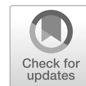
Chapter 18
Molecular Technologies for Assessment of Bioremediation and Characterization of Microbial Communities at Pollutant-Contaminated Sites



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Abstract Among the various microbial biodegradation techniques, molecular microbiology methods have revolutionized microbial biotechnology, thus leading to rapid and high-throughput methods for culture-independent assessment and exploitation of microbes present in polluted environments. Whether organic or inorganic, pollutants present in contaminated sites can cause an imbalance in the ecosystem by affecting the flora and fauna. The efficiency of naturally occurring microorganisms for field bioremediation could be significantly improved by the microbial molecular biology approach for its comparatively high efficiency and safety. Many techniques, including polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), amplified ribosomal DNA restriction analysis (ARDRA), terminal-restriction fragment length polymorphism (TRFLP), single-strand conformation polymorphism (SSCP), and ribosomal intergenic spacer analysis (RISA) can be selectively employed in microbial flora and ecology research. Recent methods such as genotypic profiling, metagenomics, ultrafast genome pyrosequencing, metatranscriptomics, metaproteomics, and metabolomics have provided exemplary knowledge about microbial communities and their role in the bioremediation of environmental pollutants.

Only 1% of the microbial diversity can be cultured by traditional techniques. Thus, the application of molecular techniques in studying microbial populations in polluted sites without the need for culturing has led to the discovery of novel and previously unrecognized microorganisms. Such complex microbial diversity and dynamics in contaminated soil offer a clear opportunity for bioremediation

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strategies. These techniques not only prove the existence of microbes in polluted sites but also reveal the undetectable complex relationships among them.

This book chapter presents an overview of the different applications of molecular methods in bioremediation of hydrocarbons and other pollutants in environmental matrices and an outline of recent advances in the applications of such techniques.

Keywords Bioremediation · Metagenomics · Metaproteomics · Metabolomics · Pyrosequencing · Biodegradation · PCR · DGGE · DNA hybridization

1 Introduction

The tremendous increases in industrialization and in the extraction of natural resources have created extreme environmental contamination and pollution. Many toxic compounds have been dispersed in numerous contaminated sites. Varied evidence shows higher risks to human health: cocktails of pollutants in nature are causing a global epidemic of cancer and other degenerative diseases. These types of pollutants are mainly classified as inorganic or organic. To make environments safe for human habitation and food consumption, developing innovative and economically low-cost solutions for decontaminating polluted environments is a challenging task. In developed countries such as the UK, USA, Canada, Australia, Japan, and European countries, much progress has been made as compared to India. In India, evaluation of the developments in laboratories is urgently needed.

New molecular techniques provide a novel opportunity to proceed with the required microbial culturing and have greatly increased the available methods for delineating bacterial diversity and functionality during the bioremediation of hazardous industrial waste. Currently, only a fraction of the potent microorganisms involved in biodegradation can be cultured by using standard laboratory agars and different pollutants from various ecosystems (Chikere 2000; Malik et al. 2008).

Comparative study between molecular/metagenomic and culture-dependent methods states that only about 1% of all microorganisms are amenable to culture (Mallik et al. 2008). In many cases, the concept of phylogenetic diversity has been key for the development of more effective microbial culture methods by the improvement of media, extended incubation period, and various growth factors such as temperature, pH, and atmospheric conditions (Rajendhran and Gunasekaran 2011). However, it is the subject of great efforts to evaluate the total microbial diversity/dynamics in various environments, a persisting challenge especially during bioremediation of industrial hazardous waste (Chikere and Ughala 2011; Chikere et al. 2011a, b). For evaluation of microbial communities, research experiments rely on new rapid methods for characterization of cellular constituents such as proteins (enzymes), nucleic acids, and specific compounds (Maila 2005; Maila et al. 2005, 2006; Montiel et al. 2009). These molecules can be extracted directly from the polluted soil and used to elucidate microbial community composition during bioremediation (Simon and Daniel 2011). This book chapter presents recent techniques

Table 18.1 Advantages and disadvantages of bioremediation

Advantages of bioremediation	Disadvantages of bioremediation
Natural process acceptable to the public as an efficient waste treatment process for contaminated sites	Limited to biodegradable compounds
Residual components are usually harmless products such as water, simpler hydrocarbons, carbon dioxide, and cell biomass	Residual components may be more toxic than the parent toxic compound
Useful for complete detoxification and biodegradation of many hazardous contaminants	These processes need specific site factors for complete success
Complete destruction of target pollutants is possible	Many techniques are difficult to perform in large-scale field operations
More economic than other physicochemical technologies used for hazardous waste cleanup	Longer treatment time than other, physicochemical options

for molecular microbiology applications in the assessment of microbial diversity and dynamics in polluted environments to identify the predominant microbial communities or derivative genes for bioremediation.

