Microorganisms for Sustainability 10
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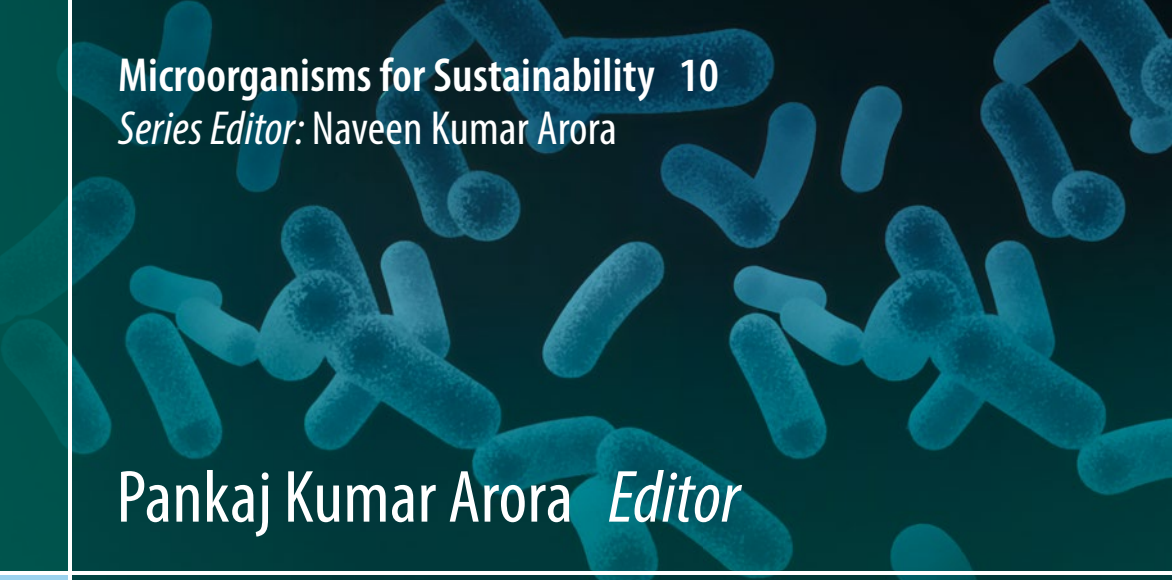

Pankaj Kumar Arora *Editor*

Microbial Metabolism of Xenobiotic Compounds

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Microorganisms for Sustainability

Volume 10

Series editor
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Microorganisms are performing diverse and huge number of tasks on our planet, most of which are making earth a habitable and sustainable ecosystem. Many of these acts of the microorganisms are being or can be utilized as low input biotechnology to solve various problems related to environment, particularly caused due to anthropogenic activities. Microbial technologies can be used for chemical free agriculture, replacing harmful pesticides as well as fertilizers for crop protection and enhancing the yields. Similarly, microbes can be used to provide green alternatives to the fossil fuels thus combating the problems related to pollution and global warming. Microorganisms can be the magic bullets for reclamation of wastelands/ stress affected regions, bioremediation of contaminated habitats and biodegradation purposes. The series proposed will cover all these wider aspects and explain how microbes can be used for sustaining our planet in a greener way. Each volume of the series will have a different Editor with his/ her own area of expertise.

The book series will focus on the role of microbes in sustaining the ecosystems. Naturally, microorganisms play so many important roles in sustaining plant and animal life on earth. Global issues such as remediation of polluted sites, degradation of pollutants, biofertilizers and biopesticides to replace chemicals in agriculture, reclamation of barren soils, combating climate change, green fuels are the major contemporary issues and focus of researchers.

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Foreword

Microbial metabolism is a key factor to determine the fate of xenobiotic compounds in the environment. Several xenobiotic compounds including persistent organic pollutants, nitro-aromatic compounds, pesticides, herbicides, pyridines, polychlorinated biphenyls, polyaromatic hydrocarbons, and chlorophenols are widely distributed in our environment. The presence of these compounds in the environment creates high risk to the living beings due to their toxicity. Microorganisms are the only living organisms on the earth which have exceptional ability to metabolize these harmful compounds as their nutrients. Some of microorganisms use these compounds as cosubstrates, thus converting them to nontoxic or less toxic compounds. This metabolic strategy can be exploited for biodegradation and bioremediation of recalcitrant organic compounds from the contaminated medium for environmental management.

Microbial Metabolism of Xenobiotic Compounds, edited by Dr. Pankaj Kumar Arora, is very useful to researchers working in the fields of biodegradation and bioremediation of xenobiotic compounds. The chapters contributed by the leading experts in the field are really excellent and provide source materials on the different aspects of biodegradation and bioremediation of recalcitrant organic pollutants. I appreciate the editor's effort in editing this valuable book that will go some way to make our planet cleaner and sustainable. I congratulate the book editor for bringing out this valuable book with up-to-date knowledge in the field of microbial metabolism of xenobiotic compounds. I wish this book a grand success and will be useful to the stakeholders, including researchers, academicians, students, environmentalists, and policy makers.

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Preface

Microorganisms play various roles in environment. They can metabolize many recalcitrant organic pollutants including both aliphatic and aromatic organic compounds, either to obtain carbon and/or energy for growth, or as cosubstrates, thus converting them to simpler products such as carbon dioxide, water, chloride, and biomass. This metabolic strategy can be exploited for biodegradation and bioremediation of recalcitrant organic compounds from the contaminated medium. Bioremediation is an eco-friendly approach that uses microorganisms, plants, or their enzymes to degrade/detoxify the recalcitrant organic compounds such as phenols, chlorophenols, petroleum hydrocarbons, polychlorinated biphenyls (PCBs), organic solvents, azo dyes, pesticides, etc. from contaminated soils and wastewaters. The removal of a wide range of organic pollutants from contaminated medium requires our increasing understanding of different degradation pathways and regulatory networks to carbon flux for their degradation and detoxification, which is utmost important for environmental safety. Therefore, this book provides a comprehensive knowledge on the basic principles and prospects of microbial metabolism of xenobiotic compounds. The book describes the microbiological, biochemical, and molecular aspects of biodegradation and bioremediation of recalcitrant organic compounds for pollution prevention and control.

This book, *Microbial Metabolism of Xenobiotic Compounds*, describes the toxicity of various recalcitrant organic compounds, their environmental impact, and biodegradation pathways for the microbes-mediated degradation and detoxification for environmental management. Many relevant topics have been contributed in this book by the experts from different universities, research laboratories, and institutes from around the globe in the area of microbial metabolism of xenobiotic compounds. In this book, extensive focus has been relied on the recent advances in microbial metabolism of xenobiotic compounds, including the microbial degradation of pyridine and pyridine derivatives, anaerobic biodegradation of pesticides, application of DNA-stable isotope probing in the examination of microorganisms involved in biodegradation, enzymatic bioremediation, biotransformation of heavy crude oil and biodegradation of oil pollution by arid zone bacterial strains, catalytic promiscuity of aromatic ring hydroxylating dioxygenases and their role in the plas-

ticity of xenobiotic compounds degradation, aromatic compounds and biofilms: regulation and interlinking of metabolic pathways in bacteria, biodegradation and bioremediation of polychlorinated biphenyls (PCBs), bioremediation of polycyclic aromatic hydrocarbons (PAHs), role of macrofungi in bioremediation of pollutants, role of microemulsions in enhancing the bioremediation of xenobiotics, microbial metabolism of melanoidins from distillery effluent; and microbial degradation of nitro group containing compounds and herbicide, etc. Researchers working in the field of microbial metabolism of xenobiotic compounds will find a good package on the progress made in bioremediation and biodegradation of recalcitrant organic compounds for environmental safety and sustainability.

In the last, I hope that this book will be useful to researchers, environmentalists and scientists, microbiologists and biotechnologists, eco-toxicologists, remediation practitioners and policymakers, industry persons and students at master's and doctoral level in the relevant field. Thus, in this book, readers will find the updated information as well as the future direction for research in the field of microbial metabolism of xenobiotic compounds.

I greatly acknowledge the Department of Biotechnology (DBT), India, for providing me Ramalingaswamy Re-Entry Fellowship, which is one of the most prestigious fellowships for Indian scientists.

Lucknow, Uttar Pradesh, India Pankaj Kumar Arora

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About the Series Editor

Naveen Kumar Arora PhD in Microbiology and Professor and Head of the Department of Environmental Science, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India, is a renowned researcher in the field of environmental microbiology and biotechnology. His specific area of research is rhizosphere biology and plant growth-promoting rhizobacteria (PGPR). He has more than 60 research papers published in premium international journals and several articles published in magazines and dailies. He is an editor of 15 books, published by Springer. He is a member of several national and international societies, is on the editorial board of four journals, and is a reviewer of several international journals. He is also the Editor in Chief of the journal *Environmental Sustainability* published by Springer Nature. He has delivered lectures in conferences and seminars around the globe. He has been advisor to 118 postgraduate and 9 doctoral students. He has also received awards for excellence in research by the Honorable Governor of Uttar Pradesh, Asian PGPR Society, and Samagra Vikas Welfare Society. Although an academician and researcher by profession, he has a huge obsession for the wildlife and its conservation and has authored a book *Splendid Wilds*. He is the President of Society for Conservation of Wildlife and is also a Secretary of the Society for Environmental Sustainability (website: www.ses-india.org).

About the Editor

Pankaj Kumar Arora is working as an Assistant Professor and DBT-Ramalingaswami Re-entry Fellow at the Department of Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, India. He is also serving as an Editorial Board Member for *Scientific Reports*, a journal of Nature Publishing Group, an Associate Editor for *Frontiers in Microbiology*, and an Academic Editor for *PLOS ONE*. He is a recipient of several national awards and fellowships including Young Botanist Award (2012) of Indian Botanical Society, Dr. Y. S. Murty Medal (2015) of Indian Botanical Society, and Ramalingaswami Re-entry fellowship (2018) of Department of Biotechnology. His specialization area is environmental microbiology and biotechnology and currently he is working on biodegradation and bioremediation of various xenobiotic compounds including nitrophenols, chlorinated nitrophenols, and indole. He completed his postdoctoral studies from the University of Hyderabad, MJP Rohilkhand University, Bareilly, and Yeungnam University, South Korea. He has totally 36 publications in reputed journals and has 8 years of teaching and research experiences at national and international reputed institutes.

Chapter 1
Microbial Degradation of Pyridine and Pyridine Derivatives

Nidhi Gupta, Edward J. O'Loughlin, and Gerald K. Sims

Abstract Pyridine derivatives belong to an important class of aromatic compounds that occur largely as a result of human activities, although they are not necessarily xenobiotic compounds. Pyridines can also be derivatized to form a wide variety of xenobiotic compounds ranging from drugs to pesticides. Analogs to phenolic compounds, pyridines exhibit properties that differ in some respects to homocyclic compounds, and this may have profound effects on their biodegradation. The presence of the ring nitrogen defines the reactivity of pyridine derivatives. After 60 years of research into biodegradation of pyridine derivatives, some themes have emerged; however, new discoveries continue to change our understanding of how pyridines are degraded in the environment. This chapter brings together the current state of knowledge on the biodegradation of pyridines.

Keywords Pyridine · Alkylpyridines · Photodegradation · Hydroxypyridines

1.1 Introduction

Simple pyridine derivatives enter the environment through natural and anthropogenic routes, and some pose documented health risks. Biodegradation of these compounds has been evaluated for more than 60 years, and these studies have revealed a number of common themes. There is, however, a considerably smaller body of literature dealing with biodegradation of pyridines than for hydrocarbons. An effort was made herein to be thorough in representing the state of knowledge regarding the

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biodegradation of pyridines. When we last reviewed this topic in 1989 (Sims GK, O' Loughlin EJ: Crit Rev Environ Control 19:309-340, 1989), there were considerable knowledge gaps in degradative mechanisms, little information on anaerobic biodegradation, and almost no evidence for any of the genes encoding degradation pathways; however, there have been a number of advances in each of these areas in the past decade.

The pyridine ring occurs in biological systems. It was originally discovered by Thomas Anderson (1851) in bone oil (Anderson 1851). Thus, both synthesis and degradative pathways exist in nature. Unsubstituted pyridine is seldom found in living organisms but has been isolated from rayless goldenrod (Buehrer et al. 1939). While pyridine and alkylpyridines generally are not present at high concentrations in living organisms, pyridine derivatives occur ubiquitously as pyridoxine (vitamin B_6) and vitamin B_3 (primarily in the form of niacin [nicotinic acid or pyridine-3-carboxylic acid] and nicotinamide [3-pyridinecarboxamide]) which is used in the synthesis of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) and less commonly as plant alkaloids (nicotine, trigonelline, arecoline, actinidine, anabasine, anatabine, ricinine, gentianine, and trigonelline). Trigonelline is also found in the urinary waste of mammals, as it is formed by methylation during niacin metabolism. Pyridine-2,6-dicarboxylic acid (dipicolinic acid) is a major component of bacterial endospores (Slieman and Nicholson 2001). Pyridine and alkyl pyridines are flavor components of beer (Harding et al. 1977) and a variety of foods (Suyama and Adachi 1980). They are formed during cooking of meats, likely due to the reaction of alkanals with amino acids (Hui 2012). Maga (1981) listed dozens of foods in which pyridines are important organoleptic compounds (including artichoke, asparagus, barley, beans, cheeses, cocoa, coffee, eggs, peanuts, pecans, rice, rum, and whiskey). In many cases, alkylpyridines found in foods are actually formed during the cooking process. Thermal decomposition of some amino acids produces pyridines, such as alpha-alanine, which produces 2-methyl-5-ethylpyridine (Lien and Nawar 1974) and cysteine, which releases pyridine, 2-methylpyridine, and 3-methylpyridine (Kato et al. 1973). Pyridines are found in the asphaltene fractions of crude oil and, as noted below, are formed during heating of fossil fuels in gasification and extraction processes. Marine crude oils generally contain lower concentrations of pyridines than terrestrial crude, owing to solubility

More commonly, pyridines are of anthropogenic origin. They are high production volume solvents and traded internationally. The current global market is estimated to be more than $500,000,000 and increasing. Pyridines have a broad range of industrial uses, such as solvents and reactants in organic synthesis, and pyridine is added to ethanol to discourage recreational consumption. The quaternary amine detergent, cetylpyridinium bromide, is a common antiseptic in consumer products like toothpastes and mouthwash. Pyridine-based conductive polymers (Yang et al. 2014) offer promise as energy storage nanoparticles. Pyridinium compounds can be used for desulfurization of fuel oils (Verdía et al. 2011) and as a stabilizing donor ligand for olefin metathesis (Occhipinti et al. 2017). Pyridine moieties are found in a number of drugs, such as isoniazid and sulfapyridine (antibiotics), altinicline

(experimental drug for Parkinson's disease), flupirtine (non-opioid analgesic), lansoprazole (anti-ulcerative), and pantoprazole (treatment of gastroesophageal reflux disease). Other drugs include nikethamide (a respiratory stimulant), eucaine (local anesthetic), demerol (analgesic), and antihistamines, chlorpheniramine maleate and pyrilamine maleate. Pyridine rings are found in herbicides across a spectrum of modes of action, such as the photosystem I inhibitors diquat and paraquat; the acetolactate synthase (ALS)-inhibitors imazamox, imazapyr, and nicosulfuron; the synthetic auxins aminopyralid, clopyralid, fluroxypyr, picloram, and triclopyr; and the aquatic herbicide fluridone, which inhibits carotenoid synthesis. They are found in insecticides, including the organophosphate chlorpyrifos, the feeding inhibitor chlorantraniliprole, and the neonicotinoid insecticide imidacloprid. Other pesticides, such as the nitrification inhibitor nitrapyrin; the fungicide boscalid, which inhibits spore germination; and the avicide starlicide, are based on pyridine chemistry. Pyridine-based pesticides are widely used and in 2013 represented approximately 10% of the global pesticide market with total sales of $5 billion, of which chlorantraniliprole, imidacloprid, and paraquat accounted for $1240, $1070, and $905 million, respectively (Guan et al. 2016).

Over the past 30 years, the US Environmental Protection Agency (EPA) Toxics Release Inventory (TRI) reported annual releases of pyridine to the environment of roughly 250,000 kg, about 90% of which was released to soil and the remainder to air and water. Of the more than 1700 current and closed sites on the EPA National Priorities List (Superfund Program), 4 have been found to be primarily contaminated with pyridine, although not all sites were tested for it. Coal tar distillation was once a common source of pyridine derivatives (most are now prepared synthetically), thus they are often found near legacy sites (Pereira et al. 1983, 1987). Pyridine; 2-, 3-, and 4-methylpyridine; as well as other more complex alkylpyridines are common contaminants associated with fossil fuels and gasification sites (Stuermer et al. 1982). Zamfirescu and Grathwohl (2001) observed that contaminant plumes associated with a legacy gasification site changed in composition along the flowpath and that N-heterocycles tended to become enriched with distance from the source. Diesel fuels derived from either fossil fuels (Hughey et al. 2001) or biodiesel (Lin et al. 2007) often contain pyridine derivatives. A part of the asphaltene component of crude oil, pyridines and other N-heterocycles contribute to fouling of catalysts used to refine petroleum, which has led to development of catalytic techniques to convert pyridines to hydrocarbons (Duan and Savage 2011). As unconventional on-land oil and gas production has increased, so has the wastewater volume, growing from 5.7 to 138 × 10^9 m^3 between 1998 and 2010 (Gregory et al. 2011). In some cases, such as a site in Rundle, Australia, the base fraction of the process water from oil shales may be limited entirely to N-heterocyclic compounds (Dobson et al. 1985). Using 2D gas chromatography, Dijkmans et al. (2015) showed that oil shales contain pyridine derivatives, indicating the compounds may naturally be present in the shales. Alkyl pyridines are reported to be used in hydraulic fracturing fluids and have been detected in flowback or produced water from unconventional oil production (Hayes and Severin 2012). Alkyl pyridines were also detected in products of

hydrothermal liquefaction of the biofuel alga, *Nannochloropsis salina* (Sudasinghe et al. 2014).

1.2 Chemical Properties

The six-membered pyridine ring is planar (the average bond angle is 120°), with aromatic character and a high resonance energy (23 kcal/mol), and its chemistry is defined by the nitrogen heteroatom at position one (Fig. 1.1). Pyridine and methylpyridines are hygroscopic and miscible with water as well as a number of organic solvents. The UV absorption spectrum of pyridine varies with substitutions and pH. Molar absorptivity is generally greater in the protonated form, and there is a general tendency for a shift to longer wavelengths with most common substitutions and protonation of the ring N.

The electronegative nitrogen heteroatom results in electron-deficient ring carbon atoms, especially at positions 2, 4, and 6. The heteroatom is thus electron rich. This alters reactivity of the pyridine ring relative to benzene. For example, pyridine ring carbons resist oxidation, which is borne out in its stability in strongly oxidizing dichromate reagents, such as pyridinium dichromate and pyridinium chlorochromate, used to oxidize alcohols to carbonyls. Unlike pyrrole, the third sp2 orbital of the pyridine heteroatom has only one pair of electrons, making pyridine a stronger base than pyrrole due to the availability of the nitrogen heteroatom to share electrons with acids. Compared to aliphatic amines, pyridine is a weak base ($pK_a = 5.17$ versus 10.56 for 4-aminobutyrate) (Dean 1987); pyridine derivatives with electron-withdrawing substituents are even weaker bases than pyridine itself (e.g., $pK_a = 0.72$, 1.01, and 1.25 for 2-chloropyridine, pyridine-2 carboxylic acid, and 2-hydroxypyridine, respectively); conversely electron-donating substituents make pyridine derivatives more basic (e.g., $pK_a = 5.96$, 6.71, and 7.43 for 2-methylpyridine, 2-aminopyridine, and 2,4,6-trimethylpyridine, respectively). Moreover, the basicity of the substituted pyridine is dependent on the position of the substituent on the ring, as illustrated by the hydroxypyridine series 2-hydroxypyridine ($pK_a = 1.25$), 3-hydroxypyridine ($pK_a = 4.80$), and 4-hydroxypyridine ($pK_a = 3.23$).

In either electrophilic or nucleophilic substitution reactions, pyridine behaves similarly to benzene substituted with electron-withdrawing groups. Thus it resists

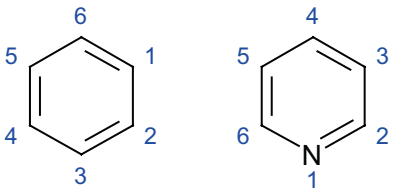

Fig. 1.1 Comparison of the structures for benzene (left) and pyridine (right). The presence of the N heteroatom causes significant changes in chemical properties relative to benzene

electrophilic reactions such as halogenation, nitration, and sulfonation, especially at positions ortho or para to the nitrogen due to the resulting positively charged nitrogen atom. Pyridine is also relatively resistant to addition reactions.

Hydroxyl groups activate pyridine to electrophilic attack, especially ortho and para to the hydroxyls (which exist predominantly as the ketone tautomer). To probe the potential reactivity toward oxygenation reactions, Houghton and Cain (1972) challenged pyridine and monosubstituted hydroxypyridines in the Udenfriend system (Udenfriend et al. 1954), which consists of Fe(II), EDTA, ascorbic acid, and molecular oxygen, which has been used to mimic biological oxidations, such as monooxygenase reactions. Pyridine was nonreactive in the Udenfriend system and in fact has subsequently been used as a solvent to conduct Udenfriend system investigations (Barton and Delanghe 1998). These findings, along with the extreme stability of pyridine toward oxidation, such as in dichromate (Holloway et al. 1951; Westheimer and Chang 1959), Kjeldahl oxidation (Dakin and Dudley 1914), or Gif reactions (Barton and Delanghe 1998), would suggest a reductive mechanism that would be more favorable for biodegradation than oxygenase attack. Houghton and Cain (1972) found that the Udenfriend system produced 2,3- and 2,5-diols from 2-hydroxypyridine, while 3- hydroxypyridine produced 2,3-, 3,4-, and 3,5- diols, and 4-hydroxypyridine produced 3,4-dihydroxypyridine and pyridine N-oxide. These results would suggest ring carbons in hydroxypyridines (or the corresponding ketone tautomer) that would be more favorable substrates for monooxygenase attack than those in pyridine and that pyridine should preferentially be attacked at the nucleophilic N heteroatom. These generalizations are consistent with early investigations into the biodegradation of pyridine and simple pyridine derivatives, in which the only oxygen-containing metabolite detected from pyridine was pyridine-N-oxide, while hydroxypyridines and alkylpyridines produced intermediates hydroxylated in the expected positions. It was generally assumed that unsubstituted pyridine could be reduced to form a dihydro- or tetrahydro-pyridine product that would be susceptible to hydroxylation by the enzymatic addition of water.

Substituted pyridines, particularly those containing hydroxyl and carboxyl groups, form complexes with a broad range of transition metals. 3-Hydroxypyridine forms complexes with Cu(II), Ni(II), Co(II), Cd(II), and Cr(III) (Koval'chukova et al. 2002). Colored complexes resulting from the reaction of dihydroxypyridines with $FeCl_2$ have been used for the identification of these compounds in culture media (Houghton and Cain 1972). Pyridine carboxylic acids including pyridine-2-carboxylic acid (picolinic acid), nicotinic acid, and pyridine-2,6-dicarboxylic acid complex metals including V(IV), Cr(III), Mn(II), Fe(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), and Ag(II)(Allan et al. 1979; Chakov et al. 1999; Chang and Foy 1982; Kleinstein and Webb 1971; Sakurai et al. 1995; Yuen et al. 1983) and several pyridine carboxylic acid complexes of Cu(II), Cr(III),V(IV), and V(V) have been investigated as therapeutic agents for the treatment of diabetes (Crans et al. 2000; Nakai et al. 2004, 2005; Preuss et al. 2008; Thompson and Orvig 2001; Willsky et al. 2011). In addition, pyridine-2,6-dicarboxylic acid is an effective extractant for the recovery of Cd(II) and Pb(II) from soils (Hong and Chen 1996; Macauley and Hong 1995). The herbicide picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic

acid) complexes Fe(II), Fe(III), Ni(II), and Cu(II) (Michaud and Hoggard 1988; Yuen et al. 1983). Pyridine-metal complexes have found use in analytical chemistry, perhaps most notably the use of 2,2′-bipyridine and the bipyridine derivative 1,10-phenanthroline as reagents in colorimetric assays for Fe(II) (Cagle and Smith 1947; Fortune and Mellon 1938).

1.3 Abiotic Factors in the Fate of Pyridines

Photodegradation: The UV absorption maximum of pyridine is 256 nm in neutral to acidic aqueous solutions and decreases to 249 nm in the vapor phase (Errami et al. 2016). The compound is susceptible to photochemical degradation in the vapor phase (Errami et al. 2016) or in aqueous solution (Elsayed 2014), whereas sonochemical degradation proceeds only very slowly (Elsayed 2014). Abiotic degradation rates increase somewhat in the presence of H_2O_2 (Elsayed 2014). While photochemical degradation appears to be only moderately effective for wastewater treatment, degradation in the atmosphere generally occurs in less than 2 days and is likely the primary fate of atmospheric pyridine (Errami et al. 2016). Several pyridine-based pesticides are also susceptible to photochemical degradation, including the nitrapyrin (2-chloro-6-(trichloromethyl)pyridine) hydrolysis product, 6-chloropyridine-2-carboxylic acid (Redemann and Youngson 1968), paraquat (Slade 1965), diquat (Slade and Smith 1967), fluroxypyr (Hu et al. 2014), imazamox (Quivet et al. 2006), fluridone (MacDonald et al. 1996), chlorpyrifos (Wu et al. 2006), picloram (Gear et al. 1982), imazapyr (Pizarro et al. 2005), and imidacloprid (Cacho et al. 1999).

Transport: Pyridines are mobile in the environment. Volatilization is a major path for loss of pyridine and alkylpyridines from soil and aquatic environments (Sims and Sommers 1985, 1986). The vapor pressure at 25 °C is approximately 0.15 MPa for pyridine (Weast et al. 1989) and 0.03 MPa for methylpyridines (Chirico et al. 1999). Pyridine derivatives occur in tobacco smoke (Schmeltz and Hoffmann 1977) and are detected in indoor environments in which smoking occurs (Jenkins et al. 2000). Pyridines are emitted into the atmosphere from shale wastewater (Hawthorne et al. 1985). Pyridine and all three methylpyridine isomers are completely miscible with water, and, as a result, pyridines occur in groundwater near coal gasification sites (Leenheer and Stuber 1981), wastewater from unconventional on-land oil and gas production (Brown et al. 2015; Dobson et al. 1985; Leenheer et al. 1982; Riley et al. 1981), and in aquifers beneath major pyridine spills (Fuller 2015; Ronen et al. 1996). Pyridine pesticides including chlorantraniliprole (Vela et al. 2017), imazapyr (Porfiri et al. 2015), picloram (Pang et al. 2000), fluroxypyr, and clopyralid (Ulen et al. 2014) have been shown to leach through soil, and picloram (Lym and Messersmith 1988) and boscalid (Reilly et al. 2012) have been detected in groundwater.

Sorption: Pyridines absorb to a broad range of geologically/environmentally relevant materials including clay minerals (Baker and Luh 1971; Laird and Fleming

1999; O'Loughlin et al. 2000; Sabah and Celik 2002; Zachara et al. 1990), zeolites (Rawajfih et al. 2010), metal oxides (Vasudevan et al. 2001), organic matter (Ahmed et al. 2014; Graber and Borisover 1998), and whole soils (Bi et al. 2006, 2007; Zhu et al. 2003). Moreover, sorption of pyridines by agricultural and geological waste materials can be an effective treatment for wastewaters contaminated with pyridines (Ahmed et al. 2014; Lataye et al. 2008a, b; Mohan et al. 2005; Zhu et al. 1988). The sorption of pyridines to soils can occur by specific and nonspecific interactions with both organic matter and mineral components. As discussed previously, the nitrogen heteroatom of pyridine is a base with pK_a values ranging from −0.4 to 9.1 depending on the substituents and their position on the ring (Dean 1987). As such, the sorption of pyridines to soils is typically dominated by cation exchange processes at pH values where the protonated form is the dominant species (i.e., below the pK_a) (Baker and Luh 1971; Bi et al. 2007; Laird and Fleming 1999; Zachara et al. 1987, 1990; Zhu et al. 2003); however, surface acidity can lead to predominance of the protonated form 1 to 1.5 pH units above the pK_a of a given pyridine (Laird and Fleming 1999). At pH values far in excess of the pK_a, as well as for pyridines with nonionizable nitrogen heteroatoms (e.g., hydroxypyridines) or with highly nonpolar substituents, sorption is primarily by means of surface complexation or van der Waals interactions (Bi et al. 2006; Laird and Fleming 1999; Sabah and Celik 2002; Vasudevan et al. 2001). Sorption of pyridines to soils and sediments has significant impacts on their environmental behavior including mobility, reduction in bioavailability (e.g., reduced toxicity and biodegradation), and increase in persistence (Bi et al. 2006; Johnson et al. 1995; Gebremariam et al. 2012; Leenheer and Stuber 1981; Loux et al. 1989; O'Loughlin et al. 2000; Starr and Cunningham 1975; Stougaard et al. 1990).

1.4 Biological Factors in the Environmental Fate of Pyridines

Biodegradability: Though often described as refractory, pyridine and alkyl pyridine derivatives are often highly biodegradable; however, as with homocyclic compounds, the nature and position of ring substituents have profound effects on biodegradation rates. Pyridine has a relatively short biodegradation half-life of 7 days or less in aqueous media. Alkylpyridines, pyridine carboxylic acids, and hydroxypyridines degrade somewhat more slowly, with half-lives ranging from 7 to 24 days. Among the most recalcitrant of the simple pyridines are halopyridines and aminopyridines (Naik et al. 1972; Sims and Sommers 1985, 1986).

Degradation in natural and engineered environments: Pyridines can be removed from wastewater using biodegradation. Phenol-fed aerobic granules have been used as supports for microbial removal of pyridine from pharmaceutical wastewater (Adav et al. 2007). Wastewater from a coke processing plant was treated with a microbial bioreactor to remove organic contaminants, including pyridine (Li et al. 2003). Pyridine biodegradation has been coupled to energy production in microbial fuel cells (Zhang et al. 2009).

A large body of literature is available on the environmental behavior of many pyridine pesticides. Biodegradation of picloram, chlorpyrifos, triclopyr, and fluroxypyr in the environment usually involves initial hydroxylation (Lee et al. 1986; Lehmann et al. 1990; Leoni et al. 1981; Meikle et al. 1974). The trichloromethyl moiety of the nitrification inhibitor, nitrapyrin, is converted to 6-chloropyridine-2-carboxylic acid in soils (Wolt 2000). The organophosphate insecticide, chlorpyrifos, exhibited faster degradation in mineral than organic soil, possibly owing to enhanced sorption (Chapman and Harris 1980). In the field, a pyridinol metabolite was shown to persist for months after chlorpyrifos was degraded (Leoni et al. 1981). Numerous authors have demonstrated biodegradation of chlorpyrifos by diverse genera of bacteria and fungi (Chishti et al. 2013). Fluridone appears to be persistent in soils, especially under flooded conditions (Marquis et al. 1982), and often remains detectable in oxic soils a year after application (Banks et al. 1979; Schroeder and Banks 1986); prior exposure enhanced degradation rates, suggesting microbial involvement (Banks et al. 1979). Degradation of imazethapyr, which also appears to be mediated by microorganisms in soils, is limited by bioavailability due to soil sorption (Loux et al. 1989). Though highly persistent, the avicide, 4-aminopyridine was sufficiently sorbed to soils to exhibit limited leaching (Starr and Cunningham 1975).

1.5 Aerobic Biodegradation of Pyridine

Early work on the microbial metabolism of the pyridine ring was motivated primarily by a need to better understand metabolism of nicotine (3-[(2S)-1-methyl-2-pyrrolidinyl]-pyridine), nicotinic acid, nicotinamide, and the coenzymes NAD and NADP. Interest in pyridines as environmental contaminants increased after a surge in the development of synfuel plants following passage of the US Energy Security Act in 1980. The Energy Security Act included the US Synthetic Fuels Corporation Act, which called for a drastic increase in synfuel production to reduce dependence on oil imports. Early research on the environmental impacts of synfuel plants identified contamination of water with pyridine and alkylpyridines as a major risk factor. This discovery spurred research on the environmental fate of pyridine and alkylpyridines throughout the 1980s. Also in the 1980, a major spill (227,000 L) of mixed pyridines resulted in significant groundwater contamination in Indianapolis, IN, which further promoted research on the fate of these compounds.

The degradation of pyridines has been reported under both oxic and anoxic conditions (Fetzner 1998; Kaiser et al. 1996; Li et al. 2001; Rhee et al. 1997) by pure or mixed cultures (Lodha et al. 2008) across a range of phylogenetically diverse microorganisms including *Nocardia* sp. (Watson and Cain 1975), *Bacillus* sp. (Watson and Cain 1975), *Paracoccus* spp. (Bai et al. 2008; Lin et al. 2010; Qiao and Wang 2010; Wang et al. 2018), *Pseudomonas* spp. (Mohan et al. 2003; Padoley et al. 2009), *Rhodococcus* spp. (Do et al. 1999; Yoon et al. 2000a), *Arthrobacter* spp. (Kolenbrander et al. 1976; Kolenbrander and Weinberger 1977; Zefirov et al. 1994), *Shewanella* sp. (Mathur and Majumder 2008), *Alcaligenes* sp. (Ronen et al. 1994),

Shinella sp. (Bai et al. 2009), *Gordonia* sp. (Yoon et al. 2000b), *Pimelobacter* sp. (Lee et al. 1994), and *Achromobacter* sp. (Deng et al. 2011), among others. The biodegradation of pyridine is reported to occur via two different mechanisms: ring reduction or ring hydroxylation followed by ring fission. Resistance of the pyridine ring to oxidation (as discussed above), as well as the frequent detection of reduced metabolites, supports the existence of a reductive mechanism. On the other hand, the identification of pyridine ring fission products containing oxygen is evidence for pathways involving hydroxylation. However, the question of biological oxidation of the unsubstituted ring remained a question for many years.

Ring reduction: Nocardia sp. Z1 was the first microbe reported to utilize pyridine as a sole carbon, nitrogen, and energy source (Houghton and Cain 1972). No hydroxyl-substituted metabolites were identified during pyridine degradation, suggesting the involvement of a reductive mechanism. *Brevibacterium* sp., *Corynebacterium* sp., *Bacillus* sp. 4, and *Streptomyces* sp. HJ02 were subsequently reported to degrade pyridine by ring reduction (Shukla 1973; Shukla and Kaul 1974; Watson and Cain 1975; Li et al. 2009). Based on radiolabeling studies and the use of mutant strains to examine pyridine metabolism by *Bacillus* sp. 4 and *Nocardia* sp. Z1, two different fission pathways were proposed (Watson and Cain 1975) resulting in the accumulation of either glutaric acid semialdehyde or succinic acid semialdehyde, respectively (Fig. 1.2). Different products have been shown to accumulate depending upon the bonds involved in ring fission. Cleavage between C-2 and C-3 resulted in the formation of succinic acid, whereas cleavage between C-1 and C-2 resulted in the formation of glutaric acid (Fig. 1.2). *Micrococcus luteus* was

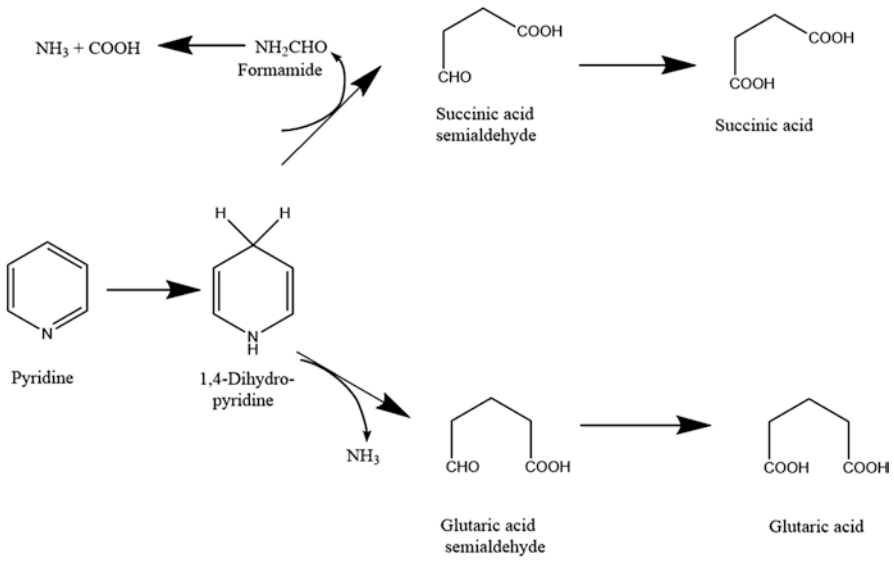

Fig. 1.2 Proposed mechanism for degradation of pyridine via hydration in *Bacillus* sp. and *Nocardia* sp. (Kaiser et al. 1996)

also reported to utilize pyridine via the pathway leading to succinate (Sims et al. 1986) as proposed by Watson and Cain (1975) for *Bacillus* sp. 4. However it was observed that in addition to pyridine degradation products, pyridine metabolism by *M. luteus* also resulted in the accumulation of riboflavin (Sims and O'Loughlin 1992). Although riboflavin was not an intermediate product of pyridine degradation, it was produced only during growth on pyridine, suggesting that pyridine may have played a role in regulating riboflavin production. Riboflavin overproduction has also been reported in hydrocarbon degradation (Sabry et al. 1989) and degradation of 2-methylpyridine (O'Loughlin et al. 1999; Shukla 1974).

The primary evidence for ring reduction prior to cleavage has been the liberation of reduced fission products. The inability to detect the initial dihydropyridine intermediates is not surprising, given their tendency to auto-oxidize to their pyridine analogs very quickly, sometimes in as little as 2 h (Baranda et al. 2006). In addition, tetrahydropyridines can be oxidized to their pyridine analogs by oxygenases (such as monoamine oxidase or MAO) without concomitant incorporation of oxygen into the product (Przedborski et al. 2001). Alternatively, the inability to detect cyclic intermediates may be explained by their absence, as it has recently been demonstrated that it is possible to open a pyridine ring without hydroxylation through direct fission of the C5-C6 bond (Perchat et al. 2018), although this has only been shown for N-methylnicotinate. However, some of the missing steps in a reductive mechanism have finally been demonstrated. Wang et al. (2018) have identified several reduced cyclic compounds produced during pyridine metabolism by *Paracoccus* sp. NJUST30, unequivocally demonstrating that the ring was initially reduced before it was hydroxylated. The hydroxylation step would only require a simple hydration of a double bond. That work, which will be discussed further below, revealed a simple "work around" to biologically produce hydroxylated metabolites from pyridine without the need to overcome pyridine's resistance to oxidation. Oxygenase activity was not evaluated by Wang et al. (2018), thus it is not clear whether simple hydration of a double bond or perhaps oxygenase activity was involved in hydroxylation. Alternatively, the absence of oxygenase activity may account for the authors' success in isolating the reduced cyclic metabolites, which otherwise may have converted to pyridine analogs, as has been shown with dihydropyridines (Baranda et al. 2006).

The *Paracoccus* sp. isolated from a sequencing batch reactor by Wang et al. (2018) was able to degrade pyridine at relatively high concentrations (500 mgL^{-1}) within roughly 1 week. Numerous metabolites were isolated that provide insight into possible variations of a reductive pathway for pyridine degradation, leading the authors to propose three possible arrangements of the metabolites detected, each beginning with a hydroxylated dihydropyridine derivative. Once aromaticity was lost due to reduction, the ring would have been susceptible to a wide variety of common catabolic reactions. Other metabolites reported in Wang et al. (2018) were consistent with alternating dehydrogenation and hydration steps, ubiquitous in hydrocarbon degradation. Fully reduced piperidin-2-ol was observed, as was succinate semialdehyde, the latter of which has been reported in other studies in which C2-C3 ring cleavage occurred. A reduced form of glutamate (4-formylamino-

butyric acid) was reported as well. Glutamate is converted to succinate via succinate semialdehyde in the gamma-aminobutyrate (GABA) shunt in bacteria. Processing pyridine metabolites by recruitment of such ubiquitous pathways likely reduces the number of novel mechanisms required for the organism to degrade pyridine.

Ring hydroxylation: The resistance of pyridine to hydroxylation is predicted by its chemistry, and early studies of pyridine biodegradation did not show evidence of formation of hydroxypyridine intermediates (Shukla and Kaul 1974, 1975, 1986; Sims et al. 1986; Watson and Cain 1975). However, since our 1989 review, the degradation of pyridine via ring hydroxylation has been reported for *Rhodococcus opacus* (VKM Ac-1333D) (Zefirov et al. 1994), *Arthrobacter crystallopoietes* (VKM Ac-1334D) (Zefirov et al. 1994), and *Arthrobacter* sp. KM-4 (Khasaeva et al. 2011). Zefirov et al. (1994) was the first to propose the degradation of pyridine via hydroxylation by *R. opacus* and *A. crystallopoietes*. Hydroxylation products 2-hydroxypyridine and 2,6-dihydroxypyridine were detected in the media containing pyridine with *R. opacus*, whereas 3-hydroxypyridine, 2,3-dihydroxypyridine, and 2,3,6-trihydroxypyridine were identified with *A. crystallopoietes* (Fig. 1.3). Catabolism of pyridine was proposed by hydroxylation at positions 2, 3, and 6 followed by C_2-C_3 bond cleavage resulting in the formation of N-formamide followed by 2,5-pyrroldione. This metabolite is finally reduced to succinate semialdehyde. A similar pathway involving initial hydroxylation to 2-hydroxypyridine, followed by hydroxylation to 2,3-dihydroxypyridine and ultimately leading to succinate semialdehyde, was proposed for pyridine metabolism by *Arthrobacter* sp. KM-4 (Khasaeva et al. 2011).

The ring hydroxylation steps in pyridine degradation pathways have been proposed based on the hydroxylated intermediates detected, but the enzymes involved

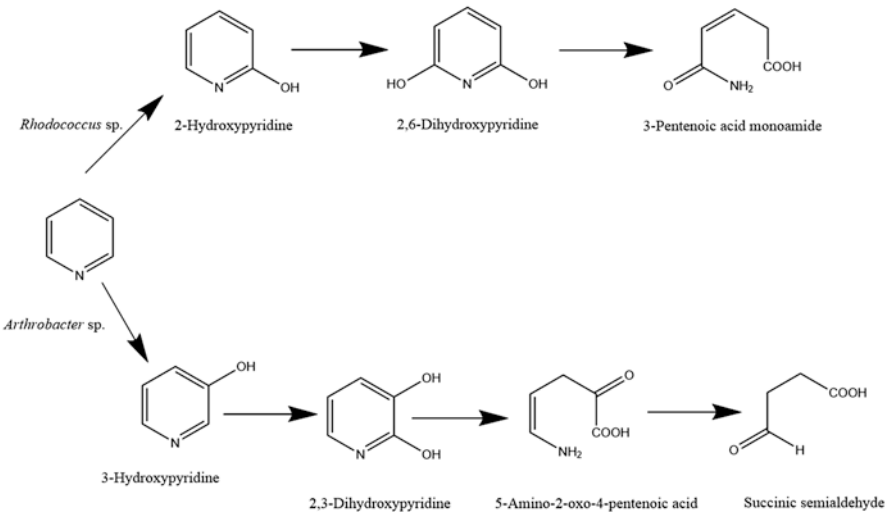

Fig. 1.3 Proposed pathways for the degradation of pyridine via hydroxylation by *R. opacus* and *A. crystallopoietes*. (After Zefirov et al. 1994)

in catalysis have not been identified as yet; however, the involvement of phenol hydroxylase has been suggested. A *Rhodococcus* sp. with the ability to utilize pyridine as the sole carbon and nitrogen source with an increased ability to utilize pyridine in the presence of phenol was isolated by Sun et al. (2011). In addition to pyridine and phenol, it also has the ability to reduce chromium (VI) simultaneously. It was reported that the phenol hydroxylase gene was induced in the presence of pyridine as a nitrogen source and the addition of pyridine with phenol induced increased expression of phenol hydroxylase. Owing to the structural similarity of phenol with pyridine, increased expression of the hydroxylase gene, and the identification of hydroxylated pyridine intermediates, it was proposed that phenol hydroxylase catalyzes the hydroxylation of pyridine leading to metabolites similar to those reported by Zefirov et al. (1994) for pyridine degradation by *R. opacus*. However, the hydroxylation of pyridine by *Diaphorobacter* sp. J5-51, *Acinetobacter* sp. SJ-15, *Acinetobacter* sp. SJ-16, *Acidovorax* sp. J5-66, and *Corynebacterium* sp. JOR-20 resulted in the accumulation of 1-hydroxypyridinium, and no growth was observed (Sun et al. 2014), suggesting that this reaction is a metabolic dead end. Indeed, heterologous expression of the phenol hydroxylase gene in a non-pyridine-degrading *Pseudomonas* sp. CO-44 resulted in the detection of metabolites observed during pyridine metabolism in *Diaphorobacter* sp. J5-51, but the metabolite accumulated was not utilized further.

Pyridine degraders may possess multiple mechanisms to produce a given metabolite within a single organism. Two initial monooxygenase reactions were described to compete for reductant during pyridine degradation via an oxidative mechanism. The first monooxygenase, which formed 2-hydroxypyridine, was observed to preferentially utilize available reductant. Competition continued even after 2-hydroxypyridine concentration exceeded that of pyridine (Yang et al. 2018). Ring hydroxylation and interactions between oxygenases and pyridine rings should be a rewarding areas for future research, as such interactions have recently been shown capable of producing unexpected results. For example, oxidation of tetrahydropyridines (Przedborski et al. 2001) as well as direct cleavage of a pyridine ring in the alkaloid trigonelline (Perchat et al. 2018) by oxygenases without the incorporation of a hydroxyl group has now been demonstrated.

1.6 Aerobic Biodegradation of Hydroxypyridines/Pyridones

As discussed above, hydroxypyridines have been identified as intermediates in the degradation of pyridine by several bacteria (Khasaeva et al. 2011; Zefirov et al. 1994). However, many microorganisms not known to degrade pyridine have been shown to degrade monohydroxypyridines (or their corresponding pyridone tautomer) including members of the genera *Achromobacter* (Cain et al. 1974), *Agrobacterium* (Watson et al. 1974a), *Arthrobacter* (O'Loughlin et al. 1999; Kolenbrander et al. 1976; Gasparaviciute et al. 2006), *Bacillus* (Sharma et al. 1984), *Burkholderia* (Stankeviciute et al. 2016), *Pseudomonas* (Zefirov et al. 1993), and

others. Most proposed 2-hydroxypyridine degradation pathways begin with hydroxylation to 2,5-dihydroxypyridine, followed by maleamic acid, maleic acid, and fumaric acid formation (i.e., the maleamate pathway) as reported for *Achromobacter* sp. G2 (Cain et al. 1974), *Bacillus brevis* (Sharma et al. 1984), *Burkholderia* sp. MAK1 (Stankeviciute et al. 2016), and *Nocardia* sp. (in which case 2,3,6-trihydroxypyridine is a purported intermediate between 2,5,-dihydroxypyridine and maleamic acid) (Shukla and Kaul 1986) (Fig. 1.4). Stankevičiūtė et al. (2016) reported the presence of an inducible 2-hydroxypyridine 5-monooxygenase enzyme in *Burkholderia* sp. MAK1 responsible for converting 2-hydroxypyridine to 2,5-dihydroxypyridine. This monooxygenase is a soluble di-iron monooxygenase encoded by a five-gene cluster (*hpdA, hpdB, hpdC, hpdD, hpdE*) (Petkevicius et al. 2018). 2,5-Dihydroxypyridine is converted by *Burkholderia* sp. MAK1 to N-formylmaleamic acid which is further degraded to fumaric acid via the maleamate pathway (Petkevicius et al. 2018) (Fig. 1.4), analogous to the degradation of 2,5-dihydroxypyridine by *Pseudomonas putida* KT2440, *P. putida* S16, and *Ochrobactrum* sp. SJY1 (Jiménez et al. 2008; Tang et al. 2012; Yu et al. 2015).

Not all 2-hydroxypyridine biodegradation pathways proceed through 2,5-dihydroxypyridine. Conversion of 2-hydroxypyridine to *cis*-5,6-dihydro-5,6-

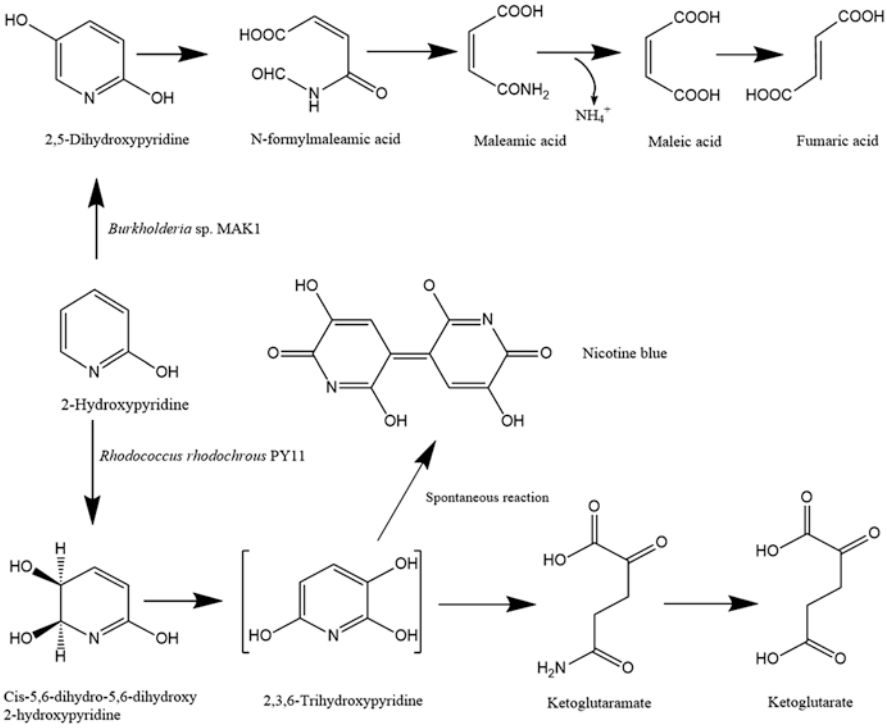

Fig. 1.4 Proposed pathway for the degradation of 2-hydroxypyridine by *Burkholderia* sp. MAK1 (Petkevicius et al. 2018) and *R. rhodochrous* PY11. (Vaitekunas et al. 2016)

dihydroxy-2-hydroxypyridine catalyzed by HpoBCDF, a four-component dioxygenase, is the first reaction of 2-hydroxypyridine degradation in *Rhodococcus rhodochrous* PY11 (Vaitekunas et al. 2016). The product of the first reaction is converted to 2,3,6-trihydroxypyridine by 2-pyridone-5,6-dihydro-cis-5,6-diol dehydrogenase (HpoE). The 2,3,6-trihydroxypyridine ring is opened by a hydrolase (HpoH) to form 2-ketoglutaramate which is subsequently transformed to α-ketoglutarate by 2-ketoglutaramate amidase (HpoI). *hpoH* encodes for the hydrolase protein catalyzing the ring opening step, and *hpoI* encodes amidase catalyzing the removal of amide group from 2-ketoglutaramate.

Although several proposed pyridine/2-hydroxypyridine pathways have included 2,3,6-trihydroxypyridine as an intermediate (Shukla and Kaul 1986; Vaitekunas et al. 2016; Zefirov et al. 1994), it is unstable under oxic conditions and has not been directly observed as an intermediate in 2-hydroxypyridine degradation; rather its formation has been inferred. The formation of a blue pigment is often reported during 2-hydroxypyridine degradation (Shukla and Kaul 1986; Vaitekunas et al. 2016), particularly among *Arthrobacter* spp. (Ensign and Rittenberg 1963; Kolenbrander et al. 1976; Kolenbrander and Weinberger 1977; O'Loughlin et al. 1999; Semenaite et al. 2003; Gasparaviciute et al. 2006; Stanislauskiene et al. 2012). The blue pigment produced during 2-hydroxypyridine degradation by *A. crystallopoietes* (Ensign and Rittenberg 1963) was identified as 4,5,4′,5′-tetrahydroxy-3,3′-diazadiphenoquinone-(2,2′)(Kuhn et al. 1965), also known as nicotine blue, and has been confirmed as the blue pigment produced during 2-hydroxypyridine degradation by *Arthrobacter pyridinolis* (Kolenbrander et al. 1976), *Arthrobacter viridescens* (Kolenbrander and Weinberger 1977), and a *Nocardia* sp. (Shukla and Kaul 1986). Since the spontaneous oxidation of 2,3,6-trihydroxypyridine in air results in the formation of nicotine blue (Holmes et al. 1972), the presence of a blue pigment during pyridine/2-hydroxypyridine degradation is often cited as evidence for the formation of 2,3,6-trihydroxypyridine (O'Loughlin et al. 1999; Semenaite et al. 2003; Shukla and Kaul 1986; Vaitekunas et al. 2016; Zefirov et al. 1994). Further support for nicotine blue serving as a proxy for 2,3,6-trihydroxypyridine formation is provided by enzymatic studies. Nicotine blue was the observed product of 2,3,6-trihydroxypyridine by 2-pyridone-5,6-dihydro-cis-5,6-diol dehydrogenase (HpoE) in the 2-hydroxypyridine degradation pathway proposed for *Rhodococcus rhodochrous* PY11 that requires 2,3,6-trihydroxypyridine as in intermediate (Vaitekunas et al. 2016). Moreover, the reaction of 2,6-dihydroxypyridine oxidase from *Arthrobacter oxydans* with 2,6-dihydroxypyridine under low oxygen conditions resulted in the transient accumulation of a product with UV-Vis spectral properties similar to 2,3,6-trihydroxypyridine, followed by accumulation of nicotine blue (Holmes et al. 1972).

Compared to 2-hydroxypyridine, the microbial degradation of 3-hydroxypyridine and 4-hydroxypyridine is less well characterized. *Rhodococcus* sp. Chr-9 can grow with 3-hydroxypyridine as a sole carbon source, but metabolite production was not investigated as the focus of the study was on pyridine degradation (Sun et al. 2011). Zefirov et al. (1993) identified 2,3-dihydroxypyridine, 3,4-dihydroxypyridine, 3-hydroxypyridine-N-oxide, and 1,2-dihyro-2,3-dihydroxypyridine and

2,3-dihydroxypyridine and 3,4-dihydroxypyridine as products of 3-hydroxypyridine transformation by *Pseudomonas fluorescens* PfE1 and *R. opacus*, respectively. Similarly, pyridine-degrading Nocardia sp. Z1 was able to convert 3-hydroxypyridine to 2,3- and 3,4-dihydroxypyridine, which were not metabolized further (Houghton and Cain 1972). *Achromobacter* spp. 7 N and 2 L were isolated by enrichment with 3-hydroxypyridine as the principal carbon source (Houghton and Cain 1972). Both strains were able to convert 3-hydroxypyridine to 2,5-dihydroxypyridine, with particularly high accumulation of this product by strain 7 N. Further study of strain 2 L determined that 2,5-dihydroxypyridine was degraded by the maleamate pathway (Fig. 1.4) (Cain et al. 1974).

Watson and Cain isolated a 4-hydroxypyridine-degrading bacterium, *Agrobacterium* sp. 35S, that transforms 4-hydroxypyridine to 3,4-dihydroxypyridine by means of 4-hydroxypyridine-3-hydrolase (Watson et al. 1974a). 3,4-Dihydroxypyridine was transformed to 3-formiminopyruvate (with 3(N-formyl)-formiminopyruvate as a purported intermediate), followed by 3-formylpyruvate and finally pyruvate and formate (Watson et al. 1974b). *Arthrobacter* sp. IN13 is able to grow on both 2- and 4-hydroxypyridine as sole carbon sources; however no intermediates for 4-hydroxypyridine degradation have been identified (Gasparaviciute et al. 2006).

1.7 Aerobic Biodegradation of Alkylpyridines

The biodegradation of alkylpyridines has been reported for a range of microorganisms including *Bacillus* sp. (Reddy et al. 2009a), *Pseudomonas* sp. (Padoley et al. 2009), *Nocardia* sp. (Padoley et al. 2009), *Gordonia* sp. (Stobdan et al. 2008), *Arthrobacter* spp. (O'Loughlin et al. 1999; Shukla 1974; Khasaeva et al. 2011), *Pseudonocardia* sp. (Lee et al. 2006), and *Saccharomyces cerevisiae* (Gulyamova et al. 2006). Alpha-substituted alkylpyridines have been the most extensively studied. An *Arthrobacter* sp. isolated by enrichment on 2-methylpyridine by Shukla (1974) was able to use both 2-methylpyridine and 2-ethylpyridine as sole carbon and nitrogen sources. No aromatic intermediates were observed during 2-methylpyridine degradation, but based on metabolite analysis and differential substrate utilization, it was proposed that 2-methylpyridine degradation was not initiated either via methyl oxidation or by hydroxylation of the pyridine ring. Several tentative 2-methylpyridine pathways were proposed, all of which converged on the formation on succinate semialdehyde. Degradation of both 2-methylpyridine and 2-ethylpyridine by a different *Arthrobacter* sp. (strain R1) was reported by O'Loughlin et al. (1999). Unlike the *Arthrobacter* sp. isolate by Shukla, R1 could also degrade 2-hydroxypyridine. Both *Arthrobacter* spp. produced riboflavin during 2-methylpyridine degradation, as did *M. luteus* during pyridine degradation (Sims and O'Loughlin 1992).

Khasaeva et al. (2011) proposed two pathways for the degradation of 2-methylpyridine by *Arthrobacter* sp. KM-4, both of which ultimately converge

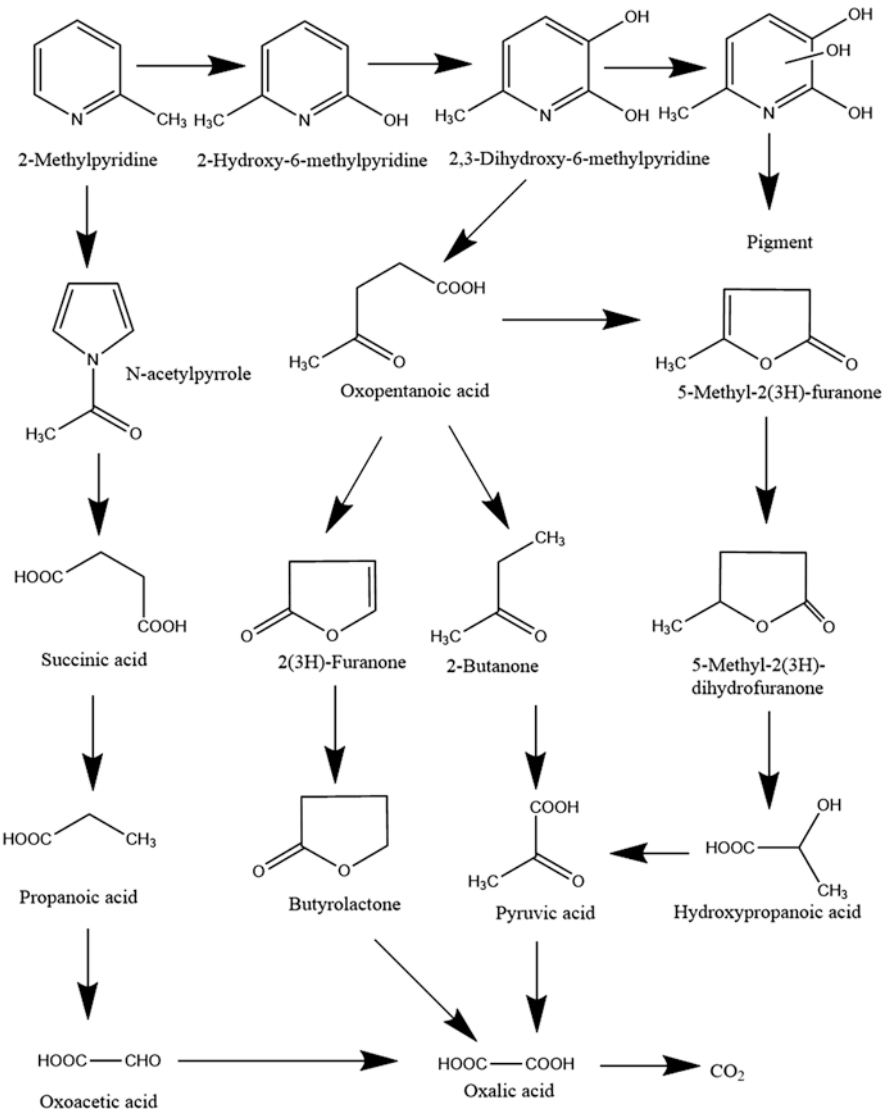

Fig. 1.5 Proposed pathway for 2-methylpyridine degradation by *Arthrobacter* sp. KM-4

with the formation of oxalic acid (Fig. 1.5). The first pathway involved formation of acetylpyrrole by means of a putative oxidative opening of the pyridine ring between C-2 and C-3 followed by cyclization to form a pyrrole ring. The second pathway involved double hydroxylation to form 2,3-dihydroxy-6-methylpyridine followed by ring cleavage between the hydroxyl groups and formation of 4-oxopentanoic acid.

2-Methylpyridine was used as the sole carbon and nitrogen source by *Bacillus cereus* GMHS (Reddy et al. 2009a). The degradation of 2-methylpyridine by *B. cereus* GMHS was initiated by hydroxylation of the methyl group resulting in 2-pyridinemethanol (Reddy et al. 2008). Reddy et al. (2009b) identified an 11 kb plasmid containing 2-methylpyridine degradation genes; elimination of the plasmid resulted in loss of 2-methylpyridine catabolism. Homology modeling and binding studies based on toluene dioxygenase from *P. putida* indicated that 2-methylpyridine was the preferred substrate after toluene, suggesting the involvement of toluene dioxygenase in 2-methylpyridine degradation by *B. cereus* GMHS (Reddy et al. 2008).

The degradation of other alkylpyridines has been less well studied than 2-methylpyridine. Although not degradation in the conventional sense, the yeast *S. cerevisiae* 913a-1 transforms 3-methylpyridine into nicotinic acid (pyridine-3-carboxylic acid) (Gulyamova et al. 2006). *Pseudomonas pseudoalcaligenes* strain KPN and a *Nocardia* sp. can use 3-methylpyridine as a sole carbon and nitrogen source (Padoley et al. 2009). Both 3-methyl- and 3-ethylpyridine can be used by *Gordonia nitida* LE31 as sole carbon and nitrogen sources (Yoon et al. 2000b). No cyclic intermediates were detected during 3-methyl- or 3-ethylpyridine degradation by LE31; however, the induction of levulinic acid degradation and the expression of levulinic aldehyde dehydrogenase activity in 3-methyl- or 3-ethylpyridine grown cells are consistent with C-2–C-3 ring cleavage (Fig. 1.6) (Lee et al. 2001). 4-Methylpyridine was used as a sole carbon and nitrogen source by *Gordonia terrae* strain IIPN1 (Stobdan et al. 2008) and *Arthrobacter* sp. strain KM-4 (Khasaeva et al. 2011). Transient accumulation of 2-hydroxy-4-methylpyridine and 2-hydroxy-4-ethylpyridine was observed during growth of *Pseudonocardia* sp. strain M43 on 4-methyl- and 4-ethylpyridine as sole carbon and nitrogen sources, respectively (Lee et al. 2006). Feng et al. (1994) reported that a mixed culture obtained from a soil enrichment was able to degrade 2-, 3-, and 4-ethylpyridine; 2-hydroxy-6-ethylpyridine and 2-hydroxy-4-ethylpyridine/4-ethyl-2-piperidinone were identified as intermediates in the degradation of 2-ethyl- and 4-ethylpyridine, respectively. Among di- and trimethylpyridines, 3,4-dimethylpyridine is degraded by *Pseudonocardia* sp. strain M43 (Lee et al. 2006); 2,6-dimethylpyridine is degraded by *P. pseudoalcaligenes* strain KPN and a *Nocardia* sp. (Padoley et al. 2009) and *Arthrobacter* sp. strain KM-4 (Khasaeva et al. 2011); and an *Arthrobacter* sp. degrades 2,4-dimethyl- and 2,4,6-trimethylpyridine (Shukla 1975).

1.8 Anaerobic Biodegradation of Pyridines

As with aerobic biodegradation studies, early work on anaerobic degradation of the pyridine ring focused on nicotinic acid and its analogs and metabolites. *Clostridium* sp. (Harary 1957a, b) and *Clostridium barkeri* (Stadtman et al. 1972) produce 6-hydroxynicotinate, acetate, propionate, ammonium, and CO_2 from nicotinic acid. *C. barkeri* was shown to have a nicotinic acid hydrolase that produced

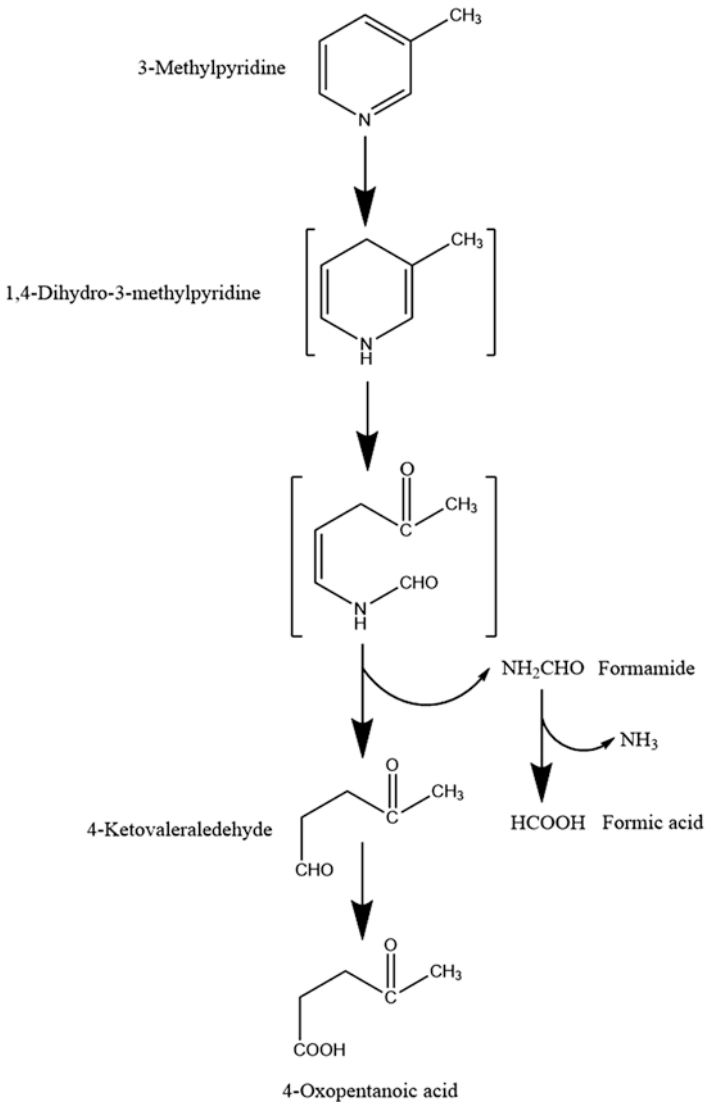

Fig. 1.6 Proposed pathway for the degradation of 3-methylpyridine by *Gordonia nitidia* LE31 (Lee et al. 2001)

6-hydroxynicotinate using oxygen derived from water. Nicotinic acid was also mineralized by *Desulfococcus niacini* under sulfate-reducing conditions (Imhoff-Stuckle and Pfennig 1983).

The occurrence of pyridine and pyridine derivatives in the subsurface near synfuel plants raised the question of the potential for their anaerobic biodegradation. Pyridine degradation has since been evaluated under fermentative, nitrate-reducing,

iron-reducing, sulfate-reducing, and methanogenic conditions, with highly variable results depending on source materials. The bulk of information available for anaerobic degradation of the unsubstituted pyridine ring has been published since 1989. In one of the earliest such studies, pyridine degraded slowly (requiring 8 months for depletion) in sulfate-reducing or methanogenic aquifer materials, whereas nicotinic acid was degraded in approximately 1 month under either redox regime (Kuhn and Suflita 1989). In that study, mono-methylpyridines exhibited variability among redox regimes as a function of the position of the methyl group. In freshwater sediments (Liu et al. 1994), pyridine degraded more rapidly under nitrate-reducing conditions (1 month) versus sulfate-reducing or methanogenic conditions (3 months). In aquifer materials from a 2-methylpyridine contaminated site, pyridine exhibited degradation under nitrate-reducing, sulfate-reducing, and methanogenic conditions (Kaiser and Bollag 1991; Kaiser and Bollag 1992; Kuhn and Suflita 1989; Bak and Widdel 1986; Ronen and Bollag 1992).

Azoarcus evansii strain pF6 degraded pyridine under both aerobic and nitrate-reducing conditions via a pathway that did not involve hydroxylated intermediates (Rhee et al. 1997). Rapid degradation of pyridine (under 60 h) was reported for acclimated sludge materials derived from coke plant wastewater (Li et al. 2001). A hydroxylated intermediate (4-methyl-2-(1H)- pyridone) was formed from 4-methylpyridine under sulfate-reducing conditions (Kaiser et al. 1993). Partial transformation of 3-hydroxypyridine and complete transformation of 2- and 4-hydroxypyridine were observed in contaminated aquifer materials incubated under sulfate-reducing conditions for 3 months (Kaiser and Bollag 1992). Pyridine was found to be persistent in an anoxic estuarine sediment for nearly a year, whereas 2- and 3-hydroxypyridines were degraded in just over 2 weeks (Kuo and Liu 1996; Liu and Kuo 1997). In this same sediment, 4-hydroxypyridine was persistent (for over 6 months). In most cases, intermediates were not detected; however it was apparent that 2,3-dihydroxypyridine was formed during dissipation of 3-hydroxypyridine. Among the dihydropyridines, 2,3-, 2,5-, and 3,4-dihydroxypyridine were dissipated over a 4-month period.

Structure-biodegradability relationships among monosubstituted pyridines in sulfate-reducing sediments showed some similarities to previous reports for aerobic environments. For example, substitution with carboxyl or hydroxyl groups favored biodegradation, whereas chlorine groups slowed biodegradation (Liu et al. 1998). The authors also observed slower degradation of methylpyridines under anoxia.

The presence of electron acceptors may be necessary for pyridine degradation under anoxia. A two-component bioreactor system (aerobic/anaerobic) was examined for potential to remove pyridine (Shen et al. 2015). Pyridine degradation was enhanced in the anaerobic system, resulting in mineralization of both pyridine carbon and nitrogen. Under anoxia, nitrogen was released as ammonium, which when recycled in the aerobic bioreactor was oxidized to nitrate. Nitrate thus formed could be used as an electron acceptor to drive pyridine degradation in the anaerobic bioreactor. Both nitrate and pyridine inhibited degradation at high concentrations. Sediment samples taken across a salt gradient in the Tsengwen River were incubated with pyridine under iron-reducing or sulfate-reducing conditions (Liu and

Kuo 1997). Supplementing with electron acceptors (amorphous $Fe(OH)_3$, MnO_2, or sulfate) promoted pyridine degradation only in the freshwater samples. A denitrifying bacterium related to *Azoarcus* (isolated from industrial wastewater) was found to degrade pyridine under either aerobic or anaerobic conditions (Rhee et al. 1997). Since the organism possessed pyridine-induced glutarate-dialdehyde dehydrogenase and isocitratase activities, it was concluded that metabolism was likely through one of the previously demonstrated reductive pathways involving cleavage between the nitrogen heteroatom and the carbon at position two. Similarly, the lack of oxygenase involvement in atrazine metabolism allows the same organism to degrade atrazine both aerobically and anaerobically (Crawford et al. 1998).

1.9 Conclusion

In the past 30 years, significant advancements have been made in understanding microbial metabolism of simple pyridine derivatives. Considerably more is known of the behavior of these compounds in anaerobic environments, including evidence for biodegradation under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic environments. Missing metabolites in the long-known reductive pathways for pyridine metabolism have been identified, and several studies have shown susceptibility of pyridine rings to oxidative degradation. The alkaloid trigonelline has even been shown to undergo ring fission by oxygenase attack without the introduction of an oxygen atom. Slow progress has been realized in the understanding of genes involved in pyridine degradation. Despite the advancements in the science of pyridine biodegradation, the routes for introduction of pyridine contaminants into the environment remain largely unchanged. Both natural and anthropogenic routes of introduction are well-established, with the latter being responsible for most of the occurrences of simple pyridines. The resurgence of energy extraction techniques associated with heterocyclic wastes ensures interest in the fate of pyridine derivatives will continue for some time.

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Chapter 2
Anaerobic Biodegradation of Pesticides

Gerald K. Sims and Ramdas Gopinath Kanissery

Abstract With the exception of those used in paddy rice, pesticides are typically applied to oxic environments but may be transported to anoxic environments through leaching, surface runoff, or eroded sediments. Pesticides are often applied to sites subject to transient flooding, eventually causing soil to become anoxic as oxygen consumptions rates exceed supply rates. This is largely due to decreased gas diffusion as pore space becomes saturated. Pesticide degradation occurs in each of the major anaerobic redox regimes, including aerobic, nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic environments. The ecology of microorganisms involved in anaerobic degradation of pesticides was poorly described until recently. Pesticide degraders (especially anaerobes) can be difficult to isolate; however, molecular biology tools allow examination of microorganisms involved in pesticide degradation without the need for isolation. In some cases, pesticide biodegradation has proved more rapid in aerobic environments, while certain substances are more labile under anaerobic conditions.

Keywords Anaerobic degradation · Biodegradation · Biotransformation · Pesticides

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2.1 Introduction

Pesticides differ from many organic contaminants in their intentional release into the environment to manage pests. Evaluation of pesticides for registration thus involves studies primarily focused on the consequences of *intended* usage over a large area (dispersed or non-point sources). However, it is also possible for pesticides to enter the environment unintentionally, typically at a point source such as a spill (Shaffer et al. 2010) or legacy sites where mixing and loading of pesticides have occurred over time. Pesticides undergo many environmental fate processes, including sorption (reversible and irreversible), hydrolysis, volatilization, transport, and degradation (biological or abiotic). Certain properties of pesticides are predictors of particular behaviors. For example, it has been shown that for a given pesticide, empirical sorption and degradation measurements may be strongly correlated among a wide range of soil types (Lehmann et al. 1993; Mervosh et al. 1995a, b; O'Loughlin et al. 2000; Sims et al. 2009). Environmental fate processes can be linked, with each component process responding differently to environmental conditions; thus, outcomes may be difficult to predict (Sims and Cupples 1999). The flow of pesticides into one environmental compartment (such as sorption to soils or sediments) may reduce flow into another.

A wealth of information has been gathered for the behaviors of pesticides in oxic environments. The role of oxygen in pesticide biodegradation is more complex than its function as an electron acceptor for aerobic microorganisms, e.g., as a substrate for oxygenases involved in biodegradation. Thus, in anoxic environments, pesticide biodegradation may involve different mechanisms relative to oxic environments. Some pesticides may be transported from the site of application to rivers, lakes, or marine environments, in which the compounds may be exposed to anoxic environments, especially if they enter a water body sorbed to sediments. Pesticides may also be subjected to transient anoxia at the site of application (such as seasonal flooding of agricultural production areas). Anoxia will result in potential for anaerobic microbial activity that may directly or indirectly affect transformation of the chemical structure of the pesticide. The duration of anoxic conditions influences the redox regime and consequently the composition of the microbial community (and accompanying physiological traits). We have endeavored to review the literature on pesticide biodegradation in a range of anaerobic environments.

When pesticides move from the site of application (crops, turf, rights of way, etc.), they may pass through a variety of redox conditions, including aerobic environments and a suite of reducing redox regimes ranging from nitrate-reducing to methanogenic. Test protocols used in the registration of pesticides with the US Environmental Protection Agency (USEPA) or the Organization for Economic Cooperation and Development (OECD) are designed to achieve highly anaerobic conditions expected at the end of the transport path, such as a lake or marine sediment environment. These tests generally are best for predicting behavior in sulfate-reducing or methanogenic conditions. However, when pesticides remaining at the site of application are subjected to transient inundation, it is likely nitrate-reducing

or iron-reducing conditions will result. Since these conditions are dominated by very different organisms with very different lifestyles, the USEPA or OECD test guidelines may not adequately predict the effects of transient anoxia on pesticide biodegradation. Flooded rice production poses a hybrid situation in which the complete range of redox regimes will potentially exist within the upper 30 cm of soil over the course of a growing season. Paddy rice is among the better-studied saturated environments and may be informative of fate under either transient or long-term flooded environments.

Consequences of variation in the nature and rates of pesticide degradation are manifold, including potential for persistence, and thus carry-over damage on-site or greater time for migration and exposure off-site. Rapid biodegradation may interfere with pesticide function at the site of application. Biodegradation usually inactivates pesticides and reduces toxicity; however, biological transformations may produce products potentially more toxic than the parent compound (Macherey and Dansette 2008). The net effect of the affinity of a pesticide for environmental compartments (solid, liquid, gas, and biological) dictates the overall environmental fate outcomes (Calamari and Barg 1992). Parameters such as sorption coefficient (K_{OC}), solubility, Henry's constant (H), or the octanol/water partition coefficient (K_{OW}) describe the affinity of a pesticide for these compartments.

2.2 Microbial Life Under Anoxia

Microorganisms respond to various environmental conditions, including the presence of oxygen, and such physiological responses tend to be stringently regulated within the cell. For example, *E. coli* is capable of aerobic respiration as well as fermentation and anaerobic respiration using nitrate, nitrite, or fumarate, as electron acceptors. A system comprised of a sensor gene (ArcB) and transcriptional regulator (ArcA) controls aerobic metabolism in response to oxygen availability (Unden et al. 1994). The same organism possesses a transcriptional sensor-regulator protein (FNR protein) which can in turn regulate anaerobic respiration as a function of oxygen availability. Fermentation in *E. coli* is regulated indirectly by oxygen via induction of the gene activator, FhlA, by formate accumulation, promoting fermentation with formate and H_2 as substrates. Many microorganisms display a cascade of responses to redox conditions in the environment. Generally, when O_2 is present, most metabolic alternatives to aerobic respiration are inactive. Furthermore, O_2 pushes the equilibrium of abiotic redox reactions in which it is involved in favor of oxidized species, regulating the physicochemical environment in which microorganisms function. Redox reactions can be described in various ways, most commonly using the Nernst equation, the abbreviated form of which is

$$E = \left(E^{o} - 59\ \text{mV} / z\right) \log 10 a_{\text{Red}} / a_{\text{Ox}} \tag{2.1}$$

in which E is the cell potential, E° is the standard cell potential, a_{Red} is the reductant's chemical activity, and a_{Ox} is the oxidant's chemical activity. The number of moles of electrons transferred in the reaction is denoted by z. When redox state is measured experimentally in an environmental sample, the observation reflects the net result of all of the redox reactions taking place in the sample. Measurement is accomplished with an electrode consisting of Pt wire, which is directly introduced into the sample as well as a reference electrode, typically calomel (Hg° and HgCl) or Ag° and AgCl in contact with the sample via a KCl salt bridge (through which ions flow). Flow of electrical current is measured with a voltmeter (often in the form of a pH meter). The half-cell reactions for common reference electrodes are described as follows:

$$\mathrm{Ag^{o} \rightarrow Ag^{+} + e^{-}} \tag{2.2}$$

$$\mathrm{Hg^{o} \rightarrow Hg^{+} + e^{-}} \tag{2.3}$$

The output from portable Hg°/HgCl or Ag°/AgCl reference electrodes is then reported relative to a standard reference, such as the standard hydrogen electrode, or SHE, defined as 0.0 mV. For example, when a calomel reference is used, + 250 mV (+200 for Ag°/AgCl) is added when the results are expressed relative to the SHE.

Ecosystems in which pesticides occur exhibit Eh and pH characteristics within the limits of the natural environment. Most environments will fall between pH 4 and 9 and exhibit Eh limits bound by the stability of water (oxidation to produce O_2 at the high end of the Eh range and reduction of H+ to produce H_2 at the low end of the Eh range). Figure 2.1 graphically represents these natural limits (Becking et al. 1960). In soils or sediments, Eh values obtained generally range from +600 mV to −400 mV. Redox (Eh) measurement in the field is subject to interferences and foul-

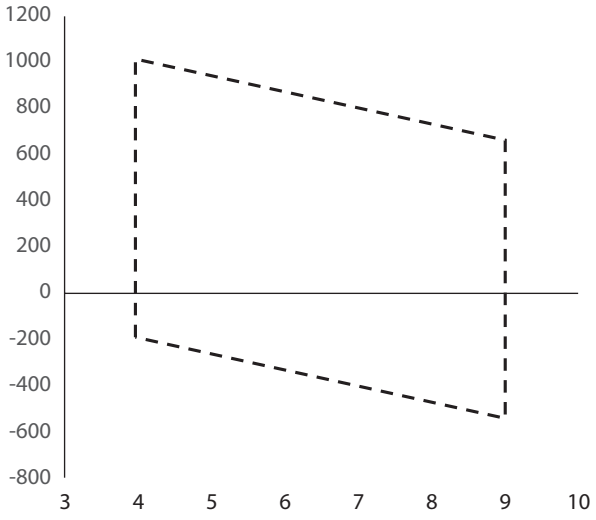

Fig. 2.1 Boundaries for Eh and pH values in the natural environment. Depicts pH range between 4 and 9 and Eh bound by the stability of water. (Adapted from Baas Becking et al. 1960)

ing of the Pt electrode (Rabenhorst and Burch 2006). In practice, Eh measurements are usually accompanied by pH measurements, and the corrected electrical potential may be expressed as the negative log of Eh or pE (Patrick et al. 1996). Detection of transient reducing conditions may also be accomplished using chemical indicators that a particular redox condition has occurred. Indicators of reduction in soils, or IRIS devices, are used for this purpose. Typically, plastic tubes coated with iron oxide films are used for such measurements in situ (Jenkinson and Franzmeier 2006; Rabenhorst and Burch 2006).

Anaerobiosis in natural settings is generally the consequence of the effect of excess water on O_2 supply and demand. Even in unsaturated soils, chemical and biological oxygen demand depletes the partial pressure of O_2 in soil pore space relative to the aboveground atmosphere. Oxygen is resupplied to soil pore space by diffusion from the surface. Since Fick's law of diffusion coefficient for oxygen (and other gases) is roughly four orders of magnitude greater for a gas phase than for water, the O_2 supply rate decreases dramatically as soils become saturated. When oxygen demand (typically driven by organic carbon) exceeds O_2 supply, anoxic conditions develop, promoting anaerobic microbial activities, which may result in reducing conditions. Since oxygen is supplied from the surface, it is expected that the soil surface will remain aerobic while conditions will become more reducing with depth so long as oxygen demand exceeds supply. Anoxia may develop below the first few centimeters of the soil surface under flooded conditions. At depth, organic carbon and demand for electron acceptors decrease; thus redox conditions may change again with depth as production of reduced metabolites declines.

Variability in other environmental conditions and microbial community composition may exist at various scales in a soil or subsurface environment. As noted, the degree of soil saturation affects gas diffusion kinetics (most notably O_2), with slower diffusion rates occurring in saturated soils. However, diffusion of nonvolatile substrates (such as a pesticide) through the water phase is positively related to water content, and this has been shown to have strong effects on pesticide bioavailability to degraders (Johnson et al. 2013). Compartmentalization imposed by pore space can promote functional redundancy in unsaturated soils, owing to the effect of limited intra-pore substrate diffusion on competition for substrates (Tiedje et al. 1999). As soil saturation increases, so does the effective diffusion of nonvolatile substrates, enhancing competition for these substrates and increasing pressure on organisms with redundant ecological functions. In addition, variations in soil structure and properties at small scales can result in microsites that differ from the bulk soil. For example, unsaturated soils have been shown to support anaerobic micro-sites capable of supporting anaerobic processes such as denitrification (Sexstone et al. 1985). Similarly, many poorly drained soils exhibit mottling from localized iron and manganese redox cycles resulting from periodically perched water tables. Conversely, aerobic organisms may be active in anoxic environments, also owing to localized aerobic conditions. *Arthrobacter* sp. (ATCC 49987), an aerobe which degrades 2-picoline, was isolated from an iron-reducing aquifer at USEAP Superfund site No. IN000807107, which was contaminated with 2-picoline (O'loughlin et al. 1999). Buried surface soils (paleosols) may contain elevated organic carbon relative

to the overburden and thus may exhibit increased biological activity and demand for electron acceptors well below the surface (Brockman et al. 1992). This may affect redox conditions of aquifers. Aquifers exhibit a range of redox conditions depending on microbial activity and the availability of electron acceptors and donors and may undergo large temporal or spatial changes in an organic contaminant plume (Christensen et al. 2000). It has been suggested that the numerical populations of pesticide degraders in soils may be significantly less than the number of pores habitable by microorganisms, indicating the bulk of soil pores lack degraders (Johnson et al. 2013). For nonvolatile substrates, degradation would be expected to be diffusion-limited in this scenario in unsaturated soils. Conversely, soil saturation has the potential to increase bioavailability of pesticides and other substrates to degraders, which could only promote degradation if the degraders can function in saturated soil.

