9
Bioremediation

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Abstract Environmental pollutants such as polycyclic aromatic hydrocarbons (PHAs), polychlorinated biphenyl's (PCBs), pesticides, petroleum hydrocarbons, and heavy metals are released into the environment, where they cause deleterious effects to wildlife and humans, owing to their inertness and being recalcitrant. However, the existence of microorganisms and plants capable of utilizing or accumulating such compounds has made the applications of such organisms in cleaning up of the environment a workable strategy. Therefore, Bioremediation (the application of bacteria and fungi) and Phytoremediation (the application of plants) to clean-up the environment are the two feasible and safe approaches that offer promise regarding environmental reclamation and sustainable use.

1. INTRODUCTION

1.1. Environmental Pollution: An Overview

The natural global environment (land, air and groundwater) is heavily polluted by human activities such as mining, discharge of industrial wastes, agrochemical usage and long-term application of urban sewage sludge in agriculture soils, waste incineration and vehicle exhausts, as well as anthropogenic organic pollutants. The above activities introduce into the environment a diverse array of pollutants including heavy metals, volatile organic compounds,

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nitroaromatic compounds, phenolic compounds, xenobiotic aromatic hydrocarbons: polycyclic aromatic hydrocarbons (PAHs), and pesticides, and polychlorinated biphenyls (PCBs) (1–6). Once the pollutants are in the environment, they pose great health risks to both humans and wildlife, which is due to their toxicity and recalcitrance. For example, PCBs, which were phased out in many countries in the mid-1980s because of their toxicity and adverse effects on humans and wildlife, are still ubiquitous all over the global environment and its biota because of their resistance to biodegradation. Similarly, pesticides and organophosphates have been or are being phased out for similar reasons. Owing to their toxicity and recalcitrance, PCBs and similar pollutants are generally referred to as persistent organic pollutants (POPS) (7). Heavy metals, on the other hand, pose the greatest health risk because of the difficulty associated with their removal from the environment, which arise from the fact that they cannot be chemically or biologically degraded and are thus ultimately indestructible (2).

1.2. Environmental Remediation Strategies

The health risks associated with environmental pollution have made it necessary to develop strategies to reclaim the environment from the various pollutants. Over time, a number of approaches or strategies have been devolved. To date, the most commonly used conventional approaches to remediate contaminated sites include, among others, landfilling, recycling, pyrolysis and incineration (8). Landfilling involves digging up contaminated soil and moving it to a landfill. Alternatively, the contaminated site is demarcated and contained (9). This method simply moves the contamination elsewhere and may create significant risks in the excavation, handling and transport of hazardous materials. Coupled with this drawback, it is very difficult and increasingly expensive to find new landfill sites for the final disposal of the material. Therefore, this method is only an interim solution since the contamination remains on site, requiring monitoring and maintenance of the isolation barriers long time into the future, with all the associated costs and potential liability (9).

Incineration at high temperature and various types of chemical decomposition (e.g., base-catalyzed dechlorination, and UV oxidation) may be effective at reducing levels of a range of contaminants but are limited in a number of ways. For instance, several technologies for in situ remediation such as chemically enhanced soil flushing using extracting solution (organic and inorganic acids) and complexation agents have been proposed for remediation. In a number of cases, these approaches are not only technologically complex, labour intensive and expensive to run, but also result in extensive changes in the physical, chemical and biological characteristics of the soil. Besides, they are often associated with an increase of exposure to contaminants for both workers at the site and nearby residents. Consequently, not only do they lack public acceptance but their applications are also limited to a small scale. Typically, they are unsuitable for very large areas such as mining sites or industrially/agrochemically contaminated soils (6, 9).

1.3. Bioremediation: A Concept

Microorganisms are ubiquitous, being widely distributed in a diverse array of habitats ranging from marine to terrestrial environments. Some of these habitats include those that have been heavily contaminated by heavy metals, as well as chemical and organic pollutants

emanating from human activities (Sect. 1.1). The inhabitation of polluted environments by microorganisms means that they are equipped with the necessary metabolic machinery to enable them survive in such environmental conditions. It is assumed that microorganisms may utilize such contaminants as carbon source and/or as terminal electron acceptors. These, in turn, enable microorganisms utilize such compounds for energy conservation and their eventual mineralization (9, 10). Besides microorganisms, some plant species are endowed with the capacity to concentrate, degrade and volatilize contaminants (6). These activities by some microorganisms and plant species are useful in reclaiming the environment of pollutants and are the basis of the bioremediation concept. Bioremediation, as a concept, relies or seeks to utilize the metabolic capacities of microorganisms and plants to decontaminate the environment of pollutants. The term bioremediation is primarily applied to the use of microorganisms (bacteria and fungi), while phytoremediation is applied with reference to the use of plants and their associated microbes in the decontamination of polluted environments. Considering the two processes, i.e., using microbes and plants, bioremediation may be defined as the process by which living organisms (bacteria, fungi, earthworms and plants) degrade or transform and detoxify hazardous organic and inorganic contaminants or waste under natural conditions into innocuous compounds such as carbon dioxide and water or to less toxic forms (8, 9, 11). Transformations of environmental pollutants are achieved through reactions that take place as part of their metabolic processes. Therefore, living organisms of potential for bioremediation possess enzymes and novel pathways that enable them to detoxify and/or mineralize those pollutants.

1.4. Advantages of Bioremediation

Bioremediation offers a number of advantages over physico-chemical approaches. Typically, bioremediation techniques are more economical than traditional methods such as incineration and other chemical methods, and can achieve complete degradation of organic pollutants without collateral destructions of the site material or its flora and fauna. Besides being economical, bioremediation can be used in situ for pollutants that are present at low but environmentally significant concentrations. This, in turn, prevents their gradual build-up in the environment. Furthermore, pollutants can be treated on site, thus reducing exposure risks for clean-up personnel or potentially wider exposure as a result of transportation accident. This approach also renders it unnecessary to transfer the contaminants from one environmental medium to another, for example, from land to water or air, as complete destruction of target pollutants is possible. Owing to the disadvantages associated with the applications of physico-chemical remediation approaches, bioremediation approaches remain the only versatile and ecologically acceptable clean-up technology (6, 7, 9, 12, 13).

Inasmuch as some instances of pollution can be readily bioremediated using existing technologies (Sect. 1.3), this is not normally the case with pollution involving toxic, inert and chemically stable compounds such as PCBs, PAHs, pesticides, heavy metals and synthetic polymers. These pollutants are not known to be degraded efficiently by many microorganisms and therefore require development of new innovative technologies (3, 6, 8, 12). These pollutants degrade slowly under natural conditions and depending on their respective half-lives, tend to enter the food web, where they are subsequently biomagnified (8, 9). The recognition

of bioremediation as clean technology and the apparent limitations of its operationalization, has directed research into innovative ways of enhancing the capability of natural bioflora to effectively mineralize the environmental pollutants at acceptable rates (expounded in Sect. 2.3).

2. ENVIRONMENTAL CONTAMINANTS

2.1. Environmental Contaminants

Environmental contaminants targeted for bioremediation may, for convenience, be grouped into six major groups comprising: (a) Chlorinated contaminants (these include chlorinated solvents, PCBs and chlorinated phenols), (b) PAHs, (c) Petroleum hydrocarbons (d) BTEX (Benzene, toluene, ethylbenzene and xylene), (e) Pesticides, and (f) Heavy metals (Table 9.1). These environmental contaminants pose serious health problems to both humans and wildlife owing to their high toxicity and persistence within the environment (6, 7, 9, 14, 15). Each group is explored in detail in the following sections:

2.2. Chlorinated Contaminants

Chlorinated contaminants comprise chlorinated solvents, polychlorinated biphenyls (PCBs) and chlorinated phenols (Table 9.1). Chlorinated organic compounds are among the most significant pollutants in the world. They comprise, among others, trichlorethene (TCE), tetrachloroethene (PCE), 1,1,1-trichloroethane (TCA) and chlorobenzene. Polychlorinated biphenyls (PCBs), on the other hand, are a class of chemicals consisting of theoretically about 209 compounds, collectively known as congeners. In PCBs, the aromatic biphenyl carbon skeleton carries between one and ten chlorine atoms. Even though there are 209 possible congeners, typical industrial preparations obtained by random chlorination of biphenyls contain 20–60 PCB congeners (7, 9, 14, 15).

Chlorinated compounds and PCBs in particular exhibit peculiar properties. For example, polychlorinated biphenyls are thermally and chemically very stable, flame- and oxidation-resistant, have low vapour pressure, are super hydrophobic and have excellent dielectric properties. These properties explain the surge in their application in a number of industrial processes such as the manufacture of flame retardants, oil condensers, dielectrics, plasticizers, heat exchangers, extender of insecticides, insulation of transformer and hydraulic fluids. It is therefore, not surprising that the annual tonnage of PCBs produced rose from 100-ton quantities in the early 1930s to a peak of 200,000 tons in 1975. By mid-1980s, about 1.5 million tons of PCBs had been produced worldwide and a substantial fraction entered the environment, while the remaining fraction will ultimately enter the environment (3, 7).

Inasmuch as PCBs possess properties desirable in a number of industrial applications, their continued use is limited by their toxicity and persistence in the environment. Environmental persistence results in their bioaccumulation in the food chain, with the accompanying disastrous effects on humans and wildlife. In humans and most mammals, incomplete degradation of most of these pollutants by the different mammalian enzymes of non-specific activity, tend to transform them into more toxic and harmful intermediates. Currently, oxygenated metabolic intermediates of some congeners are known to be teratogenic, immunogenic and/or

Table 9.1
Environmental contaminants targeted for bioremediation

Class of contaminant	Specific examples	Organism(s) involved			Microbial process	Reference(s)
		Bacterial species	Fungal species	Plants		
Chlorinated solvents	Trichloroethylene	*Methanotrophs*			Co-metabolism	(9)
	Perchloroethylene Trichloroetheure (TCE)	Dehalococcoides ethenogenes strain 195			Halorespiration	(14)
	Tetrachloroethene (PCE)	*D. ethenogenes* strain TCAI			Reductive dechlorination	(15)
	1,1,1-trichloroethane (TCA)	*D. restrictus*				
	Chlorobenzene					
Polychlorinated biphenyls (PCBs) or congeners (about 209 different compounds)	4-chlorobiphenyl	*Clostridium* sp.	*Phanerochaeta*		Meta or-ortho-cleavage oxidation route	(3)
	4,4-dichlorobiphenyl	*Burkholderia cepacia* strain-LB, 400	*Chrysosporium*			(9)
	2,6-dichlorobiphenyl	*Pseudomonas* sp.	*Tranaetes versicolor*		Co-metabolismsss	(7)
	2,5,2,S-tetrachlorobiphenyl					(15)
	2,2-, 2,3,6-and 2,4,6-chlorobiphenyls	*Rhodococcus globerulus* p6			CYP 450 monooxygenase system-dependent degradation	(5)
		Sphingomonas sp.	*Pleurotus ostreatus*			

(Continued)

Table 9.1
(Continued)

Class of contaminant	Specific examples	Organism(s) involved			Microbial process	Reference(s)
		Bacterial species	Fungal species	Plants		
Chlorinated phenol						(9)
Polyaromatic hydrocarbons (PAHs)	Naphthalene	*Alcaligenes* sp.	*Myceliopthora thermophia*		CYP 450-mixed function oxidase mediated oxidation	(9)
	Anthracene	*Pseudomonas* sp.				
	Flourene	*Mycobacterium* sp.				
	Pyrene	*Beijerinckia* sp.				(4)
	Benzo(a)pyrene	*Rhodococcus* sp.	*Phanerochaete chrysospo-rium*			
	Phenanthrene	*Sphingomonas* sp.				(5)
	Benzo(a) anthracene	*Streptomyces* sp.	*Tremetes versicolor*			(14)
	Benzo (b) flouranthene	*Burkholderia* sp.				
		Vibro sp.				
	Benzo (k) fluranthene	*Gardona* sp.				
	Dibenz (a,h) anthracene	*Cyclotrophicus* sp.				
		Stenotrophomonas sp.				
	Indeno (1,2,3-cd) pyrene	*Moraxella* sp.				
		Aeromonas sp.				
		Flavobacterium sp.				
		Bacillus sp.				
		Nocardia sp.				