2 A General Overview of Bioremediation

Bioremediation offers one of the best ways to destroy toxics or render them harmless using natural biological activity. Bioremediation is important for two reasons:

1. Chemicals are not used: Use of natural microbes and no chemicals is the most important advantage of bioremediation, because the use of chemicals in the treatment and removal of toxics can lead to further contamination of the environment. Initially, many chemicals were used for remediation but slowly their side effects to the environment were observed, which opened the way for use of microbes in remediation.
2. Option of waste recycling: Bioremediation is also preferred in that once the waste is neutralized or removed, it can be recycled, whereas in a chemical remediation some amount of the remaining waste shows incomplete neutralization and thus cannot enter the recycling process. Some of the advantages and disadvantages of bioremediation are given in Table 18.1.

3 Overview of Environmental Pollutants and Their Toxicity

3.1 Organic Pollutants

The use of synthetic chemicals has increased tremendously because of demand, their easy manufacturing, and their applications in various industries. These chemicals

include pesticides, plastics, paints, hydrocarbon fuels, soaps, detergents, and many more substances. Each chemical has a different effect on the environment and its organisms. Dichlorodiphenyltrichloroethane (DDT) affects the endocrine system of birds, leading to eggshell thinning and reduced breeding. Other chlorohydrocarbons are also highly toxic to aquatic organisms, being persistent, soluble in fat, and having very low solubility in water; thus, they quickly accumulate in the food chain, a process described as bioaccumulation. Many countries have banned the use of these chemicals and have promoted the use of other chemicals such as organophosphates, carbamates, pyrethrins, and pyrethroids. Having relatively less solubility in water and fat, these chemicals do not bioaccumulate and thus are less toxic, having a much lower impact on the environment. However, these chemicals can be highly toxic to biota including mammals and aquatic organisms. Soaps and detergents, containing surfactants, have relatively low toxicity, persistence, and bioaccumulation. Although discharged to the environment in large quantities their effects are generally low. Plastics, on the other hand, enter the environment as solid wastes with little toxicity but have other adverse environmental effects. Proper management of pollutants through bioremediation is the demand of the present time. Some important pollutants, their sources, and their effects are highlighted in Table 18.2.

3.1.1 Hydrocarbons

Hydrocarbon contamination in the environment poses a serious problem whether it comes from petroleum products, pesticides, or other toxic organic matter. Of the hydrocarbons, petroleum products are a great concern because these are toxic to all forms of life. Environmental contamination by crude oil is relatively common because of its widespread use and the associated disposal operations and accidental spills.

3.1.2 Polychlorinated Biphenyls (PCBs)

Another group of organic pollutants, polychlorinated biphenyls (PCBs), shares a common structure but differ in the number of attached chlorine atoms. The international treaty on Persistent Organic Pollutants, drafted by 122 nations in Johannesburg in December 2000, emphasized phase-by-phase removal of targeted PCBs from the world. PCBs can be carcinogenic according to the International Agency for Research on Cancer, the U.S. Environmental Protection Agency, and the National Toxicology Program. According to the National Institute for Occupational Safety and Health, PCBs are a potential occupational carcinogen leading to increased rates of melanoma, liver cancer, gallbladder cancer, biliary tract cancer, gastrointestinal tract cancer, and brain cancer. PCBs are reported to cause various types of cancer in rats, mice, and other study animals.