Utilization of each of the most important electron acceptors in soils (O_2, NO_3^-, NO_2^-, Fe (III), Mn (IV), SO_4^2, CO_2 or acetate) is associated with different redox potentials, and the presence of a particular electron acceptor may poise the observed redox potential until it is depleted. For example, so long as sufficient O_2 is present to drive aerobic respiration, redox may be poised at >300 mV until oxygen is depleted, after which redox potential will decline until microorganisms begin to utilize another electron acceptor, such as nitrate, which may again poise the observed redox measurement until that electron acceptor (or the electron donor) is depleted. As a result, upon saturation of soils, redox conditions change in a sequential pattern, the duration of each step being determined by the quantity (capacity) of the relevant electron acceptor (assuming the electron donor is not limiting). Owing to the diversity of properties of individual ecotypes, a succession of organisms accompanies the succession of redox regimes. In agricultural production areas, soil saturation is generally transient (permanently flooded soils are seldom chosen for agriculture, including rice production areas that must be drained for mechanical harvesting). During such transient saturation, dominant ecotypes shift from aerobic respiring organisms (Eh poised at around 330 mV) to organisms with the capacity to use NO_3^- as an electron acceptor (poising Eh at around 220 mV) till nitrate is depleted. If saturation continues, ecotypes able to utilize Mn(IV) become active, poising Eh at around 200 mV, until available Mn(IV) is no longer available. If saturation persists long enough, Fe(III)-reducers become active, poising Eh at around 120 mV. Iron is generally present in large concentrations in soils; thus iron-reducing conditions usually persist until transient soil saturation subsides (Tor et al. 2000). Iron-reducing conditions are also favorable to fermentation (in which the organic substrate is both the electron donor and acceptor). Figure 2.2 depicts a typical pattern of microbial process responses to saturated conditions.

In permanent or long-term flooding, the succession of redox regimes will continue upon depletion of electron acceptors. As H_2 accumulates under iron-reducing

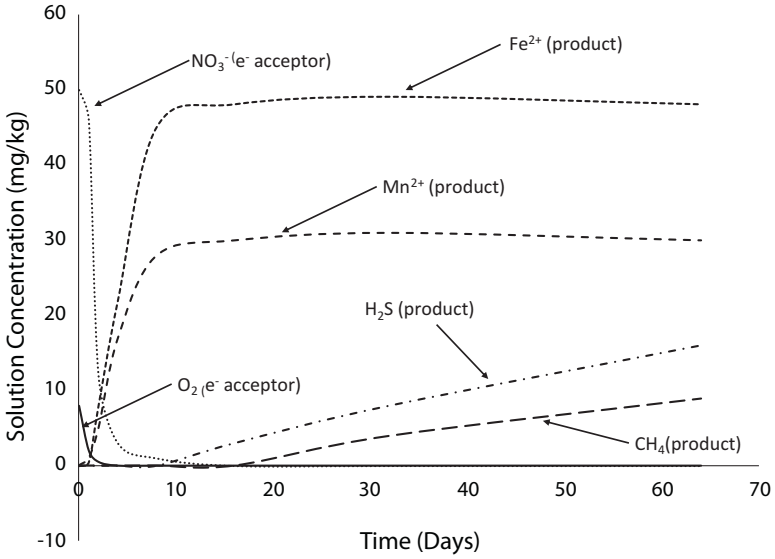

Fig. 2.2 Typical sequence of events initiated by flooding previously unsaturated soil graphical representation of the depletion of electron acceptors O_2 and NO_3^- and formation of reduced products from other electron acceptors (Fe(II) from Fe(III), Mn(II) from Mn(IV), H_2S from SO_4, and CH_4 from organic substrates). (Modified from Sims and Kanissery 2012)

conditions, sulfate-reducing bacteria (SRB) and archaea will begin to reduce SO_4^{2-} (around −150 mV), and finally methanogens will produce methane at the expense of CO_2 or acetate (approximately −250 mV) in the last stage of the succession.

Effects of microbial succession on soil processes have been studied extensively in flooded rice production systems, in which nitrogen fertilizer losses may result in significant economic impact. Unlike transient flooding related to local weather events, rice ecosystems undergo predictable patterns in flooding, which can facilitate planned experimentation. However, frequently anoxic soils undergo more rapid transitions to anaerobiosis (Pett-Ridge and Firestone 2005), and thus may not be good predictors of the behavior of infrequently flooded sites. Over time, flooded soils become spatially stratified with respect to redox conditions (Patrick and Delaune 1972). For example, in flooded rice, a relatively shallow water column allows O_2 to reach the soil surface, resulting in an oxidized surface layer, the depth of which is a result of oxygen supply to consumption ratios (Fig. 2.3). Aquatic plants, such as rice, are equipped to transport O_2 from the phyllosphere to the rhizosphere by way of specialized aerenchyma tissues. This may result in localized oxidizing conditions in the rhizosphere relative to the bulk soils (Jensen et al. 1967).

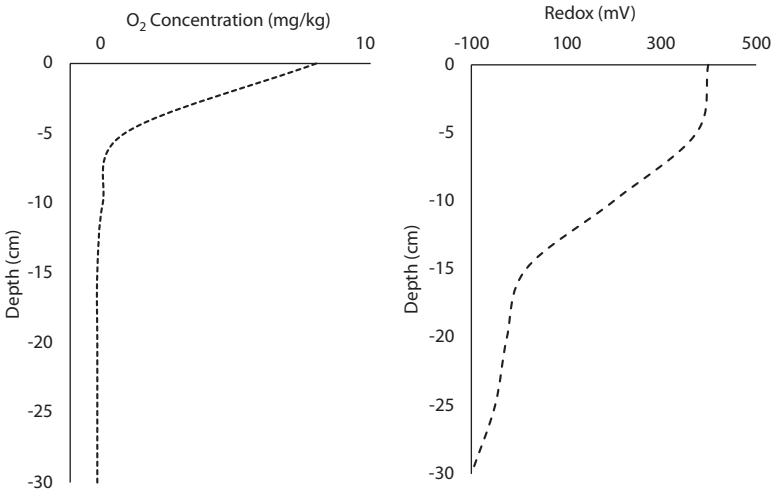

Fig. 2.3 Pattern of O_2 concentration and redox with depth in a flooded soil. The thickness of the oxidized surface layer is a function of the ratio of O_2 supply from the surface to O_2 demand in the soil

2.3 Pesticide Degradation Under Anaerobic Conditions

Studies on anaerobic fate of pesticides are often conducted under uncharacterized anaerobic conditions. For example, anaerobic degradation of the neonicotinoid insecticide paichongding (IPP) varied among soils with differing properties, though redox conditions were not measured (Cai et al. 2015). The authors noted that treatment of soil with the insecticide negatively impacted microbial diversity and that degradation rates were related to pH and organic carbon content. Numerous transformations, including hydrolysis, reduction and elimination of a nitro group, and removal of either dimethyl or ether groups, were observed (Fig. 2.4). Degradation of the insecticide, chlorpyrifos, was enhanced by repeated application to rice under either flooded or non-flooded conditions (Das and Adhya 2015). The primary metabolite, was the hydrolysis product 3,5,6-trichloro-2-pyridinol regardless of redox regime. Soil factors related to chlorpyrifos sorption, such as cation exchange capacity or clay content, tended to suppress degradation under non-flooded conditions, suggesting potential bioavailability limitation for biodegradation in upland rice. Dimethenamid, a chloroacetamide herbicide, was degraded at a similar rate with or without the inclusion of electron acceptors or glucose (Crawford et al. 2002). Acetochlor, another herbicide, exhibited similar degradation rates under iron-reducing or methanogenic conditions (Loor-Vela et al. 2003). Experimental herbicide, pyribambenz propyl, was shown to undergo anaerobic degradation, albeit more slowly than aerobic degradation in a range of different soils. Degradation products were identical to those observed under aerobic conditions, with the exception of a demethylation product, 2-(4-hydroxy-6-methoxypyrimidin-2-yloxy)

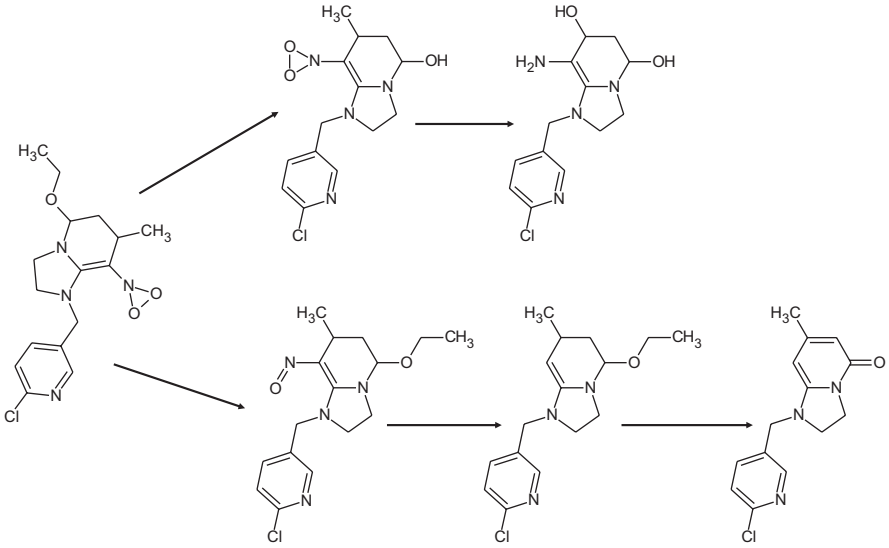

Fig. 2.4 Pathways for anaerobic biodegradation of the novel chiral insecticide paichongding. (After Cai et al. 2015)

benzoic acid (Wang et al. 2014). Similarly, for glyphosate, a globally used herbicide, anaerobic soil conditions demonstrated slower microbial degradation and mineralization kinetics than aerobic soils in the soil types studied (Kanissery et al. 2015).

The nature of anaerobic conditions (e.g., nitrate-reducing versus iron-reducing, etc.) may have a profound impact on pesticide degradation. A number of studies have either determined or controlled the redox regime in studies on anaerobic degradation of pesticides. For example, the herbicides atrazine and alachlor can both be degraded under aerobic, nitrate-reducing, sulfate-reducing, or methanogenic conditions (Wilber and Parkin 1995). We will describe some such experiments in order of decreasing redox potentials, beginning with nitrate-reducing conditions (NRCs). NRCs occur after depletion of O_2 and are characterized by a period of nitrate loss due to reductive processes, such as denitrification. Nitrate is used as an electron acceptor by many species of bacteria, a teleological explanation being that nitrate reduction yields a large change in free energy and is ubiquitous in the environment. In soil environments, nitrate quantities are seldom sufficient to support anaerobic respiration for more than 1 or 2 weeks, and in some cases, nitrate is depleted within days of a saturation event (Tor et al. 2000). NRCs are thus frequently transient in surface soils. After oxygen is depleted, a diverse array of microorganisms (more than 50 genera across in the case of bacteria) are capable of utilizing nitrate as an electron acceptor.

While the use of nitrate as an electron acceptor may allow a xenobiotic degrader to respire under anoxia, it is not a given that the organism will be able to degrade the pesticide under these conditions. In aromatic metabolism, a key role of O_2 is at the

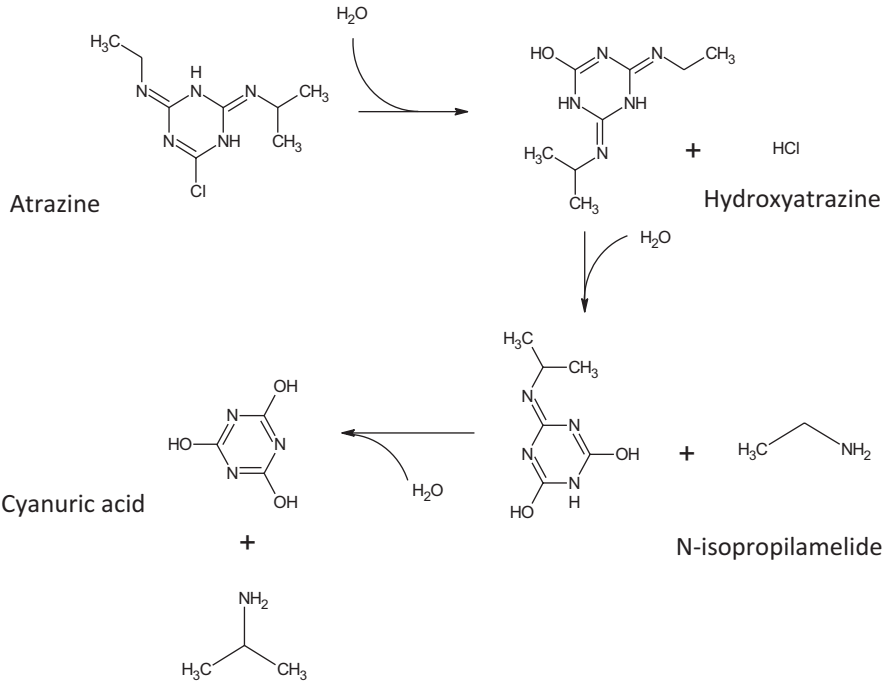

Fig. 2.5 Pathway for biodegradation of atrazine used by *Ralstonia* sp. strain M91-3 and *Pseudomonas* sp. strain ADP. (Zhang and Bennett 2005)

substrate level in oxygenase enzymes, often involved in halogen removal or ring activation and fission. There are, however, other ways to remove halogens or open aromatic rings, which do not require oxygen as a substrate. For example, the degradative pathway used by *Ralstonia* sp. strain M91-3 and *Pseudomonas* sp. strain ADP, which is depicted in Fig. 2.5, involves hydrolases rather than oxygenases for dehalogenation and activation for ring cleavage. Water rather than O_2 is required for these reactions.

Using a redox gradient apparatus (Fig. 2.6), Crawford et al. (1998) evaluated the range of conditions under which *Ralstonia* sp. strain M91-3 could function and confirmed the organism completely reduces nitrate to N_2 (using $^{15}N_2$ headspace analysis) and is able to couple atrazine degradation to nitrate respiration. *Pseudomonas* sp. strain ADP, a well-studied atrazine degrader, has also been shown to degrade atrazine under NRCs (Shapir et al. 1998). Both *Ralstonia* sp. strain M91-3 and *Pseudomonas* sp. strain ADP were initially isolated under aerobic conditions. Having the capacity to respire with nitrate as well as possessing a degradation pathway that does not require molecular oxygen allows them to degrade atrazine under either aerobic or nitrate-reducing conditions. This observation becomes more relevant when considered in the context of conditions observed in the field. Nitrate-reducing conditions typically occur in arable land due to transient flooding, which

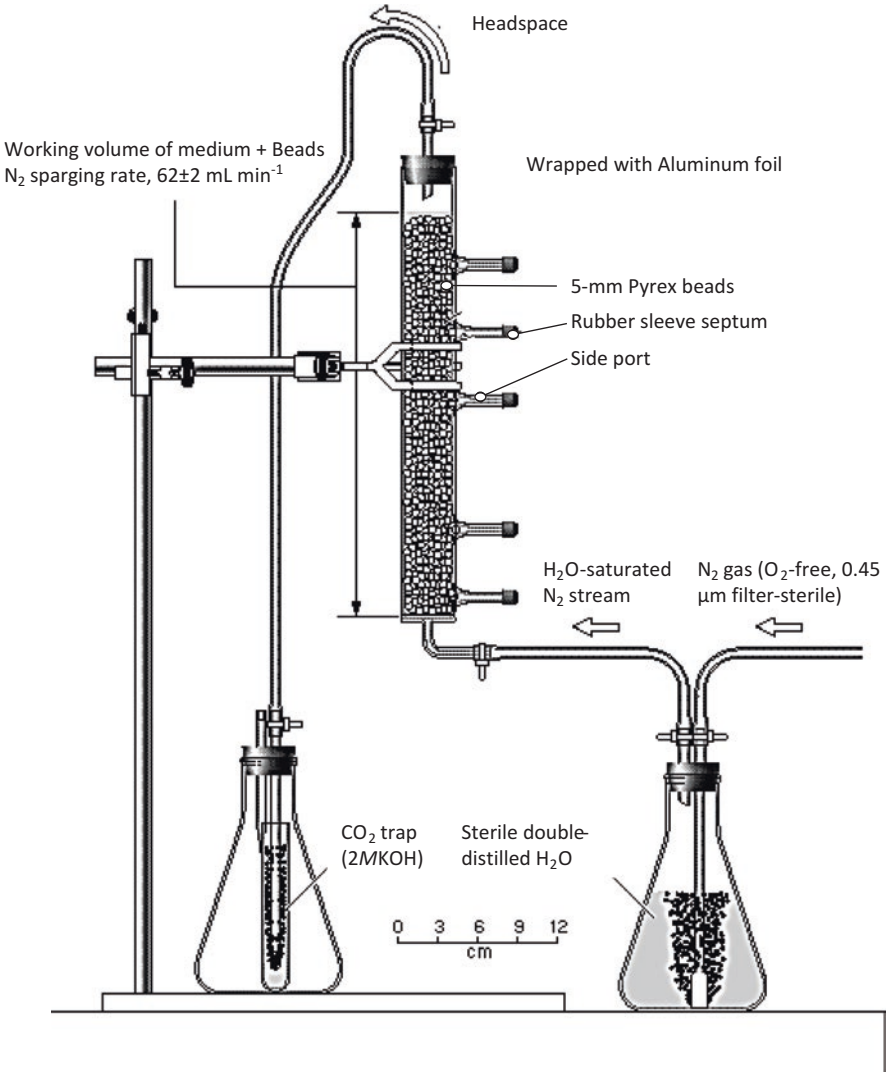

Fig. 2.6 Redox gradient apparatus for evaluating anaerobic degradation of herbicides by soil isolates. (Reproduced by permission from Crawford et al. (1998))

would favor nitrate-respiring organisms that were already active under the aerobic conditions that existed before soil was inundated. In such a scenario, by the time obligate anaerobes became active, much of the nitrate would already have been consumed by nitrate-respiring aerobes.

Pesticide biodegradation is often observed under NRCs. Differences in enantioselectivity for the racemic herbicide, mecoprop (MCPP), were observed to be a function of redox regime (Frková et al. 2016). Aerobic microbial communities

appeared to be either nonselective or selective for the R-enantiomer, whereas under nitrate-reducing conditions, communities appeared to be S-selective. The author also reported that while addition of glucose as a co-substrate had no effect on degradation, addition of nitrate (presumably as an electron acceptor) promoted degradation. Mauffret et al. reported stimulation of nitrate-reducing bacteria in groundwater by the presence of chloroacetanilide herbicides (Mauffret et al. 2017). These authors also observed an apparent increase in atrazine tolerance among groundwater communities with a history of exposure to the herbicide. Bertelkamp et al. compared behaviors of xenobiotics in three redox regimes ranging from oxic to nitrate-reducing (Bertelkamp et al. 2016). The insecticide, dimethoate, and the herbicide, diuron (as well as the pharmaceutical, metoprolol), exhibited higher degradation rates under oxic than under anoxic conditions. The herbicides, atrazine and simazine, as well as pharmaceuticals, carbamazepine and hydrochlorothiazide, persisted regardless of redox regime. NRC favored degradation of bromoxynil with complete degradation occurring in 32 days, yet no activity was detected under aerobic conditions (Preuβt et al. 1995). While addition of acetate delayed degradation of bromoxynil under NRCs (Preuβt et al. 1995), degradation of atrazine and alachlor was stimulated by acetate under NRCs (Wilber and Parkin 1995).

NRCs are not always favorable for biodegradation. Suflita et al. (1983) observed slower degradation of the herbicide linuron under nitrate-reducing conditions than under methanogenic or sulfate-reducing conditions than in aquifer materials. Similarly, Larsen and Aamand (2001) reported that mecoprop degraded very slowly in denitrifying or methanogenic sediments and atrazine, isoproturon, or metsulfuron-methyl appeared resistant to degradation under anoxia. Gu et al. (1992) also found atrazine to be recalcitrant under NRC. The presence of mineral fractions may have a profound influence on anaerobic pesticide degradation. Tang et al. (2018) described enhanced degradation of 2,4-dinitrophenol (degradation product of 2,4-D and triclosan) under NRC in the presence of goethite. The lack of biodegradation under NRC may be due to the absence of appropriate degraders. Shapir et al. (1998) demonstrated improvement in atrazine degradation rates in anoxic sediments via inoculation with *Pseudomonas* sp. strain ADP, suggesting indigenous atrazine degrader populations were limiting. Anaerobic sediments with a high level of indigenous atrazine degradation did not respond to inoculation with *Ralstonia* sp. strain M91-3, despite the organism's ability to couple atrazine degradation to denitrification (Crawford et al. 1998).

The next form of anaerobic respiration in the redox tower is iron reduction, which can be inhibited by nitrate (although some iron-reducing bacteria also use nitrate as an electron acceptor). In surface soils, iron-reducing conditions (IRC) develop upon depletion of nitrate, and this shift to IRC is associated with changes in composition of the microbial community (Lueders and Friedrich 2000; North et al. 2004). As recently as 1984, it was generally accepted that reduction of either Fe^{3+} or Mn^{4+} in soil was an indirect process, resulting from accumulation of fermentation products that accumulated after O_2 depletion (Tiedje et al. 1984). Direct usage of these metals as electron acceptors by microorganisms (dissimilatory iron reduction) became common knowledge only recently (Lovley 1997). Some authors have

reported increased degradation of pesticides as conditions transitioned from nitrate-reducing to iron-reducing. Degradation of both trifluralin (Tor et al. 2000) and metolachlor (Kanissery et al. 2018) increases as soils move into IRC. When organic compounds are degraded under IRC, it is not always clear whether the compounds were transformed directly by iron-reducing bacteria (IRB) or were indirectly transformed by minerals reduced by IRB, as is the case for reduction of 4-chloronitrobenzene by a *Geobacter* species (Heijman et al. 1993). Reduced clay minerals proved better sorbents than oxidized analogs for sorption of atrazine or alachlor (Xu et al. 2001). Conversely, when soils containing sorbed glyphosate (which has sorption properties similar to phosphate) underwent IRC, glyphosate desorption was observed (Kanissery et al. 2015). Reduced clays appeared to have the capacity to hydrolyze and dechlorinate atrazine and similarly transformed alachlor to 14 unique products (Xu et al. 2001). In the case of dinitroaniline herbicides (trifluralin, pendimethalin, nitralin, and isopropalin), transformation has been described as a microbially mediated abiotic process, in which IRB reduce iron minerals, which in turn chemically transform the herbicide (Tor et al. 2000; Wang and Arnold 2003). The ability of reduced iron minerals to carry out the transformation was confirmed using synthetically reduced minerals, such as nontronite (Tor et al. 2000) or surface-bound Fe(II) in goethite (Wang and Arnold 2003). In other cases, iron-reducing organisms have been shown to directly degrade a variety of organic contaminants, such as estrogens (Ivanov et al. 2010) and BTEX compounds (Jahn et al. 2005) in the absence of sediments that might otherwise catalyze reduction. The use of DNA-stable isotope probing demonstrated production of microbial biomass from the herbicide metolachlor by organisms closely related to IRB (Kanissery et al. 2018). *Comamonas koreensis* CY01 has been shown to couple iron reduction to degradation of 2,4- D (Wu et al. 2009). Figure 2.7 shows similar reductive transformations of trifluralin (a herbicide) and trinitrotoluene (an energetic compound). These compounds produce similar products via either biodegradation or reactions with reduced iron minerals, both of which may occur under IRC.

Like IRC, sulfate-reducing conditions (SRC) become apparent after the depletion of nitrate and may overlap with the Mn(IV) reduction (Peters and Conrad 1996). Iron-reducing bacteria (IRB) can forestall the onset of SRC activity by maintaining dissolved concentrations of hydrogen, formate, and acetate concentrations below thresholds for activity of sulfate-reducing bacteria (Chapelle and Lovley 1992). When concentrations of these substrates significantly exceed roughly 1.0 nM, 2.0 μM, and 1.0 μM, respectively, sulfate-reducing bacteria (SRB) become active. Owing to these requirements for substrates and strict anoxia, activities of SRB are expected only at very low redox potentials encountered after extended periods of anoxia in upland soils (wetland soils may exhibit sulfate reduction relatively quickly after flooding). SRB and methanogens are essentially omnipresent in soils (Peters and Conrad 1996), which explains why well-aerated soils eventually go through all of the steps of sequential reduction if maintained in a flooded condition. More than 200 species of SRB and archaea are known (Barton and Fauque 2009). SRB obtain energy by oxidizing H_2 or organic substrates using sulfate, sulfite, thiosulfate, or elemental sulfur as electron acceptors. Some may use other substrates, such as

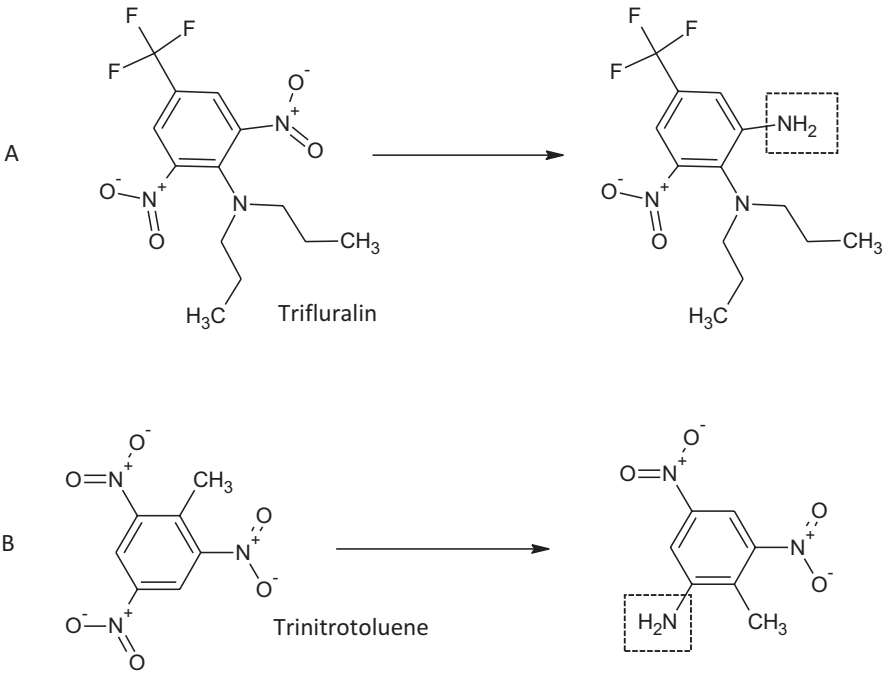

Fig. 2.7 Reductive transformations observed for (**a**) the herbicide, trifluralin (Kaufman 1974), and (**b**) the energetic compound, trinitrotoluene (Esteve-Núñez and Ramos 1998). These reactions may be the result of either direct biodegradation or indirect abiotic transformation by microbially reduced iron minerals

fumarate, nitrite, nitrate, Fe(III), or dimethyl sulfoxide, as electron acceptors. Acidic conditions favor SRB, with the redox potential (Eh) required decreasing from −50 to −200 mV as pH increases from 5 to 7 (Rabenhorst and Burch 2006). Dormant SRB and methanogens nonetheless can survive under oxidizing conditions and have been detected in oxidizing environments, such as desert soils (Peters and Conrad 1996). Boopathy (2017) demonstrated degradation of atrazine in both nitrate-reducing and sulfate-reducing soil slurries, with the latter exhibiting faster degradation rates. The addition of molasses as a co-substrate enhanced degradation under SRC. Mineralization was observed, along with accumulation of the common aerobic degradation products, hydroxyl-atrazine and cyanuric acid. The addition of sulfate enhanced the degradation of acetochlor in glucose-treated anaerobic soil, reducing the $t_{1/2}$ from 15 to 10 days (Loor-Vela et al. 2003), whereas the auxin herbicides, 2,4-dichlorophenoxyacetic acid, (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5- T), and 2-methyl-4-chlorophenoxyacetic acid (MCPA), proved recalcitrant under SRC (Kuhlmann and Kaczmarzcyk 1995).

SRB can outcompete methanogens for H_2 and when sulfate is not limiting, which is consistent with observations of sulfate reduction before methanogenesis in flooded soils. As hydrogen concentrations increase, methanogens and SRB coexist

(Lovley et al. 1982). A diverse community of anaerobes is active under methanogenic conditions, including some organisms able to transform pesticides into products that can serve as substrates for methanogens. Anaerobic fate in sewage sludge of five pesticides, including the nematicide thiabendazole, fungicides enilconazole and ethoxyquin, biocide ortho-phenylphenol, and insecticide diphenylamine was examined using waste from food packing plants (Karas et al. 2015). Of the five pesticides, thiabendazole and enilconazole, which were strongly sorbed to sludge solids, were most resistant to anaerobic degradation. Among the herbicides (Lin et al. 2006), only dicamba was substantially degraded within a month in methanogenic river sediments (Lin et al. 2006). Picloram (3,5,6-trichloro-4-amino-2-pyridinecarboxylic acid) underwent reductive dehalogenation more readily under methanogenic than SRC or NRC, and the addition of sulfate inhibited dehalogenation (Ramanand et al. 1993). As noted above, other pesticides, such as alachlor and atrazine, can also be transformed or degraded under methanogenic conditions (Wilber and Parkin 1995).

Reductive dehalogenation, also called halorespiration, is a strictly anaerobic process in which a halogenated substrate is used as an electron acceptor. Halorespiration is associated with sulfate-reducing and methanogenic environments. Jugder et al. (2015) recently reviewed the substrate specificities and properties of currently known reductive dehalogenases. It is likely halorespiration existed before the advent of xenobiotic halogenated compounds and has been demonstrated to play a major role in the fate of heavily halogenated compounds. The energetic favorability of oxygenase attack or hydrolytic removal of halogen groups decreases with the degree of halogenation; thus organochlorine insecticides, such as DDT, tend to be recalcitrant in aerobic conditions. However, under anaerobic conditions, halorespiration can be quite effective for dehalogenation of chlorinated organics. Favorability of halorespiration decreases in the order iodine, bromine, chlorine, and fluorine (Mulligan and Yong 2004). Figure 2.8 shows examples of reductive dehalogenation of pesticides substituted with chlorine, bromine, and iodine.

A number of pesticides are labile under methanogenic conditions. The neonicotinoid insecticide cycloxaprid underwent several transformations under anoxic conditions, including reductive dechlorination in the chloropyridinyl ring moiety (Liu et al. 2015). Other xenobiotic halogenated organic compounds have been shown to undergo reductive dehalogenation, including alachlor, metolachlor, and propachlor (Stamper and Tuovinen 1998), chlorinated benzoates (Suflita et al. 1983), 2,4,5, trichlorophenoxyacetic acid (Mikesell and Boyd 1985), and methoxychlor (Baarschers et al. 1982). DDT is highly recalcitrant in the environment, though a number of organisms have been shown to degrade the insecticide. The primary biodegradation of DDT in environmental samples is DDD (1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene), formed from DDT via reductive dechlorination (Aislabie et al. 1997). Herbicides containing bromine (bromoxynil) and iodine (ioxynil) have been shown to be substrates for halorespiration by *Desulfitobacterium chlororespirans* (Cupples et al. 2005). Biological reductive dehalogenation can be mimicked by abiotic transformation with bisulfide. This has been shown with the chloroacetanilide herbicides, alachlor, propachlor, and metolachlor (Loch et al. 2002; Stamper and Tuovinen 1998).

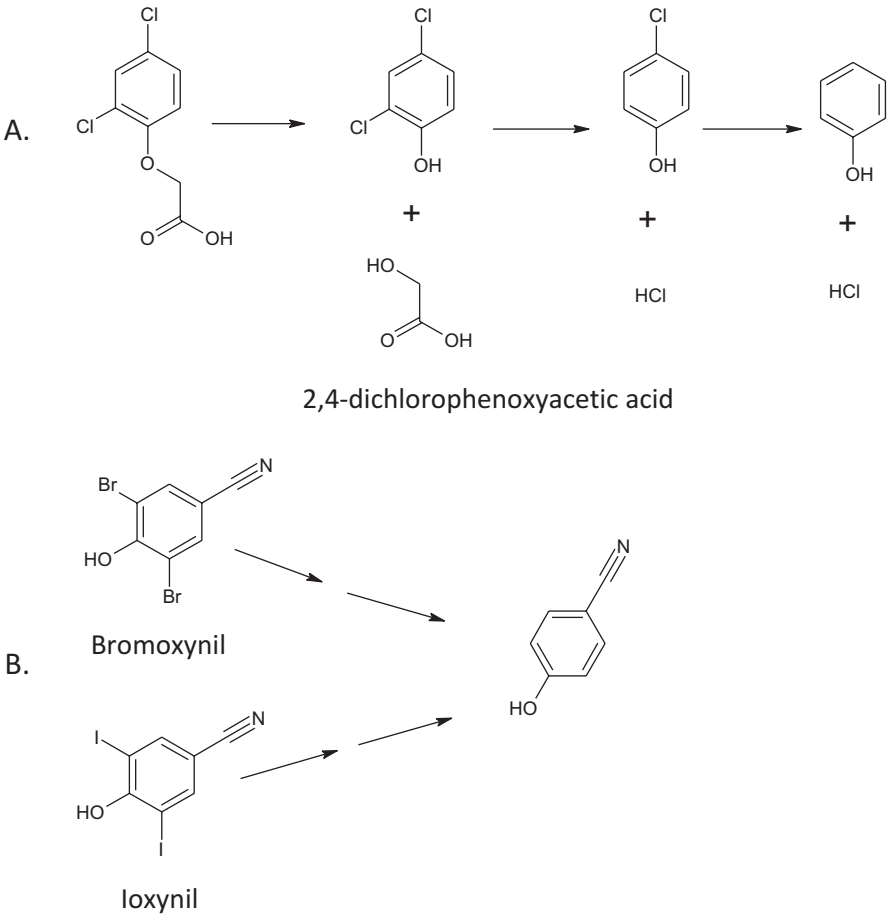

Fig. 2.8 Examples of reductive dehalogenation of the herbicides (**a**) 2,4-dichlorophenoxyacetic acid (Zhang and Bennett 2005) and (**b**) bromoxynil and ioxynil (Cupples et al. 2005)

2.4 Future Directions

The potential for pesticide degradation in reducing environments has been exploited in wastewater treatment scenarios. Derakhshan et al. (2018) demonstrated co-metabolism of atrazine under NRC using an anoxic moving bed biofilm reactor. Similarly, Chouhan et al. (2017) achieved degradation of MCPA (2-methyl-4-chlorophenoxyacetic acid) and DMCPA (dimethylamine salt of MCPA) in an anoxic sequencing batch reactor, although the product of DMCPA degradation accumulated, eventually inhibiting degradation. A bioreactor containing straw and bark mulch attenuated atrazine and nitrate concentrations in soil drainage lines (Camilo 2016).

Electron acceptors may be limiting for the particular organisms involved in anaerobic biodegradation at a given site. Microbial electroremediating cells (MERCs) utilized electrodes in lieu of chemical electron acceptors to stimulate anaerobic biodegradation in contaminated soils. This approach has been tested on a few pesticides. Mineralization of the herbicide, isoproturon, increased by 20-fold in flooded soils in which electrodes had been set to +600 mV relative to an Ag/AgCl reference electrode (Jose et al. 2016). Another herbicide, atrazine, was also remediated from contaminated soil with the aid of MERC technology, resulting in a five-fold increase in degradation rates relative to natural attenuation (Domínguez-Garay et al. 2016).

Recent advancements in molecular ecology, such as DNA-stable isotope probing (DNA-SIP), have facilitated in situ identification of microorganisms involved in pesticide degradation (Sims 2008). The first DNA-SIP study conducted with a pesticide was that of Cupples and Sims (2007), in which ^{13}C- 2,4-D was introduced into an agricultural soil. Since then, Tong et al. (2015) used ^{13}C-labeled pentachlorophenol to identify organisms in a rice paddy involved in degradation of this chlorinated pesticide. Dominant bacteria involved in PCP biodegradation were found to belong to the genus *Dechloromonas*. Members of this genus have been shown to oxidize aromatic compounds including benzene, toluene, benzoate, and chlorobenzoate in the absence of oxygen, possibly by providing the oxygen required for dioxygenase activity by dismutation of chlorate. While most SIP studies have employed a ^{13}C-labeled substrate (Cupples and Sims 2007), Shaffer et al. (2010) demonstrated the feasibility of DNA-SIP using ^{15}N-labeled pesticides as substrates. Relatively few SIP studies have been performed on pesticide substrates. Among the few DNA-SIP studies that involved anaerobic conditions, Kanissery et al. (2018) demonstrated a change in the dominant organisms responsible for metolachlor mineralization when aerobic soils that received herbicide treatment were incubated anaerobically. Liu et al. (2011) demonstrated dominance of *Alphaproteobacteria* among degraders of the herbicide, 2-methyl-4-chlorophenoxyaceticacid (MCPA), in soils.

2.5 Summary

Though pesticides are typically applied to oxic environments (with the notable exception of paddy rice), they may be transported to anoxic environments through several mechanisms, such as leaching or surface runoff. In addition, sites of application may be subject to transient flooding. Pesticide degradation has been demonstrated in each of the major anaerobic redox regimes, including nitrate-reducing, iron-reducing, sulfate-reducing, or methanogenic environments. Organisms involved have been difficult to isolate; however, tools of molecular biology have facilitated the examination of microorganisms involved in pesticide degradation without the need to isolate the active organisms. In some cases, pesticide biodegradation has proved more rapid in aerobic environments; however, in other cases, it has been shown that certain substances are more labile under anaerobic conditions.

It is important to understand the behavior of pesticides in situations other than the point of usage, as accidental spills do occur and several routes exist for transport throughout the environment. It is also important to understand on-site behavior, which may affect pesticide function or on-site nontarget impacts (such as carryover damage to sensitive crops). Such information also informs the discovery process for selection of future pesticides. Understanding optimal pesticide behavior may reduce the need for "end of the pipe" (off-site) remediation solutions.

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Chapter 3
DNA Stable Isotope Probing to Examine Organisms Involved in Biodegradation

Gerald K. Sims, Andres M. Gomez, and Ramdas Kanissery

Abstract In situ applications of molecular biology to terrestrial and aquatic ecosystems have advanced the study of activities of microorganisms without the need for cultivation. DNA-based fingerprinting tools, such as 16S-TTGE, T-RFLP, DGGE, or pyrosequencing, facilitate in assessing pesticide impacts on microbial community composition. Quantitative PCR or functional gene microarrays help us understand effects of pesticides on genes of interest. Such tools improve our understanding of environmental impacts on microbial phylogeny or function, though few can link a specific organism to its function in situ. Stable isotope probing (SIP) emerged specifically to provide this linkage. SIP approaches vary in sensitivity, specificity, and inference space, depending on biomolecules targeted to obtain phylogenetic information. Combining SIP with "omics" tools further characterizes responses of microbial communities to environmental events. Herein we have reviewed strengths and weaknesses of common SIP techniques, with emphasis on the ecology of pesticide degradation.

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3.1 Introduction

Since the first reports of Leeuwenhoek's *animalcules* and Hooke's *cells* (Lane 2015), the scientific community has been fascinated with microorganisms and what they do. For the first 100 years, the state of the art for examining microorganisms in situ was to isolate them from the environment and grow them in culture. In the case of xenobiotic degraders, this generally meant using enrichment media to isolate organisms either capable of degrading a particular substrate or resistant to a biocide of interest. The approach allowed the identification of many organisms that possessed particular functions but failed to establish a causal relationship between the isolate and the function in situ. Most environmental microorganisms, especially those in soils, are yet unidentified, let alone successfully cultivated. A wealth of information is available on microbially mediated processes though little of it has been obtained at the scales at which microorganisms function. Great effort has been directed toward assessing the capabilities of microbial species in isolation, yet few microorganisms actually exist as monocultures in the natural environment. Advances in molecular biology have made it possible to address many of these gaps in the knowledge base.

Microorganisms are clearly responsible for degradation of most organic contaminants, and it is also true that chemicals affect microbial communities. It was established more than 100 years ago, using simple plate counting techniques, that soil application of organic solvents resulted in large changes in the dominant groups, with some increasing in abundance as others declined (Buddin 1914). The application of DNA-based fingerprinting tools, such as 16S-TTGE, T-RFLP, or DGGE, provided more power to reveal impacts of contaminants or environmental conditions on microbial communities (Gomez et al. 2011; McCaig et al. 2001; Tejada et al. 2010). Quantitative PCR (Zaprasis et al. 2010) can be applied to environmental samples to detect specific organisms or their functional genes without the need for isolation. As tools such as functional gene microarrays became available, they were applied to environmental samples for functional analysis of whole communities (Cho and Tiedje 2002; McGrath et al. 2010). More recently, mass parallel sequencing techniques such as 454 pyrosequencing and Illumina MiSeq have broadened our perspective of the vast diversity harbored in soil micro-ecosystems and previously unidentified pollutant degradation dynamics (Huaidong et al. 2017; Wang et al. 2016b; Wu et al. 2017). Many of these molecular tools for microbial ecology can also be used to reveal identities or functions of microorganisms, but few can link a specific organism to a particular function. Some tools, such as quantitative dot blot, stable isotope probing, and FISH-MAR (fluorescent in situ hybridization micro-autoradiography), however, can be used to make this connection.

Considerable advancement has been made in the use of stable isotopes to label phylogenetically informative biomolecules, including proteins, phospholipid fatty acids (PLFA), DNA, and RNA, an approach generally described as stable isotope probing (SIP). SIP provides definitive evidence that a phylotype is active in a given function and is especially well suited to biodegradation research. The common

forms of SIP have been reviewed by others (Sims 2008; Uhlik et al. 2013). Here, we provide an updated overview of these tools primarily as they apply to environmental research and present findings from studies in which they were employed to identify organisms involved in biodegradation of organic contaminants.

3.2 Use of Isotopes to Measure Microbial Processes

Isotopic tracers have been used in biology for nearly a century. Stevens injected $RaCl_2$ intravenously to study lymphomas, and Calvin used $^{14}CO_2$ to unravel the C3 pathway for photosynthesis (Stevens 1926; Calvin 1948). Since the 1950s ^{14}C, ^{35}S, and ^{15}N (Dils and Hübscher 1959; Smellie et al. 1953), ^{32}P (Lajtha et al. 1954; McMurray et al. 1957), and ^{3}H (Cronkite et al. 1959; Belfrage et al. 1965) have been used to label both PLFA and DNA, which are the most common biomarkers used in SIP. Radioisotopes as well as stable isotopes have been used extensively to follow metabolic processes in environmental samples, such as the requirement for ^{14}C to demonstrate microbial degradation for the registration of pesticides (Wolt et al. 1996). ^{15}N-labeling is standard practice in tracing microbial transformations of nitrogen fertilizers. ^{13}C can be used to describe turnover and structural features of soil organic matter (Wander et al. 1996). Combinations of isotopes have been used for similar purposes. The introduction of $^{14}C/^{15}N$-labeled urea into soil resulted in label separation, with ^{14}C incorporated into amino acids and ^{15}N into nitrate, demonstrating fixation of urea-C by ammonium oxidizers, a significant impact stemming from the simple fact that many ammonium-oxidizing bacteria possess urease (Marsh et al. 2005). Isotopes have facilitated localization of organisms involved in specific environmental functions at the scale of bacterial cells (micro-autoradiography or MAR). For example, when ^{14}C-MAR was used to examine the light-dependent degradation of atrazine in the presence of the hornwort plant, it was revealed that epiphytic atrazine-degrading bacteria were either responsible for or assisted with biodegradation (Rupassara et al. 2002). While any of the above studies employing stable isotopes could be accurately described as "stable isotope probing," SIP has recently developed a very specific connotation in which an isotope is used to tag microbial biomarkers to obtain phylogenetic information on active populations without culturing them. The lack of a cultivation step allows detection of active organisms that do not grow in common media and perhaps have not yet been identified. For example, Wang (Wang et al. 2015) applied DNA-SIP to study utilization of ^{13}C-labeled exopolysaccharides and cellulose obtained from bacterial cultures grown on labeled glucose. Cellulose degraders identified in this study were closely related to known organisms, whereas those that degraded exopolysaccharides had a very low identity to any known organisms. Since the introduction of DNA-SIP (Radajewski et al. 2000), the term stable isotope probing has been used almost exclusively to describe such applications of isotopes. This narrow usage within the scientific lexicon is consistent with the exponential adoption of DNA-SIP since its inception (Fig. 3.1).

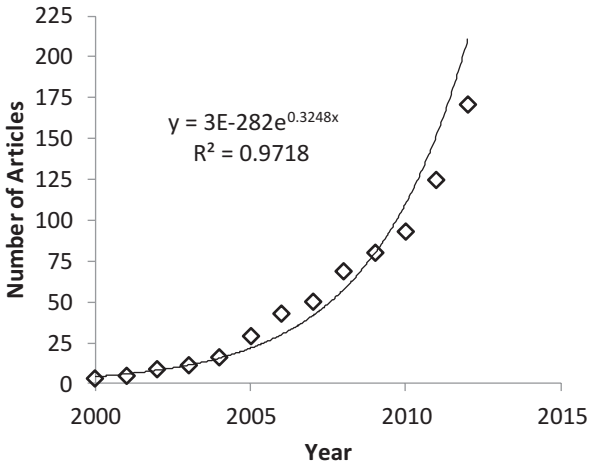

Fig. 3.1 Adoption of the term "stable isotope probing" to describe tagging microbial biomarkers identifies active microbial populations without culturing them. Data herein consists of research articles published in a given year in which stable isotope probing is used in the above context

Applications of SIP are generally denoted by the isotopes and biomarkers selected for isotopic labeling. The requirements for biomarkers include becoming labeled upon exposure of the organism to an isotope and containing useful information about the identity of the organism(s) active in the process of interest. The primary biomarkers in use for SIP include phospholipid fatty acids (PLFA), proteins, and nucleic acids (DNA and RNA). SIP may be paired with other approaches, such as high-throughput sequencing (Young et al. 2015; Youngblut et al. 2018) and metagenomics (Fortney et al. 2018; Herrmann et al. 2017), process measurements, or micro-autoradiography. Isotopes are chosen based on the composition of the biomarkers and requirements for label detection. The most common isotopes are ^{13}C, ^{15}N, and ^{18}O. Deuterium labeling is also a possibility; however, hydrogen-deuterium exchange is a risk, and the probability of exchange increases with extraction methods that alter pH. Nonetheless, Baran succeeded in using D_2O SIP to reveal unusually rapid metabolite turnover among cyanobacteria (Baran et al. 2017).

Biomarkers must be variable enough among organisms to differentiate them into taxa. Phospholipid fatty acids are found in microbial cell membranes and turnover quickly in the environment, and thus PLFA in an environmental sample is considered representative of extant organisms (Bossio et al. 1998). Composition and relative abundance of PLFAs vary among species sufficiently to be used for identification of isolates using instrumental analysis such as MALDI-TOF/MS (Santos et al. 2018). Since there is considerable overlap in composition among similar organisms, quantification of the relative abundance of individual fatty acids is important for high-level taxonomic specificity. This is possible when PLFAs are extracted from pure cultures; however, the diagnostic power diminishes sharply for environmental samples in which PLFAs derived from an entire community are pooled. PLFAs are thus far less useful for identification of individuals within mixtures of organisms than is the 16SrRNA gene, which retains its unique identity upon extraction, including within pooled DNA from complex communities. In SIP, PLFAs are used to profile the overall structure of complex microbial communities with respect to broad

groups of organisms, such as bacterial/fungal or Gram-positive/Gram-negative ratios (Quideau et al. 2016). Isolation of PLFAs from environmental samples is relatively dependable, and instrumental analysis for PLFAs by GC-IRMS is quite sensitive. Since the purpose of the isotope label in PLFA-SIP is to tag the biomarker in a mass spectrum (rather than change its buoyant density), PLFA-SIP does not require ultracentrifugation and, more importantly, requires very little label incorporation (less than 0.1 atom %). PLFA-SIP can be performed in a lab outfitted for chemical analysis since molecular biology tools like PCR and electrophoresis are not required. These advantages may outweigh limited taxonomic information provided by PLFA for certain applications.

Nucleic acids (DNA and RNA) unlike PLFAs contain relevant taxonomic information within each molecule and thus can be used for identification of individual organisms within a mixed community. DNA-SIP and RNA-SIP require significant label incorporation (>20%) to change the buoyant density enough for separation of the active pool from the rest of the community by ultracentrifugation. Achieving this may involve unrealistically high concentrations of substrate and long incubation times (incorporation into RNA is faster than with DNA). DNA isolation from environmental matrices has become relatively dependable compared to either proteins or RNA. More robust databases exist for 16S rRNA genes (roughly 26 million entries in GenBank) than for proteins, giving the edge to nucleic acids for phylogenetic resolution.

Proteins have become important biomarkers for SIP as they provide greater sensitivity and linkage to metabolic activity; however, as with RNA, isolation of protein from complex matrices like soil can be difficult. Like PLFA analysis, isotopic enrichment of protein is determined by mass spectrometry, eliminating the need for ultracentrifugation and vastly increasing sensitivity, only about 2% label incorporation (Jehmlich et al. 2008) being required. The MS data also provides a direct measurement of label incorporation, which is much less subjective and error-prone than measuring buoyant density shifts in nucleic acid-SIP.

3.3 PLFA-SIP

Owing to chemical composition and method of analysis, PLFA-SIP is generally limited to ^{13}C-labeled substrates. After incubation of an environmental sample with a labeled substrate, lipids are extracted, derivatized to methyl esters (FAME), and analyzed via gas chromatography coupled to mass spectrometry (GC-MS) or isotopic ratio mass spectrometry (GC-IRMS) to determine the identity and quantity of PFLAs that have been labeled.

Labeling PLFA in environmental samples for the purpose of microbial identification was attempted before similar efforts were made with DNA. Boshchker et al. introduced ^{13}C-acetate and methane into aquatic sediments to identify sulfate reducers and methanotrophs (Boschker et al. 1998). One of the key benefits of SIP techniques – the ability to detect minor, yet active populations – was validated in this

study. Unexpected Gram-positive taxa proved to be more active in sulfate reduction than the dominant Gram-negative members of the sulfate-reducing community. Lerch et al. employed FAME-based ^{13}C-SIP to examine microbial populations involved in 2,4-D degradation (Lerch et al. 2009). Coupling FAME analysis with soil compartment analysis revealed the flow of 2,4-D carbon and suggested a succession of organisms involved in degradation. Lu et al. used pulse-labeling of rice plants with $^{13}CO_2$ to perform PLFA-SIP in the field (Lu et al. 2004). As rice plants matured over the growing season, the active decomposers in the rhizosphere microbial community underwent succession that could be demonstrated with SIP. Seasonal and diurnal patterns of rhizosphere microbial activity in rice fields have been reported previously, though earlier studies did not identify the active populations (Sims and Dunigan 1984). A similar study employing multiple label pulses in a microcosm showed that active populations were concentrated in the rhizosphere relative to the bulk soil (Lu et al. 2007) and that the active phylotypes differed between rhizosphere and non-rhizosphere soil. This study revealed that SIP could facilitate localization of the site of activity for metabolism of a highly diffusive substrate. Using field labeling with ^{13}C-methane, Mohanty et al. (Mohanty et al. 2006) demonstrated that nitrogen fertilizers differentially stimulated type I methanotrophs (*Methylococcaceae*) while inhibiting type II methanotrophs (*Methylocystaceae*). Biotraps containing labeled benzene and toluene were used in PFLA-SIP to identify actively degrading populations (Geyer et al. 2005), though PFLA-SIP provided limited useful taxonomic information in this study.

Chang et al. introduced a ^{13}C-labeled substrate (acetate) with biotraps and combined PLFA-SIP and ^{13}C-DNA SIP to identify active degraders in a contaminated aquifer (Chang et al. 2005). Organisms identified were consistent with activities detected down-gradient, including Fe(III), U(VI), and SO_4^{2-} reduction. The limited taxonomic information provided by PFLA-SIP was in good agreement with identities provided by DNA-SIP, suggesting the two approaches detect the same groups and differ largely in sensitivity and level of taxonomic discrimination. Dias et al. combined ^{13}C-PFLA SIP with RNA-SIP to demonstrate that genetic modification of potato (to change starch content) resulted in changes to the active components of the rhizosphere community (Dias et al. 2013). Also combining ^{13}C-PFLA SIP and RNA-SIP, Macgregor et al. observed incorporation of label from acetate, propionate, amino acids, or glucose into bacterial biomarkers, indicating more ^{13}C was found in PLFAs than RNA (MacGregor et al. 2006).

The primary advantages of PLFA-SIP over other SIP techniques are simplicity, dependability, and sensitivity. The primary disadvantage is the limited amount of taxonomic information provided. In situations in which very limited label incorporation is likely, PFLA-SIP may be the preferred choice.

3.4 Protein-SIP

Protein-SIP was reviewed recently (Seifert et al. 2012), and protein-SIP was also included in a broader recent review of common SIP techniques (Uhlik et al. 2013). Jehmlich et al. published a generic protocol for protein-SIP that had been developed using ^{13}C-and ^{15}N- substrates labeled at nearly 100 atom% in laboratory cultures (Jehmlich et al. 2008). After labeling, the procedure required protein extraction followed by separation using gel electrophoresis, LC-MS or MALDI-TOF-MS, and analysis of data with appropriate software tools. Degree of label incorporation was presumed to be quite accurate, since it is determined via MS analysis. Label incorporation into proteins was faster than with DNA, and detection was far more sensitive (limit of detection estimated at 2 atom%). Important limitations include the fact that extraction of proteins from some environmental matrices, such as soil, can be challenging, and it is estimated that more than 10^5 cells are necessary for identification, which may effectively decrease sensitivity of protein-SIP. Like DNA-SIP, early papers with protein-SIP utilized cultures in the lab for proof of concept (Jehmlich et al. 2008). Taubert et al. (2012) conducted a protein-SIP experiment in microcosms containing contaminated soils from an industrial site. ^{13}C-benzene versus ^{13}C-carbonate was used to evaluate flux of carbon through the microbial community under sulfate-reducing conditions. The MS data provided information about carbon flow including intermediates. In addition to some phylogenetic information about organisms involved, it was also possible to provide functional classification of proteins that were detected. Using activated carbon biotraps to introduce ^{13}C-naphthalene, Herbst et al. conducted protein-SIP and metaproteomic analysis on a sample from a contaminant plume (Herbst et al. 2013). The authors were able to identify involvement of *Burkholderiales*, *Actinomycetales*, and *Rhizobiales* in the degrader community. Proteins involved in the naphthalene degradation pathway were labeled at 50 atom% with ^{13}C, indicating naphthalene was a primary carbon source for these organisms. Lunsmann et al. combined 16S rRNA gene sequencing, metaproteomics, and protein-SIP (^{13}C-toluene) to examine toluene degradation in a constructed wetland (Lünsmann et al. 2016). Key players in toluene degradation were identified (*Burkholderiaceae* and *Comamonadaceae*), and involvement of monooxygenases in the degradative process was verified. Using protein-based stable isotope probing coupled to metagenomics, Mosbaek et al. examined organisms involved in balancing the relative activities of acidogens and methanogens in an anaerobic digester (Mosbæk et al. 2016). Using a binned metagenome reference, it was possible to identify organisms carrying out specific functions of interest. Organisms involved in both processes were identified. It was suggested that some of the acetate degraders detected may have been involved in a syntrophic relationship with hydrogenotrophic methanogens. Hydrogenotrophic methanogens utilize hydrogen to reduce CO_2 to methane (Richards et al. 2016). Having found that harvesting lumber led to

decreased respiration and cellulolytic activity in a stand of Ponderosa pine, Wilhelm et al. employed metagenomic analysis coupled to ^{13}C-SIP (cellulose) to reveal changes in community structure (Wilhelm et al. 2017). As cellulolytic activity decreased, thermophilic cellulolytic fungi appeared to replace less stress-tolerant cellulolytic bacteria as dominant players in cellulose turnover.

3.5 Nucleic Acid-SIP

Like PLFA-SIP, nucleic acid-SIP preferentially labels active populations, which allows separation of the target organisms from background. DNA and RNA have been used as biomarkers for nucleic acid-SIP; the basic protocols for labeling and retrieval of the labeled nucleic acids are similar. Since recovery of RNA from soils can be challenging (Saleh-Lakha et al. 2005), DNA-SIP (Radajewski et al. 2000) has been the most common approach to date. Figure 3.2 provides an overview of the nucleic acid-SIP method.

A microbial community (soil or water) is treated with a labeled substrate (typically ^{13}C or ^{15}N) either in situ or in vitro. After allowing time for sufficient growth on the substrate to achieve adequate labeling of the active organisms, cellular nucleic acids are extracted from the environmental sample and subjected to density gradient centrifugation to separate nucleic acids in the active population from the unlabeled background community. If ethidium bromide is added to the gradient, the heavy band may be visualized under UV illumination, and the lower (heavy) band can be recovered from below with a syringe. This approach may not result in adequate band separation, which is needed to reduce cross contamination. Nor does this approach provide buoyant density data, the importance of which is covered below. A more robust approach, described by Lueders et al. (Lueders et al. 2004a) involves careful elution of the CsCl gradient by pumping water into the top of the centrifuge tube and collecting fractions from a small hole in the bottom of the tube (Fig. 3.3).

DNA buoyant density is proportional to GC content (Meselson and Stahl 1958) as well as extent of isotopic labeling. If buoyant density of the DNA pool of active

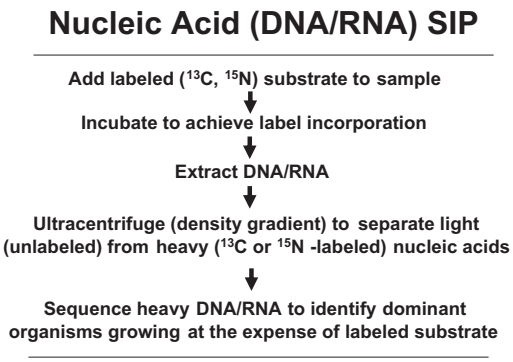

Fig. 3.2 Overview of steps in nucleic acid stable isotope probing

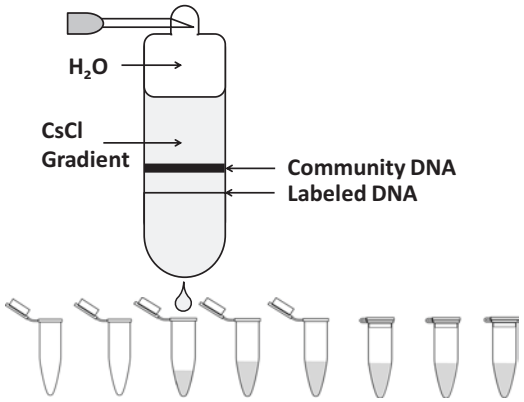

Fig. 3.3 Depiction of elution of the CsCl gradient into discrete fractions by pumping water into the top of the ultracentrifuge tube and collecting the fractions from a small hole in the bottom of the tube

organisms is known (by measuring the BD of the fraction), and an unlabeled control is available, it is possible to use the buoyant density shift to calculate the extent of label incorporation into the active organisms (Kanissery et al. 2018). If buoyant density data is available and there is no unlabeled control, sequence data may be used to estimate enrichment; however, there can be considerable discrepancy between the GC contents of 16SrRNA and the genome.

Several modifications of DNA-SIP have improved performance. For example, including unlabeled DNA (from *Escherichia coli* K-12) as internal standard can help delineate the band containing the heavy DNA pool. The presence of traces of unlabeled standard in the heavy DNA can also provide an estimate of the degree of contamination (Singleton et al. 2005). Recovery of trace quantities of bacterial ^{13}C-DNA can be facilitated by adding ^{13}C-labeled archaeal carrier DNA prior to centrifugation (Gallagher et al. 2005).

Once heavy DNA has been recovered, the process of identification begins. In most cases, heavy DNA is characterized using PCR-based approaches using (in the case of bacteria) the 16SrRNA bacterial gene as a target. Often, libraries have been constructed by cloning the heavy DNA fragment into *E. coli*, and once appropriate clones containing the 16SrRNA of interest were found, the insertions were sequenced and analyzed for similarity to sequences in the GenBank library. Additional techniques, such as T-RFLP analysis (terminal restriction fragment length polymorphism), can help find the appropriate clones for sequencing and also provide a control to verify (or at least suggest) the presence in the original sample of the organism detected in the heavy fraction. In T-RFLP analysis, the recovered DNA is subjected to restriction enzymes with known cut sites. The fragments so formed are resolved via capillary electrophoresis (Clement et al. 1998; Cupples and Sims 2007; Osborn et al. 2000). The use of multiple restriction enzymes provides different characteristic T-RFLP patterns that can be targeted for finding the correct clones to sequence from a clone library or to show the response of a detected organism to the added substrate in the original sample. Using this approach, Cupples and Sims (2007) demonstrated that T-RFLP patterns associated with DNA from the heavy fraction were not only present in the original sample but increased in

abundance in response to addition of the substrate (with or without label). Other researchers have employed microarrays to achieve this purpose. In the case of RNA-SIP, there is a tendency for RNA to diffuse within the gradient, requiring a more robust fractionation scheme to ensure separation of the heavy fraction. Different approaches may be used to analyze the heavy fraction in RNA-SIP, such as RT-PCR or DGGE (Manefield et al. 2002a). Aoyagi et al. (2018) reported that replacing T-RFLP analysis with high-throughput (Illumina) sequencing could result in increased sensitivity up to 500-fold over T-RFLP. This study was performed with RNA isolated from pure cultures, and thus may not account for issues encountered with environmental samples. As noted previously, problems can arise in extraction of RNA from environmental samples.

Isotope choice can have profound effects on the degree of buoyant density shift achieved by labeling. With roughly 20% incorporation of ^{13}C-labeled substrates, buoyant density shifts are generally sufficient to separate unlabeled high GC DNA from labeled low GC DNA. Labeling elements less abundant in DNA (such as N) result in much more subtle shifts in buoyant density. Figure 3.4 (adapted from Cupples et al. 2007) illustrates the resolution of differently labeled DNA (^{14}N versus ^{15}N) from two organisms in density gradient centrifugation. The influence of G + C content is illustrated by comparing the buoyant density shifts for *E. coli* and *Micrococcus luteus*, which have GC contents of roughly 50 and 72%, respectively. Unlabeled *M. luteus* was not separated from labeled *E. coli*, owing to the relatively limited buoyant density shift provided by ^{15}N labeling. Very high levels of DNA enrichment (at last 20 atom % for ^{13}C) are required to achieve sufficient change in buoyant density for separation; thus most studies employ highly enriched substrates (at or near 100 atom%).

DNA-SIP has been employed widely for biodegradation of a variety of substrates (typically limited to those commercially available in ^{13}C-labeled form), such as glucose, caffeine, (Padmanabhan et al. 2003), naphthalene (Padmanabhan et al. 2003; Yu and Chu 2005), anthracene (Zhang et al. 2011), phenol (DeRito et al. 2005; Manefield et al. 2002b; Padmanabhan et al. 2003), methanol (Lueders et al. 2004b), methane (Morris et al. 2002), propionate (Lueders et al. 2004a) methyl bromide, methyl chloride (Miller et al. 2004), pentachlorophenol

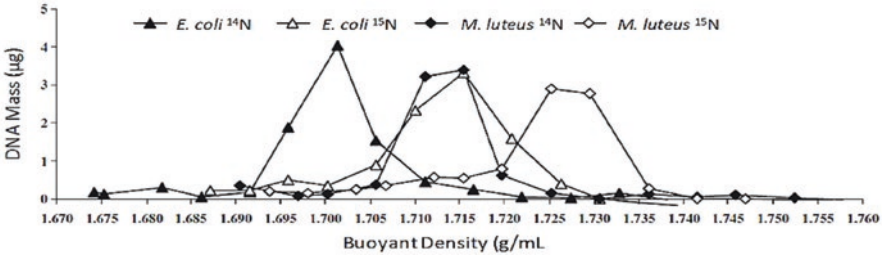

Fig. 3.4 Gradient elution of DNA recovered from *E. coli* and *M. luteus* showing the buoyant density distribution of unlabeled (^{14}N) (closed triangles) and labeled (^{15}N) (open triangles) DNA for each organism. (Modified from Cupples et al. 2007)

(Mahmood et al. 2005), para-nitrophenol (Kowalczyk et al. 2015), ammonium (Cupples et al. 2007), metolachlor (Kanissery et al. 2018), 2,4-D (Cupples and Sims 2007), and 2,4-dinitrophenol (Dallinger and Horn 2014). Among the most commonly studied substrates have been BTEX compounds (benzene, toluene, ethylbenzene, and xylene) or PCPs and PCBs owing to their importance as pollutants and commercial availability in ^{13}C-labeled forms (Herrmann et al. 2010; Sul et al. 2009; Luo et al. 2009; Oka et al. 2008; Sun and Cupples 2012; Kim et al. 2014; Chen et al. 2016). DeRito et al. coupled secondary ion mass spectrometry with ^{13}C-SIP using phenol as a substrate (DeRito et al. 2005). Through the use of ^{13}C-DNA-SIP combined with DGGE of RT-PCR-amplified extracts, an organism related to *Azoarcus* was found to metabolize benzene when nitrate was present in a hydrocarbon-contaminated aquifer (Kasai et al. 2006). Organisms active in degradation of hydrocarbons in the field (Padmanabhan et al. 2003) as well as bioreactors (Singleton et al. 2005) have been identified by DNA-SIP. Similarly, Jeon et al. (2003, 2004) discovered a novel naphthalene degrader, *Polaromonas naphthalenivorans* sp. nov., with this technology. DNA SIP has also been used to evaluate multi-trophic interactions in biodegradation. Li et al. used^{13}C-isotopic labeling to demonstrate that earthworms enhanced turnover of pentachlorophenol in soil and DNA-SIP provided identities of phylotypes which were found both in the soil and the gut of the earthworms (Li et al. 2015).

Many SIP studies have focused on anaerobic ecosystems.^{13}C-DNA- SIP has been used to identify organisms active in methane cycling in both marine and freshwater environments carbon (Borodina et al. 2005; Ginige et al. 2005; Hutchens et al. 2004; Lueders et al. 2004a; Miller et al. 2004; Radajewski and Murrell 2002). Lin et al. demonstrated that both *Methylococcaceae* and *Methylocystaceae* were represented in the methanotroph community in a soda lake, yet most were not active, and only members of the *Methylococcaceae* assimilated ^{13}C (Lin et al. 2004). Using DNA-SIP to study autotrophic denitrifying populations in a medium free of organic substrates and supplemented with ^{13}C-carbonate and nitrate, Xing et al. observed that (as evidenced by ^{13}C enrichment) an organism related to *Thiobacillus* dominated autotrophic denitrification process (Xing et al. 2017). However, the nitrate reductase (nirS) genes in the heavy DNA fraction (detected by cloning and sequencing) were not related to known autotrophic denitrifiers. In addition to identification of phylotypes, functional genes, such as those encoding methane monooxygenase, are often examined. Cross-feeding is commonly observed when methanotrophs are examined using a $^{13}CH_4$-labeled substrate (Dumont et al. 2011; Qiu et al. 2009; Redmond et al. 2010). Lu and Conrad labeled rice biomass with $^{13}CO_2$ and examined methanogenesis of carbon derived from the labeled residue with RNA-SIP, which revealed involvement of novel Archaea (Lu and Conrad 2005). Findings from ^{13}C- PLFA-SIP and DNA-SIP were used to provide complementary information on active sulfate reducers in anaerobic sediments (Webster et al. 2006).

Though ^{13}C has been by far the most commonly used isotope in DNA SIP, other isotopes have been used successfully. Despite the limited change in buoyant density resulting from ^{15}N-labeling, several studies have made use of ^{15}N-substrates in DNA-SIP studies (Buckley et al. 2007; Cadisch et al. 2005) with substrates ranging

from crop residues to RDX (Roh et al. 2009). Espana et al. used ^{15}N-DNA SIP to examine bacteria and fungi active in turnover of maize residues (España et al. 2011a, b). Conversely, Shaffer et al. (2010) observed too much label dilution for ^{15}N-DNA SIP to establish the identity of degraders at a pesticide spill site using either ring- or side chain-labeled atrazine. The soil from the spill site (Mollic Albaqualf) contained more than 160 mg/kg inorganic N. Previous studies using ^{15}N-labeled atrazine and three different isolates demonstrated that degraders vary in the degree to which exogenous N sources affect assimilation of ^{15}N from atrazine (Bichat et al. 1999). Addison et al. also experienced limited separation of ^{15}N-labeled nucleotides (Addison et al. 2010). The authors were unable to achieve complete separation of labeled and unlabeled RNA using a mixed population. This was not surprising given that labeled and unlabeled DNA from a single organism exhibited some degree of overlap in isopycnic gradient ultracentrifugation (Cupples et al. 2007). Thus, the success of ^{15}N-DNA SIP is dependent on centrifugation conditions, exogenous N competition, as well as nitrogen regulation in the organisms involved. Aanderud and Lennon examined the potential for use of heavy water (both oxygen and hydrogen were labeled) to label microbial DNA (Aanderud and Lennon 2011). The authors concluded that incorporation of ^{18}O into DNA was relatively stable. Heavy water was then used to conduct an $H_2{}^{18}O$-SIP investigation of the effects of soil rewetting on metabolically active bacteria. Using bar-coded pyrosequencing of 16S rRNA genes, they observed increases in abundance of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* and concomitant decreases in abundance of *Chloroflexi* and *Deltaproteobacteria*.

Recently, DNA-SIP has been coupled to high-throughput sequencing (HTS) of the 16S rRNA gene and metagenomics (Chen and Murrell 2010; Uhlik et al. 2013), mainly to improve the sensitivity of SIP assays, to cover higher microbial diversity, and to unravel the relationships between phylotypes and their true metabolic functions. Indeed, HTS approaches are reported to have up to 500-fold higher sensitivity for screening ^{13}C-labeled nucleic acids than fingerprinting techniques (e.g., T-RFLP). When DNA-SIP will be used for metagenomic analysis, genomic DNA is amplified by multiple displacement amplification rather than PCR as described above, and fosmid libraries are constructed (Chen et al. 2008; Neufeld et al. 2008). More recent work has also shown highly sensitive isotope probing of ^{13}C-labeled rRNA and DNA coupled with HST of bacterial phylogenetic markers (e.g., 16S rRNA Illumina MiSeq™), an approach used to identify novel (previously uncharacterized) bacteria and bacterial community dynamics involved in the degradation of 1,4-dioxane in activated sludge (Aoyagi et al. 2018), vinyl chloride in ground water (Paes et al. 2015), acetate and lactate in anaerobic digesters (Sun et al. 2018), hexahydro-1,3,5-trinitro-1,3,5-triazine in navy detonation areas (Jayamani and Cupples 2015b), pyrene in agricultural and industrial soils (Chen et al. 2018), ethyl benzene in agricultural soils (Jayamani and Cupples 2015a), and pentachlorophenol in forest, paddy, and pit (Tong et al. 2018) among other environments. Although sensitivity and diversity coverage are significantly improved with the adoption of amplicon high-throughput sequencing in SIP experiments, these techniques may still suffer from the same bias SIP amplicon-based surveys bring, in the context of

low phylogenetic resolution from short amplicons and limited available genomes and databases to infer novel diversity. To circumvent some of these limitations, recent advances on bioinformatics and statistical approaches for analyzing and interpreting HTS-SIP data have facilitated multi-window high-resolution SIP, quantitative SIP, and better buoyant density shift estimations (Youngblut et al. 2018). Likewise, the implementation of meta-transcriptomics SIP (based on labeled mRNA) provides a step closer to determining real-time microbial metabolism in situ (Jameson et al. 2017). Lastly, as the cost of other meta-OMIC methods such as proteomics and metabolomics decrease, its integration to SIP experiments should provide unprecedented depth in the elucidation of function in complex microbial communities (Marlow et al. 2016). Recent trends have also included the use of fluorescence in situ hybridization and micro-autoradiography (FISH-MAR) in DNA-SIP studies to localize specific organisms identified by SIP (Lolas et al. 2012).

Registration of pesticides in the United States or the European Union requires evidence of the role of microorganisms in degradation, which is generally obtained using sterile controls. More elegant proof is sometimes offered when pure cultures capable of growth on the pesticide are obtained. While some older pesticides (such as atrazine or 2,4-D) are amenable to enrichment culture, most modern pesticides are quite complex in structure and used at low rates (the area application rate for tembotrione produces a soil concentration of about 0.15 ppm in the surface 15 cm). Thus few labs have been successful in obtaining isolates capable of growth on such compounds. Some molecular tools, such as QPCR, high-throughput gene sequencing, or functional gene microarrays, potentially can be used for detection and even quantification of pesticide degradative potential but only if the genes involved have been identified and associated with a limited number of biodegradative functions. Fenner et al. pointed out that most degradative proteins are associated with superfamilies containing up to 600,000 similar proteins with different functions, which negates the potential to target degradative genes for novel pesticides (Fenner et al. 2013). Tools such as PICRUSt (Langille et al. 2013; Sun et al. 2018) can use 16S rRNA data to predict degradative genes present in a microbial community (Thelusmond et al. 2016); however, this is only possible for known gene families contained in the reference genomes used. SIP techniques may provide a more robust and convincing way to demonstrate biodegradation potential for pesticide registration studies, as it provides an identity of organisms involved and does not require isolation or knowledge of degradative pathways and genes. Few SIP investigations have actually been performed using pesticides as substrates, most of which were conducted in academic labs using older chemistries for which ^{13}C labels are commercially available. Since pesticide registration packages require custom synthesis of ^{14}C-labeled substrates, it is likely that most such synthesis labs could also provide highly enriched ^{13}C labels as well. Among the few pesticides and antimicrobials studied, 2,4-D (Cupples and Sims 2007), MCPA (Liu et al. 2013), metolachlor (Kanissery et al. 2018), and triclosan (Liu et al. 2016) have received the most attention.

The first pesticide to which DNA-SIP was applied was 2,4-D (Cupples and Sims 2007). It is also one of the few herbicides for which a wealth of information is

available on organisms involved in its degradation. Though a very old herbicide, introduced at the close of WWII, it is still used, and new pathways and genera capable of degrading 2,4-D have been discovered quite recently (Kitagawa and Kamagata 2014). Like most substrates examined so far by SIP, 2,4-D can be purchased readily with a ^{13}C-label. A DNA SIP study revealed a member of the β subdivision of *Proteobacteria* responded to 2,4-D treatments, as evidenced by both labeling of DNA and increases in the T-RFLP profiles of soil in both labeled and unlabeled 2,4-D-treated soils (Cupples and Sims 2007). This group had previously been implicated in 2,4-D degradation (Cavalca et al. 1999). Kanissery et al. used ^{13}C-DNA-SIP to evaluate the impact of soil flooding on organisms involved in metolachlor biodegradation and noted distinctly different bacterial phylotypes involved under the different redox regimes (Kanissery et al. 2018).

Recovery of DNA from environmental samples has become relatively dependable (as has PLFA recovery), whereas protein and RNA recovery can be problematic, especially in complex matrices, such as heavily amended feedlot soil (Maxwell and Gerald 2008). Compared with other SIP methods, nucleic acid-based SIP methods are more informative for phylogeny but require at least tenfold (or more) label assimilation (Uhlik et al. 2013). This may lead to excessive substrate application rates and lengthy incubation times to ensure sufficient enrichment for the required buoyant density shift. RNA-SIP may be accomplished with shorter incubation times owing to faster synthetic rates. Longer incubation times promote dispersal of the label among organisms recycling carbon released by the primary degraders (cross-feeding). Cross-feeding is less likely to result in high levels of enrichment than would be observed for an organism utilizing the substrate as a primary carbon source. It is possible to estimate enrichment levels from the buoyant density shift if an unlabeled treated control is included and BD measurements are taken for each fraction. For example, Hungate et al. (Hungate et al. 2015) carried out sequencing each density fraction separately and using BD shift calculated isotopic enrichment. Kanissery et al. also used BD shifts to determine enrichment of known organisms by comparing to an unlabeled control (Kanissery et al. 2018). Alternatively, measurement of cross-feeding may become an experimental goal when assessing flow of carbon through a community. Nucleic acid-SIP requires separation by ultracentrifugation, which is time-consuming and limits the number of experimental units that can realistically be analyzed.

The high levels of enrichment employed for nucleic acid-SIP will also result in labeling of intermediates and end products, which can facilitate compartment analysis to accompany the SIP data, although high-label rates may promote cross-contamination of lab ware, which can be a liability for labs also conducting trace level isotope analysis. Combining ^{14}C and ^{13}C labels for biodegradation studies can facilitate evaluation of flow of carbon into compartments that are more easily quantified using radiolabel, such as non-extractable residues (NER), mineralization, and metabolite formation (Girardi et al. 2013; Kanissery et al. 2018). The low rates of ^{14}C typically used in radiotracer studies will not appreciably affect DNA buoyant density. This supplementary data is especially useful when studying biodegradation of xenobiotic compounds (Kanissery et al. 2018).

All of the available SIP methods are intended for introduction of labeled substrates into environmental samples (either in the field or in field samples taken to the lab). When the sample matrix is soil or sediment, heterogeneous distribution of the substrate is highly likely. For example, physical mixing is not possible when undisturbed samples are used. Johnson and Sims found that degradation of 2,4-dichlorophenoxy acetic acid varied among four carrier solvents and the fraction of 2,4-D mineralized was a hyperbolic function of solvent volume (Johnson and Sims 2011). The primary factors that appeared to govern the differences were bioavailability and solvent toxicity. It was also observed that substrate concentration affected mineralization rate only when the substrate was introduced in an organic solvent, while the portion of substrate that was readily desorbed was greater with water than organic solvents. Hansen solubility parameters (Launay et al. 2007) provided insight into behavior of solvents and how they would be expected to interact with soil. Heterogeneous distribution of substrates and microorganism is also the reality for contaminants in the field and is thought to be partly responsible for the coexistence of redundant phylotypes within a soil matrix (Felske and Akkermans 1998; Zhou et al. 2004). Lab-induced heterogeneity can be turned into an asset if efforts are made to account for diffusion processes in degradation kinetics (Johnson and Sims 2011; Mervosh et al. 1995). While problems with mixing are unlikely for aqueous samples, other issues may arise, such as toxicity, stimulation of microbial activity, or influence of carrier solvents on bioavailability (Johnson and Sims 2011).

3.6 Conclusions

The past 20 years have seen a proliferation of new SIP methods as well as "omics" tools that enhance the value of the data collected. Combinations of SIP techniques or partnering SIP with other tools such as FISH-MAR or process measurements create potential to answer previously impossible questions. SIP techniques have strengths and weaknesses which may be complementary if used together. As "omics" databases expand, these tools become increasingly more powerful. In the near term, it may be possible to utilize Raman scattering techniques in SIP. Raman micro-spectroscopy can be used at the single cell level to obtain biochemical patterns without the need for label introduction. Wang et al. posited the potential for combining this approach with omics analysis (Wang et al. 2016a). Inasmuch as all SIP approaches share an initial step in which an isotopically labeled substrate is introduced into the sample, active biomass (including all of the biomarkers relevant to SIP) and intermediates and end products become labeled if the substrate is turned over. This opens opportunities for diverse combinations of techniques, including meta-OMICS, that can help unravel the complex interactions of organic contaminants with the environment.

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Chapter 4
Enzymatic Bioremediation: Current Status, Challenges of Obtaining Process, and Applications

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Abstract Enzymes play an important role for degradation of various xenobiotic compounds. In this chapter, we summarize the role of various enzymes including oxidoreductases, monooxygenases, dioxygenases, peroxidases, and laccases for bioremediation of various xenobiotic compounds. Microbial oxidoreductases are able to degrade natural and artificial pollutants, reverse toxicity caused by xenobiotics, and reduce heavy metals, through their oxi-reduction capacity. Monooxygenases and dioxygenases are able to play a central role in the degradation and detoxification of aromatic compounds through hydroxylation and ring cleavage. Peroxidases act in bioremediation processes due to their thermostability and capacity to oxidize a wide range of substrates. Laccases can act on a variety of pollutants including petroleum derivatives (PHAs), paints, plastics, dyes, estrogenic substances, and paper via oxidative reactions, decarboxylation, and demethylation and can oxidize phenols, polyphenols, metals, polyamines, and aryl diamines groups and also act on lignin degradation and on azo dyes.

Keywords Monooxygenase · Dioxygenase · Laccases · Peroxidase

4.1 Introduction

Environmental pollution is one of the main problems of the contemporary era due to the high volume of waste from anthropogenic activities. Wastes from industries including textiles, paper, plastics, petrochemicals, and pharmaceuticals can have highly toxic substances such as phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), petroleum derivatives, agrochemicals, organic pollutants, and heavy

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metals. These toxic substances can cause severe endocrine dysfunctions (Wake 2005; Murthy and Naidu 2012) due to carcinogenic, neurotoxic, teratogenic, or mutagenic effects (Gavrilescu et al. 2015; Gasser et al. 2014; Nguyen et al. 2014; Rao et al. 2014; Kapoor and Rajagopal 2011; Alcalde et al. 2006).

Among the 105 known chemical elements in nature, 30 have some toxicity to humans, and the combination of two or more of these elements can form more than 11 million chemicals. Chemicals compose the most diverse environments; thus, a particular environment consists of several substances in certain proportions. When a substance concentration is above natural limits, it is called a contaminant, and when it is harmful to living organisms, it is called a pollutant (MMA 1981).

The knowledge about the transformation of chemical substances is ancient. Studies conducted in the sixteenth century, as those from Hieronymus Brunschwig (1450–1513) and Philippus Aureolus Paracelsus (1490–1541), described methods of preparing tinctures, antimycotics, and sedatives based on plant extracts (Leicester 1971). However, it was in the eighteenth and nineteenth centuries, driven by the industrial revolution, that chemical transformations gained prominence due to the development of petroleum, pharmaceuticals, fertilizers and agrochemicals, dyes, and explosives industries (Bensaude-Vicent and Stengers 1992; Mierzecki 1991).

Until the twentieth century, the risks of chemical compounds manipulation were little known and subjugated, causing numerous health problems. As an example, DDT (dichlorodiphenyltrichloroethane), launched in the 1920s to control agricultural pests, was immediately accepted by the population due to the efficacy, apparent safety to humans, and the absence of acute effects. However, after about 30 years of its use, carcinogenic and environmental impacts were confirmed, culminating in their market banishment in the 1970s in most countries (ATSDR 2016). In addition, the industrialization and urbanization resulted in increased generation of waste and effluents, emerging the concern with their correct destination, especially of residues composed of potentially polluting substances (Rao et al. 2014).

In this way, the residues began to receive treatments before their final disposal, being first separated regarding to the pollutant potential and treated before the final disposal.

Currently, potentially polluting wastes are often treated by methods such as incineration and chemical decomposition. However, new waste treatments have been developed and applied, such as the use of biotic factors for the treatment of organic wastes, which, in general, are less complex alternatives, with greater population acceptance and less environmental impact (Brown et al. 2017). Biotic factors effectiveness against toxic compound occurs due to natural metabolic processes: mechanisms for degradation, transformation, or elimination of toxic compounds that are naturally formed.

In this context, bioremediation is a process in which organisms, such as plants and microorganisms, and/or their metabolites, as enzymes, are used to degrade or detoxify environmental pollutants. However, in general, the transformation of a pollutant into an inert substance requires series of reactions, i.e., an enzyme complex

which often means that different organisms are needed for a satisfactory result (Pereira and Freitas 2012; Ahuja et al. 2004).

In this sense, environmental conditions suitable for the rapid development of each organism are essential for effective bioremediation, which may be complex when the necessary organisms have divergent optimal growth conditions, inhibitory relations or when the contaminants are resistant to biotic attack (Rao et al. 2014; Gianfreda et al. 2006; Durán and Esposito 2000).

Thus, the application of organisms' enzymatic extracts instead of the organisms themselves may be more advantageous, since it is possible to provide a greater process control (known enzymatic concentration); faster and more homogenous action; remediation of complex compounds, including recalcitrant ones (which have removal difficulties by metabolic, chemical, or physical processes); and a more controllable and efficient treatment, according to the substrate and environmental conditions (Brown et al. 2017; Whiteley and Lee 2006; Ahuja et al. 2004; Gianfreda and Bollag 2002; Vidali 2001).

Frequently, pollutants from pharmaceutical, cosmetic, pulp, paper, and food industries are rich in free radicals such as phenolic compounds, compounds bounded to heavy metals, and reactive oxygen species (ROS) (by-products of redox reactions that have unpaired electrons, which in excess cause oxidative stress, cellular damage, and cell death) (Kovalchuk 2010). These reactive molecules can be neutralized by oxidoreductases (Karigar and Rao 2011).

In addition to free radicals, lipophilic residues are potentially polluting materials present in wastewater from the oil processing for the food industry, slaughterhouses, and dairy products. According to Hocevar et al. (2012), each liter of lipid residue is capable of compromising about 1 million liters of water. Kumar et al. (2012), Mita et al. (2010), and Rigo et al. (2008) have tested the application of lipases in bioremediation of lipid residues and cite advantages over conventional methods, such as specificity, which allows the conversion into nontoxic by-products; higher yield, allowing the use in wastes with high or low pollutants; and operation using soft conditions with less energy consumption. Lipophilic residues of agrochemicals, such as organophosphates, are composed by acetylcholinesterase (ACHE), an enzyme with inhibitory action, and act as insecticide, fungicide, and herbicide (Balali-Mood and Abdollahi 2014). This agrochemical class has high polluting potential due to high toxicity, persistence in the environment, and difficult degradation (Ely et al. 2016).

Liquid wastes from the food industry have high protein content and, when deposited in aquatic environments, causes a rapid increase of decomposing bacteria population, which leads to a substantial decrease on the aquatic fauna survival due to the increase of carbon dioxide and decrease of oxygen on the environment (Dai Prá et al. 2009).