Petroleum hydrocarbons	Paraffins (alkanes)	α-Proteobacteria		Oxidation	(14)
	Cycloalkanes	γ-Proteobacteria			
	Resin	*Syntrophus* sp.			
	Asphaltene	*Methanosaeta* sp.		Co-metabolism	(13)
		Methanospirillum sp.			(11)
		Desulfotomaculum sp.			
		ε-Proteobacteria sp.			
		Sphingomonas sp.			
		Alcahivorax sp.			(18)
		Marinobacter sp.			
BTEX	Benzene	*Dehalococcoides* sp.	Liginolytic fungi	Attenuation	(9)
	Toluene	*Methylocystis* sp.			
	Ethylbenzene	*Pseudomonas* sp. ADP	*Phanaerochaete chrysosporium*		(19)
	Xylene				

(Continued)

Table 9.1
(Continued)

Class of contaminant	Specific examples	Organism(s) involved			Microbial process	Reference(s)
		Bacterial species	Fungal species	Plants		
Pesticides	Atrazine	*Pseudomonas* sp.	White rose fungi		CYP450 monooxyagenase system dependent degradation	(5)
	Carbaryl	ADP	*Phanerochaete cyrosospo-rium*			
	Carbofuran Coumphos					(9)
	Diazinon	*Pseudomonas diminuta*	*Trametes versicolor*			(7)
	Glycophosphate Parathion					
	Propham		*Pleurotus ostreatus*			
	Organophosphate					
Heavy metals	Copper	*Rhizobacterium* sp.	Mycorrhizal fungi	Elsholzia	Metallothioneins (MTs) & phytochelatins (PCs)	(2)
	Cobalt			Splendens		
	Zinc	Cyanobacterial strains of genus *Synechococcus*				
	Cadmium			*Biscutella laevigata*	Bioaccumulation and sequestrations	(6)
	Lead Nickel					
	Mercury Arsenic			*Thlaspi* sp.	Compartmentalization	

carcinogenic. Moreover, the oxygenated metabolites may act as environmental oestrogens (the so called endocrine disruptors), thereby affecting the normal functioning of endocrine system. Accordingly, many investigators in this field think that PCBs and their oxygenated metabolic intermediates may be one of the causes of decreasing fertility in industrialized nations (3, 7, 8).

2.2.1. Microbial Degradation of Chlorinated Pollutants

In order to use microorganisms for bioremediation of chlorinated pollutants, such organisms need to be isolated and studied to evaluate their suitability. Since microbes capable of degrading chlorinated pollutants are likely to be found in environments where such pollutants are dumped, such environments have often been explored for potential chlorinated pollutant-degrading microbes. The predominant microorganisms fall into two groups: bacteria and fungi. Microbial degradation of chlorinated pollutants has widely been studied in regard to their degradability, molecular characteristics of enzymes involved, as well as the associated genes from a variety of soil microbes (15). Studies in a number of laboratories worldwide have identified microbes and enrichment cultures that metabolize and utilize PCBs as carbon and/or energy source. Through these studies, it has been established that the ability of microorganisms to degrade PCB depends heavily on their possession of the necessary enzymes and specialized pathways (7).

Microbial degradation of polychlorinated biphenyls (PCBs) occurs both aerobically and anaerobically. As a general rule, highly chlorinated congeners (which are highly stable and highly hydrophobic) are good substrates for anaerobic degradations, but are poor substrates for aerobic degradation. Anaerobic utilization of PCBs proceeds possibly via chlororespiration whereby the PCBs are initially used as electron acceptors. This process, also known as dechlorination, progressively converts higher-chlorinated congeners to lower chlorinated forms or more hydrophobic congers to less hydrophobic forms. The lower-chlorinated congers are, in turn, poor substances for anaerobic dechlorination, but are good substrates for aerobic degradation, in which they act primarily as electron donors (3). From the abovementioned, it is evident that microorganisms that are useful for bioremediation of sites polluted by chlorinated compounds are those that can couple reductive dehalogenation of chlorinated solvents and PCBs with energy conservation by electron-couple phosphorylation. In essence, these bacteria should be able to carry out what is known as halorespiration (15).

Bacterial species such as *Dehalococcoides ethenogenes*, strain 195, *D. ethenogenes* strain TCA1, *Dehalobacter restrictus* strain TEA and *Dehalococcoides* sp. strain CBDB degrade chlorinated solvents through halorespiration or reductive dechlorination processes, with an accompanying energy conservation. To date, *Dehalococcoides ethenogenes* strain 195 is the only strain known that is able to completely dechlorinate tetrachloroethene (PCE) to ethane; while strain TCA1 is capable of conserving energy for growth through the reductive dechlorination of 1,1,1-trichloroethane (TCA), converting it sequentially to 1,1-dichloroethane and chloroethane. *Dehalobacter restrictus* strain TEA, which is a strict anaerobe, couples PCE and trichloroethene (TCE) dechlorination to hydrogen oxidation for growth in a respiratory process. Such metabolic capabilities of these strains have found application in bioremediation of TCA contaminated aquifer sediment (15). Other bacterial species, especially the methanotrophs, can co-metabolize pollutants such as trichloroethylene (TCE) and aromatics using

their methane monoxygenase enzyme systems. The oxygenases have broad substrate specificity and have been shown to co-oxidize pollutants such as aromatics and trichloroethylene (TCE) (15, 16).

On the other hand, PCBs can be degraded either by microorganisms via a meta-cleavage pathway to yield tricarboxylic acid cycle intermediate and (chloro) benzoate (CBA) or are transformed by a co-metabolic process using biphenyl dioxygenase enzymes, and fungal ligninolytic enzymes (3, 7, 15). The degradation or transformation of PCBs to form chlorobenzoates involves four enzymes. They include biphenyl dioxygenase (Bph Dox), which introduces molecular oxygen to one of the biphenyl rings, usually at the 2 and 3 positions, a dehydrogenase, a dihydroxy biphenyl dioxygenase (DHBD), which cleaves the biphenyl ring, and a hydrolase (7, 15). White-rot fungi such as *Phanerochaete chrysosporium* and *Trametes versicolor* utilize three principle ligninolytic enzymes: Lignin peroxidase (Lip, E.C.1.11.1.14), Mn-dependent peroxidase (MnP, 1.11.1.13) and phenol oxidase or Laccase (LAC, E.C.1.10.3.2). Besides, Cytochrome P450 monooxygenase system may enhance the rate of biodegradation of PCBs (5).

Biphenyl dioxygenases are distributed in a number of bacteria genera and several genes have been studied. The notable species include *Pseudomonas pseudoalcaligenes* strain KF707, *Burkholderia cepacia* strain LB400, *Rhodococcus globerulus* P6, *Pseudomonas aeruginosa*, *Arthrobacter globiformis* and *Sphingomonas* sp. (3, 7). Bacterial degradation of PCBs requires the participation of a consortium of different species. This is due to the fact that each bacteria species exhibits a particular activity spectrum with regard to the type and extent of PCB congeners metabolized, with some strains having a narrow spectrum and others, notably *Burkholderia cepacia* strain LB400 and *Rhodococcus globerulus* P6, being able to transform a broad range of congeners. These differences reflect parallel differences among the respective biphenyl dioxygenases from these bacterial species. As a matter of fact, knowledge gained from comparative studies of genes encoding substrates recognition subunit of multi-component biphenyl dioxygenase enzymes, indicate that they differ greatly in substrate specificity (3, 7). It is probable that these differences in substrate specificity of biphenyl dioxygenases may explain the different capabilities these enzymes have to catabolize PCB congeners.

In order to understand the degradation pathways of PCBs, a large number of bacteria have been isolated and their capabilities to mineralize the substrate (degrade both the biphenyl rings) evaluated. From these studies, it has been established that a great majority of culturable bacterial species degrade only the least chlorinated rings, and release the second ring as chlorobenzoate. If this also applies to other unculturable microorganisms, it could then be inferred that bacteria capable of mineralizing both of the aromatic rings of chlorobiphenyls are, for some unknown reasons, rare in nature. For this reason, mineralization of chlorobiphenyls appears to require the presence of communities of chlorobiphenyl transforming and chlorobenzoate-degrading organisms at the contaminated site(s) (3, 17). For bioremediation application, an elegant system involving complementary interaction of a consortium comprising microorganisms capable of metabolizing chlorobiphenyls (such as *Burkholderia* sp. LB400 and some fungal species) to release chlorobenzoate, and a consortium comprising chlorobenzoate-mineralizing microorganisms (such as *Pseudomonas* sp. B13FR1 and some

fungal species), may be assembled. Furthermore, chlorinated pollutants are normally intermixed with other organic pollutants such as monocyclic aromatics, polycyclic aromatics, among others. This substrate overlap means that other pollutants on a site may act as co-substrates that can influence the composition and activity of biphenyl-metabolizing communities. At times, PCBs may be co-metabolized by pathways not dedicated to biphenyls. For example, it has been shown that biphenyls can be metabolized by *Pseudomonas putida* CE2010, tod (toluene) and cmt (cumate) pathways, which complement one another, thereby providing the ability to mineralize PCBs (3). Therefore, this co-dependence of different microbial communities constituting a global microbial biota could be used in remediation of heavily contaminated sites, thus reclaiming them for beneficial human activity.