Table 18.2 Pollutant sources, toxicity, and health effects

Pollutants	Toxicity	Organs affected
Hydrocarbons (Saturated alkanes, branched alkanes, alkenes, napthenes, aromatics, naptheno-aromatics, resins, asphaltenes, carboxylic acids, ethers, and others released mainly by human activities	Humans are exposed to hydrocarbon pollution directly or indirectly	Nervous system, immune system, respiratory system, circulatory system, reproductive system, sensory system, endocrine system, liver, kidney, etc.
Polychlorinated biphenyls (PCBs)	More than 90% of human exposure to PCBs is through food, mainly meat and dairy products, fish, and shellfish	Short-term exposure results in skin lesions and altered liver function Long-term exposure results in impairment of the immune system, nervous system, endocrine system, and reproductive functions
Agricultural supplements Hazardous pesticides, herbicides, synthetic insecticides	Accumulate in the food chain	Liver and kidney functions
Dyes Can be acidic, basic, disperses, azo- or anthraquinone-based and metal complex dyes	Azo dyes enter the body by ingestion; metabolized by intestinal microorganisms causing DNA damage	Skin, nervous system, liver and kidney
Arsenic Pesticides; gold, copper, nickel, iron, lead mining; coal burning; wood preservatives; pharmaceutical and glass industries; pigments; poison bait; agrochemicals; antifouling paint, electronics industry	Exposure mostly through consumption of groundwater containing high levels of inorganic arsenic, food prepared with this water, or food crops irrigated with this water	Chronic arsenic poisoning (arsenicosis); gastrointestinal tract, skin, heart, liver, and neurological damage, diabetes, bone marrow and blood diseases, cardiovascular diseases
Asbestos Mining of raw asbestos around refineries, power plants, shipyards, steel mills, vermiculite mines, building demolition	Exposure through release of microscopic asbestos fibers into the air via inhalation; can also be ingested or adsorbed on the skin	Causes parenchymal asbestosis, pleural abnormalities, lung carcinoma, and pleural mesothelioma
Cadmium Rechargeable batteries, zinc smelting, mine tailings, burning coal or garbage containing cadmium, pigments, televisions, solar panels, phosphate fertilizer, metal plating, sewage sludge	Cadmium can enter plant crops, depending on soil characteristics and pH; cadmium can enter animals at levels that are not harmful to them, but can affect humans consuming those animal products	Liver and kidney damage, low bone density

(continued)

Table 18.2 (continued)

Pollutants	Toxicity	Organs affected
Lead Mining, batteries, solder, ammunition, pigments, paint, hair colour, fishing equipment, leaded gasoline, plumbing, coal burning, water pipes	Accumulation of lead in topsoil from leaded fuel and mining activities	Nervous system, hand–eye coordination, encephalopathy, bone deterioration, hypertension, kidney disease
Mercury Thermometers, electrical switches, fluorescent light bulbs, batteries, dental fillings, mining, pesticides, medical waste, chlor-alkali industry	Humans are exposed via eating contaminated seafood; children are exposed via direct ingestion of contaminated soil	Central nervous system and gastric system, brain development, coordination, eyesight and sense of touch, liver, heart, kidney
Radionuclides Nuclear weapons program; nuclear weapons testing; nuclear power plants; uranium mining and milling; commercial fuel reprocessing; geological repository of high-level nuclear wastes; nuclear accidents	Exposed to living beings by radiation poisoning	Skin redness and hair loss, radiation burns, acute radia tion syndrome; prolonged exposure can be carcinogenic

3.1.3 Pesticides

Pesticides have been commonly used to control pests during recent decades (Timmons 1970; Chauvel et al. 2012), leading to widespread deposition of these xenobiotics into the environment (Toccalino et al. 2014). The intensive use of pesticides causes harmful effects on biodiversity, food security, and water resources (Malaj et al. 2014; Queyrel et al. 2016). Agricultural producers are using these pesticides to increase food production because the world population is expected to increase by 30%, to 9.2 billion, by 2050, with the further demand to increase food production by 70% (Popp et al. 2013). Nonpesticidal tools have been developed and will be important in the near future, but chemical pesticides are currently the best solution to pest control and food security (Popp et al. 2013; Fisher et al. 2012).

3.1.4 Dyes

Dyes are important in human lives because they impart color to our clothes, are used as food colors, and are even used in our medicines. Scientists have done much research to produce these artificial dyes: more than 10,000 dyes are available commercially and 7 lakh tons of dyes are produced annually (Zolinger 1987).

Approximately 10% to 15% of dyes are released into the environment during the dyeing process, and many of them are highly colored and aesthetically unpleasant. Dyes are difficult to remove by conventional water treatment procedures, and they persist in water because they are highly soluble. Biodegradation is highly applicable for detoxifying the toxic and carcinogenic components of dyes (Rindle and Troll 1975).