Treatments with oxidoreductases [such as oxygenases (monooxygenases, dioxygenases, and peroxidases) and oxidases (laccases)] and hydrolases (proteases and lipases) are pioneers in the enzymatic bioremediation. These enzymes are capable to convert phenols, aromatic hydrocarbons, aromatic amines, chlorinated and non-chlorinated compounds, recalcitrants, and estrogenic chemicals, among

others, into simpler or conjugated and less reactive substances (Tuomela and Hatakka 2011; Farnet et al. 2011; Yamada et al. 2006). In this context, the main oxidoreductases and hydrolases as well as their potential for application in the bioremediation of potentially polluting compounds will be described below.

4.1.1 Oxidoreductases

Oxidoreductases (EC class 1) are enzymes that catalyze electron transfer, through oxi-reduction reactions, from a reducing agent or electron donor to another molecule that acts as an oxidizing agent or electron acceptor and, usually, uses NADP or NAD^+ as a coenzyme. Microorganisms, such as fungi and bacteria, are capable of producing energy by oxidoreductase-mediated biochemical process and transforming contaminants into harmless compounds, acting as detoxifiers (Karigar and Rao 2011; Gianfreda et al. 1999; Bollag 1998).

Oxidoreductases are produced by various bacteria, fungi, and higher plants and are able to degrade natural and artificial pollutants, reverse toxicity caused by xenobiotics, and reduce heavy metals, through their oxi-reduction capacity (Mulo and Medina 2017; Rubilar et al. 2008; Husain 2006; Park et al. 2006). Among the producers of oxidoreductases are the bacteria *Bacillus safensis*, which degrade petroleum compounds (Husain 2006); white-rot fungi as *Panus tigrinus*, which produce oxidoreductases such as laccases, lignin peroxidase, and manganese peroxidase, capable of transforming phenols (D'Annibale et al. 2004); and plants (families Fabaceae, Gramineae, and Solanaceae) which degrades phenolic compounds (Rubilar et al. 2008; Newman et al. 1998). Following, we will approach the oxidoreductase, oxygenase (monooxygenases and dioxygenases and peroxidases), and oxidase (laccases) functions, which have a broad spectrum of action in the environmental bioremediation.

4.1.2 Oxygenases

Oxygenases (EC 1.13 and 1.14) are enzymes capable of catalyzing the oxygen transfer, from molecular oxygen (O_2) to an organic or inorganic substrate, using FAD, NADH, or NADPH as coenzymes. The oxygenases are classified according to the number of oxygen atoms used during the oxidation: when there is a transfer of an atom, they are classified as monooxygenases and when there is a transfer of two atoms, dioxygenases (Karigar and Rao 2011; Bugg 2003). In addition, they are able to play a central role in the degradation and detoxification of compounds through hydroxylation, epoxidation, dehydrogenation, cyclization, and rearrangement reactions (Cochrane and Vederas 2014; Arora et al. 2009). The OxDBase database provides detailed specifications of 237 oxygenases; among them, 118 are monooxygenases and 119 dioxygenases (Arora et al. 2009).

The degradation of aromatic compounds, such as phenols, is carried out mainly by oxygenases (Chakraborty et al. 2014), which cleave the aromatic ring by the addition of one to two oxygen molecules. Furthermore, pollutants from organic halogen compounds such as herbicides, fungicides, and pesticides can be degraded by dehalogenation, an elimination reaction, in which a halogen is withdrawn with the addition of a metal forming an alkene (Sivaperumal et al. 2017; Fetzner and Lingens 1994). In addition to aromatic compounds, chlorinated compounds and aliphatic hydrocarbons can also be degraded by oxygenases, being converted to acetyl-CoA and catabolized in the citric acid cycle, along with electrons transportation in the respiratory chain, until the oxidation to CO_2 and water (Madigan et al. 2016).

4.1.2.1 Monooxygenases

Monooxygenases have been characterized in a variety of microorganisms (Das and Chandran 2011). They are classified into two subgroups: flavin monooxygenases (which degrade chlorinated pesticides, possess flavin as a prosthetic group, and are dependent on NADP and NADPH) (Bajaj et al. 2010) and P450 monooxygenases (metabolize xenobiotics and contain heme prosthetic group) (Tuomela and Hatakka 2011; Galán et al. 2000). Among the baterial P450 monooxygenases, CYP102 from *Bacillus megaterium* BM3 stand out for being better characterized; able to hydroxylate a variety of alkanes, fatty acids and aromatic compounds (Karigar and Rao 2011; Grosse et al. 1999) (Ghashghavi et al. 2017).

The monooxygenases (EC 1.14.14), or mixed-function oxidases, catalyze the transfer of one O_2 atom to the substrate, and the electrons of the coenzymes NADH or NADPH reduce the other oxygen to water, being also classified as hydrolases (Arora et al. 2009). Although most of these enzymes require coenzymes, there are monooxygenases that act independently, requiring only molecular oxygen and the substrate as a reducing agent (Arora et al. 2010; Cirino and Arnold 2002), as tetracenomycin F1 monooxygenase from *Streptomyces glaucescens* and quinol monooxygenases from *Escherichia coli* (Arora et al. 2010; Shen and Hutchinson 1993).

These enzymes act primarily on alkanes, steroids, fatty acids, and aromatic and aliphatic hydrocarbons, through reactions of desulfurization, dehalogenation, denitrification, ammonification, and hydroxylation of the substrate, and are considered biocatalysts for bioremediation processes and synthetic chemistry (Roccatano 2015; Arora et al. 2010). Hydrocarbons degradation by monooxygenases is characterized by the oxidation of a methyl group to a primary alcohol, which will then be oxidized to an aldehyde and converted to a fatty acid (Das and Chandran 2011).

4.1.2.2 Dioxygenases

The dioxygenases (EC 1.13.11), or oxygen transferases, catalyze the transfer and incorporation of two atoms of molecular oxygen to the substrate (Bugg 2003). Dioxygenases are mainly used for bioremediation of wastes from the pharmaceutical, chemical, and colorant. These enzymes can act in the transformation of aromatic into aliphatic compounds by the decomposition of the aromatic ring from the positions 1 and 2 (Khatoon et al. 2017; Guzik et al. 2013). They are classified according to the mode of action (Gibson and Parales 2000): *aromatic ring-cleaving dioxygenase* (ARCD), capable of cleaving aromatic rings, or a*romatic ring-hydroxylating dioxygenase* (ARHD), capable of degrading compounds by the addition of two oxygen molecules (Ju and Parales 2011; Karigar and Rao 2011).

Dioxygenases can use iron as a cofactor; these enzymes are divided into three classes, according to how iron is incorporated in the enzyme active site:

(i) Mononuclear iron dioxygenases or non-heme iron-dependent dioxygenases: a single iron is used to incorporate one or two oxygen atoms to the substrate, performing cleavage of the bonds C-C, C-S, hydroperoxidation of fatty acid, and thiol oxidation (Visser 2011; Leitgeb and Nidetzky 2008; Abu-Omar et al. 2005). An example of these enzymes are catechol dioxygenases, which catalyze the addition of oxygen atoms to the 1,2-dihydroxybenzene (catechol) molecule, cleaving of the aromatic ring, and are found in a great diversity of microorganisms, mainly in soil bacteria, such as *Pseudomonas* (Muthukamalam et al. 2017; Madigan et al. 2016; Fetzner 2012; Van Hamme et al. 2003). The catechol dioxygenases are further classified as *extradiol* (contains Fe (II) and cleaves the binding between 2,3-CTD) or *intradiol* (contains Fe (III) and cleaves the binding between 1,2-CTD) according to the cleavage mechanism (Fetzner 2012; Visser 2011; Arora et al. 2009; Bugg and Ramaswamy 2008; Abu-Omar et al. 2005). After catechol degradation, the products are incorporated in the citric acid cycle until they are mineralized to CO_2 (Madigan et al. 2016).

(ii) Rieske dioxygenases (2Fe-2S): catalyze the degradation of aromatic compounds by cis-dihydroxylation converting them into cis-dihydrodiol, and these enzymes are also found in soil bacteria and dependent on the electron transfer to reduce two electrons from the substrate (Abu-Omar et al. 2005).

(iii) Heme dioxygenases: use iron by a heme prosthetic group, as in the oxidation reaction of L (and D-)-tryptophan into N-formyl kynurenine (Efimov et al. 2011; Thackray et al. 2008).

In addition, toluene dioxygenases (TOD) is a prominent enzyme due to the ability to act as monooxygenase, transforming monocyclic aromatics, aliphatics, and alkenes, or dioxygenases, transforming aromatic compounds (Mukherjee and Roy 2013).

4.1.3 Laccases

Laccases (EC 1.10.3.2) constitute the family of multicopper oxidases, which oxidize the substrate by transferring electrons (without incorporating them into the substrate) from one mononuclear copper center to another trinuclear copper center, reducing dioxygen to form water (Rao et al. 2014; Shraddha et al. 2011; Chakroun et al. 2010; Sakurai and Kataoka 2007).

Laccase transformation capacity was first described by Yoshida in 1883, who isolated the enzyme from Japanese lacquer (*Rhus vernicifera)* (Yoshida 1883). Laccase has several isoforms and can be found in plants, insects, bacteria, and fungi, especially white-rot ones as *Trametes versicolor*, *Lycoperdon* sp., *Marasmius quercophilus*, *Coriolus versicolor*, *Pleurotus ostreatus*, and *Phellinus igniarius*; approximately 100 isoenzymes were isolated from fungi and biochemically characterized until 2009 (Farnet et al. 2009; Liu et al. 2009; Canfora et al. 2008).

Among the enzymes for bioremediation purposes, laccases are the most attractive one, with wide possibilities of applications (Rao et al. 2014). They are able to act in a great diversity of organic and inorganic substrates of industrial processes. These enzymes are stable and require only molecular oxygen for catalysis, i.e., without needing an additional co-substrate (Stadlmair et al. 2017; Gasser et al. 2014; Tran et al. 2010). Redox mediators, such as 2,2′-azino-bis (3-ethylbenzothia zoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), 3–5,dimethoxy-4-hydroxyacetophenon (DMHAP), and violuric acid, can optimize the electron transfer mediated by laccases. However, the real advantages of a mediator application in these reactions are uncertain and can cause contamination (Kobakhidze et al. 2018; Ashe et al. 2016; Nguyen et al. 2014; Rodríguez-Rodríguez et al. 2012; Prieto et al. 2011; García-Galán et al. 2011; Marco-Urrea et al. 2010).

Laccases can detoxicate pollutants such as petroleum derivatives (PHAs), paints, plastics, dyes, estrogenic substances, paper, and cellulose (Upadhyay et al. 2016; Viswanath et al. 2008, 2014; Cabana et al. 2007); oxidize phenols, polyphenols, metals, polyamines, and aryl diamines groups; and act on lignin degradation and on azo dyes, performing not only oxidative reactions but also substrate decarboxylation and demethylation (Legerská et al. 2016; Mai et al. 2000).

Studies, aiming to understand some of the challenges inherent in the use of laccases, such as the inhibition by halogenated reagent, azide, cyanide, and hydroxide, are still needed. In addition, there are some difficulties for fungal laccase production, such as the demand of high nitrogen concentrations, the control of the ionization potential of some molecules (such as p-hydroxybenzoic acid and naphthalene), and the alteration control of the enzyme oxidation capacity in certain phenol concentrations (Rao et al. 2014; Farnet et al. 2009).

4.1.4 Peroxidases

Peroxidases (EC 1.11.1.7) catalyze the reduction of peroxides, such as hydrogen peroxide (H_2O_2) by the oxidation of an electron donor (Bansal and Kanwar 2013). The peroxidases act in bioremediation processes due to their thermostability and capacity to oxidize a wide range of substrates. They can be applied in the removal processes of aromatic compounds, such as phenols, cresols, chlorinated phenols, polyphenols, amines, and primary aromatic polyamines, and nitroaromatic compounds, TNT (2,4,6-trinitrotoluene), and in the degradation of lignin and dyes (Cameron et al. 2000; Regalado et al. 2004; Bansal and Kanwar 2013; Ely et al. 2016). Peroxidases act on the compounds' precipitation, on the disruption of the aromatic ring, and on the total mineralization of compounds until the formation of CO_2 and water (Husain 2010; Maciel et al. 2007).

Peroxidases can be produced by animals, plants, bacteria, and fungi and are classified according to the family or presence or absence of a heme group (Bansal and Kanwar 2013; Husain 2010; Koua et al. 2009) (Fig. 4.1). Horseradish peroxidase, cultivated in cold weather countries, is the most studied peroxidase and has wide action capacity in diverse pollutants; however, it is active at low temperatures, which makes its application unfeasible for the treatment of liquid effluents and recalcitrants' removal (Husain 2010; Maciel et al. 2007; Kim et al. 2005; Mohan et al. 2005).

Among fungal peroxidases, lignin peroxidase (Lip) and manganese peroxidase (MnP) have a high potential for transformation of toxic substances, high yield, and low cost of production. Fungal peroxidases are advantageous in comparison to plant peroxidases, which have production limitations due to low yield and high cost. However, due to the wide use of these enzymes as environmental biocatalysts and the growing interest in their application in bioremediation, studies regarding the sources and low cost of obtaining processes with equal or greater efficiency are needed (Zancan et al. 2015; Dec and Bollag 1994).

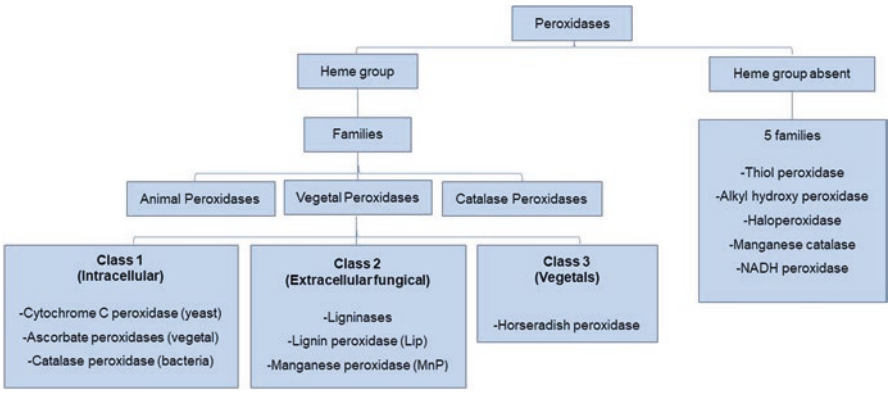

Fig. 4.1 Classification of peroxidases

4.1.5 Class II of Heme Peroxidases

Among class II of heme peroxidases are Lip and MnP, which catalyze oxi-reduction reactions with H_2O_2 as the substrate. These enzymes are mainly produced by bacteria (*Basidiomycetes*) and white-rot fungi such as *Phanerochaete* (Xu et al. 2015; Hofrichter 2002) and play a central role on lignin degradation (Ruiz-Dueñas et al. 2007), as MnP oxidizes the phenolic structures of lignin, whereas LiP mainly acts on the non-phenolic components (Falade et al. 2016).

Lip is a glycoprotein that possesses iron as a prosthetic group and catalyzes the oxidation reaction of hydrogen peroxide with veratryl alcohol as a mediator (Abdel-Hamid et al. 2013; Baptista et al. 2012; Tang et al. 2006). Although closely related to the reduction potential of non-phenolic structures, Lip also acts on aromatic compounds such as phenols, aromatic carbohydrates, and also halogenated compounds (Souza and Rosado 2009; Martínez et al. 2005; Baciocchi et al. 2001), what justifies its wide application on the bioremediation of diverse pollutants (Tuomela and Hatakka 2011).

MnP is also a glycoprotein that uses Mn^{2+}/Mn^{3+} as an intermediate redox pair (Embrapa 2004), producing oxalate chelate of Mn^{3+} as a final product and capable of acting on xenobiotic pollutants (ten Have and Teunissen 2001). MnP acts exclusively on the phenolic portions of lignin and acts on aromatic amines, PHAs, and various dyes (Zhang et al. 2016; ten Have and Teunissen 2001).

Currently, immobilization studies with chitosan beads and sol-gel matrix have been performed aiming higher activity and stability of these enzymes (Bilal et al. 2017).

Thus, several studies demonstrate the efficiency of enzymes in the treatment of environmental pollutants; however, future research should be carried out aiming a low-cost production and higher yield (Peixoto et al. 2011), greater enzymatic stability (Giandreda and Rao 2011), application control, research for new enzymes, use of isolated enzymes (Stadlmair et al. 2018), synergistic action (Touahar et al. 2014), microbiome research using sequencing techniques as metagenomics (Drewes et al. 2014) and metatranscriptomic (Yu and Zhang 2012), as well as analysis of the mechanisms of action by mass spectrometry (MS) and characterization of the formed products which can be performed by nuclear magnetic resonance (NMR) and MS (Stadlmair et al. 2017; Lee et al. 2014).

4.1.6 Hydrolases

Hydrolases perform mainly hydrolysis reactions, i.e., they transfer functional groups to water (Nelson and Cox 1970). In bioremediation, hydrolases act reducing the toxicity of pesticides and insecticides composed by organophosphates and carbamates (Sharma et al. 2018). For organophosphates degradation, some microorganisms have specific hydrolases known as parathion hydrolases (Iyer et al. 2013).

Hydrolases also act in the decomposition of oil residues, petroleum derivatives (Kumar et al. 2012; Karigar and Rao 2011), and industrial effluents (Verma et al. 2012), acting on the chemical bonds of esters, peptide, and carbon. The enzymatic remediation for the degradation of toxic organic compounds is safe, economic, and controlled due to the substrate specificity and advantageous when compared to the physicochemical treatment, which depends on predetermined conditions such as temperature, has environmental restrictions, and eventual production of unwanted by-products (Jardine et al. 2018; Sharma et al. 2018; Karigar and Rao 2011).

Strains of *Stenotrophomonas maltophilia* were isolated from soils with agricultural activity and produced hydrolases capable of decomposing organophosphates (Iyer et al. 2016). Hydrolases from the fungus *Cladosporium cladosporioides* showed bioremediation potential in soils contaminated with chlorpyrifos, an organophosphorus insecticide used in various agricultural crops (Gao et al. 2012). Below, we will approach some already known groups of hydrolases (amylases, lipases, proteases, and cellulases) that are used in the chemical and food industries and have also been applied in the bioremediation of several pollutants (Sharma et al. 2018; Sánchez-Porro et al. 2003).

4.1.7 Amylases

Amylases are one of the most important groups of industrial biocatalysts and represent approximately 25% of the market for enzymes (Cotârlet et al. 2011). There are several types of amylases. The α-amylases (EC 3.2.1.1) catalyze the hydrolysis of 1,4-α-D-glycosidic bonds in simple starch and carbohydrate components (Cotârlet et al. 2011). Most α-amylases are metalloenzymes, which require calcium ions for their activity, stability, and structural integrity (Kuddus and Ramteke 2012). The β-amylases (EC 3.2.1.2) are independent of calcium ions and hydrolyze α-1,4-glycosidic bonds in starch, removing maltose units from the nonreducing ends of the chains (Vajravijayan et al. 2018). Glycoamylases (EC 3.2.1.3) hydrolyzes1,4 and 1,6 glycosidic bonds, releasing d-glucose from the nonreducing ends of starch, polysaccharides, and oligosaccharides (Abidi et al. 2017). Isoamylases (EC 3.2.1.68) and pullulanases (EC 3.2.1.41) can debranch the starch, hydrolyzing α-1,6-glycosidic bonds in amylopectin to produce amylose and oligosaccharides (Li et al. 2017; Zheng et al. 2013). Amylases have biotechnological applications in food processing, pharmaceutical production, and bioremediation (Kuddus and Ramteke 2012).

Vegetable residues from agricultural activities and markets are just a part of the whole amount of garbage produced, mainly in large cities. Bacteria isolated from plant residues are proved produce amylase (Janarthanan et al. 2014), showing that the enzymatic production can be an alternative for bioremediation of plant residues (Janarthanan et al. 2014).

N-alkanes are a group of hydrocarbons found in fossil and fuel products, when released to the environment they become contaminants of soils and water. An

in vitro study showed that amylases from *Bacillus subtilis* degraded all the n-alkane from a contaminated biomass in 72 h (Karimi and Biria 2016).

In the textile industry, amylases are used to break down starch residues, improving product quality (Vaidya et al. 2015).

Amylases from *Streptomyces* sp. are an alternative for bioremediation of polluted soils and wastewater from cold regions due to their predominant enzymatic activity at low temperatures (10–20 °C) (Cotârleț et al. 2011), while amylases from *Bacillus* spp. catalyze starch decomposition at 53 °C (Jardine et al. 2018).Thus, these amylases can be viable alternatives for the bioremediation of wastes from paper and food industries (Jardine et al. 2018; Vaidya et al. 2015).

4.1.8 Lipases

Lipases (EC 3.1.1.3), also known as triacylglycerol acylhydrolases, are enzymes that catalyze reactions of hydrolysis and synthesis as esterification, alcoholysis, and interesterification of fatty acids, esters, and glycerides (Miranda et al. 2015; Kumar et al. 2012; Pandey et al. 1999).

Lipases are applied in the bioremediation of oil residues, petroleum contaminants, effluents, and soil recovery (Ghafil et al. 2016; Kumar et al. 2012; Verma et al. 2012).

Vegetable oils are used as food due to their composition of polyunsaturated fatty acids that are proven beneficial to human health (Borsonelo and Galduróz 2008); however, after cooking these polyunsaturated chains become saturated fatty acids, which are often dispensed to the environment, becoming an environmental pollution problem. Lipases can be a viable and eco-friendly alternative for the treatment of these residues, being able to modify the fatty acids chains and reduce the toxicity of cooked oil (Okino-Delgado et al. 2017; Kumar et al. 2012).

Through transesterification, an in vitro assay proved that orange waste lipases were capable of modifying cooked soy oil, reducing its toxicity on human epithelial cells (Okino-Delgado et al. 2017).

Soil pollution by mineral oil hydrocarbons, derived from petroleum products, is a common environmental problem and a side effect of industrial activities (Karigar and Rao 2011). Fungal lipases proved to be the best biochemical markers for the bioremediation soils contaminated with diesel oil with high hydrocarbons concentration, reducing the hydrocarbon concentration and the increasing soil aerobic activities (Riffaldi et al. 2006).

Bacterial isolates from soil contaminated by automobile engine oil showed the ability to produce lipases and degrading hydrocarbons, the main soil contaminant (Mahmood et al. 2017).

Lipases from *Pseudomonas* spp. were immobilized in coal and applied to the bioremediation of soil contaminated with industrial waste oils. Lipases were more effective for soil decontamination in comparison to the immobilized bacteria, acting directly in the fatty acids and causing a reduction of approximately 75% of the

residual oils concentration (Ghafil et al. 2016). The bioremediation of wastewater contaminated with crude oil through the application of lipases from *Pseudomonas aeruginosa* was confirmed, by the gradual decrease of the oil concentration and reduction of approximately 80% of waste toxicity over a period of 7 days (Verma et al. 2012).

4.1.9 Proteases

Proteases (EC 3.4.21.12) are a group of multifunctional enzymes that catalyzes the hydrolysis of peptides bonds and can perform synthesis reactions on ester and amide bonds. Proteases are present in all living forms as bacteria and fungi (microorganisms), plants, and animals (Kuddus and Ramteke 2012) and have been the main enzyme obtained from marine sources, mainly from marine microorganisms (Sivaperumal et al. 2017). In in vitro assay, proteases from *Pseudomonas fluorescens* acted in the degradation of the hydrocarbons, reducing in 54% the diesel oil concentration, a fossil fuel often found as a soil pollutant soils (Kumar et al. 2014).

Nonionic ethoxylated surfactants are compounds present in general cleaning products as detergents, shampoos, and hygiene products. In large quantities, these compounds can cause environmental pollution. Fungal proteases from *Cladosporium cladosporioides* and *Geotrichum candidum* were related to the decomposition of approximately 55% of the nonionic ethoxylated surfactants in water samples, showing to be an alternative for bioremediation of effluents with high levels of hydrocarbons and phenolic residues (Jakovljević and Vrvić 2016). Proteases isolated from *Penicillium verrucosum* showed a potential performance in the bioremediation of synthetic detergents reducing in 80% and 260% the concentration of sodium tripolyphosphate and oleyl ethoxylated alcohol, respectively (Jakovljević and Vrvić 2017).

Plant proteases have been described as low-cost enzymes, stable at different temperatures and pH that are easily obtained; they have been used as biomarkers of heavy metal contamination (Halmi et al. 2016; Shukor et al. 2006). Proteases are closely related to the decomposition of dead animals, industrial discards, and any protein residues that cause environmental pollution (Karigar and Rao 2011). Most of the leather industries use lime and sodium sulfide to remove animal skin residues from leather, causing significant environmental pollution (Majumder et al. 2014; Jaouadi et al. 2008). Lime produces an environmentally toxic sludge, while sodium sulfide, in addition to the unpleasant odor, is highly toxic to human health; thus, protease-mediated enzymatic treatment is an alternative to the chemical treatment of leather (Majumder et al. 2014). Broiler farms produce as main residues carcasses and animal feathers that without the appropriate treatment end up polluting the environment (Pillai and Archana 2008). In both cases, proteases could diminish the toxicity and effluents quantity, through the keratinolytic activity, i.e., the ability to eliminate the protein residues by breaking the peptide bonds (Majumder et al. 2014).

4.1.10 Cellulases

Cellulases (EC 3.2.1.4) are the main enzymes for the degradation of cellulose, the most abundant biopolymer and one of the most abundant sources of organic carbon on Earth (Sharma et al. 2018, 2016; Mewis et al. 2013). Cellulose family is composed of at least three enzymatic groups: endo-(1,4)-β-D-glucanases (EC 3.2.1.4), exo-(1,4)-β-D-glucanases (EC 3.2.1.91), and β-glycosidases (EC 3.2.1.21). Endoglucanases randomly break internal O-glycosidic bonds, resulting in glucan chains of different lengths. Exoglucanases act at the ends of the cellulose chain and release β-cellobiose as the final product. β-Glycosidases act specifically on the disaccharides of β-cellobiose and produce glucose (Kuhad et al. 2011).

Endoglucanases can be used in the bioremediation of dicofol, a pesticide of low toxicity used in several agricultural crops such as apple and cotton, whose permanence in the soil has been causing environmental impact, by degrading it in 4,4-dichlorodibenzophenone (nontoxic compound) (Wang et al. 2015).

Acid-thermophilic cellulases from *Bacillus sonorensis* were effective in the bioconversion of lignocellulosic residues into biofuel, directing plant residues to a sustainable production chain (Chang et al. 2014).

The exploitation processes, extraction, refining, transportation, storage, and use of oil, commonly lead to oil spills that can affect rivers, oceans, and coasts, causing a great environmental impact. As an alternative to repair the damages, among many other methods, the use of oil absorbents is an economic and ecological way of dealing with this problem (Peng et al. 2013).

The use of agro-industrial residues as an alternative to oil absorbents is common, but the high cellulose content (hydrophobic compound) limits its absorbent capacity; thus, treatments with cellulases can be a viable alternative to increase the specific surface area, favoring the absorption of petroleum residues. In comparison to the chemical method, the modification of plant material by cellulases is efficient and sustainable (Peng et al. 2013).

4.2 Conclusion and Future Perspectives

Treatments with oxidoreductases and hydrolases have been pioneers in the enzymatic application for bioremediation (Fischer and Majewsky 2014; Silva et al. 2016; Naghdi et al. 2018). These enzymes are of particular interest of environmental issues due to their ability to transform phenols, aromatic hydrocarbons, aromatic amines, chlorinated and non-chlorinated compounds, recalcitrants, and estrogenic chemicals, among others (Tuomela and Hatakka 2011; Yamada et al. 2006; Farnet et al. 2011).

Enzymes can aid the bioremediation process by precipitating pollutants, without altering the compound chemistry; degrading pollutants by oxidative reactions, turning the compounds to CO_2, N, P, and water (mineralization); and biotransforming

and reducing the pollutants to harmless or easily treatable substances for bioconversion in added value products, such as biomass (Karam and Nicell 1997; Durán and Esposito 2000; Rodrigues 2003). Moreover, enzymes are advantageous when compared to the chemical treatments due to the specificities that allow their performance in a wide spectrum of pH, temperature, and salinity (Durán and Esposito 2000).

However, an efficient enzymatic application in different environmental conditions is still a challenge since it depends on the enzymatic suitability to chemical and physical conditions of the system (such as pH and temperature) and enzymatic properties, as the catalytic site, mechanism of action and potential activity. In a future perspective, it is necessary to study the complexes behavior and to identify the metabolites and their degradation pathways, in order to find the adequate solution for each pollutant (Sharma et al. 2018).

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Chapter 5
Biotransformation of Heavy Crude Oil and Biodegradation of Oil Pollution by Arid Zone Bacterial Strains

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Abstract The present century has seen tremendous progress in various types of renewable fuels and its possible applications. Fossil fuel, such as crude oil remained as prime source of energy, and it still fuels industries and households. In fact the demand for fossil fuels has increased in the last decade or so, because of increased population and demand due to industrial revolutions. It also leads to increased incidences of crude oil-related pollutions, oil spills, pipeline damages, accidental or intentional spillage, release from tankers, etc., which are quite persistent and very difficult to remediate. Heavy crude oil spillage is even more difficult to remediate, due to its hydrophobic, toxic constituents, and its partial or incomplete degradation leads to even more toxic intermediates in the affected environment. Harmful effects of crude oil spills are often observed in marine mammals, birds, and land-based animals, including humans. Commonly used remediation practices are often not quite effective and lead to only partial removal. Microbial biodegradation is reported to be an effective and environment-friendly alternative, which could be applied under both aerobic and anaerobic conditions onshore or offshore. Bacteria from marine and arid region are reported to be better biodegrader as compared to fungi. Several types of enzymes are reported to be quite effective for catalytic conversion of heavy crude oil and its derivative constituents. Recent progress in genetic engineering and omics techniques will be quite useful for further identifying the metabolomic routes and devising an efficient biodegradation to completely harmless end products.

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Keywords Heavy crude oil · Asphaltenes · Biotransformation · Biodegradation

5.1 Heavy Crude Oil

Crude oil is generally defined as a complex mixture of hydrocarbons and other organic/inorganic compounds, derived from the gradual degradation of organic matter at high pressures and temperatures, buried in sedimentary rocks for millions of years. Those organic matters were generated from living systems such as higher plants and macro- and microorganisms (Durand 1988). The constituents of crude oil vary depending on organic sources, thermal and pressure, geological, and reservoir compositions. In general different products such as gasoline, diesel, jet fuel, coke, kerosene, asphalt, and other products are made from refined crude oil (Fig. 5.1). Crude oils differ in their characteristics such as polarity, molecular mass, molecular size (carbon numbers), solubility, and elemental composition (Tissot and Welte 2012). Different forms of crude oil are present in nature: light crude, intermediate crude, heavy oil, tar sand, bitumen, and oil shale (Santos et al. 2014). In general carbon (>85%) and hydrogen (>10%) are the major elements of crude oils, and the rest are non-hydrocarbon elements, such as sulfur, nitrogen, and oxygen (0.1–5%), and traces of different metallic compounds (such as chloride, magnesium, lead, vanadium, arsenic, nickel, etc.) (Chaudhuri 2010; Mansoori 2009; Moustafa and Morsi 2012). The hydrocarbons present in crude oil are generally grouped into alkanes (e.g., paraffins), alkenes (e.g., olefins), cycloalkane (e.g., naphthenes), and

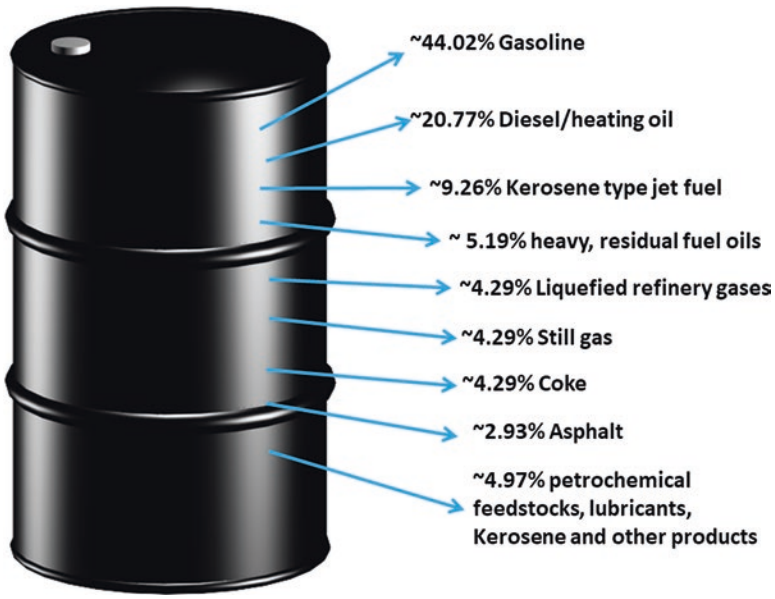

Fig. 5.1 Typical products made from a 42 gallon barrel of refined crude oil

aromatics (e.g., benzenes) in varying proportions. Paraffins (C_nH_{2n+2}) are saturated hydrocarbons, olefins (C_nH_{2n}) are unsaturated hydrocarbons, naphthenes (C_nH_{2n}) are cyclic saturated hydrocarbons, and aromatics (C_nH_{2n-6}) are generally very active hydrocarbons. Complex hydrocarbons such as polycyclic aromatic hydrocarbons (PAH) and alkyl aromatics are also present in crude oil, along with different metals (Akmaz et al. 2011; Chaudhuri 2010).

By visible explanation, heavy or extra heavy crude oil is a type of crude oil which is very difficult to flow, due to "higher density or specific gravity," than that of light or intermediate crude oil. Heavy crude oil has been defined as any liquid petroleum with API gravity <20°, specific gravity >0.933, and sulfur content higher than 2% (w/w) (Speight 2006; Santos et al. 2014). However, the definition of heavy crude oil based on API gravity, viscosity, or density is quite arbitrary. The oil viscosity is the main fluid property that highly affects its production; however, there is no standard relationship between density and viscosity, but "heavy" and "viscous" terms tend to be used often to describe heavy oils. Conventional oil viscosity may range from 1 to 10 centipoise (cP), whereas heavy and extra heavy oil viscosity may range from <20 cP to >1,000,000 cP (Alboudwarej et al. 2006). In general, crude oils of API gravity <10° API are in solid or semisolid state and considered as extra heavy oil (Speight 2006; Santos et al. 2014). There could be more than 100 carbon atoms in the molecular structure of heavy oil, resulting in high boiling point and molecular weight.

Heavy oil, tar sands, bitumen, and oil shale contain high concentrations of asphaltenes, resins, acidic components, wax, and other components (Mansoori 2009). Generally, structure of resins and asphaltenes vary from one crude oil to another (Ghollami et al. 2013). Asphaltenes are among the heaviest and most polar components of crude oil (Goual 2012; Leon and Kumar 2005). The physical and physicochemical properties of resins are different from the asphaltenes, and its molecular weight is also significantly lower than the asphaltenes. Generally resins have a wide range of molecular weight and polarities and are completely soluble in light fractions of crude oil (Mansoori 2009). Resins are a heavier fraction than aromatic and saturate fractions of crude oil (Akmaz et al. 2011). Based on those four main hydrocarbon components/categories, quite a few analytical methods were developed to quantify a petroleum mixture composition, such as PONA (paraffins, olefins, naphthenes, and aromatics), PNA (paraffins, naphthenes, and aromatics), PIONA (paraffins, isoparaffins, olefins, naphthenes, and aromatics), SARA (saturates, aromatics, resins, and asphaltenes), and elemental analysis (C, H, S, N, O) (Al-Sayegh et al. 2016; Riazi and Eser 2013). However, SARA analysis is more commonly reported method for crude oil characterization.

5.2 Heavy Crude Oil Pollution

Nowadays different types of environmental pollution issues such as contamination of land, water, and air are of much concern, and globally researchers are faced with the overwhelming challenge of overcoming damaging effects. Crude oil pollution is

one among those pollutants (Joshi 2016). The increasing demand for crude oil as a major energy source for society and industries has resulted in an increased production, transportation, and refining which in turn lead to environmental pollution (Ajagbe et al. 2012). Globally, leakage and spillages of crude oil from damaged pipelines, storage tankers and offshore platforms, careless spills and disposal, and dumping in drilling pits (after completion of oil well) are major sources of petroleum contamination in the environment, which often contaminate the impacted soil and surrounding ecosystem (Santisi et al. 2015). It adversely impacts marine habitats, migratory birds, agricultural practices, underground water tables, flora, and fauna. The crude oil components pose an immediate threat to humans, animals, and the environment, as they are mutagens and carcinogens (Bezza and Chirwa 2015). Accidental or intentional crude oil spills or discharges into water systems will ultimately end up on land and may contaminate the properties of the surrounding soil and change its physical and chemical properties (Urum et al. 2006). The use of this oil-contaminated soil in agricultural lands typically affects plant metabolism, often causing a reduced crop yields. It also poses a great hazard for human health through the direct contact or through the consumption of contaminated crops. Furthermore, this contaminated soil may be used in making of concrete to erect structures or may be used in road and tunnel constructions. The degree of contamination could also affect the tensile quality of the concrete made from such soil (Ajagbe et al. 2012). Crude oil affects feathers and furs of marine birds and mammals by reducing their insulating ability, thus making them more exposed to harsh temperature changes and less buoyant (Balseiro et al. 2005; Dunnet et al. 1982). The adverse effects of oil spill are also reported to reduce the ability of soil to support plant growth, contaminate ground water, and increase the heavy metal concentration causing adverse health effects due to bioaccumulation, causing respiratory distress and pose fire hazard (Campbell and Krauss 2010; Robertson and Krauss 2010; Adams et al. 2011; Malik 2000). One of the most critical objectives in an oil spill location is to access the level of threat and to eradicate it in a harmless and most efficient manner. Complete removal is not practically achievable mostly due to implementation cost or scale. Thus the immediate objective is generally to remediate the site to the acceptable levels that is harmless to surrounding flora and fauna.

Various technological approaches are reported for treating land contaminated by petroleum hydrocarbons: chemical, physical, biological, and thermal (Brassington 2008). Most of these treatment technologies have limited full-scale applications to treat the contamination. Sometimes physical cleanup and chemical treatment methods may further lead to more complicated side effects (Sei and Fathepure 2009). During chemical treatment, direct injection of chemical oxidants into contaminated soil and water is performed, which thereby changes the native balance between chemistry and biology. Biological treatment generally includes the breakdown of contamination into nonhazardous structures utilized by microorganisms (Mukred et al. 2008). In field-scale applications, some of these treatment methods are time-consuming and not cost-efficient when handling huge amount of such contaminated material (Urum et al. 2006). Biosurfactants are reported to be effective in the removal of oil from oil-contaminated sands (Portet-Koltalo et al. 2013; Bezza and

Chirwa 2015; Joshi and Desai 2010). For marine oil spill treatments, evaporation, followed by photooxidation and by geochemicals reactions, helps at an early stage to remove lighter fractions. Turbulent marine currents also results in "water-in-oil" (micro)emulsions. Heavy fractions are scattered or gets broken up, and just a little portion may be removed by the process of biodegradation. Although chemical-physical phenomena assume a critical role in detoxification, a definitive and complete degradation is mainly accomplished by marine microflora, mainly by bacteria (McGenity et al. 2012; Santisi et al. 2015). There are some factors, such as mechanical (waves, air current), physical (temperature, solar radiation), and chemical (pH, dissolved oxygen, and nutrient concentration), that affect the fate of crude oil by natural transformation and biodegradation (Nikolopoulou and Kalogeraki 2010). In land-based treatment, sedimentation happens when oil adsorbs to soil particles. Indigenous free-living bacteria are reported to be the most suitable PAH degraders (Dasgupta et al. 2013). Those suitable microbes are able to oxidize crude oil hydrocarbons and utilize it as a source of carbon, nitrogen, and energy (growth) and further convert the complex, toxic hydrocarbon compounds to simpler, nontoxic forms (She et al. 2011).

5.3 Analysis of Heavy Crude Oil

Heavy crude oil is a complex organic compound with high molecular weight, where major parts are aromatic and an aliphatic part (the branches). An essential step for compositional analysis of crude oil is to separate it into fractions of hydrocarbon (saturates and aromatics), resins, and asphaltenes (SARA). The asphaltene fraction and its intermolecular exchanges are reported to be mainly responsible for oil properties such as high viscosity and the tendency to form "water-in-oil" emulsions and coke (Shi et al. 2010). The efficiency of different degradation processes, including bioremediation and biotransformation, can be assessed based on the qualitative and quantitative analysis of different/individual components present in the crude oil before and after treatment.

Different techniques used to analyze crude oil composition are crude oil such as thin-layer chromatography-flame ionization detector (TLC-FID), high-performance liquid chromatography (HPLC), pyrolysis-gas chromatography-mass spectrometry (PyGC-MS), carbon (^{13}C) and proton (^{1}H) nuclear magnetic resonance (NMR), and other spectroscopic techniques (Fourier transform infrared (FTIR), X-ray photoelectron spectroscopy (XPS), extended X-ray absorption fine structure (EXAFS), X-ray absorption near edge structure (XANES), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), etc.) (Merdrignac and Espinat 2007). The pyrolysis coupled to Gas chromatography/Mass spectrometry (Py/GCMS) is reported to be a qualitative or semi-quantitative technique, where the pyrolysis products are directly analyzed by GC-MS. The main drawback of this technique is the difficulty to connect the pyrolysate fragments to the original structure of the compounds.

High-performance liquid chromatography (HPLC) could be used to analyze PAHs of heavy crude oil, and it could be coupled to different types of detectors such as ultraviolet (UV), fluorescence, infrared (IR), refractometer, or mass spectrometer (MS). However, the limitation is that it has to be used in a normal phase because of the compound solubility and compatibility with organic solvents. Fan and Buckley (2002) reported effective use of TLC and HPLC methods for SARA analysis for medium-gravity crude oils. Bissada et al. (2016) reported an automated multidimensional high-performance liquid chromatography (AMD-HPLC) method to enhance group-type characterization of crude oil or bitumen. The AMD-HPLC system was developed based on a combination of adsorption and partition chromatography. They reported that when compared to traditional methods, the AMD-HPLC was highly efficient, eliminating cross-contamination, and highly reproducible. NMR techniques could be used to obtain structural information from different types of crude oil samples. Different types of protons can be distinguished using ^{1}H NMR, and with ^{13}C NMR, two major carbon types like aromatic and aliphatic may be distinguished. Nevertheless, to extract more data, spectral edition has to be used, and several structural parameters need to be calculated from linear spectral combinations. Sanchez-Minero et al. (2013) reported SARA composition and characterization of five crude oils with different API gravities using NMR. Rudyk et al. (2017) also reported use of NMR for characterization of crude oil extracted by supercritical CO_2, from Gorm field in the Danish North Sea. Zhang et al. (2015) reported high-resolution MALDI-TOF-MS technique in combination with solvent-free sample preparation to characterize a petroleum pitch and a coal-tar pitch. Kim et al. (2016) also successfully demonstrated MALDI-TOF spectroscopy analysis to investigate the molecular weight distribution variation of heavy oil during visbreaking. FTIR could be used to obtain detailed information on functional group distributions of heavy crude oil (CH_n, OH, NH, and various CO groups) in combination with other techniques. XPS, EXAFS, and XANES techniques can be used to obtain information about chemical functional groups of sulfur, nitrogen, and metal elements. More recently, ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is reported to be the latest technique to characterize and identify polar and nonpolar species in petroleum without any chromatographic preseparation steps (Rodgers et al. 2002; McKenna 2009).

Even though several advanced techniques are available for compositional analysis of crude oils, gas chromatography (GC) is still undoubtedly one of the widely used techniques. The better separation combined with a wide range of detectors employing various detection principles, such as TCD, FID, MS, etc., makes GC an important, often an irreplaceable tool for crude oil analysis (Blum et al. 1990; Olesik 1991). The basic steps involved in GC analysis of heavy crude oil include extraction, cleanup, sample introduction, separation, detection, and identification or quantification of compounds. Sample preparation for the GC analysis is the most crucial step for getting the required result parameters (Halket et al. 2005; Ternes et al. 2002).

5.4 Bacterial Biotransformation and Biodegradation of Heavy Crude Oil

Several reports focusing on biotransformation and biodegradation of crude oil and PAHs are already published. Biotransformation or biodegradation of crude oil is the process in which heavier fraction of crude oil are converted to lighter compounds (Al-Sayegh et al. 2015, 2016; Shibulal 2017). Although PAHs are relatively stable chemical compounds, their millions of years of presence in the environment has prompted the advance of numerous microorganisms ready to initiate and utilize them as carbon and energy source (Prince et al. 2010). Biotransformation of heavy crude oil is the process of transforming heavier components of crude oil into lighter ones that is achieved by living organisms or enzyme preparations derived from there. The goal of biological upgrading is to make the heavy crude oil easier to produce and transport, as well as to increase the economic value of oil (Leon and Kumar 2005). Different chemical routes such as oxidizing aromatic ring of a large molecule, thus releasing smaller-trapped molecules, chelating metals present in asphaltene aggregates, severing internal bond between asphaltene molecules and large aliphatic chains, and/or originating two resins or aromatic molecules could change the physical properties of heavy crude oil and improve its viscosity (Leon and Kumar 2005). The general microbial mechanisms to access hydrophobic substrates are either by direct contact of the cell with the hydrocarbon or by biosurfactant-mediated accession through cell contact with emulsified hydrocarbons at the "oil-water-cell" interface (Wentzel et al. 2007). Biosurfactants enhance substrate bioavailability by increasing oil surface area (Das and Chandran 2011). During biodegradation process, microorganisms either transform or mineralize organic contaminants to less harmful elements or completely metabolize it and assimilate/recycle it into the natural biosphere. The biodegradation of petroleum and other hydrocarbons in the environment is an intricate process, which depends on the nature and amount of oil or PAHs present, environmental conditions, and the structure of the autochthonous microbial community (Atlas and Bartha 1992; Shibulal 2017; Van Hamme et al. 2003). In crude oil, PAHs are the major components which accounts for about 50–98% and 20–50% are n-alkanes (Van Beilen et al. 2003). Microbial degradation of crude oil has been shown to be effective by action on aliphatic and light aromatic fractions, whereas high molecular weight aromatics, resins, and asphaltenes are difficult to be degraded (Leahy and Colwell 1990).

The appropriate sampling and proper carbon sources are two main limitations while enriching and isolating hydrocarbon-degrading bacteria, as proper understanding of the microbial ecology from oil fields requires a carefully monitored sampling procedure. Collecting samples without contamination of the oil reservoir samples is the most challenging step for a petroleum microbiologist (Magot 2005). It is well known that the presence of PAHs in oil-contaminated environment selectively enriches hydrocarbon-utilizing microorganisms (Rosenberg and Gutnick 1981; Geetha et al. 2013). Some microbes can grow utilizing crude oil, whereas, sometimes, it is necessary to provide trace metals and other carbon sources to

enhance their growth (Ciric et al. 2010). In bioremediation, a mixture of different cultures more than pure one is preferable. There are two possible explanations for the benefit from the synergistic interaction. First, one of the species may get rid of toxic metabolite to help the other bacteria proceed doing the job without hindrances. Second, each species can degrade specific compounds which the other species can't (Mukred et al. 2008). Moreover, engaging more than one bacterial strain – microbial consortia – to enhance biotransformation is a major realized opportunity. Generally, anaerobic microbial growth with hydrocarbons is slow to an extent of days or weeks in case of anaerobic microbes (Widdel and Grundmann 2010). However, that is not always the case, and some reports suggested comparable results under both aerobic and anaerobic conditions (Xu et al. 2009).

Because of their flexibility, bacteria play a major role during biotransformation of hydrocarbons; however, there is no single species that will totally degrade any complex class of hydrocarbons (Brooijmans et al. 2009; Rahman et al. 2003). Several genera of bacteria are reported to utilize PAHs alone or when blended with different nutrients (Al-Sayegh et al. 2015, 2016). There are various marine bacteria able to degrade hydrocarbon of crude oil. Those are classified under various subphyla which are *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (Roling et al. 2002). *Pseudomonas* and *Rhodococcus* genera are known for their ability of degrading hydrocarbon, but common microorganisms of the biodegradation process in marine habitats are belonging to *Alcanivorax* genus (Santisi et al. 2015). Microorganism's ability of degrading oil spill is different due to many factors affecting its activity. Those possible factors are temperature and nutrient's quantity in environment, oil structure, and degree of weathering. At the same side, microbes have different ways of degradation of oil, for example, *Alcanivorax* sp. and *Cycloclasticus* sp. are reported to degrade straight-chain and branched alkanes and PAHs, respectively (McGenity et al. 2012). Hydrocarbonoclastic bacteria exhibit higher biodegradation rates than the microbes introduced to hydrocarbon-containing sites. Many microbes have been reported to have the potential for biodegradation such as *Achromobacter*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Actinomyces*, *Corynebacterium*, *Nocardia*, *Aeromonas*, *Alcaligenes*, *Beneckea*, *Brevibacterium*, *Beijerinckia*, *Pseudomonas*, *Flavobacterium*, *Moraxella*, *Micromonospora*, *Vibrio*, and yeasts (Britton 1984; Piemonte et al. 2013). *Garciaella petrolearia* TERIG02 was reported to have preference to asphalt and aromatic compounds and was able to reduce heavy oil viscosity by 37–50% in the absence and presence of molasses at 50 °C (Lavania et al. 2012). Darvishi et al. (2011) reported *Enterobacter cloacae* ERCPPI-1 strain utilizing heavy crude oil as the sole carbon source and degrading 76.3% of heavy crude oil after 21 days of incubation. Al-Mujaini et al. (2018) reported partial degradation of C_{12}-C_{30} carbon compounds from crude oil, using three *Pseudomonas* strains. Shibulal et al. (2017) reported biotransformation of heavy crude oil (67%) by isolate *Paenibacillus ehimensis* BS1, under aerobic conditions, in 9 days of incubation.

Biodegradability of different components of heavy crude oil in order of preference could be presented as n-alkanes> branched-chain alkanes> branched alkenes> low molecular weight n-alkyl aromatics> monoaromatics> cyclic alkanes> PAHs>

asphaltenes (Tyagi et al. 2010; Salanitro 2001; Kopytov et al. 2014). Lower rate of aromatic biodegradation could be attributed to low solubility, biotransformation to toxic metabolites, catabolic repression, presence of easily utilizable substrates, and absence of co-metabolic substrates (Chikere et al. 2011). Those microbes capable of biodegradation are able to survive under the limitation of nutrients in dormant state and can again become active when crude oil is available. Under substrate limiting conditions, microbes selectively utilize straight-medium-chain alkanes than long-branched-chain alkanes. Several reports are published in recent past, describing microbial degradation of alkanes, aromatics and paraffinic hydrocarbon degradation (Binazadeh et al. 2009; Etoumi et al. 2008; Grishchenkov et al. 2000; Sabirova et al. 2006; Wentzel et al. 2007; Atlas 1981; Atlas and Bartha 1992; Leahy and Colwell 1990). It was reported in a study that nC_8-nC_{11} were degraded completely followed by nC_{12}-nC_{40} with percentage efficiency of 100% and 98–57%, respectively (Rahman et al. 2003). *Gordonia amicalis*` strains were reported to be to be a potent degrader of large alkanes under both aerobic and anaerobic conditions (Hao et al. 2008). Hydrocarbon-degrading and biosurfactant-producing *B. subtilis* strains were reported to degrade alkanes ($>C_{27}$), where concentration of lower alkanes ($<C_{25}$) was increased after degradation period under anaerobic conditions (Bachmann et al. 2014). Indigenous *Bacillus* strains isolated from Daqing Oil Field were reported to degrade the higher fractions of crude oil and improve flow characteristics (She et al. 2011). *Acinetobacter* sp. M-1 could utilize paraffinic wax as a sole carbon source (Wentzel et al. 2007). The presence of oxygen is reported to be required to initiate biodegradation of PAHs for effective enzyme action (Chikere et al. 2011). Thermophilic hydrocarbon degraders belonging to *Bacillus*, *Thermus*, *Thermococcus*, and *Thermotoga* species, reported to be present in high-temperature oil reservoirs, could also be beneficial for oil biodegradation (Feitkenhauer et al. 2003). *B. stearothermophilus, G. jurassicus, and B. thermoleovorans* were isolated from oilcontaminated sites and high temperature petroleum reservoirs, which were capable of utilizing alkanes from C_{15}-C_{23} (Sorkoh et al. 1993; Nazina et al. 2005; Kato et al. 2001). Al-Bahry et al. (2013) reported 33 genera and 58 species identified for the first time from Omani oil wells. For successful biodegradation, sometimes other nutrients also need to be incorporated, such as nitrogen, phosphorus, and iron source, along with some trace metals and co-substrates (Das and Chandran 2011; Salanitro 2001; McGenity and Gramain 2010). However, addition of disproportionate nutrient concentrations could inhibit the biodegradation activity altogether (Das and Chandran 2011).

5.5 Aerobic and Anaerobic Biodegradation: Enzymes

There are at several bacterial genera isolated from arid regions that can use hydrocarbons as a sole source of carbon and energy, to degrade or transform PAHs (Head et al. 2006). Bacteria are reported to be more adaptable to extreme environments exposed to PAHs than fungi, yet there is no single reported species that can

completely degrade all types of hydrocarbons and heavy crude oil (Chikere et al. 2011). Heavy crude oil could serve as a good carbon and energy sources for the microbes capable to metabolize under aerobic or anaerobic conditions. However, biodegradation mechanisms differ for aerobic and anaerobic conditions (Shibulal 2017).

5.5.1 Aerobic Biodegradation

Oxidation is the first step in crude oil biodegradation, carried out by group of enzymes (monooxygenases, dioxygenases laccases, soluble cytochrome (P450 – CYP153), membrane-bound cytochrome (P450 – CYP52)), where alkanes are metabolized to carboxylic acids, which eventually further catabolized via β-oxidation (Hawumba et al. 2010). The aromatic hydrocarbon rings are hydroxylated to form diols, which are then eventually cleaved to form catechols and degraded further to intermediates via β-oxidation (Rojo 2010; Sierra-Garcia and Oliveira 2013). Biodegradation rates differ depending on the temperature of the environment in which the activity occurs (Das and Chandran 2011). Aliphatic hydrocarbons (short and long chain) are oxidized to the alcohol by substrate-specific enzymes – monooxygenases/hydroxylases. The alcohol is then further oxidized and finally processed in β-oxidation (R Margesin et al. 2003; Rojo 2010; Van Hamme et al. 2003; Wentzel et al. 2007). In bacteria, enzymes belonging to different classes, propane/butane monooxygenase, CYP153 monooxygenases, AlkB-related non-heme iron monooxygenase, flavin-binding monooxygenase AlmA, flavin-dependent monooxygenase LadA, and copper flavin-dependent dioxygenase, are involved in the initial terminal hydroxylation, the most characterized enzyme gene being the alkB gene (Hamamura et al. 2008; Kuhn et al. 2009; Margesin et al. 2003; Salminen et al. 2008). Bacterial catabolism of aromatic hydrocarbons under aerobic conditions involves a set of pathways that convert diverse type of substrates into a small number of intermediates. These intermediates are further cleaved and catabolized by enzymes of metabolic pathway system and further utilized by cell (Carmona et al. 2009). The genes encoding the enzymes responsible for degradation of aromatic compounds have been characterized from *Proteobacteria* to actinobacteria: Rieske non-heme iron-dependent oxygenases (RNHO), flavoprotein monooxygenases (FPM), soluble di-iron multicomponent monooxygenases (SDM), CoA-ligases, and intradiol and extradiol dioxygenases (Brennerova et al. 2009; Jouanneau 2010; Vilchez-Vargas et al. 2010; Hamamura et al. 2008; Kuhn et al. 2009; Margesin et al. 2003; Salminen et al. 2008; Vilchez-Vargas et al. 2010). Ideally complete biodegradation of hydrocarbons ends up as carbon dioxide and water and microbial biomass. Sometimes hydrocarbon-degrading bacteria utilize multiple enzymes to degrade and consume mixture of substrates. The enzyme activity is also reported to be lost or downregulated under extended exposure to n-alkane (Wentzel et al. 2007). Although microbial fractionation of hydrocarbon is reported as pervasive in nature and many long- and short-chain hydrocarbon-utilizing bacteria have been isolated,

only few studies are there about the comprehensive genetic buildup responsible for the same. Osmotic shock method was reported to collect the enzymes from microbial consortium, which could degrade naphthalene, phenanthrene, pyrene and crude oil (Xu et al. 2013). It was reported that metabolic efficiency of secreted enzymes differed according to the utilized substrate and cytoplasmic enzymes outperformed extracellular enzymes. The authors also reported biosorption of those PAHs using live and dead microbial cells.

5.5.2 Anaerobic Biodegradation

Anaerobic growth of microbes is too slow in alkanes, and the cultivation of cells on higher carbon alkanes also poses difficulty due to poor solubility (Widdel and Grundmann 2010). Most of the initial studies of anaerobic PAH metabolism were done using benzene, toluene, ethylbenzene, and xylenes (BTEX) because of their classification as priority pollutants (Callaghan et al. 2010). Bacteria capable of utilizing n-alkanes with six or more carbon atoms and anaerobic pathways for PAH degradation have also been reported (Heider 2007; Grossi et al. 2008; Widdel and Grundmann 2010; Gieg et al. 2010; So et al. 2003). Different enzymes involved in catabolism of hydrocarbons under anaerobic conditions are glycyl radical enzymes, fumarate-adding enzymes (FAE), succinate synthases or methyl-alkyl succinate synthases (MAS), and naphthylmethyl succinate synthases (NMS) (Kuntze et al. 2011; Beller et al. 2002; Winderl et al. 2007; Callaghan et al. 2008, 2010; Grundmann et al. 2008; Heider 2007). Nitrate-reducing bacteria (NRBs) are also reported having same mechanism of carboxylation for the activation of C-chain in PAHs (Callaghan et al. 2009). Other proposed mechanisms for anaerobic degradation of alkanes hypothesized to release oxygen during chlorate respiration for the activation of alkanes and in methanogenic system by an anaerobic hydroxylation (Head et al. 2010; Mehboob et al. 2009).

The aromatic hydrocarbon compounds are utilized by microbes anaerobically by using reductive reactions (Fuchs et al. 2011). Although biochemistry of anaerobic degradation of aromatic hydrocarbons has been revealed, the genes responsible for the enzymes involved in the process and the actual mechanism behind are still unclear (Feng et al. 2007). Six different bacterial strains have been genetically characterized for the aromatic hydrocarbon degradation efficiency (Feng et al. 2007). Toluene degradation under anaerobic conditions by the enzyme benzylsuccinate synthase (encoded by *bbs* and *bss* operons), is the most studied pathway. The radical-catalyzed addition of fumarate produce substituted succinate derivatives, which is the preliminary step in the activation of benzene derivatives and n-alkanes (Kube et al. 2004). The remaining anaerobic degradation reactions for both toluene and ethyl benzene are similar which includes reductive dearomatization, ring cleavage by hydrolysis, followed by integration in β-oxidation (Boll et al. 2002; Carmona et al. 2009; Kube et al. 2004).

5.6 Heavy Crude Oil Biodegradation Genes

Different types of catabolic genes, such as alkM, alkB, xylE, xylene monooxygenase (xylM), catechol 2,3-dioxygenase ($C_{23}O$), and benzoyl-CoA reductase (bcr), are reported to be responsible for biodegradation of alkanes and aromatic hydrocarbons (Rajaei et al. 2013; Higashioka et al. 2009; Shibulal 2017). Some of the genes responsible for producing PAH-degrading enzymes are reported to be harbored on plasmids (Oct, Nah7, dox, TOL) (Prince et al. 2010). In *P. putida* GPo1, alkB gene complex is reported to be located on the OCT plasmid (Van Beilen et al. 1994). A long-chain alkane hydroxylase - LadA (flavin-dependent oxygenase) was characterized in *Geobacillus thermodenitrificans* NG80-2 with some strains with homologous alkB gene was described as present due to homologous gene transfer among the bacteria (Feng et al. 2007; Tourova et al. 2008). Al-Sayegh (2017) reported different genes responsible for crude oil and PAH biodegradation found in the locally isolated *B. subtilis* AS2 strain: aromatic compound gene, pcaC, that is also involved in the degradation of the xenobiotic (benzoate); aminobenzoate degradation gene, atoD; aromatic compound degradation genes, catE, praC, hpaB, and frmA; benzoate degradation genes, fadA, catE, praC, fadN, fadB, paaH, and E2.3.1.9; aminobenzoate degradation genes acyP, cypD_E, E3.1.3.41, E3.1.3.1, and phoD; chloroalkane and chloroalkene degradation genes, frmA, ALDH, fdhA, and E3.8.1.2; chlorocyclohexane and chlorobenzene degradation genes, catE and E3.8.1.2; xylene and dioxin degradation genes, praC/xylH; ethylbenzene degradation gene, fadA/fadI; styrene degradation gene, catE; atrazine degradation genes, urea, ureB, and ureC; and naphthalene degradation genes, frmA, ADH5, and adhC. Similarly he also reported different degradation genes found in another locally isolated *B. licheniformis* AS5: styrene degradation genes, E3.5.1.4/amiE and catE; aromatic compound degradation genes, catE, dmpC, praC, dmpH, pobA, hpaD, E1.1.1.1, frmA, and adhE; benzoate degradation genes, fadA, catE, dmpC, praC, dmpH, pobA, fadN, fadB, paaH, and E2.3.1.9; aminobenzoate degradation genes, acyP, atoD, cypD_E, E3.1.3.41, E3.1.3.1, and phoD; chloroalkane and chloroalkene degradation genes, E1.1.1.1, frmA, adhE, ALDH, and E3.8.1.2; chlorocyclohexane and chlorobenzene degradation genes, catE and E3.8.1.2; xylene degradation genes, catE, dmpC, praC, and dmpH; ethylbenzene degradation gene, fadA; and naphthalene degradation genes, E1.1.1.1, frmA, and adhE. He reported that both isolates efficiently degraded heavy crude oil and also produced biosurfactant (Al-Sayegh et al. 2017).

5.7 Conclusion and Future Recommendations

In the near future, heavy crude oil resources will be exploited heavily, due to increasing demand for liquid fuel and scarcity of easily recoverable light oil. This will eventually lead to increased instances of crude oil-related pollutions, pushing

for innovative and effective ways to handle the pollution. Heavy crude oil contains numerous hydrocarbons with varying structural complexities, which makes it resistant and quite difficult for faster biodegradation using microbes, and the residual mixture after partial biodegradation may become further recalcitrant and toxic. Although laboratory studies claim to be very effective, heavy crude oil is almost difficult to be completely degraded and always results in some complex end products in field-scale treatments. The toxicity and bioavailability of those residual mixtures could stay for a long time affecting surrounding flora and fauna. Bacterial enzymes are reported to play a crucial role in biodegradation of heavy crude oil and PAHs. However, most of the enzyme families which may play a crucial role in aromatic metabolism are still not revealed, which is still a challenge. The exact method of hydrocarbon uptake by bacteria is still unclear. For low molecular weight alkanes, the direct uptake by the microbes is a possible way, and medium-long-chain alkanes may be reduced due to the adherence to the hydrophobic cell surface, by a surfactant-mediated access or by other emulsification processes, or due to the passive diffusion through bacterial cell. Presently the potential discovery of new genes, synergetic action of group of enzymes, and metabolic pathways of degradation under anaerobic conditions need to be further explored using latest tools and developments in omics.

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Chapter 6
Catalytic Promiscuity of Aromatic Ring-Hydroxylating Dioxygenases and Their Role in the Plasticity of Xenobiotic Compound Degradation

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Abstract Persistent organic pollutants pose one of the most critical challenges to the humankind due to their well-established hazardous effects to the ecosystem and various life forms including the human being. Many of these pollutants are anthropogenic in nature and exhibit the tendency of recalcitrance toward natural biodegradation. Most of these pollutants belong to chemical scaffolds that were never present in the environment in the past. Consequently, the evolution of the enzymes and metabolic pathways for their metabolism and degradation over the short geological time span that these compounds have been present in the environment is considered to be rather challenging. Interestingly, microorganisms belonging to diverse taxonomic groups have been identified and characterized with the metabolic potential to degrade the anthropogenic compounds and utilize them as a source of carbon/energy. Evolution of such degradative potential is widely accepted to have occurred with one of the following mechanisms: (i) horizontal gene transfer and (ii) genome reorganization and domain shuffling. An alternative and recent theory in this regard suggests that the evolution of degradative enzymes for anthropogenic compounds may have exploited the "catalytic promiscuity" of metabolic enzymes evolved for degradation of structurally related yet distinct compounds. Noticeably, such catalytic promiscuity of metabolic enzymes has been reported for a number of enzymes involved in degradation of anthropogenic compounds. "Aromatic ring-hydroxylating dioxygenase" is one prominent group of enzymes which exhibit catalytic promiscuity, a potential that has been exploited for technological application in the field of biocatalysis as well as for enhancing the plasticity of anthropogenic xenobiotic compound degradation. The present book chapter aims to present a comprehensive account of aromatic ring-hydroxylating dioxygenases with respect to their basic

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introduction, classification, the molecular mechanism of action, structure-function relationship, catalytic promiscuity, and applications with respect to expansion of biocatalysis and biodegradation.

Keywords Dioxygenase · Aromatic compounds · Xenobiotic compounds · Biodegradation

6.1 Introduction

Anthropogenic activities pertaining to industrialization during the past century have resulted in extensive degradation and deterioration of the environment through depletion of quality and quantity of resources such as air, water, and soil (Jones and de Voogt 1999). One of the major factors responsible for such extensive environmental deterioration has been non-judicious production and disposal of the plethora of persistent organic pollutant such as anthropogenic aromatic compounds (Keane et al. 2002; Inczefi-Gonda 1999). These compounds are synthesized for applications in the chemical manufacturing industry as well as agriculture. Anthropogenic aromatic compounds have exceptional physical and chemical properties which make them useful for application in several industries including dyes, paints, explosives, cosmetics, solvents, drugs, etc. The chemical structure of aromatic compounds is characterized by delocalized pi electrons between carbon atoms, which make them extremely stable and thus suitable for application in a variety of industrial and agricultural process. The same structural characteristics also make aromatic compounds extremely recalcitrant to natural degradation. Consequently, the aromatic compounds exhibit a strong tendency to persist in the environment and also cause deleterious effects (mutagenesis, carcinogenesis, etc.) to different life forms (Benigni and Passerini 2002; Kovacic and Somanathan 2014; Shahin 1987). It is alarming that since the industrial revolution, an excess of anthropogenic aromatic compounds has been released into the earth's atmosphere. Many of these compounds belong to chemical skeletons that were never present in the environment in past. Therefore, it could be argued that the evolution of degradative enzymes and metabolic pathways for degradation of anthropogenic aromatic compounds over a relatively short geological timescale is rather challenging (van der Meer 1997; Russell et al. 2011).

Till date, only a handful of microorganisms have been characterized for their ability to degrade a few specific class of anthropogenic aromatic compounds, viz., insecticides, pesticides, dyes, explosives, etc. (Phale et al. 2007; Jindrova et al. 2002; Zylstra and Gibson 1991). These microorganisms are valuable resources for bioremediation technology development as well as for basic studies addressing questions related to evolution and diversity of metabolic enzymes, their mode of action, their molecular regulation, etc. Among the microorganisms characterized for metabolism of anthropogenic aromatic compounds, it is suggested that they have acquired new

metabolic functions via accessing genetic repertoire through (i) horizontal gene transfer, (ii) evolution of degradative functions through stress-induced mutagenesis, and (iii) genome reorganization and domain shuffling (Russell et al. 2011; Phale et al. 2007; Diaz 2004). An alternative and recent theory in this regard suggests that the evolution of degradative enzymes for a new group of anthropogenic compounds often exploits the "catalytic promiscuity" of other metabolic enzymes. According to this theory, the evolution of new degradative enzymes may have occurred from enzymes for which natural products serve as the substrate and that may act upon anthropogenic chemicals via promiscuous activity and analogous mechanism (Russell et al. 2011; Kabumoto et al. 2009; Wackett 2009; Janssen et al. 2005).

Enzymes with the inherent structural capability to act on more than one substrate are referred to be catalytically promiscuous. This property has been reported for a number of enzymes involved in degradation of anthropogenic compounds and potentially acts as the fundamental resource for evolution and expansion of the degradative/metabolic functions. The promiscuity of metabolic functions is not only restricted to degradative enzymes but also encompasses the regulatory elements involved in the degradation process. An example highlighting this phenomenon has been observed and reported in case of nitrotoluene degradation by *Acidovorax* sp. strain JS42, wherein the degradation of 2-nitrotoluene is regulated by the transcriptional activator NtdR that is nearly identical to NagR, the activator of the naphthalene degradation operon in *Ralstonia* sp. strain U2. Both regulators respond to salicylate, an intermediate of naphthalene degradation, but NtdR also recognizes a wide range of nitroaromatic compounds (Maia 2009). Having said that, the worth of promiscuous degradative enzymes is perhaps significantly more for the evolution and distribution of new metabolic functions leading to the degradation of anthropogenic compounds (Khersonsky and Tawfik 2010; Janssen et al. 2005; Copley 2009). Conspicuously, a large number of enzymes are involved in a variety of cellular functions ranging from metabolism of complex substrates to DNA damage repair (O'Brien 2006). The catalytic promiscuity of enzymes is a widespread phenomenon as several reports have indicated that it is observed with different enzymes found among organisms of diverse origin including archaea, bacteria, and lower and higher eukaryotes such as human beings (Unterlass et al. 2017; Srinivasan et al. 2016; Martinez-Nunez et al. 2017; Verma and Pulicherla 2016). Microorganisms bestowed with unique characteristics, e.g., large population size, very short generation time, efficient mutation fixation system, access to more diverse genetic resources, etc., have benefitted most out of such promiscuous enzymes and evolved several new metabolic functions for degradation of complex and recalcitrant anthropogenic compounds. There are a number of microbial metabolic enzymes that have been characterized for their catalytic promiscuity. Noticeably, promiscuous activity has been observed with diverse anthropogenic compounds including aliphatic compounds, cyclic aliphatic compounds, monoaromatic compounds, polyaromatic compounds, and heterocyclic aromatic compounds. A summarized account of some of the model enzymes characterized with catalytically promiscuous activity is presented in Table 6.1.

6.2 Aromatic Ring-Hydroxylating Dioxygenases

6.2.1 Important Enzymes for Metabolism of Aromatic Compounds

As evident from the cited examples in Table 6.1, the aromatic ring-hydroxylating dioxygenases (ARHDs) is one of the most important groups of enzymes involved in the metabolism of natural as well as anthropogenic aromatic compounds. They are a subclass of a bigger group of enzymes referred as "ring-hydroxylating oxygenases (RHOs)" that catalyze the initial oxidation step of a broad range of aromatic

Table 6.1 A list of a few model enzymes with well-established catalytic promiscuity

Sl. no.	Enzyme (classification)	Origin	Catalyzed reaction	References
1	Toluene-1,2-dioxygenase (EC 1.14.12.11)	*Pseudomonas putida* F1	Enzymatic dihydroxylation of aromatics, aliphatic compounds (catalyze 109 different reactions using monocyclic aromatic substrates, polycyclic aromatic substrates, aliphatic substrates, miscellaneous substrates)	Gao and Ellis (2008) and Gao et al. (2010)
2	Naphthalene 1,2-dioxygenase (EC 1.14.12.12)	*Pseudomonas* sp. strain NCIB 9816	Enzymatic catalysis of dioxygenation, monooxygenation, desaturation, dealkylation, and sulfoxidation reaction (catalyze 76 different reactions using monoaromatic, substituted aromatic, and heterocyclic aromatic substrate)	Gao and Ellis (2008), Gao et al. (2010) and Resnick and Gibson (1996)
3	Polyaromatic hydrocarbon dioxygenase (PdoA2B2)	*Mycobacterium vanbaalenii* strain PYR-1	Catalysis of hydroxylation of HMW and LMW polyaromatic hydrocarbons including fluoranthene, pyrene, acenaphthene, acenaphthylene, and phenanthrene	Kweon et al. (2014)
4	Para-nitrophenol monooxygenase (PnpA)	*Arthrobacter* sp. strain JS443 and *Bacillus sphaericus* strain JS905	Catalysis of sequential monooxygenation/hydroxylation of *p*-nitrophenol and 4-nitrocatechol	Kallubai et al. (2015) and Kadiyala and Spain (1998)
5	Para-nitrophenol monooxygenase (PnpA)	*Burkholderia* sp. strain SJ98	Catalysis of monooxygenation of 11 monoaromatic compounds with nitro/chloro/methyl substitutions	Vikram et al. (2013)
6	Haloalkane dehalogenase (LinB)	*Sphingobium* sp.	Catalysis of dehalogenation of hexachlorocyclohexane and its multiple degradation intermediates (pentachlorocyclohexane, pentachloro-cyclohexanol, tetrachloro-cyclohexene-1-ol, etc.)	Reviewed by Lal et al. (2010)

hydrocarbon including complex aromatic compounds such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorinated aromatic compounds (CACs), and nitroaromatic compounds (NACs). The ARHDs occupy a central figure in microbial metabolism of natural as well as anthropogenic aromatic compounds due to the fact that aromatic compounds are rather complex structure and their degradation is severely impaired due to the bottleneck phenomenon of the very first step, i.e., the initial oxidation of the aromatic ring. It is often suggested to be the most catalytically challenging reaction in the aerobic degradation of aromatic compounds (Gibson and Parales 2000). The reaction product(s) are usually less stable and less recalcitrant for further reactions including the key step of ring cleavage (Boyd et al. 2001; Gibson and Parales 2000). For catalyzing the oxygenation reaction that is considered rather difficult and catalytically challenging, the ARHDs use molecular oxygen as the substrate and add them to the aromatic ring as the substituent leading to the generation of oxygenated products including quinones and catechols through conversion of closed-ring structures to nonaromatic cis-diols (Boyd et al. 2001).

As suggested above, the catalysis of the oxygenation reaction of the electronically destabilized reaction of the aromatic ring is potentially one of the most challenging catalytic reactions; yet, some of the bacterial strains are capable of doing it. Such strains are best owed with a unique set of an enzyme called ARHDs, also referred as "Rieske non-heme iron dioxygenase," that can catalyze the addition of hydroxyl group(s) (-OH). It is an enzyme system that consists of ferredoxin, ferredoxin reductase, and a terminal ring-hydroxylating dioxygenase. It mostly uses NAD(P)H as the electron donor and catalyzes the same oxygenation reaction. The terminal ARHDs belong to a large family of iron oxygenases and itself consist of two different functional components, viz., (i) hydroxylase components and (ii) electron transfer components. The hydroxylase component may be heteromultimeric containing $(\alpha\beta)_n$ or homomultimeric proteins containing $(\alpha)_n$ oligomers. The α subunit of the hydroxylase component remains bound to the prosthetic group (i.e., Rieske-type center Fe_2S_2 and a mononuclear iron). Among the best characterized ARHDs, the hydroxylase component, alpha-subunit is about 50 kDa and a beta-subunit of about 20 kDa. The electron transport components also consist of two subunits: (i) ferredoxin and (ii) ferredoxin reductase. In some ARHDs, the electron transport system consists of only a single bifunctional ferredoxin/reductase subunit. Characteristically, among all the ARHDs, the Rieske cluster [2Fe-2S] acts as a redox center for receiving electrons from the electron transfer component(s), and mononuclear iron acts as a catalytic site for dioxygen activation. This intricate tripartite electron transfer leads to the generation of catalytic potential that is capable of catalyzing a rather complex chemical reaction. A graphical summary of this widely accepted reaction mechanism of the ARHDs is presented in Fig. 6.1.

The electron transfer in the above transport system begins with the action of a FAD- or FMN-type reductase followed by the action of a plant-type ferredoxin domain before entering the ARHDs. Within ARHDs, the electron transporter system receives electrons and uses them to activate the Rieske cluster to catalyze deoxygenation with the help of the iron-sulfur protein center and the mononuclear iron.

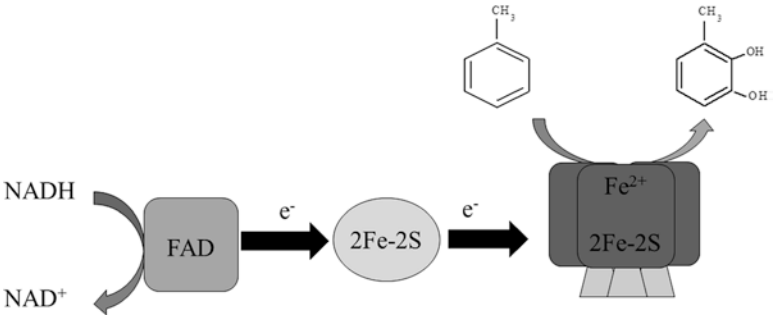

Fig. 6.1 Schematic representation of the widely acknowledged reaction mechanism of aromatic ring-hydroxylating dioxygenases (ARHDs), for example, toluene dioxygenase

6.2.2 Diversity and Classification

Due to the importance of the ARHDs in both biodegradation and biocatalysis, recent past has observed a surge in the studies focusing on identification and characterization of ARHDs from microorganisms belonging to diverse taxonomic lineages. Some of the representative bacterial isolates from which the ARHDs have been characterized include strains belonging to genera *Citrobacter*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, and *Sphingomonas* (Zeng et al. 2017; Schuler et al. 2009; Jakoncic et al. 2007; Selvakumaran et al. 2011; Karandikar et al. 2015; Kimura et al. 2006). With regard to the host bacterial strains, the ARHDs harboring strains have been widely reported from *Actinobacteria* and *Proteobacteria* as indicated above, yet the understanding of sequence diversity and structure-function relationship of ARHDs has largely come from the "naphthalene"- and "toluene"-degrading *Pseudomonads* only. With the emergence of culture-independent metagenomic approaches, the characterization of ARHDs from other taxonomic lineages has gained significant interest. Consequently, a number of studies have reported the characterization of ARHDs from metagenomic DNA samples of varied ecosystems (Martin et al. 2013; Yavas and Icgen 2018; Phale et al. 2007). With such metagenomic studies, many ARHDs have been identified entirely depending on their sequence alignments with previously reported ARHDs. Thus with ever-increasing information about the sequence diversity and complementing structure-function information about the characterized ARHDs, their classification is now being proposed on the holistic basis rather than on the basis of sequence alignments alone. Such holistic classification is still being developed; meanwhile, according to the present methods, the ARHDs are classified on the basis of the amino acid sequence alignments of the α subunits of the ARHDs, wherein, they are broadly classified into four groups proposed as phthalate, benzoate, naphthalene, and toluene-/biphenyl-degrading dioxygenases, respectively (Yavas and Icgen 2018).

The most widely acknowledged method for classification of ARHDs is based on the presence and type of the prosthetic groups (reductase, ferredoxin, and oxygenase). Accordingly, the ARHDs, as well as other ring-hydroxylating oxygenases

(RHOs), have been classified into five classes termed as (Class IA, Class IB, Class IIA, Class IIB, and Class III). A summarized account of this classification scheme highlighting the difference among different classes of the ARHDs is presented in Table 6.2. Since the development of the first classification system for ARHDs in 1990, there have been a lot of developments with respect to DNA sequence analyses as well as protein structure-function analyses of ARHDs; consequently, a need to formulate a new classification system for these enzymes has been suggested during recent years. A new system is all the more needed for classification of newly identified and characterized ARHDs which do not belong to any group in the previous classification. One such example was reported by Chemerys et al. (2014). They reported the identification and characterization of four novel ARHDs from the metagenomic DNA of a polluted soil sample (Chemerys et al. 2014). Noticeably, in this report, the authors showed that the ARHD-specific amplicon differed from one another by up to 321 nucleotides (17%). Thus, the authors suggested that there is the existence of a significant genetic diversity of ARHDs (Chemerys et al. 2014), and further methods for diversity analyses and classification of the ARHDs need to be

Table 6.2 A summarized account of different classes of ARHDs, classified on the basis of the composition of prosthetic groups

Sl. no.	Class of ARHDs	Component and prosthetic group			Example enzyme system reference
1	IA	**Reductase**	–	**Oxygenase**	Phthalate dioxygenase (*Burkholderia cepacia*)
		FMN Cys_4 [2Fe-2S]		Cys_2-His_2 [2Fe-2S]	3-chlorobenzoate-3,4 dioxygenase (*Alcaligenes* sp.)
				Fe^{2+}	
2	IB	**Reductase**	–	**Oxygenase**	Benzoate 1,2-dioxygenase (*Acinetobacter* sp.)
		FAD Cys_4 [2Fethe -2S]		Cys_2-His_2 [2Fe-2S]	Anthranilate dioxygenase (*Acinetobacter* sp.)
				Fe^{2+}	
3	IIA	**Reductase**	**Ferredoxin**	**Oxygenase**	Dioxin dioxygenase (*Sphingomonas* sp.)
		FAD	Cys_4[2Fe-2S]	Cys_2-His_2 [2Fe-2S]	Pyrazon dioxygenase (*Pseudomonas* sp.)
				Fe^{2+}	
4	IIB	**Reductase**	**Ferredoxin**	**Oxygenase**	Toluene dioxygenase (*Pseudomonas putida*)
		FAD	Cys_2 His_2 [2Fe-2S]	Cys_2-His_2 [2Fe-2S]	
				Fe^{2+}	
5	III	**Reductase**	**Ferredoxin**	**Oxygenase**C	Naphthalene dioxygenase (*Pseudomonas putida*)
		FAD	Cys_2 His_2 [2Fe-2S]	ys_2-His_2 [2Fe-2S]	2-Nitrotoluene dioxygenase (*Pseudomonas* sp.)
				Fe^{2+}	

Adapted from Nam et al. (2001)

developed. According to the recent method for ARHD classification, the emphasis is given on phylogenetic and evolutionary characteristics, which might be independent for each constituent electron transport components. Due to the emergence of such classification method, the ARHDs that were previously considered and classified as to be members of different classes are now classified together (Chakraborty et al. 2012).

6.2.3 Phylogenetic Diversity and Evolution

Due to the catalytic significance of the ARHDs, their phylogenetic diversity and evolution have been a matter of great scientific interest ever since very early days of the studies with ARHDs; however, pertinent studies in this direction were conducted only during the past two decades. Initial studies were carried out with PCR amplification of the DNA fragments using gene-specific primers or degenerate primers to determine the genetic diversity of ARHDs in aromatic compound degrading as well as the environmental metagenomic DNA samples (Zhou et al. 2006). The degenerate primers were initially designed on the basis of the conserved segment of ring-hydroxylating dioxygenase of the representative dioxygenases including *nahAc*, *phnAc*, and *nidA* (Zhou et al. 2006). Noticeably, with initial studies, several novel ARHDs were identified from environmental metagenomic DNA samples; however, such novel ARHD genes could not be identified from the aromatic compound-degrading bacteria. These studies indicated that there might be a need for designing new primers to assess the entire diversity of ARHDs. Subsequently, with the advent and advancement of the next-generation sequencing (NGS) based on whole genome sequencing and metagenome sequencing analyses, several novel ARHDs have been identified and characterized (Chemerys et al. 2014; Zafra et al. 2016; Martin et al. 2013; Singleton et al. 2012). Accordingly, the phylogenetic diversity of the ARHDs has also changed significantly over the years. According to the phylogenetic tree for ARHDs till mid of the last decade, the phylogenetic tree showed two subgroups of ARHDs based on the constituents of the α subunit of the dioxygenase gene. The major representative ARHDs belonging to these two subgroups are *nahAc* and *nidA*, respectively (Nam et al. 2001).