2.3. Polycyclic Hydrocarbons and Petroleum Contaminants

Polycyclic aromatic hydrocarbons (PAHs) are aromatic compounds made up of two or more fused benzene rings. Polycyclic aromatic hydrocarbons found in the environment originate from a number of activities comprising, among others, (a) incomplete combustions of organic fuels e.g. emission sources such as automobiles exhausts, (b) stationary matter e.g., coal-field, electricity-generating power plants, (c) domestic matter e.g., tobacco smoke and residential wood or coal combustion, and (d) area source matter e.g., forest fires and agricultural burning (4, 7). Like PCBs, polycyclic aromatic hydrocarbons (PAHs) are recalcitrant and can persist in the environment for long periods. Likewise, PAHs are also grouped among pollutants generally referred to as persistent organic pollutants (POPs). Their wide distribution in the environment is directly linked to their utilization in a number of industrial and domestic products whereby they also form major waste products. Some products in which PAHs like naphthalene and phenanthrene are constituents include pesticides, fungicides, detergents, dyes and mothballs (4, 7). Major groups of PAHs are summarized in Table 9.1. Examples include naphthalene, phenanthrene, acenaphthene, fluranthen, benzo(a)pyrene, benzo(a)anthracene, benzo(b) flouranthene, benzo (k) flurantheru dibenz (a, h) anthacene, 1-nitropyrene, and indeno (1,2,3-c,d pyrene) (4, 9).

Petroleum contaminants, on the other hand, are categorized into four divisions: saturates, which are hydrocarbons containing no double bonds, aromatics, which are hydrocarbons having one or more aromatic rings with or without alkyl substitution(s), and the resins as well as the asphaltenes. In contrast to the saturate and aromatic divisions, both resins and asphaltenes contain non-hydrocarbon polar compounds. The elements present in resins and asphaltenes, in addition to carbon and hydrogen, are trace amounts of nitrogen, sulphur and/or oxygen. Resins and asphaltenes are largely solids, and not only are their chemical structures complex but they also have remained, to a greater extent, unknown. Furthermore, according to chemical structures, saturates are classified into alkanes (paraffin) and cycloalkanes (naphthalenes) (18).

Environmental contamination by petroleum hydrocarbons can be attributed to oil-tanker accidents, rupture of storage tanks, pipeline leakages and transport accidents. Oil contaminants, which are a complex mixture of hydrocarbons, often enter into the ecosystem where they are exposed to a number of abiotic and biotic factors. These factors may either alter or lead to loss of some components. For example, abiotic factors such as evaporation, dissolution, and photochemical oxidation significantly alter the composition of petroleum hydrocarbons

whereby low molecular weight volatile fractions and water-soluble components are removed. Such volatile petroleum components as n-alkanes with chain lengths shorter than C14 and monocyclic aromatic hydrocarbons (e.g., benzene and xylene) are subjected to both evaporation and dissolution. Under sunlight, petroleum undergoes photochemical modification resulting in an increase in the polar fraction and decrease in aromatic fraction (13, 18). After these physical processes, long chain and complex hydrocarbons are left in the environment. These are recalcitrant and are slowly degraded by microorganisms: bacteria and fungi. In the process, microorganisms remediate the environment of these pollutants.

Polycyclic aromatic hydrocarbons as well as petroleum hydrocarbon contaminants pose pubic health concern owing to their persistence in the environment and they have potentially deleterious effects on both wildlife and humans. Many PAHs, for example, have toxic, mutagenic and/or carcinogenic properties. Naphthalene, a common micro pollutant in potable water, exhibits cataractogenic activity. Studies conducted on laboratory animals have revealed that naphthalene binds covalently to molecules in the liver, kidney and lung tissues, thereby enhancing its toxicity through its inhibitory effects on mitochondrial respiration. In humans, acute naphthalene poisoning can lead to haemolytic anaemia, nephrotoxicity as well as dermal and ophthalmologic changes among occupationally exposed workers. Besides naphthalene, phenanthrene is known to be a photo sensitizer of human skin, a mild allergen, a potent inhibitor of gap-junction intracellular communication, and mutagenic to bacterial systems under specific conditions. Little information is available on PAHs such as acenaphthene, fluranthene and flourene with respect to their toxicity in animals. However, the toxicity of benzo (a) anthracene, benzo (b) flouranthene, benzo (k) fluranthene, dibenz (a, h) anthracene and indenol (1,2,3-d,c) pyrene has been studied and there is sufficient experimental evidence to show that they are carcinogenic (4).

One important property of PAHs is their high solubility in lipids. This makes them readily absorbed from the gastro intestinal tract of mammals. As a result they are distributed in a wide variety of tissues with marked tendency for localization in body fat (4). Owing to their toxicity, PAHs and petroleum-based hydrocarbon have been listed by the US Environmental Protection Agency as priority pollutants for bioremediation.

2.3.1. Microbial Degradation of Polycyclic Aromatic and Petroleum Hydrocarbons

In order to enhance the bioremediation processes, a number of microorganisms capable of growth on various PAHs and petroleum hydrocarbons from contaminated sites have been studied for their suitability for application in bioremediation of contaminated environments. For example, a large number of naphthalene-degrading microorganisms including *Alcaligenes denitrificans*, *Mycobacterium* sp., *Pseudomonas putida*, *P. flourescens*, *P. paucimobilis*, *P. vesicularis*, *P. cepacia*, *P. testosteroni*, *Rhodococcus* sp., *Corynebacterium venale*, *Bacillus cereus*, *Moraxella*, sp., *Streptomyces* sp., *Vibrio* sp., *Sphingomonas*, *Burkhodelria*, *Methanosaeta* sp., *Methanospirillum*, *Desulfotomaculum*, *Geobacter* sp., and *Cyclotrophicus* sp. have been isolated and examined for mineralization of PAHs and petroleum hydrocarbons (4, 14). Among fungi, a few genera have been isolated and studied. They comprise species such as *Phanerochaete chrysosporium*, *Tremetes versicolor*, *Pleurotus ostreatus* and *Myceliophthora thermophia* (5, 7).

Bacterial and fungal degradation of PAHs and petroleum hydrocarbons is dependent on their ability to grow on such compounds as carbon and energy sources. Alternatively, these pollutants may be co-metabolized in the presence of other substrates or transformed into less toxic degradation products. Therefore, several enzyme systems in the past several years have been identified, and their genes are characterized. Enzymes such as oxidoreductase (laccases and cytochrome P450 mono-oxygenase (CYPs)) are being exploited for the enzymatic degradation of PAHs and have been isolated in a diverse species of bacteria and fungi (7).

The first step in the microbial degradation of PAHs involves the incorporation of oxygen atoms on two carbon atoms of the benzene ring of a PAH by dioxygenase enzymes. The *cis*-dihydrodiol formed, undergoes re-aromatization by a dehydrogenase to form dihydroxylated intermediates, which subsequently undergo ring cleavage to form tricarboxylic acid (TCA) cycle intermediates. Specifically, PAHs can be oxidized by CYP enzymes to form catechols, which are then oxidized by dioxygenases (catechol dioxygenase) to harmless products and incorporated into the TCA cycle of microorganisms. Besides the CYP enzymes, PAHs are also oxidized by ligninolytic enzymes and particularly the Laccases. These enzymes, belonging to a group of multicopper enzymes, also catalyze the oxidation of a variety of phenolic compounds. A laccase from a thermophilic fungus, *Myceliophthora thermophia* (MtL) for example, has been extensively studied. The gene for laccase was subjected to several rounds of gene shuffling in order to improve its catalytic activity and stability. The improved enzyme exhibited a 22-fold increase in the K_{cat} for 2,2-azinobis (3-ethylbenzthiazoline-6 sulphonic acid) (ABTS) and a 170-fold higher total activity than the wild type. These findings indicate that the MtL enzyme holds a great potential for bioremediation of PAHs. This is due to its high thermal stability that enables it to work at elevated temperatures needed to increase the solubility of highly recalcitrant PAHs as well as the highly improved catalytic activity (4, 7).

The effectiveness of these enzyme systems in degrading PAHs pollutants is limited to PAHs with at most five rings. For example, although benzo (a) pyrene (BaP), a five-ring molecule abundantly present as an active component of coal tar has been detected in a variety of environmental samples, so far, no microorganisms has been reported that can use BaP as a sole source of carbon and energy. However, a partial degradation of BaP in a six component PAHs mixture by *Mycobacterium* sp. may allude to the possibility that complex PAHs are degraded via co-metabolism strategy with other substances. This strategy is also employed by several microorganisms to metabolize recalcitrant and less bio-available environmental pollutants (4).

2.4. BTEX and Pesticides Contaminants

BTEX contaminants comprise benzene, toluene, ethyl benzene and xylene, while pesticides contaminants comprise atrazine, carbaryl, carbofuran, coumphos, diazinon, glycophosphate, parathion, propham and organophosphate (Table 9.1) (9, 19). BTEX and pesticide pollutants mostly originate from anthropogenic sources, which include, among others, oil production and storage facilities, gas work sites, paint manufacturing plants, chemical manufacturing industries, timber treatment plants and pesticide manufacturing industries. BTEX and pesticide pollutants from these sources are released into the environment as waste from the various industries or as a result of accidents occurring at a manufacturing or storage facility.

Pesticides such as atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-5-triazine) belong to a class of s-triazine herbicides first introduced in the 1950s. It has since been widely used for weed control in agricultural production of crops such as maize, sorghum and sugarcane. Despite containing only one chlorine constituent, atrazine is recalcitrant to biodegradation, with a reported half-life of greater than 170 days in soils containing atrazine degrading microorganisms. Due to its recalcitrance, atrazine is frequently detected in surface and ground water samples, posing a direct risk to humans via potable water consumption (7). Organophosphates (OP) are highly toxic neurotoxins used in insecticides and chemical warfare agents. Included in the organophosphate group are paraxon, parathion, chloryrifos disulfoton, ruelene, carbophenothion and dimeton. The neurotoxic properties of this class of compounds are mainly due to its ability to suppress acetyl cholinesterase. As a result, the breakdown of acetylcholine at the synaptic junction by acetyl cholinesterase is inhibited. Further, these compounds have also been associated with pathology and chromosomal damage connected with bladder cancer (7).

The main problem associated with these pollutants is their long half-lives, which means that they persist for long periods in the environment. Like other pollutants already described, the danger associated with recalcitrance is the eventual accumulation of the pollutants in the food chain. This, therefore, calls for their removal or reduction to acceptable levels by remediation processes. As pointed out earlier (Sect. 1.3) bioremediation offers promise to completely detoxify the pollutants. For BTEX and pesticide pollutants, several species of bacteria have been isolated from contaminated environments and several genes of interest have been studied.

2.4.1. Microbial Degradation of BTEX and Pesticides

BTEX can be biodegraded under both aerobic and anaerobic conditions. This means aerobic and anaerobic bacteria with capabilities of degrading BTEX for carbon and energy exist. This is of great importance in the development of bioremediation strategies for soil pollutants and groundwater pollutants, which may require aerobic and anaerobic degraders, respectively (20, 21). Aerobic BTEX degraders have been isolated from surface soils at contaminated sites as well as from non-contaminated soils. Two mains groups of BTEX degraders exist. They comprise *Actinobacteria*, encompassing strains such as *Rhodococcus* sp., *Microbacterium*, *Mycobacterium* sp., *Arthrobacter* strains and *Proteobacteria*, encompassing strains such as *Pseudomonas* sp., *Azoarcus* sp. and *Bradyrhizobium*. These species constitute the culturable BTEX degrading bacteria, most of which utilize benzene as the only carbon source. However, there are strains that utilize toluene as the only carbon source, which indicate that the ability to utilize TEX compounds as carbon source is not always accompanied by the ability to utilize benzene in the bacterial community (21).

