadler, L. B., & Love, N. G. (2016). Impact of microbial physiology and microbial community structure on pharmaceutical fate driven by dissolved oxygen concentration in nitrifying bioreactors. *Water Research, 104*, 189–199. https://doi.org/10.1016/j.watres.2016.08.001

Stevenson, B. A., Hunter, D. W. F., & Rhodes, P. L. (2014). Temporal and seasonal change in microbial community structure of an undisturbed, disturbed, and carbon-amended pasture soil. *Soil Biology and Biochemistry, 75*.https://doi.org/10.1016/j.soilbio.2014.04.010

Sui, Q., Liu, C., Zhang, J., et al. (2016). Response of nitrite accumulation and microbial community to free ammonia and dissolved oxygen treatment of high ammonium wastewater. *Applied Microbiology and Biotechnology, 100*, 4177–4187. https://doi.org/10.1007/s00253-015-7183-z

Tignat-Perrier, R., Dommergue, A., & Thollot, A., et al. (2020). Seasonal shift in airborne microbial communities. *Science of The Total Environment, 716*.https://doi.org/10.1016/j.scitotenv.2020.137129

Vázquez-Padín, J. R., Fernández, I., Morales, N., et al. (2011). Autotrophic nitrogen removal at low temperature. *Water Science and Technology, 63*, 1282–1288. https://doi.org/10.2166/wst.2011.370

Wang, S., Holzem, R. M., & Gunsch, C. K. (2008). Effects of pharmaceutically active compounds on a mixed microbial community originating from a municipal wastewater treatment plant. *Environmental Science & Technology, 42*, 1091–1095. https://doi.org/10.1021/es072026x

Wang, X., Chen, Z., Shen, J., et al. (2020). Effect of carbon source on pollutant removal and microbial community dynamics in treatment of swine wastewater containing antibiotics by aerobic granular sludge. *Chemosphere, 260*, 127544. https://doi.org/10.1016/j.chemosphere.2020.127544

Wilhelm, S. W., LeCleir, G. R., & Bullerjahn, G. S., et al (2014) Seasonal changes in microbial community structure and activity imply winter production is linked to summer hypoxia in a large lake. *FEMS Microbiology Ecology, 87*.https://doi.org/10.1111/1574-6941.12238

Wilpiszeski, R. L., Gionfriddo, C. M., & Wymore, A. M., et al. (2020). In-field bioreactors demonstrate dynamic shifts in microbial communities in response to geochemical perturbations. *PLoS One, 15*.https://doi.org/10.1371/journal.pone.0232437

Win, T. T., Kim, H., Cho, K., et al. (2016). Monitoring the microbial community shift throughout the shock changes of hydraulic retention time in an anaerobic moving bed membrane bioreactor. *Bioresource Technology, 202*, 125–132. https://doi.org/10.1016/j.biortech.2015.11.085

Woodcock, S., Curtis, T. P., Head, I. M., et al. (2006). Taxa–area relationships for microbes: The unsampled and the unseen. *Ecology Letters, 9*, 805–812. https://doi.org/10.1111/j.1461-0248.2006.00929.x

Xu, J., Tang, W., Ma, J., & Wang, H. (2017). Comparison of microbial community shifts in two parallel multi-step drinking water treatment processes. *Applied Microbiology and Biotechnology, 101*.https://doi.org/10.1007/s00253-017-8258-9

Zhang, T., Liu, Y., & Fang, H. H. P. (2005). Effect of pH change on the performance and microbial community of enhanced biological phosphate removal process. *Biotechnology and Bioengineering, 92*, 173–182. https://doi.org/10.1002/bit.20589

Zhang, L., Ban, Q., Li, J., & Wan, C. (2019a). Functional bacterial and archaeal dynamics dictated by pH stress during sugar refinery wastewater in a UASB. *Bioresource Technology, 288*, 121464. https://doi.org/10.1016/j.biortech.2019.121464

Zhang, D., Xu, S., & Antwi, P., et al. (2019b). Accelerated start-up, long-term performance and microbial community shifts within a novel upflow porous- plated anaerobic reactor treating nitrogen-rich wastewater *via* ANAMMOX process. *RSC Advances, 9*.https://doi.org/10.1039/C9RA04225C

Zhou, H., Wang, G., Wu, M., et al. (2018). Phenol removal performance and microbial community shift during pH shock in a moving bed biofilm reactor (MBBR). *Journal of Hazardous Materials, 351*, 71–79. https://doi.org/10.1016/j.jhazmat.2018.02.055

Zhou, J., Zhang, R., Liu, F., et al. (2016). Biogas production and microbial community shift through neutral pH control during the anaerobic digestion of pig manure. *Bioresource Technology, 217*, 44–49. https://doi.org/10.1016/j.biortech.2016.02.077

Zhu, Y., Wang, Y., Jiang, X., et al. (2017). Microbial community compositional analysis for membrane bioreactor treating antibiotics containing wastewater. *Chemical Engineering Journal, 325*, 300–309. https://doi.org/10.1016/j.cej.2017.05.073

Index

M. P. Shah (ed.), *Genomics of Antibiotic Resistant Bacteria in Industrial Waste Water Treatment*, https://doi.org/10.1007/978-3-031-44618-4