With further advancement in the understanding of the sequence diversity of the newly discovered ARHDs from aromatic compound-degrading bacterial isolates and more specifically from the environmental metagenomic DNA and the structural characteristics, the phylogenetic classification system for ARHDs has also evolved significantly. According to one of the recent systems proposed by Kweon et al. (2008), the classification of aromatic ring oxygenases is according to a system that analyzes RHO enzymes as a whole rather than analyzing only the conserved sequences as suggested by earlier classification systems. Additionally, this system also proposed to have the following characteristics: (i) it is dynamic such that it can adapt to the growing pool of ring-hydroxylating oxygenase enzymes; (ii) it can be applied even to the oxygenases with incomplete sequence information; (iii) it has

direct applicability to newly generated experimental data; and (iv) it provides insights into the evolution of ring-hydroxylating oxygenases based on enzyme interaction (Kweon et al. 2008). Thus according to this phylogenetic classification system, nearly 130 ring-hydroxylating oxygenases are now phylogenetically classified into 5 different categories (Kweon et al. 2008). A representative phylogenetic dendrogram showing this latest method for classification of ring-hydroxylating oxygenases is shown in Fig. 6.2. From this dendrogram, it could be clearly inferred that with respect to the phylogenetic classification of ring-hydroxylating oxygenases, although their grouping tendency seems to follow their substrate specificities, yet their phylogenetic affiliations are determined mainly by their relationship with the components of the electron transport components. This could be easily observed with example of oxygenases classified within type V, e.g., PhtAa (phthalate

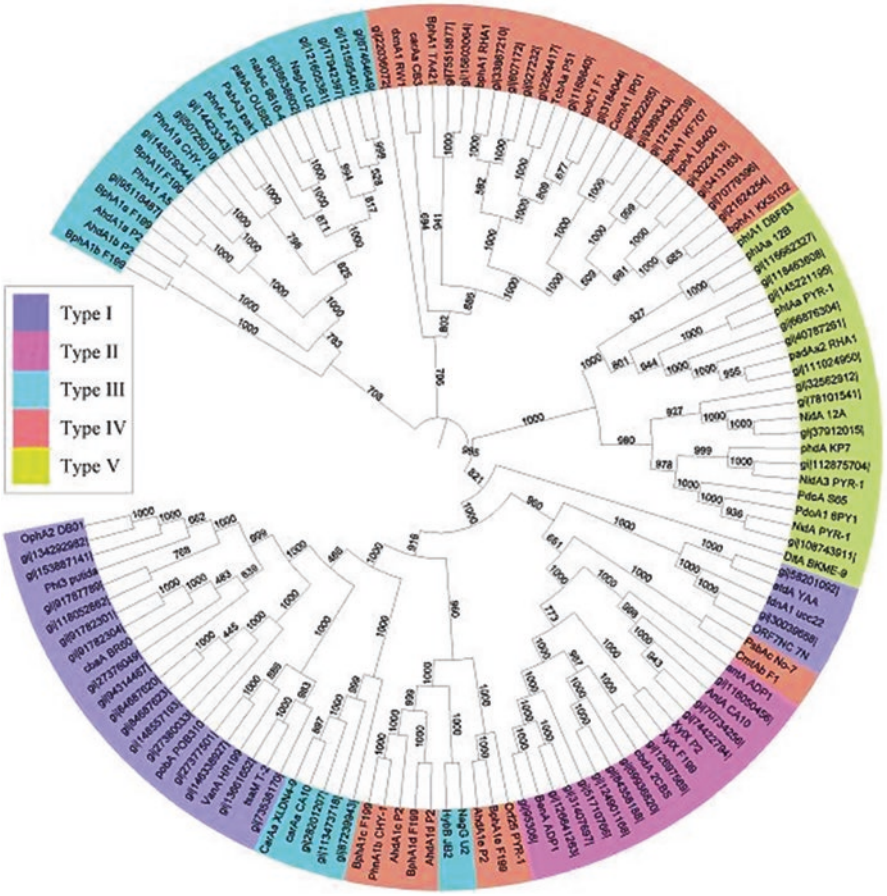

Fig. 6.2 Phylogenetic classification of model ring-hydroxylating oxygenase enzymes showing their classification under five phylogenetic groups/types. (Taken from open source article: Kweon et al. (2008))

dioxygenase) and NidA3 (fluoranthene dioxygenase) from PYR-1. Although their substrates are significantly different, both of these oxygenases are grouped together because their electron transport components are experimentally shown to be the same, i.e., [3Fe-4S]-type ferredoxin and GR-type reductase.

In accordance with the phylogenetic classification, the evolution of ARHDs has also been the focus of several studies during the last one to two decades. While the precise evolutionary lineage for all of the reported ARHDs is not yet explicitly defined, it is agreed that it is possible that the evolution of the α and β subunits might have occurred from a common ancestral two-subunit component. Even with only a few comprehensive studies with regard to the evolution of ARHDs, it has been experimentally represented that the evolution of the ARHDs, especially those which act upon structurally similar aromatic compounds, must have evolved from the common ancestor. One of the very recent studies presenting direct evidence in this regard carried out PCR amplification, sequencing, and alignment analyses of ARHD gene from nine different bacterial genera (including *Raoultella*, *Stenotrophomonas*, *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, *Serratia*, *Comamonas*, *Pantoea*, *and Micrococcus*) that were previously reported for degradation of monoaromatic hydrocarbon. The sequence alignment of ARHD-specific amplicons showed a high degree of homology (ranging between 81% and 99% homologies), thus presented evidence for the evolution of divergent sequences from a common ancestor (Yavas and Icgen 2018).

In one of the relatively earlier studies with regard to evolution of ARHDs, carried out on the characterization of anthranilate 1,2-dioxygenase (AntDO) in *Acinetobacter* sp. strain ADP1, Eby et al. (2001) reported that the gene cluster encoding for AntDO shares a high degree of sequence similarity with a gene cluster encoding or dioxygenase involved in the metabolism of benzene (viz., BenDO). Therefore, it suggests for a possible common evolutionary origin for both sets of genes (Eby et al. 2001). This study also highlighted that at the level of nucleotide sequences, both AntDO and BenDO are also closely related to several other ARHDs that are involved in the metabolism of methyl-benzoate, halobenzoate, aminobenzene sulfonate, and trichlorophenoxyacetic acid (Eby et al. 2001). Another study reported the presence of ten genes in *Mycobacterium* sp. strain KMS which encode for large subunits having significant homology to phenyl propionate dioxygenase genes. Additionally, 16 pairs of adjacent genes encode alpha- and beta-subunits of dioxygenase and 2 genes encode beta-subunits. Noticeably, these genes are orthologs of *nid* genes in *M. vanbaalenii* isolate PYR-1. The authors suggested that the presence of multiple copies of the genes corresponding to homologs or orthologs of *nid* gene could be explained by an evolutionary process which involved gene duplication (Zhang and Anderson 2012). Further, authors also showed that strain KMS harbors four dioxygenase beta-subunit *nid* genes with promoter sequences that are almost identical to those of the PYR-1 (Zhang and Anderson 2012).

With examples of the abovementioned reports and several other studies, it has been clearly demonstrated that the evolution of different ARHDs must have occurred

from common origin/ancestor. However, there is experimental evidence that suggests for the evolution of aromatic compound degradation pathway and ARHDs through elaborate mechanisms such as "patchwork assembly" of genes. Liu et al. (2011) reported about the evolution of the 2-chloronitrobenzene catabolism pathway in *Pseudomonas stutzeri* ZWLR2-1 through recruiting two catabolic clusters encoding a nitroarene dioxygenase and a chlorocatechol degradation pathway (Liu et al. 2011).

As an alternative approach for studying the evolution of ring-hydroxylating oxygenases, Chakraborty and Dutta (2011) followed the origin and evolution of the catalytic domain of large subunit of ring-hydroxylating oxygenases, belonging to the Bet v1-like superfamily was assessed on the basis of structural alignment of representative proteins of the superfamily (Chakraborty and Dutta 2011). Noticeably, this study highlighted that the evolution of example ring-hydroxylating oxygenases, belonging to the Bet v1-like superfamily, might have occurred through multiple evolutionary events of convergence and divergence. A graphical summary of the same is presented in Fig. 6.3.

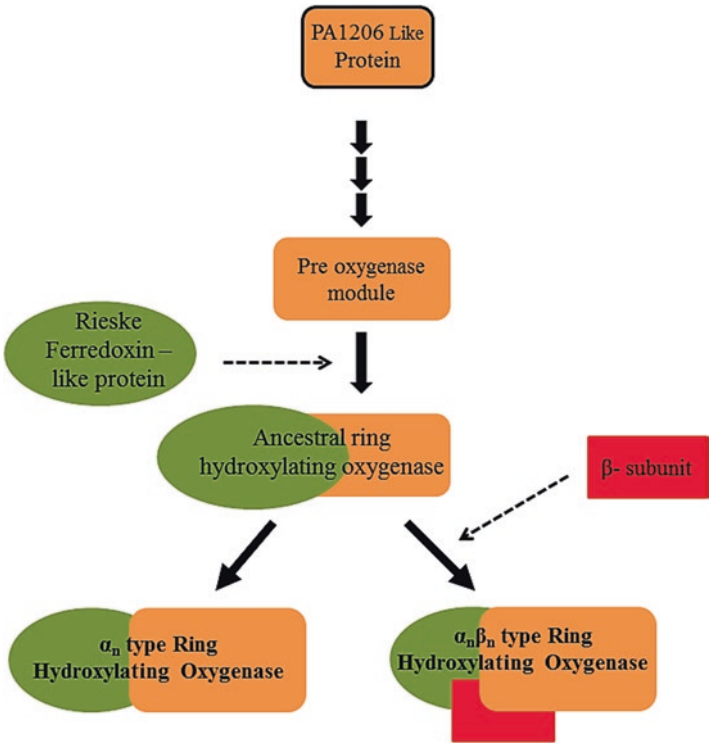

Fig. 6.3 Schematic representation of the proposed evolutionary events that might have taken place for the evolution of α_n and $\alpha_n\beta_n$ types of aromatic ring-hydroxylating oxygenases. (Adapted from Chakraborty and Dutta (2011))

6.3 Relaxed Substrate Activity of ARHDs and Plasticity of Aromatic Compounds Metabolism

Evolution of metabolic pathways for degradation of anthropogenic aromatic compounds has transpired through myriads of evolutionary events reported within various studies. At the same time, degradation of aromatic compounds has also greatly benefitted from the occurrence of relaxed substrate activity of ARHDs. Relaxed substrate activity of ARHDs has recently been suggested as one of the most important driving forces underlying the rapid evolution of metabolic functions toward anthropogenic compounds that have been introduced in the environment only very recently (Boyd et al. 2001; Overwin et al. 2016; Diaz 2004; Janssen et al. 2005). The phenomenon of relaxed substrate activity of ARHDs and its significance in the plasticity of xenobiotic compound degradation has been observed in several cases; however, for a long time, the underlying mechanism such as plasticity degradation was not completely understood. With increasing interest in the evolution and plasticity of microbial degradation of anthropogenic xenobiotic compounds, several studies pertinent to investigation of the mechanistic aspects of this phenomenon have been undertaken in the past two decades. These studies have greatly benefitted from the advancements in the field of biophysics, structure biology, and bioinformatics (in silico protein structure modeling and analyses).

One of the earliest studies with regard to plasticity of degradative functions was reported for a phenol-degrading *Pseudomonas putida* strain EKII. This strain was isolated from an enrichment culture for utilization of phenol; however, subsequently, it was reported that phenol-induced resting cells of strain EKII were capable of metabolizing cresols, chlorophenols, 3,4-dimethylphenol, and 4-chloro-m-cresol as sole substrates (Hinteregger et al. 1992). Although, the transformation of the above compounds could be achieved only in co-metabolic transformation in the presence of phenol, this observation could indicate the possible relaxed substrate activity of phenol-degrading enzymes in strain EKII. Similarly, the plasticity of aromatic degradation function has been observed with other microbial isolates capable of degrading a wide variety of both monochromatic and polyaromatic compounds. Another model bacterial strain worth citing in this regard is *Mycobacterium vanbaalenii* PYR-1 that was first isolated and characterized as the bacterial strain capable of degrading high molecular weight polyaromatic hydrocarbon (HMW PAHs) (Khan et al. 2002; Moody et al. 2004). In subsequent studies, it was reported that strain PYR-1 could also degrade several other HMW PAHs such as fluoranthene, benzo[*a*]pyrene, benz[*a*]anthracene, and 7,12-dimethylbenz[*a*]anthracene as well as low molecular weight (LMW) PAHs, viz., naphthalene, fluorene, phenanthrene, and anthracene. Characteristically, the degradation of each of these MHW and LHW PAHs primarily occurred via deoxygenation (Kweon et al. 2014). In light of the versatile degradative capabilities of strain PYR-1, a genetic perturbation model in *M. vanbaalenii* PYR-1 was developed which proved the presence of two types of V-ring-hydroxylating oxygenases, viz., NidAB and NidA3B3 (Kim et al. 2012).

These oxygenases are responsible for catalyzing the hydroxylation of pyrene and fluoranthene, respectively. Additionally, they also exhibit a pleiotropic hydroxylating activity for LMW PAH substrates (Kim et al. 2012). In a follow-up study, it was reported that loss of pdoA2 gene, which encodes for an aromatic ring-hydroxylating oxygenase, rendered loss of degradation of HMW PAHs (Kweon et al. 2014). The same report also showed that Pdo system is capable of oxidizing biphenyl and other LMW PAHs naphthalene, phenanthrene, anthracene, and fluorene and HMW PAHs pyrene, fluoranthene, and benzo[a]pyrene (Kweon et al. 2014). A graphical representation of this metabolic scheme, highlighting the unique pleiotropic hydroxylating activity of PdoA2B2 (a model AHRD), is presented in Fig. 6.4.

The relaxed substrate activity has also been reported in case of ARHDs involved in degradation of monoaromatic hydrocarbons and their substituent derivatives. One such commonly referred example of ARHDs is nitrobenzene dioxygenase (NBDO). The microbial degradation of nitrobenzene is well documented to proceed via two distinct metabolic pathways, i.e., (i) reductive metabolic pathway as reported in case of *Pseudomonas pseudoalcaligenes* JS45 and *Pseudomonas putida* HS12 and (ii) oxidative metabolic pathway first reported in strain JS765 belonging to *Comamonas* sp. The oxidative degradation pathway involves a multicomponent ring-hydroxylating dioxygenase (viz., nitrobenzene dioxygenase, NBDO) (Lessner et al. 2002). This enzyme was reported to oxidize a wide range of substrates and identified as the first member of the naphthalene family of Rieske non-heme iron oxygenases capable of oxidizing all isomers of mono- and dinitrotoluenes (i.e., 2-nitrotoluene, 3-nitrotoluene, 2,4-dinitrotoluene) with the concomitant release of nitrite (Lessner et al. 2002).

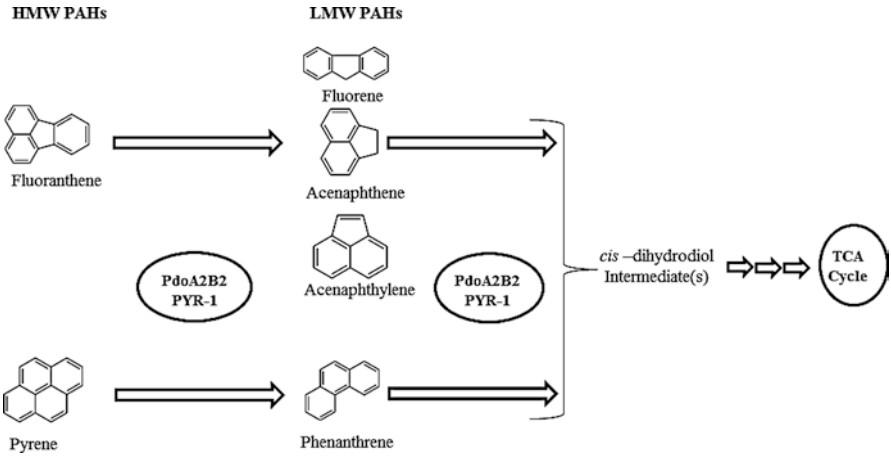

Fig. 6.4 Schematic representation of relaxed substrate activity of PdoA2B (a pleiotropic ring-hydroxylating oxygenase system) in the metabolism of HWM PAHS (viz., fluoranthene and pyrene) and LMW PAHs (fluorene, acenaphthene, acenaphthylene, and phenanthrene) in *Mycobacterium vanbaalenii* strain PYR-1. (Adapted from Kweon et al. (2014))

In case of the abovementioned examples, the relaxed substrate activity of the concerned dioxygenases is observed with aromatic substrates and/or their degradation intermediates. Such a phenomenon is observed with a number of another ring-hydroxylating oxygenase involved in degradation of monoaromatic as well as polyaromatic compounds. A noticeable example of such oxygenase includes *para*-nitrophenol monooxygenase of *para*-nitrophenol (PNP)-degrading isolate, viz., *Arthrobacter* sp. strain JS443. According to the evidence available from genetic analyses as well as in silico analyses, the oxygenase component of the enzyme hydroxylates PNP as well as 4-nitrocatachol (4-NC) using a two-step sequential monooxygenations (Kallubai et al. 2015; Perry and Zylstra 2007; Kadiyala and Spain 1998). Another similar example of para-nitrophenol monooxygenase having a relaxed substrate activity was reported with PnpA of *Burkholderia* sp. strain SJ98 that was reported for relaxed monooxygenation activity toward a total of 11 monoaromatic compounds including nitrophenols, nitrocatachol, chloro-nitrophenol, and methyl-nitrophenol (Vikram et al. 2013). There are several other examples of the ARHDs that are promiscuous with regard to the specificity of the substrates. This characteristic has been extensively investigated at microbiological, biochemical, and molecular genetics levels. These studies have provided significant insights about their functional relevance with respect to adaptation of microbial degradation of anthropogenic aromatic compounds over a short geological time.

6.4 Structure-Function Characteristics for Relaxed Substrate Activity of ARHDs

The functional promiscuity of ARHDs has been widely regarded as a valuable biotechnological resource that has been explored for applications in bioremediation as well as biocatalysis studies. To further exploit the biotechnological potential of relaxed substrate specificity of ARHDs, the assessment of their structure-function characteristics is critical. Therefore, several structure-function studies have been carried out; however, the diversity of such studies is rather limited as majority of the thorough studies have concentrated on the structure-function analyses of toluene dioxygenase (TOD) and naphthalene dioxygenase (NOD). There are relatively fewer discreet studies/reports about the structure-function characterization of other representatives of the ARHDs. Based on one of the earliest publications with respect to structure-function characteristics of representative ARHDs, it is clearly shown that their oxygenase components are either homomultimeric with α_n structure or heteromultimeric with $\alpha_n\beta_n$ structure. The α subunit contains an iron-sulfur protein (ISP) domain along with a conserved Rieske [2Fe–2S] center at the N-terminal, whereas C-terminal harbors a catalytic domain with a conserved mononuclear iron-binding site (Butler and Mason 1997). Several other studies have further highlighted the structure-function relationship of ARHDs involved in metabolic degradation of anthropogenic aromatic compounds.

Chronologically, some of the earliest studies were performed with naphthalene dioxygenase (NDO) of *Pseudomonas* sp. NCIB 9816 which is utilized for oxidizing naphthalene to (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (Kauppi et al. 1998). The molecule was reported to have $\alpha_3\beta_3$ hexamer, wherein alpha-subunit has a beta-sheet domain that contains a Rieske [2Fe-2S] center and a catalytic domain. This study also defined the structural characteristics of the active site of the enzyme (Kauppi et al. 1998). However, this report did not address the phenomenon of the relaxed substrate activity of NDO. A follow-up study carried out with the refinement of structure and cyclic averaging of the abovementioned NDO showed that its active site has electron density corresponding to a flat aromatic compound (an indole adduct) and further docking studies with indole, naphthalene, and biphenyls inside the active site pocket indicated for the presence of sub-pockets where the one close to the active site iron is reserved for the binding of the aromatic ring which is hydroxylated upon catalysis (Carredano et al. 2000). This structural characteristic was reported to be in accordance with the structure-activity relationship and relaxed substrate specificity of the enzyme. With subsequent studies, NDO has been characterized as one of the most promiscuous ARHDs and reported to be capable of catalyzing as many as 76 different reactions. It is listed as one of the most important and most promiscuous degradative/metabolic enzymes on the EAWAG-Biocatalysis/Biodegradation Database (previously known as University of Minnesota Biocatalysis/Biodegradation Database; website link: http://eawag-bbd.ethz.ch/) (Gao et al. 2010).

The 3-D of NDO has been experimentally determined with X-ray crystallography using protein crystals alone as well as co-crystals with a few model aromatic compounds that act as the substrate for hydroxylation. Figure 6.5 represents the molecular docking characteristics of the active site of NDO and its interaction with a few example catalytic substrates. The binding sites and interactions are defined with the use of PDB IDs listed in the parentheses. From these co-crystal structures, it is clearly observed that catalytic site of NDO maps around the iron center and His208 is critical in interaction with most of the substrates, whileAsp-205 is involved in electron transfer.

Another example of ARHD, wherein the structure-function relationship in light of the relaxed substrate activity is relatively well characterized, is nitrobenzene dioxygenase (NBDO) of *Comamonas* sp. strain JS765. The crystal structure of NBDO was solved by molecular replacement using coordinates of the NBDO in complex with two different nitroaromatic substrates (viz., nitrobenzene – NB; and 3-nitrotoluene – 3NT). The analyses of NBDO and its substrate binding highlight that both NB and 3NT bind in the same fashion and interaction with same hydrophobic residues within the active site (Friemann et al. 2005). The structural characteristics of active site of NDO and NBDO indicate that the overall structures are very similar and the basis of their substrate specificity resides within a few amino acid residues (Friemann et al. 2005). Several other ARHDs have been characterized for their structure-function and substrate specificity, and furthermore ARHDs are still being characterized.

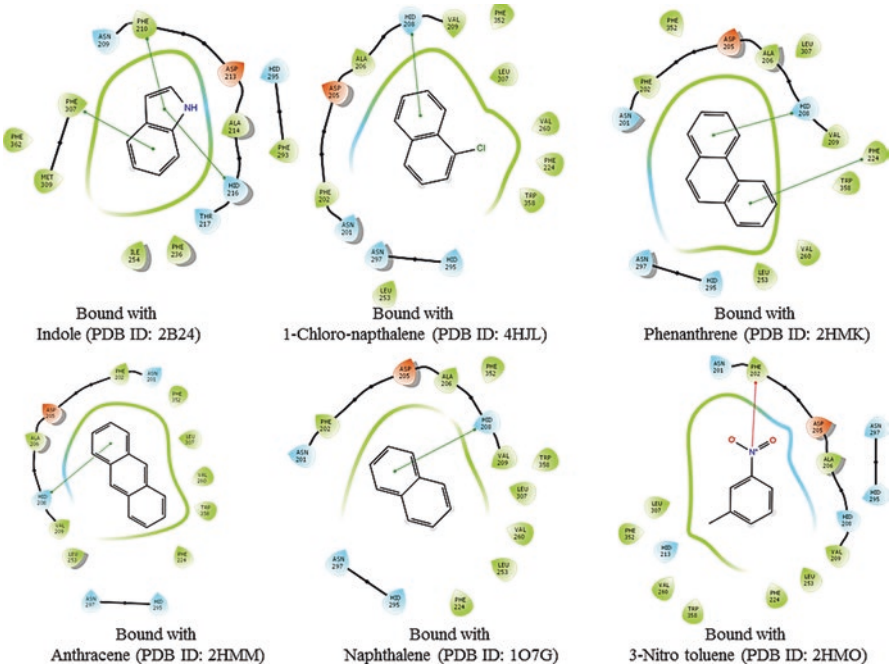

Fig. 6.5 Molecular docking representations showing characteristic binding of naphthalene dioxygenase with naphthalene, indole, 1-chloro-naphthalene, phenanthrene, anthracene, and 3-nitrotoleune. The interaction maps are computed with Schrodinger 11.0 using selected 3-D structures of naphthalene dioxygenase co-crystalized with the substrates

6.5 Plasticity of Anthropogenic Compound Degradation

Microbial metabolism of natural and anthropogenic compounds is the cornerstone supporting the global carbon cycle. Microorganisms in general and bacteria in particular are bestowed with remarkable degradative capabilities which enable them for transforming and/or metabolizing nearly all known organic materials. Noticeably, microbial metabolism is also capable of recycling even those anthropogenic compounds that are produced by synthetic organic chemistry methods and that do have not existed in nature for long. The possible explanation for such observation underlies in the phenomenon that microorganism, particularly bacteria, rapidly evolved the metabolic capability in response to new synthetic anthropogenic growth substrates. This explanation is adequately supplemented with results obtained with molecular biology and biochemistry investigations which reveal that there is an ongoing evolution of the microbial metabolic capabilities. In addition to the well-established/well-documented evolutionary events (i.e., remnants of genetic material from lateral gene transfer), one of the critical evolutionary events in this regard is substrate adaptation of potentially promiscuous degradative enzymes. In case of the bacterial degradation of model anthropogenic aromatic compounds (viz.,

chlorobenzene by *Pseudomonas* sp. strain P51, *Burkholderia* sp. strain PS12, *Ralstonia* sp. strain JS705), the degradative pathway seems to have come into existence through transposon-mediated insertion of broad substrate ARHDs and modified ortho-ring-cleavage dihydrodiol dioxygenase (Beil et al. 1997, 1999; Werlen et al. 1996; van der Meer et al. 1998).

Similarly, the microbial metabolic pathway for pentachlorophenol, another anthropogenic compound, has also been reported to have evolved via gene shuffling and recruitment strategy. The first step of this degradation pathway involves a broad substrate-specific flavin-dependent aromatic ring-hydroxylating monooxygenase which catalyzes hydroxylation of pentachlorophenol to tetrachlorobenzoquinone (Dai et al. 2003). Subsequent degradation proceeds via two consecutive dehalogenation reactions that are catalyzed by a relaxed substrate dehalogenase (Xu et al. 1999).

Evolution of the microbial degradation of 2,4-dinitrotoluene (2,4-DNT) has also been suggested to have evolved through the involvement of two ring-hydroxylating oxygenases (i.e., 2,4-DNT dioxygenase) that catalyze the removal of the two nitro substituents leading to formation of 2-hydroxy-5-methyl-benzoquinone as the prominent intermediate. The molecular genetic characterization of 2,4-DNT degradation pathways evolved by assemblage of multiple modules around the above oxygenase. The 2,4-DNT dioxygenase also exhibits promiscuous substrate activity as it was reported that the heterologous expression system (*Escherichia coli* clone) expressing 2,4-DNT dioxygenase converted DNT to MNC and also converted naphthalene to (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (Suen et al. 1996).

With examples presented above and several other works discussed, reviewed elsewhere, there are abundant evidences that aromatic ring-hydroxylating dioxygenases (ARHDs) are bestowed with remarkable relaxed substrate activity and it has a critical role in enhancing the plasticity of the microbial degradation of anthropogenic aromatic compounds.

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Chapter 7
Aromatic Compounds and Biofilms: Regulation and Interlinking of Metabolic Pathways in Bacteria

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Abstract Bioremediation is the powerful eco-friendly technique for the remediation of toxic aromatic pollutants. However, the activity of augmented organisms in freely suspended form often decreases at the contaminated sites due to number of stress factors. Bacterial biofilms are efficient systems, recently being applied in bioremediation, as they warrant enhanced bioavailability, protection of cells from toxic shocks and optimum microenvironment for the degradation reactions to occur. Recent studies suggest the involvement of biofilm in biodegradation process. However, the regulation and interconnection of the degradation pathways through biofilms are still unclear. The present chapter suggests the interlinking of biofilm process and degradation of aromatic compounds through various mechanisms like chemotaxis, HGT events and EPS production. The interference of QS sensing genes and their regulators in the biodegradation of various aromatic compounds and EPS synthesis are also discussed. Hence, this would come up with a better understanding of biofilm-based processes during biodegradation, which in turn aids in consortia development and bioremediation potential.

Keywords Aromatic · Metabolism · Biofilm · Microbes · Quorum sensing · Biosurfactant · Chemotaxis

7.1 Introduction

The rapid development and progress of industries in the last few decades has prompted in poisoning of the environment with hazardous aromatic compounds. The toxic waste effluents have brought the probability of indelible environmental setbacks into the public beliefs (Paul et al. 2005; Pandey and Jain 2002). Therefore, various chemical, physical and biological strategies are being developed for

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environment restoration. Bioremediation is an environmentally "green" method of using microbes – either naturally occurring or introduced – to break down pollutants to non-toxic substances. It can be in situ or ex situ. Some examples of bioremediation and its related technologies include natural attenuation, bioaugmentation, bioventing, rhizofiltration, phytoremediation, landfarming, bioreactor, bioleaching, composting, biostimulation and electrokinetic-mediated bioremediation, where direct current is applied to porous media within subsurface to accelerate transport phenomena (Gill et al. 2014). It can be carried out by applying any one or a combination thereof (Deshmukh et al. 2016).

The biological methods for treating noxious effluents are preferable than physical and chemical processes, concerning their economy and efficacy (Pandey and Jain 2002), where instead of toxic byproducts generated under non-biological methods, useful and relatively less toxic intermediates are produced, thus entering biogeochemical cycles. This has been aptly demonstrated during the bioremediation of petroleum hydrocarbons (Lien et al. 2015; Qin et al. 2013) and other toxic aromatic pollutants in contaminated niches (Claus 2014; Nolvak et al. 2013). Use of metabolically versatile microbes has been accepted as an ecofriendly and efficient solution for partial or complete mineralization of recalcitrant and toxic compounds (El Fantroussi and Agathos 2005). Biodegradation relies on enzymatic attack on the compounds and converts them into less toxic intermediates. It can be beneficial only when the environmental conditions allow microbial activity and growth. Many bacterial genera and species were isolated to be efficient in biodegradation only under laboratory conditions; when introduced into the contaminated sites, their activity and viability declines. This obstacle in bacterial growth is accustomed of many environmental factors, such as temperature, pH, limited bioavailability of substrates, oxygen, moisture and interference of other toxic compounds. Although some exceptions do exist, they prefer an optimal condition which is hard to attain in situ. Biodegradation is a gradual mechanism. Only certain species of fungi and bacteria have ability to degrade certain compounds, which include *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, etc.

The aim of this chapter is to decipher the enhanced capacity of naturally immobilized cells called biofilms over conventional planktonic cells in the biodegradation of hazardous aromatic pollutants. The QS regulates biodegradation of various aromatic pathways through influencing various processes like chemotaxis, EPS production, HGT events and biosurfactant production. Furthermore, the inter-regulation of biofilm and aromatic degradation genes provides direct evidence of involvement of quorum sensing genes in biodegradation enhancement of toxic aromatic pollutants, which serves a better understanding in development of biofilm-based remediation technologies.

7.2 Metabolism of Aromatic Compounds in Bacteria

Aromatic compounds could be defined as organic compounds containing one or more aromatic rings, preferably benzene rings. They are regarded as priority pollutants as they are resistant to biodegradation (Singh et al. 2006). They are introduced into the natural systems as runoffs from industrial activities, commonly possessing amino, halogen, nitro and alkyls as functional groups, which are responsible for imparting toxicity to the molecule. The resonance energy of aromatic rings stabilizes the carbon-carbon bonds and renders microbes with a substantial biodegradation challenge (Harwood and Parales 1996). Both anaerobic and aerobic biodegradation pathways of microbes have been deciphered; adequate studies have been carried out on aerobic degradation. In general, the aerobic biodegradation proceeds via two phases. Firstly, an aromatic compound is initiated for transformation by peripheral pathways, which employs variety of ring modification reactions, thereby producing dihydroxylated benzene ring or substituted dihydroxylated benzene ring (Fig. 7.1). The second stage allows ring cleavage and further modification reactions, leading to the formation of central carbon intermediates. Different categories of aromatic compounds including PAH are being degraded by this mechanism.

7.2.1 Microbes Associated with Biodegradation in Environment

Diverse bacteria of the genus *Acinetobacter*, *Alcaligenes*, *Rhodococcus*, *Nocardia* and *Pseudomonas* can degrade toxic aromatic compounds aerobically. Substantial studies were carried out on *Pseudomonas* and its close neighbours, on account of its remarkable performance in biodegradation of aromatic compounds ranging from unsubstituted (benzene) to highly substituted (benzopyrene). Moreover, diverse phyla of microorganisms can meticulously degrade wide variety of toxic compounds. Essentially, the biodegradation rates are low and accumulate in the environment. In situ bioremediation of hazardous and toxic wastes containing various kinds of aromatics, aliphatics, heavy metals and complex hydrocarbons is an overall challenge worldwide (Das and Chandran 2011; Pal and Paul 2004). Extensive work on microbial community analysis through metagenomics of diverse contaminated environments has implicated to natural abundance of catabolic communities, particularly archaebacteria and bacteria, capable of degrading hydrocarbons (Hazen et al. 2009; Head et al. 2014; Fowler et al. 2012). During degradation of carbamate and organophosphate insecticides, bacteria such as *Pseudomonas* sp., *Chromobacterium* sp. and *Bacillus* sp. and fungi such as *A. niger*, etc. were found to be abundant, which catalyse the hydrocarbon degradation in contaminated soil.

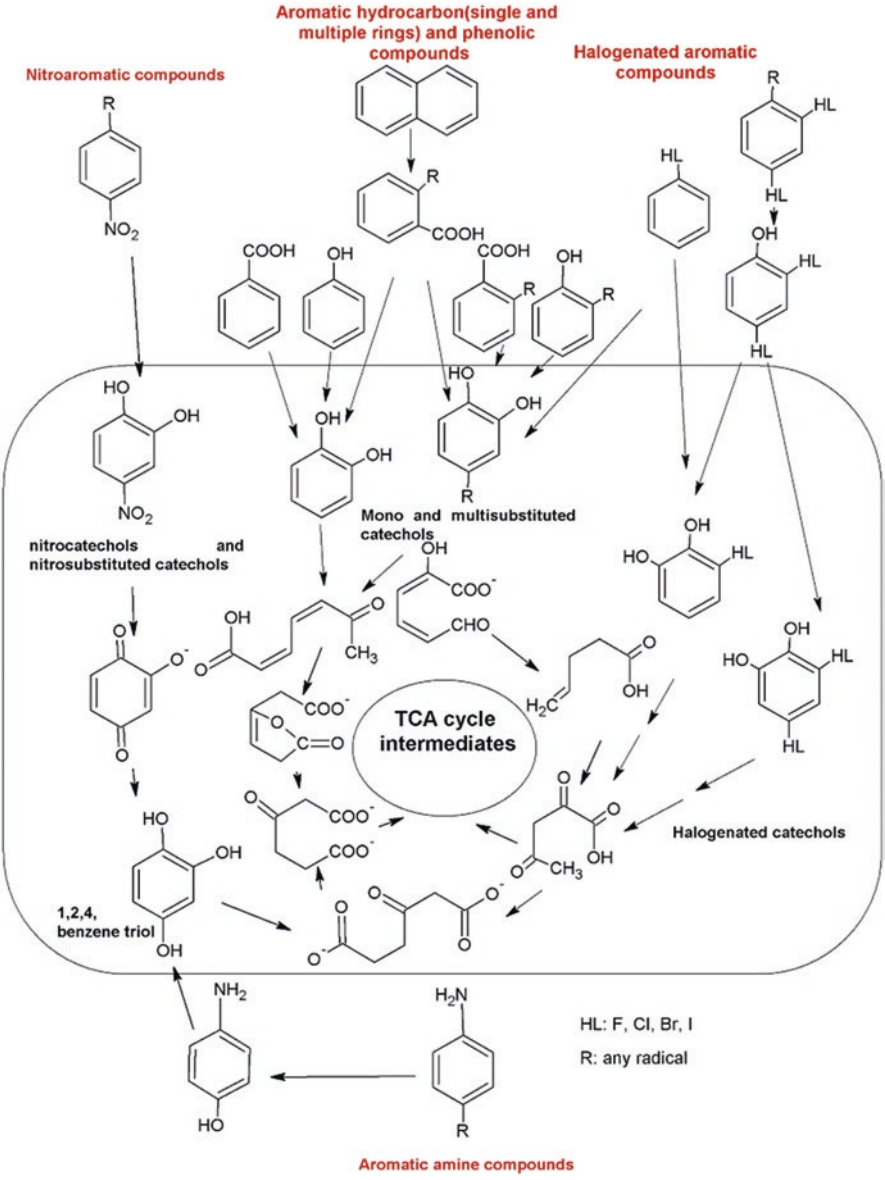

Fig. 7.1 Strategies for aromatics and substituted aromatic compound degradation and interlinking of pathways in bacteria

Different species of *Pseudomonas* (*P. stutzeri*, *P. putida*) and *Bacillus* (*B. endophyticus*, *B. pumilus*, *B. subtilis*) were abundant in oil-contaminated soil, indicating their compelling role in its degradation. These bacterial strains produced lipase extracellularly, which hydrolyse triglycerides (the main component of fats and oils)

glycerol and fatty acids (Karigar and Rao 2011; Bestawy et al. 2005). It has been reported that *Bacillus* strains together with *Pseudomonas* sp. are efficient in bioremediation of oil-contaminated soil (Ahmad et al. 2015). Apart from *Bacillus* and *Pseudomonas* sp., *Corynebacterium* sp., *Acinetobacter* sp. and *Micrococcus* sp. play important role in the pollutant degradation (Cappello et al. 2007; Archaya et al. 2014). Bacteroidetes are another group of potent bacteria capable of biodegradation, having three major classes (*Cytophagia*, *Flavobacteria* and *Sphingobacteria*). *Gammaproteobacteria* dominates in soil followed by α *β-Proteobacteria*. Associated strains of this family include *Xanthomonas Chromobacterium*, *Pseudomonas*, *Acinetobacter* and *Burkholderia* were found to possess various toxic aromatic compound-degrading capabilities (Cappello et al. 2007; Bestawy et al. 2005). Several microbes able to degrade oil and petroleum-rich products, including species of *Arthrobacter*, *Flavobacterium*, *Nocardia*, *Micrococcus*, *Alcaligenes* and *Mycobacterium*, are soil isolates (Malik and Ahmad 2012). Biodegradation by intrinsic microbial populations could also be reliable system for remediation for variety of organic pollutants which are contaminating the environment (Cappello et al. 2007).

Bioremediation of oil-contaminated sludge having hydrocarbons using gram-negative microbes was found to be improved (Zhang et al. 2011). Among the gram-negative bacteria, *Proteobacteria* predominated, which harbours *γ-Proteobacteria* and *β-Proteobacteria* (Tan and Ji 2010). Available literature stated that organisms having enhanced PAH, nitro and chloroaromatic-degrading capacity belong to the genera *Pseudomonas*, *Bordetella*, *Brucella*, *Stenotrophomonas*, *Ochrobactrum*, *Achromobacter*, *Advenella*, *Klebsiella*, *Mesorhizobium*, *Mycobacterium*, *Raoultella* and *Pusillimonas* in the sewage sludge. These organisms have also accentuated the abilities to utilize crude oil as a carbon and energy source in oil-contaminated sludge (Molina et al. 2009; Mishra et al. 2014). This could be accounted to their ability to proliferate and tolerate crude oil sludge as a toxic organic material. Some reports have classified *Bacillus* and *Pseudomonas* genera as bioemulsifiers having the ability to elevate the availability of PAHs through biosurfactant production (Xu et al. 2007; Mishra et al. 2014). Other bacterial species also have the ability to degrade toxic organic solvents and petroleum wastes. Essentially, other genera such as *Ochrobactrum*, *Advenella* and *Achromobacter* have also been reported as efficient degraders of crude oil-contaminated sludge (Zhang and Huang 2005; Katsivela et al. 2005; Santisi et al. 2015; Veeranagouda et al. 2006; Rajaei et al. 2013).

7.2.2 Biodegradative Pathways of Aromatic Compounds

During biodegradation, well-defined transporters or channels in the biodegradation pathway gene clusters have evolved for common aromatic compounds confronted by bacteria. This development is responsible for their ample turnover in the carbon cycle. Structurally different aromatic compounds of various classes are first converted into catechol and substituted catechol-based intermediates through the

peripheral pathways, which are further transformed or channelled via few central metabolic pathways leading to TCA cycle intermediates (Fig. 7.1). The associated enzymes are broadly categorized as peripheral (upper pathway) and ring cleavage (lower pathway) (Hayashi 2008). Ring cleavage is catalysed by different dioxygenases depending upon the position of oxygen insertion in the ring. Comparing major catabolic pathways for aromatic compounds in bacteria has disseminated that, initially, different enzymes carry out the transformation steps into some basic metabolites, termed as central metabolites such as catechols and protocatechuate. These dihydroxylated metabolites are funnelled into central metabolic routes via different ring cleavage pathways enzymes. This generalized strategy of catabolic pathways for aromatic compounds degradation advocates that microbes have expanded their substrate span by evolving "peripheral" enzymes, which could convert initial substrates into one of the central metabolites (Fuchs et al. 2011). The ring-cleavage enzymes from varied genera exhibit significant functional analogy as they serve to narrow down the intermediates from different pathways into dihydroxylated compounds. On the other hand, the peripheral enzymes are functionally diverse as they catalyse the conversion of diverse range of aromatic compounds into central dihydroxylated metabolites, which reduces the need to have different enzymes, during biodegradation of variety of aromatic compound (Whiteley and Lee 2006). In ortho-cleavage (intradiol cleavage), the oxygen insertion occurs inbetween the hydroxyl groups, which undergoes beta-ketoadipate pathway, as beta-ketoadipate is a key intermediate in this pathway. In meta-pathway (extradiol cleavage), oxygen gets inserted adjacently to one of the hydroxyl group in the molecule. A third ring fission pathway called the gentisate pathway is also followed, when the two hydroxyl groups are in para position on the aromatic ring, and cleavage occurs in between the adjacent hydroxylated carbon and carboxyl-substituted carbon (Dagley 1971). However during anaerobic biodegradation, the peripheral pathways usually funnelled to benzoyl-CoA but occasionally to phloroglucinol or resorcinol. Specific multicomponent reductase catalyses ATP-dependent de-aromatizing reaction. In some cases, aerobic hybrid pathways are also found, where the aerobic and anaerobic features are combined in degradation pathways. The degradation of phenylacetate is a classical example, where initial step is marked by the formation of phenylacetyl-CoA, which is subjected to aromatic ring oxygenation reaction (Luengo et al. 2007).

7.2.3 Gene Clusters Involved in Ring Cleavage

Aromatic compounds are metabolized via anaerobic and aerobic pathways. Reduction of compounds occurs in presence of other electron acceptors in anaerobic mechanism, whereas oxidation occurs in aerobic pathway. Consequently, the gene clusters involved will be different in their structure and function in bacteria. (Fuchs et al. 2011). Benzoyl-CoA is the most common intermediate during

anaerobic degradation of aromatic pollutants, in which an electron-withdrawing substituent comes into play, where carboxy-thioester group serves the purpose, whereas catechols and protocatechuate are the central carbon intermediates of the aerobic pathway. Problem arises when phenol is converted to benzoyl-CoA. This pathway involves 4-hydroxybenzoate, phenylphosphate and 4-hydroxybenzoyl-CoA where phenol is carboxylated at the para position to 4-hydroxybenzoate. The whole pathway is assumed to involve interplay of several proteins, as indicated from genes involved in the synthesis of phenol-induced proteins. Gene clusters encoding the above genes were similar to phosphoenol pyruvate synthase which may synthesize phosphorylating enzyme. Other four genes were similar to ubiquinone biosynthesis genes of *E. coli* that decarboxylates a derivative of 4-hydroxybenzoate (Rost et al. 2002; Lipscomb 2008). However, organisms undergo different pathways depending upon the size and arrangement of genes. Analyses of the nucleotide sequences of the *R. palustris* and *T. aromatica* benzoate degradation genes point out two different routes of benzoyl-CoA metabolism, while their benzoyl-CoA reductases have similar structures. After reduction of benzoyl-CoA, the diversification of the degradation pathways in the organisms is manifested, with the hydration being the next consequent step in *T. aromatica*, whereas a second 2-electron reduction occurs in *R. palustris*. Diversity in ring-cleavage substrates renders differences in gene sequences of the enzymes. *T. aromatica* and *R. palustris* ring-cleavage genes are dissimilar in size, where *oah* gene of *T. aromatica* is 40% longer than *badI* gene of *R. palustris*. The deduced amino acid sequences of *Oah* and *BadI* are only 30% identical. HGT events confer aromatic compound-degrading ability to the organisms which allows the bacteria to undergo similar pathway irrespective of their genus.

7.3 Biofilm-Forming Ability in Bacteria

The biofilm formation and development proceeds through three apparent stages, namely, (a) attachment, (b) EPS and microcolony formation and (c) maturation of biofilm and dispersal (Ghosh et al. 2017a, b; Pal et al. 2016). Due to lack of olfactory, visual and auditory perception, initially, adherent organism respond to the stresses arising from slight deformations on the surface they senses non-covalent forces such as van der Waals, electrostatic or hydrophobic forces and becomes aware of their adhering state, thereby initiates themselves from planktonic to a biofilm phenotype (Busscher et al. 2012). The microbes then start to communicate with each other and synthesize extracellular polymeric substances and channels resulting into the development of the film. When the concentration of signals gets levelled up, nutrient decreases and cells initiate dispersing signals (Ghosh et al. 2017a, b). All of these processes are performed through an interaction of different biofilm-forming pathways.

7.3.1 Biofilm Pathways in Bacteria

Proposed mechanisms explaining the increased resistance of biofilms to various stress are the induction of stress response mechanisms, slow growth rate of underlying cells and selective penetration of substances in the biofilm (Flemming et al. 2016; Ghosh et al. 2016). The factors and molecular mechanisms involved in biofilm development are the interplay of various cell surface proteins and proteins involved in stress response (Franklin et al. 2011). However biofilm processes are modulated by quorum sensing EPS production and biosynthesis of extracellular structures in gram-negative and gram-positive bacteria (Franklin et al. 2011; Abee et al. 2011). Bacteria undergo sequential mechanisms mediating intra- and cross-species cell-cell communication and cell to surface attachments or interspecies quorum sensing (QS). It has been observed that mostly biofilm communities responsible for aromatic compound-contaminated sites are harboured by proteobacteria and gram-negative bacteria (Shrout and Nerenberg 2012). Hence, QS responsible for biofilm formation are *las*-based signalling systems. In gram-negative bacteria, AHL QS signalling requires AHL synthase protein, AHL signal molecule and a regulator that responds to the local AHL concentration (Qureshi et al. 2015). The regulatory protein, when cued with the threshold AHL signal, initiates the transcription of several varied genes such as EPS synthesis, biosurfactant production, interspecies competition, etc. (Shrout and Nerenberg, 2012; Mangwani et al. 2017; Pal et al. 2016; Ghosh et al. 2017a, b). *Pseudomonas aeruginosa* synthesizes three polysaccharides: Alginate, Psl and Pel-polysaccharides. Biosynthesis of *psl* occurs by transport of polysaccharide across the membrane through a lipid carrier protein, while *pel* and alginate synthesis occurs in the cytoplasm and transported across the membrane through their glycosyltransferase proteins, which are analogous to cellulose biosynthesis enzymes (Franklin et al. 2011). The production of activated sugars for transport and modification is regulated by *las* QS system or by some indirect mechanisms (Goodman et al. 2004). *Sinorhizobium meloti* synthesizes two extracellular matrix polysaccharides called EPS I and EPS II, where EPS II production, is regulated by AHL-mediated QS. However, role of *rhl* in biofilm cannot be ruled out. Quorum sensing mediated by *las* and *rhl* indirectly activates the biosynthesis of *pel* polysaccharides in *P. aeruginosa*. Here exists a strategy which indirectly controls complex circuitry of *pel* production. It involves RetS and GacS/GacA as two-component systems, RsmZ (small RNA molecule), RsmA (RNA-binding protein). RetS intercepts the GacS/GacA, a two-component regulatory protein complex that elevates transcription of RsmZ. RsmZ binds and inhibits RsmA, which in turn binds the leader sequences of target mRNA and downregulates their expression (Romeo 1998; Heurlier et al. 2004; Dubey et al. 2005; Goodman et al. 2004). Also, RsmA negatively regulates the translation of rhlI and lasI (Pessi et al. 2001). Therefore, *pel* expression is regulated at the post-transcriptional level by RsmA, which serves as repressor of QS systems in gram-negative organism (Sakuragi and Kolter 2007). In gram-positive bacteria, LuxS synthesizes autoinducer-2 (AI-2) which positively regulates biofilm formation in *B. subtilis* (Lombardia et al. 2006). Recent report

suggested the involvement of AI-2 signal and *luxS* gene both of which are responsible biofilm formation in several gram-positive bacteria like *B. cereus* group (Hardie and Heurlier 2008). Autoinducer-2 when exogenously added into the medium resulted in biofilm disassembly in *L. monocytogenes*, *B. cereus* and *S. aureus*. *S. aureus* employs *ica* operon, responsible for intracellular adhesion (Hochbaum et al. 2011). However, *luxS* positively regulates *lsrI* and *lsrK genes*, which allows biofilm formation under toxic aromatic compound stress (unpublished). So AI-2-mediated signalling may not be the principal QS mechanism in gram-positive bacteria. Many organisms employ a complex interplay of various QS mechanisms and other processes which synergistically interacts and produces EPS (Abee et al. 2011). Among them, motility appeared to play an important role for static biofilm formation. It controls the transition between the motile planktonic and the sessile biofilm mode of growth. Reports point out that this transition was under control of the DegU (response regulator), whose phosphorylation is directly regulated by acetyl-phosphate levels inside the cells this reflects that central carbon metabolism also controlled biofilm formation (Gueriri et al. 2008). Degree of DegU phosphorylation advocates whether cells activate EPS synthesis (exoprotease production; high DegU) or swarming motility (low DegU) (Kobayashi 2007; Verhamme et al. 2007). EpsE is encoded in an operon required for synthesis of biofilm matrix and is capable of arresting flagellar rotation in biofilm cells by disassembly of elements responsible for motor force generation (Blair et al. 2008). In *Bacillus*, motility, sporulation and biofilm formation are strictly connected and controlled by global regulators. However, the role of QS cannot be ruled out in this regulatory process. When the quorum of cells is high, quorum sensing signals accumulate and induce surfactin biosynthesis within a subpopulation of cells (Lopez et al. 2009) which is intercepted by neighbouring cells, but not by synthesizing cells. It triggers potassium leakage followed by KinC (sensor kinase) activation, which is localized in membrane microdomains (similar to lipid rafts in eukaryotic cells) and represents a kind of paracrine signalling pathway, where a certain number of target cells regulates and enables specific distribution of signal in sensing cell types (Lopez and Kolter 2010). Activated KinC modulates Spo0A (master regulator) through sequential phosphorylations. Spo0A-P induces SinI production, which inhibits the function of SinR, which serves as the repressor of several biofilm operons (Lopez et al. 2009; Abee et al. 2011). Hence, biofilm operons can be expressed by escalating the inhibitory effect of sinR.

7.3.2 Mechanism Used by Degradative Biofilm Bacteria During Stress Conditions

Bacteria construct communities with elevated complexity and plasticity in a biofilm, which affords increased feasibility of survival and adaptation of cells, as they are cemented in EPS. It provides optimum micro niche, which allows growth of

distinct microbes, quorum sensing (QS) and sharing of metabolic products; thereby usage of aromatic compounds is accelerated, and cells get protection from toxic chemical surroundings, fluctuating pH, etc. (Flemming and Wingender 2010). This increased tolerance of the biofilm state is of particular pertinence to biotransformation and biodegradation process, in terms of sustainability of cells, as planktonic cells may not be able to persist in a toxic environment (Johnsen and Karlson 2004). Quorum sensing allows cell-cell communication, which regulates certain key mechanisms in bacteria, biofilm formation, exopolysaccharide biosynthesis, horizontal gene transfer (HGT), biosurfactant production, motility catabolic gene expression and chemotaxis, which are important mechanisms employed by bacteria during degradation processes and hence are essential for bioremediation (Huang et al. 2013). As a result cells may have altered phenotype in an invariable environment with respect to gene expression and growth as compared to the planktonic cells. Biofilms possess a multitude of surface-active molecules that are beneficial for solubilization and degradation of hydrophobic compounds (Mangwani et al. 2014; Shukla et al. 2014; Zhang et al. 2011). In some cases, gram-negative bacteria adapt uniquely to cope with the hydrocarbon stress. Underlying cells of biofilm release membrane vesicles (MVs) which are capable in terminating competition by annihilating its neighbouring bacteria and exploiting the nutrients from their lysed cells (Mangwani et al. 2017).

7.3.3 Genes Expressed During Aromatic Compound Stress

Transcriptomic and proteomic approaches served as powerful tools to elucidate functional mechanisms and discover specific molecular markers (Griffin et al. 2002; Kim et al. 2009). However, using single approach alone could not ascertain adequate information. The degradative genes of aromatic compounds are often found in clusters. The complete degradation pathway is composed of transport genes responsible for entry of the substrate into the cell followed by their degradation by catabolic enzymes encoded by catabolic genes. Also, regulatory genes are required which controls transport and catabolic genes expression (Khomenkov et al. 2008). Available reports point out that organization of catabolic genes for aromatic degradation can be encoded in mobile genetic elements such as transposons or plasmids integrated in the chromosomes. Generally, biodegradation of substrate and metabolite is subjected to regulation in coordination. However, they are controlled by a set of structural genes, which also encodes for regulatory proteins at the gene level. LysR-type transcriptional regulators are one of the largest family of regulatory proteins identified in bacteria so far. They are involved in the regulation of degradation pathways of chlorobenzoates, phenols and benzoate (Aldrich et al. 1987; Henikoff et al. 1988; Parsek et al. 1994). Furthermore, other regulators belonging from IclR family and AraC/XylS family were reported to play significant roles in the regulation of catabolic genes. Differential gene expression under catechol and phenol degradation conditions the involvement of these regulatory proteins in *Bacillus subtilis*

(Tam et al. 2006). Transcriptome studies revealed 331 differentially expressed genes (DEGS) were annotated by the KEGG during phenol degradation by *Acinetobacter* sp. DW-1. Crucial genes of phenol degradation pathway including phenol hydroxylase, catechol 1,2 dioxygenase and catechol 2,3 dioxygenase were simultaneously expressed.

Genes involved in biofilm formation, including EPS synthesis, predicted to have transmembrane transporter activity were significantly upregulated under phenol degradation conditions. In addition, various membrane proteins were also found to be significantly upregulated. However, they are responsible for establishment of localization, protein binding, transition metal ion binding, iron-sulphur cluster and nucleic acid binding. Furthermore, integral membrane protein which belongs from TerC family, outer membrane OmpA family protein and outer membrane autotransporter barrel domain protein were also upregulated (Franklin et al. 2011). Consequently, these proteins along with some cell surface projections like the proteins involved in fimbriae production were found to be implicated in biofilm formation. During PAH degradation by *P. aeruginosa* PAO1, the PAH degradative gene clusters were spaced nearer to biofilm gene clusters. Genes responsible for quorum sensing and providing oxygen to the upper layers of biofilm were upregulated simultaneously along with PAH degradation genes (Yan and Wu. 2017).

7.4 Interaction and Regulation of Biodegradative Pathways with Biofilm

7.4.1 Applicability of Biofilms in Biodegradation Processes

Biological approaches for treating effluents contaminated with toxic compounds are better than physical and chemical methods concerning their efficacy and economy (Qureshi et al. 2009). Nowadays, ability of biofilm populations or communities in remediation processes has currently been understood. Biofilm-mediated remediation offers safer and proficient alternative to bioremediation, in comparison with conventional suspended microorganisms, because cells encased in a biofilm have increased ability of survival and adaptation during stress, as they are shielded from toxic chemical surroundings on account of their EPS (Singh et al. 2006). Consequently, synergistic physiological and physical interactions occurs among cells in a biofilm, as a result the consumption of pollutant is escalated. Therefore, biofilms are currently being employed in industrial treatment plants for immobilization of cells and efficient degradation of pollutants. Atkinson (1981) reported the applicability of biofilms for the treatment of wastewater in the early 1980s. However, biofilm reactors have gained attention in recent decades in bioremediation sector.

Chlorinated and nitro aromatic compounds are rendered as recalcitrant pollutants, which are ubiquitously found in the effluents of chemical industries (Salinero et al. 2009). To degrade DCP (2,4-dichlorophenol) from synthetic wastewater, Kargi

and Ekker (2005) used a biofilm reactor composed of rotating perforated tube, which harboured activated sludge augmented with *P. putida* capable of degrading DCP. It degraded 100% of DCP. Similarly, bacteria enriched to get adhered in polyaromatic hydrocarbons (PAHs) allow PAH biodegradation (Lendenmann and Spain 1998). This has been well documented for diclofop-methyl (methyl 2-(4-(2,4-dichlorophenoxy) phenoxy) pyruvate), a two-ring chlorinated herbicide, which got accumulated in the EPS of the degrading community. The biofilm community metabolized herbicide effectively. Lendenmann and Spain (1998) used a mixed culture, which degraded a mixture of two isomers of dinitrotoluene (DNT) in a fluidized bed biofilm reactor. The reactor was fed with mixed solutions of 2,6-DNT (10 mg L-1) and 2,4-DNT (40 mg L^{-1}). Degradation efficiencies were achieved to be 94% for 2,6-DNT and more than 98% for 2,4-DNT at all loading rates. Biodegradation of 4,6-dinitro-ortho-cresol, which was reported to be a recalcitrant synthetic pesticide, was successfully achieved in fixed-bed column reactors as well as in batch cultures (Gisi et al. 1997). Ghosh et al. (2016) revealed enhanced 2-4-D degradation and 4-chlorocatechol accumulation in biofilm-associated cells compared to planktonic cells.

7.4.2 Regulation of Aromatic Compound Degradation Pathways Through QS and EPS Genes

The accomplishment of a certain biodegradation pathway depends on two crucial measures that bacteria must get control of, in order to encounter the selective pressure levied by the chemical species regarded as carbon sources. Firstly, the accessibility of the substrates to the cells and expression of right complement of genes, which initiates optimal chain of successive transformations, leading to assimilation and mineralization of the compound. Regulated promoters are the key elements that warrant transcription of catabolic opérons in response to their requirement and at levels enough to guarantee ample metabolic return under high concentration of the substrate (Fernandez et al. 1994). Under such circumstances, biofilms serve an added advantage, as they confer bioavailability of compounds and distribution of catabolic genes in a microbial community through horizontal gene transfer events, enable sequential regulation of catabolic operons and accelerate biodegradation rates, as cells in a biofilm are shielded from toxic loads through EPS (Flemming et al. 2016).

The role of quorum sensing in regulating aromatic catabolic pathways and the later as a source of precursors for generation of signals or quorum quenching process is currently being deciphered (Diaz et al. 2013; Mangwani et al. 2017) (Fig. 7.2). Likewise, in *P. aeruginosa*, anthranilate is generated during tryptophan degradation, which can be directed for activation of *Pseudomonas* quinolone signal through (PQS) biosynthesis pathway or can be routed to energy generation via ant-cat pathway. It is tightly controlled by AHL-mediated QS systems which depend on

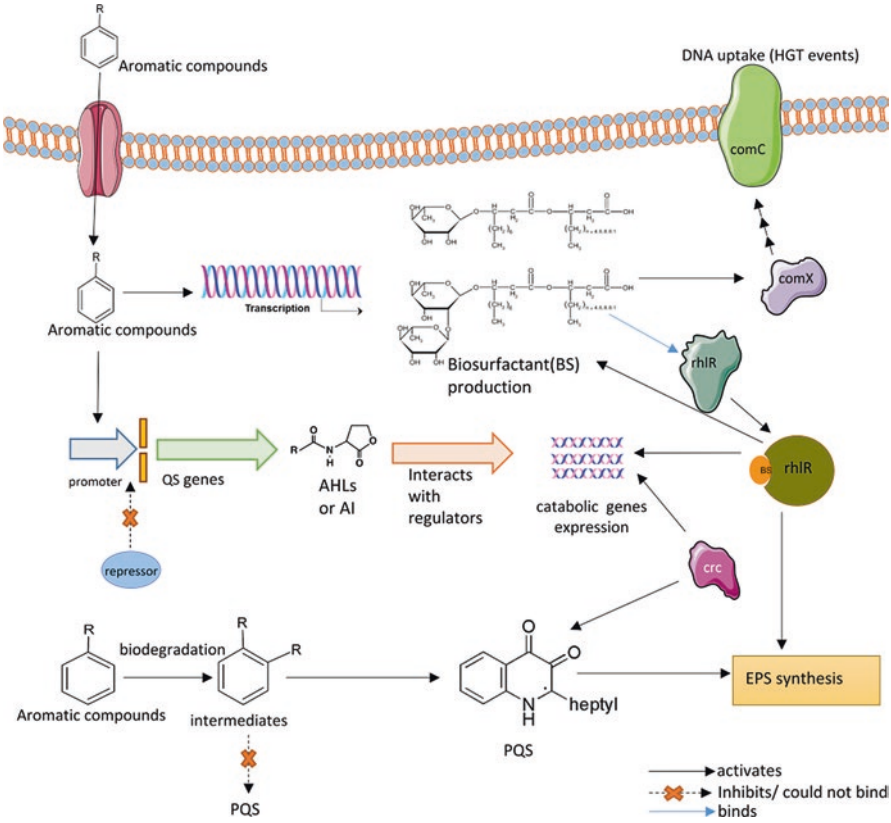

Fig. 7.2 Graphical representation of regulation of aromatic compound degradation through various biofilm mechanisms

LuxR-type signal receptors as well as regulatory mechanisms at transcriptional and post-transcriptional level. The intermediates generated during aromatic compound degradation play a significant role in the synthesis and degradation of such QS signals and vice versa (Chugani and Greenberg 2010). During aerobic catabolism of some chloroaromatic compounds, protoanemonin, a secondary metabolite generated by misrouting the ß-ketoadipate pathway, is a quorum quencher. It is revealing an interesting new function in many *Pseudomonas* sp.-dominated consortia in potent concentrations (Suenaga et al. 2009). Certain enzymes or metabolites from catabolic pathways may inhibit quorum sensing. During phenylacetate degradation different communication signals and bioactive compounds are generated. In addition, some ring cleavage enzymes behave as quorum quenchers, for example, Hod can cleave PQS, revealing an unknown function for these key degradation enzymes (Karpinets et al. 2009). QS directly regulates PAH degradation in *P. aeruginosa* N6P6. It was manifested by enhanced expression of QS genes (*rhl*I and *las*I). QS regulation involves AI binding to a sensory cell surface receptor, which either

activates a signal transduction cascade resulting in the transcription of catabolic genes or interacts with transcriptional regulators, which in turn express the relevant catabolic genes. However, distinct quorum sensing mechanism is involved in the biodegradation of specific aromatic compounds. As biodegradation of phenanthrene and pyrene progress, higher levels of QS gene(s) are expressed which enhances EPS production followed by biofilm growth and expression of PAH catabolic genes resulting in efficient degradation of PAH. Also, *rhl*I, not *las*I expression, was significantly increased during pyrene degradation, but *las*I expression was increased during phenenthrene degradation (Mangwani et al. 2015). Available reports point out that biofilm genes either directly initiate the transcription of catabolic genes or they indirectly regulate by interacting with the regulators which activate their transcription. RhlI/rhlR are responsible for emulsifiers or biosurfactant production, which alleviates biodegradation capacity in the bacterial biofilm by conferring increased hydrocarbon solubility (Stankowska et al. 2012). They allow the development of conditioning film and recruit other organisms like *Ulva intestinalis*, which need a conditioning surface for attachment (Atkinson and Williams 2009), thereby allowing the recruitment of various species in a biofilm. Hence, the cell-cell communication is not limited among the AHL-producing species but also in other organisms of varied taxa as co-inhabiting species able to sense QS signals and responding through behavioural adaptations. This communication potential escalates bioremediation capability in a community (Atkinson and Williams 2009; Stankowska et al. 2012). Reports suggest the crucial involvement of rhl QS genes in gene transfer events in a biofilm. Apart from biosurfactant production, they allow extracellular DNA release, where *rhl*R interacts with *com*X, which is responsible for extracellular DNA uptake by the cells and allows transformation (Mangwani et al. 2014). However, AHL genes itself are also being transferred by HGT events in the biofilm as evidenced by the occurrence of multiple mobile genetic elements in the *lux*R homologous region upstream to it (Singh et al. 2006). Horizontal gene transfer of AHL allows cross talk with the neighbouring species, which in turn accelerates HGT events and enhances biodegradation efficacy in a degradative biofilm community (Huang et al. 2013; Singh et al. 2006). Apart from QS genes, other EPS-producing genes also regulate the biodegradation of several aromatic compounds. Ghosh et al. (2017a, b) reported enhanced utilization of catechol in biofilm adapted cells of *P. mendocina* EGD-AQ5 and activation of ortho-cleavage pathway. There was a strong correlation between *pelA* and *cat*1,2D expression. Similarly, Yong and Zhong (2013) reported the activation of catechol 2, 3-dioxygenase (C23O) expression by *rhl*I/R in biofilm-associated cells of *P. aeruginosa*, with no effect on the first degradation step of phenol hydroxylation (unpublished data). Catabolic plasmids get transferred through HGT events in biofilms. It allows evolution of metabolic pathways in population(s) or communities which gains the ability to degrade wide variety of aromatic compounds. This auto-gene relocation is more pronounced among the biofilm members which are densely packed. Genes responsible for EPS and QS mechanisms often allow cell-cell communication, which activates HGT events (Shukla et al. 2014). Transformation efficacy is 600-fold higher in biofilm as compared to suspended cells (Molin and Tolker-Nielsen 2003). Once the cells sense

the surface, EPS and surfactant production commences which is regulated by rhl QS mechanism. This allows solubility and drives the chemotaxis of recalcitrant PAH into the cells. It governs biofilm formation in several microorganisms through flagellum, which serves as a principal locomotory organ in several species. Apart from regulation by QS, another global regulator called *crc* has been recently found. It renders carbon sources available inside the cell and inhibit carbohydrate catabolism enzymes, intriguably through BkdR, the transcriptional activator of branched-chain keto acid dehydrogenase, which is inducible multienzyme complex. It was previously shown to influence pyocyanin synthesis, regulated by the *Pseudomonas* quinolone signal (PQS) QS system. It releases extracellular DNA via autolysis (Yang et al. 2007).

In the presence of aromatic compound stress, crc represses carbohydrate catabolism but allows chemotaxis of aromatic compounds by affecting type IV pili structural subunit expression. PilA promotes microcolony formation on biofilms through type IV-dependent twitching motility. Mutation studies indicate that *P. aeruginosa* crc mutants could form thin film of cells such as monolayers, instead of aggregating into a number of micro and macrocolonies. Therefore, Crc may also represent a connection between carbon availability and the decision of entering into the biofilm mode of growth (Shirtliff et al. 2002). Hence, aromatic compounds drive biofilm formation processes by inducing QS and EPS synthesis and vice versa.

7.5 Conclusion

Bacterial biofilms are efficient systems recently being applied in bioremediation, as they warrant enhanced bioavailability of aromatic compounds to the underlying cells, by producing bioemulsifiers, which allow solubilization of number of hydrophobic compounds and protect the cells from the toxic shock. Its optimum microenvironment for the degradation allows reactions to occur. It allows sharing of catabolic genes through HGT events. The present chapter suggests interlinking of biofilm process and degradation of aromatic compounds through various mechanisms like chemotaxis, HGT events and EPS production. Involvement of QS as well as other EPS-producing genes in biofilm growth cycle was amply being studied in few bacterial groups, but involvement of these biofilm mechanisms in biodegradation has not been studied extensively. Hence, this would come up with a better understanding of inter-regulation of biofilm and aromatic compound biodegradation. This kind of communication helps in design and generation of consortia with enhanced bioremediation ability, which would escalate the development of biofilm-based remediation technologies.

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Chapter 8
Polychlorinated Biphenyls (PCBs): Environmental Fate, Challenges and Bioremediation

Seethalaksmi Elangovan, Sathish Babu Soundra Pandian, Geetha S. J., and Sanket J. Joshi

Abstract Synthetic chlorinated organic compounds—polychlorinated biphenyls (PCBs)—have been used in several industrial applications for over 50 years and are among the most persistent classes of xenobiotic pollutants. PCBs remain in the environment for a long period due to their low reactivity and stability in harsh environmental conditions. Samples of PCBs can be analysed using chromatographic methods (gas or liquid) coupled with mass spectrometry after various pre-treatment and extraction methods. Hydrophobicity and a chemically stable nature cause them to break down very slowly under natural conditions. Catabolism by microbial enzymes is an efficient route for environmental biodegradation of PCBs, but as chlorination substitution in the biphenyl ring increases, the microbial degradation rate decreases. Different types of microbes are reported to degrade PCBs under anaerobic and/or aerobic conditions by reducing and oxidizing dechlorination mechanisms, respectively. Four main enzymes are reported for the biodegradation pathway of PCBs: biphenyl dioxygenase (bphA), dihydrodiol dehydrogenase (bphB), 2,3-dihydroxybiphenyl dioxygenase (bphC) and 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (bphD). Different types of bacteria are reported to successfully degrade PCBs, but only a few fungi are possible degraders in the absence of alternative carbon sources.

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8.1 Introduction

The industrial boom after World War II saw a range of recalcitrant chemical compounds enter into environment, and each year over thousands of different xenobiotic chemicals are added to the market. Such compounds have poor degradability and thus accumulate in air, water and soil. Polychlorinated biphenyls (PCBs), also called polychlorobiphenyls, are among the most recalcitrant of these pollutants. They have been used for more than 50 years in different industrial applications (Abramowicz 1990). Schmidt and Schultz (1881) first reported the synthesis of PCBs; they are a group of synthetic chlorinated organic compounds in which chlorine is attached to the basic structural unit, the biphenyl, which is composed of two benzene rings. Benzene is a by-product of gasoline, which is extracted from crude oil and heated under a controlled condition to form biphenyls, which by electrophilic chlorination use chlorine gas to produce PCBs. The degree of chlorination determines the chemical and physical properties of PCBs. PCB compounds have quite low reactivity and are non-flammable; they have high electrical resistance and good insulating properties and are quite stable at high temperatures and pressures. They enter into the environment through spills, leaks and improper disposal of electrical and other equipment, where they persist for an extended period, since they are not readily degradable. Because of their chemical stability and low degradability, PCBs possess long distance mobility and are highly accumulative in organisms. For this reason, PCBs have been designated as persistent organic pollutants (POPs), together with dioxins and nine chlorine-based agricultural chemicals (such as, DDT and chlordane) with properties similar to PCBs. An international convention concerning usage regulations and disposal of POPs (The Stockholm Convention on POPs 2010) was adopted in May 2001 in order to prevent this pollution spreading globally. In year 2007, a methodology was developed by the United Nations Environment Programme (UNEP) Chemicals Branch, by which countries following its protocol released their POPs inventories as a starting point for developing interventions that would reduce or eliminate these pollutants altogether (Fiedler 2007).

Toxicity of PCBs has been reported in incidents such as the Kanemi oil poisoning syndrome, caused by PCB-contaminated edible oil (Miyata et al. 1977). These toxic contaminants have the ability to bioaccumulate in the food web. Larsson (1987) reported the bioaccumulation of PCBs from the sediments of an artificial freshwater pool containing PCB concentration of 2.7 μg/g by macroalgae *Cladophora glomerata* to a level of 3.6 μg/g dry in a period of 2 months. PCBs have a range of toxicity, and people exposed to high levels of them respond with skin and other conditions, such as chloracne (Ju et al. 2009) and liver damage. PCBs are recycled between air, water and soil. Their airborne deposit onto plants and aquatic sources cause PCBs to be assimilated by surrounding fauna, and since they are

lipophilic in nature they accumulate in fatty biological tissue and the food web (Cohen 2010). The main source of exposure to PCBs for the general public is via consumption of food, particularly animal and dairy products. Toxicity varies depending on the specific PCB. For these reasons, the study of different methods of degradation to remove PCBs is of great significance, and microbial biodegradation plays a major role.

8.2 Chemical Properties of PCBs

PCBs are synthetic organic chemical compounds in which up to 2–10 chlorine atoms have attached to two linked benzene rings (the biphenyl) and some or all of the hydrogen atoms have been substituted by chlorine atoms. The basic chemical structure is shown in Fig. 8.1.

The chemical formula of PCBs is $C_{12}H_{10}$-nCl_n, where n ranges from 1 to 10. More than 209 distinct PCB *congeners* are reported. Lipophilicity and melting point increase with increasing degree of chlorination, whereas water solubility and vapour pressure decrease. PCBs with a lower degree of chlorination are more volatile than those with a higher degree. PCB congeners in pure form are colourless, often crystalline and have high flashpoints (170–380 °C); they are either oily liquids or solids with no discernable taste or odour. As the number of chlorines in a PCB mixture increases, flashpoint also rises, leading to less combustible substances. PCBs have low electrical conductivity, high resistance to thermal degradation and high thermal conductivity. The International Union of Pure and Applied Chemists (IUPAC) adopted the PCP numbering system proposed by Ballschmiter and Zell (1980).

8.3 Production and Application

The production of PCBs began in1929, manufactured as mixtures of 60–90 different congeners. In that year, Monsanto was the only American company to manufacture PCBs, selling them in the USA under the trade name Aroclor. An Aroclor PCB mixture contains over 100 different individual PCBs. As the number of chlorines in PCBs increases, the compound becomes more stable and thus resistant to biodegradation. PCBs produced commercially are used in transformers, printing inks, plasticizers in paints and cements, capacitors, pesticides, hydraulic fluids, lubricating

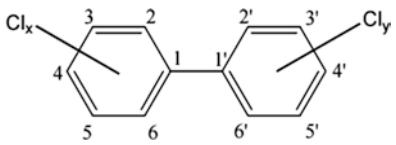

Fig. 8.1 Basic chemical structure of chlorinated biphenyls

and cutting oils, reactive flame retardants and sealants for caulking, wooded floor finishes, as de-dusting agents, waterproofing compounds and casting agents.

8.4 Analytical Methods to Determine PCBs

Many analytical methods for PCB analysis from different samples are approved by federal agencies and organizations such as Environmental Protection Agency (EPA), the American Public Health Association (APHA) and the National Institute for Occupational Safety and Health (NIOSH). For food and feed sample analysis, the Association of Official Analytical Chemists (AOAC) has standardized methods. Additionally, modified analytical methods are included to obtain higher sensitivity and/or to improve accuracy and precision. In general, PCB analysis methods can be categorised into: specific methods (gas and liquid chromatography coupled with mass spectrometry-MS); and non-specific methods, which includes PCB screening kits, X-ray fluorescence (XRF) spectrometry, and micro coulometric titration, which provides total PCBs. PCB analysis includes several steps as shown in Figs. 8.2 and 8.3 and as briefly reported in the following section of this chapter (Barska et al. 2005; Cranor et al. 2005; Rejczak and Tuzimski 2015; Ahmed 2003; Muir and Sverko 2006; Sanchez-Rojas et al. 2009; Silva et al. 2012; Namiesnik and Szefer 2009; National Research Council 2001; Riaz and Zamorani 1988; Tan and Chai 2011; Llompart et al. 1998; Bjoerklund et al. 2002; Halfadji et al. 2003; Criado et al. 2003).

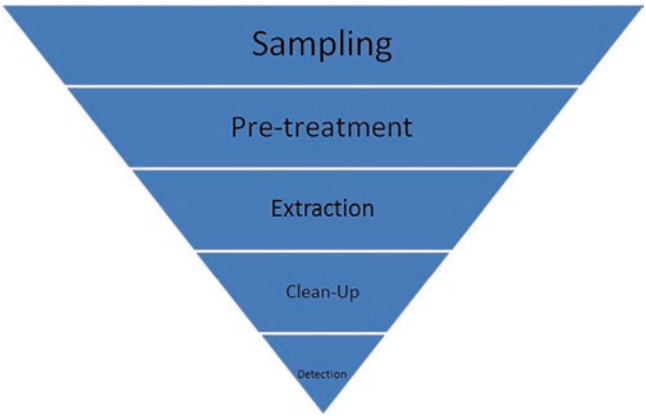

Fig. 8.2 Steps in PCB analysis

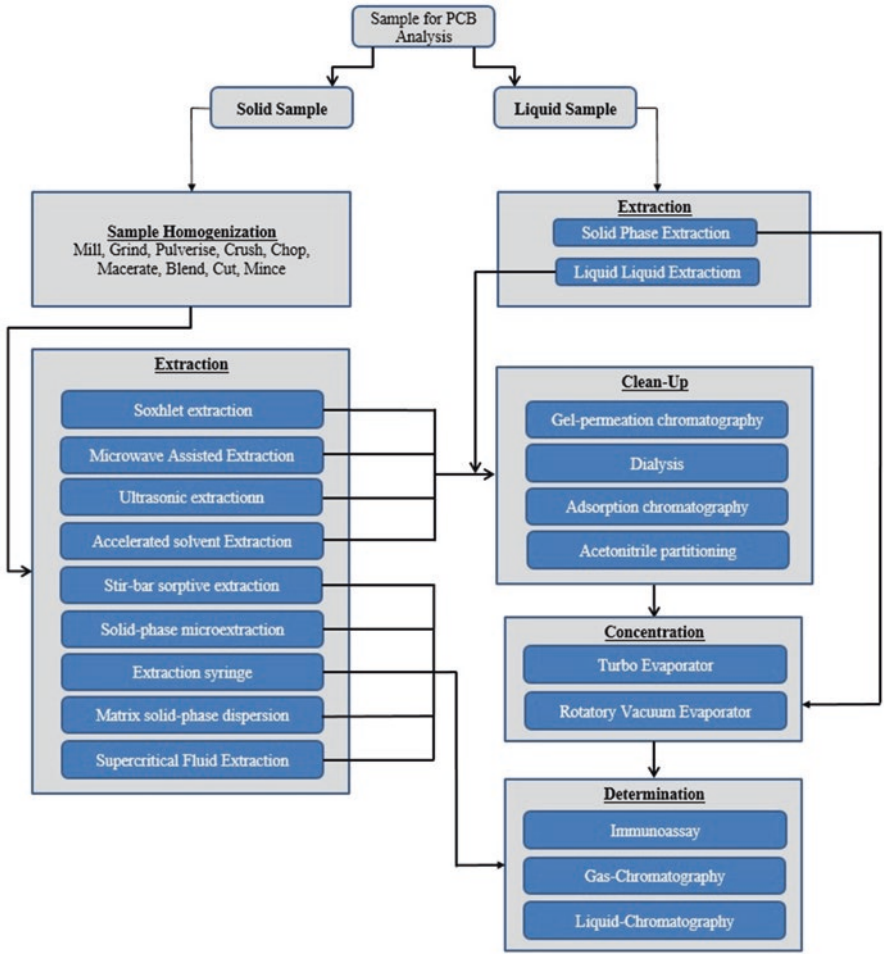

Fig. 8.3 Different analytical methods to determine PCBs

8.4.1 Extraction

PCBs are extracted from the sample matrix by liquid/liquid extraction (LLE), solid-phase extraction (SPE), Soxhlet extraction, stir-bar sorptive extraction (SBSE), solid-phase microextraction (SPME), extraction syringe (ESy), matrix solid-phase dispersion (MSPD), ultrasonic extraction (USE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and automated accelerated solvent extractor. MAE has already been successfully applied in the extraction of PCBs and other semi-volatile pollutants from solid matrices and samples. Quantitative extraction of solutes from solid matrices is possible using MAE by employing microwave energy as a source of heat in closed vessels, with comparable extraction efficiency

in shorter extraction time and with smaller solvent volume. Fully automated methods such as accelerated solvent extraction considerably simplify sample preparation, using less time for fast and easy extraction with lower solvent consumption. In this method, the extraction solvent is pumped into an extraction cell containing the sample, and temperature and pressure are increased. The extract is further transferred to a typical collection vial for the next step of clean-up or analysis.

Liquid-liquid extraction (LLE) is an extraction method to separate compounds based on their relative solubility in two different immiscible fluids: aqueous and non-polar organic solvent. Generally compounds get transferred from aqueous to organic phase. LLE extraction technique can be performed with a variety of apparatuses, from separatory funnels to counter-current distribution equipment. Samples for PCB analysis are first homogenized with sodium sulfate to remove the water present in the sample, and then dried overnight, followed by extraction with a suitable solvent. Non-polar solvents are used for extractions from oily matrices, and medium-polarity solvents (acetone or dichloromethane) are often combined to achieve the desired viscosity and solvency strength for the particular extraction. Stir bar sorptive extraction (SBSE) is a novel technique for sample enrichment of volatile and semi-volatile organic compounds from aqueous and gaseous media. SBSE consists of a glass-lined magnetic stirring bar covered with a thick layer of polydimethylsiloxane (PDMS). Once exposed to a sample solution, the solution's components are enriched in the PDMS, which is subsequently removed. The sorbed compounds are then both thermally desorbed by means of a liquid and analysed by GC-MS or LC system. The technique is easy to use, with better sensitivity and accuracy, and it has been applied successfully for trace analysis from different samples.

Soxhlet extraction is one of the most frequently used of liquid-solid extraction methods, being a fairly simple and cheap method that provides good reproducibility. Worldwide, laboratories still routinely use this technique for extraction of PCBs from food matrices. The disadvantages of this extraction method are long extraction time (6–18 h), difficulty of the extraction itself, generation of dirty extracts and extensive clean up. In 1989, Pawliszyn and co-workers invented solid-phase microextraction (SPME) (Kusch 2018). SPME is a simple, efficient and solvent-free sample preparation, which boasts simplicity and low cost. Analytes could be directly extracted and concentrated to the stationary phase (such as polydimethylsiloxane (PDMS)) coated on a fused-silica optical fiber, thus integrating sampling, extraction, concentration and sample introduction in a single step. SPME reduces sample preparation time with improved detection limits. It has been routinely used in combination with gas chromatography (GC-MS) for the extraction of volatile and semi-volatile organic compounds, and with high-performance liquid chromatography (HPLC) and HPLC-MS. SPME is reported to be an ideal choice which yields relatively clean and concentrated extracts. The polarity and volatility of analytes dictates the selection of sampling method and type of chromatography technique to be used.

Ultrasonic extraction is a green, environment-friendly method of extraction. The sample is mixed with anhydrous sodium sulfate in a vessel and submerged in an appropriate organic solvent and placed in an ultrasonic bath. The mixture is extracted

using ultrasonic extraction thrice, and the extract is vacuum-filtered or centrifuged. The extract is ready for final concentration, clean-up and/or analysis. The extraction efficiency depends on the solvent polarity, sample homogeneity and extraction time.

Extraction syringe (ESy) is a most recent technique for the handling of liquid samples. It combines online microporous membrane liquid-liquid extraction and GC analysis. ESy integrates sample pre-treatment, enrichment, clean-up and GC injection. In ESy, a mini flat-sheet membrane holds both sides by polypropylene (PP) plastic like a sandwich; this unit is called an ESy extraction card. A syringe pump is attached to one side of the PP plastic—the acceptor side of the ESy card—so as to impregnate and fill the acceptor side with solvent. The sample (1–3 ml) is delivered by a syringe pump to the other side of the extraction card—the donor side. Once the extraction is complete the card holder needle moves down and injects the whole extract directly into the GC system. The amount of solvent used is fairly negligible, and the sample handling does not need extra clean-up steps.

Supercritical fluid extraction (SFE) apparatus contains a stainless steel extraction cell, flash stainless steel extraction collector, cooler, CO_2 pump, co-solvent pump and heater. Liquid CO_2 ($\geq$ 99.98%, UltraPure) from a cylinder is pumped at 3×10^{-3} kg/min. Extractions are generally carried out at high temperatures and pressure for 1–3 h, as controlled by proprietary software. Solid-phase extraction (SPE) is a sample preparation technique used for the extraction of analytes from liquid samples. This technique facilitates the extraction, clean-up and concentration of analytes. It is extensively used as an alternative extraction or clean-up method to LLE to determine pollutants in liquid samples. The affinity difference between an analyte and interferents for a solid phase allows the separation of the target analyte from the interferents in SPE. Matrix solid-phase dispersion (MSPD) was introduced by Barker (2007). One of the main advantages of this technique is that extraction and clean-up are done at the same time, which helps to reduce sample contamination during the process. This technique has been applied for the extraction of PCBs from different matrices, food samples, biota and environmental samples.

8.4.2 Sample Clean-Up

Different techniques are used to clean extracted sample, either singly or in combination, influenced by the selectivity and sensitivity of the final technique used to detect PCB and by the extraction method employed. Clean-up steps are necessary, since extracted tissue or sediments will contain many compounds other than PCBs; suitable clean-up is necessary to remove interference. Different techniques such as gel permeation, silica gel, florisil, activated carbon and HPLC are often used to remove interference.

Adsorption chromatography is the most commonly used clean-up method. It involves passing extracts through several adsorbent columns such as alumina, silica and florisil. These absorbents are used either separately or in combination, with different mesh sizes, activity and column type. As PCBs are stable under acidic

conditions, columns may be used to remove lipid from extract using sulfuric acid or acid-impregnated silica. For sulfur-free sediment, alumina columns give a satisfactorily clean extract for GC analysis. Additional clean-up steps may be required for PCB analysis by ion-trap GC-MS. Activated florisil microcolumns have also been used to separate planar PCBs from non-planar PCBs. The column is first eluted with hexane followed by dichloromethane in order to obtain two fractions: non-planar and planar PCB congeners, respectively. The use of activated carbon and carbon dispersed on silica gel are also reported.

Dialysis is a membrane-based sample preparation technique, which has been used for sample preparation for chromatography applications. It uses a semi-permeable membrane device (SPMD) in an organic solvent phase for the separation of contaminants from lipid matrix prior to organic chemical analysis. This method is quite useful for various types of oily samples and other matrices that are difficult to clean up by traditional clean-up procedure. It is an efficient means of removal of lipids in the determination of bioaccumulative, persistent, halogenated organic compounds. Polychlorinated biphenyls with minimal lipid carryover are determined in lipid matrix. The advantage of this technique is that is has very satisfactory and highly reproducible analyte recoveries in a single operation; however, it requires a large volume of solvent. Acetonitrile-hexane partition is also commonly used to remove lipids from sample extracts, where PCBs get partitioned into the acetonitrile phase, and lipids get partitioned into the hexane phase. Acetonitrile partitioning needs 5 extractions with 10 times the volume of the lipid sample.

Gel-permeation chromatography (GPC) technique is primarily used for the analysis of biological samples. Since PCB is lipophilic in nature, tissue samples are extracted with non-polar solvent for lipid extraction. Cross-linked dextrans (e.g., Sephadex or agarose) are generally used as cross-linked polymer material in GPC columns. GPC method can be fully automated, and it is also more applicable for the isolation of unknown contaminants. GPC can also handle a large quantity of lipid in each sample when a second GPC column is added in parallel.

8.4.3 Determination

Immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) are also reported to screen PCBs from sample, also known as competitive ELISA. This method is based on the ability to bind PCBs to specific binding sites on antibodies in the well plate, which requires less effort for sample clean-up. This is a less expensive, rapid method, useful for screening PCBs from environmental samples in ppm concentration. However, the method has several disadvantages that limit its application for screening PCBs.

'Competitive ELISA' is a technique, based on which several immunoassay test kits are commercially available to measure different organic contaminants such as: pesticides, and PCBs from environmental samples.