Initial degradation of BTEX requires the concerted action of monooxygenases and dioxygenases enzymes to form catechol. Catechol 2,3 dioxygenase, thereafter cleaves the aromatic ring, converting it into intermediates that are further degraded via the Krebs cycle. BTEX catabolic genes have been isolated from various bacterial strains and also from metagenomes-contaminated soil. The genes relevant to BTEX degradation have been identified and included are *xyl* (*xylA*, $xylE_1$ and $xylE_2$), *tbu* (*tbuA*, *tbuE*), *tmo* (*tmoA*), *tmb* (*tmbD*), and *tod* (*todC*1, *todE*). These genes encode for either BTEX monooxygenases or dioxygenases. Proteins

involved in BTEX degradation can be found in subfamilies 1.2.A, 1.2.B and 1.2.C within family 1.2 and in subfamily 1.3.B within family 1.3 of the catechol 2,3 dioxygenase (C23O) amino acid sequences. Subfamily 1.2.A contains the C23O sequences of mainly fluorescent Pseudomonas bacteria, whereas subfamily 1.2.B contains C23O sequences of mainly Sphingomonas bacteria (21). Subfamily 1.2.C comprises two C23O sequences involved in the BTEX degradation i.e., the *cdo* gene encoding for the C23O II Cdo in *Pseudomonas putida* MT15 and the *tbuE* gene encoding for the C23O TbuE in *Rastonia pickettii* PKO1. The subfamily 1.3.B contains the 3-methylcatechol 2,3 dioxygenase TodE similar to those found in *Pseudomonas putida* F1 and *Pseudomonas putida* DOT-T1, as well as TodE of *Pseudomonas putida* PB4071, which are involved in toluene degradation (21). Complete remediation of BTEX contaminated environments would therefore require the interplay of metabolic activities of different bacterial genera, whereby the different metabolic pathways operate synergistically to completely mineralize these pollutants from contaminated environments.

As with BTEX pollutants, pesticides degradation by microbes has attracted attention, and several microorganisms have been recommended and their metabolic capacity to mineralize pesticides evaluated. Several enzyme systems have been studied for their suitability in bioremediation application. A typical example is a study conducted using *Pseudomonas* sp. ADP. In this study, the genes and encoded enzymes responsible for atrazine metabolism were isolated and characterized. From these studies, it is now known that degradation of atrazine to cyanuric acid requires the action of three different enzymes; AtzA, B and C enzymes. In the biodegradation of atrazine, *Pseudomonas* sp. ADP enzyme (AtzA) transforms atrazine to hydroxyatrazine while AtzB catalyses the hydrolytic deamination of hydroxyatrazine to yield *N*-isopropylammelide. Finally, the enzyme AtzC converts *N*-isopropylammelide to cyanuric acid, which is subsequently mineralized to carbon dioxide and ammonia by other soil microorganisms (7). For organophosphates, bacterial phosphotransferases (PTE), also known as organophosphorus hydrolase (OPH), are highly efficient enzymes that hydrolyze the cleavage of P–O, P–F or P–S bonds in a number of organophosphates (7).

2.5. Heavy Metal Contaminants

Pollution of the environment with heavy metal is a global environmental problem. Heavy metal contaminants result from human activities such as mining and smelting, agricultural activities such as agrochemical usage and long term application of urban sewage sludge in agricultural soils, industrial activities such as sewage disposal, waste incineration, as well as from anthropogenic sources (2, 6, 22). Heavy metals ions of health concern include lead, arsenic, cadmium, copper, zinc, nickel, selenium, cobalt and mercury (Table 9.1). Heavy metal speciation in the environment is determined by their mobilities and solubilities, which in turn, determine their relative effects on soil ecosystems, and the associated ill-health effects. Once in the environment, metals and metalloids often accumulate in the agricultural soils and water, ending up in food due to transfer from soil to plant. The co-existence and persistence of heavy metals in soils as multiple contaminants and human exposure to them through ingestion of heavy metal contaminated food or drinking water, can lead to their accumulation in humans, plants and animals (6).

Heavy metals such as cadmium (Cd), mercury (Hg), and lead (Pb) induce deregulation of a number of physiological activities resulting in ill-health. Lead intoxification, for example, interferes with the synthesis of haem in humans. This is through its inhibitory effects on enzymes of haem synthesis pathway. Apart from interference with haem synthesis, lead toxicity is also associated with renal function impairment including interstitial fibrosis, tubular atrophy and decreased glomerular filtration at concentrations $\geq .40\ \mu g/dL$. In addition, exposure to high doses of lead during foetal development is currently associated with adverse effect among children. Elevated blood Pb levels in children ($\geq 70\ \mu g/dL$) can lead to mental retardation due to brain injury (23, 24). Cadmium toxicity, on the other hand, is also associated with renal tubular dysfunction, cardiovascular disease and malignant neoplasm, such as prostate cancer and lung cancer (25).

Besides the effect of individual metal intoxication, mixed metal contaminations seem to exert a synergistic effect on the overall toxic effects. For example, exposure to multimetals such as Lead and Arsenic may cause inhibition of myeloperoxidase release, thus further decreasing the immune competence of the splenic macrophages. Further, high degree of DNA fragmentations of splenic macrophages on exposure to multimetals indicates that a greater number of cells undergo apoptosis on heavy metal exposure and thus disturb their functional integrity (26).

2.5.1. Remediation of Metal Contaminants

Owing to their inertness to both chemical and biological degradation, heavy metals are extremely persistent in the environment, where they readily accumulate to toxic levels. The accumulation of metal ions, therefore, becomes not only an environmental hazard, but also a public health concern. It is because of these concerns that it is necessary to devise strategies of removing metal contaminants from the environment to acceptable levels.

Various physico-chemical and biological remedial technologies have been developed over the last three decades in order to address metal contamination problems. The selection of each technology is dependent on the specific site of contaminants and the type of metal contaminant(s). Physico-chemical technologies involve chemically enhanced soil flushing using extraction solutions such as organic and inorganic acids, and use of complexation agent. However, these technologies are associated with many problems. Typically, they are expensive, labour intensive, and result in extensive changes to the physical, chemical and biological characteristics of treated soil (6). Further, the health hazards associated with soil contamination with heavy metals, together with the high cost of removal and replacement of polluted soil require that alternative and cheaper technologies be developed to reclaim or recover the degraded land. Current research has been focused on the use of both microorganisms (bacteria and fungi) and plants to remediate metal ion-polluted soils. This would later on facilitate improvement of soil structure, and hence its usability for productive human activities (2, 6, 27).

2.5.2. Microbial Removal of Heavy Metal Contaminants

Microbial removal of heavy metal contaminants from contaminated water, wastewater streams and soil involves sequestering of metals from soils and sediments, and/or solubilizing

metals to facilitate their extractions (2). Bacteria and other organisms inhabiting metal contaminated niches possess resistance mechanisms to toxic metals, which make metal toxicity harmless to them, and in other instances, microorganisms may use various defence systems. Resistance mechanisms, such as the active efflux pumping of the toxic metal out of the cell as well as the enzymatic detoxification (generally redox chemistry), convert toxic ions into less toxic or less bioavailable metal ions. Defence systems, on the other hand, involve exclusion, compartmentalization, metal complexion using metallothioneins (MTs), and enzymatic transformation of metals. Therefore, it is possible to find naturally occurring organisms with unique abilities of metal absorption, accumulation and precipitation. Further, these systems can be utilized in engineering microorganisms for bioremediation of polluted water and soil (2, 27).

2.5.2.1. Microbial Defence Systems

Many microorganisms inhabiting heavy metal contaminated environments have developed a number of defence systems, which they use to detoxify or remove the toxic metal ion(s) from the environments. Detoxification of toxic metals is achieved either enzymatically through transformation of metals to metalloids or through synthesis and production of metal binding proteins such as metallothioneins (MTs).

Enzymatic activities of various microorganisms transform certain metal species through oxidation, reduction, methylation and alkylation reactions. These biological processes have important implication for bioremediation applications because they generate less poisonous metal species. Valls and Lorenzo (27) described the enzymatic process of detoxification of mercury and arsenic. The mechanisms for bacterial resistance to mercury (Hg^{+}) depend on the reduction of mercury by the enzyme mercury reductase to a less toxic and volatile mercury (Hg^{o}) species which is released into the atmosphere. Sometimes mercury derivatives or compounds such as organo-mercurials (methyl mercury for example), which are highly poisonous, exist among contaminants. These organo-mercurials are transformed to mercury (Hg^{+}), which is subsequently transformed to volatile Hg^{o}. The reductase activity thus provides a means of mercury removal by mobilization of the metal to the atmosphere (27).

The proficiency of natural mercury tolerant bacterial isolates in mercury volatilization is being investigated under different conditions and experimental systems. For instance, a *Pseudomonas putida* strain was shown to remove over 90% of the metal from a 40 mg/L solution in 24 h. The gene encoding for mercury reductase (*merA*) activity has been cloned and introduced into *E. coli* as well as *Deinococcus radiodurans* strains. The later strain, being resistant to radiation, is instrumental in the decontaminations of a mixture of mercury and radioactive waste, since it can grow in the presence of both radiation and ionic mercury (27), effectively volatilizing the metal.

Anaerobic microbial transformation of metalloids through reactions such as the methylation has also been reported for arsenic, selenium and tellurium. The process of methylation may be coupled to methane biosynthesis among arsenic-transforming methanogenic bacteria, which converts arsenic to volatile compounds, dimethyl-or trimethyl arsine. Arsenic volatilization may thus be used as a mechanism for its detoxification. Alternatively, arsenic (As (III)) may be oxidized to the more readily absorbed species As (V), which subsequently forms insoluble sulphides upon exposure to hydrogen sulphide (H_2S). For bioremediation

purposes, microbial oxidation would be useful in precipitating As from solution if combined with a separate step of exposure to hydrogen sulphide produced by sulphate-reducing bacteria (SRB). As for arsenite, its oxidation to arsenate in nature is predominantly a microbially driven process. This is due to the fact that chemical oxidation is slow under most environmental conditions. For instance, As (III) was oxidized by *Thermus* sp. at a rate approximately 100-fold greater than abiotic rate (27). However, making these mechanisms operational to bioremediation applications awaits further knowledge of their molecular basis (27).

Heavy metals toxicity may also be removed by use of metal chelating proteins such as metallothioneins (MTs) and phytochelatins (PCs). MTs are low molecular weight (6–7 kDa), cystein (Cys) rich proteins found in animals, higher plants, eukaryotic microorganisms and some prokaryotes. Further, MTs are divided into three different classes on the basis of their cysteine content and structure. The Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs (in which X denotes any amino acid) are characteristic and invariant features of MTs. Like MTs, phytochelatins are also cystein (Cys) rich peptides that are enzymatically synthesized from glutathione (GSH) by phytochelatin synthase (PC synthase). They also chelate heavy metals and have a general structure (γ-Glu-Cys) n-Gly where $n = 2$–11 (28). PCs, however, are so far found in some plant species and none have been identified in animals and prokaryotic microorganisms (2, 27).