Gas chromatographic (GC) technique is the most often used technique for identification and quantitation of PCBs, since GC detectors possess high selectivity and sensitivity for the PCBs. Many different detectors are used to determine PCBs by gas chromatography, like electron capture detector (ECD), electrolytic conductivity detector (ELCD), flame ionization detector (FID) and mass spectrometry detector. GC coupled with FID is used much less often than ECD, having exceptional sensitivity to multiple chlorinated compounds compared with FID. Mass spectrometry has sensitivities lower than ECD, but greater selectivity for PCBs, and MS can distinguish and individually measure homologs even if they are co-elutes. Recently, use of the capillary column has made it possible to achieve even lower detection limits and better separation of individual PCBs. LC-MS is becoming an essential technique for the analysis of environmental pollutant, as its user-friendliness, productivity and high selectivity often allows simplified sample preparation. Procedures are relatively highly reproducible and comparatively simpler. However, specific care should be taken on matrix-induced ion suppression, which can be minimized by good sample preparation, good chromatographic separation and optimizing the MS-operating conditions.

8.5 Bacterial Biodegradation

Since PCBs are hydrophobic, inert and stable compounds, their natural breakdown is quite sluggish. Despite its stable nature, much research has been done to study its biodegradation. Bacterial enzymatic degradation is one of the major means of PCB removal (Seeger et al. 1997), but as chlorine substitution increases, the microbial degradation rate of PCBs in soils generally decreases (Furukawa et al. 1979). Several researchers have reported microbial degradation of PCBs in contaminated water, sediments and soils. They have. mainly reported reductive dechlorination of the PCBs leading to less chlorinated compounds, repetitively up to the non-chlorinated biphenyl molecule. Natarajan et al. (1999) reported complete mineralization of the biphenyl congener by a PCB-dechlorinating anaerobic consortium. Dechlorination is reported to occur mainly on meta- and para-chlorine positions and could consequently affect the toxic properties (Lang 1992). Aerobic metabolic pathways as well as the genes involved in biodegradation have also been described (Abramowicz 1990; Chaudhry and Chapalamadugu 1991; Mukerjee-Dhar et al. 1998). Aerobic pathway involves a biphenyl-dioxygenase, which converts PCBs to chlorinated benzoic acids and chlorocatechols (Abramowicz 1990). Bedard et al. (1987) reported the production of a 3,4-dioxygenase by *Pseudomonas* sp. LB400 and *Alcalignes eutrophus* H850, and enzymes need a high amount of oxygen to be effective. Those xenobiotic compounds could spread throughout the municipal system, thus, wastewater treatment plants could also play an important role in removal of PCBs from the environment. Due to its hydrophobic properties, it could accumulate by sorption onto sludge particles during settling processes. Microbiological treatment of the contaminated sludge could establish a conjunction point where

different bacterial metabolic processes could enable minimizing the release of PCBs to local land or water bodies.

Microorganisms are known to play a key role in PCB biodegradation. Biologically, PCBs can be degraded by two major metabolic steps: anaerobic (reducing) dechlorination and aerobic (oxidising) dechlorination (Aken et al. 2009; Field and Sierra-Alvarez 2008; Vasilyeva and Strijakova 2007; Pieper 2005). Higher chlorinated PCBs are degraded anaerobically and then turned into less chlorinated congeners. These lower-chlorinated PCBs are degraded under aerobic conditions. Thus, either only aerobic or anaerobic or sequential anaerobic and aerobic conditions might also help improve or complete biodegradation (Ahmed and Focht 1973; Bedard et al. 1986; Evans et al. 1996; Furukawa and Matsumura 1976; Furukawa and Chakrabarty 1982; Furukawa 1982; Furukawa and Miyazaki 1986; Fukuda 1993; Kimbara et al. 1988). Aerobic biodegradation of PCBs by various bacteria has been reported (Abramowicz 1990; Abramowicz and Olson 1995; Bedard et al. 1987; Boyle et al. 1992; Chen and Pinatello 1997; Flanagan and May 1993; Yadav et al. 1995). Under anaerobic conditions, PCBs can be reductively dechlorinated by a variety of anaerobes (Berkaw et al. 1996; Beurskens and Stortelder 1995; Kim and Rhee 1997; Nies and Vogel 1990, 1991; Quensen III et al. 1990; Rhee et al. 1993; Tiedje et al. 1993; Williams 1994).

Biodegradation depends on the enzymes produced by microorganisms that can modify organic pollutants into less toxic forms (Dobbins 1995; McEldowney et al. 1993). The process of degradation can be carried out in two ways: as mineralisation when microorganisms use it as a source of carbon and energy; or as co-metabolism where microorganisms depend on the secondary substrate as their source of carbon and energy. For complete degradation the products of co-metabolism are further mineralised. The efficiency of biodegradation depends on several factors (Borja et al. 2005), which include: structure of pollutant; solubility; pollutant concentration; physical and environmental parameters (such as, oxygen content, temperature, intensity of light, pH and conductivity); biological parameters (such as the presence, type and number of microorganisms).

8.5.1 Anaerobic Biodegradation

Anaerobic microorganisms are suitable for the degradation of pollutants with high carbon concentration due to controlled oxygen diffusion. Anaerobic bacteria mainly attack the meta and para positions of PCBs, resulting in the formation of aerobically degradable ortho-chlorinated PCBs. Van Dortand and Bedard (1991) identified that the ortho position was also involved in dehalogenation of PCBs. This is the first experimental report to demonstrate the anaerobic ortho-dehalogenation of PCBs. In anaerobic degradation PCBs act as electron acceptors. During this process hydrogen atoms replace chlorine atoms by removal of chloride ions ($R\text{-}Cl + 2e^- + H^+ \rightarrow R\text{-}H + Cl^-$).

Reductive dechlorination was first observed in the upper Hudson River (Flanagan and May 1993). This process occurs in many environmental matrices like river and

pond sediments and flooded soils. Reductive dechlorination of PCB is the only known process to have significant impact on complex PCB mixtures. Several anaerobic bacteria have been identified. These include *Dehalococcoides ethenogenes*, *Desulfomonile tiedjei*, *Desulfitobacterium*, *Dehalobacter restrictus*, *Dehalospirillum multivorans*, *Desulforomonas chloroethenica ethenica* and the facultative anaerobes *Enterobacter* strain MS-1 and *Enterobacter agglomerans*.

In sediments, PCB dechlorination was generally carried out by anaerobes like methanogens, sulfate-reducing bacteria (SRBs), etc. The anaerobic reductive para-dechlorination of Aroclor 1242 was reported to be inhibited by the addition of eubacterium-inhibiting antibiotics. These antibiotics directly prevented the activity of fermentative bacteria and indirectly the methanogens. Because of inactive bacterial strains, there was no dechlorination and methane production. Dechlorination and methane production could only occur in the presence of substrate for methanogenic bacteria along with antibiotics. The reports suggest that methanogenic bacteria play a vital role in anaerobic reductive dechlorination of PCBs (Dingyi et al. 1995; Adrian et al. 2009; Matturro et al. 2016).

Fava et al. (2003) concluded that the biodegradation of PCBs (such as mono- and dichlorinated PCBs, and compounds similar to Aroclor 1242 and 1254) in contaminated estuarine and marine sediments, was mediated by indigenous SRBs and methanogenic bacteria. For this study three sediments were collected from Porto Marghera contaminated area of Venice Lagoon (Italy). Under anaerobic condition, the sediments were supplemented with sodium bicarbonate and sodium sulfide and monitored for dechlorination. After 6 months of incubation, the highly chlorinated biphenyls were depleted along with the cumulation of low-chlorinated biphenyls. The biologically active microbial consortia utilised sulfate, and the production of methane was also detected.

The addition of a single PCB congener also increases the meta- and ortho-dechlorination of PCBs. In a previous study, Aroclor 1260 was investigated in anaerobic slurries of estuarine sediments from Baltimore Harbor (Wu et al. 1997, 1998). The anaerobic culture was enriched by the addition of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-CB) or 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB). After the incubation period, lag time was depleted in sediments enriched with 2,3,4,5-CB. The 2,3,5,6-CB congener also enhanced dechlorination, though not as 2,3,4,5-CB did. These results suggest that each enrichment contained different organisms with high substrate specificities. As a result, the added congeners enhanced the reductive dechlorination found. The results demonstrated that the addition of single congener also increases the anaerobic reductive dechlorination of PCB.

The genus *Clostridium* was effectively involved in PCB degradation. Based on the ability of dechlorinating meta and para PCBs, nine species were identified (*Clostridium hydroxybenzoicum*, *Cl. botulinum*, *Cl. proteolyticum*, *Cl. beijerinckii*, *Cl. intestinalis*, *Cl. thermolacticum*, *Cl. paraputricum*, *Cl. cellulosi and Cl. cellobioparum*). In this study the phylogenetic relationship with PCB-degrading clostridium using 16S ribosomal RNA (rRNA) genes was identified. The variations in small subunits of rRNA genes were examined using defined operational taxonomic units in samples from contaminated sediments from Lake Medina, New York. Further molecular biology analysis revealed that 75% of all the 16SrRNA clones

having anaerobic para- and meta-PCB dechlorinating activity (Hou and Dutta 2000).

Dehalococcoides ethenogenes strain 195 was reported to reductively dechlorinate PCBs (Fennell et al. 2004). In marine sediment, *Dehalococcoides mccartyi* is also involved in reductive dechlorination of PCB. After incubation for 200 days, the decrements (higher to lower) were observed. In this process the pcbA4 and pcbA5 and, to a lesser extent, pcbA1 genes enriched under saline conditions were observed.

The major microbial biodegradation pathway of PCBs reports four specific enzymes: biphenyl dioxygenase (bphA), dihydrodiol dehydrogenase (bphB), 2,3-dihydroxybiphenyl dioxygenase (bphC) and 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (bphD) are sequentially reported for the oxidative degradation of PCBs into chlorobenzoates and 2-hydroxypenta-2,4-dienoate (Furukawa and Miyazaki 1986).

Desulfomonile tiedjei, strain dcB-1, was reported to gain ATP for growth by using aromatic dechlorination as its sole electron acceptor (Dolfing and Tiedje 1987; Dolfing 1990; Mohn and Tiedje 1991). This organism can be grown on hydrogen or acetic acid as its sole electron donor using the conversion of 3-chlorobenzoate to benzoate, and HC1 as its electron acceptor (Dolfing 1990; Mohn and Tiedje 1990). Their report established the basic model and underlying biochemical mechanism, which showed that microorganisms could grow using chlorinated substrates as their sole electron acceptor.

8.5.2 Aerobic Biodegradation

Aerobic transformation of PCBs is a widely known and well-studied technique. It occurs in an oxygen-rich environment. Aerobic transformation is a co-metabolic pathway, which means the microorganisms involved require an additional source of carbon apart from PCBs. The common enrichment factor for PCB-degrading bacteria is biphenyl or monochlorobiphenyls. PCB degradation involves two gene clusters: the first involves the sparsely chlorinated PCB congeners formed from anaerobic degradation of the higher congeners being degraded by co-metabolic aerobic oxidation via the 2,3-dioxygenase pathway, converting the low-chlorinated PCBs to the corresponding chlorobenzoic acids. The second involves chlorobenzoic acid, which is completely mineralised to Cl, water, CO_2 and cell biomass by indigenous bacteria (Abramowicz 1995; Urbaniak 2013).

Several bacterial strains that mediate PCB degradation have been isolated and characterised; they are *Pseudomonas*, *Alcaligenes*, *Burkholderia*, *Comamonas*, *Sphingomonas*, *Ralstonia*, *Cupriavidus*, *Achromobacter*, *Acidovorax*, *Norcardia* and *Acinetobacter* as Gram-negative strains, and *Rhodococcus*, *Corynebacterium* and *Bacillus* as Gram-positive strains. Ahmed and Focht (1973) reported that two species of Achromobacter are suitable for degrading ‘dichlorobiphenyl’ and ‘monochlorobiphenyl’ to ‘chlorobenzoic acid’. Clark et al. (1979) described how the presence of acetate (co-substrate) can increase the co-metabolism of PCB. In their study,

three different enriched mixed cultures—*Alcaligenes odorans*, *Alcaligenes denitrificans* and an unidentified bacterium—were reported. Several researchers (Abramowicz 1990; Arnett et al. 2000; Bedard et al. 1987; Billingsley et al. 1997; Furukawa 1982) summarised the correlation between PCB structures and microbial breakdown as follows:

1. Low-chlorinated PCB congeners are more easily degraded. As chlorine substitution increases, the degradation rate of PCBs decreases.
2. PCBs containing two chlorines in the ortho-position of a single ring (i.e., 2,6-) and in each ring (i.e., 2,2′) are poorly degraded.
3. PCBs containing all chlorines on a single ring are degraded much more easily than those containing the same number on both rings.
4. The bacterial strains involved are responsible for both the relative rate of primary degradation and the appropriate ring attacked.
5. PCBs having two chlorines at the 2,3-position of one ring are more susceptible to microbial attack than tetra- and penta-chlorobiphenyls. This series of PCBs could be metabolised through an alternative pathway.
6. Initial dioxygenation followed by ring cleavage of the biphenyl molecule occurs with a non-chlorinated or less chlorinated ring.

Degradation of PCB by *Alcaligenes xylosoxidans*, *P. stutzeri*, *Ochrobactrum anthropi* and *P. veronii* were found to be more efficient with the addition of glucose and biphenyls (Murinova et al. 2014). The addition of glucose alone increases the degradability of PCBs more than the addition of biphenyl. The addition of biphenyl only increases the degradation of highly chlorinated PCBs for some strains. Among other strains *Alcaligenes xylosoxidans*, was reported to have highest degradability with the addition of glucose.

Generally, bacterial strains with PCB-degrading ability degrade congeners having five or fewer chlorine substitutions and very low or no degrading ability on higher chlorinated PCBs, but *Enterobacter* (LY 402), a Gram-negative bacterium, could effectively degrade both the higher and lower chlorinated PCBs under aerobic conditions (Cao et al. 2011). *Enterobacter* (LY 402) strain has the ability to transform 92% of the penta-CBs, 76% of the hexa-CBs and 37% of the hepta-CBs, and it also degrades some of the octa-CBs. On the other hand, the strain LY402 also showed an ability to degrade both ortho- and para-chlorinated congeners. LY402 was able to degrade 97% of 2,3,5,6,2′,5′-CB (ortho-) and 46% of 2,3,4,5,2′,3′,5′-CB (ortho-); 89% of 2,4,5,2′,4′,5′-CB (para-) and 21% of 2,3,4,6,2′,3′,4′-CB (para-); and 73% of 3,4,3′,4′-CB. LY 402 strain also has the ability to degrade the intermediate metabolites (2-CBA and 2,3-CBA) involved in of PCB degradation. Strain LY402 also found to be effective in degrading complicated PCB mixtures, Aroclors 1242, 1254 and 1260. LY402 strain was found to have a stronger PCB transformation activity from the environment.

Metabolism of several types of chlorobiphenyls by the enzymes of the upper biphenyl catabolic pathway encoded by the bph locus of *Pseudomonas* sp. strain LB400 (Seeger et al. 1995) was reported by using recombinant strains harboring gene cassettes containing ‘bphABC’ or ‘bphABCD’. The enzymes of the upper

pathway were generally able to metabolise mono- and dichlorinated biphenyls, but they could only partially transform most trichlorinated congeners.

The strong and unique ability of the transformation of PCB by *Rhodococcus* species (Strain RHA1) have been identified. The RHA1 strain extensively degrades highly chlorinated congeners, including hepta-chlorobiphenyl. Intermediate metabolites di- and trichlorobenzoic acids were also identified. During incubation, the RHA1 strain gradually degraded these chlorobenzoic acids. Compared with *Pseudomonas* sp. LB400 and *P. pseudoalcaligenes* KF707, RHA1 strain exhibited a good transformation activity on both ortho and para positions. The degrading activity was a superior characteristic of RHA1 compared with previously reported strong PCB degraders (Masai et al. 1995; Seto et al. 1995).

Pseudomonas CH07, a marine mercury-resistant bacterium, was able to degrade a variety of highly chlorinated biphenyls from the technical mixture Clophen A-50 (De et al. 2006). Devrukhkar et al. (2017) reported that the benzoate-enriched *P. mendocina* strain CL-10.4 also has the ability to completely degrade the commercial mixture of PCB-Aroclor 1242.

Based on the examination of 36 pure isomers of PCB, 23 compounds were metabolised by *Alcaligenes* species Y 42 (Furukawa et al. 1979), and 33 species were metabolised by *Acinobacter* sp. strain P6. The Alcaligenes sp. converted 2,4,6-trichlorobiphenyl, and Acinobacter converted 2,4,2′,4′- and 3,4,3′,4′-tetrachlorobiphenyls to chlorobenzoic acid, subsequently. The meta cleavage products of PCB were accumulated through the oxidative route of 2,4′-, 2,4,4′- and 2,5,4′-chlorobiphenyls. Those groups of PCBs were chlorinated at the 2,4′-position. The dihydroxy compounds are accumulated by *Acinobacter* species. *Alcaligenes* sp. was not capable of metabolising this group of PCBs. PCBs that possess chlorine atoms at the 2,3-position in the molecule were alternatively metabolised. In this reaction, two metabolites were produced and accumulated. One of the products was chlorinated benzoic acid and another is not yet identified. Trichlorobiphenyl was also metabolised by different methods by two organisms. *Alcaligenes* metabolise very slowly compared to *Acinobacter* species. In this study it was proved that chlorine substitution also affected the metabolic behaviour of PCB. Tu et al. (2011) investigated rhizobia, nitrogen-fixing bacteria also as an emerging candidate to degrade PCB. *Sinorhizobium meliloti*, a rhizobial strain, played a key role in the bioremediation of contaminated soil.

8.6 Fungal Biodegradation

Apart from bacteria, fungi also participated in the degradation of PCB. Fungi were rarely reported as PCB degraders in the absence of an alternative carbon source; however, the growth capability on biphenyl has been considered an indicator of the ability to degrade PCBs. Only a little was known about fungal degradation of PCBs, regardless of fungus' ability to biodegrade a variety of aromatic pollutants, and a few species have been identified which were already involved in the degradation of

aromatic pollutants. These include *Phanerochaete chrysosporium*, *Trametes versicolor*, *Lentinus edodes*, *Pleurotus ostreatus*, *Grifola frondosa* and *Coriolopsis polyzona*. In contrast, mitosporic fungi have been rarely reported for PCB biodegradation. In liquid mineral medium, the removal of 2-chlorobiphenyl, 4,4′-dichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl by six selected fungal species (*Aspergillus fumigatus*, *Penicillium chrysogenum*, *P. digitatum*, *Fusarium solani* and two strains of *Scedosporium apiospermum*) was reported. PCB biosorption abilities of six fungi were different: where *A. fumigatus* was the least efficient PCB-removing strain, and the *P. chrysogenum* strain potentially degraded three PCB congeners with high efficiency. High-chlorinated PCB biodegradation (up to 48%) was also displayed by the two strains of *S. apiospermum*. The strains *P. digitatum* and *F. solani* showed moderate degradation (up to 24%). These strains degrade the highly chlorinated and most recalcitrant biphenyl 2,2′,5,5′-tetrachlorobiphenyl; however, possible routes of biodegradation were not available (Krcmar et al. 1999; Ruiz-Aguliar et al. 2002; Kamei et al. 2006; Takagi et al. 2007; Rabinovich et al. 2004; Singh 2006; Novotny et al. 1997; Seto et al. 1999; Kubatova et al. 2001; Verdin et al. 2004; Sietmann et al. 2006; Mancera-Lopez et al. 2008).

Phanerochaete chrysosporium was the first white-rot fungus reported to degrade a wide range of PCB congeners. The degradation of three PCB congeners (4,4′-dichlorobiphenyl [DCB], 3,3′,4,4′-tetrachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl) by *P. chrysosporium* in liquid culture were reported. The dichlorobiphenyl mineralised in considerable volume (11%) as compared to tetra and hexachlorobiphenyl. Intermediates like 4-CBA and 4-CBAlc were identified from the degradation of 4,4′-DCB by *P. chrysosporium*. This is the first report providing information about the intermediates in PCB degradation by white-rot fungus. Based on the degree of chlorination and the PCB metabolism pattern and also the intermediate characteristics, similarities were described between *P. chrysosporium* and bacterial systems (Dietrich et al. 1995). In the presence of carbon sources and nitrate as a nitrogen source (non-white-rot conditions), the *Phanerochaete chrysosporium* also able to degrade 2,2′,4,4′,5,5′-hexa-CB by the enzyme nitrate reductase were also determined.

The degradation of lower chlorinated biphenyls by six white-rot fungi was reported by Kubatova et al. (2001). *P. chrysosporium* and *T. versicolor* showed no PCB degradation in soil. In contrast, the four strains of *P. ostreatus* removed 40% of Delor 103 (the commercial mixture of PCB) in 2 months. The rate of degradation decreased with increasing chlorination.

Some studies showed that the filamentous fungi can also degrade PCBs. Their hyphal structure (fungal highways) can easily penetrate environmental matrices. The oxidative enzymes produced by white-rot fungi can degrade a wide range of aromatic organo-pollutants. In extracellular environment, this non-specific, radical-based system of fungi is active, easily penetrating a contaminated matrix and also poorly bioaccessible pollutants (Stellaa et al. 2017). PCB-degrading abilities of two white-rot fungi (*Pleurotus ostreatus* and *Irpex lacteus*) in real contaminated soil were investigated. A total of 18.5–50.5% removal was observed after 12 weeks of treatment. Numerous transformation products were also detected, which also indicates that both fungi were able to degrade PCBs in soil. Compared to *I. lacteus*

species, *P. ostreatus* was more efficient for degrading PCBs. These results demonstrated the capability of using this fungus for possible bioremediation applications.

In another study, three white-rot fungi, *T. versicolor*, *P. chrysosporium* and *L. edodes*, utilized to degrade a mixture of PCBs in the presence of non-ionic surfactant (Tween-80) were examined. Compared with other surfactants, Tween-80 had no inhibitory effect on the growth of fungi. Apart from *T. versicolor* and *L. edodes*, *P. chrysosporium* was the most effective degrader. In contrast to *T. versicolor*, which degrades both low- and high-chlorinated PCB congeners, *P. chrysosporium* and *L. edodes* accumulated low-chlorinated congeners (Ruiz-Aguliar et al. 2002).

8.7 Biochemical Pathway

8.7.1 Aerobic Pathway

Aerobic degradation of PCB is reported to contain two pathways (Hofer et al. 1994; Seeger et al. 1995; McKay et al. 1997, 2003; Hulsmeyer et al. 1998; Sakai et al. 2002; Taguchi et al. 2001): the biphenyl upper pathway and the biphenyl lower pathway. In general, the lower-chlorinated PCB congeners are more easily transformed than higher-chlorinated PCB congeners; also, chlorines present in one aromatic ring are more easily degraded than chlorines present in both aromatic rings. The degradation is carried out by biphenyl (bph) catabolic enzymes. Many PCB degrading bacteria have been reported and enzymes involved in biphenyl degradation have also been characterised. The bph operon has multiple genes for the degradation of chlorinated biphenyls to chlorobenzoate, followed by pyruvates and acetyl-coA (Fig. 8.4).

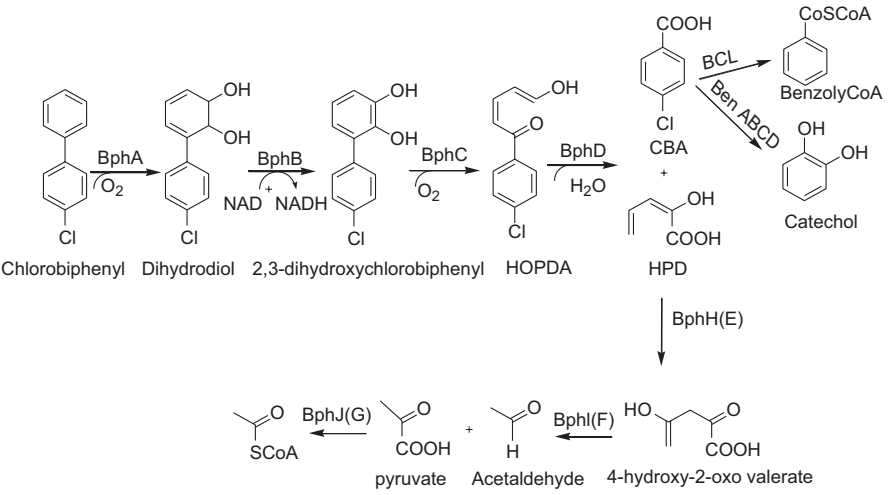

Fig. 8.4 Aerobic PCB biodegradation pathway

8.7.1.1 The Biphenyl Upper Pathway

The upper pathway involves four steps in the transformation of chlorobiphenyls to chlorobenzoic acid. The process is catalysed by four bph enzymes, namely, biphenyl 2,3-dioxygenases (bphA), *cis*-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenases(bphB), 2,3-dihydroxybiphenyl-1,2-dioxygenases (bphC) and 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA)hydrolases (bphD). The first step of the PCB metabolic pathway involves the conversion of chlorobiphenyl to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (dihydrodiol) by a multicomponent biphenyl dioxygenase (bphA). The enzyme bphA consists of large and small subunits (bphA1–biphenyl dioxygenase large subunit, bphA2–biphenyl dioxygenase small subunit, bphA3 – ferredoxin component and bphA4–ferredoxin reductase. The addition of molecular oxygen at the 2,3-position of the chlorinated ring transforms biphenyl to *cis*-hydrodiols.

The dehydrogenation of dihydrodiol (chlorinated) to 2,3-dihydroxybiphenyl (chlorinated) is catalyzed by the enzyme bphB. The ring cleavage step is catalysed by bphC resulting in the formation of the meta cleavage product HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid). The final step involves the transformation of HOPDA to chlorobenzoic acid (CBA) and 2-hydroxypenta-2,4-dienoate (HPD).

8.7.1.2 The Biphenyl Lower Pathway

The final products of upper pathway HPD and CBA are further metabolised in the lower pathway. HPD, a five-carbon aliphatic compound, is transformed by 2-hydroxypenta-2,4-dienoate hydratase (bphH(E)) to 4-hydroxy-2-oxo-valerate, which is further converted to acetaldehyde to pyruvic acid by 4-hydroxy-2-oxovalerate aldolase. An acylating acetaldehyde dehydrogenase (bphJ(G)) converts acetaldehyde to acetyl-CoA, which then can enter the Krebs (TCA) cycle. CBA is also transformed to benzoyl-CoA by benzoate-CoA ligase (BCL). The benzoyl-CoA is further mineralised to 2,3-dihydroxydihydrobenzoyl-CoA by benzoyl-CoA dioxygenase. This is followed by non-oxygenolytic cleavage of the aromatic ring and the beta oxidation-like pathway of the ring cleavage product. This pathway produces 3-hydroxyadipyl CoA and 3-ketoadipyl CoA, and then finally is converted to succinyl-CoA and acetyl-CoA. CBA could also be converted to catechol by the enzymes benzoate 1,2-dioxygenase and benzoate dihydrodiol dehydrogenase.

8.7.2 Anaerobic PCB Degradation Pathway

The anaerobic degradation of PCB involves the reductive dehalogenase enzyme resulting in the removal of chlorine, which is based on positions like meta, para, ortho and doubly flanked, singly flanked and unflanked single chlorine. But still, the proper pathway for anaerobic dechlorination of PCB was not elucidated

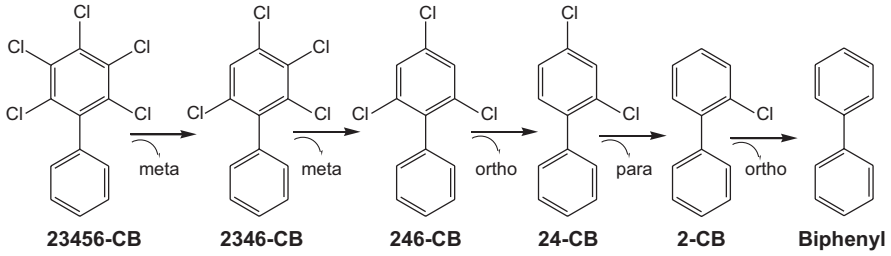

Fig. 8.5 Anaerobic PCB biodegradation pathway

(Bedard et al. 2006). The proposed pathway of the dechlorination of 2,3,4,5,6-pentachlorobiphenyl dechlorinated to 2-chlorobiphenyl by the removal of chlorine atoms from all the three positions of the biphenyl ring was elucidated by M. R. Natarajan et al. (1999). Fennell et al. (2004) described dechlorination of double-flanked chlorines of 2,3,4,5,6-pentachlorobiphenyl by *Dehalococcoides ethenogenes* strain 195.

A sequential dechlorination 2,3,4,5,6-chlorobiphenyl pathway proceeds as follows (Fig. 8.5): 2,3,4,5,6-chlorobiphenyl to 2,3,4,6-chlorobiphenyl (one meta chlorine removed) → 2,3,4,6-chlorobiphenyl to 2,4,6-chlorobiphenyl (one meta chlorine removed) → 2,4,6-chlorobiphenyl to 2,4-chlorobiphenyl (one ortho chlorine removed) → 2,4-chlorobiphenyl to 2-chlorobiphenyl (one para chlorine removed) → 2-chlorobiphenyl to biphenyl (one ortho chlorine removed).

8.8 Conclusion and Future Outlook

PCBs were first synthesized and introduced to the market in the late 1920s. Due to their stability at high temperatures and other harsh environmental conditions, they were widely used in various industries. But this same excellent stability began haunting humankind with its severe negative effects and recalcitrant nature. PCBs were reported to be toxic to the immune system and, as highlighted by EPA, to be a probable human carcinogenic agent, one which could also cause tumors. The main source of exposure to PCBs is either working in an agricultural or at an industrial site already exposed to PCBs, or by consuming food contaminated with it (especially fatty food and meat). Due to those issues, industrial production and usage of PCBs was stopped all over the world, and the EPA phased out PCBs starting in 1979; since 1984, their use has been banned. Several bacteria and fungi are reported to degrade PCBs under aerobic and/or anaerobic conditions. Those microbes produce a specific set of enzymes capable of almost completely degrading PCBs. Although due to its complex structure and very low solubility in water, biodegradation rates and efficiency in large-scale applications are still limited. Further extensive research is still needed to enhance the biodegradation efficiency of PCB microbes and enzymatic degradation using novel technologies to make it work on a large scale with better economics.

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Chapter 9
Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs): Current Practices and Outlook

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are active members of the group of multi-aromatic organic compounds, considered to be the most ubiquitous environmental pollutants, mainly engendered from partial combustion of wood, coal, oil or other organic materials. Currently, more than 500 PAHs are prevalent in the atmosphere; reactions between PAHs and various chemicals such as ozone, sulfur dioxide and nitrogen oxides lead to the formation of more toxic chemicals such as diones, nitro- and dinitro-PAHs and sulfonic acids. Due to high global concern, studies are being carried out by researchers to remove PAHs in an eco-friendly and cost-effective manner. Biodegradation of PAHs is a widely used strategy in which diverse types of bacterial, fungal, algal, earthworms, protozoans, plant species and their derived compounds such as biocatalysts, and biosurfactants are being used. Though the microbial degradation of PAHs has been extensively explored, it is a quite progressive area with many research findings being added to the literature. This chapter focuses on a critical overview of current knowledge around the biodegradation of PAHs. It also discusses the recent advancement including 'omics' approaches in bioremediation techniques to illuminate fundamental challenges and future prospects.

Keywords Bioremediation · Polycyclic aromatic hydrocarbons (PAHs) · Bacteria · Fungi · Algae · Omics

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9.1 Introduction

Soil contamination through industrial and agricultural activities is the main cause of environmental and health-related issues. Prevalent soil or water contaminants include petroleum hydrocarbons (PHCs), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, chlorophenols, heavy metals, metals and salt. An increase of various pollutants in the soil or water, contributing to detrimental effect on human and environmental health, is well documented. PAHs are aromatic organic compounds containing two or more fused aromatic rings, specifically benzene rings, in linear, angular or cluster arrangements (Latimer and Zheng 2003; Baklanov et al. 2006). These compounds are ubiquitously distributed in the environment, persistent with various structures; they bear toxic, carcinogenic and mutagenic properties (Arey and Atkinson 2003; Di Toro et al. 2000; Gupta et al. 2015).

9.1.1 Characteristics of PAHs

Polycyclic (or polynuclear) aromatic hydrocarbons (PAHs) fall under the category of persistent organic hydrocarbons that result when two or more aromatic rings fuse together. The benzene ring usually contains only carbon (C) and hydrogen (H) atoms, the latter of which may readily substitute nitrogen (N), sulfur (S) and oxygen (O) atoms to form heterocyclic aromatic compounds. They are mostly colourless to white or pale-yellow solids. The water solubility of PAHs decreases with each additional aromatic ring. PAHs are high melting and boiling solids with low vapour pressure (Arey and Atkinson 2003; Di Toro et al. 2000; Phillips 1999). These properties decrease with increasing molecular weight; as a result, the resistance property towards oxidation and reduction also increases. PAHs are hydrophobic in nature, making them very-low solubility in aqueous medium and high persistence in soil.

9.1.2 Environmental Distribution and Toxicity of PAHs

PAHs are one of a series of the most hazardous pollutants released into the environment primarily during the incomplete combustion and pyrolysis of organic materials throughout natural (biogenic and geochemical) and anthropogenic activities. Natural sources of PAHs generation are forest and rangeland fires, oil seeps, volcanic eruptions and exudates from trees. Most remarkable anthropogenic sources of PAHs are the ones that include burning of fossil fuels, coal tar, wood, garbage, used lubricating oil and oil filters, electric power generation, home heating, municipal solid waste incineration and petroleum spill and discharge (Di Toro et al. 2000;

Haritash and Kaushik 2009). Other anthropogenic activities that release PAHs include internal combustion engines (driving), agricultural burning, roofing or working with coal tar products, sound- and waterproofing, coating pipes, steelmaking and paving with asphalt.

Wide distribution of PAHs from anthropogenic and environmental sources results in its bioaccumulation in food chains at various trophic levels. However, their accumulation in foods is substantial, depending on the mode of handling, and is detected in a wide range of vegetables, fruits, meats and fishes (Phillips 1999). PAHs can be easily detected in their forms and their metabolites in blood and urine, mutagenicity in urine and faeces, chromosome aberrations in peripheral blood lymphocytes and DNA, and protein adduct formation in the latter and in other tissues (Baird et al. 2005). Severe hematological disorders have been noticed in animals when they are being exposed to elevated concentration of PAHs. Aplastic anaemia, pancytopenia, decrease in peripheral blood leukocytes and bone marrow depression with almost complete destruction of pluripotent haematopoietic stem cells were observed in non-responsive mice after oral administration of benzo[a] pyrene (BaP), while responsive mice were shown resistance to bone marrow toxicity (Novosad et al. 2002; Page et al. 2004).

PAHs are in a priority pollutant list of the U.S. Environmental Protection Agency (USEPA) and the European Community (EC) (Anyakora et al. 2005). On the basis of abundance and toxicity, USEPA has listed 16 PAH compounds as priority environmental pollutants in water, soil and sediments (Liu et al. 2001).

Several physical, chemical and biological methods are currently available for the remediation of PAHs in water/soils (Abdel-Shafy and Mansour 2016; Kuppusamy et al. 2017). Biological approaches are considered the most efficient and low-cost technique for total removal of PAHs. In biological processes, known collectively as bioremediation, microbes and their combinations are being used for the degradation of hazardous chemicals present in soil, water and/or other contaminated sites (Wang et al. 2014). PAHs-contaminated sites have been remediated in several countries for many years using biological methods. Over the past decade, several reviews and book chapters (Abdel-Shafy and Mansour 2016; Aitken and Long 2004; Haritash and Kaushik 2009; Kuppusamy et al. 2017; Morelli et al. 2013) have focused on potential biological remediation strategies and their distinct metabolic pathways for the degradation of PAHs. This chapter focuses on current knowledge around various technologies such as microbial remediation (bacteria and fungi), phyco (algae) remediation and phyto (plant)-remediation including recent advancements of PAHs bioremediation on bench-, pilot- and field-scale. Further, it also discusses genetic, transcriptomic, proteomic and metabolomic approaches in the field of PAHs bioremediation.

9.2 Biological Remediation Technologies

Bioremediation is the emerging application process of biological means (including bacteria, fungi, algae, plants, etc.) for cleaning up contaminated soil, groundwater and wastewater that contains a mixture of contaminant types including salts, organic compounds, radionuclides and heavy metals at widely varying concentrations. It is a cost-effective, alternative pollutant removal method for the degradation or/and transformation (mineralisation) of contaminants without deleterious effects on the environment. Bioremediation technologies hasten biodegradation processes originating principally from biodegradation—a natural process that takes place without human intervention (Congress 1991). In the process of biodegradation, complex organic contaminants may have completely degraded through biotransformation into less complex metabolites and through mineralisation into inorganic materials, water, carbon dioxide (aerobic) or methane (anaerobic) (Das and Chandran 2011; Dean 1999) by the metabolic activity of microbes. Nevertheless, the microbial activity can be affected by many factors such as temperature, oxygen, pH, water, bioavailability of PAHs, salinity, toxicity of endproducts, microbial community and nutrients; these are believed to play a significant role in the performance process (Abdel-Shafy and Mansour 2016; Kuppusamy et al. 2017). There are several biological remediation techniques (bioremediation; bacteria and fungi, phycoremediation; algae, phytoremediation; plants and rhizoremediation; plant and microbe) for the treatment of PAHs-contaminated soil (Fig. 9.1). Based on the selection of the proper remediation approach, these remediation techniques are carried out by two basic types: (i) in-situ (land farming, biostimulation, bioaugmentation, composting and phytoremediation) and (ii) ex-situ (bioreactors) (Kuppusamy et al. 2017).

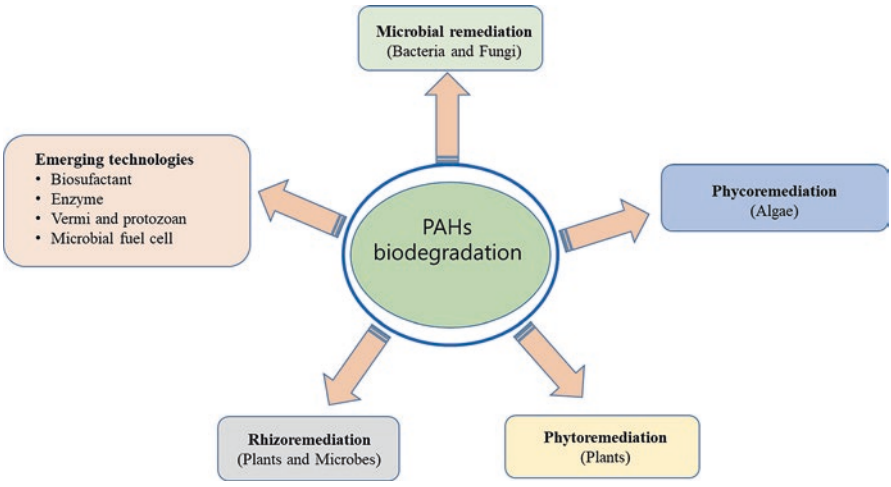

Fig. 9.1 Schematic diagram summarising different biological methods for PAHs degradation

9.2.1 In-Situ Remediation

This method involves an on-site remediation process where the pollutant is spread vertically and horizontally. In such a method, excavation and transport of the contaminated soil is not required to go to off-site treatment facilities and is therefore considered less expensive. However, the status of weather, soil permeability, contamination depth and potential deep leaching of chemicals, moisture content, nutrient availability, pH and temperature are amongst the critical environmental conditions that must be carefully considered for success with in-situ bioremediation (Gupta et al. 2016). In-situ remediation methods involve cleaning the PAHs contamination site mainly in four ways: (i) land farming (ii) biostimulation, (iii) bioaugmentation and (iv) bioventing. *Land farming* is a process in which contaminated soils are mixed with fertilizer, then tilled and irrigated to provide aeration, moisture and promote soil homogeneity for biodegradation; and to stimulate indigenous microbes (to enhance natural attenuation process) (Hansen et al. 2004; Juhasz and Naidu 2000; Wang et al. 2016). *Biostimulation* involves the application of nutrients or substrates to stimulate the growth and metabolic rate of indigenous microorganisms to substantially improve the degradation process (Sayara et al. 2011). *Bioaugmentation* is the addition of laboratory cultured indigenous or exogenous biodegraders (bacteria, fungi or algae) into contaminated soils to stimulate the degradation of a contaminant in the soil (Sayara et al. 2011; Vidali 2001). *Bioventing* is a combined process of bioaugmentation and biostimulation. This technique was successfully used to treat organic pollutants such as PAHs in soils and aquifers (Kuppusamy et al. 2016).

9.2.2 Ex-Situ Remediation

These methods involve a transfer of pollutants from contaminated sites to an off-site for the treatment purpose. Although these processes incur extra costs for soil excavation, transport, treatment, disposal and site refilling, these treatments can be precise and controlled, and as a result attaining good outcomes in significantly less time. Bioreactors are some of the examples of ex situ treatment methods (Mohan et al. 2006) used for PAHs remediation.

9.3 Bacteria Degradation of PAHs

Bacteria are known to be involved in the degradation of PAHs in contaminated sites. Many studies on successful applications of different bacterial strains, individually or as consortia, isolated from soil or sediments have been done in the treatment of PAHs-contaminated soils. A group of microbes (consortia) have diverse enzymatic

activities, which can degrade more complex organic pollutants than their individual applications. The principal metabolic pathways, those of degradation-related enzymes and catabolic genes of bacteria for the degradation and metabolism of PAH, have been widely studied (Haritash and Kaushik 2009; Nzila 2018; Peng Jing-Jingwang Ningli 2011). In nature, bacteria degrade PAH contaminants by aerobic and anaerobic processes (Qin et al. 2018). Aerobic degradation of PAHs have been extensively studied in various environments (soil, sea, sediments and bioreactor), but the ability of bacteria to degrade PAHs under strictly anaerobic conditions is limited to a few strains (Nzila 2018). Many polluted environments such as aquifers, sediments and submerged soils are often of lead anoxic condition, where anaerobic bacteria can play a key role in PAHs biodegradation (Callaghan 2013; Lu et al. 2011; Nzila 2018).

Several studies have reported using bacteria as pure culture or consortium for the degradation of PAHs. The most commonly reported PAHs-degraded bacterial genera are *Arthrobacter*, *Bacillus*, *Stenotrophomaonas*, *Vibrio*, *Corynebacterium*, *Flavobacterium*, *Marinobacter*, *Micrococcus*, *Nocardia*, *Ochrobactrum*, *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Mycobacterium* and *Sphingomonas* (Kumari et al. 2018; Singh and Tiwary 2017; Varjani and Upasani 2016).

Tarafdar et al. (2017) reported the degradation of anthracene by *Bacillus thuringiensis* strain isolated from the fly ash deposition. Eskandari et al. (2017) identified two bacterial species *B. licheniformis* and *B. mojavensis* among studied oil-contaminated soil microorganisms that are able to remove a mixture of PAHs. The strain *B. licheniformis* was capable of destroying cenaphtylene, acenaphtene and indeno pyrene in 72, 96 and 96 h, respectively while the species *B. mojavensis* was able to destroy naphthalene in 72 h and acenaphtene, acenaphtylene, benzo(ghi)prylene, dibenzo(ah)anthracene and indeno pyrene in 96 h.

Jauhari et al. (2018) studied three bacterial strains, *Pseudomonas aeruginosa*, *Cronobacter* sp. and *Rhodococcus* sp., isolated from petroleum-contaminated soil with very high efficiency (94, 84 and 78%, respectively) for degradation of anthracene (1000 mg/L) over 10 day growth periods. It was confirmed that anthracene gets mineralised by these strains through the *O*-phthalate pathway with specific activities of catabolic enzymes like C120, C230, 3,4-PCD and 4,5-PCD. However, aromatic rings of anthracene cleaved by *Rhodococcus* sp. mainly through meta-cleavage pathway were also confirmed. Recently, a novel species of the genus *Gordonia,* isolated from oilfield-produced water, utilized naphthalene and pyrene as its sole carbon source and was degraded with mixed naphthalene, phenanthrene, anthracene and pyrene (each at a concentration of 500 mg/L) in ratios of 100, 95.4, 73.8% and 53.4%, respectively, over 7 days (Qi et al. 2017).

Kamil and Talib (2016) reported potential of a *Corynebacterium urealyticum* in degrading artificially contaminated phenanthrene soil at different concentrations. A bacterial community from Actinobacteria, Firmicutes, a- and g-Proteobacteria, and Bacteroidetes isolated from the tidal flats near Sinduri Beach in Taean, Korea, able to degrade PAHs has been demonstrated by Lee et al. (2018). A microbial consortium can improve PAH degradation due to their synergetic coordinated metabolic activities (Tauler et al. 2016). Pugazhendi et al. (2017) studied the application of

bacterial consortium for degradation of high molecular weight PAH compounds present in crude oil. Kumari et al. (2018) investigated the ability of a consortium, *Stenotrophomonas maltophilia*, *Ochrobactrum anthropi*, *Pseudomonas mendocina*, *Microbacterium esteraromaticum* and *Pseudomonas aeruginosa* for biodegradation of multiple PAHs. It was found that a consortium of these bacteria showed enhanced biodegradation of naphthalene (89.1%), fluorine (63.8%), phenanthrene (81%) and benzo(b)fluoranthene (72.8%) compared to their individual bacterial activity.

Many natural habitats such as aquifers, sediments and submerged soils contaminated with large amounts of PAHs are often lead anoxic where PAHs can degrade by anaerobic bacteria via anaerobic catabolism (Callaghan 2013; Lu et al. 2011). Researchers from a different group have reported the degradation of PAHs without oxygen by addition of alternative final electron acceptors such as nitrate, sulfate or ferric ions (Ambrosoli et al. 2005; Chang et al. 2008; Coates et al. 1996; Liang et al. 2014). Qin et al. (2018) isolated a novel strain *Cellulosimicrobium cellulans* CWS2 from PAHs-contaminated soil, capable of utilizing BaP (2.5–50 mg/L) as the sole carbon and energy source under nitrate-reducing conditions (1 g/L of $NaNO_3$). The capacity of CWS2 on the removal of BaP was 78.8% in 13 days when the initial BaP concentration was 10 mg/L.

Li et al. (2010) demonstrated the use of $NaHCO_3$ (20 mM) for the anaerobic biodegradation of four mixed PAHs (10 mg/kg of sediment; fluorene, phenanthrene, fluoranthene and pyrene) in mangrove sediment with enriched PAHs-degrading bacterial consortium. PAH degradation was increased by consortium compared to without consortium in medium. However, no effect of $NaHCO_3$ was observed in the biodegradation of PAHs, leading to the conclusion that the presence of other terminal electron acceptors such as nitrate and sulfate or CO_2 produced by anaerobes might be utilised by consortium. Marozava et al. (2018) initiated an anaerobic degradation of 1-methylnaphthalene by a member of the *Thermoanaerobacteraceae* in an iron-reducing enrichment culture. Zafra et al. (2016) investigated the two microbial (bacteria and fungi) mixed consortia with the ability to degrade the phenanthrene (92%), pyrene (64%) and BaP (65%) in the PAHs-polluted soils. Some cyanobacteria can also break down PAHs in water environments. Ibraheem (2010) reported five cyanobacterial species *Phormidium*, *Anabaena*, *Nostoc*, *Aphanothece conferta*, and *Synechocystis aquatilis* could enable degradation of phenanthrene by 12% (on day 60), 51% (on day 40), 43% (on day 60), 40% (on day 40) and 46% (on day 40) (Ibraheem 2010).

9.4 Fungal Degradation of PAHs

Fungi are potential candidates for degradation of PAHs due to their certain advantage over bacteria with respect to resistance to different environmental conditions, ability to grow on a variety of media and to produce enzymes for the degrading of organic pollutants (Harms et al. 2011; Messias et al. 2009; Venkatesagowda et al. 2012). Unlike bacteria, few fungi can only utilize PAHs as a sole source of carbon

and energy, and co-metabolise them into a wide variety of detoxified-oxidised metabolites (Bamforth and Singleton 2005; Hadibarata et al. 2012; Hadibarata and Kristanti 2012; Wu et al. 2010). Several groups of fungi, namely, *Phanerochaete*, *Pleurotus*, *Trametes*, *Bjerkandera*, *Chrysosporium*, *Cunninghamella*, *Coriolopsis*, *Aspergillus*, *Penicillium*, *Trichoderma*, *Mucor* and *Cladosporium*, were found to be potential degraders of PAHs (Aydin et al. 2017; Gupta et al. 2017; Kadri et al. 2017; Marco-Urrea et al. 2015). However, many fungal species with undefined potential for remediation of PAHs remain in nature, still to be identified. Fungal metabolism of PAHs is usually mediated by ligninolytic and non-ligninolytic enzymes (Gupta et al. 2016; Marco-Urrea et al. 2015). Nevertheless, many fungi can produce both types of enzymes, but one cannot rule out what levels of each enzyme secreted and contributed to the breakdown the PAH (Chigu et al. 2010; Marco-Urrea et al. 2015; Ning et al. 2010; Wu et al. 2010).

9.4.1 Ligninolytic Fungi

Most white-rot fungi are ligninolytic, producing extracellular ligninolytic enzymes such as lignin peroxidase, manganese-dependent peroxidase, phenol oxidase (laccases and tyrosinases) and H_2O_2-producing enzymes to oxidise lignin present in wood and other organic matter (Mester and Tien 2000). These ligninolytic enzymes have been proven to degrade PAHs (Kadri et al. 2017; Lee et al. 2014). PAH biodegradation by ligninolytic white-rot basidiomycete fungi has been studied mostly in *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *T. versicolor* and *Bjerkandera adusta P. chrysosporium* has been shown to degrade the pyrene, anthracene, phenanthrene, benz[a]anthracene and BaP in various levels in soils and solutions (Bishnoi et al. 2008; Haemmerli et al. 1986; Kadri et al. 2017; Wang et al. 2014; Wang et al. 2009). Andersson et al. (2003) assessed the efficacy of *Pleurotus ostreatus* and *Antrodia vaillantii* to degrade several PAHs in the artificially contaminated soil. *P. ostreatus* strains were *employed* for biodegradation of different PAHs in lab experiments (Dubrovskaya et al. 2017; Pozdnyakova et al. 2018), soil (Márquez-Rocha et al. 2000) and cultivation substrates in a pilot plant (Di Gregorio et al. 2016). Phenanthrene-degrading fungus *T. versicolor*, isolated in Korea, was able to remove 76% of phenanthrene from the fungal culture (Han et al. 2004). Moreira et al. (2000) reported the ability of three fungi, *Bjerkandera adusta*, *Irpex lacteus* and *Lentinus tigrinus*, to degrade phenanthrene, fluoranthene, pyrene and chrysene in forest and salt marsh soils. In a study by Valentín et al. (2006), biodegradation of four different PAHs by the white-rot fungus *Bjerkandera adusta* in spiked marsh soil in a bioreactor was around 30 mg PAH/kg soil. Juhasz and Naidu (2000) reported the capability of *Bjerkandera* sp. in degradation of BaP and benzo(a)anthracene.

Hidayat and Yanto (2018) isolated a new tropical fungus, *Trametes hirsuta*, shown to degrade 0.8, 0.17 and 0.46% of phenanthrene, chrysene and BaP per mg dry weight of biomass, respectively. Agrawal and Shahi (2017) isolated a white-rot

fungus *Coriolopsis byrsina* from the Surguja district of Chhattisgarh, India, and were able to degrade 96.1% of pyrene in mineral salt broth and 51.85% in soil. Vieira et al. (2018) observed an efficient degradation of pyrene (100%) by marine-derived basidiomycete fungus *Marasmiellus* sp. under saline conditions. It was reported that *Ganoderma lucidum* was capable of degrading 99.65% of phenanthrene and 99.58% of pyrene in mineral salt broth (Agrawal et al. 2018). Pozdnyakova et al. (2018) studied the ability of *Pleurotus ostreatus* and *Agaricus bisporus* for mineralisation of phenanthrene and anthracene in the presence of laccase and versatile peroxidase enzymes.

9.4.2 Non-ligninolytic Fungi

Non-ligninolytic fungi metabolize PAHs mostly mediated by Phase I and Phase II detoxification metabolism by cytochrome P450 monooxygenase and epoxide hydrolase-catalyzed reactions (Aydin et al. 2017; Kadri et al. 2017; Marco-Urrea et al. 2015). However, extracellular enzymes such as laccase were also produced by some of these fungi (Aydin et al. 2017; Kadri et al. 2017; Marco-Urrea et al. 2015). Non-ligninolytic fungi for PAH degradation include strain(s) of *Aspergillus*, *Cladosporium*, *Cunninghamella*, *Fusarium*, *Mucor*, *Penicillium*, and *Trichoderma* (Bamforth and Singleton 2005; Marco-Urrea et al. 2015). Biotransformation of fluoranthene by *Cunninghamella elegans* in a bioreactor under biofilm-based and niche-mimicking conditions was demonstrated (Mitra et al. 2013).

Saraswathy and Hallberg (2005) reported a *Penicillium ochrochloron*, which was found effective in degrading pyrene. The strain was able to degrade a maximum of 75% of 50 mg/L pyrene over 28 days of incubation. Moreover, *Penicillium* sp. isolated from Antarctic soil was able to degrade acenaphthene (10.0%) and BaP (2.0%) under low-temperature conditions (at 20 °C) (Govarthanan et al. 2017). Liu et al. (2010) showed that *Penicillium* sp., *Aspergillus niger* and white-rot fungus could remove 48, 58 and 16% of BaP, respectively, in a sterile, artificially polluted soil after 35 days. Potin et al. (2004) performed degradation studies in a PAHs-contaminated soil with two inoculation treatments by spore or mycelial inoculum of 21 native filamentous fungi. However, the extent of total PAH degradation occurred with *Coniothyrium* sp. and *Fusarium* sp. mycelial inoculum.

Hesham et al. (2017) demonstrated that *Fusarium Solani* could degrade naphthalene (84.82%), phenanthrene (40.09%), chrysene (57.84%) and BaP (71.06%) at the end of 10 days. Zafra et al. (2015) found that *Trichoderma asperellum* was able to degrade phenanthrene (74%), pyrene (63%), and BaP (81%) at a concentration of 1000 mg/kg in 14 days. Moreover, *Trichoderma* genus was found to utilize pyrene, resulting in its degradation, and it was further enhanced using an alternative carbon source such as yeast extract, sucrose or lactose (Mineki et al. 2015). Mao and Guan (2016) reported a fungus *Scopulariopsis brevicaulis* isolated from an aged PAHs-contaminated soil, having the ability to degrade phenanthrene (60%), fluoranthene (62%), pyrene (64%) and BaP (82%) over 30 days incubation with PZ-4 strain. In

microcosm studies for 28 days, 77% of total PAHs was removed from the soil, and the highest removal was observed for phenanthrene (89%) and BaP (75%).

Birolli et al. (2018) demonstrated the degradation of anthracene (14 days, 50 mg/mL initial concentration in malt 2% medium) by five marine-derived fungi, *Trichoderma harzianum* CBMAI 1677, *Cladosporium* sp. CBMAI 1237, *A. sydowii* CBMAI 935, *Penicillium citrinum* CBMAI 1186 or *Mucor racemosus* CBMAI 847). Among them, *Cladosporium* sp. CBMAI 1237 showed the highest degradation of anthracene and 16% more in the presence than in the absence of artificial seawater. Further experiments with different PAHs (50 mg/L) in malt for 21 days resulted in *Cladosporium* sp. CBMAI 1237 biodegrading anthracene 71%, anthrone 100%, anthraquinone 32%, acenaphthene 78%, fluorene 70%, phenanthrene 47%, fluoranthene 52%, pyrene 62% and nitropyrene 64%. In another study, *M. racemosus* and *T. harzianum* strains were reported for having potential biodegradation activity of anthracene (Jové et al. 2016). de Lima Souza et al. (2017) isolated *Hypoxylon* sp. from sediments contaminated with various levels of PAHs and showed the lowest growth of inhibition rates over the other six species tested for tolerance to phenanthrene and pyrene.

Fungi have been shown to efficiently degrade PAHs by producing ligninolytic enzymes under anaerobic conditions (Aydin et al. 2017). Silva et al. (2009) studied the soil fungi which could remediate PAHs and produce ligninolytic enzymes under microaerobic and very low-oxygen (<1%) conditions. In this study, naphthalene (46.5%), phenanthrene (25.1%), perylene (37%) and decacyclene (37.7%) by *Aspergillus* sp., chrysene (40.9%) by *Trichocladium canadense* and naphthol[2,3-a] pyrene (40.8%) by *Verticillium* sp. were degraded under microaerobic conditions. Under very low oxygen, *T. canadense* removed 22.1% of naphthalene, 9.8% of chrysene, 18.8% of decacyclene; *F. oxysporum* removed 13.2% of phenanthrene; *Achremonium* sp. removed 20.5% of perylene; and Basidiomycete strain H2 and *Verticillium* sp. removed 12.5% of naphthol[2,3-a]-pyrene. Cobas et al. (2013) studied phenanthrene remediation both in an aqueous medium (90% after 14 d) and in soil (70% after 28 d) using *Trichoderma longibrachiatum*-mediated permeable reactive biobarriers (PRBBs).

9.5 Microalgae-Mediated Degradation of PAHs

PAHs are subject to removal or degradation by a range of naturally occurring microorganisms, but research has focused on the removal of these contaminants using bacteria and fungi, and microalgae have been explored to a limited extent. However, particular attention should be paid to microalgae as they play an important role in wastewater treatment processes and biodegradation of toxic organic pollutants. Multiple microalgae help in the degradation of PAHs. Hong et al. (2008) assessed the accumulation and biodegradation of phenanthrene and fluoranthene, by the diatoms *Skeletonema costatum* (Greville) Cleve and *Nitzschia* sp., enriched from a mangrove aquatic ecosystem. In a another study, the removal and transformation of seven high-molecular weight PAHs, namely, benz[a]anthracene, benzo[b]

fluoranthene, benzo[k]fluoranthene, BaP, dibenzo[a,h]anthracene, indeno[1,2,3-c,d]pyrene and benzo[g,h,i]perylene, by a freshwater microalga *Selenastrum capricornutum* under gold and white light irradiation was studied. A PAH compound-dependent removal efficiency was observed (Luo et al. 2014).

Warshawsky et al. (2007) studied the combination of *Mycobacterium* sp. and *Sphingomonas yanoikuyae* and a green alga *Selanastrum capricornutum* on the degradation of BaP to various intermediates. The rate of degradation was greater in bacterial cultures grown with the algal/BaP extract than those grown with BaP alone. Takáčová et al. (2014) reported the biodegradation efficiency for BaP by *Chlorella kessleri*. In a separate study, sorption and degradation of BaP was determined by two microalgal species, *Selenastrum capricornutum* and *Scenedesmus acutus* (García de Llasera et al. 2016). It was found that that the removal rate of BaP was 99% by *S. capricornutum* after 15 h exposure, whereas it was 95% by *S. acutus* after 72 h exposure. Biodegradation efficiency for the anthracene and pyrene was determined by *Anabaena fertilissima* (Patel et al. 2015). In another study, a microalga *Chlorella* sp. was capable of degrading 70% of 50 μM pyrene in 7 days incubation (Subashchandrabose et al. 2017). Further, the rate of degradation was nearly 20% increased with use of Tween-80 when compared with Triton X-100.

9.6 Phytoremediation of PAHs

Phytoremediation is the use of a variety of plants to degrade, extract and immobilize contaminants considered capable of eliminating PAHs from the soil. Based on processes and applicability, there are various categories like phytoextraction, phytovolatilisation, phytofiltration, phytostabilisation, phytotransformation and rhizoremediation. Phytotransformation, or phytodegradation, and rhizoremediation are two primary mechanisms involved in the degradation of the organic compounds into simple molecules in the soil or plant tissue. However, some studies reported that the accumulation of PAHs by plants is associated with a phytoextraction process (Alagić et al. 2015; Košnář et al. 2018; Sivaram et al. 2018) . In phytodegradation process, plant roots exude various enzymes such as dehalogenase and oxygenase, which can directly degrade organic contaminants (Dubrovskaya et al. 2017).

Various plant species such as fescue grass (*Festuca arundinacea*), switch grass (*Panicum virgatum*), maize (*Zea mays* L.), soybean (*Glycine max* L.), Sorghum (*Sorghum bicolor* L. Moench) and alfalfa (*Medicago sativa* L.) have the ability to phytodegrade the PAHs. Guo et al. (2017) examined the influence of maize root and soybean root exudates on pyrene degradation. D'Orazio et al. (2013) reported a pot culture study on three plant species for pyrene degradation. The results showed that the amount of pyrene in soils decreased by 32, 30 and 28%, with *Medicago sativa*, *Brassica napus*, and *Lolium perenne* , respectively, and 18% in the control soil without plants. Košnář et al. (2018) showed the removal of 16 individual PAHs using maize from ash-treated soil ranged between 4.8 and 87.8% in a 120-day pot experiment. The phytoremediation potential of Fire Phoenix on the degradation of

8 PAHs was investigated (Liu et al. 2014b). This study demonstrated that plants have a prominent role in enhancing the degradation of 8 PAHs with an increase in planting time. After a 150-day culture growth, the concentrations of the 8 PAHs by Fire Phoenix were decreased to 96.18%. Sivaram et al. (2018) studied bioaccumulation and biodegradation ability of 14 plant species grown in soils spiked with BaP and pyrene in a glasshouse. The rate of PAHs removal efficacy of BaP and pyrene were varied among the plant species over a period of 50 days for BaP (6–26%) and pyrene (14–40%) and the maximum removal of both PAHs was observed in Sudan grass (C4), vetiver (C4), maize (C4) and sunflower (C3).

9.7 Rhizoremediation of PAHs

Rhizoremediation is a combined process of micro- and phytoremediation, considered as a promising technology because it offers ecological and natural aesthetic benefits at low cost. Rhizodegradation can also be referred as plant-based bioremediation technology, rhizosphere bioremediation or microbe-assisted phytoremediation technology (MAPT). Microbe-assisted phytoremediation has been used for removal of PAHs contaminants synergistically. Rhizodegradation of polluted soils mainly relies on stimulating the population of degrading microorganisms through plant rhizospheric effects (Merkl et al. 2004; Newman and Reynolds 2004; Truu et al. 2015). Many studies have attempted rhizoremediation of PAHs-polluted soils by the combination of plants with bacteria, or/and fungi-assisted bioremediation; this combination has shown to enhance the rate of rhizosphere degradation of PAHs. Guo et al. (2017) investigated rhizoremediation of 12 PAHs in rhizosphere and non-rhizosphere soils using *Mycobacterium* sp. and ryegrass (*Lolium multiflorum* L.) under glasshouse conditions. After 60 days, the removal percentages of PAHs in the bacteria added rhizospheric soil were higher (5-ring PAHs, 25.6%; 6-ring PAHs, 27.6%; and 3- and 4-ring PAHs only 2.2–9.7%) compared to the non-inoculated soil.

Liu et al. (2014a) investigated a phenanthrene-degrading endophytic bacterium, *Massilia* sp., for their ability to promote plant growth and phenanthrene uptake as well as community structure of root endophytic bacteria in wheat. They indicated that *Massilia* sp. is able to reduce the phenanthrene levels in wheat, enhance biomass and change the bacterial community structure in phenanthrene-contaminated wheat in a contamination level-dependent manner. A study performed in the combination of Sorghum plant and *Pseudomonas aeruginosa* to improve phytoremediation of soil contaminated with pyrene showed that inoculated bacterium play a key role in pyrene removal (66–82% for pyrene concentrations of 150 and 300 mg/kg, respectively) from contaminated soil. The results of the current study also showed that *P. aeruginosa* inoculation increased pyrene removal rate and number of bacteria in the soil (Rostami et al. 2017).

Biotransformation and conjugation of phenanthrene in pak choi at the subcellular level by endophytic bacteria *Pseudomonas* sp. was investigated (Sun et al. 2018). Inoculation of *Pseudomonas* sp. reduced the subcellular distribution of phenanthrene

to plant subcellular fractions (i.e., cell wall, cell membrane, cell solution and cell organelles). Eskandary et al. (2017) demonstrated the effective use of *B. licheniformis* and *B. mojavensis* isolated from oil-contaminated soils to ameliorate 10 PAHs in oil-contaminated soil grown with *Festuca arundinacea* under controlled greenhouse conditions. They stated that the concentration of some PAHs in rhizosphere soil samples inoculated with both bacteria was decreased, and even some PAHs (e.g., naphthalene, phenanthrene, benzo[a]anthracene and dibenzo[a,h]anthracene) reached below the detection limit of the method.

Košnář et al. (2018) observed a synergistic association of maize and ligninolytic fungus *P. ostreatus* in soil contaminated with 16 PAHs and wood chip of waste apple tree trunks as a lignocellulosic substrate for fungus. It was found that fungus in the treated soil improved fungal biomass, enzyme and microbial activities, and it removed total PAHs by 36.2% after the 120-day period.

Mycorrhizal fungi have also been known to promote plant growth and PAHs biodegradation and rhizoremediation (Gao et al. 2017; Ren et al. 2017). Ren et al. (2017) conducted a greenhouse experiment to evaluate the potential interaction between legume (*Sesbania cannabina*), rhizobia (*Ensifer* sp.) and AM fungus (*Glomus mosseae*) on PAHs dissipation in spiked soil. The triple symbiosis showed more than 97 and 85% degradation of phenanthrene and pyrene, respectively, in soil, whereas it showed 81 and 72% degradation in only plant-treated soil. In treatments where *Glomus etunicatum* or *Glomus lamellosum* inoculation were combined with alfalfa (*Medicago sativa* L.), the loss of pyrene was increased with 38.3–68.9% and 39.4–71.3% in soils, respectively (Gao et al. 2017).

9.8 Recent Advancements in PAHs Degradation

Recently, novel approaches such as combined organic composting, enzyme-mediated bioremediation, biosurfactant-enhanced bioremediation, and microbial fuel cells for the process of PAHs biodegradation have received particular attention.

9.8.1 Vermi- and Protozoan Degradation of PAHs

Many bioremediation studies have reported on bacteria, cyanobacteria, fungi and algae. However, only scant information is available on earthworm and protozoan, which are part of the aquatic and terrestrial community involved in PAHs degradation. Earthworms play a significant role in removal of PAHs from soil. They absorb PAHs from soil to biotransform or biodegrade them, rendering them harmless. Earthworms can accumulate PAHs, mainly the 3- or 4-ringed PAH compounds (Achten and Andersson 2015). In another study, Sinha et al. (2008) confirmed PAH removal from soil with 11,820 mg/kg PAH concentration by a consortium of earthworms (*E. fetida*, *E. euginae* and *Perionyx excavates*). Schmidt et al. (2017)

demonstrated that *E. fetida* was able to transform pyrene and phenanthrene to conjugated phase II metabolites in hydroponic culture. The results of Deng and Zeng (2017) demonstrated that the inoculation of alfalfa with earthworms and/or white-rot fungus prompted the growth of alfalfa and removed the phenanthrene in the soil, and the removal rate in soil was highest (93%) under alfalfa + earthworms + white-rot fungus treatment.

According to Kachieng'a and Momba (2018), protozoa are considered more active agents in PAHs degradation and could be potential candidates for remediation of PAHs-contaminated soil and water. They reported a consortium of symbiotic protozoan isolates (*Paramecium* sp., *Vorticella* sp., *Epistylis* sp. and *Opercularia* sp.) able to degrade approximately 70%, while individual isolates could degrade 65% at the end of the study.

9.8.2 Enzymes in PAHs Degradation

Enzymes are biocatalytic macromolecules that facilitate the conversion of substrates into products by lowering the activation energy of molecules. In the last few years, degradation of contaminants with enzymes separated from their cells has been used for PAHs bioremediation. At present, fungal enzymes are studied most for remediation of organic-contaminated soils. Wu et al. (2008) studied the direct application of free laccase enzyme for the transformation of 15 priority PAHs in soil. Similarly, Zhang et al. (2016) studied degradation of PAHs using purified manganese peroxidase form *Trametes* sp. and found it very efficient at degrading both individual PAHs (fluorene, fluoranthene, pyrene, phenanthrene and anthracene) and PAHs in mixtures. Although enzymatic remediation is an alternative to conventional bioremediation, the cost of purification and enzymes is the major constraints of this method. Immobilization of enzymes with various carrier materials allows an alternative technology that enables an increase in stability and repeat utilization and it reduces degradation. Wang et al. (2018) demonstrated that immobilised laccase could increase degradation of pyrene (Pyr) and BaP around 10–30% compared with free laccase. In the past few years, researchers have paid more attention to molecular docking in environmental remediation to find the suitable orientation of molecules in the enzyme active sites to predict binding affinity.

9.8.3 Combined Organic Addition for PAHs Degradation

Traditional bioremediation techniques combined with organic additives such as biochar, compost, sludge residue and poultry manure incorporation positively enhance overall PAHs remediation. Lignocellulosic materials such as wheat straw, corncobs and straw pellets as carriers have been shown to improve the growth capacity and soil PAH degradation performance of fungi (Covino et al. 2010).

Fernández-Luqueño et al. (2016) observed a rapid biodegradation rate of phenanthrene and anthracene in the soil amended with wastewater sludge. Kong et al. (2018) observed a significant reduction of PAH content when wheat straw biochars was applied to petroleum-polluted soil. Additionally, adding biochar to soils increased specific taxa, including PAH degraders. Han et al. (2017) reported that biomass wastes from wheat stalk, mushroom compost and cow manure accelerated the degradation of aged PAHs and significantly increased abundances of the bacterial community. Košnář et al. 2018 studied the combination of biostimulation and bioaugmentation showed superior removal efficiencies of phenanthrene, fluoranthene and pyrene. Phytoremediation of PAHs derived from biomass fly ash-soil using maize (*Zea mays* L.) amended with compost or vermicompost was studied. The results of this study showed that compost and vermicompost applied in planted ash-soil efficiently removed PAHs in a range between 62.9 and 64.9%, respectively.

9.8.4 Biosurfactant-Enhanced Degradation of PAHs

PAHs bioavailability in soil is usually low and can be overcome with the application of biosurfactants to enhance PAHs bioavailability for microbial degradation in soil/aqueous phase. Biosurfactants are amphiphilic secondary compounds (both hydrophobic and hydrophilic) derived from microorganisms and plants. Compared to chemical surfactants, biosurfactants are readily biodegradable and perform with excellence in the remediation process (Lamichhane et al. 2017; Liang et al. 2017). In general, two different types of biosurfactants (microbial-based and plant-based) have been widely used in surfactant-enhanced bioremediation (SEBR) and surfactant-enhanced phytoremediation (SEPR). A number of researchers have studied the remediation of PAHs-contaminated soil by various biosurfactants (Lamichhane et al. 2017; Liang et al. 2017).

The use of rhamnolipid biosurfactant at low concentration (25 mg/L) increased (96.2%) the degradation of fluorene (280 mg/L) by *Paenibacillus* sp., but increased it only 90.6% with Tween-40 (3% v/v) and 96.5% with Tween-60 (3.5% v/v) ((Reddy et al. 2018). (Bezza and Chirwa 2017), recognised the degradation enhancement potential of pyrene by lipopeptide biosurfactant produced by *Paenibacillus dendritiformis* and found that lipopeptide at 600 and 300 mg/L enhanced pyrene degradation to 67.5 and 51%, respectively, compared to its absence.

PAHs biodegradation was 86.5% in the presence of biosurfactant, which was dramatically higher.

Moreover, the addition of 0.2 and 0.6% biosurfactant enhanced the removal of 13 PAHs by 34.2 and 63%, respectively, from only 6% without surfactant (Bezza and Chirwa 2017). Liao et al. (2015) investigated the biosurfactant-amended phytoremediation of phenanthrene and pyrene by maize plant. This study suggest

that the use of rhamnolipid and saponin could increase desorption of phenanthrene (10 and 29%) and pyrene (9 and 28%, respectively) in soil.

9.8.5 Microbial Fuel Cells (MFC) in PAHs Degradation

A microbial fuel cell is a bio-electrochemical device that generates power through the oxidation of organic and inorganic matter by microbes. This technique is increasingly considered for the remediation of (in)organic-contaminated soils including PAHs. However, very few studies have reported the ability of MFCs in remediation of PAHs (Gambino et al. 2017; Hamdan et al. 2017). Sherafatmand and Ng (2015) demonstrated a sediment microbial fuel cell (SMFC) for the degradation of PAHs under aerobic or anaerobic environment. A significant removal of naphthalene (41.7 and 76.9%), acenaphthene (31.4 and 52.5%) and phenanthrene (36.2 and 36.8%) in the aerobic and anaerobic environment, respectively, was observed. Gambino et al. (2017) investigated consortia of *Bacillaceae*, *Enterobacteriaceae*, *Staphilococcaceae*, *Xanthomonadaceae* and *Pseudomoniadaceae* to study the degradation ability of PAHs in MFCs. The results showed that anode enrichment with microelectrogenic bacteria decreased overall PAH concentration to 90%. However, this technique may not be suited for the large scale and the suitability of this technique for the degradation of PAHs will need to be investigated further.

9.8.6 Omics Approaches in Degradation of PAHs

Omics (metagenomics, transcriptomics, proteomic and metabolomic) approaches to understanding the role of rhizosphere microbiome and plants for the development of genetically modified microorganisms (GMOs) and plants play a significant role in the enhancement of bioremediation/transformation of PAHs (Aydin et al. 2017; Kotoky et al. 2018). These techniques are used to investigate total genome content (also known as metagenomics), catabolic gene expression (mRNA collection; transcriptomics), protein profile (proteomics) and key metabolites (metabolomics) from the microbiome including uncultured microbes during the bioremediation practice. The potential of "omics" approaches suggests the use of engineered rhizospheric microbiome and plants for decreasing the toxicity of PAHs (El Amrani et al. 2015). However, an insufficient amount of studies have focused on omics approaches for enhancement of bioremediation/transformation of PAHs, and so more practice performance is still needed.

Metagenomic approaches provide total genetic content of a microbial community for identification of different groups of microbes and their functional genes involved in a particular habitat. Metagenomics analysis of soils contaminated with anthracene increased the percentages of sequences belonging to the *Actinobacteria*

and reduced the percentage of Proteobacteria while increasing percentages of sequences belonging to Proteobacteria in unamended soil after 14 days of study (Castro-Silva et al. 2013). Zafra et al. (2016) reported metagenomics sequence analysis of a fungal-bacterial consortium on degradation of PAH in soil. The results from this analysis demonstrated that the inoculated consortia could change the native microbial diversity of soil and enhance the degradation rate of PAHs in soil presumably due to co-metabolic degradation. Thus, metagenomics analysis enabled researchers to exploit knowledge about uncultivable microorganisms and their various probable metabolic pathways for the degradation of PAHs in polluted soils.

Transcriptomics or *metatranscriptomics* provides examination of mRNA expression of a single microbe or microbiome with a change in environmental situations (Hautefort and Hinton 2000). de Menezes et al. (2012) studied the microbial expression analysis of dioxygenase genes in soil stressed with phenanthrene and reported that a higher abundance of transcripts in the soil was due to stress response and detoxification activity of soil microbial communities. Similarly, rhizosphere soil of willows significantly enriched in transcripts was related to PAH degradation (Yergeau et al. 2018). Pagé et al. (2015) revealed that *Salix purpurea* growing in PAHs-contaminated soil stimulated the expression of 4 oxygenase genes out of the 10 studied within the bacterial orders *Actinomycetales*, *Rhodospirillales*, *Burkholderiales*, *Alteromonadales*, *Solirubrobacterales*, *Caulobacterales* and *Rhizobiales.* Herbst et al. (2013) confirmed that *Burkholderiales*, *Actinomycetales* and *Rhizobiales* were the most abundant microbes in the communities of groundwater amended with either C13-naphthalene or C13-fluorene by metaproteomic analysis and protein-stable-isotope probing (SIP).

Proteomics analysis deals with information about proteins and their functions in microbial communities involved in the bioremediation of pollutants in a contaminated environment. The proteogenomics approach was applied to *Mycobacterium vanbaalenii* for investigation of aromatic hydrocarbon catabolic pathways in presence of high molecular-PAHs (Kim et al. 2009). The various expressed proteins were identified as catalase-peroxidase, putative monooxygenases, dioxygenases, naphthalene-inducible dioxygenases and aldehyde dehydrogenase. Verdin et al. (2005) reported the overexpression of cytochrome P450 monooxygenase enzyme in *Fusarium solani* fungus under the presence of benzopyrene.

Metabolomics provides information of degradation products and primary metabolites in response to pollutant exposures. Transcriptomic and proteomic studies can be helpful in unweaving important information about the different metabolic pathways. Keum et al. (2008) studied metabolic intermediates during degradation of phenanthrene by *Sinorhizobium* sp. Metabolic pathways such as catechol, gentisic acid and protocatechuic acid pathway of phenanthrene degradation and expression of catabolic genes involved in these pathways by halophilic consortium were studied under different salinities (Wang et al. 2018).

GMOs are any organisms whose genetic material has been altered to enhance the catabolic efficiency associated with pollutant-degrading pathways. Such strategies are now common to accelerate the rate of biological degradation of PAHs using genetically modified bacteria and plants. Cytochrome P450 monooxygenases

(CYPs) for the degradation of naphthalene, fluorine, acenaphthalene and acenaphthylene were altered at the different active sites within the enzyme which enhanced oxidation potential of the enzyme in *Pseudomonas putida* and *B. megaterium* (Carmichael and Wong 2001; Harford-Cross et al. 2000). Peng et al. (2014) generated transgenic plants with enhanced tolerance to and uptake of phenanthrene by transferring the complex dioxygenase system of *Pseudomonas* into *Arabidopsis* and rice.

9.9 Summary and Future Outlook

Natural and anthropogenic activities contribute to generate PAHs, which are becoming a great concern due to their persistence in living beings and the environment. Several strategies have been employed for effective bioremediation of PAHs over the past century. Bioremediation technologies discussed in this chapter have been recognised as suitable technologies that contribute to PAH remediation. However, current PAHs biodegradation rates are low due to several environmental, biological and physico-chemical factors (Kuppusamy et al. 2017). Despite having multiple bioremediation technologies for PAHs removal, no single remediation technology can be the ultimate solution for different types of PAHs (Mohan et al. 2006). Thus, depending on the severity of contaminant and remedial objectives, a proper remediation approach has to be carefully considered. In the past few decades, a plethora of microbes have been screened and characterised with PAHs-degrading capabilities. However, microbial interactions, measurement and control of biochemical pathways within different PAHs-degrading microbial consortium (bacteria, bacteria and fungi, bacteria and algae or cyanobacteria and algae) have yet to be explored. This is the case because different types of consortium are shown to be much more beneficial in the remediation of PAHs than their single or individual consortium (García de Llasera et al. 2016).

It has been observed that in contaminated soils and sediments, bioavailability of PAHs is one of the most limiting factors, strongly affecting the feasibility of remediation; but this can be overcome moderately by the use of biosurfactants. Some biosurfactant producer microbes can also be used to enhance PAHs bioavailability for microbial degradation; however, application for field-level remediation is quite expensive. Thus, cost-effective production of biosurfactant is required for its broader application in the field (Bezza and Chirwa 2017). In addition, many unique enzymes associated with PAHs-degradation have been isolated and purified, and their role in PAHs-degradation has been characterised. Nevertheless, larger production and wide application in enzymatic bioremediation is a time-consuming process and has financial constraints; this problem can be overcome by the use of omics approaches. The use of organic additives is promising for an effective PAHs-degradation strategy. However, their characterisation, quantification and interactions must be considered because complex composition of natural

amendments may affect the metabolism of soil microbiota as well as of the plants involved in phytoremediation.

An effective bioremediation strategy requires selection of efficient microbes, algae, plants or combinations thereof. So far, undefined pathways of many potential microbial species are related to PAHs-remediation, so there is a need to use an omics approach to study the complex behaviour of novel species and their degradation pathways. Advances in omics technologies have provided an opportunity to develop genetically modified organisms and plants to boost bioremediation of PAHs. Researchers, to some extent, are convinced of the safe use of GMOs as a potential alternative for biodegradation of PAHs at reasonably low cost. Some non-technical factors such as environmental laws and mandates, aside from technical constraints, limit the use of GMOs. Therefore, it can be concluded from the present review that the application of coupled green degradation methods such as various types of microbial consortium, rhizoremediation, combined organic addition, and microbes with biosurfactants may be enough to manage the cleanup of the PAHs-contaminated sites.

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Chapter 10
Microbes Are Essential Components of Arsenic Cycling in the Environment: Implications for the Use of Microbes in Arsenic Remediation

Sudhakar Srivastava and Kavita Shukla

Abstract Arsenic (As) is a ubiquitously distributed toxic element, and it has been present in the environment since the very beginning of evolution. Hence, microbes to higher organisms possess mechanisms to tackle arsenic that include conversion of arsenic from one form to another including inorganic to organic and vice versa. Microbes present in different environments possess a number of pathways for arsenic conversion and therefore play a crucial role in arsenic cycling in the environment. Arsenic contamination has emerged as a serious problem in some parts of the world in the past few decades. These include Bangladesh, India, China, Vietnam, Pakistan, etc. The presence of arsenic in soil and groundwater in affected areas also leads to the entry of arsenic in plants. The level of arsenic accumulation in plants including edible portions depends on the arsenic species. In this scenario, microbes, which affect arsenic speciation, can play a role in regulating arsenic accumulation and consequently arsenic stress in plants. The microbes can therefore be utilized effectively to safeguard crop plants from arsenic. If the microbes also possess plant growth-promoting ability, this strategy can impart further benefits. A number of plant growth-promoting microbes (PGPMs) have been identified, characterized and utilized for the improvement of growth of plants in arsenic-contaminated environment as well as for the reduction of arsenic levels in plants. This review presents the role of microbes in arsenic cycling in the environment and discusses efforts for their utilization in the amelioration of arsenic stress in plants.

Keywords Arsenic · Plant growth-promoting microbes · Remediation

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10.1 Introduction

Arsenic (As) is an element present ubiquitously in the environment since the beginning of evolution, and consequently the pathways of its detoxification are found in almost every organism from bacteria to humans (Yang and Rosen 2016). Arsenic occurs in the environment in different inorganic [arsine (As^{-3}), elemental arsenic (As^0), arsenite [As(III)] and arsenate [As(V)]] and organic forms [dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), trimethylarsine oxide (TMAO), arsenocholine, arsenobetaine, etc.]. These different inorganic and organic species of arsenic have variable toxicities to biological systems. Hence, the processes of arsenic detoxification include conversion of more toxic inorganic form either to less toxic inorganic or to organic forms, followed by efflux from cells or vacuolar sequestration in different microbes (Bentley and Chasteen 2002; Upadhyay et al. 2018). Arsenate reduction to As(III) and vice versa As(III) oxidation to As(V) are catalysed by arsenite oxidase and arsenate reductase, respectively. Arsenic methylation is performed by As(III) S-adenosylmethionine (SAM) methyltransferases (Yang and Rosen 2016). The final fate of arsenic conversion to various organic forms can be the formation of volatile arsenic species like arsine (AsH_3) and trimethylarsine (TMA) (Paez-Espino et al. 2009). The potential of microbes for the conversion of arsenic to different methylated and volatile arsenic species depends on a number of factors including soil chemistry, organic matter, etc. (Mestrot et al. 2011).

Arsenic contamination is a widespread problem today with threats associated with arsenic toxicity being global. The problem is severe in Southeast Asian countries including Bangladesh, India, China, Pakistan, etc. These causes of arsenic contamination in these areas are mainly natural biogeochemical processes, although enhanced and worsened by human intervention (Srivastava et al. 2012; Rodriguez-Lado et al. 2013; Podgorski et al. 2017). Arsenic levels in the groundwater and soil are higher than maximum permissible limits set by WHO. The major accepted hypothesis for the presence of high arsenic in groundwater states that chemical changes induced by digging of shallow tube wells and hand pumps stimulated microbial reactions. This in turn led to the mobilization of arsenic present in bound form in sediments in the groundwater. The role of the number of microbes was identified in the process of arsenic mobilization in groundwater. The use of contaminated groundwater for irrigation leads to the entry of arsenic in soil and subsequently in plants. Rice is one of the worst affected crop plants with arsenic accumulation in its grains (Awasthi et al. 2017). The hunt for feasible low-cost strategies to mitigate arsenic contamination of rice is on. However, no suitable technology is available. The use of plant growth-promoting microbes (PGPMs) for the amelioration of arsenic toxicity and accumulation in rice is a deserving eco-friendly and sustainable method (Upadhyay et al. 2018). Further, suitable options are required for the remediation of highly contaminated and even abandoned sites. To this end also, plant- and microbe-based remediation methods have been considered viable options that can give desirable results in more time but at an affordable cost.

Hence, microbial utilization, due to its unique potential to handle even the high arsenic concentrations, is an approach that needs to be studied and optimized for commercial purposes.

In the present review article, the basic scheme of microbial metabolism of arsenic is presented. The studies demonstrating the utilization of microbes or their inherent pathways (genes) are discussed, and future perspectives are outlined.

10.2 Microbes: Role in Arsenic Cycling

Similar to cycling of various elements like carbon, nitrogen, oxygen and sulphur, a toxic element like arsenic also undergoes various ecological spheres in a cyclic manner. The microbes hold a crucial position in arsenic cycling due to the fact that they possess several possible ways to either detoxify and efflux arsenic or utilize arsenic species for metabolic purposes (Mukhopadhyay et al. 2002; Yang and Rosen 2016). It is now well known that arsenic-related genes in microbes are present in several clusters (Andres and Bertin 2016). The most simple arsenic operon is *ars* operon with basic constituent genes as *arsRBC*, where ArsR is an As(III)-responsive repressor, ArsB is an As(III) efflux permease and ArsC is an As(V) reductase (Yang and Rosen 2016; Andres and Bertin 2016). In this operon, other genes may also be present like ArsA (the As(III)-stimulated ATPase) and ArsD (As(III) metallochaperone); the operon thus becomes large: *arsRDBAC*. The binding of ArsA to ArsB increases the efficiency of As(III) efflux by ArsB. The function of ArsC becomes important when As(V) is the major species entering the cells that has to be reduced to As(III) for the efflux. Yet more genes may be present in ars operon, e.g. arsH and arsP, having variable functions. Several variations of the set of genes in ars operon in different microbes have been found (Andres and Bertin 2016). The eukaryotic counterpart of *arsRBC* in *Saccahromycescerevisiase* includes *ACR1 (ARR1)*, *ACR2 (ARR2)* and *ACR3 (ARR3)* that encode for transcriptional activator, arsenate reductase and efflux pump, respectively (Ghosh et al. 1999).

Another important microbial arsenic-related operon system is *aioBA* that encodes two subunits of arsenite oxidase. Other genes like *aioC* (encoding c-type cytochrome), *aioD* (encoding molybdenum cofactor) and *nitR* (encoding nitroreductase) and regulatory genes, *aioXSR* (encoding As(III)-binding protein, histidine kinase and response regulator, respectively), are present in some proteobacteria (Andres and Bertin 2016). Another operon meant for the respiration of As(V) is *arrAB* (encoding two subunits of respiratory As(V) reductase), while an operon for anaerobic As(III) oxidation is *arxB'AB* (where *arxA* and *arxB* encode for two subunits of anaerobic As(III) oxidase). Two other important genes of arsenic metabolism in microbes include *arsM*, encoding for arsenite methyltransferase, and *arsI*, encoding for C-As lyase. These two genes carry out the function of arsenic methylation and demethylation and organoarsenical degradation. Hence, there is an array of pathways through which microbes can handle arsenic infiltration. A few microbes are arsenotrophs which means they utilize As(III) oxidation and As(V) reduction for

their growth (Zargar et al. 2012). The basic reactions of arsenic metabolism and/or detoxification are presented in Fig. 10.1.

In arsenic microbial cycling, the reduction of As(V) to As(III) is catalysed by respiratory or cytoplasmic arsenate reductase. Most of the microbes reduce As(V) to As(III) for the purpose of its subsequent efflux back to the medium as a part of arsenic detoxification mechanism. In this As(V) reduction, microbes do not get any energy. However, in respiratory As(V) reduction, microbes gain energy to support their growth. The microbes (dissimilatory arsenate-reducing prokaryotes, DARPs) involved in arsenic release in groundwater through reductive dissolution of minerals use arsenate as a terminal electron acceptor in arsenic respiration under reducing conditions (Laverman et al. 1995; Krafft and Macy 1998). In this way, microbes are able to synthesize ATP. The electron donors may be various small organic molecules like pyruvate, glycerol, malate, etc. (Newmann et al. 1998; Oremland and Stolz 2005; Paez-Espino et al. 2009). DARPs belong to diverse group like *Firmicutes* and proteobacteria. The two-enzyme complex of ArrA and ArrB acts as an arsenate reductase in DARPs. In case of As(III) oxidation, As(III) acts as the electron donor and the process is coupled to respiration. The As(III) is oxidized, while oxygen is reduced along with energy gain that is then utilized for cell growth (Santini and vanden Hoven 2004; Muller et al. 2003). Arsenate-reducing microbes and DARPs are important players in groundwater release of arsenic in the form of As(III).

Arsenic also goes through cycles of methylation and demethylation in its cycle. The methylation of arsenic in a stepwise manner leads to the formation of mono-, di- and tri-methylated species, which are less toxic than inorganic arsenic species. Hence, arsenic methylation is also a detoxification mechanism (Upadhyay et al. 2018). The methylation of arsenic has been detected in bacteria, archaea, fungi, protozoan, cyanobacteria and algae and higher animals and humans (Wang et al. 2014; Yin et al. 2011; Qin et al. 2006). The methylation is catalysed by ArsM. The last product of methylation is trimethylarsine [TMAs(III)] which is a gas. However, it was surprising for years that even with capabilities of arsenic methylation being present so widely, arsenic still exists mostly as inorganic species in nature. The reason for this was revealed upon discovery of arsenic demethylation (Feng et al. 2005; Maki et al. 2006). ArsI is a C-As lyase that cleaves the C-As bond converting MAs(III) into As(III). ArsI may also cleave C-As bond in aromatic arsenic compounds. Thus, the use of even organic arsenicals, like roxarsone and p-arsanilic acid that are being used presently, is harmful as their eventual fate is demethylation and conversion to inorganic arsenic species (Yang and Rosen 2016). Another enzyme, ArsH, which is an NADPH-dependent FMN reductase, can oxidize trivalent organic arsenicals like MA(III) and Rox(III) to their pentavalent less toxic forms. This enzyme is crucial for arsenic tolerance of some bacteria.

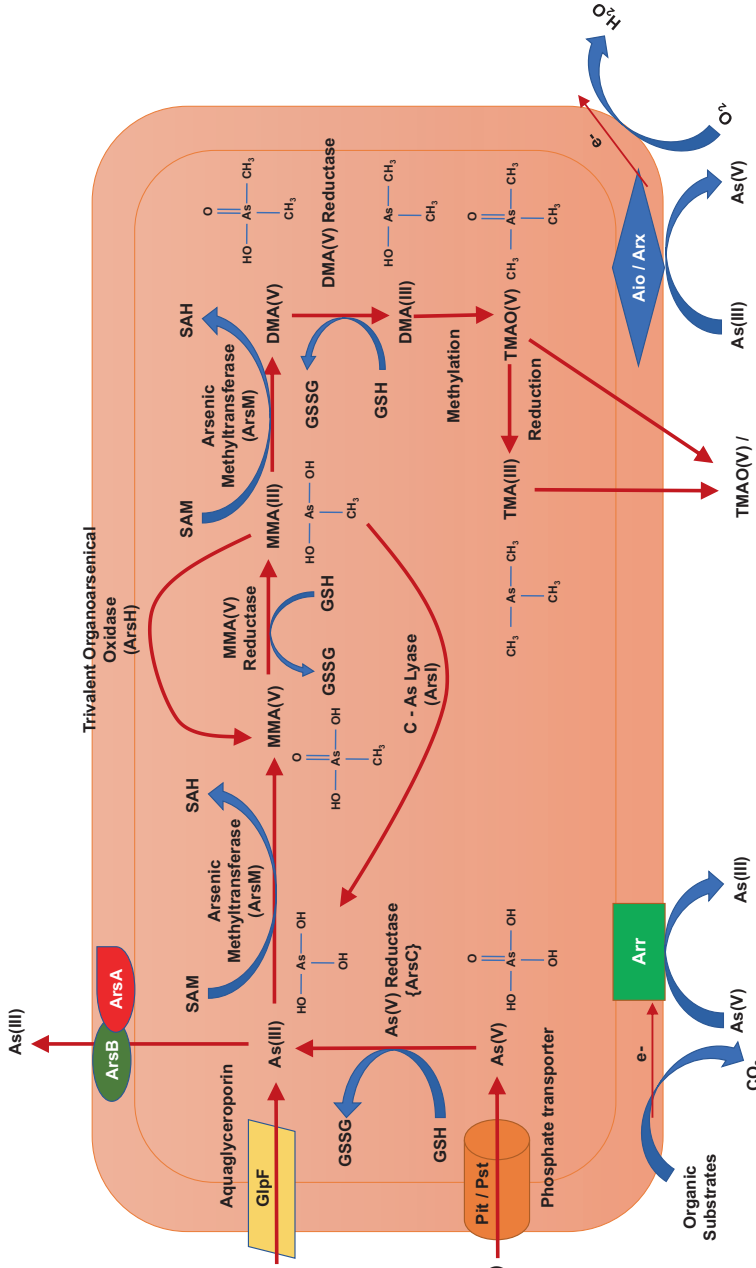

Fig. 10.1 Metabolism of arsenic in a microbial cell. There are different arsenic operons: Ars operon represents cytoplasmic arsenate reductase (ArsC), arsenite efflux ATPase (ArsA, ArsB) and arsenic methyltransferase (ArsM). There are other enzymes also like C-As lyase (ArsI) and trivalent organoarsenical oxidase (ArsH). Arr represents respiratory arsenate reductase, while Aio/Arx represents respiratory arsenite oxidase. Abbreviations: *As(V)* arsenate, *As(III)* arsenite, *MMA(V)* monomethylarsonic acid, *MMA(III)* monomethylarsonous acid, *DMA(V)* dimethylarsinic acid, *DMA(III)* dimethylarsinous acid, *TMAO(V)* trimethylarsine oxide, *TMA(III)* trimethylarsine, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *SAM* S-adenosylmethionine, *SAH* S-adenosylhomocysteine

10.3 Role of Microbes in Arsenic Regulation in Plants in Lab and Field

Combating the problem of arsenic contamination of the environment requires simultaneous two-sided attention like the two sides of a coin. At one end, high arsenic present in abandoned land and water sites needs to be removed in a cost-effective manner, viz. through plant-based remediation. On the other end, arsenic present in agriculturally productive land and water needs to be prevented from entering plants. Since microbes can cycle arsenic into different inorganic and organic forms, they can potentially be utilized alone or in consortia to regulate arsenic accumulation and achieve desirable results. A number of studies have been performed to identify arsenic-tolerant microorganisms from arsenic-contaminated environments and have led to successful identification of several potential microbes that can be utilized to either increase the arsenic accumulation of phytoremediator plants or decrease the arsenic level in edible tissues of plants.

10.4 Microbe-Mediated Growth Improvement and Arsenic Level Reduction in Crop Plants

Roychowdhury et al. (2018) identified ten arsenic-resistant bacterial isolates from the fly ash (pond ash sample) belonging to genera like *Bacillus*, *Kytococcus*, *Staphylococcus*, etc. (e.g. *B. subtilis*, *B. thuringiensis*, *K. sedentarius*, *S. pasteuri*). These strains were capable of As(III) to As(V) and As(V) to As(III) conversion and store arsenic in their biomass. Prum et al. (2018) analysed the arsenic removal efficiency of several plants (*Echinodorus cordifolius*, *Cyperus alternifolius*, *Acrostichum aureum*, *Colocasia esculenta*) and found *E. cordifolius* to be the best arsenic remover. They also tested *E. cordifolius* along with an arsenic-tolerant microbe *Arthrobacter creatinolyticus* for arsenic removal in pot experiments. Authors found that dipping bacteria on plant roots for 5 min significantly increased plants' efficiency for arsenic removal both in lab and in field in a constructed wetland.

Bacterial inoculation has also been tested to reduce arsenic toxicity to and arsenic accumulation in rice seedlings. In a study, rice seedlings were inoculated with different strains of cadmium-tolerant bacteria (KKU2500-1, KKU2500-2, KKU2500-3, KKU2500-9, KKU2500-12, KKU2500-16 and KKU2500-22) and As(III)-oxidizing bacteria (4.25, 4.27, 4.40 and 4.44) in various combinations. These bacteria possessed As(III)-oxidizing ability and produced a high amount of inorganic sulphide and thiol compounds. The strain combinations like KKU2500-3/4.25 and KKU2500-3/4.44 not only improved the growth of rice seedlings in the presence of As(V)/As(III) but also significantly reduced arsenic translocation to shoot (Thongnok et al. 2018). Das and Sarkar (2018) isolated an arsenic-resistant microbe, *Acinetobacter lwoffii* (RJB-2), from arsenic-contaminated soil of North 24 Parganas, West Bengal, and used it as inoculum to *Vigna radiata*.

The bacterium inhibited arsenic uptake by plants and showed significant improvement in plant growth and also decreased oxidative stress to plants. Mallick et al. (2018) isolated *Kocuria flava* and *Bacillus vietnamensis* as resistant bacteria from Sunderban, West Bengal. The application of these bacteria led to improved growth and decreased arsenic in rice plants. An arsenic-tolerant microbe *Micrococcus luteus* has been found to improve growth and biomass of grapevine plants (Ivan et al. 2017).

Apart from bacteria, fungi and algae have also been tested as potential inoculants for improving the growth of plants and for reducing arsenic accumulation at the same time. The effect of the application of arbuscular mycorrhizal fungi (AMF), *Rhizoglomus intraradices* and *Glomus etunicatum*, on *Triticum aestivum* plants was analysed by Sharma et al. (2017). The mycorrhizal inoculation resulted in improved growth of plants, reduced arsenic translocation and maintained phosphorus to arsenic ratio. An improved growth and a decline in arsenic accumulation have also been found in soybean plants upon inoculation of *Rhizophagus intraradices* (Spagnoletti et al. 2016). *R. intraradices* has also been able to reduce inorganic/organic arsenic ratio in rice plants (Li et al. 2016). The application of the fungal strain of *Trichoderma*, *T. reesei* NBRI0716, was demonstrated to increase the yield of chickpea plants and reduce inorganic arsenic concentration when grown in arsenic (100 mg kg^{-1})-contaminated soil (Tripathi et al. 2013). Algal inoculants, *Chlorella vulgaris* and *Nannochloropsis* sp., have also been found to reduce arsenic accumulation in rice seedlings and ameliorate arsenic toxicity in a study by Upadhyay et al. (2016). In a recent work, Awasthi et al. (2018) studied a consortium of alga (*C. vulgaris*) and bacterium (*Pseudomonas putida*) against arsenic stress in rice seedlings. They demonstrated significant improvement in the growth of rice seedlings along with a decline in arsenic in root and shoot of seedlings when rice plants were grown with the consortium.

10.5 Microbe-Mediated Growth Improvement and Increased Arsenic Accumulation in Phytoremediator Plants

The use of microbes in increased arsenic removal and phytoremediation efficiency of plants has also been tested. In a recent work, Mukherjee et al. (2018) isolated endophytic microbes from arsenic-tolerant plant, *Lantana camara*, from Nadia, West Bengal, and applied these microbes as consortia in *Solanum nigrum*. These bacteria included *Enterobacter* sp., *Kocuria* sp. and *Kosakonia* sp. The microbial consortium helped plants maintain good growth and have higher shoot arsenic accumulation. The increased conversion of As(V) to As(III), glutathione levels and changes in transporter expression were major mechanisms behind the consortium-mediated increased arsenic tolerance of *Solanum* plants. Mesa et al. (2017) isolated four potential bacterial strains isolated from rhizosphere (*Ensifer adhaerens*) and roots (*Variovorax paradoxus*, *Phyllobacterium myrsinacearum*) of *Betula*

celtiberica in Spain and found that while *E. adhaerens* enhanced the growth of plants, *V. paradoxum* and *P. myrsinacearum* increased the accumulation of arsenic in plants. Arsenic-resistant microbes from rice fields (*Lysinibacillus* sp., *Bacillus altitudinis*, *B. megaterium*) have been reported to increase arsenic removal by *Pteris vittata* plants (Singh et al. 2015). Other potential arsenic-tolerant bacterial strains (*Pseudomonas* sp., *Delftia* sp., *Bacillus* sp., *Variovorax* sp. and *Pseudoxanthomonas* sp.) have also been found to enhance arsenic accumulation by *P. vittata* plants along with increase in growth, thus improving arsenic removal (Lampis et al. 2015). In *Populus deltoides*, the application of *Agrobacterium radiobacter* led to an increase in arsenic removal and root-to-shoot translocation (Wang et al. 2011).

10.6 Biotechnological Tools to Utilize Microbial Genes for the Regulation of Arsenic Accumulation in Plants

Extensive research on microbial strategies and pathways of arsenic tolerance has resulted in the identification of several potential genes that can be used for the development of transgenic plants for altering arsenic accumulation properties of plants. Such a strategy would reduce cost associated with microbial inoculation during each cropping period. Further, attempts have also been made to augment the arsenic resistance potential of microbes themselves through the expression of their own or foreign gene and then utilize such transgenic microbes for the amelioration of arsenic toxicity in plants.

A gene of huge interest for the past few years has been *arsM* whose enzyme product is involved in arsenic methylation and volatilization in microbes. Since organic methylated arsenicals have less toxicity and volatile arsenicals are expelled from plants, this gene has been the gene of choice in several studies. An *arsM* gene from *Chlamydomonas reinhardtii* (*CrarsM*) was expressed in the symbiotic bacterium, *Rhizobium leguminosarum*, that resulted in higher arsenic methylation potential of symbiont. The use of transgenic *R. leguminosarum* in symbiotic association with red clover plants resulted in an increased level of methylated arsenic in red clover plants. In addition, arsenic volatilization (0.01%–0.02% of total As) was also observed (Zhang et al. 2017). The *arsM gene* from the alga, *Chlamydomonas reinhardtii*, has also been used for the development of transgenic plants of *Arabidopsis thaliana*. It was found that the methylation of inorganic arsenic species to organic species and volatile arsenic species was increased in transgenic plants (Tang et al. 2016). A soil fungus, *Westerdykella aurantiaca*, has also been utilized for the isolation of *arsM* gene, *WaarsM*, by Verma et al. (2016). The *WaarsM* gene was expressed in the yeast, *S. cerevisiae*, which showed higher arsenic methylation as well as arsenic volatilization. The application of transgenic yeast cells to rice plants also improved the growth of rice in arsenic-stressed conditions. In an earlier work, Meng et al. (2011) utilized *arsM* gene from *Rhodopseudomonas palustris* to develop

transgenic rice plants and found an increased level of organic arsenic species including volatile arsenic.

10.7 Future Prospects

Microbes are the treasure of metabolic pathways and have several potential mechanisms that can be utilized to assist plants in handling arsenic stress more effectively. Most of the studies till date have studied microbial application with a single microbe or with a single group of microbes. Future studies need to focus on consortia containing different types of microbes like bacteria, algae and fungi so as to holistically improve plants' growth and alter arsenic accumulation. Further, microbial genes need to be utilized in combination to achieve better results in terms of arsenic level change in crop plants/phytoremediator plants.

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Chapter 11
Biodegradation of Synthetic Pyrethroid Insecticides

Shaohua Chen and Hui Zhan

Abstract Synthetic pyrethroid insecticides have been used extensively for controlling indoor and outdoor insect pests, posing a great threat to humans and the ecosystem. Pyrethroid residues are often detected in aquatic and terrestrial environments; therefore developing a suitable bioremediation strategy is urgent. Since biodegradation is considered as an economical and safe approach, lots of work have been studied about pyrethroid-degrading microorganisms. This chapter summarizes the toxicity and environmental safety of pyrethroids, microbial degradation of pyrethroids, and biodegradation pathway of pyrethroids, pyrethroid-degrading enzymes, and bioremediation of pyrethroid-contaminated environments. This chapter will provide an instructive direction to apply pyrethroid-degrading microorganisms in the environment for bioremediation.

Keywords Pyrethroids · Biodegradation · Pathways · Enzymes · Bioremediation

11.1 Introduction to Synthetic Pyrethroids: State of the Art

Synthetic pyrethroids, chemical analogs of natural pyrethrins extracted from *Chrysanthemum cinerariaefolium*, have been sold as commercial insecticide globally for indoor and outdoor utilization against varieties of pests in agriculture, public and commercial buildings, veterinary facilities, and household for over several decades (Casida 1980; Katsuda 1999). Pyrethrins are limited for indoor use due to its instability to direct sunlight, heat, and oxygen; therefore pyrethroids are designed and synthesized more stable and effective which have predominant efforts to combat the transmission of malaria and other mosquito-induced diseases (Katsuda

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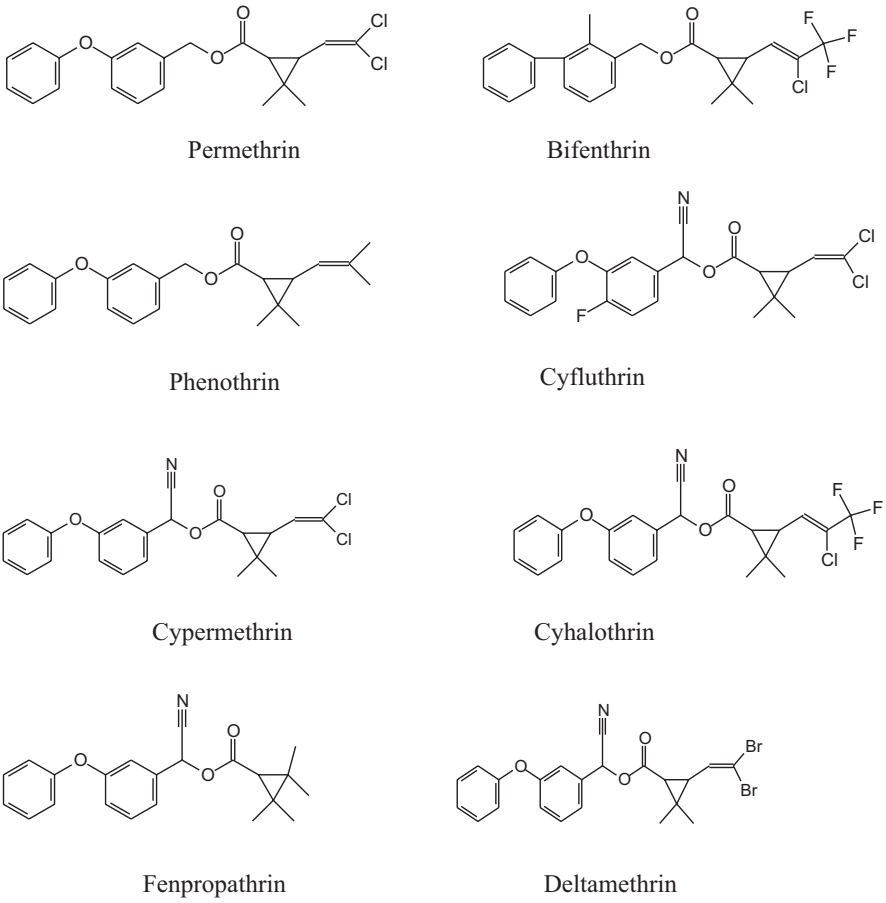

Fig. 11.1 Chemical structures of pyrethroids

2011; Soderlund 2012; Ujihara et al. 2011). Allethrin is the first synthetic pyrethroid against household pests which was synthesized by Milton S. Schechter in 1949 and commercialized into insecticide market in 1952. Pyrethroids contain an acid and an alcohol moiety with an ester bond in its basic chemical structure (Fig. 11.1). To increase the insecticidal toxicity and stability of pyrethroids, chemical modifications have been developed on alcohol moiety, acid moiety, and ester linkage (Katsuda 1999). Pyrethroids are categorized into two types, Type I and Type II, based on physical and chemical properties (Laskowski 2002). Type I synthetic pyrethroids, including allethrin, bifenthrin, d-phenothrin, permethrin, resmethrin, tefluthrin, and tetramethrin, do not have cyano group. On the contrary, Type II synthetic pyrethroids have an α-cyano group which is more neurotoxic than Type I, including cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, fenpropathrin, flucythrinate, flumethrin, fluvalinate, and tralomethrin (Kaviraj and Gupta 2014;

Palmquist et al. 2011). Pyrethroid insecticides show broad spectrum, quick knockdown, and negative temperature coefficient characteristics and lower acute toxicity to mammals. Therefore, these properties made them replace parts of organophosphorus insecticides gradually (Katsuda 1999; Ray and Fry 2006). The pyrethroid insecticides exhibit high efficacy in controlling organophosphorus-resistant and carbamate-resistant pests, due to the different mode of action. Mounting evidence suggests that pyrethroids target on voltage-gated sodium channels and disrupt the normal functions of both central and peripheral nervous systems; furthermore, the voltage-gated calcium and chloride channels are the potential secondary sites leading to insecticidal neurotoxicity (Davies et al. 2007; Palmquist et al. 2011; Soderlund 2012). Although pyrethroids exhibit low mammalian and avian toxicity compared to organophosphorus insecticides, the potential negative effects resulting from the extensive and increasing use of these compounds cannot be ignored.

11.2 Toxicity and Environmental Safety of Pyrethroids

Pyrethroids can enter the aquatic and terrestrial environment through various ways directly or indirectly; thereby pyrethroid residue has been detected frequently in the soil, surface water, sediments, and indoor environment (Chinen et al. 2016; Deziel et al. 2015; Liu et al. 2016; Mimbs et al. 2016). Pyrethroids can bind strongly to soil particles and transport into aquatic environments because of their high hydrophobicity (Cycoń and Piotrowska-Seget 2016). Allethrin, bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenpropathrin, fenvalerate, permethrin, d-phenothrin, resmethrin, and tetramethrin are the main commercial pyrethroid insecticides and have been used widely. Cypermethrin and permethrin residues were observed frequently in soil environment and indoor environment, respectively (Tang et al. 2017b). Due to their lipophilic properties, synthetic pyrethroids are able to bioaccumulate in organisms via food chains and hard to remove. Beta-cypermethrin and its metabolite were observed to accumulate in egg and edible tissues of laying hens (Liu et al. 2017), which indicates humans are mainly exposed to pyrethroids not only through residential environments but also food chains.

Most pyrethroids have low or moderate toxicity to insects and invertebrates. Two basic neurotoxicity syndromes of pyrethroids to insects are distinguished, Type I pyrethroids causing reflex hyperexcitability followed by prostration and paralysis while Type II with an α-cyano group producing convulsive phase (Davies et al. 2007; Ray et al. 2000). Moreover, pyrethroids have neurotoxicity to mammals, since the high conserved structure and function of voltage-gated sodium channels between insects and mammals (Soderlund 2005, 2010). The signs of high-level pyrethroid intoxication in mammals and insects are similar, as explained in earlier posts, Type I pyrethroids are associated with T (tremor) syndrome and Type II with CS (choreoathetosis with salivation) syndrome (Clark and Symington 2008; Lawrence and Casida 1982). Pyrethroid insecticides exhibit extremely acute toxicity to fish and aquatic and benthic invertebrates, which limits their application in

aquatic environments (Bradbury and Coats 1989; Khan 1983; Li et al. 2017; Mugni et al. 2013). However, the massive use of pyrethroids increases their possibility contaminating the aquatic ecosystems by runoff or spray drift and inducing negative effects on aquatic nontarget organisms directly or indirectly (Crossland 1982; Crossland et al. 1982; Weston and Lydy 2010). There is another study which shows that deltamethrin and fenvalerate have chronic and cytotoxic toxicity to soil invertebrates and pose a potential risk to soil community at low concentrations (Song et al. 2015). Research indicates there is a certain connection between exposure to pyrethroids and obesity, for instance, *cis*-bifenthrin could give rise to lipid accumulation in human hepatoma cells (Xiang et al. 2018). Additionally, pyrethroid insecticides can disrupt endocrine system in fishes and mammals with different mechanisms, such as obstructing, imitating, or synergizing endogenous hormones in endocrine signaling pathways (Brander et al. 2016). For example, it has been researched that λ-cyhalothrin, fenvalerate, and permethrin are able to disrupt endocrine system in zebra fish embryo at a natural environmental concentration (Zhang et al. 2017). The phytotoxicity of pyrethroids toward *Cucumis sativus* has been investigated, and the results showed that cypermethrin and deltamethrin affected seedling development, and the metabolite of pyrethroids 3-phenoxybenzoic acid (3-PBA) hindered germination (Bragança et al. 2018). Pyrethroid metabolite 3-PBA is frequently detected in human urine samples; furthermore, exposure to pyrethroids induces potential adverse effect on sperm concentration and sperm DNA (Han et al. 2008; Ji et al. 2011; Xia et al. 2008). Some health issues from the long-term and low-concentration pyrethroids exposure may occur, as the continuous and copious use of pyrethroids increases their residues in food, indoor, and natural environment.

Insecticides can be removed via chemical, physical, physicochemical, and biological degradation approaches in the natural environments. It is widely agreed that microbial degradation plays a significant role in pyrethroid breakdown, meanwhile soil microorganisms are the key decomposers. Therefore, it is worthwhile to investigate the potential of pyrethroid-degrading microorganisms for bioremediating pyrethroid-contaminated environment.

11.3 Microbial Degradation of Pyrethroids

Microbes are viewed as excellent eco-friendly tools for the degradation and detoxification of organic and inorganic pollutants (Gautam et al. 2017; Saxena et al. 2016). Recently, research of pyrethroid biodegradation is mainly focused on isolation and identification of pyrethroid-degrading organisms, pyrethroid-degrading genes and enzymes, metabolic pathways of pyrethroids, and application of pyrethroid-degrading organisms for bioremediation. Various microorganisms have been isolated and determined their ability of degrading pyrethroid insecticides, including bacteria, fungi, actinomycete, algae, and so on, while bacterial strains play the most predominant role. Most pyrethroid-degrading microorganisms were isolated from

soil, particularly from the contaminated environment, using enrichment culture techniques or screening directly (Akbar et al. 2015a, b; Pankaj et al. 2016; Zhang et al. 2016). To date, the isolation and identification of pyrethroid-degrading microorganisms are at a pronounced abundant level, especially the bacterial strains. Under the optimal conditions, many bacterial genera are found to be efficient in degrading pyrethroids, such as *Achromobacter* sp., *Acidomonas* sp., *Bacillus* sp., *Catellibacterium* sp., *Clostridium* sp., *Micrococcus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Sphingobium* sp., *Stenotrophomonas* sp., *Streptomyces* sp., and so on (Akbar et al. 2015a; Chen et al. 2011d; Grant and Betts 2004; Grant et al. 2002; Jilani and Khan 2006; Guo et al. 2009; Lin et al. 2011; Maloney et al. 1988; Paingankar et al. 2005; Tallur et al. 2008; Tiwary and Dubey 2016; Zhang et al. 2011a, b; Zhao et al. 2013; Cycoń et al. 2014). Although the exploiting of pyrethroid-degrading fungi is not abundant as bacteria, several fungi strains exhibit prominent degradation capability, such as *Candida pelliculosa* ZS-02 and *Cladosporium* sp. HU, whose degradation rates to some pyrethroids are more than 90% (Chen et al. 2011b, 2012a). Table 11.1 shows the pyrethroid-degrading strains isolated from various environments.

Most pyrethroid-degrading strains are capable to utilize pyrethroids as sole carbon source and growth substances, which belong to catabolic degradation mode. Meanwhile, part of isolates are unable to grow or decompose pyrethroids without other carbon sources or nutrients, belonging to co-metabolic degradation mode, for instance, the growth of *Pseudomonas fluorescens* SM-1, *Achromobacter* sp. SM-2, and *Bacillus cereus* SM-3 was dependent on Tween 80 as primary carbon source (Maloney et al. 1988). Due to the chemical structure similarity, many pyrethroid-degrading isolates are found to degrade a wide spectrum of pyrethroid insecticides including both Type I and Type II, which suggests they are promising and potential to bioremediate complex contaminated environments. For instance, *Bacillus thuringiensis* ZS-19 was able to degrade cyhalothrin, fenpropathrin, deltamethrin, cypermethrin, cyfluthrin, and bifenthrin effectively and efficiently under the optimum conditions; especially cyhalothrin was completely degraded within 72 h at the concentration below 100 μg/mL (Chen et al. 2015). Remarkably, only several strains exhibit the capability of complete mineralization and splendid detoxification of pyrethroids. *Micrococcus* sp. strain CPN 1 utilized cypermethrin as sole carbon source and mineralized it completely (Tallur et al. 2008). As cypermethrin is a large-scale class in synthetic pyrethroid insecticides, most isolates that have been reported are cypermethrin-degrading strains. In laboratory conditions, factors that affect the degradation efficiency of pyrethroids in liquid media are mainly including temperature, pH, and inoculum biomass (Zhang et al. 2010, Chen et al. 2013b, 2011a, c; Xiao et al. 2015). Generally, the consortia consisting of various degrading isolates exhibit the higher efficiency than sole strain (Zhan et al. 2018a), for example, the co-culture of *Bacillus cereus* ZH-3 and *Streptomyces aureus* HP-S-01 completely metabolized cypermethrin at 50 mg/L within 72 h under the optimal degradation conditions (Chen et al. 2012b).

Table 11.1 Pyrethroid-degrading microbes

Pyrethroids	Strains	Taxonomy	References
Cypermethrin	*Pseudomonas* sp.	Bacterium	Zhang et al. (2011a) and Tang et al. (2017a)
	Serratia sp.	Bacterium	Zhang et al. (2010) and Cycoń et al. (2014)
	Alcaligenes sp.	Bacterium	Yu and Fan (2003)
	Aspergillus sp.	Fungus	Liang et al. (2005)
	Micrococcus sp.	Bacterium	Tallur et al. (2008)
	Sphingobium sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Klebsiella sp.	Actinomycete	Wu et al. (2006)
	Bacillus sp.	Bacterium	Chen et al. (2012b) and Xiao et al. (2015)
	Escherichia sp.	Bacterium	Murugesan et al. (2010)
	Corynebacterium sp.	Bacterium	Murugesan et al. (2010)
	Streptomyces sp.	Actinomycete	Chen et al. (2012b, 2013b) and Lin et al. (2011)
	Catellibacterium sp.	Bacterium	Zhao et al. (2013)
	Achromobacter sp.	Bacterium	Chen et al. (2011e)
Fenvalerate	*Achromobacter* sp.	Bacterium	Maloney et al. (1988)
	Bacillus sp.	Bacterium	Maloney et al. (1993)
	Alcaligenes sp.	Bacterium	Yu and Fan (2003)
	Aspergillus sp.	Fungus	Liang et al. (2005)
	Sphingobium sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Klebsiella sp.	Actinomycete	Wu et al. (2006)
	Pseudomonas sp.	Bacterium	Fulekar (2009)
	Owenweeksia sp.	Bacterium	Boricha and Fulekar (2010)
	Stenotrophomonas sp.	Bacterium	Chen et al. (2011d)
	Cladosporium sp.	Fungus	Chen et al. (2011b)
Deltamethrin	*Rhodococcus* sp.	Bacterium	Khan et al. (1988)
	Pseudomonas sp.	Bacterium	Maloney et al. (1988)
	Achromobacter sp.	Bacterium	Maloney et al. (1988)
	Bacillus sp.	Bacterium	Maloney et al. (1993)
	Alcaligenes sp.	Bacterium	Yu and Fan (2003)
	Aspergillus sp.	Fungus	Liang et al. (2005)
	Klebsiella sp.	Actinomycete	Wu et al. (2006)
	Streptomyces sp.	Actinomycete	Chen et al. (2011c)
Fenpropathrin	*Alcaligenes* sp.	Bacterium	Yu and Fan (2003)
	Ochrobactrum sp.	Bacterium	Wang et al. (2011)
	Sphingobium sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Klebsiella sp.	Actinomycete	Wu et al. (2006)
	Bacillus sp.	Bacterium	Chen et al. (2014)
	Clostridium sp.	Bacterium	Zhang et al. (2011b)

(continued)

Table 11.1 (continued)

Pyrethroids	Strains	Taxonomy	References
Bifenthrin	*Sphingobium* sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Klebsiella sp.	Actinomycete	Wu et al. (2006)
	Stenotrophomonas sp.	Bacterium	Lee et al. (2004)
	Aeromonas sp.	Bacterium	Lee et al. (2004)
	Erwinia sp.	Bacterium	Lee et al. (2004)
	Candida sp.	Yeast	Chen et al. (2012a)
Cyfluthrin	*Achromobacter* sp.	Bacterium	Maloney et al. (1988)
	Bacillus sp.	Bacterium	Maloney et al. (1988, 1997)
	Pseudomonas sp.	Bacterium	Saikia et al. (2005)
	Aspergillus sp.	Fungus	Saikia and Gopal (2004)
	Trichoderma sp.	Fungus	Saikia and Gopal (2004)
	Phanerochaete sp.	Fungus	Saikia and Gopal (2004)
	Brevibacterium sp.	Bacterium	Chen et al. (2013a)
Permethrin	*Pseudomonas* sp.	Bacterium	Maloney et al. (1988)
	Achromobacter sp.	Bacterium	Maloney et al. (1988)
	Bacillus sp.	Bacterium	Maloney et al. (1988, 1992)
	Alcaligenes sp.	Bacterium	Yu and Fan (2003)
	Sphingobium sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Acinetobacter sp.	Bacterium	Zhan et al. (2018b)
Fluvalinate	*Pseudomonas* sp.	Bacterium	Maloney et al. (1988)
	Achromobacter sp.	Bacterium	Maloney et al. (1988)
	Bacillus sp.	Bacterium	Maloney et al. (1988, 1992)
Cyhalothrin	*Alcaligenes* sp.	Bacterium	Yu and Fan (2003)
	Sphingobium sp.	Bacterium	Guo et al. (2009) and Wang et al. (2009)
	Bacillus sp.	Bacterium	Chen et al. (2015)
	Ochrobactrum sp.	Bacterium	Zhai et al. (2012)
Allethrin	*Acidomonas* sp.	Bacterium	Paingankar et al. (2005)
D-phenothrin	*Pseudomonas* sp.	Bacterium	Yang et al. (2018)

11.4 Biodegradation Pathways of Pyrethroids

The degradation metabolites and proposed pathways of different synthetic pyrethroids in different microorganisms are not exactly the same. The biodegradation pathways of pyrethroids that are degraded by microorganisms, even the same pyrethroid, show significant differences, which may result from not only different biochemical properties and incubation periods of the microorganisms but also the stereoisomers of the pyrethroids (Cycoń and Piotrowska-Seget 2016). Pyrethroids are esters with an acid and alcohol moiety according to the chemical structure. Consequently, the detoxification of pyrethroids is mainly focused on the ester linkages, as hydrolysis of the ester bonds by esterase is the most efficient strategy (Sogorb and Vilanova 2002). As is often the case, pyrethroids are initially

hydrolyzed by carboxylesterases in microorganisms, producing a carboxylate and an alcohol. Accordingly, the degradation mechanism is generally the same, but there are significant individual differences. So far, the biodegradation pathways of Type II pyrethroids in bacteria strains have been studied in detail, such as cypermethrin, cyfluthrin, cyhalothrin, and fenpropathrin (Chen et al. 2013a, 2015; Pankaj et al. 2016; Wang et al. 2011). 3-Phenoxybenzoic acid (3-PBA) and 3-phenoxybenzaldehyde are the most common intermediates in the degradation pathways of Type II pyrethroids by bacteria, except the cyfluthrin degradation in *Brevibacterium aureum* DG-12, the beta-cyfluthrin degradation in *Pseudomonas stutzeri* S1, and the fenpropathrin degradation in *Clostridium* sp. ZP3 (Chen et al. 2013a; Saikia et al. 2005; Zhang et al. 2011b). The degradation pathway of fenpropathrin in *Bacillus* sp. DG-02 has been proposed by Chen et al. (2014). Fenpropathrin was primarily hydrolyzed via carboxylester linkage cleavage to yield 2,2,3,3-tetramethylcyclopropanecarboxylic acid phenyl ester and α-hyroxy-3-phenoxybenzeneacetonitrile which transformed to 3-phenoxybenzaldehyde spontaneously, followed by the oxidization of 3-phenoxybenzaldehyde via diaryl cleavage producing. The detected metabolites, 3,4-dihydroxybenzoic acid, 3,4-dimethoxyphenol, and phenol, were formed from 3-phenoxybenzoate (Fig. 11.2). Not all Type I pyrethroids are metabolized in bacteria without formation of 3-PBA and 3-phenoxybenzaldehyde. The metabolism of permethrin in *Pseudomonas fluorescens* SM-1 produced 3-PBA, and 3-phenoxybenzaldehyde was detected in permethrin degradation by *Acinetobacter baumannii* ZH-14 (Maloney et al. 1988; Zhan et al. 2018b). But *Candida pelliculosa* strain ZS-02 degraded bifenthrin without formation of 3-PBA and 3-phenoxybenzaldehyde (Chen et al. 2012a). This isolate first degraded bifenthrin by hydrolysis of the carboxylester linkage to produce cyclopropane carboxylic acid and 2-methyl-3-biphenylyl methanol. Subsequently, 2-methyl-3-biphenylyl methanol was further transformed by biphenyl cleavage to form 4-trifluoromethoxy phenol, 2-chloro-6-fluoro-benzyl alcohol, and 3,5-dimethoxy phenol (Fig. 11.3).

All synthetic pyrethroids contain one to three chiral centers resulting in number of stereoisomers, which exhibit their individual degradation properties (Qin et al. 2006). Generally, the *trans*-isomers of pyrethroids are hydrolyzed more quickly than corresponding *cis*-isomers (Sogorb and Vilanova 2002). A study found that after hydrolysis of ester bond, the R-enantiomer of permethrin was mineralized faster than the S-enantiomer no matter its *trans*- or *cis*-isomers (Qin and Gan 2006).

11.5 Pyrethroid-Degrading Enzymes

Bioremediation using microbial enzymes is considered as an excellent strategy to degrade/detoxify the environmental pollutants (Bharagava et al. 2017, 2018; Saxena and Bharagava 2015, 2017). The hydrolysis of synthetic pyrethroid insecticides by carboxylesterases that belong to the α, β-serine hydrolase multigene family is one of the major metabolic pathways in human, so is it in pyrethroid-degrading microorganisms (Crow et al. 2007; Cygler et al. 1993). To date, several

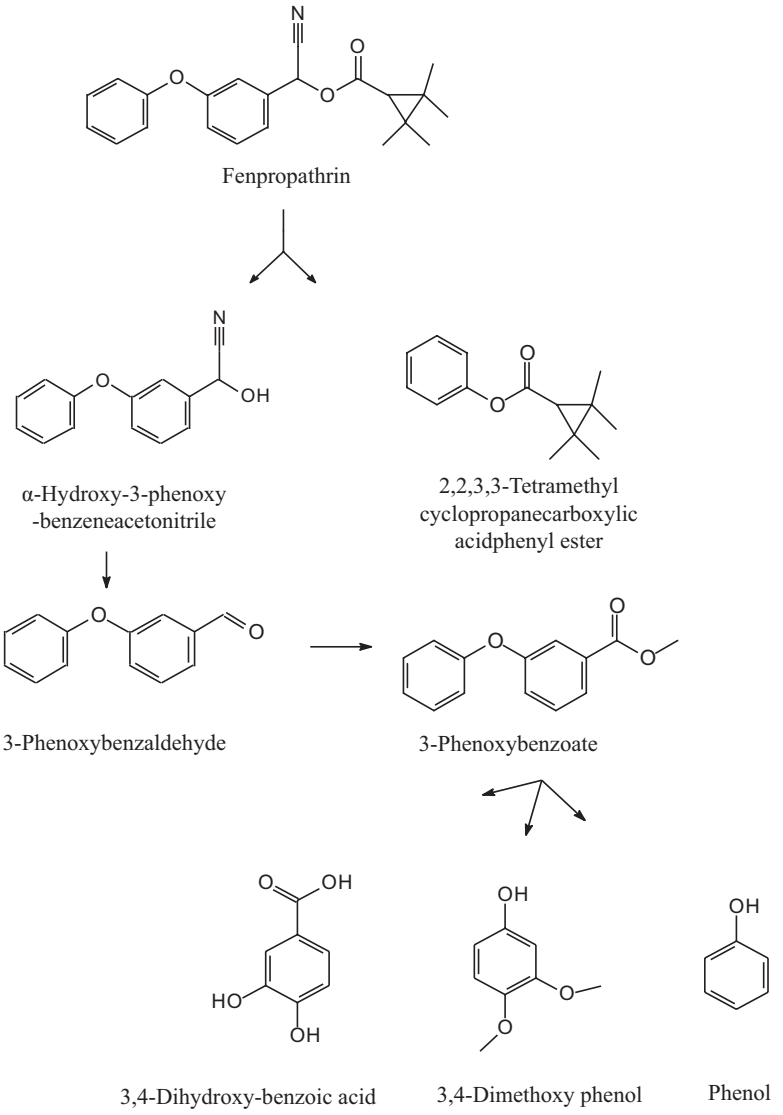

Fig. 11.2 Pathway for degradation of Type II pyrethroid fenpropathrin in *Bacillus* sp. strain DG-02. (Chen et al. 2014)

pyrethroid-degrading enzymes have been successfully purified and characterized from corresponding pyrethroid-degrading microorganisms, including monooxygenase CMO from *Streptomyces* sp. (Chen et al. 2013b), esterase from *Rhodopseudomonas palustris* PSB-S (Luo et al. 2018), aminopeptidase from *Pseudomonas aeruginosa* GF31 (Tang et al. 2017a), carboxylesterase permethrinase from *Bacillus cereus* SM3 (Maloney et al. 1993), EstP from *Klebsiella* sp.

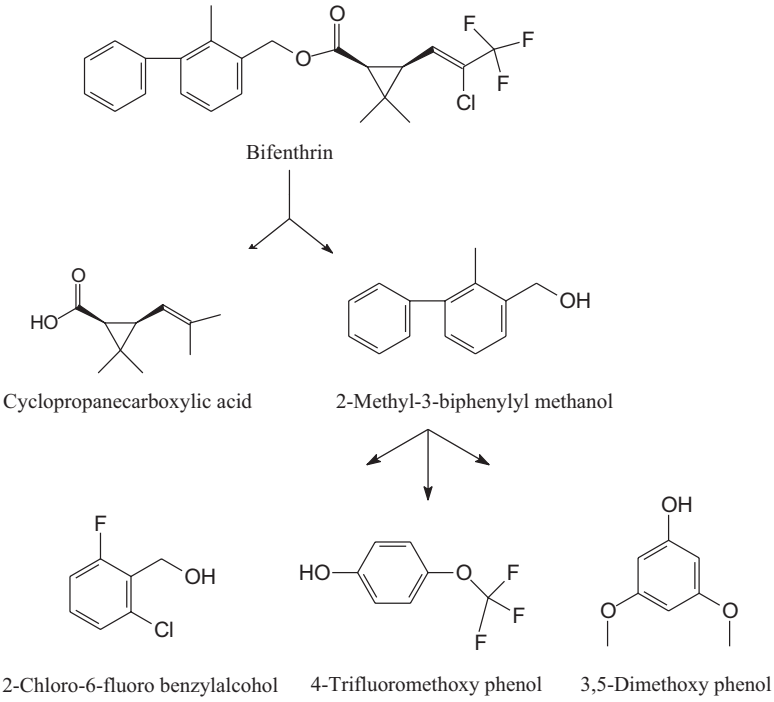

Fig. 11.3 Pathway for degradation of Type I pyrethroid bifenthrin in *Candida pelliculosa* strain ZS-02. (Chen et al. 2012a)

strain ZD112 (Wu et al. 2006), carboxylesterase PytZ and PytY from *Ochrobactrum anthropi* YZ-1 (Ruan et al. 2013; Zhai et al. 2012), carboxylesterase PytH from *Sphingobium* sp. JZ-1(Wang et al. 2009), pyrethroid hydrolases from *Sphingobium* sp. JZ-2 and *Aspergillus niger* ZD11 (Guo et al. 2009; Liang et al. 2005), Pye3 from metagenomic library of soil (Li et al. 2008), and thermostable esterase/lipase Sys410 from Turban Basin metagenomic library (Fan et al. 2012). Most pyrethroid-degrading enzymes are carboxylesterase with monomeric structure, and no cofactors or coenzymes are required for their enzymatic activity. Both monooxygenase and aminopeptidase are firstly reported to degrade β-cypermethrin, which may provide new enzymic resources for bioremediation (Chen et al. 2013b; Tang et al. 2017a). A few pyrethroid-degrading enzymes were able to degrade various pyrethroids, and some enzymes were even capable to hydrolyze ρ-nitrophenyl esters of various fatty acids such as EstP, Pye3, and Sys410 (Fan et al. 2012; Li et al. 2008; Wu et al. 2006). The pyrethroid-hydrolyzing esterase EstP also degraded organophosphorus insecticide malathion, and its molecular mass was about 73 kDa (Wu et al. 2006). Pye3 degraded all pyrethroid insecticides and exhibited superior catalytic properties than carboxylesterases originated from resistant mammals and insects (Li et al. 2008).

11.6 Conclusions and Future Perspectives

Bioremediation is an eco-friendly, inexpensive, effective, and efficient strategy to alleviate environmental contamination, which refers to the transformation of the toxic contaminants to inactive and harmless compounds by microorganisms with effective enzymes (Chen et al. 2011e, 2012c). Almost all pyrethroid-degrading isolates displayed high degradation efficiency in the liquid medium under laboratory conditions; however only part of them have been found to utilize pyrethroids in the soils and remediate the contaminated soils in laboratory. The most promising pyrethroid-degrading microorganisms should be available not only in the laboratory but also in the natural environments. Generally, it is not suitable to use the degrading strains directly in the natural environment, because the degradation efficiency is subject to environmental conditions and hard to control. Coexpression of two pesticide-degrading enzymes in a genetically engineered bacterium expands the degradation spectrum, which is potential to eliminate multiple contaminants in the same time (Lan et al. 2006). Enzymatic degradation is also promising to remove pyrethroids in the environment, because enzymatic bioremediation may be more efficient than microbial bioremediation. Although plentiful pyrethroid-degrading microorganism has been isolated, there is still a lot of work to do for their application in bioremediation of pyrethroid-contaminated environments.

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Chapter 12
Microbial Degradation of Polyethylene: Recent Progress and Challenges

Shiv Shankar, Shailja Singh, Anuradha Mishra, Manju Sharma, and Shikha

Abstract Polythene or polyethylene is the most commonly used polymer for the manufacturing of geomembranes, plastic bags, containers, bottles, and plastic films. The main properties of the plastics suited for its application include durability, inertness, light weight, flexibility, and low cost. Over the last three decades, the indiscriminate use of the polyethylene in transportation, packaging operations, agriculture, and industry has increased the problem of its accumulation in soil sediments and aqueous streams. Accumulation of polyethylene has emerged as a significant environmental issue nowadays. The improper disposal of solid waste containing plastic has increased the extent of the problem manifold. Used plastic packing materials and improperly disposed plastic bags prevent the entry of water and air into the earth, thereby causing depletion of groundwater and negative impacts on soil fauna. Adverse biochemical effects are caused to soil and water fauna upon ingestion of these toxic compounds. Upon unintentional ingestion, polythene causes intestinal blockage in aquatic biota like fishes, sea turtles, and seabirds. Existing conventional physical and chemical methods for the disposal of polyethylene are costly and result in formation of toxic compounds. Biodegradation of plastic is proposed as a more environmentally sound technology for disposal of plastic waste as compared to its recycling, incineration, and landfilling. In the light of the aforesaid context, the present chapter is an attempt to highlight various issues of microbial degradation of plastic, viz., general chemistry of polyethylene, its classification, role of microbes and their enzyme systems in degradation of polyethylene, and stages and obstacles in microbial degradation of the polyethylene.

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12.1 Introduction

Polythene or polyethylene is the most commonly used plastic across the world. Globally, 80 million tons of the plastic is produced every year. Different types of plastic are being used for the manufacturing of geomembranes, plastic bags, containers, bottles, and plastic films (Ahmed et al. 2018). Majority of plastics have the general formula $(C_2H_4)_n$. It is generally a combination of similar polymers of ethylene with varying numbers of *n*. Plastic was synthesized accidently in 1898, when a German chemist Hans von Pechmann was investigating diazomethane.

In the year 1933, Eric Fawcett and Reginald Gibson synthesized first industrially practical plastic accidently at Imperial Chemical Industries (ICI), Northwich, England.

The synthesized plastic was diazomethane which was an obnoxious unstable substance unsuitable for frequent industrial applications. They prepared a white and waxy material by combining benzaldehyde and ethylene at high pressure. In the year 1935, another worker at ICI named Michael Perrin converted this accidental discovery into a reproducible process of synthesis of low-density polyethylene for industrial and other applications in

Over the last three decades, the indiscriminate use of the polythene in transportation, packaging operations, agriculture, and industry has increased the problem of accumulation of polyethylene in soil sediments and aqueous streams. The main properties of the plastics suited for its application include durability, inertness, light weight, flexibility, and low cost. Its principal disadvantage is its recalcitrance rendering it non-biodegradable. Globally, 0.15 billion tons of synthetic polymer is produced. The use of plastic is growing at the rate of 12% annually (Das and Kumar 2014; De Bomfim et al. 2019). The rate, at which the plastic is being accumulated in the environment, is deemed a serious environmental issue by scientists. In environment, the plastic is accumulating at the rate of 25 million tons/year (Kaseem et al. 2012). In the United States and Germany, plastic constitutes 20% of municipal solid waste (MSW), while in Western Europe and Australia it constitutes 25% of MSW. Plastic is a highly recalcitrant material. Its complete degradation takes about 1000 years. Burning of plastic waste results in emission of greenhouse gases (carbon dioxide) and dioxins (Pramila and Vijaya Ramesh 2011).

Such gases result in hazardous health risks like pulmonary diseases and cancer to human beings.

Used plastic packing materials and improperly disposed plastic bags prevent the entry of water and air into the earth, thereby causing depletion of groundwater and negative impacts on soil fauna. In addition, polythene degradation under sunlight results in formation of toxic compounds which contaminate soil and water ecosystem. Adverse biochemical effects are caused by soil and water fauna ingesting these toxic compounds. Upon unintentional ingestion, polythene causes intestinal

blockage in aquatic biota like fishes, sea turtles, and seabirds. Soil pollution affects fertility of soil by preventing the microbial decomposition of soil organic matter. Plastic in soil also interrupts the movement of soil water (Denuncio et al. 2011; Kumar et al. 2013).

Over the past few years, the contamination of environment with polyethylene has emerged as a significant environmental problem. The improper disposal of solid waste containing plastic has increased the extent of the problem. Microbial degradation of plastic at disposal site is proposed as a more environmentally sound practice as compared to its recycling, incineration, and landfilling. Biodegradation of polyethylene is a sustainable technology which results in formation of less toxic substances as compared to physicochemical methods of plastic disposal (Ahmed et al. 2018).

In the light of the aforesaid context, the present chapter is an attempt to highlight various issues of microbial degradation of plastic, viz., general chemistry of polyethylene, its classification, role of microbes and their enzyme systems in degradation of polyethylene, stages and obstacles in microbial degradation of the polyethylene, etc.

12.2 Properties of Plastic

There are several thermal, chemical, mechanical, electrical, and optical properties of the polyethylene.

12.2.1 Thermal Properties

Melting point of polythene varies with the type of the polyethylene. Generally, low-density polyethylene melts at 80 °C, but common grades of high- and medium-density polyethylene have melting point ranging from 105 to 115 °C.

12.2.2 Mechanical Properties

Strength, hardness, and rigidity of the polythene are low, but it has high ductility, friction, and impact strength. Under consistent force, it shows strong creep, which can be lowered by summation of small fibers. The surface of the polythene is waxy. Due to its symmetrical structure, polyethylene has the tendency to undergo partial crystallization. High crystalline nature of the polythene increases its density as well as mechanical stability.

12.2.3 Chemical Properties

The chemical behavior of polyethylene is similar to paraffin. It comprised of high-molecular-weight, nonpolar, and saturated hydrocarbons which are not covalently linked with each other. Majority of high-density polyethylene (HDPE), medium-density polyethylene, and low-density polyethylene are chemically resistant. These polyethylenes are not easily oxidized or reduced. Polyethylene crystals do not dissolve at room temperature, but these are dissolved easily in aromatic solvents like xylene and toluene or in chlorinated solvents such as trichlorobenzene and trichloroethane. Polyethylene is hydrophobic in nature and has low permeability for water vapors and gases like carbon dioxide and oxygen. Polyethylene may undergo brittling when it is exposed to sunlight. Carbon black is generally used as a UV stabilizer. Polyethylene burns slowly with generation of paraffin odor. It keeps on burning even after removal of flame source forming drip.

12.2.4 Electrical Properties

Polyethylene acts as a good insulator for electricity rendering good tracking resistance; however, it gets easily charged electrostatically. The tendency to get electrically charged can be reduced by additions of carbon black, graphite, or antistatic agents.

12.2.5 Optical Properties

Polyethylene can be transparent opaque and milky opaque depending on the thickness of the film and thermal history. Low-density polyethylene has highest transparency, while high-density polyethylene has least transparency. The transparency of the polyethylene is reduced if the crystallites are larger than the wavelength of the visible spectrum.

12.3 Classification of the Polyethylene

On the basis of density and branching, polyethylene has been categorized into different types. Variables such as type and extent of branching, molecular weight, and structure of the crystal determine its mechanical properties. High-density polyethylene (HDPE), linear low-density polyethylene (LLDPE), and low-density polyethylene (LDPE) are the widely used and sold categories of the polyethylene.

There are several types of polyethylene:

- Ultra-low-molecular-weight polyethylene (ULMWPE or PE-WAX)
- Ultra-high-molecular-weight polyethylene (UHMWPE)
- High-molecular-weight polyethylene (HMWPE)
- High-density cross-linked polyethylene (HDXLPE)
- High-density polyethylene (HDPE)
- Low-density polyethylene (LDPE)
- Medium-density polyethylene (MDPE)
- Cross-linked polyethylene (PEX or XLPE)
- Linear low-density polyethylene (LLDPE)
- Chlorinated polyethylene (CPE)
- Very-low-density polyethylene (VLDPE)

12.4 Degradation of Polyethylene

12.4.1 Thermal Degradation

Different types of polyethylene can be chemically degraded during processing of the polymer under the influence of the heat. Thermal degradation of polyethylene at high temperature leads to deleterious effects on thermal properties and mechanical as well as surface morphology of polyethylene-based plastic products due to thermal degradation.

12.4.2 Photooxidative Degradation

Ultraviolet radiation of low wavelength results in cleavage of chemical bonds in polyethylene, thereby leading to its photodegradation. Photooxidation is an autooxidative process based on free radical. Chromophoric species present in polyethylene such as hydroperoxides and carbonyl groups are responsible for the absorption of ultraviolet radiation (Khabbaz et al. 1999). These chemical species are added intentionally into polymer structures during the processing of polyethylene.

12.4.3 Environmental Stress Cracking (esc)

In response to mechanical stress, polyethylene forms crazes in the presence of polar vapors of liquid and detergents which are called environmental stress cracking (esc).

12.4.4 Hydrolytic Degradation

Due to the degradation of the principal chain scission, hydrolysis of polyethylene occurs.

At low pH, polymers like polyacetals, polycarbonates, and polyamides can be degraded in the medium. Majority of biodegradable polymers in general and synthetic polymers in particular are degraded by hydrolysis.

12.4.5 Chemical Degradation

Polyethylenes are chemically degraded by corrosive liquids and gases except polyether ketone (PEEK) and polytetrafluoroethylene (PTFE). Several environmental pollutants such as ozone, oxides of sulfur, and acids like nitric acid, sulfuric acid, and hydrochloric acid attack and degrade polyethylene polymers.

12.4.6 Degradation of Plastic by Radiation and Weathering

Majority of high-energy radiations like X-rays, alpha rays, and beta rays cause degradation of polyethylene. However, the rate of the degradation of polyethylene is lower in the case of lower-energy (UV) radiation.

12.4.7 Biodegradation of Polyethylene

Biological degradation is a process wherein microbes like algae, bacteria, fungi, yeasts, and their enzyme systems consume the polymer as source of energy resulting in the degradation of polyethylene (Amaral-Zettler 2019). Biodegradation of the plastic is caused by enzymatic activities that lead to a chain cleavage of the polymer into monomers. Microbes colonize on the surface of the polyethylene films forming a biofilm and use it as a sole source of carbon leading to its partial degradation. For the formation of biofilm on the surface of the polyethylene, cell surface hydrophobicity of microbes play a very important role in biodegradation of polyethylene. After forming the biofilm on the surface of the polyethylene, they start using the polymer as a sole source of carbon. In preliminary stages of the degradation, the cleavage of main chain results in the formation of low-molecular-weight fragments (oligomers), dimers, or monomers. The extracellular enzymes secreted by the microbes catalyze the process of the degradation (Sangeetha Devi et al. 2019).

The process of biodegradation is carried out by different types of microorganisms like bacteria and fungi which convert complex organic matter as well as synthetic polymers (polyethylene and polyurethane) into simple units (Fig. 12.1). Microorganisms have the capability to derive nutrition from synthetic polymers.

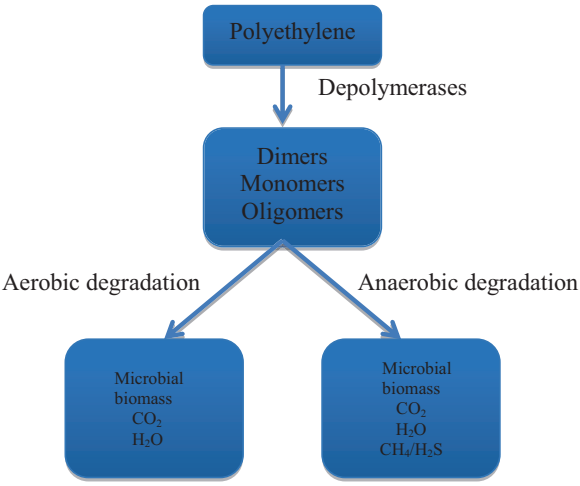

Fig. 12.1 Aerobic and anaerobic conditions in polyethylene biodegradation

12.5 Properties of Polyethylene as Obstacles for Their Biodegradation

The microbial degradation of polyethylene is affected by different biological, chemical, and physical factors in nature. High crystallinity, hydrophobicity, and surface topography hamper the biodegradation of polyethylene (Thompson et al. 2009; Restrepo-Flórez et al. 2014). Polymers like PVC, PE, PP, and PS are highly resistant to degradation than PET-based polymers. The chemical bonds present in PET are more susceptible for hydrolysis (Krueger et al. 2015). Oxidation of stable carbon-carbon double bond in polyethylene is required for its degradation (Restrepo-Flórez et al. 2014). Degradation of polyethylene is affected by environment factors like oxygen, UV radiation, and temperature. The degradation of polyethylene is also impacted by the presence of chemical oxidants (Arkatkar et al. 2010). Hydrophobicity and high molecular weight reduces the formation of biofilm and assimilation of polyethylene by microbes. The assimilation of polyethylene by microbes is reduced by hydrophobic nature and high molecular weight of the polyethylene (Wei et al. 2014; Restrepo-Flórez et al. 2014; Krueger et al. 2015). The effective adsorption and catalytic efficiency of microbial enzymes involved in polyethylene degradation are reduced by hydrophobic surface and low surface-to-volume ratio of the polyethylene (Sammond et al. 2014). Small particle size of polyethylene is more accessible to microbes and their enzymes. The smaller-sized polyethylene particles ranging from 0.25 mm to 0.5 mm have been demonstrated to be easily degraded by microbial polyester hydrolase (Gamerith et al. 2017). The degree of crystallinity has profound impact on the degradability of the polyethylene. Polyethylene containing amorphous region is more susceptible to microbial degradation than the polyethylene with crystalline region (Restrepo-Flórez et al. 2014). The rate of the degradation of low crystalline PET film by a fungal polyester hydrolase has been reported to be linear insinuating crystalline region is also attacked by the enzyme (Ronkvist et al. 2009).

12.6 Microorganisms and Their Enzyme Systems Involved in Degradation of Polyethylene

The term microorganism covers several groups of living beings (e.g., bacteria, algae, and fungi), which have a microscopic size and, for the most part, are unicellular. The biodegradation of polyethylene by microorganisms is an outcome of the production of enzymes which catalyze breakdown of polymers in order to get nutrient for their survival. Environmental factors like ambient temperature and availability of nutrients and water are essential for improved degradation of polyethylene by microorganisms. Enzymes as biological catalysts perform biochemical reactions.

The interaction of enzyme and substrate takes place at active site (specific for a given substrate or a series of substrates) resulting in formation of specific product. Enzymes present in microbial cell may require cofactors like metal ions and organic cofactors for optimum enzyme activity. The amount of the enzymes produced by the microbes may vary based on species to species and strains to strains. A wide spectrum of microbial enzymes is involved in degradation of different types of polyethylene. Some polyethylene-degrading prominent enzymes are as under:

12.6.1 Laccases

Laccases (benzenediol/oxygen oxidoreductase, E.C 1.10.3.2) are oxidoreductases produced by bacteria, fungi, and actinomycetes. They are also distributed in some insects and plants. Among fungi, white rot fungi are the dominant producers of the laccase. Laccases are deemed as a promising green enzyme which catalyzes the oxidation of different aromatic and nonaromatic compounds in the presence of oxygen. During oxidation of different substrates, they do not result in the formation of any toxic intermediate. This property of laccase makes it suitable for different biotechnological applications such as in detection of phenol, delignification and bleaching of the wood pulp, and removal of organic dyes from waste water (Shankar and Shikha 2012).

Several authors have reported the enzymatic degradation of polyethylene by microbial laccases (Table 12.1).

12.6.2 Lipase

Lipase (EC 3.1.1.3) produced from *Rhizopus delemar* under submerged culture conditions and polyurethane esterase from *Comamonas acidovorans* have been reported to degrade low-molecular-weight polylactic acid.

Table 12.1 Laccase-mediated degradation of polyethylene

S.N.	Source of laccase	Polyethylene	References
1	*Trametes versicolor*	LDPE	Fujisawa et al. (2001)
2	*Rhodococcus ruber*	LDPE	Santo et al. (2013)
3	*Bacillus cereus*	LDPE	Sowmya et al. (2014)
4	*Pleurotus ostreatus*	LDPE	Da Luz et al. (2015)
6	*Cochliobolus* sp.	PVC	Sumathi et al. (2016)
7	*Pseudomonas aeruginosa*, *Bacillus* sp. and fungi *Fusarium graminearum*	LDPE	Ganesh et al. (2016)
8	*Pleurotus ostreatus*	LDPE	Gómez-Méndez et al. (2018)

12.6.3 Papain and Urease

Papain (ECv3.4.22.2) and urease (EC 3.5.1.5) have been found to degrade medical polyester polyurethane. Papain catalyzes the hydrolysis of urethane and urea linkage resulting in the formation of free hydroxyl and amine group.

12.6.4 Proteases

Protease (ECv3.4.21) produced from *Brevibacillus* spp. and *Bacillus* spp. has been shown to degrade polyethylene.

12.6.5 Serine Hydrolase

Serine hydrolase (ECv3.1) uses polyurethane as a substrate.

The precise mechanism of microbial degradation of polyethylene is not well understood. In literature, two approaches have been elaborated. First approach highlights degradation of polyethylene using identified pure cultures of microbes under defined environmental conditions. The use of this approach helps in understanding the impact of environmental factors on microbial degradation of polyethylene. However, this approach does not clearly depict collective role of microbial strains in degradation of polyethylene (Tribedi and Sil 2013).

In second approach, complex microbial communities are employed for degradation of polyethylene under complex and natural environmental samples like soil, water, and compost (Nowak et al. 2011). For the last few decades, different types of microorganisms have been reported to degrade polyethylene. Few promising polyethylene-degrading microbes (bacteria and fungi) have been presented in Tables 12.2 and 12.3. Until now, members from 17 bacterial genera and 9 fungal genera have been reported to degrade polyethylene. Employment of molecular character-

Table 12.2 Polyethylene-degrading bacteria

S.N.	Bacteria	Polyethylene	References
1	*Rhodococcus ruber*	PE	Sivan et al. (2006)
2	*Pseudomonas* sp., *Alcanivorax* sp., *Tenacibaculum* sp.	PCL	Sekiguchi et al. (2011)
3	*Arcobacter* and *Colwellia* spp.	LDPE	Harrison et al. (2011)
4	*Pseudomonas* sp. E4	PE	Gyung Yoon et al. (2012)
5	*Pseudomonas aeruginosa* PAO1 (ATCC 15729), *Pseudomonas aeruginosa* (ATCC 15692), *Pseudomonas putida* (KT2440 ATCC 47054), and *Pseudomonas syringae* (DC3000 ATCC 10862	LDPE	Kyaw et al. (2012)
6	*Kocuria palustris* M16, *Bacillus pumilus* M27, and *Bacillus subtilis* H1584	LDPE	Harshvardhan and Jha (2013)
7	*Rhodococcus ruber* C208	UV-irradiated PE films	Santo et al. (2013)
8	*Chelatococcus* sp. E1	LMWPE	Jeon and Kim (2013)
9	*Pseudomonas aeruginosa* E7	PE	Yang et al. (2014)
10	*Vibrio alginolyticus*, *Vibrio parahaemolyticus*	PVA-LLDPE	Raghul et al. (2014)
11	*Stanieria*, *Pseudophormidium*	PET	Oberbeckmann et al. (2014)
12	*Pseudomonas* sp.	LDPE	Bhatia et al. (2014)
13	*Bacillus amyloliquefaciens*	LDPE	Das and Kumar (2015)
14	*Pseudomonas* sp. AKS2	LDPE	Tribedi et al. (2015)
15	*Pseudomonas* sp.	PE	Tribedi et al. (2015)
16	*Phormidium*, *Lewinella*	PET	Oberbeckmann et al. (2016)
17	*Bacillus subtilis*	PE	Vimala and Mathew (2016)
18	*Lysinibacillus fusiformis* strain VASB14/WL and *Bacillus cereus* strain VASB1/TS	PE	Shahnawaz et al. (2016)
19	*Zalerion maritimum*	PE	Paco et al. (2017)
20	*Bacillus subtilis* V8, *Paracoccus aminophilus* B1 4-, *Pseudomonas putida* C 2 5, *Pseudomonas aeruginosa* V1, and *Acinetobacter calcoaceticus* V4	LDPE	Pathak and Kumar (2017a)
21	*Zalerion maritimum*	PE	Paco et al. (2017)
22	*Bacillus*, *Pseudomonas*	LDPE	Pathak and Kumar (2017b)

(continued)

Table 12.2 (continued)

S.N.	Bacteria	Polyethylene	References
23	*Klebsiella pneumoniae* CH001	HDPE	Awasthi et al. (2017)
24	*Microbacterium awajiense*, *Rhodococcus jostii*, *Mycobacterium vanbaalenii*, and *Streptomyces fulvissimus*	LDPE	Huerta Lwanga et al. (2018)
25	*Sphingobacterium moltivorum*	LDPE	Montazer et al. (2018)
26	*Brevibacillus* sp. and *Aneurinibacillus* sp.	LDPE	Skariyachan et al. (2018)
27	*Bacillus vallismortis* bt-dsce01, *Pseudomonas protegens* bt-dsce02, *Stenotrophomonas* sp. bt-dsce03, and *Paenibacillus*	HDPE	Skariyachan et al. (2018)
28	*Bacillus* spp.	HDPE	Sangeetha Devi et al. (2019)

Table 12.3 Polyethylene-degrading fungi

S.N.	Fungi	Polyethylene	References
1	*Lasiodiplodia theobromae*	LDPE	Sheik et al. (2015)
2	*Aspergillus* spp.	HDPE	Sangeetha et al. (2015)
3	*Aspergillus clavatus* strain JASK1	PE	Gajendiran et al. (2016)
4	*Zalerion maritimum*	PE	Paço et al. (2017)
5	*Penicillium oxalicum* NS4 (KU559906) and *Penicillium chrysogenum* NS10 (KU559907)	HDPE & LDPE	Ojha et al. (2017)
6	*Trichoderma viride* and *Aspergillus nomius*	LDPE	Munir et al. (2018)
7	*Aspergillus* sp. and *Penicillium* sp.	LDPE	Alshehrei (2017)
9	*Streptomyces* species	PET	Farzi et al. (2019)

ization techniques is expected to increase this number. Molecular characterization techniques offer a wide spectrum for the evaluation of the composition of microbial communities in general and fraction of non-culturable microorganisms in particular.

12.7 Different Stages in Biodegradation of the Plastic

There are four important steps involved in the degradation of plastic:

(a) Biodeterioration
(b) Bio-fragmentation
(c) Assimilation
(d) Mineralization

12.7.1 Biodeterioration

The process of biodeterioration involves few alterations in physical and mechanical properties of the plastic. This process incepts the formation of microbial biofilm on the surface and inside the polyethylene. However, the formation of the biofilm depends on structure and composition of the polyethylene. Environmental conditions also play a crucial role in biodeterioration of the polyethylene. Microbes secrete high-molecular-weight extracellular polymeric substances which control the formation of biofilm which helps in its attachment to the surface of the plastic. The structural integrity of microbial biofilms is maintained by extracellular polymeric substances secreted by the microbes.

EPS make their way to the pores wherein microorganism grows resulting in an increase in pore size leading to the formation of cracks. Cracks reduce the mechanical strength of the polyethylene. Chemo-lithotrophic bacteria present in biofilm formed over the surface of the polyethylene secrete some acids like HNO_2, HNO_3, and H_2SO_4 which lower the pH inside the pores resulting in alterations in the microstructure of the plastic.

12.7.2 Bio-fragmentation

The fragmentation of polymers at low pH results in monomerization of polyethylene. In the biodegradation of polyethylene, fragmentation plays a very important role. Without fragmentation, polymers are not capable of entering into cytoplasm via plasma membrane. Different microbes produce extracellular enzymes which catalyze the monomerization of polyethylene.

12.7.3 Assimilation

Monomers formed during the process of fragmentation are ingested by microbial cells and undergo oxidation via catabolic reactions. The process of assimilation involves addition of atoms inside the cells of microorganisms. At this step, the process of degradation of polyethylene is not completed.

12.7.4 Mineralization

Organic substances are converted into inorganic substances like water and carbon dioxide during mineralization. Different types of secondary metabolites are produced during the process of assimilation. The unutilized secondary metabolites are

transported out of the microbial cell which can be mineralized by other microbes into metabolites like CO_2, N_2, CH_4, and H_2O as a final product.

12.8 Impact of Microbial Activity on Polyethylene

The properties of the polyethylene are severely affected by microbes colonizing the surface of the polyethylene. Generally, the impact of microbial activity on polyethylene is gauzed on the basis of seven characteristics which include hydrophobicity/hydrophilicity, functional groups on the surface, surface topography, crystallinity, molecular weight distribution, mechanical properties, and mass balance.

12.8.1 Hydrophobicity/Hydrophilicity

In any material, the nature of the functional groups, their exposition, and concentration determine its hydrophobicity and hydrophilicity. It has been reported that microorganisms prefer to colonize hydrophilic surfaces as compared to hydrophobic surface for biofilm formation. Hydrophobicity of a polymer is determined on the basis of contact angle of the surface with a pro liquid like water. Hydrophilic surfaces generally have smaller contact angle with water (Roy et al. 2008; Sudhakar et al. 2008).

12.8.2 Functional Groups on the Surface

FTIR is generally used for the analysis of different functional groups present on the surface of the polyethylene. During functional group analysis of polyethylene, function groups such as carbonyls (1715 cm^{-1}), vinyls (1650 cm^{-1}), and esters (1740 cm^{-1}) are taken into more consideration. Researchers have reported that such functional groups are more evident on the surface of the polyethylene undergoing microbial degradation. In the presence of the microorganisms, the increase in the number of double bonds has been observed (Nowak et al. 2011). The investigation of polyethylene surface is important since the functional groups susceptible for oxidation are much easily attacked by microbes during the degradation of polyethylene. Such oxidized groups also increase the hydrophilicity of the polyethylene surface (Tribedi and Sil 2013).

12.8.3 Topography of the Surface

Surface topography of the polyethylene is altered when microbes colonize its surface. Different microorganisms develop microcolonies over the surface of the polyethylene as well as hyphal structures which have also been shown to invade the surface of the polyethylene (Tribedi and Sil 2013).

12.8.4 Crystallinity

Polyethylene is comprised of crystalline microstructures and is a semicrystalline polymer. Amorphous region in polyethylene is firstly attacked by the microorganisms as compared to crystalline region. It has been reported that the percent crystallinity of polyethylene increases due to microbial degradation of amorphous region.

12.8.5 Molecular Weight of the Polyethylene

The microbial degradation of polyethylene is limited due to high molecular weight. Microbes prefer average and low-molecular-weight polyethylene as compared to high-molecular-weight polyethylene. Some authors have reported contradictory results (Fontanella et al. 2010). Few authors have demonstrated that the principal factor affecting the molecular weight is the effect of environmental factor such as ultraviolet radiation instead of direct microbial attack (Fontanella et al. 2010).

12.8.6 Mechanical Properties

Majority of studies on the effect of microbial activity on the degradation of polyethylene have been focused on thin films. The impact of microbial activity on thick-walled polyethylene has not been studied extensively. Therefore, the change in mechanical strength of the polyethylene due to microbial activity is still an active research area.

12.9 Conclusion and Future Perspectives

Accumulation of plastic in environment has emerged as significant environmental issue across the world. Its sustainable use and safe disposal are a need of the hour. Microbial degradation of polyethylene is an eco-friendly means to dispose plastic in an environmentally sound manner.

Several authors have reported that the pace of microbial degradation of polyethylene by pure strains as well as by microbial consortia is very slow. The microbial degradation of polyethylene is significantly affected by abiotic factors like ultraviolet radiation, physicochemical properties of the polymer, and different oxidizing agents. Such factors govern the microbial utilization of polyethylene. Research in the area of biodegradation of polyethylene is descriptive in nature. Only some researchers have demonstrated the mechanism of degradation of polyethylene including role of microbial enzymes on degradation of polyethylene. Down the line, there is a need to adopt more mechanistic approach in degradation of polyethylene. The mechanism of polyethylene degradation can be well elucidated if there is a precise identification of enzymes responsible for the degradation of polyethylene. Identification of the fate of the polyethylene inside microbial cell is also a significant area of the research. However, some findings reveal that the polyethylene inside microbial cell is metabolized in tricarboxylic acid cycle (TCA). There is a need to address the issue of impact of microbial degradation on surface morphology of polyethylene. Several authors have reported that the amorphous region is more susceptible for microbial degradation than crystalline region. The degradability of crystalline region by microbes has not been researched extensively. Ultimately, it would be of great importance to correlate the role of environmental factors with microbial degradation of polyethylene.

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Chapter 13
Biodegradation of Polychlorinated Biphenyls

Soumya Nair and Jayanthi Abraham

Abstract Polychlorinated biphenyls (PCBs) are organic molecules that are stable in nature. They were widely used in the early 1940s. PCBs have entered the ecosystem due to their wide applications via legal and illegal use. Due to its insoluble nature, these environmental contaminants are persistent in the environment, thereby contaminating different ecosystem. This affects the flora and fauna. The environmental persistence of these chlorinated molecules results mainly in the inability of aquatic fauna and soil biota to utilize the compound at a substantial rate. PCBs pose a toxicological risk to the environment and the human due to its ubiquitous distribution. PCBs are linked with many genetic diseases such as cancers, birth defects, tumours, etc. to name a few. Conventional methods of removal such as incineration or desorption are unsafe, expensive and time consuming. The application of microorganisms in the degradation process of PCBs is an excellent alternative which began in the early 1990s. Much research has been conducted on PCB degradation assisted by the microorganisms to determine the methods by which the degradation rate can be improvised. PCB molecule can be utilized and degraded using the aerobic and the anaerobic method. The route of degradation completely depends on the PCB molecule, type of microbial strain, and the interaction between them. The current book chapter reviews the different ways via which the PCB molecule can be biodegraded.

Keywords Polychlorinated biphenyls · Incineration · Desorption · Bioaccumulation · Carcinogenic · Microbe-mediated degradation or biodegradation

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13.1 Introduction

Polychlorinated biphenyls or PCBs are organic compounds with their characteristics analogous to that of dichlorodiphenyltrichloroethane (DDT) (Nemerow and Agardy 1998). They are produced on a commercial scale by the catalytic control of chlorination of the biphenyls, thereby generating a complex mixture of different isomers. The degree of chlorination differs yielding above 200 products (congeners) (UNEP 1999). PCB congeners which are produced with the same number of chlorine atoms in it are known as homologs. The production of PCBs first started in the early 1930s which gradually increased in the 1970s (Lee 1995). These PCBs were able to produce isomers containing 60–90 congeners (Watts 1998). Such chlorinated biphenyls were in extensive use for their application in the industrial sector because of their excellent physico-chemical properties. PCBs are marketed in the USA under the trademark "Aroclor" which is branded by a four-digit identification number. Amongst the four, the first two digits signify the number of carbon (C) atoms, whereas the last two digits signify the amount of chlorine present in the mixture in percentage. Apart from Aroclor, PCBs have other international trade names such as Kanechlors in Japan, Pyralene in France, Fenclorin in Italy and Clophen in West Germany (Watts 1998). Statistical analysis suggests that between the 1930s and 1980, more than 6 billion kilograms of chlorinated biphenyls were used in and around North America alone (Laukers 1986). Out of this, 15% of the PCBs enter the ecosystem through legal and illegal practice and disposal by industrial or anthropological use (Cookson Jr 1995). A portion of it also enters the environment due to accidental releases mainly via leakage in the production site (Erickson 1997). PCBs are highly resistant to degradation by any means. As a result, they tend to persist in soils and wetlands for many years to come. Moreover, these chlorinated compounds are lipophilic in nature. As a result, they cause bioaccumulation in the cells of flora and fauna, which indirectly gets transferred to the food chain. Due to the growing concerns of the impact of PCBs on the ecosystem, their persistence in nature, manufacturing and usage, it was banned in Sweden and Japan in the year 1970 and 1972, respectively. In the USA, the production of PCBs was banned in the year 1976 under the Toxic Substances Control Act. According to the act, the electrical applications of PCBs have been discontinued. The severe requirements for handling and disposal of these chemical compounds have been identified and quantified. Recently, the Chemical Treaty based on the persistent organic pollutants (POPs) states that PCBs are priority chemicals which will eventually be eliminated by the year 2025 (Global chemical treaty 2001). The major reason behind the environmental persistence of these chlorinated compounds is due to the inability of the soil and the wetland biota to degrade the compound at a substantial rate. The ongoing studies on the degradation of PCBs, with special reference to the microbe-mediated degradation, show that the compound can either be converted to lesser toxic chemical or can be mineralized. In this regard, there are two microorganism-mediated degradation processes of PCBs. They are the anaerobic reductive

dechlorination and the aerobic oxidative degradation (Cookson Jr 1995). The current book chapter reviews the related literature and studies on the microbial-mediated PCB degradation.

13.2 Properties of PCBs

Polychlorinated biphenyls are made up of a biphenyl nucleus surrounded by 1–10 chlorine atoms. The elementary structure of PCBs was demonstrated by Wiegel and Wu in the year 2000. The properties of each PCB compound are directly dependent on the number of chlorine atoms (degree of chlorination). The industrial scale production of the PCBs ranges from highly mobile colourless liquids that are oily to viscous and dark-coloured liquids (yellow to black resins). Usually PCB compounds with lesser number of chlorine atoms (i.e. mono-, di-, tri- and tetra-chlorinated compounds) tend to be colourless oily liquids. For example, penta-chlorobiphenyls are very viscous, honey-coloured oils (Sylvestre 1985). But when it comes to PCB compounds with higher degree of chlorination, they are greasy or waxy in nature. For such compounds, flash points can vary as low as 140 °C–200 °C. However, it was observed that most PCBs have no flash points at all. This was concluded based on the measurements by the standard test. The vapours of such chlorinated compounds are invisible and have a characteristic strong odour (Safe management of PCBs, code of practice 1989). PCBs are less soluble in water but extremely soluble in oils and fats. Their solubility in water decreases with increase in the number of chlorine atoms present in its structure. For example, the solubility ranges from 6 ppm for a mono-chlorobiphenyl to 0.007 ppm for an octa-chlorobiphenyl (Albro and McKinney 1981). Most importantly, for a deca-chlorobiphenyl compound, its solubility is twice that of octa-chlorobiphenyl, in spite of the fact that the former has higher chlorine content. The solubility also varies amongst the PCB congeners with the same number of chlorine atoms (Sawney 1986). The properties of PCBs which makes it valuable for industrial applications includes stability even at higher temperature, its inertness to most of the chemical reactions, inflammable, high electrical resistivity and low acute toxicity (Hutzinger 1974).

13.3 Uses of PCBs

PCBs are exploited extensively in a wide range of industrial applications for several decades, for example, dielectrics in capacitors, oil in transformers, hydraulic fluids in hydraulic tools and heat exchange liquids. PCBs are also used as lubricants for turbines and motors and in metal treatment, as plasticizers, adhesives, surface coatings, carbonless copy paper, pesticides, dyes and waxes (National Research Council 1979).

13.4 Sources of PCBs

There are no records of known natural sources of the polychlorinated compounds. As mentioned earlier, these compounds persist in the ecosystem and are found in air, water, soil and food. Due to the accidental release, use or disposal, PCBs tend to enter the environment via air, water and soil. These chlorinated compounds also tend to get released during their manufacturing process or leak during transportation and from fire outbreaks. PCBs have the ability to travel long distances via air and get deposited in areas remote to where they were released (Agency for toxic substances and disease registry 1993). Municipal and medical hazardous waste combustion or incineration accounts for a substantial portion of PCB release in the environment. Additional sources of PCB emissions include its treatment, storage and disposal facilities or landfills, hazardous waste disposal sites, steel and iron recovery facilities, accidental releases and environmental sinks (U.S. Environmental protection agency 1996). PCB concentration in aquatic system near shorelines with human activity is generally higher. The major sources of PCB contamination in surface waters are from environmental cycling, i.e. from contaminated sediment, air or land. It can be stated that the sediments at the bed of wetlands act as a reservoir from which these chlorinated compounds are released in trace amounts to the water. PCBs tend to get accumulated in the aquatic flora and fauna (bioaccumulation). It is seen that the concentration of the accumulated PCBs in fish is hundreds and thousands of times higher than that present in water. PCBs have the tendency to strongly attach to soil and persist in that environment for several years. Environmental cycling is suspected to be the current major source of PCB contamination in soil outside the disposal or spill sites (Agency for toxic substances and disease registry 1993).

Another source of PCB contamination is at workplace. Workplace PCB exposure may occur during repair and maintenance of transformers, accident spills, fires or its disposal. PCB exposure can also take place by breathing the contaminated air or touching any PCB-contaminated materials. Old electrical appliances and machinery are also understood to be one of the prime sources of household contamination.