The biosynthesis of MTs is regulated at transcription level and is induced by several factors which comprise, among others, hormones, cytotoxic agents, and metals such as cadmium (Cd), zinc (Zn), mercury (Hg), Copper (Cu), gold (An), silver (Ag), Cobalt (Co), Nickel (N) and Bismuth (Bi). Like MTs, biosynthesis of PCS is also induced by metals including Cd, Hg, Ag, Cu, Ni, Pb, and Zn. Once synthesized, MTs and PCs sequester heavy metals by forming complexes with them. Consequently, the environment is mitigated of the heavy metal toxicity (2, 27). While MTs are essentially metal-chelating protein from higher animals and eukaryotic microorganisms, they have been only found in a few cyanobacterial strains of the genus *Synechococcus*. The MT from this strain is encoded by *smtA* gene and contains fever cysteine residues than mammalian MTs.

In view of the fact that other bacterial metal resistance mechanisms such as active metal efflux mechanisms protect only the bacteria without necessarily remediating the contaminated environment, it is desirable for bioremediation purposes, to enhance the defence mechanisms that may accompany active removal of metal contaminants from the environment and thus its mitigation. Enhancement of such capabilities may be achieved by genetic engineering of bacteria to produce MTs or enhancement of their capacity to transform toxic heavy metals or metalloids into less toxic or completely harmless products. Toward this goal, several bacterial genes responsible for conferring a metal resistance phenotype have been cloned and expressed in *E. coli* as fusion protein to other proteins (Table 9.2). This is advantageous because it makes it possible to target MTs to cell surface of the bacteria, thus greatly enhancing their capabilities to complex metal contaminants from the environments (2, 27).

The first studies in genetic engineering of metal chelating proteins involved the cloning of human MTs and their intracellular expression in bacteria. This involved fusing the human MT to an arabinose (*araB*) gene of *E. coli*. The resultant cytoplasmic production human MTs fused to *araB* in *E. coli* brought about a three- to fivefold increase in Cd and Cu

Table 9.2
Recombinant metal binding proteins and their effect on the cell upon exposure to metal contaminants

Protein	Expressed site	Host	Effect	References
Monkey MT	Intracellular	*Escherichia coli*	Though metal accumulation was effected expressed protein has short half-lines and were less stable	(2)
Yeast MT	Intracellular	*Escherichia coli*	Though metal accumulation was effected expressed protein has short half-lines and were less stable	(2)
Human MT-11	Intracellular	*Escherichia coli*	Increase in metal resistance reported	(2)
Mouse MT-I	Intracellular	*Escherichia coli*	Increase in metal resistance reported	(2)
Rainbow Trout MT	Intracellular	*Escherichia coli*	Increase in metal resistance reported	(2)
Plant MT	Intracellular	*Escherichia coli*	Increase in metal resistance reported	(2)
Yeast (CUP1) MT	Fusion to LamB	*Escherichia coli*	Increased metals binding capacity	(2)
Mammalian (HMTIA) MT	Fusion to LamB	*Escherichia coli*	Increase metal binding capacity 15–20-fold	(2)
Neurospora crassa MT	Maltose-binding protein in the periplasm	*Escherichia coli*	Cd-binding capacity increased 65-fold	(2)
Recombinant MT	LamB	*Escherichia coli*	Increase 15–20-fold in $Cd2^{+}$ binding	(27)
Recombinant MT	β-domain of IgA protease auto transporter	*Escherichia coli*	Increase in $Cd2^{+}$ binding	(27)
Recombinant MT	β-domain of IgA protease auto transporter	*Escherichia coli*	Increase in $Cd2^{+}$ binding	(27)
Recombinant MT	β-domain of IgA protease auto transporter	*Pseudomonas putida*	Increase in $Cd2^{+}$ binding	(27)
N. Nacrassa MT	Maltose-binding protein	*Escherichia coli*	6.5-fold enhancement in metal uptake	(27)

bioaccumulation. In addition, the chelating efficiency of MT was proven to be higher when targeted to the periplasmic space. However, targeting to the cell membranes or periplasmic space was shown to circumvent the problems associated with cytoplasmic expression such as: metal uptake limitation, toxicity associated with intracellular metal accumulation, and interference with redox state of the cytosol. For that matter, systems that target MTs to either the periplasmic space or to the other membrane compartments have been developed in *E. coli*, *R. metallidurans*, and *Pseudomonas putida* (2, 27) (Table 9.2).

An alternative to the surface display coordinating moieties is cytoplasmic expression combined with the introduction of specific heavy metal transporter. This approach further overcomes metal uptake limitations across the cell membrane. Unfortunately, it too, is restricted to those metals for which there are active transport systems such as mercury, copper, lead, and nickel. This approach has been used with reasonable success when yeast and pea MTs fused to glutathione S-transferase gene, were cloned into *E. coli* together with a nickel transporter from *Helicobacter pylori*. A threefold increase in Ni accumulation was produced in cell expressing MTs. Similarly, genetically engineered bacteria co-expressing the merT–merP mercury transporter with MTs or metal-binding peptides in the cytoplasm showed an Hg bioaccumulation comparable to that of cells directly expressing the binding peptides on the cell surface (27).

2.5.2.2. Plant Removal of Heavy Metals

Apart from microorganisms, plants too, are endowed with the ability to accumulate metal ions and concentrate them into harvestable parts (phyto-extraction), absorb metals from contaminated water (Rhizo-filtration), immobilize and reduce the mobility and bioavailability of contaminants (phyto-stabilization), and volatilize the contaminants from soil to the atmosphere (phyto-volatilization). These bioremediation strategies are chiefly achieved by plants and may further be enhanced by plants-associated microbes (rhizo-microorganisms). Besides, plant-microbe associations have also been used to degrade chloronito aromatic pollutant such as 4-chloronitrobenzene (4CNB): thus the application of plants in bioremediation is not limited to heavy metals (104). Collectively, plant based remediation process are referred to as phytoremediation (2, 6).

For a plant to be useful for phytoremediation purpose, it should possess the following attributes: (a) the plant should be able to accumulate high levels of metal and translocate it to the harvestable segments of the plant; (b) it should grow rapidly and reach a high biomass; (c) the plant should be metal tolerant, thus allowing it to grow in high metal concentrations. Another category includes metal-tolerating plants which may not be metal accumulators. Such plants also offer possibilities for bioengineering by introduction of metal-binding protein/peptides genes (2, 6). In nature, it is not common to find plants that combine all these attributes. It is, therefore, not surprising that many metal hyper accumulating plants not only grow very slowly, but also have a low biomass owing to their small sizes. Moreover, many fast growing and high biomass producing plants such as Vetiver grass and hemp, though metal tolerant, they are not metal accumulators. Besides these factors that are intrinsic to plants, phytoremediation may be restricted by limitation of Contaminants bioavailability. In order to enhance metal uptake, soil amendments with metal-chelator such as EDTA, citrate, and

hydroxylamine may be applied to make metals bioavailable and thus absorbed by plant roots. Even then, the type of chelator and its time of application are important considerations (6).

To make phytoremediation practicable, both plant biomass and metal accumulation capabilities should be enhanced. Efforts to have plant biomass increased have centred on the use of plant growth regulators (PGR) such as auxins, cytokinins and plant hormone indo-acetic acid (IAA). Auxins and cytokinins enhance phytoremediation abilities of non-hyper-accumulating plants by increasing their growth and biomass. Indo-acetic acid, on the other hand, encourages hyper-accumulation of metals through enhancement of the bioavailability of metal contaminants to plants. Typically, IAA enhances bioavailability of iron (6). While some plant growth regulators and hormones are produced by some plants, some PGRs are produced by rhizobacterial (PGPR) strains and mycorrhizal fungi that live symbiotically with plant-root system. Plant growth promoting rhizobacterial strains such as Pseudomonands and Acinetobacter produce IAA, which results in enhanced uptake of iron, zinc, magnesium, calcium, potassium, and phosphorus by crop plants. Furthermore, PGPR fix nitrogen, produce phytohormones and specific enzyme activities, lower ethylene levels, protect plants from diseases by producing antibiotics as well as other pathogen-depressing substances such as siderophores (6).

Like in bacteria, metal accumulation may also be enhanced by genetically modifying plants capable of growing in metal contaminated environments to express MTs and PCs. Transgenic plants that express MTs have been scored to enhance Cd tolerance, Cd accumulation, or modified Cd distribution. For example, a human MT-11 gene introduced into tobacco and oilseed rape, enabled growth of these transgenic seedlings in Cd contaminated environments at concentrations of 100 μM. In some instances, an increased Cd tolerance of up to 200 μM Cd^{2+} or an altered distribution of Cd have been observed in transgenic plants expressing MTs, while in other instances, expression of MTs achieved a modified distribution of the accumulated metal (2). For instance, the human MT-11 gene fused to the β-glucuronidase gene was expressed in tobacco. In vitro grown seedlings expressing the fusion protein accumulated 60–70% less Cd in their shoots than the control plants. In the control plants, 70–80% of the Cd was translocated to the leaves whereas in the MT-expressing plants only 40–50% was translocated (2). Reduced translocation to leaves was accompanied with increased Cd levels in both roots and stem. A modified distribution is of a particular interest for crops in the objective of translocating of metal contaminants to non-consumed segments of the plant or to harvestable parts for phytoremediation. Apart from introducing mammalian MTs into plants, modifications on plant detoxifying proteins, the phytochelatins (PCs), or over-expression of enzymes that are involved in the synthesis of glutathione and PCs have been used to further enhance heavy metal tolerance and accumulation in plants (2).

It is comprehensible from the above discussion that the successful application of plants to reclaim environments heavily contaminated with heavy metals would require careful integration of plant-types of divergent capabilities to accumulate or tolerate metals. While it may be necessary to develop transgenic plants, it would be more beneficial to exploit natural means of enhancing growth and increasing biomass especially through the integral use of plant growth regulators and hormones, as well as free-living or symbiotic plant growth-promoting rhizobacteria and mycorrhizal fungi.

3. BIOREMEDIATION STRATEGIES

As already pointed out (Sect. 1.3), bioremediation is a natural process by which microorganisms either immobilize or transform environmental contaminants to innocuous end products. Bioremediation includes all processes and actions that take place in order to biotransform an environment, already altered by contaminants, such as pesticides, herbicides, insecticides, cleaning chemicals and chemicals used in the food chain, to its original status. There is variation in the processes employed; however, similar principles apply as in the use of microorganisms or their enzymes. The enzymes may be indigenous which may be stimulated by the addition of nutrients or optimization of conditions, or may be seeded into the soil. The objective is to transform the contaminants into substances that can be absorbed and used by the autotrophic organisms with no toxic effect on them (29, 30). Bioremediation has been used in the treatment of contaminated soil and ground water through: (a) stimulation of the activity of indigenous microorganims by the addition of nutrients, regulation of redox conditions, optimizing pH conditions, (b) inoculation of the site by microorganisms with specific biotransforming abilities, (c) application of immobilized enzymes, and (d) use of plants (phytoremediation) to remove and/or transform pollutants (31). Specific methods used for bioremediating contaminated soil and water include: landfarming, compositing, intrinsic bioremediation, and slurry bioreactor (Table 9.3).

3.1. Landfarming

Landfarming is a managed treatment and disposal process that involves the controlled application of waste to soil or soil-vegetation system (32). It relies on agricultural principles and aims to control the biocycling of natural compounds. Conditions of soil microbial populations are optimized by the dilution of contaminated soil with clean soil, tilling of the soil to reduce initial toxicity, as well as by controlling physical parameters, such as aeration, pH, soil moisture content, and temperature. Aeration is obtained by tilling the soil, or by forced aeration after covering the soil and exiting air cleaned through filters. Temperature control is achieved through the introduction of hot air, or the 'greenhouse effect' in a closed system.

3.2. Composting

Composting is a biological aerobic decomposition of organic matter under strictly controlled conditions. This helps thermophilic microorganisms transform organic materials into a stable, soil-like product (33, 34). The process is natural in soil where microorganisms decompose materials. However, the natural processes may be so slow that some materials hardly get decomposed. In order to increase the rates and use composting for industrial purposes, microbial growth may be optimized through optimizing oxygen concentration, pH, moisture content, carbon to nitrogen (C:N) ratio, and particle size (33, 34). Composting could also be enhanced through the use of bulking agents such as wood chips and vermiculite, which through increasing the void space in the compost (35), would allow for the maintenance of adequate oxygen to enable the obligatory process to proceed.

Table 9.3
Bioremediation technologies and their application

Technology	Principals	Advantages	Disadvantages	Applications	References
Land farming	Solid-phase treatment system for contaminated soils; may be done in situ or in a constructed soil treatment cell	Simple procedure. Inexpensive. Currently accepted method	Slow degradation rates. Residue contamination often removed. High exposure risks. May require long incubation periods	Surface contamination. Aerobic process. Low to medium contamination levels	(32)
Composting	An anaerobic microbial driven process that converts solid organic wastes into stable, sanitary, humus-like material	More rapid reaction rates. Inexpensive. Self-heating	Need bulking agents. Requires aeration. Nitrogen addition often necessary. High exposure risks. Residual contamination. Incubation periods are months to years	Surface contamination. Aerobic process. Agricultural and human wastes. Sewage sludges, industrial wastes, yard wastes, municipal solid wastes	(33) (34) (35) (36)
Intrinsic bioremediation	Relies on the natural assimilative capacity of the ground to provide site remediation and control contaminant migration	Relatively inexpensive. Low exposure risks. Excavation not required	Low degradation rates. Less control over environmental parameters. Needs good hydrogeological site characterization. Incubation periods are months to years	Deep contamination. Aerobic or nitrate reducing conditions. Low to medium contamination levels. Oil and gasoline. Chlorinated hydrocarbons	(39) (40) (44) (45) (46)
Slurry bioreactor	Soil and water agitated together in reactor	Good control over parameters. Good microbe/compound contact. Enhance desorption of compound from soil. Fast degradation rates. Incubation periods are days to weeks	High capital outlay. Limited by reactor size. High exposure risks	Surface contamination. Recalcitrant compounds. Soil that binds compound tightly. Aerobic and anaerobic processes	(35) (48) (49) (50)

Table 9.4
Composting methods

Method	Composting time	Cost	Usage	Disadvantages
Windrow	2–6 months for municipal solid waste	Low	Used mainly in combination with in-vessel technology for curing the compost	Difficult control of conditions, temperature, water concentration odour
Aerated piles	6–12 weeks	Medium	Used for sewage sludges, municipal solid waste, yard wastes and industrial organic wastes	Continued electrical costs
In-vessel	Less than a week to 2 weeks	High due to installation costs	All types of waste	High costs, intense and skilful management

Composts constitute a valuable soil amendment and may be used, as a fertilizer substitute to supplement plant nutrient needs because of the high organic matter content. Therefore, composting can be used as a method to stabilize and decrease sewage sludges, industrial wastes, yard wastes, and municipal wastes. It has also been used in the treatment of hazardous waste such as explosives (36). There are three types of composting including: windrow, aerated static pile, and in-vessel (37, 38) (Table 9.4). The three types of composting share similar stages, but differ in the time to complete the tasks, capital and operating costs, and the ways in which to achieve the necessary conditions for bacterial growth.

3.3. In Situ Intrinsic Bioremediation

In situ or intrinsic bioremediation is a natural process, which exploits natural ways of recycling nutrients through the cycles of nitrogen and carbon (39). The decomposition of the contaminant is carried out by indigenous microorganisms, which grow on the contaminated soil and can only survive in that environment by using the contaminants as a source of energy (40, 41). The process could be exploited to enhance the degradation and recycling of wastes and to clean contaminated soils (42). To enhance the process of decomposition, the microorganisms could be genetically modified (43) or strictly selected nutrients could be added to the soil (39). The requirement for no excavation and special equipment means low cost of operation and no disturbance of the natural environment. The method is therefore suitable for treating rocky or underground water areas (39, 44). A major disadvantage of in situ bioremediation is that it is slow and may not be suitable for use where immediate site clean up is required. The method also produces toxic by-products in some cases. Addition of nutrients may not reach the target, hence prolonging the process of remediation (39, 45). The process is also more difficult to keep under control than *ex situ*, or engineered bioremediation due to the lack of experimental conditions in the contaminated soils (46).

3.4. Ex Situ or Slurry Bioremediation

In *ex situ* or slurry bioremediation, the contaminated soils are excavated and mixed with water to form a slurry that is mechanically aerated in a reactor vessel. Agitation of the reactor ensures the breakdown of soil aggregates, desorption of contaminants from soil, increased contact between wastes and microorganims, and improved oxygenation of the slurry (35). In order to improve the treatment of the contaminated soils and to increase the biodegradation capability, use of surfactants, dispersants and materials supporting microbial growth, control of temperature, and concentration of biomass is key (35, 47). To ensure efficiency, the contaminated soils are pre-treated before introduction into the reactor, the soils are graded physically to reduce the cost of mixing and agitation, soils may be fractionated to reduce the total volume to be treated and increase the rate of biodegradation of the contaminants (48). Alternatively, sodium hydroxide and sodium chloride may also be added to neutralize soil acidity and dispersion of clay particles, and to trap the contaminants. *Ex situ*/slurry bioremediation is faster than the *in situ* method, although higher costs than for the *in situ* systems are involved because of the high degree of engineering (49).

3.5. Bioaugmentation

Bioaugmentation involves the use of specialized competent strains or consortia of microorganisms, which may be indigenous or genetically modified organisms, to improve the capacity of a contaminated environment. The process relies on the immense metabolic capacities of the microbes to transform organic man-made pollutants into harmless or, less dangerous compounds. Biodegrading microorganisms do occur in nature, however, their potential to degrade and mineralize target pollutants may be limited by low numbers, unfavourable local conditions, and the presence of complex molecules or a mixture of compounds that require specific microorganisms and/or pathways (50).

Bioaugmentation may be attained through: the addition of pre-adapted pure bacteria strains (51, 52); pre-adapted consortia, i.e. degrading enrichment cultures (53); genetically engineered bacteria, to avoid the accumulation of potentially toxic pollutants and biodegradation-relevant genes transferred by conjugation into microorganisms in the biotope under remediation (54).

Bioaugmentation has been used to (a) improve the flocculation of activated sludge, and (b) to enhance the removal efficacy of recalcitrant compounds. Bioaugmentation enhances the removal of 3-chorobenzoate, 4-methyl benzoate, toluene, phenol, and chlorinated solvents (55, 56). However, the technique has not yet received wide application due to the fact that the bioaugmentation of activated sludge is less predictable and controllable than direct physical or chemical destruction of pollutants. The removal of refractory and inhibitory compounds in coke plant wastewater, that was unachievable by conventional methods, such as solvent extraction, steam stripping, and/or biological treatment, was achieved recently using bioaugementation, with a quinoline-biodegrading aerobic bacterium, *Burkholderia pickettii*, obtained from activated sludge (57).

4. APPLICATION OF BIOREMEDIATION

4.1. Case Studies of Bioremediation

Bioremediation is key in the food industry, having been used in the treatment of wastes from processing of fruits and vegetables, olive oil, fermentation, dairy, meat, and poultry products.

4.1.1. Fruit and Vegetable Processing Industry

The fruit and vegetable processing industry includes among others: fruit and vegetable canning, frozen vegetables, vegetable dehydration, fruit and vegetable drying, fruit pulping, tomato juice and fruit concentrates, etc. Since fruit and vegetable production are seasonal, environmental pollution from waste generated from the industry is equally seasonal. A big proportion of the waste from the fruit and vegetable industry is solid suspensions and high biochemical oxygen demand (BOD). Other parameters also affected by such waste include pH, chemical oxygen demand (COD), dissolved oxygen, and total solids. The pH is mainly acidic. The chemical composition varies depending on the type of fruits and vegetables processed, and the pesticides, herbicides and cleaning chemicals used during production. Separate treatment is therefore used for the different wastes. For solid waste treatment, composting, slurry bioreactors and landfarming may be used. The waste is pretreated to remove the water and neutralize the pH to allow for efficient microbial growth and development. Bulking agents such as sawdust, paper, mature compost, straw, and coffee residuals may be added to improve the porosity of the sludge and decrease the bulk density (38). Increased porosity helps in the drainage of water. The bulking agents have the double effect of also increasing the C: N ratios of the waste due to their high carbon content and the pH (58).

4.1.2. Olive Oil Industry

The olive oil industry generates wastewater, a liquid waste that contains dark-coloured juice, organic substances such as sugars, organic acids, polyalcohols, pectins, colloids, tannins and lipids. These products have very high BOD, COD, and concentration of organic substances, such as phenols, which are difficult and expensive to degrade (59, 60). Biotreatment of the olive oil mill wastewater (OMW) may be conducted aerobically or anaerobically.

In the aerobic process, the oxygen is provided by an external source. However, the biodegradation proceeds very slowly due to operational problems and requires a high concentration of the feed to operate more efficiently (61). The aerobic process cannot efficiently remove certain persisting pollutants, such as polyphenols and colouring substances. Suggestions have been made to mix sewage wastewaters with OMW to improve biodegradation and reduce the cost as well (62). In order to improve biodegradation of OMW, the polyphenols and lipids have to be removed prior to the aerobic treatment. In addition the colouring substances could be removed using the fungus Pleurotus.

The anaerobic process has been shown to produce better results than the aerobic process on organic pollutants, sugars, polyphenols, and pectins. The growth rates of the microorganisms are lower than the corresponding rates for the aerobes. Examples of anaerobic processes include: anaerobic lagooning, anaerobic contact and the upflow anaerobic sludge blanket.

4.1.3. Fermentation Industry

Waste from the fermentation industry may be generated from brewing, distilling and wine manufacture. Fermentation waste is characterized by high BODs and CODs, although differences have been observed in the concentration of the organic compounds. The high concentrations of tannins, phenols and organic acid in fermentation wastewater enhance the anaerobic bioremediation processes (63). These processes may be enhanced further by optimizing the acidity (5–6 pH) and temperature (40°C) of highly concentrated brewery wastewater using the upflow anaerobic sludge blanket (64). Treatment of winery waste is limited by the presence of vinasse, which must be biologically treated for 4–8 days to reduce the COD by 90% (65).