13.5 Environmental and Health Effects of PCBs

PCBs are said to possess low to moderate toxicity. Animal models treated with PCBs show an LD_{50} value ranging from 0.5 g/kg to 11.3 g/kg of the total body weight (Sullivan and Krieger 1992). Most of the side effects of PCB contaminations are as a result of repetitive or chronic exposure. Humans and animals tend to absorb PCBs via their skin, lungs or gastrointestinal tract (Clark 1997). Once they are absorbed inside the body, the PCBs get transported directly to the liver, muscles or adipose tissues via the bloodstream, where they tend to accumulate. Studies show that PCB

contamination causes a range of antagonistic health effects which depends on the route of PCB entry, rate of exposure, age, sex and the area affected. Studies on animal models treated with PCBs provide convincing evidences that PCBs are in fact carcinogenic in nature. With the increase in the rate of exposure of PCBs, the carcinogenicity increases. In one particular study, the animals were exposed to food contaminated with higher amount of PCBs. It was observed that when these animals consumed food with large amount of PCB contamination, for a short period of time, they had mild to severe liver damage. As a result of which, some of them even died. PCBs have also been associated with the mass mortality in seabirds (O'Riordan 1995).

Environmental concerns related to PCBs first surfaced in the year 1960. This was exactly around 30 years post the introduction of PCBs in the market. In a particular study with the seabirds, a Swedish scientist observed the thinning of the eggshell of the seabirds. This was confirmed to be due to the bioaccumulation of PCBs. Bioaccumulation also resulted in the impairment of reproduction leading to a reduced or nil reproductive capability. PCBs have antioestrogen properties that can inhibit calcium deposition during eggshell development, leading to insufficiently strong shells and premature loss. Antioestrogen effects of PCBs may lead to adverse effects on male reproductive capabilities of birds and animal species.

Research has been conducted with the workers who have been exposed to the PCB contamination. In addition to animal studies, there are a number of epidemiological studies whose results conclude that the PCBs are likely to be carcinogenic in nature (US Environmental protection agency 1996). Workers exposed to high concentration of PCBs were found to have liver cancers and malignant melanoma which are usually rare in nature. Similar results were observed when the same concentration of PCBs was exposed to the animal models. Epidemiological studies also confirm that due to the exposure of the contaminant PCB, the subject is prone to acute side effects such as skin lesions, better known as chloracne, liver damage, clinical hepatitis and other short-term effects such as weight loss, compromised immunity and medically diagnosable impairment to the central nervous system. These may lead to side effects such as headaches, giddiness, despair, uneasiness and fatigue. Other acute health effects of PCB contamination are as follows: injury to the most of the major organs of the body, such as the liver, stomach and thyroid gland, changes in the behaviour and impaired reproduction (Agency for toxic substances and disease registry 1993).

Apart from human beings, PCB contamination is also seen to affect the productivity of the marine community, most importantly, the phytoplankton community and its major composition. The reason being, the phytoplanktons are the primary food source of all sea fauna. In fact, it is the major source of oxygen in the atmosphere. The bioaccumulation of the PCB in the food chain ultimately results in the human exposure to the said contaminant via food consumption (fish and mammals contaminated with PCBs) (Agency for toxic substances and disease registry 1993).

13.6 Degradation Process

A number of microorganisms are involved in the degradation process of PCBs which occurs in two stages: aerobic and anaerobic. The entire degradation process is similar to that of the degradation of a biphenyl compound (Passatore et al. 2014). However, the presence of the chlorine atoms on PCB structure prevents them from being exploited as a substrate of the biphenyl degradation. Moreover, due to the high chemical stability of the compound, PCBs cannot be used as sources of energy (Ohtsubo et al. 2004). However, due to chlorination, these compounds can be used as electron acceptors in the anaerobic respiration pathway. This is done to store energy which is the primary stage of its degradation pathway, also termed as the reductive dechlorination (Ohtsubo et al. 2004). Once these chlorinated biphenyls have been dechlorinated to a certain degree, they undergo the biphenyl degradation pathway. This usually occurs when there is lower than five chlorines atom followed by one aromatic ring with no chlorine atom in the structure. The degradation pathway, also termed as the BP pathway, degrades the compound to accessible carbon molecule or CO_2 gas in the aerobic environment. The biphenyl degradation pathway (BP pathway) utilizes a series of enzymes in order to convert biphenyls to the TCA cycle intermediate products or benzoate. These enzymes are the BphA, B, C, D, E, F and G. The TCA cycle intermediates are the pyruvate and acyl-CoA molecule (Ohtsubo et al. 2004). Under natural conditions, there are few organisms that have the ability to dechlorinate the substrate – biphenyl chlorine. It is seen that in presence of the selective media, the bioaccumulation of PCB-dechlorinating microorganisms is still slow when compared to other systems. This is one of the basic reasons behind the slow degradation rate. As a result, PCBs usually undergo the co-metabolism pathway which involves the different types of the microorganism (Vasilyeva and Strijakova 2007).

Generally speaking, there are four steps in the degradation process of the biphenyls. They are as follows (Ohtsubo et al. 2004):

1. The PCBs tend to be solubilized first before they enter the cell.
2. PCBs are first dechlorinated by the anaerobic bacteria, followed by the transportation of the metabolites via a biofilm to the aerobic bacteria or fungi.
3. The presence of the PCB intermediate metabolites triggers the expression of the different enzymes in the BP pathway.
4. PCBs are then finally broken down to acyl-CoA which is further utilized.

13.7 Biological Transformation of PCBs

There are many ways of degradation which includes physical, chemical and biological treatment (Centeno et al. 2003). Microorganisms have the capability of modifying PCB, an organic pollutant, in a way that the destructive aftereffects are minimized. These organisms degrade PCB via the production of extracellular

enzymes. These enzymes function in modifying PCB into simpler compounds which can be easily taken up by the environment.

Microbe-mediated degradation or biodegradation takes place in two forms, i.e. mineralization and co-metabolism (Dobbins 1995; McEldowney et al. 1993). In the process of mineralization, the proficient organisms use the organic pollutant PCB as the sole source of carbon and energy resulting in the conversion of the pollutant to its simpler elements, whereas, in the process of co-metabolism, a second substrate is required as the source of carbon and energy by the organisms. But there is a slight deviation in the way co-metabolism functions, i.e. unlike mineralization; this process converts the target pollutant as well at the same time. If the end products of co-metabolism are pliable to further degradation, they can be mineralized in the next step. If not, an incomplete degradation takes place. As a result, the secondary metabolites tend to form more in quantity and accumulate. This results in an increased toxicity than the parent molecule. Such a situation can be managed if a consortium of microorganisms can be used, where the target substances can act as the source of nutrients. The efficacy of any biodegradation process depends on the substance itself as well as many other environmental factors as they are co-related (Abraham and Chauhan 2018). These factors are as follows: compound structure, presence of substituent molecule and its position in the structural conformation and solubility and its concentration. The rate of degradation may also vary depending on the situations present in the surroundings. In case of aromatic halogenated compounds, a high degree of halogenation as well as energy is required to break the stable carbon-halogen bonds (Dobbins 1995). Presence of chlorine molecule as a substituent in the structure modifies the resonant characteristics of the aromatic substances. The electron density of specific sites may also result in the deactivation of the key oxidation process of the compound mediated by the microorganisms (Furukawa 1986). Additionally, the positions occupied by the substituted chlorines atoms in the structure have stereo chemical effects on the affinity between enzymes and their substrate molecule (Furukawa 1986; Sylvestre and Sandossi 1994). The solubility of the chlorinated compound has a vital role in the degradation process. Compounds with high solubility in water are easily accessible by the microorganisms when compared to the ones with low solubility. Highly chlorinated congeners of PCB molecule are insoluble in water at any given condition. This could be one of the reasons why the highly chlorinated PCB congeners are resistant to the biodegradation process. Concentration of the pollutant PCB, is also a major limiting factor affecting the whole process of biodegradation. In general, the existence of low concentration of the pollutant may be very little or rather insufficient for the stimulation of the degradative enzymes or to weather the growth of competent microbial cells, whereas, on the other hand, a high concentration of the pollutant may render the compound toxic to the microorganisms (Sylvestre and Sandossi 1994). At a low concentration, biodegradation increases linearly with increase in the pollutant (PCB) concentration till a time when the rate fundamentally becomes constant regardless of any further increase in the pollutant concentration (Dobbins 1995).

There are other environmental factors that affect the process and the rate of degradation. They are as follows: temperature, pH, availability of suitable electron

acceptors, interfaces amongst the microorganisms, presence of inhibitory or toxic compounds, competing substrates, etc. to name a few (Chakraborty and Abraham 2017). All these factors combine and interplay to make the rate of PCB biodegradation unpredictable. The use of aerobic and anaerobic microorganisms is the only known process to degrade the environmental pollutant PCB in the terrestrial soil systems or wetlands. Anaerobic bacteria retain characteristics that are well adapted to these organic pollutants with high concentration of carbon. This is due to the diffusional limit of the oxygen molecule in high concentration systems (Dobbins 1995). The anaerobes are favourable to reductive transformations where the chlorine molecule is displaced by hydrogen molecule (McEldowney et al. 1993). This indirectly transforms the entire structural conformation of PCB. The dechlorinated PCB is appropriate for an oxidative attack by the aerobic bacteria. Aerobic bacteria grow faster than the anaerobes. Hence, they are capable of sustaining high degradation rates resulting in the mineralization of the PCB compounds. Theoretically, the microbial-mediated degradation of PCBs results in the evolution of CO_2, chlorine molecule and water. This process involves the removal of chlorine from the phenyl ring followed by a cleavage and an oxidation of the resulting end product (Boyle et al. 1992).

13.8 Anaerobic Transformation of PCBs

Anaerobic transformation of PCB compounds involves reductive dehalogenation. In this process the halogenated organic compounds serve as the sole electron acceptor (Morris et al. 1992). The halogen substituent is further replaced by hydrogen molecule (Quensen III et al. 1990).

Electron acceptors are the one of the major factors limiting the metabolism process in an anaerobic environment. Therefore, any microorganism that is able to utilize PCB as a terminal electron acceptor would beat a selective advantage (Brown et al. 1987). Anaerobic dechlorination is prone to attack a large assortment of the chlorinated aliphatic or aromatic hydrocarbons. Several anaerobic bacteria have been isolated which have the capability of dechlorinating PCB compounds (Holliger et al. 1998). These include *Dehalobacter restrictus*, *Desulfomonile tiedjei* (Mohn and Tiedje 1992), *Dehalococcoides ethenogenes*, *Desulforomonas chloroethenica*, *Desulfitobacterium* sp., *Dehalospirillum multivorans*, *Enterobacter* strain MS-1 and *Enterobacter agglomerans*. The latter two are facultative anaerobes. Some of these microorganisms reductively remove the chlorine atom from the chlorinated compound in a co-metabolism reaction, whereas others utilize the chlorinated compounds as the sole electron acceptors in their energy metabolism.

The most common trait of dehalogenators is as follows:

(a) Inducible enzymes help in catalyzing the aryl reductive dehalogenation.
(b) The enzymes taking part in the reductive dehalogenation process exhibit distinct substrate specificity.

(c) Aryl dehalogenators function in syntrophic or cross feeding communities and may be dependent on them.
(d) Energy required for the metabolic activity performed by the aryl dehalogenators is derived from the reductive dehalogenation pathway.

Microorganisms with definite and specific dehalogenating enzymes display an exclusive and a rare configuration of congener activity. Reductive dechlorination of PCBs takes place in soil under anaerobic conditions. Organisms with such dehalogenating enzymes are completely responsible for the dechlorination activities and routes. Factors such as the rate, route and extent of dehalogenation reaction are completely dependent on the composition and distribution of the microbial community that actively take part in the dehalogenation reaction. These factors are in turn influenced by different environmental factors such as pH, temperature, carbon and nitrogen sources, hydrogen or other electron donors, the presence or absence of electron acceptors, etc. to name a few (Wiegel and Wu 2000).

13.9 Aerobic Biodegradation of PCBs

The partially chlorinated PCB molecules resulting due to the complete removal of chlorine atom from highly chlorinated congeners are the major substrates for the aerobic bacteria (Cookson Jr 1995; Kuiper et al. 2004). Aerobic biodegradation of PCBs involves two groups of genes.

- The first cluster of gene is responsible for transforming the PCB subunits to chlorobenzoic acid.
- The second cluster of gene is solely responsible for the complete degradation of the chlorobenzoic acid.

Usually the common growth substrates for PCB-degrading aerobic bacteria are biphenyl ormonochlorobiphenyl molecules. When a biphenyl molecule is utilized by these aerobic bacteria, a metal ring-cleaved product is produced. Such a phenomenon has been observed in many of the bacterial sp., for example, *Pseudomonas* sp. and *Micrococcus* sp. (Boyle et al. 1992; Benvinakatti and Ninnebar 1992). In most of the degradation processes assisted by microbes, the end product is a benzoate (result of 1,2-dioxygenative ring cleavage). Reports suggest that many bacterial species have the capability to produce benzoate as the end product via PCB metabolism. Breakdown of benzoate molecule appears to differ amongst these microbes. However, the end products produced from the breakdown are less lethal contaminant to affect human and the environment. As mentioned above, PCBs are more obstinate with increase in the chlorine atom in the congener. As a result, the aerobic degradation mediated by the microbes involving a biphenyl ring cleavage is solely constrained to the lightly chlorinated congeners (Benvinakatti and Ninnebar 1992).

As mentioned earlier, biphenyl and monochlorobiphenyl compounds can serve as growth substrates for the aerobic microbes to utilize and degrade, but the

breakdown of PCB molecules with more than one chlorine atom in it proceeds by a pathway known as the co-metabolic pathway (Komancova´ et al. 2003). In this process, biphenyl molecules are used as the sole carbon and energy source while oxidizing PCB compounds. These molecules also function as an inducer of the degrading enzymes.

In a research study conducted by Ahmed and Focht (1972), they first reported that two species of *Achromobacter* were capable of utilizing biphenyl and 4-chlorobiphenyl compound as substrate for growth. Microbial-mediated degradation of PCB compounds increased to a large extent upon the addition of biphenyl. Such kind of degradation was observed in species of *Nocardia* and *Pseudomonas* (Baxter et al. 1975).

In another study performed by Clark et al. (1979), it was observed that the co-metabolism of Aroclor 1242 increased in presence of acetate and a consortium of microorganisms. In this study they used mixed cultures of *Alcaligenes odorans*, *A. denitrificans* and an anonymous bacterium. In a similar study conducted by Focht and Brunner (1985), it was observed that the mineralization of Aroclor 1242 increased in presence of *Acinetobacter* strain P6 and biphenyl. It has been reported that *Acinetobacter* strain P6 and *Arthrobacter* strain B1B were able to grow well and utilize biphenyl and 4-chlorobiphenyl (Furukawa et al. 1978). These microorganisms could also co-metabolize Aroclor 1254.

The oxidation of PCB compounds by the microorganisms in the presence of a second substrate was accredited to the increased biomasses that eventually lead to its complete degradation (Cookson Jr 1995). In one such research study, a new bacterium named *Janibacter* MS3-02 was isolated from the soil environment (Sierra et al. 2003). It was observed that the degradation of Aroclor 1242 was ominously higher in the absence of biphenyl and showed 70–100% degradation after 7 days in liquid medium. In the presence of biphenyl in the medium, degradation was observed to be only 84%. Quite a few studies on the microbe-mediated degradation of the commercially available PCBs show a definite pattern of chlorine replacement which hinders the PCB degradation. For light chlorinated PCB compounds, progressive steps involving enzymes for degradation have been established (Ahmed and Focht 1972; Ahmad et al. 1991; Furukawa 1982; Masse' et al. 1984; Yagi and Sudo 1980).

Normally, highly chlorinated PCB contaminants showed resistance to biodegradation (Furukawa et al. 1978; Bedard et al. 1986; Bedard and Harbel 1990; Comandeur et al. 1996; Furukawa and Matsumura 1976). As a result, its complete breakdown requires innumerable microorganisms with high specificity for particular molecule. In addition to the number of chlorine atom present in the molecule, its position can also affect the rate of the oxygenase attack. In a study proposed by Unterman et al. (1988), the mechanism for PCB oxidation by *Alcaligenes eutrophus*, *Pseudomonas putida* and *Corynebacterium* sp. has been proposed. *Alcaligenes eutrophus* and *Pseudomonas putida* strains were able to degrade tetrachlorobiphenyl, while *Corynebacterium* sp. was able to degrade the contaminant via 3,4-attack.

13.10 Effect of Temperature and pH on the Biodegradation of PCBs

Much research has been conducted regarding the effect of temperature on the degradation of PCB compounds. Temperature is indeed a crucial factor which affects the rate of dechlorination. It is seen that temperature has a significant effect on the growth of the microorganisms, followed by the catalytic activity of the microbial enzymes (Wiegel and Wu 2000). A study regarding the dechlorination of 2,3,4,6-chlorobiphenyl and the residual Aroclor 1260 in Woods Pond area revealed that Aroclor 1260 was a tad dechlorinated over a wide temperature ranging from 8 to 34 °C and between 50 and 60 °C with an optimal temperature of 18–30 °C. Wiegel and Wu in one such study in the year 2000 proposed a microbial reductive dechlorination which was temperature-dependent. The reductive dechlorination spiked 2,3,4,6-chlorobiphenyl in Woods Pond.

The study on the effect of pH on the dechlorination of PCB compounds in terrestrial soil is complex. The primary cause of this complexity is due to varying interactions of the different dehalogenating and non-dehalogenating microbial population that are present in the soil. The bioavailability of PCBs is also affected by the equilibrium between PCBs that are in a dissolved state and those that are adsorbed to the underlying organic matter (Sullivan and Krieger 1992). The dechlorination of the PCBs in Woods Pond and that of the residual 2,3,4,6-chlorobiphenyl was studied at pH values ranging between 5.0 and 8.0. It was further observed that the dechlorination at such a pH took place at the optimum temperatures (Wiegel and Wu 2000).

PCB dechlorination can be observed at all temperatures and pH except at 34 °C and at pH 5.0. But the optimum pH for dechlorination was observed between 7.0 and 7.5. The dechlorination of the flanked meta-, unflanked para- and ortho-chlorine atom can be seen in between the pH values 5.0 and 8.0, pH 6.0–8.0 and 6.0–6.5, respectively. At pH 7.0 and 15 °C, orthodechlorination dominated, whereas at 18 and 25 °C, unflanked para-dechlorination outpaced the other dehalogenation reactions. The optimal pH for overall chlorine removal was at 6.0–7.5.

13.11 PCB and Environment

A consortium of mixed microorganisms is also required for microbial synergism and co-metabolism. The phenomenon of synergism is very significant in augmenting the overall rate of biodegradation of PCBs using these microbial consortiums. The increasing rate of degradation is due to the collective attack at dissimilar sites on the chlorinated compound, thereby increasing the overall rate of degradation. In a study performed by Rodrigues et al. in the year 2006, it was estimated that 31% of PCB contaminants were released into the environment. PCB contamination in the ecosystem usually tends to be around industrialized zones. These contaminants tend

to enter the environment predominantly through wetlands, as a result of leakage from landfill sites, agricultural lands, incineration of waste, discharge and sewage effluents from a number of industries, etc. to name a few. The chlorinated compounds are widely spread in the atmosphere and are transported to great extent via wind or may fall to the surface in the form of rainfall, snow or precipitation in any kind. Roughly around 99% of the contaminants entering the ocean are from the atmosphere. Many of the environmental parameters such as temperature, storm frequency, wind speed, rainfall rates and/or the volatility of PCB molecule influence the rate of its movement in the atmosphere. The primary and the foremost transport route for PCBs through oceans and the other major wetlands are from effluent streams which ultimately move downstream and mix with the marine sediments. As a result it leaves the marine biota as one of the major sinks for PCB contaminants. The entry of PCB can be due to direct dumping of waste into a river or may be due to polluted air. From the air, the contaminants settle onto the flora, eventually finding their way into any of the wetland. These contaminants further get transported around the planet via ocean currents. Bioaugmentation and biomagnification also take place in the tissues of migrating fauna such as fishes. One such example is the Inuit Eskimos. They are known to carry the highest PCB concentrations in their bodies due to their increased fish diet. PCBs are insoluble in water, but they tend to get dissolved in oil and fats. This makes the marine fauna a high-risk group for consuming these contaminants. Mammals do not have the ability to detoxify PCB from their bodies. As a result, they tend to store it in their muscles and blubber (ATSDR 2000).

13.12 Conventional Treatment of PCBs

Incineration has caused more release of the chlorinated compound into the atmosphere in vapour form and/or accumulates in the soil. The method is achieved at a higher temperature, i.e. 1200°C. At such a temperature, 99.99% of the contaminants get removed. Nevertheless incineration of the hazardous waste causes exposure to lethal emissions. Moreover, the incineration process is very expensive and time consuming (Mikszewsk 2004).

Landfilling remediation is another mechanism of eliminating PCB contamination. The danger of landfilling includes PCB volatilization, leachate from the landfills, etc. into the surrounding soil and wetland. This technique leaves the aquatic flora and fauna contaminated with the chlorinated compounds and other impurities.

Dredging is another conventional technique of PCB removal (Katers 2000). This process involves the stirring of the sediment, causing it to resurface, thereby increasing the level of the contaminant on surface waters. The combination of dredging and incineration has been proven to be an effective tool for the removal of PCBs. However, the entire set-up is expensive and may cost more than half a million dollar

to construct a site of an adequate size for remediating these environmental contaminants.

Other methods for PCB removal are as follows (Rahuman et al. 2000):

1. Low temperature thermal desorption
2. Extraction
3. Chemical dehalogenation
4. Bioremediation

PCBs are worldwide obstinate contaminants. The conventional methods such as incineration, landfilling and thermal desorption are often expensive and time consuming. Due to this, bioremediation has been introduced as an alternate strategy for PCB removal. This process is assisted by the microorganisms that are capable of utilizing PCBs as their source of energy (Leigh et al. 2006).

13.13 Environmental Biodegradation of PCB

PCBs are environmental contaminants that are generated during diverse types of natural and industrial processes. Their occurrence in the ecosystem and in food products causes serious menace to human health and the environment. They present as genotoxic for the flora and fauna (Doughtery et al. 1993). Therefore, natural transformation of these PCBs is a life-threatening event in determining their fate in the environment.

13.14 Phytoremediation

Macek and his associates have defined phytoremediation as the use of plants to assist in the remediation of the environmental contaminants (Van den Berg et al. 2006; Macek et al. 2000). In other words, phytoremediation is defined as the plant-mediated remediation of the contaminants by rendering them harmless to the ecosystem (Cunningham et al. 1995; Cunningham et al. 1996). In the process of phytoremediation, one uses the flora/plants for the remediation of contaminated mediums including soil, dregs, slurry and/or water. This technique can be used in situ as well as ex situ by the elimination, degradation or evening out of a given environmental contaminant (Mackova et al. 2007). According to some authors, phytotechnology comprises of different sets of technologies which makes use of the plants to remediate different contaminated sites (Schnoor et al. 1995; Macek et al. 2002; Macek et al. 2004; Schnoor 2002; Mackova et al. 2007). Phytoremediation is currently divided based on the means of remediation. They are mentioned below:

- Phytoextraction
- Phytodegradation

- Phytostabilization
- Phytovolatilization
- Rhizofiltration

These phytotechnology techniques are used as an alternate to the far and widely used methods of physical, chemical, physico-chemical and/or thermal remediation (Buczkowski et al. 2002; Newman and Reynolds 2004; Gerhard et al. 2009). The major advantages of using these techniques are as follows:

- Ex situ and in situ application
- Low capital investment
- Cheaper operating costs
- Increased effectiveness
- Non-invasive technique

The major disadvantages of using phytoremediation techniques are as follows (Buczkowski et al. 2002; Newman and Reynolds 2004; Gerhard et al. 2009):

- Longer operational time.
- Many of these techniques are still in their experimental stage.

The dawn of the phytoremediation process was perceived by the increased rate of degradation of organic compounds in the soil in the presence of the vegetation cover. Based on the obtained results, it could be concluded that in the presence of the vegetation, there is a reduction of the total organic substances in soil. There are many research works which indicates the constructive effects of using developed plants to degrade the organic compounds (Gregor and Fletcher 1988; Pradhan et al. 1999; Nedunuri et al. 2000; Robinson et al. 2002; Banks et al. 2003; Siciliano et al. 2003; Vervaeke et al. 2003; White Jr et al. 2006; Jou et al. 2007). In one particular work, Siciliano et al. (2003) demonstrated the decrease of organochlorine substances by 30% approximately in 2 years of crop growth. Whereas, it was observed that on the soil without the vegetation cover, the reduction was two times lower than the previously observed result (with the vegetation cover). In a similar work conducted by Nedunuri et al. (2000), it was reported that the aromatic compounds could be reduced by approximately 42% and 50% over a period of 21 months by using *Lolium annual* (flax) and *Stenotaphrum secundatum* (St. Augustine grass), respectively. Other such examples of soil remediation (soil contaminated with crude oil) are by the combined use of grass and fertilizers (Nedunuri et al. 2000; Robinson et al. 2002; Banks et al. 2003; White Jr et al. 2006). In one particular research, Vervaeke et al. (2003) reported a reduction of approximately 57% of the existing aromatic compounds as well as the mineral oils. This increased rate of degradation was observed during the willow (*Salix viminalis*) cultivation for 1.5 years. In another study, Pradhan et al. (1999) had demonstrated the usage of phytoremediation technique as one of the primary remediation process as well as the final step for treating soils contaminated with PAHs. The authors had recorded an increased rate of degradation of the PAHs after 6 months of green vegetation cultivation including *Medicago sativa* (alfalfa), *Panicum virgatum* (switch grass) and *Schizachyrium*

scoparium (bluestem grass) growth. Gregor and Fletcher (1988) demonstrated the ability of the plant cells to adsorb and break down PCBs in one of their study. Jou et al. (2007) in one such study had described the uptake of PCB by *Broussonetia papyrifera.* This plant had the ability to grow very well on highly contaminated soil. The authors had previously reported similar concentrations and distributions of PCB units in plant cells and soil sediments. Many other research studies conducted by different groups of scientists demonstrated that several plants belonging to the genus *Cucurbita* such as pumpkin, squash, etc. were capable of readily taking up PCDD and PCDF from soil and translocating them to the leaves and fruits (Hülster and Marschner 1993; Hülster et al. 1994; Engwall and Hjelm 2000).

13.15 Rhizoremediation

Rhizoremediation is another technique used to remove the organic pollutants from the environment using plants. It is one of the effective remediation techniques present and used. This is mostly due to the interactions existing in the rhizosphere between roots of the plants, its exudates, soil particulates and the microorganisms. Mackova et al. (2007) had reported that the plants were able to support bioremediation by releasing its exudates and enzymes, thereby stimulating both microbial and the biochemical activity in the surrounding sediments and finally mineralizing in the rhizosphere. Plants can also speed up the remediation process in the surface soils by stimulating the growth and metabolism of soil microorganisms. This is done via the release of important nutrients, followed by the transport of oxygen to the roots (Cunningham et al. 1995; Schnoor et al. 1995; Macek et al. 2000). A study conducted by Whipps (1990) demonstrated that one gram of soil rhizosphere contains increased amount of microorganisms when compared to the plants devoid of soil. Organisms present in the rhizosphere play a vital role in protecting plants against different pathogens and stress induced as a result of the presence of higher concentration of PCB (Rainey 1999; Lugtenberg et al. 2001; Gianfreda and Rao 2004; Liu et al. 2007; Dams et al. 2007). Bacterial colonies present in the rhizosphere of the plants function in remediation by secreting different enzymes such as phosphatase, peroxidase, P_{450} monooxygenase, nitroreductase, etc. These cellular enzymes are involved in the microbe-mediated degradation process of the PCBs present in the environment. Such essential enzymes were also detected in many of the plants and fungal strains which colonize the roots completely. This association results in the interaction between the plants and the microorganisms, thereby completely degrading a particular PCB contaminant (Lamoureux and Flear 1979; Macek et al. 1998; Susarla et al. 2002; Kuiper et al. 2004; Singer 2004; Chaudhry et al. 2005; Dams et al. 2007). The entire process is thus called rhizo-degradation. The efficacy of the plant rhizosphere-mediated degradation depends completely on the ability of the prevailing microorganisms to adsorb to a given chlorinated biphenyl concentration (Singer 2004). The mutual symbiotic relationship between the organism, rhizosphere and PCB contaminant is therefore responsible for the amplified

reduction or complete removal of the PCB-contaminated soil surface in the presence of plants (Shimp et al. 1993; Yateem et al. 2007). In a study conducted by Kuiper et al. (2004), it was clearly demonstrated that naturally occurring biodegradation can be enhanced by the addition of microorganisms to the rhizosphere. Many important chemical groups are present in the rhizosphere. They are present in the form of complexes of aromatic compounds, for example, flavonoids and coumarins. The above-mentioned compounds are used by the microflora as their source of carbon and nitrogen (Leigh et al. 2002; Siciliano et al. 2003; Kuiper et al. 2004; Chaudhry et al. 2005; Yateem et al. 2007). Flavonoids and coumarins are structurally similar to organic environmental contaminants such as PCBs and PAHs. This clearly indicates the potential of these compounds to be used up by the organisms from the rhizosphere for the remediation of organic pollutants using its evolutionary metabolic pathways (Holden and Firestone 1997). Because of this capability, many researchers are interested in working with the microorganisms inhabiting the rhizosphere for their ability to degrade organochlorinated environmental pollutants and the role of major flavonoids and coumarins in the degradation process (Ferro et al. 1999; Pillai and Swarup 2002; Thoma et al. 2003; Chaudhry et al. 2005; Leigh et al. 2006; Yateem et al. 2007). Studies have described and categorized the environmental pollutants based on their nature. Some of the pollutants are as follows: PCBs, PAH, petroleum hydrocarbons, pesticides like pentachlorophenol, etc. These pollutants were observed to degrade rapidly in the rhizosphere when compared to the whole soil (Mackova et al. 1996, 1998; Nichols et al. 1997).

13.16 Conclusion

Polychlorinated biphenyls (PCBs) pose one of the most stimulating complications in the environment affecting the flora and fauna severely. The fate, transport and degradation in the ecosystem occur through complex interactions with other contaminants and microbial strains. This includes different physical, chemical and biological processes. These degradation techniques can be used and modified in order to reduce their environmental concentration. Both anaerobic and aerobic modes of degradation help in transforming the PCB molecule. Different microbial strains show their specific patterns of degradation. The degree of chlorination in PCBs is one of the major factors, which influence the biodegradation capability. In addition, environmental parameters such as temperature, pH, presence or absence of the secondary substrate, etc. also affect the composition and growth of the microorganisms. The above-mentioned parameters must be optimized in order to obtain high degradation effectiveness.

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Chapter 14
Role of Macrofungi in Bioremediation of Pollutants

Pratima Vishwakarma

Abstract The macrofungi in the galaxy of living beings have newly found biological status. The macrofungi are exploited by industries for enzymes, organic acids, vitamins, antibiotics, and other useful substances and have an active role in biodegradation. They are the factories of enzymes that decompose all types of organic waste. They have a role in solving metal pollution that causes serious effects on human life. Macrofungi play important role in biogeochemical cycling of elements. They have the ability to accumulate and recycle organic and inorganic materials which also include toxic metals. They may be new cost-effective technology for the removal of heavy metals from wastewater by the process of biosorption. They have potential in the management of polycyclic aromatic hydrocarbons. It is able to metabolize and mineralize several PAHs and their analogs. In this way, they have vast potential in the management of ecosystem.

Keywords Biodegradation · Bioremediation · Fungi · Organic pollutants · Inorganic pollutants

14.1 Introduction

Rapid increase in industrialization leads to addition of various proportions of harmful pollutants, viz., pesticides, toxic xenobiotic compounds, metals, metalloids, halogenated, polycyclic aromatic hydrocarbons, dyes, phthalates, etc. in the environment. (Goutam et al. 2018; Gautam et al. 2017; Limón-Pacheco and Gonsebatt 2009). Environmental pollution caused by the release of this wide range of compounds, i.e., persistent organic pollutants (POPs), from industries is creating disturbance to the ecosystem and results in climatic changes, reduction of water levels in the ground as well as oceans, melting of ice caps, global warming, ozone layer

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depletion due to photochemical oxidation, etc., and this made ecologists focus more on impacts of pollution and its reduction (Varsha et al. 2011).

Bioremediation using microbes and plants is an excellent strategy to degrade/detoxify the organic and inorganic pollutants from the contaminated matrix. Remediation of pollutants by microbes is a complex process that depends on the chemistry of metal ions, cell wall composition of microorganism, cell physiology, and physicochemical factors like pH, temperature, time, ionic strength, and metal concentration (Mishra et al. 2012). Various environmental pollutants are released into the biosphere by various industrial and agricultural practices. They get accumulated in the air, water, and soil and remain there as such for a long time without any change in them. Their degradation mainly depends upon the physical and chemical condition with the association of soil microorganisms and macrofungi harboring in it. Generally, two types of processes, i.e., chemical degradation and biological (microbial) degradation, are involved for removal of the chemical pollutants from a particular system, and its degradable efficiency depends upon the environmental conditions of the system. Microbial transformation is considered as the best technique as it is very environment-friendly. This process does not add any chemicals to the environment. This process occurs in any one of the five different ways, which include co-metabolic degradation, detoxification, polymerization and binding with natural compounds, extracellular or intracellular accumulation, and mineralization (Guthrie and Davis 1985).

The fungi are unique among the living organisms and are omnipresent in the biosphere. They are eukaryotic, spore-bearing, achlorophyllous microorganism and entirely heterotrophic. Fungi possess the biochemical and ecological capacity to degrade environmental organic chemicals. It decreases the various risks associated with metals, metalloids, and radionuclide loading of the surrounding either by chemical modification or by influencing chemical bioavailability. Most fungi produce extracellular enzymes which help in the assimilation of complex carbohydrates without prior hydrolysis, and this makes it possible to degrade a wide range of pollutants (Prakash 2017). Fungal species possesses the incredible abilities to degrade various persistent and toxic industrial waste products and chemical contaminants and convert it to less toxic form or nontoxic form (Prakash 2017). Fungal mycelium contains different enzymatic mechanisms which help to reduce toxins from nature and help to maintain normal flora and fauna. White rot fungi have successfully been utilized in degradation of environmental pollutant like polyaromatic compounds, pesticides, etc. (Prakash 2017). The macrofungi are the scavengers of vegetables Kingdom and play a major role in the ecological system. Macrofungi also play an active role in biodegradation; their distribution relates to the humus containing soil like of forest, in which the litter, decaying wood, hay, hips of straw, etc. are present in abundant quantity. Macrofungi helps in decomposition of these complex products by various enzymatic activity on cellulose, lignin, nitrogenous compound etc., convert it into simpler form and hence helps in nutrient recycling. (Sultana et al. 2007). This complex process is carried out by the enzymatic activity of mushrooms on cellulose, lignin, nitrogenous compound, etc. The macrofungi are the factories of enzymes that decompose all type of organic waste. The biotechnology

has uncovered white rot-causing fungi on trees for this purpose *Phanerochaete chrysosporium* that can digest the tough lignin components of wood. This mushroom is the best to carry out for bioremediation work on natural carbon-containing substances called hydrocarbons and chemicals resembling natural substances. Macrofungi reduce the healthy components which are present in DDT, dieldrin, aldrin, and even radioactive compounds, all of which have some structural similarities to lignin. This is a relatively cheap and environmentally sound way of disposing of noxious compounds which in the past were valued for their stability and used extensively. White rot fungi promise to provide a low-cost method of treating effluent from some particular industries. For example, pulp and paper mills have a specific problem with toxic effluent from bleached paper production in kraft process with chlorine bound to the lignin component being the main pollutant (Sultana et al. 2007). So the fungi are excellent candidates for toxic waste cleanup.

14.2 Xenobiotics in the Environment and Their Degradation by Macrofungi

Xenobiotics are anthropogenic compounds found in living systems or in the environment which are not natural but are unusually present in very high concentration. The possible health hazard associated with xenobiotic compounds is their persistence nature in the environment as well as their toxicity.

14.2.1 Sources of Xenobiotics

There are two ways by which xenobiotics enter into our surrounding:

(a) *Direct Source*: The main sources of xenobiotics are wastewater and solid residual releases from the industries like chemical and pharma, plastics, paper and pulp mills, textile mills, and agricultural. Common residual compounds in the wastewater and other effluents are phenol, hydrocarbons, different dyes, paint effluents, pesticides and insecticides, etc.
(b) *Indirect Source:* Indirect sources of xenobiotics include nonsteroidal anti-inflammatory drugs, pharmaceutical compounds, pesticide residues, etc.

Several methods, viz., physicochemical and biological methods, have been employed in the treatment or removal of xenobiotics. The physicochemical methods are costly and often produce undesirable products which are toxic, requiring further treatment steps. Such type of techniques often adds fragmented elements which cannot be degraded easily and will make the environment still worse. To overcome these problems, many other eco-friendly techniques have been reported such as bioremediation, phytoremediation, etc. (Varsha et al. 2011).

Mycoremediation is a form of bioremediation, the process of using fungi to return an environment (usual soil) contaminated by pollutants to a less contaminated state. The term mycoremediation was coined by Paul Stamets and refers specifically to the use of fungal mycelia in bioremediation.

The mushroom can be successfully utilized in mycoremediation technologies, where their feature concerning the uptake of heavy metal is beneficial. A number of mushrooms have been proven to mycoremediate heavy metals which include *Pleurotus platypus*, *Agaricus bisporus*, *Calocybe indica*, *Calvatia excipuliformis*, *Hygrophorus virgineus*, *Boletus edulis*, *Lepiota rhacodes*, *Lepista nuda*, *Pleurotus sajor-caju*, *Pleurotus ostreatus*, *Psalliota campestris*, and *Russula delica* (Vimala and Das 2009; Das 2005). Xenobiotics degraded by macrofungi are listed in Table 14.1.

The process of mycoremediation can be classified under the following categories: degradation by ligninolytic fungi, fungal biosorption, and mycorrhizal fungal degradation.

(a) *Degradation by Ligninolytic Fungi:* Many fungal species like basidiomycetes and ascomycetes possess the high potential to degrade lignocellulose materials present in dead wood and paper and pulp effluents (Kenneth 1996). Basidiomycetes are considered as one of the most important lignocellulose decomposers and include various ecological groups, viz., brown rot and leaf litter fungi. It contains laccases (copper-containing enzymes) which help in degradation of polycyclic aromatic hydrocarbons produced from natural oil deposits (Cho et al. 2009). Ligninolytic fungi show higher potentials to degrade organic pollutants including synthetic dyes.

Nowadays immobilized fungal cultures in semisolid state, trickling-bed, and rotating disk reactors are used for efficient biodegradation of textile dyes, because of the advantages which include long retention time of biomass in the system, ease of use in a continuous reactor, and their ability for scale-up (Varsha et al. 2011).

(b) *Fungal Biosorption:* Biosorption is a property of certain types of inactive, dead microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solutions. Yeast and fungi are unique in metal biosorption, and this process is known as mycosorption. This is one of the most promising technologies which is involved in the removal of toxic metals from industrial waste streams and natural waters and offers a potential alternative method against various remediation methods. Through this process, contaminants bind passively onto the cellular structure of fungi. Biosorption process has many advantages over the bioaccumulation process as accumulated metal or waste can be desorbed easily by simple physical methods without damaging the sorbent's structural integrity (Gurel et al. 2010).

(c) *Mycorrhizal Fungal Degradation:* Mycorrhiza is a symbiotic association between fungi and root zone of the vascular plant. This type of association increases the organic carbon content of the soil. Mycorrhizal fungi grow in a symbiotic association with plants and hence encourage degradation of organic contaminants present in soil. The typical mycorrhizons which naturally biodegrade the organic pollutants are *Morchella conica* and *Tylospcno fibrils* (Bennet et al. 2002).

Table 14.1 Xenobiotic degradation by some macrofungi

S. no.	Macrofungi	Xenobiotic compound	References
1.	*Agaricus bisporus* (J.E. Lange)	Anthracene, fluoranthene, phenanthrene, pyrene	Gramss et al. (1999)
2.	*Agaricus bisporus* (J.E. Lange)	Polycyclic aromatic hydrocarbons (PAH)	Gąsecka et al. (2012)
3.	*Agrocybe aegerita* (V. Brig.) Singer	Benz[a]anthracene	Martens and Zadrazil (1998)
4.	*Agrocybe praecox* (Pers.) Fayod	Phenanthrene, pyrene	Gramss et al. (1999)
5.	*Bjerkandera adusta* (Willd.) P. Karst.	Azo dyes, phthalocyanine dyes	Heinfling et al. (1998)
6.	*Clitocybula duseni* (Bres.) Singer	Lignite	Hofrichter et al. (1999)
7.	*Coprinus comatus* (O.F. Müll.) Pers.	Anthracene, fluoranthene, phenanthrene, pyrene	Gramss et al. (1999)
8.	*Coriolopsis gallica* (Fr.) Ryvarden	Anthracene, phenanthrene, pyrene	Pickard et al. (1999)
9.	*Ganoderma applanatum* (Pers.) Pat.	Benz[a]anthracene	Martens and Zadrazil (1998)
10.	*Gloeophyllum striatum* (Fr.) Murrill	2,4 Dichlorophenol and pentachlorophenol	Fahr et al. (1999)
11.	*Gloeophyllum striatum* (Fr.) Murrill	Fluoroquinolone Enrofloxacin	Wetzstein et al. (1997)
12.	*Gloeophyllum striatum* (Fr.) Murrill	2,4 Dichlorophenol	Schlosser et al. (2000)
13.	*Gloeophyllum trabeum* (Pers.) Murrill	2,4 Dichlorophenol and pentachlorophenol	Fahr et al. (1999)
14.	*Gloeophyllum trabeum* (Pers.) Murrill	Polyethylene glycol	Kerem et al. (1999)
15.	*Hypholoma fasciculare* (Huds.) P. Kumm.	Anthracene, fluoranthene, pyrene	Gramss et al. (1999)
16.	*Kuehneromyces mutabilis* (Schaeff.) Singer & A.H. Sm.	Anthracene, fluoranthene, phenanthrene, pyrene	Gramss et al. (1999)
17.	*Lentinus edodes* (Berk.) Singer	Benz[a]anthracene	Martens and Zadrazil (1998)
18.	*Lentinus edodes* (Berk.) Singer	Polycyclic aromatic hydrocarbons (PAH)	Gąsecka et al. (2012)
19.	*Lenzites betulina* (L.) Fr.	Anthracene, phenanthrene	Gramss et al. (1999)
20.	*Marasmiellus troyanus* (Murrill) Dennis	Benzo[a]pyrene	Wunch et al. (1999)
21.	*Marasmiellus troyanus* (Murrill) Dennis	Benzo[a]pyrene	Wunch et al. (1999)
22.	*Marasmius rotula* (Scop.) Fr.	Pyrene	Lange et al. (1996)
23.	*Morchella conica* Pers.	Anthracene, fluoranthene, Phenanthrene	Gramss et al. (1999)

(continued)

Table 14.1 (continued)

S. no.	Macrofungi	Xenobiotic compound	References
24.	*Phanerochaete chrysosporium* Burds.	2,4 Dichlorophenol	Valli and Gold (1991)
25.	*Pleurotus pulmonarius* (Fr.) Quél.	Crude oil	Olusola and Anslem (2010)
26.	*Pleurotus dryinus* (Pers.) P. Kumm.	Benz[a]anthracene	Martens and Zadrazil (1998)
27.	*Pleurotus eryngii* (DC.) Quél.	Azo dyes, Phthalocyanine dyes	Heinfling et al. (1998)
28.	*Pleurotus eryngii* (DC.) Quél.	Benz[a]anthracene	Martens and Zadrazil (1998)
29.	*Pleurotus flabellatus* Sacc.	Benz[a]anthracene	Martens and Zadrazil (1998)
30.	*Pleurotus fossulatus* (Cooke) Sacc.	Benz[a]anthracene	Martens and Zadrazil (1998)
31.	*Pleurotus pulmonarius* (Fr.) Quél.	Benz[a]anthracene	Martens and Zadrazil (1998)
32.	*Pleurotus sajor-caju* (Fr.) Singer	Benz[a]anthracene	Martens and Zadrazil (1998)
33.	*Pycnoporus cinnabarinus* (Jacq.) P. Karst.	Dibenzofuran	Martens and Zadrazil (1998) and Jonas et al. (1998)
34.	*Stropharia rugosoannulata* Farl. ex Murrill	Anthracene, fluoranthene, phenanthrene, pyrene	Martens and Zadrazil (1998) and Gramss et al. (1999)
35.	*Trametes hirsuta* (Wulfen) Lloyd	Textile dyes	Abadulla et al. (2000)
36.	*Tylospora fibrillosa* (Burt) Donk	Fluorobiphenyl	Green et al. (1999)

14.2.2 Litter Decomposition by Macrofungi

Litter is a transition zone between biotic and abiotic systems. Litter is generally composed of dead remains of plants like leaves, twigs, fruits, seeds, etc. It is black in color with lots of humus in it. Litter macrofungi play important role in decomposition of litter; they release and transform the nutrient present in litter in available form to the plants.

Macrofungi may be defined as primary producer, secondary producer, and tertiary producer on the basis of their presence on the forest floor. Primary producers are the first recycler as they soon start to grow when coming in contact with the host plant; examples of them are oysters *(Pleurotus* spp.) and shiitake (*Lentinus edodes*). Secondary producers generally grow on the previously grown fungi; they only partially decompose the substrate on which they grow like compost forms for the cultivation of various mushrooms, e.g., button mushrooms (*Agaricus bisporus)*,

Stropharia species, etc. Tertiary decomposers are soil dwellers; they grow on a substrate already harbored by other macrofungi like primary, secondary, and tertiary macrofungi. Some examples of tertiary decomposers are *Aleuria aurantia*, *Panaeolus* species, *Coprinus* species, *Conocybe* species, *Agrocybe* species, *Pluteus* species, and some *Agaricus* species. They are the successor in macrofungi and help in easy degradation of litter and humus of forest (Sultana et al. 2007).

Basidiomycetes macrofungi have the capacity to break down complex compounds into simpler form by the use of enzymes they have like ligninolytic and cellulolytic enzymes. Ascomycetous and basidiomycetous macrofungi differ in their ability to decompose litter, for example, *Mycena* spp. decompose lignin and carbohydrate more rapidly than other wood-inhibiting macrofungi (Bennet et al. 2002).

Mycorrhizas are mutualistic associations between fungi and plant roots; ectomycorrhizas (ECM) are formed by the association of *Basidiomycetes* and *Ascomycetes* with higher plants (Dell 2002). Litter-inhibiting ectomycorrhizal fungi (ECM) produce protease enzymes and distribute soluble amino compounds through hyphal networks into the root (Read et al. 1989). Litter is a very important reservoir of nutrient in the forest. In this condition ECM help in the mobilization of phosphorus, nitrogen, and other nutrients from litter to the tree root (Attiwill and Adams 1993; Perez-Moreno and Read 2000). It is estimated that ECM account for 43% of the annual turnover of N in a *Pseudotsuga menziesii* forest in Oregon (Fogel 1980). High diversity of fungal partners with tree allows optimal foraging and helps in the mobilization of various N and P forms from organic soil layers (Buscot et al. 2000).

14.3 Metal As Pollutant and Its Remediation by Macrofungi

Heavy metals are another group of toxins of environmental concern with a possible solution arising from fungal treatments. Unlike organic pollutants, metals remain in the soil for thousands of years and are not degraded biologically but can only be transformed from one oxidation state to another (Gisbert et al. 2009). Nowadays the scientific attention is mainly focused on four sources of heavy metals, as a consequence of their environmental impact – acid mine drainage (AMD), associated with mining operations, electroplating industry waste solutions, coal-based power generation (high coal quantities), and nuclear power generation (uranium mining and waste generation) (Prakash 2017). Macrofungi interact with heavy metals physiologically and morphologically. Some heavy metals play important roles in the fungal metabolism, while some are considered as toxic at a certain concentration. In recent years heavy metal pollution becomes one of the serious problems for the environment. The presence of metals in trace becomes toxic for the environment and causes a worse effect on flora and fauna. With rapid development of many industries (like mining, surface finishing, energy and fuel production, fertilizers, pesticides, metallurgy, iron and steel electroplating, electrolysis, electroosmosis, leather, photography, electric appliance manufacturing, metal surface treating, aerospace, atomic energy installation), wastes containing heavy metals are directly or

indirectly discharging into the environment. These in return causes serious environmental pollution and ultimately affect the human life (Das et al. 2008). The industrial waste may be either polluted or not polluted; its improper handling, dumping, transportation, and disposal is bound to cause harm to nature. Some of the responsible elements are toxic, explosive, and corrosive substances.

Macrofungi play important role in biogeochemical cycles of elements. They have the ability to accumulate and recycle organic and inorganic materials which also include toxic metals like Rb, Cs, Al, Cd, Ag, Au, Hg, Pb, etc. (Borovička and Řanda 2007). The main factors which influence the accumulation of heavy metals in mushroom are environmental factors such as metal concentrations in the soil, pH, organic matter, contamination by atmospheric deposition and fungal factors, biochemical composition, decomposition activity, mycelium development and fruit bodies, and a morphological portion of macrofungi (García et al. 1998). Macrofungi need a certain amount of some elements for proper growth and reproduction; mycelia of macrofungi mobilize and uptake elements from substratum on which it grows and translocate it to fruiting bodies; due to this reason, fruiting bodies of macrofungi become rich in metals and sometime also metalloids (Jarzyńska and Falandysz 2012). Accumulation of metals in macrofungi is generally species-specific; it depends upon the habitat on which it grows like parent soil bedrock geochemistry, metal ore smelters, metal refineries, metal ore mines or depositions, etc. (Zhang et al. 2010). Elements from macrofungi are determined by validated methods like inductively coupled plasma atomic emission spectroscopy (ICP-AES) and cold vapor atomic absorption spectrometry (CV-AAS). In Boletes, K and Mg concentrations are very high (41 and 1.2 mg/g dry weight (DW) respectively while Na, Rb, Zn, and Ca are also present in good quantity (560, 350, 210, and 110 μg/g DW, respectively) (Jarzyńska and Falandysz 2012).

Ectomycorrhizal fungi help trees/plants to survive in heavy metal-contaminated soil. They immobilized heavy metals in their hyphae or fruiting bodies and hence made it possible for trees to show fewer harms toward metal contamination (Dell 2002). Different macrofungi accumulate different heavy metals from substratum on which it grows.

Macrofungi are new cost-effective technology for removal of heavy metals from wastewater by the process of biosorption. Macrofungi play important role in biosorption of metal due to its metal-sequestering property. This process involves a solid phase (sorbent, or biological material) and a liquid phase (solvent normally water) which contains dissolved substance to be sorbed. Sorbent has a high affinity for sorbate; sorbate is attracted and removed by different means till equilibrium is attained by the amount of solid bound to sorbate, and its portion remains in the liquid phase (Das et al. 2008).

Industrial and domestic wastewater discharge into natural water bodies is the main source of cadmium in the environment. It has a very serious effect on human beings. In humans, it accumulates in the kidney and disrupts potassium metabolism in them by spilling protein in the urine (Patterson and Passino 1987). If we look in history, we observe that cadmium toxicity causes itai-itai disease in Japan which was very chronic (Friberg et al. 1979).

Macrofungi accumulate silver from the substratum on which they grow. Two species of *Amanita*, viz., *A. strobiliformis* and *A. solitaria*, have been found to hyper-accumulate silver. They are the first known eukaryotic organisms able to hyper-accumulate silver at quantity, up to 800–2500 times that concentration of Ag in the underlying soil. In both highest concentration of silver is found in *A. strobiliformis* which is 1024 μg g^{-1} dry weight (Borovićka et al. 2007).

Gold in macrofungi is determined by using long-term instrumental neutron activation analysis (INAA). Borovićka et al. (2006) reported the highest concentrations (expressed in dry weight) of gold in ectomycorrhizal species *Russula nigricans* (235 ng/g) and *Suillus variegatus* (1070 ng/g); in saprobic macrofungi, the highest value of 2250 ng/g was found in *Lepiota* cf. *clypeolaria*. The gold content of saprobic macrofungi originated from the auriferous area was obviously higher than that of macrofungi from non-auriferous areas. The highest contents were found in *Agaricus silvaticus* (4230 ng/g) and in two samples of *Lycoperdon perlatum* (6955 and 7739 ng/g) (Borovićka et al. 2006). Table 14.2 shows the list of some macrofungi with high metal accumulation capacity.

14.4 Polycyclic Aromatic Hydrocarbon and Its Degradation

Polycyclic aromatic hydrocarbons (PAHs) represent a vast group of organic molecules which have a broad range of properties. The distribution of PAH and its fate in the environment are of increasing interest because compounds with four or more aromatic rings are recalcitrant, they are often carcinogenic, mutagenic, or toxic and also poses serious risk to human health (Sack et al. 1997). They differ in their molecular weight, structural configuration, water solubility, aromatic ring number, volatility nature, absorption coefficients, etc. (Venkata et al. 2006).

Polycyclic aromatic hydrocarbon is formed by incomplete burning of fossil fuels, and it enters the soil through atmospheric deposition. Anthropogenic sources like gasoline and diesel fuel combustion, oil spills, former gas plant facilities, etc. contribute PAHs to the environmental matrix. Biotransformation of polycyclic aromatic hydrocarbon in human and microorganism led to the formation of carcinogenic substances in them (Juhasz and Naidu 2000). PAHs are organic compounds containing only carbon and hydrogen and composed of multiple aromatic rings.

Study of the possible role of microorganisms in PAH degradation revealed that two main groups of microorganisms are involved in the oxidation and subsequent mineralization of these compounds: soil bacteria and white rot fungi. The PAHs are limited by their low water solubility, whereas soil bacteria were found to effectively degrade low molecular weight PAHs; white rot fungi can also oxidize more condensed PAH molecules with up to six aromatic rings and limited water solubility and therefore decrease their toxicity (Baldrian et al. 2000). The white rot fungus *Pleurotus ostreatus* is able to degrade the polycyclic aromatic hydrocarbons (PAHs) benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a*,*h*]anthracene, and benzo[*ghi*]perylene in the nonsterile

Table 14.2 List of macrofungi with different metal accumulation abilities

S. no.	Macrofungi	Metal accumulation	References
1.	*Agaricus bisporus* (J.E. Lange) Imbach	Cu (107), Pb (21), Zn (57.2) (μg/g)	Isildak et al. (2007)
2.	*Agaricus bisporus* (J.E. Lange) Imbach	Hg (0.03), Pb (0.28), Cd (0.78) Fe (31.3) (mg/kg)	Demirbaş (2001)
3.	*Agaricus bisporus* (J.E. Lange) Imbach	Cu (72.81), Zn (75.83) (mg/kg)	Alonso et al. (2003)
4.	*Agaricus bisporus* (J.E. Lange) Imbach	K (0.54–1.58%), Na (37.2–61.9 μg/g), Fe (143.6–396.0 μg/g), Cu (54.6–163.4 μg/g), Zn (36.3–58.0 μg/g), Mn (56.2–91.1 μg/g), Cr (0.23–0.30 μg/g), Pb (0.15–0.22 μg/g)	Mohiuddin et al. (2015)
5.	*Agaricus bisporus* (J.E. Lange) Imbach	As (0.097 mg/kg)	Maihara et al. (2008)
6.	*Agaricus campestris* (L) Fries	Cu (126.8), Zn (215.0) (mg/kg)	Alonso et al. (2003)
7.	*Agaricus campestris* (L) Fries	Fe (127.94), Zn (89.53), Cu (38.09) (mg/Kg)	Ivan et al. (2016)
8.	*Agaricus macrospores* Mont.	Cu (242.4), Zn (267.0) (mg/kg)	Alonso et al. (2003)
9.	*Agaricus silvicola* (Vittad.) Peck	Cu (193.5), Zn (209.4) (mg/kg)	Alonso et al. (2003)
10.	*Agaricus* species	As (0.125 mg/kg)	Maihara et al. (2008)
11.	*Agrocybe cylindracea* (DC.) Maire	Cu (42.24), Zn (79.29) (mg/kg)	Alonso et al. (2003)
12.	*Amanita rubescens* Pers.	Cu (63.93), Zn (195.9) (mg/kg)	Alonso et al. (2003)
13.	*Armillaria mellea* (Vahl. ex Fr.) Karst	Fe (62.08), Zn (41.99), Cu (19.39) (mg/Kg)	Ivan et al. (2016)
14.	*Boletus aereus* Bull.	Cu (80.07), Zn (160.0) (mg/kg)	Alonso et al. (2003)
15.	*Boletus aestivalis* Paulet ex Fries	Fe (84.99), Zn (81.04), Cu (19.19) (mg/Kg)	Ivan et al. (2016)
16.	*Boletus edulis* Bull. ex Fries	Cu (13.01), Se (12.25), Mn (9.19), Al (4.62), Ag (2.34), Hg (2.18), Cd (1.22), Ni (0.99), Pb (0.79), Sb (0.60), As (0.41), Ba (0.38), Cr (0.22), Co (0.07) (mg/kg)	Ivan et al. (2015)
17.	*Boletus edulis* Bull. ex Fries	Cu (85.76), Zn (133.4) (mg/kg)	Alonso et al. (2003)
18.	*Boletus edulis* Bull. ex Fries	Fe (69.39), Zn (82.93), Cu (22.56) (mg/Kg)	Ivan et al. (2016)
19.	*Boletus griseus* Frost	Ti (8.68), Sr (0.15), Bi (298.13), Mn (0.0) (mg/kg)	Elekes and Busuioc (2010)
20.	*Boletus pinophilus* Pilát & Dermek	Cu (85.76), Zn (146.4) (mg/kg)	Alonso et al. (2003)

(continued)

Table 14.2 (continued)

S. no.	Macrofungi	Metal accumulation	References
21.	*Boletus reticulatus* Schaeff.	Cu (69.56), Zn (195.4) (mg/kg)	Alonso et al. (2003)
22.	*Calvatia excipuliformis* (Scop.) Perdeck	Ti (5.76), Sr (0.28), Bi (235.86), Mn (25.39) (mg/kg)	Elekes and Busuioc (2010)
23.	*Calvatia utriformis* (Bull.) Jaap	Cu (251.9), Zn (281.1) (mg/kg)	Alonso et al. (2003)
24.	*Cantharellus cibarius* Fr.	Cu (70.39), Zn (108.2) (mg/kg)	Alonso et al. (2003)
25.	*Clitocybe inversa* (Scop.) Quél.	Fe (54.33), Zn (62.96), Cu (19.65) (mg/Kg)	Ivan et al. (2016)
26.	*Clitocybe nebularis* (Batsch) P. Kumm.	Cu (92.35), Zn (158.3) (mg/kg)	Alonso et al. (2003)
27.	*Clitocybe nebularis* (Batsch) P. Kumm.	Fe (67.73), Zn (63.48), Cu (28.48) (mg/Kg)	Ivan et al. (2016)
28.	*Collybia butyracea* (Bull.) P. Kumm.	Ti (15.29), Sr (0.15), Bi (445.73), Mn (0.0) (mg/kg)	Elekes and Busuioc (2010)
29.	*Coprinus comatus* (O.F. Müll.) Pers.	Cu (147.3), Zn (139.7) (mg/kg)	Alonso et al. (2003)
30.	*Cortinarius armillatus* (Fr.) Fr.	Ti (15.26), Sr (0.30), Bi (623.44), Mn (0.0) (mg/kg)	Elekes and Busuioc (2010)
31.	*Cortinarius largus* Fr.	Ti (8.10), Sr (2.76), Bi (1251.66), Mn (0.0) (mg/kg)	Elekes and Busuioc (2010)
32.	*Fistulina hepatica* (Schaeff.) With.	Cu (39.51), Zn (50.33) (mg/kg)	Alonso et al. (2003)
33.	*Ganoderma lucidum* (Curtis) P. Karst.	K (0.91% µg/g), Na (37.2 µg/g), Fe (303.0 µg/g), Cu (72.5 µg/g), Zn (52.5 µg/g), Mn (64.0 µg/g), Cr (0.21 µg/g), Pb (0.13 µg/g)	Mohiuddin et al. (2015)
34.	*Hydnum repandum* L.	Cu (42.83 mg/kg), Zn (52.50 mg/kg)	Alonso et al. (2003)
35.	*Hygrophorus virgineus* (Wulfen) Fr.	Ti (15.65), Sr (0.89), Bi (571.09), Mn (15.04) (mg/kg d.w.)	Elekes and Busuioc (2010)
36.	*Hypholoma capnoides* (Fr.) P. Kumm.	Ti (19.94), Sr (3.70), Bi (990.72), Mn (87.34) (mg/kg d.w.)	Elekes and Busuioc (2010)
37.	*Lactarius deliciosus* (L.) Gray	Cu (32.62), Zn (309.8) (mg/kg)	Alonso et al. (2003)
38.	*Lactarius deterrimus* Gröger	Fe (49.25), Zn (86.12), Cu (7.41) (mg/Kg)	Ivan et al. (2016)
39.	*Leccinum scabrum* (Bull.) Gray	Cu (49.67), Zn (142.8) (mg/kg)	Alonso et al. (2003)
40.	*Lentinula edodes* (Berk.) Pegler	As (0.210), Cd (0.190) (mg/kg)	Maihara et al. (2008)
41.	*Lepista nuda* (Bull.) Cooke	Cu (117.7), Zn (182.1) (mg/kg)	Alonso et al. (2003)

(continued)

Table 14.2 (continued)

S. no.	Macrofungi	Metal accumulation	References
42.	*Lycoperdon perlatum* Pers.	Cr (1.94), Mn (13.90), Fe (782.0), Ni (1.96), Cu (10.90), Zn (134.0), Se (14.20), Cd (1.73), Pb (3.47) (mg/kg)	Stihi et al. (2011)
43.	*Macrolepiota procera* (Scop.) Singer	Cu (235.8), Zn (106.8) (mg/kg)	Alonso et al. (2003)
44.	*Macrolepiota* procera (Scop.) Singer	Fe (105.99), Zn (84.55), Cu (78.18) (mg/Kg)	Ivan et al. (2016)
45.	*Marasmius oreades* (Bolton) Fr.	Cu (116.1), Zn (152.9) (mg/kg)	Alonso et al. (2003)
46.	*Marasmius oreades* (Bolton) Fr.	Ti (6.89), Sr (0.36), Bi (8894.29), Mn (12.16) (mg/kg)	Elekes and Busuioc (2010)
47.	*Pleurotus eryngii* (DC.) Quél.	As (0.009), Cd (0.011) (mg/kg)	Maihara et al. (2008)
48.	*Pleurotus florida* Singer	Cu (53.56), Fe (243.92), Zn (95.26), Mn (41.29) (mg/kg)	Gebrelibanos et al. (2016)
49.	*Pleurotus florida* Singer	As (0.073), Cd (0.220) (mg/kg)	Maihara et al. (2008)
50.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	As (0.056), Cd (0.117) (mg/kg)	Maihara et al. (2008)
51.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	Cu (26.28), Zn (96.56) (mg/kg)	Alonso et al. (2003)
52.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	K (0.75–2.03%), Na (12.06–81.6 μg/g), Fe (69.5–626.2 μg/g), Cu (39.2–160.5 μg/g), Zn (30.4–75.5 μg/g), Mn (52.9–104.4 μg/g), Cr (0.20–0.30 μg/g), Pb (0.14–0.59 μg/g)	Mohiuddin et al. (2015)
53.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	Cu (51.19), Fe (220.87), Zn (89.68), Mn (47.55) (mg/kg)	Gebrelibanos et al. (2016)
54.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	Cr (1.81), Mn (12.40), Fe (387.0), Ni (1.85), Cu (12.50), Zn (41.30), Se (2.64), Cd (0.95), Pb (0.64) (mg/kg)	Stihi et al. (2011)
55.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	As (0.027), Cd (0.074) (mg/kg)	Maihara et al. (2008)
56.	*Pleurotus salmoneostramineus* Lj.N. Vassiljeva	As (0.043), Cd (0.229) (mg/kg)	Maihara et al. (2008)
57.	*Pleurotus tuber-regium* (Fr.) Singer	85 ppm	Anyakorah et al. (2015)
58.	*Russula cyanoxantha* (Schaeff.) Fr.	Cu (85.18), Zn (112.5) (mg/kg)	Alonso et al. (2003)
59.	*Schizophyllum commune* Fries	Ni (9.0), Cu (21.27), Zn (4.83), Cr (18.54) (mg/g)	Javaid et al. (2010)
60.	*Tricholoma portentosum* (Fr.) Quél.	Fe (153.96), Zn (80.23), Cu (11.82) (mg/kg)	Ivan et al. (2016)
61.	*Tricholoma columbetta* (Fr.) P. Kumm.	Cu (91.68), Zn (238.0) (mg/kg)	Alonso et al. (2003)

(continued)

Table 14.2 (continued)

S. no.	Macrofungi	Metal accumulation	References
62.	*Tricholoma equestre* (L.) P. Kumm.	Cu (72.14), Zn (233.5) (mg/kg)	Alonso et al. (2003)
63.	*Tricholoma portentosum* (Fr.) Quél.	Cu (66.76), Zn (164.6) (mg/kg)	Alonso et al. (2003)
64.	*Tricholoma terreum* (Schaeff.) P. Kumm.	Pb (2.4), Cd (1.6), Hg (0.06), Cu (35.8), Mn (24.8), Zn (48.0), Fe (169.0) (mg/kg).	Demirbaş (2001)
65.	*Tricholoma terreum* (Schaeff.) P. Kumm.	Fe (83.53), Zn (90.56), Cu (15.41) (mg/kg)	Ivan et al. (2016)
66.	*Volvariella volvacea* (Bull.) Singer	K (1.35%), Na (44.6 μg/g), Fe (322.5 μg/g), Cu (101.8 μg/g), Zn (36.5 μg/g), Mn (78.5 μg/g), Cr (0.24 μg/g), Pb (0.25 μg/g)	Mohiuddin et al. (2015)
67.	*Xerocomus badius* (Fr.) E.-J. Gilbert	Cu (61.58), Zn (225.7) (mg/kg)	Alonso et al. (2003)
68.	*Xerocomus chrysenteron* (Bull.) Quél.	Cu (77.56), Zn (162.3) (mg/kg)	Alonso et al. (2003)

soil. It is able to metabolize and mineralize several PAHs and their analogs when grown in the presence of them (Cohen et al. 2002). In the environment phenanthrene and pyrene are the two most abundant PAHs (Cerniglia 1992); the molecular structures of phenanthrene and pyrene are found to be potent carcinogenic PAHs (Pothuluri and Cerniglia 1994). The white rot fungi *Trametes versicolor* and *Kuehneromyces mutabilis* mineralized 15.5 and 5.0% of phenanthrene, respectively, in a period of 63 days (Sack et al. 1997).

The veterinary fluoroquinolone enrofloxacin (synthetic antimicrobial agents which have found wide application in human and veterinary medicine) is degraded in vitro by four species of wood-rotting fungi (*Gloeophyllum striatum*, *Stropharia rugosoannulata*, *Phanerochaete chrysosporium*, *Irpex lacteus)* growing on wetted wheat straw containing carbonyl- ^{14}C -labeled drug. A maximum $^{14}CO_2$ production of 17% per week was observed with the brown rot fungus *Gloeophyllum striatum* (Martens et al. 1996).

14.4.1 The Spent Mushroom Substrate as a Bioremediation Agent

The substrate which is left after the harvesting of mushroom is known as the spent mushroom substrate. It is a mixture of various industrial wastes, agricultural wastes, etc. and prepared by controlled fermentation. SMS is widely used as a biodegradable agent and also as vermicompost and organic fertilizer and in plant disease management. The process of composting is divided into four groups on the basis of microbiological stages with respect to temperature like mesophilic, thermophilic, cooling, and maturation (Fogarty and Tuovinen 1991).

The spent substrate obtained after different mushroom cultivations differs in its physical, chemical, and biological properties. SMS has the capability of absorbing various organic and inorganic substances for the substrate on which it is applied. It also contains various microorganisms which help in easy bioremediation process. The spent substrate obtained from button mushroom cultivation is nutritionally rich in N/P/K (1.9:0.4:2.4%) contents and shows high cation exchange capacity. It has a high capacity to replace farmyard manure for the purpose of raising horticultural and cereal crops. Addition of SMS on pesticides present in soil degrades it at various rates by means of its physical property and microbes present in it (Ahlawat and Sagar 2007). The mixing of spent mushroom substrate at 10, 20, and 30%, w/w in the soil, results in faster degradation of malathion, bavistin, and mancozeb in comparison to soil without any amendment of SMS.

SMS also has the ability to chemically adsorb the organic and inorganic pollutants, while the diverse category of microbes it harbors has the capability of the biological breakdown of the organic xenobiotic compounds present in soil and water (Buswell 1995; Semple and Fermor 1995; Hofrichter et al. 1997). Button spent mushroom substrate (SMS) and its dominating microbes help in easy biodegradation of commonly used agricultural fungicides carbendazim and mancozeb. All the microbes (*Trichoderma* species, *Aspergillus* species, some bacteria, etc.) present in SMS both individually and in different combinations are able to grow well. They produced extracellular ligninolytic enzymes on SMS, which help in the degradation of fungicides (Ahlawat et al. 2010). SMS is also in use for bioremediation of hydrocarbon-contaminated soil due to its high working efficiency and low cost. SMS act as a very good amendment for remediation for hydrocarbon-contaminated soil. SMS contains a high level of nutrients and enzymes which help in easy biodegradation (Eramo and Brennan 2009). It degrades three to six unsubstituted aromatic hydrocarbons, i.e., polycyclic aromatic hydrocarbon, and also other organopollutants in composting system (Cajthaml et al. 2002).

14.4.2 Enzymatic Activities of Various Mushrooms

Lignocellulose is a major portion of biomass, and its degradation is essential for maintenance of carbon cycle at the global level. It generally consists of a mixture of cellulose (ca. 40%), hemicelluloses (ca. 20 ± 30%), and lignin (ca. 20 ± 30%) (Tuomela et al. 2000).

Lignin is an aromatic polydispersed polymer which provides rigidity, water impermeability, and resistance to microbial attack to plant cell walls. Structural complexity of lignin restricts its biodegradation, so for its degradation initial work must be oxidative, nonspecific, and extracellular (Cohen et al. 2002).

Lignin decomposed by white rot fungus belongs to family *Ascomycotina* and *Basidiomycotina*. *Basidiomycotina* attack on heartwood or softwood while *Ascomycotina* only on heartwood. Lignin decomposition is faster by white rot fungi and is responsible for most of the lignin decomposition in nature. It causes selective

and nonselective lignin degradation, e.g., *Phanerochaete chrysosporium* and *Phlebia radiata* are selective in nature, while *Trametes versicolor* degrade it nonselectively.

White rot fungi helps in degradation of lignin by means of oxidative enzyme. Extracellular enzymes of white rot fungi which help in lignin degradation are lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccase. Different white rot fungi produce a different combination of enzymes (Tuomela et al. 2000). These enzymes have the ability to degrade environmentally persistent xenobiotics and endocrine-disrupting chemicals such as pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), and dioxins (Nagai 2002). White rot fungi also help to protect the environment in many ways, for example, during paper manufacturing, chlorine remains bound to the lignin which causes a serious environmental pollution after disposal, so pre-treatment of the wood with the lignin-digesting white rot fungus offers an economically and environmentally acceptable solution (Sultana et al. 2007).

Pleurotus is a saprobic, ligninolytic mushroom which uses its ligninolytic property for biodegradation of organic pollutant into valuable by-products. The main advantage of using Pleurotus spp to upgrade lignocellulosic waste is their selective degradation of lignin and hemicelluloses, as a result of which the cellulose is exposed and can be utilized by ruminants (Cohen et al. 2002). *Pleurotus eryngii* degrade nearly 50% Klason lignin during a solid-state fermentation (SSF) experiment (Martinez et al. 1994).

Some ectomycorrhizal fungi inhabiting in litter produce protease enzyme. By this enzyme, they degrade protein and distribute various soluble amino compounds into the root of plants by means of their hyphal networks like *Glomus* transport the amino acids glycine and glutamine into wheat (Dell 2002).

Laccase has the property of catalyzing single-electron oxidation of phenolic substrates or aromatic amines in various forms even in the presence of proper redox mediator; it can also oxidize nonphenolic compounds. *Lentinula edodes*, an important edible mushroom, has the highest laccase activity, with no lignin peroxidase and weak MnP activities. Enzymes produced from Lentinula edodes act as good source of environmental bioremediation agents, and also enzymes produced by it are safe for human (Nagai 2002). *Pleurotus* spp. have the ability to convert cellulose into important proteins which can be very useful and important for humans (Mane et al. 2007).

14.5 Conclusion

With the advancement of science and technology, lots of new inventions are made day by day. These inventions made our life easier but at the same time add various types of undesirable by-products in nature. Even urbanization and industrialization lead to an increase in environmental pollutants. Harmful chemicals are released into the environment by various ways like through industrial discharge, agricultural practice, daily life activities, etc. These substances persist in nature for a longer time

and are not removed easily in simple ways. They are destructive to our ecosystem and also cause various diseases in human beings, animals, and plants also. Some xenobiotics, viz., polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and trichloroethylene (TCE), accumulate in the environment due to recalcitrant properties of themselves and hence are a major cause of environmental pollutants.

Bioremediation is the process of using microorganisms to remove the harmful pollutants from the environment. Macrofungi can be used as an alternative way to remove these xenobiotics from the environment. Remediation by macrofungi is very cheap, effective, and an environmentally sound way of removing a wide array of toxins from the polluted environment. Macrofungi help in decontamination of the environment. The by-products of remediation can be valuable material for mushroom itself, so remediation process is even profitable in this term to macrofungi. Utilization of macrofungi both by bioremediation and biotransformation method results in degradation and the transformation of accumulated environmental pollutants including hydrocarbons, polychlorinated biphenyls, polyaromatic hydrocarbons, heterocyclic compounds, radio-nuclear and heavy metals in to undisruptive stuffs. Macrofungi helps in the conversion of these toxic compounds to nontoxic form (by process of detoxification) which is harmless to the environment.

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Chapter 15
Microemulsions as a Novel Tool for Enhancing the Bioremediation of Xenobiotics

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Abstract Bioremediation is an advantageous and sustainable technology to remediate contaminated environments since it is cost-effective and environmentally safe. However, some pollutants such as most organochlorine pesticides and hydrocarbons are poorly soluble in water and thus tend to adhere tightly to soil particles. Therefore, the degradation of hydrophobic compounds is usually slow and frequently unsatisfactory due to the difficulties related to their transfer from soil particles to the aqueous phase, where these compounds are more available for degradative microorganisms. In this relation, a fundamental issue for the bioremediation processes is to overcome the limited accessibility of these hydrophobic pollutants for the microorganisms. As an alternative to synthetic surfactants, which are usually introduced into bioremediation processes with the aim of enhancing the bioavailability of hydrophobic pollutants, microemulsions have attained increasing significance both in basic research and environmental applications. Microemulsions consist of a combination of surfactants, co-surfactants, and oil phase and have demonstrated to be promising candidates due to its much higher solubilization capacity

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than surfactant micelles. This chapter compiles updated data related to general characteristics of microemulsions, with a special emphasis on the application of these systems as biotechnological tools for enhancing the solubilization and biodegradation of hydrophobic compounds, such as organochlorine pesticides, especially lindane.

Keywords Microemulsion · Solubilization · Bioremediation · Removal · Hydrophobic organic compounds · Pesticides

15.1 Introduction

Environmental pollution has been on the rise in the past decades due to increased human activities on energy reservoirs, unsafe agricultural practices, and rapid industrialization. Remediation technologies involve any operation that alters the characteristics of hazardous or polluting wastes in order to reduce their toxicity, volume, or mobility through the application of physical, chemical, and/or biological processes (Betancur-Corredor et al. 2013). Some of the physical and chemical technologies that have been used are oxidation for the treatment of a great variety of pollutants such as fuels, solvents, and pesticides (Huling and Pivetz 2006), reduction of heavy metals such as Cr (VI) using zerovalent Fe nanoparticles (US EPA 2011), stabilization or solidification based on the addition of binders in order to generate a solid material in which the contaminants are immobilized (no leaching occurs) (Al-Tabbaa and Stegemann 2005), incineration at high temperature, UV oxidation, decomposition catalyzed by acids or bases, soil washing, photocatalysis, adsorption, filtration, and precipitation, among others (Byrne et al. 2017; Carolin et al. 2017). All these technologies can be very effective in reducing the levels of a large number of pollutants, but they also have several drawbacks, such as high specificity, complexity, high costs of implementation, and lack of acceptance by the population (Niti et al. 2013). In contrast, biological treatments have received considerable attention in recent years as an effective biotechnological tool to restore contaminated environments. These eco-friendly remediation technologies are called bioremediation and include processes based on the use of biological mechanisms to reduce (degrade, detoxify, mineralize, or transform) the concentration of pollutants to an innocuous state (Azubuike et al. 2016). For this purpose, diverse biological systems may be used, including bacteria, filamentous fungi, yeasts, algae, and plants and also their derivatives (Robles-González et al. 2008; Wood 2008). Bioremediation is considered relatively cost-effective and environmentally friendly compared to other physicochemical methods such as chemical decomposition, incineration, and photodegradation (Niti et al. 2013). In addition, diverse strategies were developed to enhance the biological mechanisms and improve the bioremediation performance with the ultimate goal to effectively restore polluted environments in an eco-friendly approach, at a very low cost. In this connection, due to the unique properties of microemulsions, varied formulation possibilities, and numerous applications, these systems have attracted attention in numerous important fields, including soil

washing and bioremediation (Karunaratne et al. 2017; Salam and Das 2013). On this basis, the present chapter consists of a short compilation regarding the generalities of microemulsions, such as its classification, components, and applications, with a special emphasis on the study of these systems as biotechnological tools for bioremediation purposes.

15.2 Microemulsions: Definition and General Characteristics

Microemulsions are defined as homogeneous systems formed by two immiscible fluids, hydrocarbon and water, stabilized by a surfactant or a mixture of surfactants, frequently in combination with a co-surfactant (Bera and Mandal 2015; Fanun 2012). Exhibiting a pseudo-biphasic behavior, these systems allow the solubilization of highly hydrophilic substances in oil-based systems and highly hydrophobic substances in water-based systems (Karunaratne et al. 2017).

Unlike conventional emulsions, whose microstructure is static, microemulsions are dynamic systems, with a constantly fluctuating interface. In addition, microemulsions are thermodynamically stable and spontaneously formed under a specified set of experimental conditions; i.e., a change in these conditions may lead to phase separation, and, once the original conditions are restored, they reform spontaneously. Their properties are time-independent and not influenced by the production process, such as the order of mixing or mechanical energy, making them easier to prepare. In contrast, emulsions are thermodynamically unstable and require a high input of mechanical energy for their formation; therefore the cost of preparation is higher (Bragato et al. 2002; Hloucha 2014; Talegaonkar et al. 2008). Another main difference between emulsions and microemulsions is the size of the droplets of the dispersed phase; in microemulsions, it ranges between 5 and 100 nm, while in emulsions it is generally higher (>100 nm) so that they often take on a milky appearance (Karunaratne et al. 2017). On the opposite, microemulsions usually appear transparent and translucent, although they contain high amounts of water and oil (Hloucha 2014). Other significant differences between emulsions and microemulsions are described in Table 15.1.

The viscosity of microemulsions is generally low, although it is a function of its composition. For instance, it has been demonstrated that the addition of a salt such as NaCl can increase the viscosity of a microemulsion system (Moulik and Rakshit 2006). The rheology of microemulsions varies depending on the phase point. However, it is often crucial in their application because it will affect the processability, kinetics, and stability under various conditions (Karunaratne et al. 2017).

Several experimental methods are used for the characterization of the structure of microemulsions, which are often very complex systems. Some of the techniques used for this purpose include freeze-fracture electron microscopy, light scattering, and nuclear magnetic resonance spectroscopy, among others. However, it is often an advantage to use a combination of methods to obtain a complete characterization (Hloucha 2014).

Table 15.1 Differential characteristic between microemulsion and emulsion

Characteristics	Microemulsion	Emulsion
Stability	Thermodynamic	Kinetic
Micellar size	<100 nm	>100 nm
Aspect	Transparent	Opaque, milky
Formation	Spontaneous	Mechanical energy required
Order of addition of components	Indifferent	Key
Optical isotropy	Isotropic	Anisotropic
Phases	Monophasic	Biphasic
Interfacial tension	Ultralow	High
Viscosity	Low	High

Adapted from Muñoz Hernández et al. (2005) and Talegaonkar et al. (2008)

15.3 Classification of Microemulsions

According to the nature of the components used in the preparation of the microemulsions and their proportions, different types of microemulsions may be formed, which, depending on their structure, can be classified in three basic types:

- *Oil in water (o/w) or direct microemulsions*: consist of oil droplets dispersed in the aqueous phase. The surfactant molecules are organized so that their nonpolar tails associate with each other resulting in a globular structure with a hydrophobic core (McClements 2012), also being called oil-swollen micelles (Zheng et al. 2012a). In general, they are formed when the oil concentration is low (<30%) (Flanagan and Singh 2006) (Fig. 15.1a).
- *Water in oil (w/o) or inverse microemulsions*: consist of drops of water dispersed in the oil phase. The ordering of polar and nonpolar zones of the surfactant molecules is inverse to that mentioned above so that the polar portion of the surfactant is oriented toward the inside of the micelle, while the hydrophobic chains are oriented to the outside (Muñoz Hernández et al. 2005). They are generally formed when the aqueous concentration is low (Flanagan and Singh 2006) (Fig. 15.1b).
- *Bicontinuous or middle phase microemulsions*: are formed when the amounts of water and oil are similar (Talegaonkar et al. 2008) (Fig. 15.1c).

Regarding the rheological properties of microemulsions, direct and inverse microemulsions show Newtonian behavior over a wide range of shears, while bicontinuous may undergo breakage upon medium shear forces, leading to thinning (Karunaratne et al. 2017).

Salinity can also reverse the structure of microemulsions. At high salinity levels, direct microemulsions change to reverse microemulsions, whereas at low salinity the system remains in water external phase (Bera and Mandal 2015).

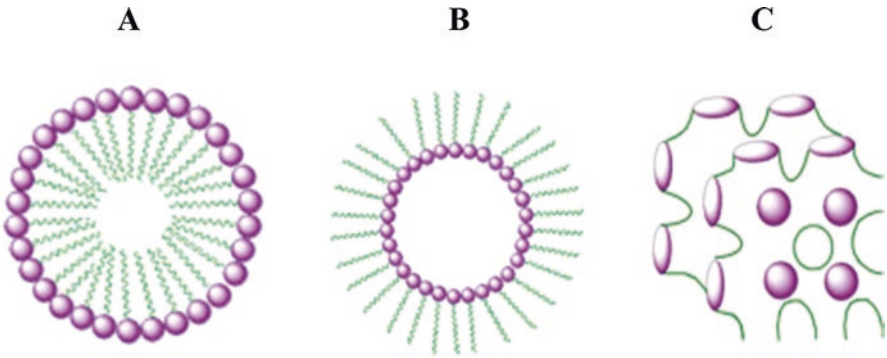

Fig. 15.1 Schematic diagram of the types of microemulsions. (**a**) Oil in water (o/w) or direct microemulsion, (**b**) water in oil (w/o) or reverse microemulsion, and (**c**) bicontinuous microemulsion. (Adapted from Mehta and Kaur 2011)

15.4 Components of Microemulsions

The physicochemical properties of microemulsions depend on the nature of its components (Bera and Mandal 2015). A large number of oils, surfactants, and cosurfactants can be used for the preparation of microemulsions; however, special emphasis is put on the use of substances generally recognized as safe or GRAS. Besides, all the components should be biocompatible, nontoxic, clinically acceptable, and used in an appropriate concentration, so that they will not be aggressive (Talegaonkar et al. 2008).

15.4.1 Surfactant

Surfactants are considered to be the principal constituents of microemulsions. They consist of amphiphilic molecules, i.e., they contain a hydrophilic portion and a hydrophobic portion, and have the ability to reduce the interfacial tension between the medium in which they are dissolved and any other fluid in contact, which facilitates the dispersion during the preparation of the microemulsion (Muñoz Hernández et al. 2005; Talegaonkar et al. 2008). Surfactants can be classified based on the charge of the head group into ionic and nonionic. Ionic surfactants can also be divided into cationic, anionic, and zwitterionic. The choice of surfactants will depend on the future application of the microemulsions (Bera and Mandal 2015).

The hydrophilic-lipophilic balance (HLB) is the most characteristic parameter of a surfactant, and it relates molecular structure to interfacial packing and film curvature. The HLB of a surfactant is a measure of the degree of its hydrophilicity or lipophilicity on a scale of 0–20, where an HLB of 0 corresponds to a completely

lipophilic/hydrophobic molecule and a value of 20 corresponds to a fully hydrophilic/lipophobic molecule. In general, surfactants with HLB < 12 favor the formation of inverse microemulsions, whereas surfactants with high HLB (> 12) are preferred for the formation of direct microemulsions. For bicontinuous structures, i.e., zero curvature, it was shown that HLB is around 10 (Talegaonkar et al. 2008).

Furthermore, regarding the length and volume of their hydrophobic tail, surfactants with linear aliphatic hydrocarbon chains of moderate length preferably form direct microemulsions, surfactants with voluminous hydrophobic tails form bicontinuous microemulsions, and surfactants with branched hydrophobic tails form inverse microemulsions. Often combinations of two or more surfactants are used for the formulation of microemulsion systems, and the resulting geometry of the system will depend on the geometry of the species involved (Muñoz Hernández et al. 2005).