4.1.4. Dairy Industry

Dairy industrial waste is one of the most important pollutants of soil and surface water. It may contain proteins, salts, fatty substances, lactose, and different cleaning chemicals, which may be alkaline or acid (66, 67). It is mainly characterized by: high organic load (e.g. fatty acids and lactose), large variations in waste supply, considerable variations in pH (4.2–9.4), and relatively large load of suspended solids (SS) (400–2,000 mg/L) (67). The cleaning chemicals comprise the biggest pollutants, since in addition to either being alkaline or acid; they also may contain phosphates, sequestering agents, surfactants, dispersing agents, anti-foaming agents, and inhibitors (68). Although the presence of detergents in dairy wastewater hardly influences the total COD in contrast to milk, cream, or whey, it presents some difficulties in their treatment. According to Wildbrertt (69), sodium carbonate passed through a treatment process almost unchanged. Both aerobic and anaerobic treatment systems have been employed in the bioremediation of dairy wastes (70–74). A new promising technology in diary wastewater treatment is thermophilic aerobic treatment, which could be used for treating high-strength organic waste streams. The technology combines the advantages of low biomass yields and rapid kinetics associated with high temperature operation and stable process control of aerobic systems. Additionally, the technology has potential for producing pathogen-free products and for the exchange of energy generated by the process (75).

4.1.5. Meat, Poultry and Fish Industries

The meat, poultry, and fish industries produce the highest loads of waste within the food industry. In the meat industry, wastes are generated in the slaughterhouses and processing units. The slaughterhouse wastes, which is separated into wastewater and solid waste, contains various quantities of blood, fats, residues from intestines, paunch grass, and manure (76). The slaughterhouse wastewater is rich in moisture (90–95%), nitrogen, BOD, and is odorous. The management of nitrogen in the meat processing industry is key in waste treatment. The waste must be pretreated to reduce the moisture to 60–75%, and bulking agents must be used to increase the porosity of the waste for efficient aeration. The pre-treatment also aids in the control of pathogens that may interfere with the process (76). According to Starkey (77), a treatment system for poultry waste should consider land availability, previous site history, publicly owned treatment work discharge, conventional waste treatment systems, and land application systems. Similarly, pre-treatment of poultry waste to reduce moisture and kill

pathogens and the use of bulking agents to increase the porosity, which also increase aeration and carbon levels in the wastewater, are considered *a sine qua non*.

4.1.6. Oil Refinery Sludge

The petrochemical industry generates a series of liquid effluents during the petroleum-refining process. These effluents are treatable through depuration processes. The oil refinery sludges that result from this depuration process have a high content of petroleum-derived hydrocarbons, which may be alkanes and paraffin of 1–40 carbon atoms, cycloalkanes and aromatic compounds (78). This makes it a potentially very dangerous waste product, which may have serious environmental consequences (79). Petroleum hydrocarbon wastes may be treated using natural biological, chemical, and physical processes (80).

4.1.7. Coke Plant Wastewater

Coke plant wastewater is generated in the coal coking, coal gas purification, and by-product recovery processes of coke plants. The wastewater contains ammonia, thiocyanate, phenolics, and other organic compounds, such as mono- and poly-cyclic nitrogen-containing aromatics, oxygen- and sulphur-containing heterocyclics, and polynuclear aromatic hydrocarbons (PAHs) (81, 82). These wastes are very harmful and carcinogenic. Conventional treatment of coke plant wastewater includes solvent extraction, steam stripping and biological treatment. However, due to the presence of refractory and inhibitory compounds, the conventional biological treatment is not efficient in removing COD. Use has been made of anoxic–oxic (A–O) and anaerobic–anoxic–oxic (A1–A2–O) processes to treat coke plant wastewater with good results (82). However, this could not reduce the effluent COD to less than 200 mg/L.

Bioaugmentation of activated sludge systems with specialized microorgamisms could be used to improve the flocculation of activated sludge and to enhance the removal efficiency of recalcitrant compounds. Bioaugmentation has been reported to enhance the removal of 3-chlorobenzoate, 4-methly benzoate, toluene, phenol, and chlorinated solvents. However, bioaugmentation of activated sludge is less predictable and controllable than direct physical or chemical destruction of pollutants.

Quinoline, a heterocyclic compound, which is poorly removed in the A1–A2–O system, was isolated from activated sludge of a coke oven wastewater treatment plant by enrichment shaking culture (57). This was achieved through bioaugmentation with a quinoline-degrading bacterium, *Burkholderia pickettii*. *B. pickettii* has a degradative role and is tolerant to refractory and inhibitory organic compounds in coke plant wastewater.

4.1.8. Marine Bioremediation

Sources of pollution in the marine environment could be due to: nutrients; sediments; pesticides; sewage outfalls; stormwater; exotic species; coastal development; hydrocarbons; heavy metals; litter and aquatic organisms (83). Three approaches to reduce marine associated environmental health risks have been suggested as: cleanup, isolation, and prevention. Marine bioremediation efforts often target hydrocarbon contaminants, but do have applications also to nutrient loading, heavy metals, haloorganic compounds and other pollutants.

Nutrient loading is a widespread phenomenon in many coastal areas. Although generally not directly toxic to indigenous organisms, it could promote excessive algal growth resulting

in hypoxia or anoxia (84). The removal of nitrate from wastewater helps prevent downstream eutrophication and can be accomplished using wastewater treatment systems, modified to remove organic compounds under anaerobic conditions. By switching to anaerobic conditions with methane as a carbon and energy source, methylotrophic bacteria convert the nitrate to nitrite and then to molecular nitrogen. Denitrifying bacteria have now been shown to also contribute significantly to biological phosphate removal through processes in which the organisms are cycled between anaerobic conditions that favour nitrate removal and the aerobic conditions that favour phosphate removal (85). This results in reduced chemical oxygen demand and expands the operational range of the biological process (86).

Metals are not degradable by microorganisms. However, microorganisms could detoxify heavy metals and radionuclides from contaminated waters by precipitating, volatilizing, solubilizing or adsorbing them (87, 88). Bacterial strains are known, which have the capacity to concentrate or remediate the metal contaminants into forms that are precipitated or volatilized from solution and hence less toxic and easily disposable. For example, sulphate-reducing bacteria were used to immobilize metals at what was once a zinc-refining site at Budelco in the Netherlands. Contaminated groundwater was pumped through a bioreactor in which ethanol, ammonia and phosphate support the growth of sulphate-reducing bacteria. The bacteria converted the sulphate in the water to hydrogen sulphide, which reacted with the heavy metal contaminants to form insoluble metal sulphides. Biosurfactants such as glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants enhance the desorption of heavy metals in two ways:

(i) They may complex free forms of the metal residing in solution, which decreases the solution phase activity of the metal and, promotes desorption according to Le Chatelier's principle.
(ii) Alternatively, through direct contact to sorbed metal at solid solution interface under conditions of reduced interfacial tension, allows biosurfactants to accumulate at solid solution interface.

The effectiveness of the use of biosurfactants for metal remediation increases in terms of cost involved at sites co-contaminated with organic compounds. However, the addition of biosurfactants may also inhibit some microorganisms. Therefore, the best strategy would be to stimulate biosurfactants produced by indigenous population present at the contaminated site. This is not only environmentally compatible but also more economical than using metal chelators such as EDTA.

Haloorganics such as polychlorinated biophenyls (PCBs), solvents and pesticides are recalcitrant to degradation. However, others may be mineralized or only partially biodegraded under anaerobic conditions. For example, consortia of indigenous microorganisms were able to degrade the PCBs in Hudson River (89), in which both anaerobic and aerobic biodegradation played roles in the otherwise slow process. Increased degradation rates were obtained on addition of inorganic nutrients, the organic co-metabolite biphenyl and oxygen. Dehalogenation is a key initial step in degradation, which may occur by oxygenolytic, hydrolytic or reductive mechanisms (90).

Crude oil or refined petroleum includes hundreds of different alkanes and aromatic hydrocarbons, among which are polycyclic aromatic hydrocarbons (PAHs), which are carcinogenic. Marine ecosystems may be affected by disastrous oil spills, spills that occur during refuelling

in ports, terrestrial spills and run-off, which are major sources of oil pollution (91). The biodegradation of petroleum compounds occurs through diverse enzymatic capabilities within bacterial populations that are ubiquitous in the marine environment and rapidly increase, in relative proportions, in the presence of petroleum contamination (92). PAHs with fused aromatic rings are refractory to biodegradation because they are hydrophobic and hence they tend to adsorb to the soil and sediment. In nature, bioemulsifiers and biosurfactants may play a role in desorption and bioavailability of the hydrophobic contaminants (93). The biodegradation of floating oil is limited by surface area (94). In order to stimulate biodegradation in such circumstances, a dispersant may be added to the oil slick. This dramatically increases the surface area available for microbial colonization at the oil–water interface. Surfactants used in some dispersants have been shown to further enhance biodegradation of dispersed floating oil by serving as a biodegradable substrate and stimulating growth of biodegradative bacteria (94).

4.2. Factors for Designing a Bioremediation Process

The design of improved biocatalysts involves different aspects of optimization, including: creating new metabolic routes; expanding the substrate ranges of existing pathways; avoiding substrate misrouting into unproductive routes or to toxic or highly reactive intermediates; improving the substrate flux through pathways to avoid the accumulation of inhibitory intermediates; increasing the genetic stability of catabolic activities; increasing the bioavailability of hydrophobic pollutants; and improving the process-relevant properties of microorganisms. A variety of strategies for designing new or improved catalysts for bioremediation are available including in vivo and in vitro strategies.

4.2.1. Biodegradative Performance

Consortia that exhibit novel catabolic activities can be obtained by sustained selective pressure in a chemostat. The consortia could be developed for the mineralization of chlorinated biphenyls, chlorinated dibenzofurans (95), and aminonaphthalenesulfonates (101). One member of the consortium transforms the substrate into the corresponding chlorinated benzoate or salicylate and grows at the expense of the initially attacked aromatic ring. Thereafter, a second member mineralizes the formed benzoate or salicylate.

4.2.2. Anaerobic–Aerobic Processes

Another approach to the mineralization of highly chlorinated congeners is the development of anaerobic–aerobic processes. Since microbial degradation of PCBs occurs in sediments, and anaerobic dehalogenation is enhanced by an increase in halogen substitution, in contrast to aerobic degradation, for which the persistence increases with increasing halogen substitution, the process could be used to transform highly chlorinated biphenyls into less-chlorinated congeners, which are more amendable to aerobic degradation. There are, however, only a few cultures that are able to dechlorinate PCBs reductively to date.