15.4.2 Co-surfactant

Although co-surfactant is not considered as a main component of microemulsions, it is generally added to the surfactant to prepare microemulsions because it presents several well-documented roles. For instance, the co-surfactant prevents the formation of rigid structures such as gels, liquid crystals, and precipitates; alters the viscosity of the system; reduces the interfacial tension; and increases the fluidity of the interface and the mobility of the hydrocarbon tail, thus allowing greater penetration of the oil into this region, among others (Bera and Mandal 2015).

The co-surfactant inserts between the surfactant molecule constituent of the interfacial film. Short-, intermediate-, or long-chain alcohols can be used as co-surfactants. Usually, short- to medium-chain alcohols (C3-C8) such as propanol, butanol, isoamyl alcohol, pentanol, etc. are used as co-surfactants for the preparation of microemulsions, as they are able to further reduce interfacial tension and increase the interfacial fluidity. The solubility of alcohols in water depends on the aliphatic chain length. Short-chain alcohols (ethanol, propanol, and isopropanol) are more hydrophilic and slightly increase the affinity of the surfactant for the aqueous phase, whereas the longer-chain alcohols (pentanol, hexanol) show very low solubility in water; hence they are localized mainly toward the oil. Intermediate alcohols (butanol, isobutanol) have almost the same affinities for oil and water and do not significantly modify HLB (Bera and Mandal 2015; Talegaonkar et al. 2008).

Besides the components forming the microemulsion, the ratio among them is also a very important factor influencing the microemulsion existence domain. In fact, Zheng et al. (2011) postulated that the co-surfactant to surfactant ratio (C/S) is the major factor influencing the microemulsion formation and seriously influences the microemulsion area. Their results demonstrated that higher oil content was incorporated in all microemulsion systems tested when the C/S ratio increased.

15.4.3 Oil

The oil has the ability to penetrate the microemulsions and thus increase the region of the surfactant monolayer. Short-chain oils penetrate the tail group region to a greater extent than long-chain oils and thus increase this region to a greater extent, resulting in the effective reduction of HLB. Saturated fatty acids (e.g., lauric and capric acid) and unsaturated fatty acids (such as oleic and linoleic acid) have been used as the oil phase for microemulsion preparation. Fatty acid esters, such as ethyl or methyl esters of oleic acid, can also be employed for microemulsion formulation (Talegaonkar et al. 2008).

As mentioned before, the ratio of the components of the microemulsion system also plays a key role in determining its properties. In this sense, increasing the oil content of microemulsion may enhance its solubilizing capacity for a hydrophobic solute by increasing the oil volume fraction (Zheng et al. 2011).

15.5 Applications of Microemulsions

The applications of microemulsions are plenteous as they have attracted attention in various fields, including drug delivery, cosmetics, dry cleaning, food, fuels, lubricants and coatings, detergents, agrochemicals, analytical chemistry, nanoparticle synthesis, and biotechnology (Karunaratne et al. 2017). They have also been used as reaction media, as a stationary phase for capillary chromatography, and as biological membrane models, and new applications are constantly being reported (Flanagan and Singh 2006).

Microemulsions present many attractive properties. For instance, one of the interesting advantages of microemulsion-based fuels is that they contribute to the reduction of the emission rate of nitrogen oxides and carbon monoxide with an improvement in fuel economy (Worakitkanchanakul et al. 2008). Microemulsions also show extraordinary water solubilization capacity which makes them capable of injecting fluids in chemical oil recovery (Bera and Mandal 2015).

In the pharmaceutical industry, microemulsions can serve as delivery systems for both hydrophobic and hydrophilic drugs and also allow the sustained or controlled release of drugs such as chemotherapeutic agents or insulin, among others (Fanun 2012; Talegaonkar et al. 2008).

The use of microemulsions in the food industry has been extensively studied. For instance, microemulsions can be used to solubilize essential oils but are only able to deliver certain of them, and the composition of microemulsions must be carefully chosen in order not to affect their antimicrobial activities. Other practical applications of the microemulsions may be to coat semisolid foods such as cantaloupes whose surface is not consumed (Ma et al. 2016).

15.6 Microemulsions as Bioremediation Tools

Recent advances in the field of environmental restoration techniques have led to the application of microemulsions in remediation and bioremediation processes of organic and inorganic compounds (Bragato and El Seoud 2003; Castro Dantas et al. 2009; Melo et al. 2015; Vargas-Ruiz et al. 2016).

When hydrophobic pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and organochlorine pesticides reach the soil, they are difficult to remove due to their strong adsorption onto soil particles through adsorption, electrostatic interaction, and/or covalent bonding. Therefore, there is an intense interest in soil decontamination by microemulsions (Bragato et al. 2002).This is an attractive technique due to the following reasons: high efficiency since the microemulsion decreases the interfacial tension between the contaminant and the soil and the desorbed contaminants dissolve more easily in microemulsions than in conventional surfactants emulsions; convenience based on the much smaller volumes handled in soil washing with microemulsions compared to those produced using the same technology with water or aqueous micellar solutions; and recycling possibility because the separation of microemulsions can be achieved in an aqueous surfactant-rich phase (for recycling) and an organic phase containing pollutants (for their elimination), simply by changing the temperature (Bragato and El Seoud 2003). For their application in soil remediation, nonionic surfactants are more suitable for the preparation of microemulsions than anionic or cationic surfactants; anionic surfactants can precipitate with divalent cations present in the soil, while cationic surfactants can adsorb onto the soil, both resulting in significant surfactant loss (Wang and Mulligan 2009). Also, for this type of processes, vegetable oils are more appropriate than mineral oil because of their biodegradability (Bragato et al. 2002).

Oil in water microemulsions composed of a nonionic surfactant (Tween 80 or Triton X-100), vegetable oil (linseed oil or soybean oil), and 1-pentanol as the cosurfactant has shown to effectively enhance the solubility of hydrophobic organic contaminants such as DDT and lindane compared to the solubilizing capacity of their respective surfactant solutions alone (Zheng et al. 2011). Besides pesticides, the removal of polycyclic aromatic hydrocarbons (PAHs) using microemulsions has been also studied. In this context, Zhao et al. (2005) reported a high and fast desorption of phenanthrene from contaminated soil by using a direct microemulsion prepared with sodium castor oil sulfate, due to its high solubilization capacity compared with the conventional surfactant solutions. The authors propose it as a potential agent for ex situ washing for PAH-contaminated soils since the castor oil is not toxic and the commercial sodium castor oil sulfate costs around the half of the cost of other commercial surfactants, such as Tween 80, for instance.

Microemulsions have been also applied to remove heavy metals from aqueous solutions and sludges (Castro Dantas et al. 2003; Dantas Neto et al. 2004). In fact, the extraction of heavy metals by microemulsions results advantageous, compared to conventional treatment techniques, mainly in relation to contamination with solvents and energy consumption. Castro Dantas et al. (2009) reported a very high

efficiency in chromium extraction from a metal-rich solution obtained by acid digestion of a leather tannery sludge, reaching up to 93.4% of chromium removal with only one extraction stage, thus showing the high potential of microemulsions to treat tannery sediments.

On the other hand, it is known that pollutants are degraded mainly in solution since they are more available for microbial action. However, in the case of hydrophobic pollutants, they are poorly soluble in water and, therefore, tend to adhere strongly to soil particles by adsorption, electrostatic interaction, and covalent binding (Zheng and Wong 2010). Hence, degradation of this kind of pollutants in soils is usually slow and often unsatisfactory. Surfactants have been used to improve the solubility and bioavailability of hydrophobic organic compounds in soils, thus facilitating their degradation by microorganisms (Mulligan 2005; Quintero et al. 2005). In this regard, Salam and Das (2013) studied the biodegradation of lindane by the yeast *Pseudozyma* VITJzN01 using microemulsions prepared from a biosurfactant (BS) produced by this yeast and olive oil as the oil phase. In minimal medium, lindane degradation rate was much higher in the presence of the bio-microemulsions, reaching the complete elimination of the pesticide on the sixth day of incubation, whereas with the biosurfactant alone, the 100% removal was achieved after 10 days of incubation. The addition of the BS as well as the bio-microemulsion increased the solubility and, thus, the substrate availability for the yeast. In slurry systems, the degradation of lindane was less and slower. After 30 days of incubation, the yeast could only degrade 40% of lindane when no emulsifying agent was added to the system. The lower degradation rate in the absence of solubilizing agent clearly indicated that lindane may be adsorbed on the soil particles and therefore was not available for *Pseudozyma* VITJzN01. When BS was used as a solubilizing agent together with the yeast inoculum, 50% of lindane degradation was observed, whereas when the bio-microemulsion was used as a solubilizing agent, the pesticide removal reached 80% after 30 days of incubation. Consequently, the authors confirmed that the bio-microemulsion prepared with a biosurfactant of glycolipid nature, olive oil, and water, without the addition of any co-surfactant, improved the lindane degradation capacity of the yeast *Pseudozyma* VITJzN01 in both liquid culture and soil slurry, which can be interpreted as an eco-friendly approach.

Furthermore, the biodegradation of lindane by *Sphingobium indicum* B90A was also evaluated with the addition of surfactants and microemulsions. The pesticide degradation was accelerated by the addition of both the surfactant Tween 80 and microemulsions formed with Tween 80, being the microemulsions much more effective than the surfactant alone, while microemulsions formed with Triton X-100 totally inhibited the biodegradation of lindane by *S. indicum* B90A due to the toxicity of Triton X-100 for the bacteria (Zheng 2011). Later, Zheng et al. (2012b) also demonstrated that the use of microemulsions formed with Tween 80 favored the degradation of DDT by the fungus *Phanerochaete chrysosporium* around two times compared to the obtained with the Tween 80 solution. This could have occurred possibly through transporting DDT from crystalline phase to mycelium as well as the possible use of the components of the microemulsion as additional carbon source to the fungus, thus providing a positive effect on the fungal growth.

Recently, Saez et al. (2017) demonstrated for the first time the enhancement of lindane removal by an actinobacterium through the use of microemulsions as bioremediation tools. First, they obtained stable direct microemulsions using Tween 80, 1-pentanol, and three different vegetable oils, while Triton X-100 and Brij L-23 did not form stable microemulsions. Then, the ratio between the components in the microemulsions was evaluated in order to improve lindane solubilization. Thus, the authors found that an increase in the C/S ratio favored the pesticide solubilization, while an increase in the oil phase with respect to the surfactant (O/S) negatively affected the stability of the microemulsions. The microemulsion prepared with soybean oil allowed the solubilization of 66% of lindane in the aqueous medium, i.e. 4.5 times higher than the obtained by the surfactant solution at the same concentration, and the desorption of 85% of lindane in soil systems, representing around 3 and 3.5 times the obtained by the surfactant solution or water, respectively; hence this microemulsion was selected for the bioremediation studies. This microemulsion system enhanced lindane removal by *Streptomyces* sp. M7 in the liquid medium almost twice the achieved with the surfactant alone. This may be not only by increasing the bioavailability of the pesticide in the aqueous medium but also because the components forming the microemulsion could have exerted a stimulation effect on the microbial growth of the actinobacterium. In soil system, the addition of the microemulsion allowed an 87% of lindane removal by *Streptomyces* sp. M7, increasing almost 50% of the removal with respect to the obtained without the addition of surfactant agents, although it did not present significant difference with respect to the obtained with the surfactant solution. Therefore, this microemulsion could be used as potential tools in soil washing technologies or ex situ bioremediation processes of wastewaters containing not only lindane but also other hydrophobic organic compounds.

15.7 Concluding Remarks

Microemulsions are promising alternatives to synthetic surfactants in enhancing the solubilization, and hence increasing the bioremediation efficiency, of hydrophobic pollutants from wastewaters, soils, and sediments. This innovative technique combining microemulsions and bioremediation expands the scope of bioremediation and provides an efficient and safe way for the remediation of different matrices contaminated by heavy metals and hydrophobic organic compounds, such as organochlorine pesticides. However, the information available is only related to laboratory scale. Thus, deeper research is still needed in order to expand the usage of microemulsions for bioremediation purposes in field scale, assessing its effect on indigenous microorganisms, among other factors to evaluate.

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Chapter 16
An Overview of Nitro Group-Containing Compounds and Herbicides Degradation in Microorganisms

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Abstract Basically, nitro functional group-containing chemicals have been used to synthesize various useful products like dyes, pesticides and solvents, and also military products and so on. Hence, many nitroaromatics (including nitrophenols) have been continuously released into the environment and appear in the soil and water. Some are known to be toxic due to their great impact on living systems (especially on health). Most such chemicals (nitroaromatic compounds) are listed as priority chemicals by the Environmental Protection Agency (EPA). The vast use of such chemicals and their toxic effects had led to the study of the degradation of nitro group-containing chemicals by microbes (an easily available and cost-effective treatment). In view of this, we discuss the degradation of a few nitro group-containing compounds and herbicide(s) by microorganisms from published literature, and we consider the future perspective.

Keywords 3,5-dinitro-*ortho*-cresol · 4-nitrophenol · Picric acid · Microorganisms · Degradation

16.1 Introduction

Over the last several decades, natural and manmade chemicals, including antimicrobial agents, antibiotics, personal care products, pesticides, herbicides, chlorinated aromatics and nitroaromatics, among others, have been used for various purposes in day-to-day life (Hoskeri et al. 2011, 2014; Megadi et al. 2010; Mulla et al. 2011a, b, 2016a, b; Tallur et al. 2015; Talwar et al. 2014). The long-term use of consumer products has released these chemicals to the environment, where they continually show up at levels from less than 1 nanogram to more than 1 microgram per litre (Mulla et al. 2012, 2014, 2016c, d, 2018). In recent years, several (including chloro and nitro group-containing aromatics) were found to be toxic to living beings—from humans to aquaculture organisms (Kovacic and Somanathan 2014; Edalli et al. 2018; Mulla et al. 2014, 2016c, d, 2017; Tallur et al. 2015). Various researchers from many countries have thus been prompted to investigate decontamination methods that would be effective on such chemicals (Arora et al. 2014, 2017; Burkul et al. 2015; Li and Yang 2018; Osin et al. 2018). Among the avenues considered, of the use of non-harmful biological mediators to detoxify toxic chemicals in wastewater treatment plants gained significantly more importance. In this chapter, we discuss some nitro group-containing compounds as used for degradation of toxic substances in microbes.

16.2 Uses of and Environmental Pollution by Nitro Group-Containing Compounds

In general, nitro group-containing chemicals go into the synthesis of certain useful products, for example, explosives, dyestuffs, insecticides, herbicides and rubbers (Arora et al. 2014; Douglas et al. 2011; Haizhen et al. 2009; Khalid et al. 2009; Spain 1995; Wan et al. 2007; Ye et al. 2004). In addition, they are also found in the preparation of products like medicines, fuels for vehicles, wall paints, solvents for polish, electronic batteries, coloured glass, etc. (Beard and Noe 1981; Dunlap 1982; Hirai 1999; Plunkett 1966; Windholz et al. 1976; Ware 1994).

On the other hand, most of nitro group-containing chemicals (including nitrophenols), are stable, but, some possess toxic properties (especially carcinogenicity) (Ju and Parales 2010; Kovacic and Somanathan 2014; Nishino et al. 2000; Padda et al. 2003; Purohit and Basu 2000). Hence, several (including nitrophenols) are included in the U.S. EPA list (Ju and Parales 2010). Some of these nitro group-containing chemicals have also been detected in food products. Likewise, mutagenic property-containing nitroaromatic compounds have been found in the atmosphere, especially by way of cigarette smoke and vehicle fuels (Kinouchi and Ohnishi 1983). Most of these have similar functional properties that make them beneficial for industrial use, on the one hand, while causing them to be harmful to the health of living systems. In general, the widespread use of nitroaromatics impacts the environment by contaminating soil and water (including groundwater) (Rieger and Knackmuss 1995; Ju and Parales 2010). Likewise, nitro functional group-containing insecticides are purposely applied to crops, leaving surrounding fields open to possible contamination. Additionally, inadequate management and/or loading practices by both manufacturers and customers results in their unintended injection into the environment (Mulla et al. 2014). For example, a Toxics Release Inventory report 2002 reported that nearly 11500 kg of 2,4-Dichloro-1-(4-nitrophenoxy)-benzene, 5100 kg of nitrobenzene and 1100 kg of 2,4-dinitrotoluene have been discharged into territory of United States (White and Claxton 2004). Ecological contamination from explosives manufacturers also occurs in Germany (Spain et al. 2000). The toxicological effects of nitro group-containing chemicals are well discussed in the literature (Kovacic and Somanathan 2014).

16.3 Biodegradation of Nitro Group-Containing Chemicals

Among multipurpose industrial organic compounds, nitro group-containing chemicals are among those most used in pesticides, pharmaceuticals, pigments, dyes, etc. (Haghighi-Podeh and Bhattacharya 1996). These chemicals may stay in the soil as

the by-products of insecticides like parathion, methyl-parathion and other more complex nitroaromatics (including herbicides) through hydrolysis. Hence, many researchers with the aim of detoxification of such chemicals have demonstrated that various organisms are able to utilize different types of nitrophenols as a growth substrate. Generally, the nitro group-containing chemical degradation process involved oxidation (monooxygenase and/or dioxygenase) and/or reduction (reductase) or Meisenheimer complex generation.

16.3.1 Microbial Degradation of Mononitrophenols

The bacterial culture *Pseudomonas putida* B2 utilizes both *ortho*- and *meta*-nitrophenol as a growth substrate and degrades both substrates by producing different pathways (Zeyer and Kearney 1984). In *Alcaligenes* sp. NyZ215 (Xiao et al. 2007) and *Pseudomonas putida* B2, enzyme monooxygenase initiates oxidative mechanism of 2-nitrophenol and is transformed to catechol with the elimination of nitrite ions. Catechol is further degraded by catechol 1,2-dioxygenase and finally enters into TCA cycle (Zeyer and Kearney 1984; Zeyer et al. 1986) (Fig. 16.1).

On the other hand, there are two different degradative pathways for 3-nitrophenol. However, both initiate with reduction of 3-nitrophenol to form 3-hydroxylaminophenol (Fig. 16.2).

During the degradation of 3-nitrophenol in *Pseudomonas putida* B2, the compound is initially transformed to 3-hydroxylaminophenol by reductase enzyme and subsequently transformed to 1,2,4-trihydroxybenzene with the release of ammonia (Meulenberg et al. 1996) (Fig. 16.2). In contrast, in *Ralstonia eutropha* JMP134, 3-nitrophenol is transformed to 3-hydroxylaminophenol through aminohydroquinone molecule (Fig. 16.2), and ammonia is released in the ring-cleavage pathway (Schenzle et al. 1997). Moreover, the enzymes involved during the degradation of 3-nitrophenol in strain JMP134 also help the bacterium to utilize 2-chloro-5-nitrophenol as the growth substrate (Schenzle et al. 1999).

On the other hand, various microbial strains are isolated and identified on the basis of their capacity to utilize 4-nitrophenol as a growth substrate. In most of cases it has been observed that 4-nitrophenol is degraded into either only 4-nitrocatechol by oxidative mechanism and/or further altered to 1,2,4-trihydroxybenzne with the release of nitrite (Fig. 16.3) in different genus bacteria like *Achromobacter xylosoxi-*

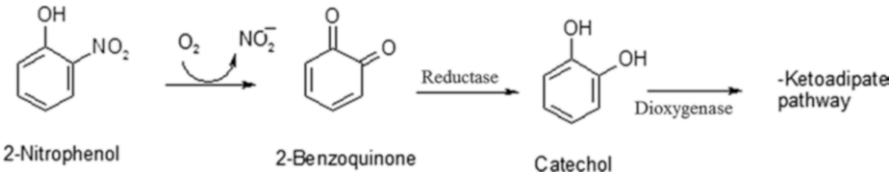

Fig. 16.1 Bacterial degradation of 2-nitrophenol

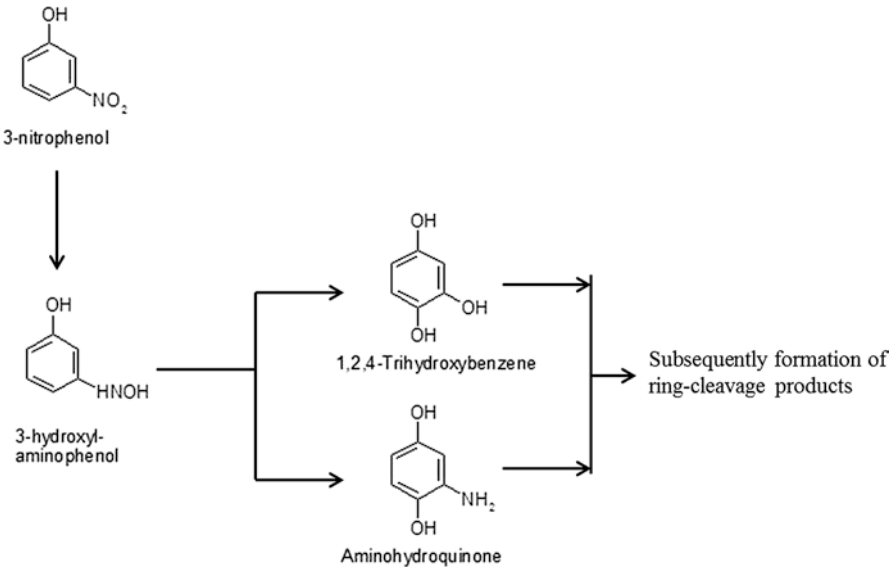

Fig. 16.2 Bacterial degradation of 3-nitrophenol

dans Ns, *Arthrobacter chlorophenolicus* A6, *Arthrobacter* sp. CN2, *Arthrobacter* sp. strain JS443, *Arthrobacter* sp. Y1, *Arthrobacter protophormiae* RKJ100, *Bacillus sphaericus* JS905, *Burkholderia cepacia* RKJ200, *Ralstonia* sp. SJ98, *Rhodococcus opacus* AS2, *Rhodococcus erythropolis* AS3, *Rhodococcus imtechensis* strain RKJ300, *Rhodococcus opacus* SAO101 and *Serratia* sp. strain DS001 (Arora et al. 2014; Gosh et al. 2010; Jain et al. 1994; Ju and Parales 2010; Kadiyala and Spain 1998; Kitagawa et al. 2004; Li et al. 2008; Pakala et al. 2007; Unell et al. 2008; Wang et al. 2016).

Kadiyala and Spain (1998) demonstrated that enzymes like oxygenase and flavoprotein reductase are involved in the first two oxidation steps of 4-nitrophenol to 2-hydroxyl-1,4-quinone by the release of nitrite in *Bacillus sphericus* JS905 (Fig. 16.3). However, bacterial strains like *Arthrobacter aurescens* TW17, *Arthrobacter* sp. YUP-3, *Pseudomonas putida* JS444, *Pseudomonas* sp. strain WBC-3, *Methylobacterium* sp. C1, *Moraxella* sp., *Rodococcus opacus* SAO101 and *Rodococcus* sp. PN1 (Hanne et al. 1993; Kitagawa et al. 2004; Nishino and Spain 1993; Spain and Gibson 1991; Takeo et al. 2008; Tian et al. 2018; Yue et al. 2018; Zhang et al. 2009) transformed 4-nitrophenol to benzoquinone with the help of enzyme monooxygenase. The by-product further converted to hydroquinone molecule (Spain and Gibson 1991) (Fig. 16.3). Furthermore, the pathways are similar at the ring cleavage, where hydroquinone and 1,2,4-trihydroxybenzene molecules individually transform into maleylacetate and finally enter into TCA cycle (Fig. 16.3). Hanne et al. (1993) studied the degradation of 4-nitrophenol by *Nocardia* sp. strain TW2 in the presence of different chemical inducers, and their results suggest that 1,2,4-trihydroxybenzene and hydroquinone pathways are expressed differ-

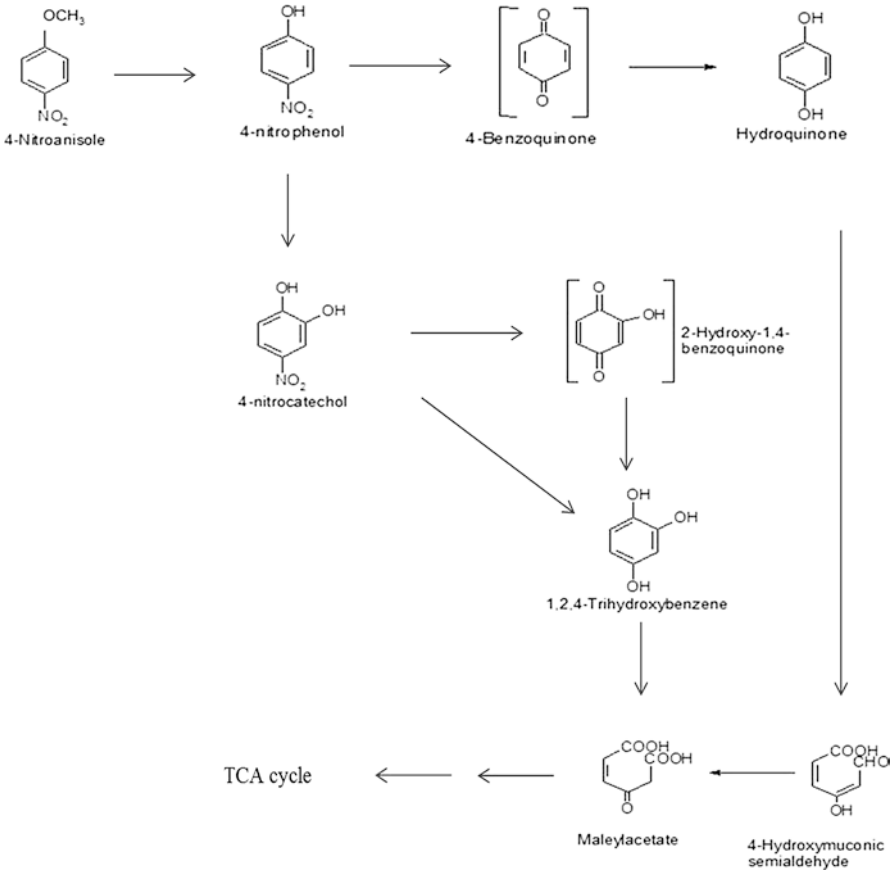

Fig. 16.3 Bacterial degradation of 4-nitrophenol and 4-nitroanisole

ently. Likewise, a similar process was also observed in both *Rhodococcus* strain PN1 and QAO101. Hence, an in-depth study of these bacterial strains is essential in order to know the exact mechanism of regulation of 4-nitrophenol catabolism. Yue et al. (2018) studied the bioaugmentation of *Methylobacterium* sp. C1 towards 4-nitrophenol removal, and their results suggest that *Methylobacterium* sp. C1 with other two bacterial cultures (*Bacillus megaterium* T1 and *Bacillus cereus* G5) biofilm were shown to be highly resistant as well as more efficient for the removal of 4-nitrophenol (up to 0.6 g/L). On the other hand, Min et al. (2017a) demonstrated by applying *Burkholderia* sp. strain SJ98 to artificially contaminated soil (4-nitrophenol, 3-methyl-4-nitrophenol and 2-chloro-4-nitrophenol), and their results suggest that the bacterial culture efficiently degrades all three compounds, thereby decreasing the concetration of nitrophenol, leading significantly to enhancement of several genera of rich microorganisms like *Nonomuraea*, *Kribbella* and *Saccharopolyspora*. Recently, Subashchandrabose et al. (2018) demonstrated 4-nitrophenol degradation by *Rhodococcus wratislaviensis* strain 9, and their results

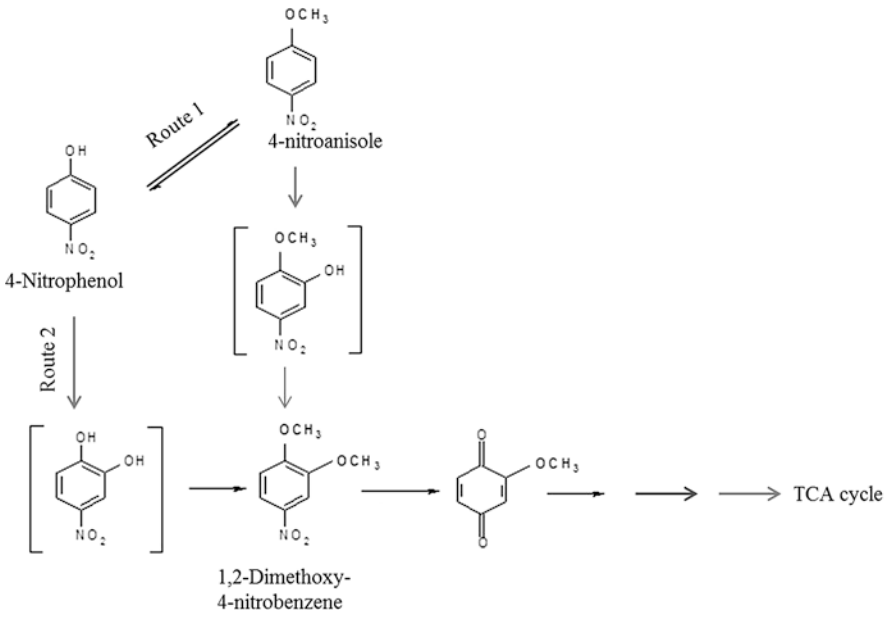

Fig. 16.4 Fungal degradation of 4-nitrophenol

showed the organism is able to degrade 900 μM of 4-nitrophenol within 14 h. Likewise, 4-nitroanisole degradation in two individual bacterial strains (AS2 and AS3) was studied, showing that 4-nitrophenol as a key intermediate occurs during degradation (Schäfer et al. 1996) (Fig. 16.3).

Teramoto et al. (2004) studied how the fungal culture *Phanerochaete chrysosporium* can utilize 4-nitrophenol as a sole source of carbon and energy. However, basically under ligninolytic conditions, 4-nitrophenol transforms to 1,2-dimethoxy-4-nitrobenzene (DMNB) through by-product formation of 4-nitroanisole by the fungus (Fig. 16.4).

16.3.2 Microbial Degradation of Di- and Trinitrophenol

Very few known microbes are able to utilize both dinitrophenols such as 2,4-dinitrophenol and 2,6-dinitrophenol and trinitrophenols like picric acid (2,4,6-trinitrophenol) as growth substrate. Ecker et al. (1992) demonstrated a bacterium is able to utilize 2,6-dinitrophenol as a growth substrate. Interestingly, in *Cupriavidus necator* JMP134, the degradative pathway of 2,6-dinitrophenol is different than the 3-nitrophenol degradation pathway.

In bacterial strain JMP134, degradation initiates with the oxidation step to transform 2,6-dinitrophenol to 4-nitro pyrogallol with the help of a dioxygenase enzyme that releases the first nitrite ion from the benzene ring. In the next step, the by-

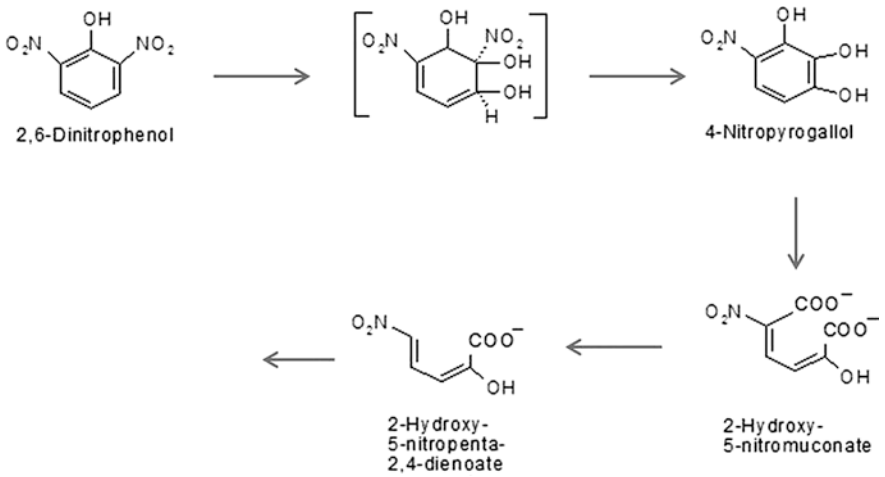

Fig. 16.5 Bacterial degradation of 2,6-dinitrophenol

product is further converted to 2-hydroxy-5-nitro muconate in a ring-cleavage and subsequently transforms to 2-hydroxy-5-nitropenta-2,4-dienoic acid by decarboxylation (Fig. 16.5). However, it was predicted that the other nitro radical present on the broken benzene ring might be released during subsequent steps. On the other hand, various genera of bacterial cultures like *Anabaena variabilis*, *Anabaena cylindrica*, *Burkhorderia* KU-46, *Haloanaerobiumpraevalens* DSM 2228, *Janthinobacterium* sp., *Nocardioides* sp. strain CB22–2, *Methanococcus* sp. B, *Nocardioides simplex* FJ2-1A, *Rhodococcus erythropolis* strain HL24–1, *Rhodococcus erythropolis* strain Hl24–2, *Rhodococcus imtechensis* strain RKJ300, *Rodococcus* sp. strain RB1, *Rhodococcus* sp. strain NJUST16 and *Sporohalobacter marismortui* ATCC 35420 (Behrend and Heesche-Wagner 1999; Blasco et al. 1999; Boopathy 1994; Gosh et al. 2010; Hess et al. 1993; Hirooka et al. 2006; Iwaki et al. 2007; Lenke and Knackmuss 1992; Oren et al. 1991; Rajan et al. 1996; Shen et al. 2009; Zin et al. 2018) utilize either 2,4-dinitrophenol and/or picric acid as a growth substrate. Initially, Knackmuss and research group studied the catabolic pathway of 2,4-dinitrophenol and picric acid in microorganism(s) (Ju and Parales 2010) (Fig. 16.6).

During the degradation process, a hydride-Meisenheimer complex is formed by the reduction of picric acid and produces 2,4-dinitrophenol with the release of a nitrite radical. Again, by hydride-Meisenheimer complex and reduction, hydrolytic cleavage of 2,4-dinitrophenol occurs, producing 4,6-dinitrohexanoate and finally entering into TCA cycle through various by-product formations. Yet, few of the responsible genes and enzymes of the degradative pathway have been determined with respect to their functional characterization and regulation mechanism (Arora et al. 2014; Ju and Parales 2010). Still, it is necessary to know the exact mechanism involved in catabolic regulation of 2,6-dinitrophenol, 2,4-dintrophenol and picric acid in different bacterial strains.

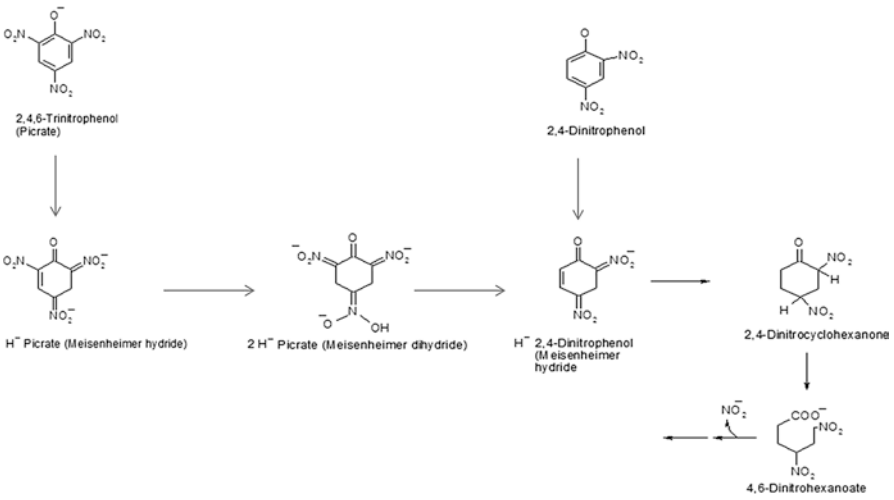

Fig. 16.6 Bacterial degradation of 2,4-dinitrophenol and picric acid

16.3.3 Microbial Degradation of Chloro and Nitro Group-Containing Chemicals

Very few microbes (*Arthrobacter nitrophenolicus* SJCon, *Burkholderia* sp. RKJ800, *Burkholderia* sp. strain SJ98, *Cupriavidus* sp. strain CNP-8 and *Rhodococcus imtechensis* RKJ300, etc.) are known to utilize 2-chloro-4-nitrophenol as a growth substrate (Arora et al. 2014, 2017; Min et al. 2018). A *Rhodococcus imtechensis* strain RKJ300 was isolated from pesticide-contaminated soil by enrichment with 4-nitrophenol as a growth substrate (Gosh et al. 2010). The organism also utilizes 2-chloro-4-nitrophenol as a growth substrate. In *Cupriavidus* sp. strain CNP-8 and *Rhodococcus imtechensis* strain RKJ300, the degradation of 2-chloro-4-nitrophenol initiates with oxidation to form chlorohydroquinone with the release of nitrite. In the next step, the metabolic by-product is dechlorinated and forms hydroquinone and subsequently converts to *gamma*-hydroxymuconic semialdyhyde and finally enters into TCA cycle (Fig. 16.7).

In *Arthrobacter nitrophenolicus* SJCon, 2-chloro-4-nitrophenol degraded to chlorohydroquinone and was further converted to maleylacetate by oxidation, which finally entered into TCA cycle. On the other hand, in *Burkholderia* sp. SJ98, 2-chloro-4-nitrophenol degraded into 4-nitrophenol with the release of chloride radicals, which was further sequentially converted into maleylacetate via 4-nitrocatechol and 1, 2, 4-benzenetriol and finally entered the TCA pathway (Fig. 16.7).

Schenzle et al. (1999) demonstrated that *Ralstonia eutropha* JMP134 (also known more recently as *Cupriavidus necator*) utilizes 2-chloro-5-nitrophenol as a growth substrate. Initially, 2-chloro-5-nitrophenol is reduced to 2-chloro-5-hydroxylaminophenol, then follows an enzymatic Bamberger rearrangement

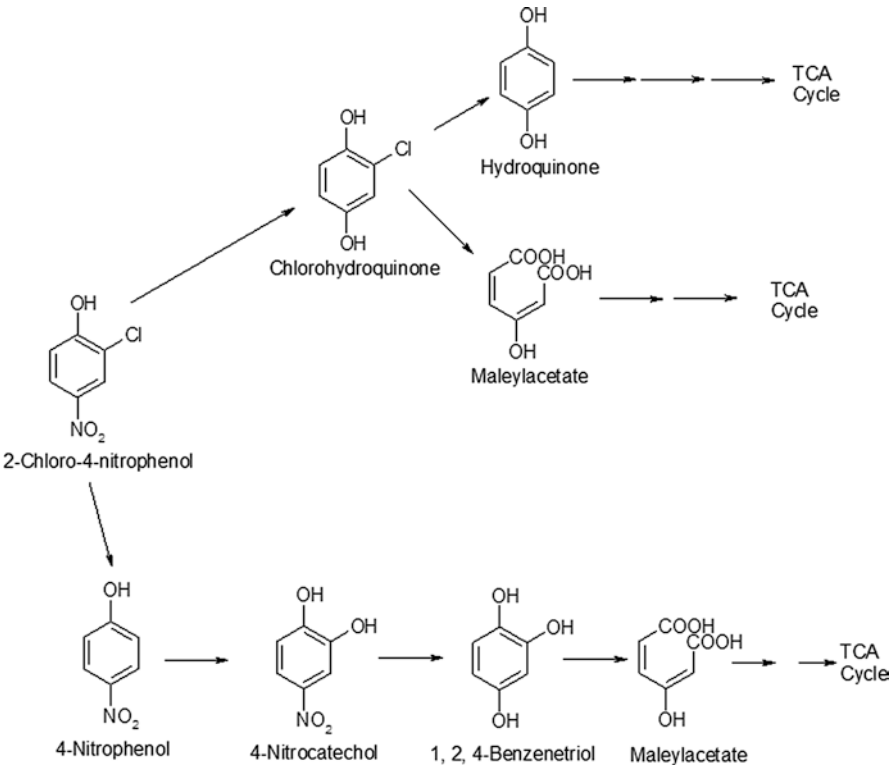

Fig. 16.7 Bacterial degradation of 2-chloro-4-nitrophenol

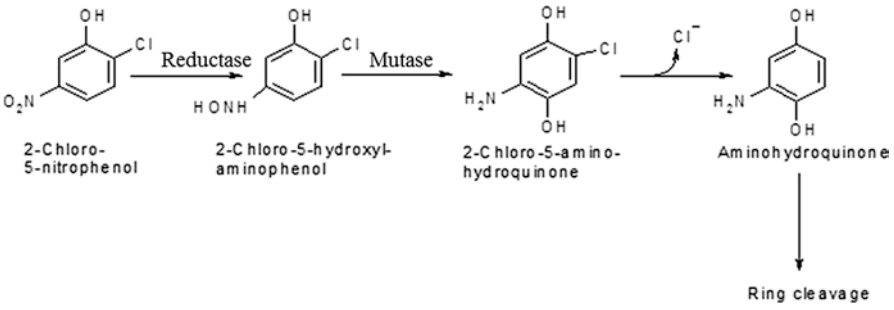

Fig. 16.8 Bacterial degradation of 2-chloro-5-nitrophenol

forming 2-amino-5-chlorohydroquinone, which is further converted to aminohydroquinone with the release of chlorine (Fig. 16.8). Similarly, in *Cupriavidus* sp. strain CNP-8, 2-chloro-5-nitrophenol degradation is initiated by reduction and finally enters into TCA cycle via sequential transformation to different by-products (Min et al. 2017b).

16.3.4 Microbial Degradation of Nitro Group-Containing Herbicides

Different genera of bacterial cultures like *Arthrobacter*-like organisms, *Corynebacterium simplex*, a *Pseudomonas*-like strain and two unidentified bacterial strains isolated from soil showed the ability to degrade a well-known herbicide 3,5-dinitro-*ortho*-cresol (Gundersen and Jensen 1956; Jensen and Gundesen 1955; Jesnen and Lautrup-Larsen 1967; Tabak et al. 1964). Interestingly, in all of these studies the only intermediate by-product detected was a nitrite radical. Tewfik and Evans (1966) reported concisely on a *Pseudomonas* sp. that metabolized 3,5-dinitro-*ortho*-cresol by a reduction process. During degradation, both nitro functional groups were reduced to amino groups, which were further oxidatively deaminated to form 2,3,5-trihydroxytoluene before entering into the ring-cleavage pathway (Fig. 16.9). On the other hand, in *Arthrobacter simplex*, 3,5-dinitro-*ortho*-cresol initially was converted to 3-methyl-5-nitrocatechol and subsequently transformed to 2,3,5- trihydroxytoluene and finally entered into TCA cycle via ring fission (Tewfik and Evans 1966) (Fig. 16.9).

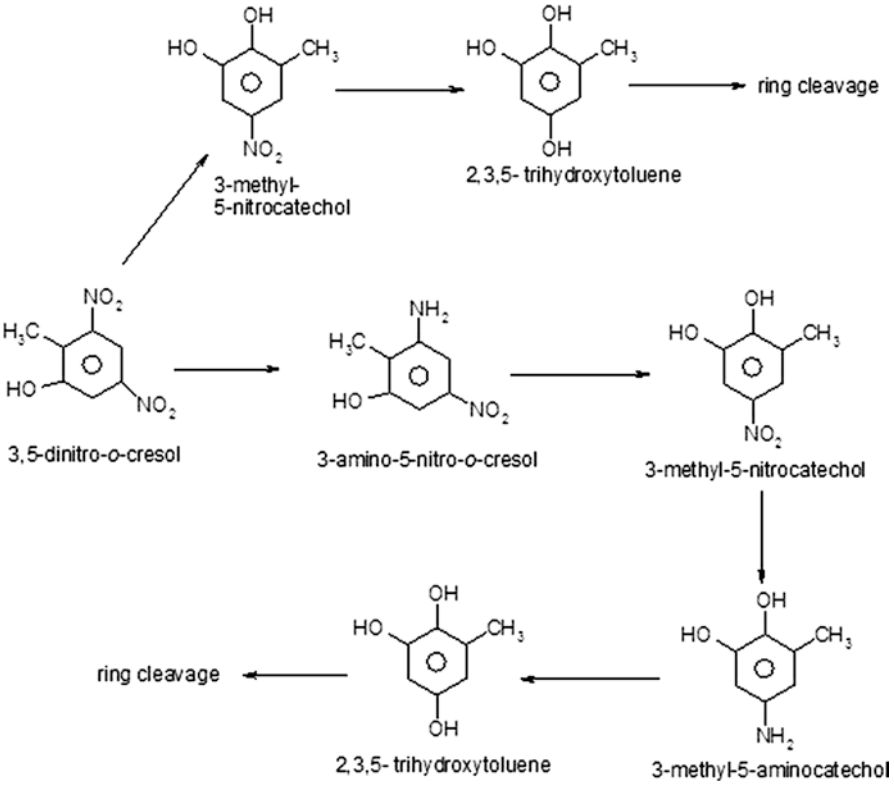

Fig. 16.9 Bacterial degradation of 3,5-dinitro-*ortho*-cresol

Meanwhile, the same research group also found that in the *Corynebacterium simplex* strain (reported earlier by Gundersen and Jensen 1956), 3,5-dinitro-*ortho*-cresol is degraded by essentially the same metabolic route; however, apart from that, reduced by-products probably were not formed as no nitroreductase activity could be confirmed in this organism (Fig. 16.9). Likewise, degradation of the other herbicide, 2-sec-butyl-4,6-dinitrophenol (dinoseb) by bacterial isolates was studied and reported (Stevens et al. 1991). However, the isolates in this case were unable to degrade the herbicide without a co-substrate. But, when the medium contained glucose/ammonium chloride with dinoseb under unshaken conditions, the medium turned a bright red colour, indicating formation of by-product(s). Various transformed products were detected and identified by GC-MS (Stevens et al. 1991). Similarly, Kaake et al. (1995) demonstrated a dinoseb degradation mechanism by bacterial cultures under reducing conditions, and they proposed a degradative pathway for dinoseb on the basis of reduction of nitro groups (attached on the benzene ring of dinoseb) with the formation of amino groups, which subsequently were replaced with hydroxyl groups.

16.4 Conclusions and Perspectives

The information reviewed in the present chapter represents the immense effort undertaken in the investigation to carry out the detoxification of nitro group-containing chemicals and herbicides. In recent times, the topic of biodegradation of such chemicals in biological living systems has emerged in the scientific community. The applications of microbes (individual pure and mixed cultures) towards catabolism of nitro group-containing chemicals and herbicides are being used now, especially in wastewater treatment plants, because, the method is freely available and cost-effective. Degradation mechanisms (especially genomic profile and enzymatic characterization) of several nitro group-containing chemicals have been comprehensively investigated in microbes (especially in bacteria). However, a few key questions still need to be clarified, among others: What is the behaviour of microbes towards a shock load of such chemicals? Can these microbes remediate such chemicals under various environmental conditions? Can a pure culture co-operate with other organism(s) (especially in a bioaugmentation process) during the degradation of such chemicals? The present substantial information for understanding the mechanisms of microbial degradation of nitro group-containing chemicals will help future studies.

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Chapter 17
Distillery Effluent: Pollution Profile, Eco-friendly Treatment Strategies, Challenges and Future Prospects

Vineet Kumar and Deepak Chand Sharma

Abstract In India, distilleries are one of the largest industries, generating vast quantities of effluent (known as raw effluent or spent wash), which is potentially a great cause of aquatic and soil pollution. Distillery effluent (DE) is characterized by its high biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and non-biodegradable inorganic and organic pollutants and highly recalcitrant dark brown colour. It also contains a complex mixture of numerous recalcitrant organic pollutants such as butanedioic acid, 2-hydroxyisocaproic acid and vanillyl propionic acid and various heavy metals, which are reported as endocrine-disrupting chemicals (EDCs) by the U.S. Environmental Protection Agency (USEPA). DE disposed even after conventional treatment processes (activated sludge and biomethanation) poses a serious threat to the environment. Thus, various physicochemical processes have been reported for its decolourization and detoxification, but these techniques are not practicable on an industrial scale due to expensive high chemical consumption, high water requirement and resulting production of a vast quantity of toxic sludge and other secondary by-products. Hence, biological approaches that use microorganisms present a highly attractive alternative for decolourization and detoxification of distillery effluent. This chapter provides a comprehensive review of DE pollutants, their ecotoxicological hazards as well as various ecofriendly treatment techniques. In addition, different challenges and future prospects of DE treatment processes are discussed towards establishing sustainable development.

Keywords Endocrine-disrupting chemicals · Activated sludge · Vermifiltration · Upflow anaerobic sludge blanket reactor

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17.1 Introduction

The safe disposal of wastes discharged from various industries is a serious problem worldwide. Wastewater discharged from industries is regarded as a key source of environmental pollution (Chandra et al. 2015; Chandra and Kumar 2015a). In India, industrial pollution has increased due to the large number of distilleries (Chandra et al. 2018a, b; Chandra and Kumar 2017a, b). A typical distillery, which uses sugarcane-molasses (a by-product of the sugar manufacturing process containing 10–15% minerals (ash), 15–20% non-sugar organic substances, 20% water and 45–50% residual sugars) as raw material for the generation of ethanol; it produces over 1 million litres of wastewater (effluent) daily. Generally in distilleries, the production of 1 litre of ethanol generates around 12–15 litres of effluent (also known as spent wash or raw effluent) (Chandra et al. 2018a; Chandra and Kumar 2017a). The characteristics of spent wash vary significantly according to the nature of the fermentation-feedstock and fermentation/distillation processes adopted during ethanol production. Spent wash contains dissolved impurities from the sugarcane juice, by-products of the fermentation, nutrients added during fermentation of sugarcane molasses and the breakdown products of various sugars present in sugarcane juice. The suspended impurities such as cellulosic fibers and dust are usually separated prior to the evaporation of sugarcane juice. However, non-sugars and minerals, water-soluble hemicelluloses, gums and proteins from the sugarcane juice are present in the spent wash in their converted or original forms, exerting an oxygen demand during subsequent treatment (Jain and Srivastava 2012a, b). There are generally two types of effluent/wastewater produced from distilleries: one is concentrated wastewater or high-strength process effluent (spent wash) that originates from the distillation process during ethanol production; the second is diluted effluent or low-strength process effluent that originates from equipment cleaning and floor washing. The high-strength effluent containing high total dissolved solids (TDS), total soluble solids (TSS), chemical oxygen demand (COD) and biological oxygen demand (BOD) and contains various toxic organic compounds (i.e., butanedioic acid, benzenepropanoic acid, 2-hydroxyisocaproic acid, vanillyl propionic acid, 2-furancarboxylic acid, benzoic acid and tricarballylic acid) and several recalcitrant organic pollutants (ROPs) (Chandra and Kumar 2017a). In addition, it has low pH, an unpleasant odor and a dark brown colour, which seriously impact aquatic organisms. The unpleasant odor of spent wash is due to the existence of skatole, indole and other sulfur-containing compounds that are not effectively degraded by the methanogens in biomethanation treatment of spent wash. The low-strength process effluent has a low concentration of COD, BOD, TSS and TDS. The intense colour in high-strength spent wash is mainly due to the existence of a dark brown polymeric pigment compound known as melanoidin, which is formed by Maillard reaction (MR), a non-enzymatic browning reaction between amino and sugars compounds (Chandra et al. 2008a; Chowdhary et al. 2018; Kumar and Chandra 2018a, b). Melanoidin is characterized by a low and high molecular weight (MW) complex organic polymer, possessing an antioxidant, antimicrobial, and

antihypertensive activity properties; it is not readily degradable by physical and biological means (Rufián-Henares and Morales 2007; Vignoli et al. 2011). Therefore, the presence of these antimicrobial compounds and the removal of colour in DE together pose a major challenge to scientists and researchers for sustainable development.

The conventional treatment of spent wash is carried out by means of anaerobic digestion followed by aerobic (activated sludge) process. This treatment process is efficient in the removal of BOD and COD from spent wash up to a certain extent (Pant and Adholeya 2007). However, the dark brown colour remains or even darkens in the anaerobically digested spent wash due to re-polymerization of melanoidin pigments. Additional drawbacks of conventional treatment systems include the complexity of spent wash and generation of waste sludge and other by-products as toxicants (Kumar and Chandra 2018a; Chandra et al. 2018b; Fan et al. 2011). Considering the strict environmental norms imposed by the Indian government's Central Pollution Control Board (CPCB) on freshwater utilization (maximum consumption of 15 litres of fresh water per litre of ethanol production) and zero-liquid discharge from distilleries, alternatives to existing treatment options such as reverse osmosis, incineration and anaerobic digestion continue to be of interest. Discharge of untreated and partially treated DE in a water aquifer results in significant water quality deterioration due to the high content of pollutants, resultant reduction of sunlight penetration and associated photosynthetic activities, a decrease in dissolved oxygen concentration and alteration of alkalinity and pH of river, lake and pond (Ramakritinan et al. 2005; Chauhan and Rai 2010; Mohana et al. 2009;). Disposal of DE on land causes a decrease in manganese availability and soil alkalinity, and inhibits germination of seeds (Chandra et al. 2008b, c; Pandey et al. 2008). Hence, disposing of partially treated or untreated DE into the environment is hazardous to the ecosystem (Chowdhary et al. 2018; Pant and Adholeya 2007). Figure 17.1 shows the distillery waste discharged before and after secondary treatment and its impact on the environment.

A wide range of physicochemical processes has been efficient for decolourization of DE, including: coagulation with aluminium chlorohydrate ($Al_2Cl(OH)_5$), ferric chloride ($FeCl_3$), magnesium chloride ($MgCl_2$) and lime; a low MW poly(diallyldimethylammonium chloride) (PDADMAC) (Liang et al. 2009a, b; Fan et al. 2011; Zhang et al. 2017); adsorption on sugarcane bagasse, peat, fly ash and zeolite (Onyango et al. 2011; Ojijo et al. 2010; Bernardo et al. 1997); osmosis with biomimetic membranes (Singh et al. 2018); oxidation with ozonation/hydrogen peroxide and manganese oxides (Arimi et al. 2015; Dwyer and Lant 2008); and photocatalyst processes with aluminium oxide, nanoparticle and kaolin clay electrochemical methods (Cañizares et al. 2009; Kobya and Delipinar 2008; Prasad and Srivastava 2009) and nanofiltration (Rai et al. 2008). However, these methods are associated with high operational cost, excess use of chemicals, sensitivity to variable water input, less effective decolourization rate and sludge generation in huge quantity with subsequent disposal problems (Liakos and Lazaridis 2014; Liang et al. 2009a, b). Therefore, sufficient treatment is essential before DE is disposed into the environment. Many criteria—low water input, high removal

Fig. 17.1 Distillery waste (**a**) spent wash dischrged after alcohol production; (**b**) solid material settle down in the bottom of storage tank as spent wash sludge; (**c**) large view of the spent wash sludge (**d-e**) anaerobically digested spent wash in collection tank (**f**) digested sludge discharged after biomethanation of spent wash

efficiency, low energy consumption, small land requirement, high possibility for recovery of useful by-products, eco-friendliness and low operational cost—must be considered when choosing a process for treating DE. Hence, cost-effective and eco-friendly treatment techniques are urgently required for the efficient treatment of DE (Chandra and Kumar 2018; Chandra et al. 2018a, b). In recent years, biological processes have attracted the attention of researchers/scientists and helped the development of an efficient eco-friendly effluent treatment system (Kaushik et al. 2010; Kumar and Chandra 2004). Biological processes have utilized various organisms such as algae, fungi, yeast, bacteria and plants or their enzymes for effective decolourization of distillery effluent. However, biological methods treat DE only to a certain extent; until now, no appropriate technique has been developed for the full-scale treatment of DE (Chandra et al. 2018b).

Hence, the aim of the present chapter is to provide a concise discussion of the various inorganic and organic pollutants present in DE and their ecotoxicological hazards. We describe various aerobic and anaerobic processes with special emphasis on vermifiltration technology associated with the use of biological agents to remediate hazardous complex DE. In this chapter, we also discuss challenges and future prospects for decolourization and detoxification of DE.

17.2 Pollution Profile and Ecotoxicological Hazards of Distillery Effluent

Safe disposal of DE is a menace to the environment due to the presence of various toxic heavy metals (HMs) and mixture of numerous ROPs (Chandra and Kumar 2017a; Chowdhary et al. 2018). Pollutants of various types that exist in DE react to each other, making the effluent more toxic and complex. The main components of DE are carbohydrates, lipids, proteins, amino acids, fatty acids, organic acids, lignin, melanoidins and their fragmentary products, while the inorganic constituents comprise a large amount of potassium (K^+:8766), sodium (Na^+:211), chloride (Cl^-:748.07–2200), sulfate (SO_4^{2-}:16.86–5760), phosphate (P:5.36-8766), calcium (Ca^{2+}:1816) and HMs such as iron (Fe:163.947–1017.37), zinc (Zn:10185–2.487), nickel (Ni:BDL–1.175), manganese (Mn:4.556), copper (Cu:BDL–0.337), chromium (Cr:BDL–0.172), lead (Pb:BDL–1.24) and cadmium (Cd:BDL–0.025 mg L^{-1}) (Narain et al. 2012; Chandra and Kumar 2017a; Tiwari et al. 2014; Jain and Srivastava 2012a, b). Besides the above compounds, melanoidin is the main organic contaminant present at high concentrations in DE. One of the most crucial aspects of DE treatment is to stop the release of toxic androgenic-mutagenic and endocrine-disrupting chemicals (EDC) compounds to the environment (Chandra and Kumar 2017a, b). Some other toxic chemicals such as 2-hydroxyisocaproic acid, di-butyl phthalate, benzenepropanoic acid, butanedioic acid, 2-hydroxyisocaproic acid, vanillyl propionic acid, 2-furancarboxylic acid, benzoic acid, tricarballylic acid and di-n-octyl phthalate are also reported in distillery effluent by various researchers (Chandra and Kumar 2017a). These compounds are listed as a potential EDCs compounds by US Environmental Protection Agency (USEPA 2012; Chandra and Kumar 2017a). Such untreated or partially treated DE can generate a very wide range of problems in an ecosystem, including colour, oxygen depletion, aesthetic pollution, eutrophication and perturbation. In addition, the undiluted or raw DE are noxious to organisms and exhibit a strong androgenic and mutagenic effect in aquatic and terrestrial organisms.

17.2.1 BOD and COD

DE has high BOD (5980.83–42,000 mg L^{-1}) and COD (21999.47–90000 mg L^{-1}) values, far larger than the industrial discharge permissible limit (Narain et al. 2012; Chandra and Kumar 2017a; USEPA 2002). High COD and BOD values of the DE indicate presence of inorganic and organic pollutants at elevated concentrations. From these values, BOD/COD ratio is calculated, representing the organic composition of effluent; this ratio generally used as an indicator for wastewater degradability index (Metcalf and Eddy 2003).

17.2.2 Solids

Solids are present in DE at high concentrations, enhancing the magnitude of sedimentation in the aquatic ecosystem, choking off animals and plants and limiting the use of receiving water. The term "solids" includes numerous components such as total solids (TS), suspended solids (SS) and dissolved solids (DS). DS are particles that pass through a filter and are not visible; only when the water is evaporated from the effluent is the amount of dissolved material apparent. SS refers to particles that may be visible, add turbidity and may be filtered out. TS refers to the quantity of a substance that would be recovered, including dissolved materials and particulates, if water is evaporated from effluent. In DE, the concentrations of TDS (3029.55–77,776 mg L^{-1}), TSS (233.74–550.25 mg L^{-1}) and TS (3263.29–83,084 mg L^{-1}) are very high (Narain et al. 2012; Chandra and Kumar 2017a, b).

17.2.3 Nutrients

Nitrogen (N) and phosphorus (P) are basic nutrients vital to every living organism; when they are present in DE at higher concentrations, when discharged in aquatic resources, lead to lower dissolved oxygen levels, reduction in sunlight penetration, eutrophication resulting in changing rates of reproduction pattern of aquatic organisms and overall deterioration of water quality. However, organisms involved in the elimination of organic contaminants from DE require N and P for their growth and survival, but these nutrients are not bioavailable to the microorganism. It is, therefore, necessary to add extra N and P to get the bioremediation process to work properly.

17.2.4 Heavy Metals

HMs like Fe, Zn, Cu, Ni, Mn, Cd and Pb are major toxic inorganic contaminants of freshwater reservoirs due to their high solubility in the aquatic environment (Barakat 2011). DE is the chief source of HMs pollution being introduced into diverse segments of the environment including soil, sediment and water (Chandra and Kumar 2017c; Chandra et al. 2018a; Tiwari et al. 2014; Bharagava and Chandra 2010). It has been reported that various HMs such as Pb^{2+}, Zn^{2+}, Fe^{3+}, Cr^{3+} and Cu^{2+} combine with melanoidins, a major colourant, to form an organo-metallic (OM) complex (Hatano et al. 2016; Migo et al. 1997). Consequently, the HMs binding affinity of melanoidin also enhances the vulnerability of the OM complex towards its toxicity in the ecosystem. Hence, it is essential to treat OM complex-contaminated DE prior to its disposal into the environment (Chandra et al. 2018c; Hatano et al. 2016).

17.2.5 Ecotoxicological Hazards

DE contains a mixture of diverse inorganic and organic compounds that may be hazardous to plant, animal and human life. Jagdale and Sawant (1979) and Joshi et al. (2000) reported that non-judicious use of spent wash adversely affects the growth of crop plants and impairs soil characteristics by increasing salinity of the soil. The adverse effect on water-stable aggregates, hydraulic conductivity and the water retention properties of soil with the application of spent wash at a high amount has been reported by Jadhav and Savant (1975). Several reports revealed that the application of spent wash inhibited seed germination and suppressed seedling growth of mung beans (*Vigna radiata*) and rice (*Oryza sativa* L.). These studies suggested that pre-treatment of DE to degrade OM prior to application to agricultural crops might yield better results (Arora et al. 1992; Kannan and Upreti 2008). However, continuous irrigation/disposal of soil with DE adversely affects the soil microorganisms and alters groundwater and soil quality also (Juwarkar and Dutta 1989). Untreated DE probably led to deterioration of soil microorganisms, and soil pollution results in low productivity. The impact of DE discharged from a sugar factory on the biochemical characteristics and growth of maize (*Zea mays* CO-1) and green gram (*Phaseolus aureus* CO-4) has been studied by Ayyasamy et al. (2008). A study was also carried out using water lettuce, water hyacinth and aquatic plants to observe the impact of DE. They noted a gradual decline in total chlorophyll contents, protein, free amino acid and shoot length in both aquatic and terrestrial plants when irrigated with different concentrations of DE compared to the control. Jain and Srivastava (2012a, b) also studied the impact of spent wash on sugarcane growth and their biochemical attributes. Results indicated that spent wash application at very low concentration showed stimulatory effects on sugarcane growth attributes, enzyme activity and chlorophyll content, while high concentration of spent wash application exhibited inhibitory effects on these attributes. Inhibitory effect of spent wash at high concentration on the shoot and root growth of sugarcane led to a change in mitotic activity, metabolic activities and mineral composition, which in turn caused a reduction in sugarcane growth (Srivastava and Jain 2010). Bharagava and Chandra (2010) also noted that at high concentration anaerobically treated post-methanated distillery effluent (PMDE) act as an inhibitor for auxin and gibberline and inhibiting germination of green gram (*Phaseolus mungo* L.) seeds. Recently, a study conducted by Chandra and Kumar (2017a) also reported the toxic effect of spent wash on seedling growth of *P. mungo* L. and *Triticum aestivum* L. seeds at different concentrations. They demonstrated that at high concentrations the spent wash showed a toxic effect on seedling growth of *P. mungo* L. and *T. aestivum*. Jain et al. (2005) and Chauhan and Rai (2010) demonstrated that indiscriminate use of DE for a long-term could lead to significant leaching of inorganic salts, which have the potential to affect groundwater quality. Partially treated or untreated discharged DE also affects the life of aquatic organisms (Kumar and Gopal 2001; Saxena and Chauhan 2003; Matkar and Gangotri 2003). In water bodies, it reduces penetration of sunlight, decreases photosynthetic activity, and dissolves oxygen concentration,

causing the death of aquatic organisms (Kumar and Chandra 2006; Raghukumar et al. 2004). Raw DE has a lethal effect on fish and other aquatic organisms. A biotoxicity assay on freshwater fish *Cyprinus carpio* var. *communis* has been studied to estimate the LC_{50} for DE. In addition, the adverse effect of DE on carbohydrate metabolism and the respiratory process of *C. carpio* were observed by Ramakritinan et al. (2005).

17.3 Biological Treatment Methods for Industrial Wastewater

17.3.1 Anaerobic Digestion

Anaerobic digestion (AD), also known as biomethanation, a most attractive primary distillery spent wash treatment method because it is an eco-friendly, low-cost and socially acceptable microorganism-based technology (Mohana et al. 2007). Generally, the anaerobic digestion process results in eliminations of 65–70% COD and 80–95% BOD and in significant energy recovery as biogas (Tewari et al. 2007; Arimi et al. 2014). In the AD process, any organic compound present in the spent wash is digested (metabolized) by the microorganisms in an oxygen-free environment to produce methane. Other products of the AD process are digested sludge and treated DE; both are highly rich in organic and inorganic nutrients. This anaerobically digested distillery sludge can be used as fertilizer. The AD process of DE can be subdivided into four successive process stages, each requiring its own characteristic group of microorganisms:

(i) Hydrolysis: This stage involves the conversion of high MW non-soluble organic compounds into soluble compounds, appropriate for use as a source of carbon and energy by growing microorganisms.
(ii) Acidogenesis: This involves the transformation of the soluble organic compounds that were the result of the first stage into low MW intermediate compounds.
(iii) Acetogenesis: In this stage, lower chain volatile fatty acids produced during acidogenesis are utilized by a group of bacteria (acetogens) to produce acetate and carbon dioxide.
(iv) Methanogenesis: This stage involves the bacterial conversion of acetate and carbon dioxide plus hydrogen to methane.

A schematic representation of AD of spent wash is given in Fig. 17.2. Anaerobic digestion consumes one- to three-fold the amount of freshwater for dilution to spent wash to ensure the appropriate condition of the anaerobic digester. The main challenges of AD of spent wash are elimination of the colour-contributing organic compounds and HMs. However, even after AD, the treated spent wash may have a high organic loading and a dark colour, requiring additional treatment steps.

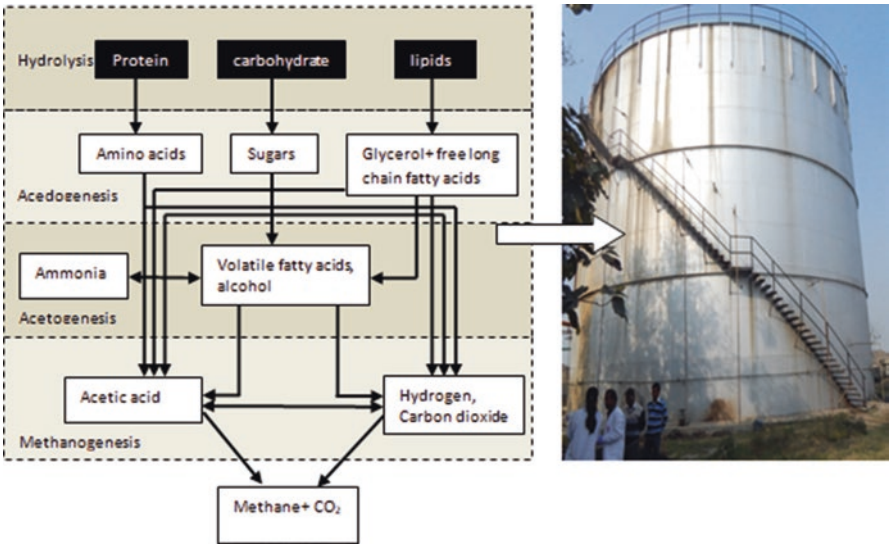

Fig. 17.2 Schematic presentation of anaerobic digestion (biomethanation) process and view of anaerobic sludge blanket reactor. (Lettinga et al. 1999)

17.3.1.1 Upflow Anaerobic Sludge Blanket Reactor

Various workers have studied many high-rate anaerobic reactors/digesters, including expanded granular sludge bed (EGSB), hybrid reactors, granular-bed anaerobic baffled reactor (GABR), upflow blanket filter, internal circulation reactor (ICR), upflow anaerobic sludge blanket (UASB) reactor, fluidized bed reactor, fixed bed reactor and upflow anaerobic filter for the treatment of DE. Among all these reactors, UASB is the most popular for anaerobic treatment of high-strength distillery spent wash due to its higher organic loading rates (OLRs) capacity and requires a shorter hydraulic retention time (HRT) (Fig. 17.2). The UASB reactor is a methane-producing digester forming a blanket of granular sludge under an anaerobic condition to facilitate contact with the substrate; the sludge is processed by anaerobic microorganisms. Vlissidis and Zouboulis (1993) reported a full-scale thermophilic (50–55 °C) AD of DE to achieve 60% COD removal with a recovery of 76% of biogas. Harada et al. (1996) carried out thermophilic (55 °C) AD of DE using a 140-liter UASB reactor for a period of 430 days. They achieved 39–67% COD and more than 80% removal of DE. The cheap performance of the reactor for COD elimination can probably be attributed to the low degradability of the waste itself. A two-stage mesophilic treatment system consisting of a UASB reactor and an anaerobic filter was found appropriate for AD of DE, enabling better conditions for the methanogens (Blonskaja et al. 2003). Sharma and Singh (2001) investigated the effect of the addition of micronutrients and macronutrients in the DE treatment performance of the UASB system. Calcium and phosphate were found to be detrimental to treatment efficiency. Wolmarans and De Villiers (2002) have reported greater than 90%

COD removal efficiency in a UASB reactor treating distillery effluent. The treatment of high-strength spent wash was studied in a bench-scale UASB reactor (Saner et al. 2014). The reactor operated at mesophilic temperature (37 °C) at different OLRs and constant HRT in two-day periods over a research period of 635 days. The maximum BOD and COD removals achieved were 89.11% and 68.35%, respectively, at optimum OLR of 15.34 kg COD/m^3 day. Musee et al. (2007) indicated that the integration of the UASB reactor and an aerobic treatment process provides an improvement in COD removal from DE. An overall 96.5% COD removal was achieved through this hybrid (anaerobic/aerobic) effluent treatment.

17.3.2 Aerobic Treatment

In DE treatment, aerobic biological processes that follow post-treatment to improve the quality of the pre-treated DE to discharge limits by the utilization of specific microorganisms, either pure strains or combination strains, with air supply. The aerobic treatment of DE usually depends on the oxidative and enzymatic activities of microorganisms viz. bacteria, cyanobacteria, fungi and yeast. Organic contaminants in effluent limit the carbon and energy required to encourage microbial growth and reproduction. In many distilleries, partial removal of COD and BOD is achieved using biological treatment by a bioreactor system to treat DE. The most common types of aerated DE treatment systems are activated sludge system.

17.3.2.1 Activated Sludge Treatment

Activated sludge (AS) process is a biological treatment method that primarily removes dissolved organic matter (DOM), as well as non-settleable and settleable suspended solids from the DE. AS is the biomass produced in incoming effluent in the presence of dissolved oxygen by the growth of bacteria and other microorganisms in an aeration tank in order to reduce the DOM in the effluent (Gernaey et al. 2004). In the AS process, microorganisms decompose the DOM and convert it to CO_2 and H_2O. The basic principle of AS processes is that as microorganisms grow in the effluent, they form particles known as floc that clump together. Further, these floc are allowed to settle to the bottom of the aeration tank, leaving a relatively clarified liquid, free of DOM and DSS. Generally, the floc are produced from aggregates of non-living organic polymers, which are most likely secreted by growing bacterial species. Floc have an open spongy structure that varies in size from <10 μm up to 1000 μm and are sufficiently robust to withstand the shear forces created by water movement during aeration of the tanks. They are shaped by physicochemical sticking of microbial cells and pollutants from effluent, as well as by the formation of colonies of microorganisms.

The AS treatment process consists of two separate phases: aeration and sludge settlement. In the first phase, effluent from the primary settlement tanks is added to

the aeration tank containing a mixed microbial population. Aeration is the most commonly used process to remove BOD from effluent and is also used to manage the AS processing units. Aeration serves two significant purposes in the AS process: (i) to supply the oxygen required for the growth and survival of microorganisms in the aeration tank; (ii) to maintain the floc in a continuous state of agitated suspension, allowing maximum contact between effluent and microbial floc surface. The successful removal of BOD in an AS process depends on effluent characteristics, effluent quantities and effluent sources. The AS process operation typically allows no settlement in the aeration tank, while a completely separate settlement tank (situated after the aeration tank) with continuous sludge exclusion and activated sludge return to the aeration tank is operated. As the settled effluent enters in the aeration tank, it displaces the mixed liquor (ML) or mixed liquor suspended solids (MLSS: the mixture of microbial mass and effluent) into a sedimentation tank. This is the second stage, where the flocculated microbial biomass rapidly settles out of suspension to form sludge. The effluent from the clarified liquid, which is virtually free from solids, is discharged as the final treated effluent. Fig 17.3 illustrates the conventional AS process for the treatment of DE.

The AS process relies on the cultivation of many different types of microorganisms suspended in the effluent as the effluent travels through an aeration tank. This suspension referred to as ML or MLSS is supplied oxygen and kept mixed by aerated oxygen (air) through the entire aeration tank. The most dominant naturally occurring microorganisms grown in the AS treatment process are heterotrophic and autotrophic bacteria. Nitrifying bacteria that make up the autotrophic group of bacteria play a key role in the removal of ammonial nitrogen from the effluent. A relatively small number of autotrophic bacterial species are grown in the effluent due to their slow growth rates; they tend to be out-competed by the faster-growing heterotrophic bacteria. In a well-maintained AS treatment system, the bacterial species are aggregated in the flocculent substance in the aeration tank, although some bacterial species occur free in the effluent. The average C:N:P ratio close to what is ideal for bacteria growth in the AS is diversely stated as either approximately 100:17:5 or 100:19:6.

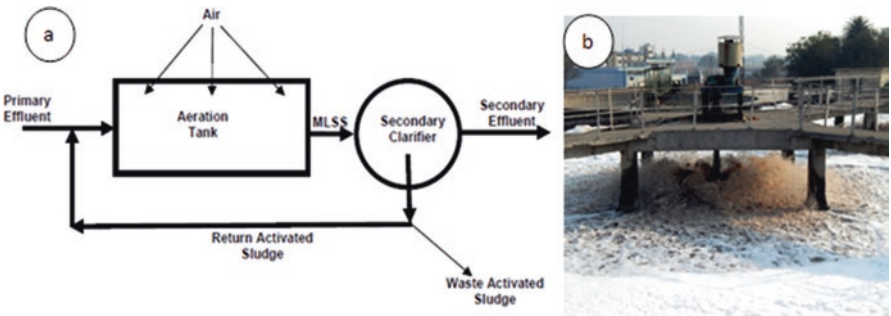

Fig. 17.3 Conventional activated sludge treatment system process: (**a**) illustration of activated sludge treatment process (**b**) a view of the aerated activate sludge treatment process

A typical wastewater treatment plant comprises primary, secondary and tertiary treatment processes. The primary treatment process involves settlement of all solids in a clarifier tank. After this, the DE then passes to the aeration tanks or the secondary treatment process. This is the main biological treatment phase wherein the AS bacteria decompose organic matter in the effluent. The AS system takes place in the capacity in three large aeration basins, which are aerated with surface aerators to maintain the required oxygen levels in the effluent for microbial growth. Following a sufficient period of treatment time (about 10 h), the ML flow from the aeration tank is transferred to the secondary clarifier, where the bacterial biomass is allowed to settle out of the DE, and the partially treated DE passes to the next treatment step. The settled activate biomass is returned to the AS process (also known as returned AS or recycled AS) to provide organisms that continue to degrade pollutants. For proper functioning of a process, the quantity of biomass is controlled by removing excess biomass from the AS system in each day. This excess microbial biomass is known as waste-activated sludge (WAS). However, a tertiary treatment may be used to further improve the quality of the secondary treated DE by removing pathogens, suspended solids, phosphates or nitrogen, as required.

17.3.2.2 Biocomposting

Biocomposting using press mud is the most popular exercise in distilleries for the safe disposal of DE. Generally, in India, press mud is used as manure in the agricultural field. The high-strength DE, either without treatment or after anaerobic digestion, is sprayed over on sugarcane press mud in a controlled manner. However, this option is constrained by the seasonal availability of sugarcane-press mud and a requirement of large land area; it cannot be carried out during the rainy season. Biocomposting of the spent wash by sugarcane press mud is shown in Fig. 17.4.

Fig. 17.4 A view of the spent wash bio-composting using sugarcane press mud

17.3.2.3 Vermifiltration

Vermifiltration is a low-cost, socially accepted, ecologically non-intrusive technology in which the joint action of earthworm activity and the adsorption properties of gravel, sand and soil particles on the organic pollutants are applied to treat effluent. Manyuchi et al. (2018) reported the significant reduction (more than 90%) of TDS, TSS, TKN, BOD and COD in DE after applying the vermifiltration process over a period of 40 h. They recommended the treated DE be used for irrigation purposes. In addition, vermicompost rich in N (1.87%), P (0.66%) and K (0.87%) was also obtained from the vermifiltration process.

17.3.2.4 Constructed Wetland

Constructed wetland (CW), also known as treatment wetland, is a dynamic and variable engineered system designed to utilize the natural processes of biological wetlands to eradicate a wide variety of contaminants, viz. suspended solids, organic compounds, nutrients, pathogens, metals and emerging contaminants from effluent (Vymazal 2014; USEPA 2000; Chandra and Kumar 2015b). Various inorganic and organic pollutants are removed from complex wastewater by physicochemical and microbial mechanisms in constructed wetlands. However, the main pollutant elimination mechanisms in CW include physicochemical processes, viz. plant-water interfaces, root-sediment, precipitation of waste-sediment, volatilization, filtration, adsorption and sedimentation, as well as biological processes like plant uptake and microbial metabolic activities (Kadlec and Knight 1996; Kadlec et al. 2000). Although filtration is considered a key process in the removal of pollutants, some additional interactions occur among water, plants and media.

For removal of contaminants from DE, some significant work was done by Billore et al. (2001), who used a horizontal flow gravel bed CW after the distillery effluent was treated conventionally. Concentrations of BOD_5 and COD in distillery effluent amounted to 13,866 and 2540 mg L^{-1}, respectively, so tertiary treatment was essential. The CW treatment system achieved BOD_5, COD, total P (TP) and total Kjeldahl nitrogen (TKN) removal up to 84, 64, 79 and 59%, respectively. Their study suggested CW might be a promising tertiary treatment technique for the remediation of contaminants present in distillery effluent. The wetland plants and their rhizospheric microorganisms in CW play a major role in effluent treatment, wherein plants directly contribute to the reduction of pollutants through assimilation and uptake and indirectly by stimulating the growth of microorganisms that degrade the pollutant in the plant rhizosphere (Stottmeister et al. 2003; Glick 2003). However, plants not only directly assimilate pollutants into their tissues but also act as catalysts for effluent purification reactions by increasing the rhizospheric microbial communities via direct transport of oxygen from the atmosphere through their

stems and roots and secretion of root exudates to the rhizosphere, or by release of oxygen from the plant through photosynthesis (Stottmeister et al. 2003). Wetland plants have high biomass production, fast growth and fibrous root systems which provide large surface areas for attachment of microorganism and the ability to accumulate large concentrations of heavy metal in their roots and shoots compared to plants in the surrounding water (Vymazal and Kropfelova 2005; Chandra et al. 2018c). Trivedy and Nakate (2000) treated DE in *T. latipholia*-planted CW, resulting in 47 and 78% decrease in BOD and COD, respectively, over a 10-day incubation period. They demonstrated that increasing concentration (100%) of DE significantly reduced the biomass of growing plants with the highest Fe accumulation. *Potamogeton pectinatus*, an aquatic microphyte, was also reported to accumulate Mn, Zn, Cu and Fe and efficiently improve the quality of DE (Singh et al. 2005). DE in a two-stage treatment process involving biodegradation of recalcitrant colouring components of the effluent by *B. thuringiensis* followed by a subsequent decline of remaining pollutants by a *Spirodela polyrrhiza* L. Schleiden was reported by Kumar and Chandra (2004). Similarly, a two-step treatment of PMDE was carried out with *B. thuringiensis* followed by *T. angustata* L.-planted CW resulted in 98–99% reduction of BOD, COD and colour after seven treatments. They suggested that bacterial pre-treatment of PMDE, integrated with phytoremediation processes, improved decolourisation and detoxification of PMDE.

Hatano et al. (2016) studied the chelating property of melanoidin-like product (MLP) and to assess the facilitatory influence on the phytoextraction potential of *Raphanus sativus* var. *longipinnatus* (Japanese radish). They reported that MLP binds with all tested metals, viz. Pb^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+} and Co^{2+}; the metal ion-binding capability of MLP toward Cu^{2+} was found to be the maximum among them. Similarly, Hatano and Yamatsu (2018) assessed the facilitating effect of MLP on the phytoextraction potential of three *Brassica* species grown in a medium containing Pb or Cd. They reported that plant biomass and Pb^{2+} uptake were considerably increased by the addition of MLP in a nutrient medium, and all the Pb^{2+} from the medium was accumulated in the root tissues of *Brassica* species. They concluded that MLP was able to detoxify Pb^{2+} and to improve bioavailability in the root tissues. The complex microbial communities associated with wetland plant root, created by interactions with effluent, is mainly responsible for biodegradation efficiency of organic pollutants and ecosystem stability (Stottmeister et al. 2003; Kumar and Chandra 2018c). The microbial community grown in CW consists of allochthonous (foreign) and autochthonous (indigenous) microorganisms. Allochthonous microorganisms, including pathogens, entering with effluent usually do not survive or have any functional significance in the wetland ecosystem. On the other hand, autochthonous microorganisms show adaptive features—they are capable of survival and growth in a high-stress environment when they participate in purification processes in wetland systems due to their high metabolic activity. Chaturvedi et al. (2006) isolated and characterized roots associated rhizospheric bacterial communities of *Phragmites australis* grown on distillery waste contaminated site. They characterized 15 culturable rhizospheric bacterial species of *P. australis*; they are *Bacillus anthracis*, *Microbacterium hydrocarbonoxydans*, *B. subtilis*, *Achromobacter*

xylosoxidans, *B. megaterium*, from the upper zone; *A. xylosoxidans*, *B. subtilis*, *B. thuringiensis*, *B. licheniformis*, *Achromobacter* sp., *B. licheniformis Alcaligens faecalis*, *P. migulae*, *Staphylococcus epidermidis* and *B. cereus.* All isolated bacterial species grown on DE-supplemented medium utilized pollutants as a sole carbon and nitrogen source, resulting in the reduction of BOD, COD, phenol and HMs values, including the colour of the distillery effluent. Chandra et al. (2012) reported 96.0 and 94.5% reduction in COD and BOD values, respectively, of post-methanated distillery effluent in the two-stage sequential treatment by bacteria followed by *Phragmites communis*.

17.4 Challenges and Future Prospects

The discharge of organic and inorganic pollutants into the aquatic and terrestrial ecosystem from distilleries creates a risk to organisms, causing significant environmental disruption. Conventional biological treatment approaches can accomplish the degradation of the melanoidins up to only 67%; however, low and high MW pigments probably repolymerize during biological processes, therefore, decolourization of DE is a crucial concern. Understanding the structure and chemical characteristics of melanoidins is essential prior to development of a suitable degradation technique. However, DE contains a large number of antioxidant compounds that could be recovered and serve as additional value-added resources. During chlorine disinfection of effluents, polyphenols and melanoidins may also lead the development of aromatic halogenated disinfection by-products (DBPs) which are highly toxic and inhibit growth (Liu and Zhang 2014). In most DE decolourisation and degradation studies, melanoidins were considered to be the main colouring pigments (Hatano et al. 2008; Liang et al. 2009a, b). Thus, advanced treatment techniques are required to degrade compounds that contribute to colour from DE. This has been increasingly recognized as a tough challenge. Melanoidins are colouring brown organic compounds; the maximum absorbance (λ_{max}) of melanoidins present in DE has been recorded at 475 nm by spectrophotometer, but this value was not steady; it sometimes varied depending on the interaction of melanoidins with co-pollutants. The changeable λ_{max} thus complicates understanding of the mechanism of decolourization and degradation of melanoidin and characterization of its fragmentary products. In distillery, freshwater is essential for different non-process applications (as described previously). However, to minimize water use, some methods were recommended by Kumar (2004) and Saha et al. (2005). These include: (i) employing continuous fermentation instead of batch, which can reduce the freshwater requirement for dilution of sugarcane-molasses; (ii) recycling of cooling-water from the water jackets of the heat exchanger, fermenter plate, condensers, blowers, coolers, etc.(the non-process wastewater can be used for horticultural and cleaning purposes); (iii) minimizing the dilution of spent wash during anaerobic digestion; (iv) increasing the effectiveness of boilers and utilization of steam in distillery. Several ROPs discharged from distilleries travel several kilometers in

aquatic streams and behave like persistent organic pollutants (POPs). In addition, during degradation or microbial action, several compounds are also transformed into more noxious compounds in the environment. These compounds may also bind to other pollutants and convert them into more vulnerable chemical forms. Consequently, the reproductive system of aquatic flora and fauna is adversely affected along with accumulation of organic polluatnst and HMs in their fatty tissue. But, there is no detailed data nor any adequate monitoring device for POPs discharged from distilleries as wastewater. Hence, for screening, any programme for remediation and monitoring of these pollutants at a regional level/national level should be initiated for environmental protection and sustainable development.

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