Additionally, the metabolic division of labour in co-cultures of aerobic microorganisms may not constitute the most effective situation and prolonged selection may lead to the transfer of genetic determinants of catabolic functions between members of the consortium and the

emergence of a single organism with the complete catabolic sequence. These natural gene-transfer events are the basis of numerous in vivo design experiments and are facilitated by the fact that naturally occurring pathways for the metabolism of organic compounds are often encoded by broad-host-range plasmids (98). Plasmid cloning vectors may, however, suffer from the same instability as natural plasmids and moreover, have antibiotic-resistance selection markers, which are undesirable for environmental applications. In order to circumvent these problems, mini-transposon cloning vectors have been developed to insert heterologous genes stably into the chromosomes of host bacteria without the use of antibiotic-resistance markers or, with markers that can be selectively eliminated after gene transfer.

4.2.3. Catalyst Performance

An increase in the rate of pollutant removal may be obtained through identification of enzymatic or regulatory step of the pathway that is rate limiting, followed by experimental elevation of the activity of the rate-limiting protein. The activity of the rate-limiting protein could be elevated through an increase in the transcription or translation of its genes, or in its stability or kinetic properties. This involves the use of mutants of regulatory proteins that either mediate higher levels of transcription than the wild-type regulator or respond to new effectors (96). The use of artificial regulatory systems allows the expression of catabolic genes to be uncoupled from the signals that ordinarily control their expression and offers considerable flexibility for process control (100).

Protein engineering could be exploited to improve an enzyme's stability, substrate specificity and kinetic properties. The rational design of proteins performed by site-directed mutagenesis requires an understanding of structure–function relationships in the molecule and a detailed knowledge of the three-dimensional structure of the enzyme itself. However, the number of degradative enzymes whose structure has been elucidated is still small and this constitutes a major limitation for rational protein design. Additionally, proteins with new activities could be developed through combining the best attributes of related enzymes by exchanging subunits or subunit sequences, or through shuffling their genes sequences (97).

4.2.4. In-Complete and Complete Metabolic Pathways

In bioremediation, co-metabolic processes need an input of energy, which may present a metabolic burden for the microorganism involved. Further, the end metabolites produced by incomplete pathways may be toxic or subject to further transformation by other microorganisms, forming reactive or toxic molecules. For example in PCB metabolism, microorganisms usually metabolize only one aromatic ring and accumulate the others as the corresponding chlorobenzoates, which have been shown to be inhibitory to further PCB metabolism (46, 47). The use of complete pathways could help overcome the problem associated to incomplete pathways. Although a complete pathway for a particular substrate may not exist in a single organism, partial and complementary pathway segments may exist in different organisms (Sect. 2.2.1). In order to form a complete pathway sequence for a target substrate for an organism exhibiting a desired catabolic phenotype, determinants for complementary pathway segments may be combined.

4.2.5. Pollutant Bio-availability

Bioremediation is limited not only by the recalcitrance of the target pollutants but also by the toxicity of such compounds and, in particular, the limited bio-availability of hydrophobic, poorly water soluble pollutants such as PCBs. Biological reactions occur in or at the interface of the aqueous phase and the surfactants have the ability to desorb and disperse poorly soluble compounds in small, high-surface-area micelles within the water phase. Surfactants can thus improve the accessibility of these substrates to microbial attack (102). The high surface activity, heat and pH stability, low toxicity and biodegradability of bio-surfactants constitute important advantages over synthetic surfactants, particularly for environmental applications. However, a major limitation of the application of bio-surfactants is the high cost involved. Efforts are currently geared towards the design of recombinant biocatalysts that exhibit a desired catabolic trait and that produce a suitable bio-surfactant (99).

4.2.6. Catalyst Survival in the Environment

Improving inoculant survival is an important goal in the further development of bacterial inocula for biotechnological applications in the environment, where the microorganisms are exposed to a variety of stresses such as toxic metals, solvents and extremes of temperature and pH. A combination of resistance to environmental stresses and catabolic phenotypes in appropriate bacterial strains, such as strains of *Deinococcus radiodurans*, solvent-resistant bacteria able to mineralize hydrophobic pollutants would yield microbial catalysts with significantly improved survival characteristics in hostile habitats.

4.3. Bioremediation Process Design and Implementation

Bioremediation process design depends on a clear understanding of the nature of the polluted environments. These environments, which include soil, surface and ground water, need to be assessed for constituent pollutants as well as natural flora. Pollutants may be classified as either organics or heavy metal, while the natural flora include microbial consortia, which comprises microbial flora (bacteria and fungi) and plants. Assessment of the polluted environment is essential in determining the nature of the pollutant and associated natural flora (Fig. 9.1). Other factors of importance include pH, temperature, and nutrient availability. Subsequent to careful assessment of these factors it is possible to determine the bioremediation strategy to undertake. For example, an environment polluted by organics would require the action of microbial consortia, while that polluted by heavy metals would require the action of both microbial consortia and plants for remediation. Issues concerning cost-effectiveness of any bioremediation process design should be addressed, before the implemetation of the process.

5. LIMITATION OF BIOREMEDIATION STRATEGY

1. It is often difficult to evaluate the success of an in situ bioremediation programme. This is true whether using genetically engineered or intrinsic microorganisms. For instance, it is not easy to deduce to what extent a certain microbe is actually contributed to the degradation process. Where genetically engineered microorganisms (GEMs) are used, it is difficult to distinguish between GEM-specific degradation and biodegradation due to indigenous microbial consortia.

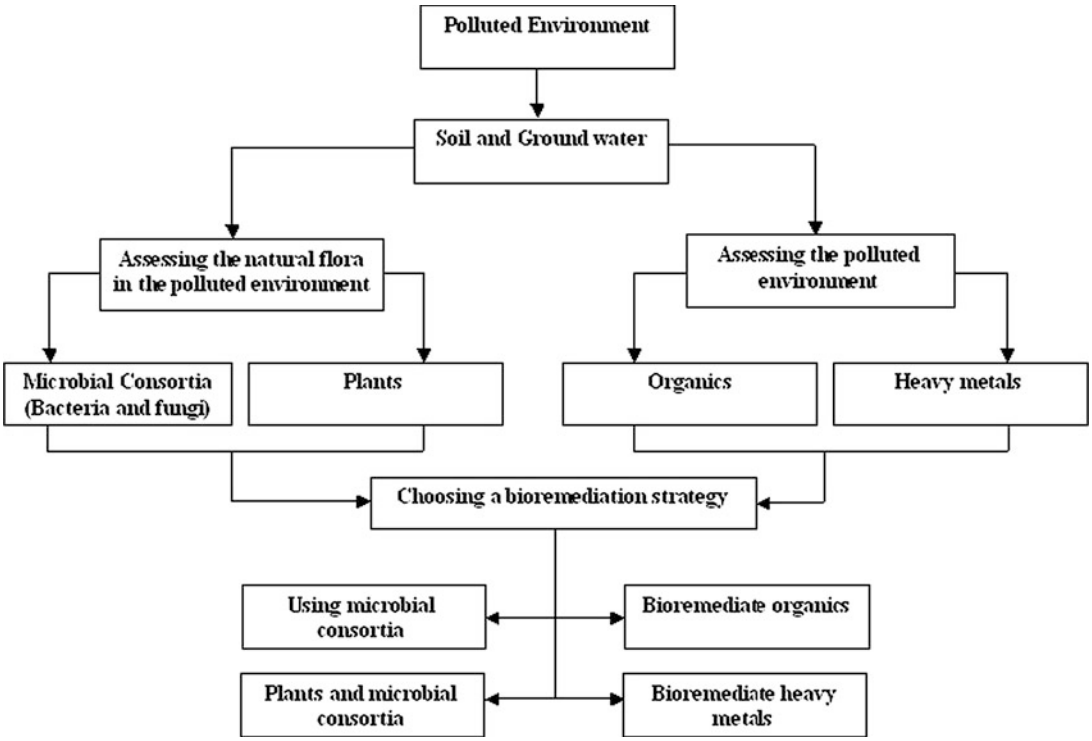

Fig. 9.1. Schematic representation of factors to consider in designing a bioremediation process.

2. Due to the highly heterogeneous distribution of the contaminants in the environment, it is difficult to statistically gauge bioremediation efficacy.
3. There are no rules to predict if a contaminant can be degraded.
4. Some contaminants such as chlorinated organic or high aromatic hydrocarbons are resistant to microbial attack. They are degraded slowly or not at all; which makes it not easy to predict the rates of clean up for a bioremediation exercise.
5. The mineralization of pollutants by cultivable bacteria has not been reported because the fraction of microbial diversity that is culturable does not contain the metabolic potential for mineralizing all the different xenobiotic pollutants present in the environment.
6. Recalcitrant and toxic xenobiotic compounds such as highly nitrated and halogenated aromatic compounds as well as some pesticides and explosives are highly stable and chemically inert under natural conditions.
7. Environmental concern on the use GEMs. It has generally not been agreed on the use of GEMs over concerns of their uncontrolled survival/dispersal into the environment.

6. FUTURE PROSPECTS

The future of bioremediation lies in the use of genetically engineered microorganisms (GEMs) (103). GEMs have shown potential for application in bioremediation of contaminated soil, groundwater, and activated sludge environments. Rate limiting steps in known metabolic

pathways could be genetically manipulated to yield increased degradation rates. More recent developments on bioremediation can be found from the literature (104–108).

NOMENCLATURE

ABTS = 3-ethylbenzthiazoline-6 sulphonic acid
Ag = Silver
Au = Gold
araB = Arabinose
BaP = Pyrene (BaP)
Bi = Bismuth
BOD = Biochemical oxygen demand
BTEX = Benzene, toluene, ethylbenzene and xylene
C23O = Catechol 2,3 dioxygenase
CBA = Benzoate
Cd = Cadmium (Cd)
Co = Cobalt
COD = Chemical oxygen demand
Cu = Copper
CYPs = Cytochrome P450 mono-oxygenase
Cys = Cystein
DHBD = Dihydroxy biphenyl dioxygenase
DNA = Deoxyribonucleic acid
EDTA = Ethylenediamine tetraacetic acid
GEMs = Engineered microorganisms
GSH = Glutathione
H_2S = Hydrogen sulphide
Hg = Mercury (Hg)
IAA = Indo-acetic acid
LAC, E.C.1.10.3.2 = Laccase
Lip, E.C.1.11.1.14 = Lignin peroxidase
merA = Mercury reductase
MnP, 1.11.1.13 = Mn-dependent peroxidase
MtL = *Myceliophthora thermophia*
MTs = Metallothioneins
N = Nickel
OMW = Olive oil mill wastewater
OP = Organophosphates
OPH = Organophosphorus hydrolase
PAHs = Polycyclic aromatic hydrocarbons
Pb = Lead
PCBs = Polychlorinated biphenyls
PCE = Tetrachloroethene = Tetrachloroethylene = PERC

PCs = Phytochelatins
PGPR = Rhizobacterial
PGR = Plant growth regulators
POPs = Persistent organic pollutants
PTE = Phosphotransferases
SRB = Sulphate-reducing bacteria
SS = Suspended solids
TCA = 1,1,1-trichloroethane
TCA = Tricarboxylic acid
TCE = Trichloroethylene
UV = Ultraviolet

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