3.2 Inorganic Pollutants

Inorganic pollutants include metals, metal compounds, mineral acids, inorganic salts, trace elements, organic metal complexes, cyanides, and sulfates, which have long-term adverse effects on aquatic flora and fauna as well as on terrestrial organisms. High concentrations of heavy metals and other inorganic pollutants from various industries contaminate the water. These compounds are nonbiodegradable and persist in the environment. Metals at concentrations above the threshold value are toxic to biota; for example, copper is toxic to microbes at concentrations greater than 0.1 mg/l.

3.2.1 Radionuclides

Radioactive pollutants are toxic to all life forms because they accumulate in the bones and teeth, causing serious disorders. Radioactive isotopes such as ^{131}I, ^{32}P, ^{60}Co, ^{45}Ca, ^{35}S, and ^{14}C originate from mining and processing of ores, research labs, agriculture, medical, and industrial activities, and as radioactive discharge from nuclear power plants and nuclear reactors (^{90}Sr, ^{137}Cs, ^{248}Pu, ^{238}U, ^{235}U) and during testing of nuclear weapons. The safe concentration for lifetime consumption is 1×10^{-7} microcuries per milliliter (μCi/ml).

3.2.2 Heavy Metals

Heavy metals are categorized as environmental pollutants because of their toxic effects on plants, animals, and humans. Heavy metals belong to a very heterogeneous group of elements that vary widely in their chemical properties and biological functions. Heavy metal contamination of soil results from both anthropogenic and natural activities. Anthropogenic activities such as mining, smelting operations, and agriculture activity increase the levels of heavy metals such as Cd, Co, Cr, Pd, As, and Ni in soil above the threshold limit. Because heavy metals are persistent in nature, they are easily accumulated in soils and plants, with long-term detrimental effects on human health when ingested as a part of the diet when consuming vegetables and plant foods. The impact of heavy metals on aquatic organisms results from the movements of pollutants from various diffuse or point sources, giving rise

to coincidental mixtures in the ecosystem. Thus, these metals pose a great threat to aquatic fauna, especially to fish, which constitute one of the major sources of protein-rich food for mankind. Thus, we studied the acute and sublethal toxic effects of heavy metals in Krishna River sediment, water, and aquaculture using atomic absorption spectroscopy. The aquatic environment becomes contaminated with a variety of pollutants generated from diverse sources (industry, agriculture, domestic). Among these pollutants, pesticides, heavy metals, and detergents are the major causes of concern for the aquatic environment because of their toxicity, persistency, and tendency to accumulate in organisms. The 19 elements that constitute the heavy metal group have many similar physical and chemical properties and are remarkably different from the other 97 known elements. Among these 19 heavy metals, lead, cadmium, and mercury are extremely toxic. Other metals, such as chromium, copper, manganese, nickel, tin, and zinc, when once dispersed in the biosphere cannot be recovered or degraded and thus cause permanent environmental damage.

Metal pollution has harmful effects on biological systems and does not undergo biodegradation. Toxic heavy metals such as Pb, Co, Cd, and Hg can be differentiated from other pollutants, because they cannot be biodegraded but can be accumulated in living organisms, thus causing various diseases and disorders even at relatively lower concentrations.

4 Molecular Fingerprinting Techniques in Microbial Identification

Fingerprinting methods rely on sequence variations in the genome of different organisms. Those differences result in different melting behaviors as well as different restriction enzyme recognition sites. Different species have sites at unique positions along the whole genome. Two commonly used methods to analyze environmental samples of unknown microbial community composition are denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) and restriction fragment length polymorphism (RFLP). Both these methods, which were introduced by Muyzer et al. (1993), make use of sequence variations of polymerase chain reaction (PCR) products amplified from environmental DNA on a gradient of either increasing denaturants or temperature. Both methods rely on differences in the sequence-dependent melting behavior of double-stranded DNA. For this, the extracted nucleic acids must be amplified using primers that target specific molecular markers such as the 16S rRNA gene. For DGGE/TGGE, the use of a 50-GC clamped (30–50 nucleotides) forward primer is essential to avoid complete dissociation of the double strands. After loading the PCR product onto the gel, an electric current is applied, pulling the DNA fragments through the gel. Depending on the sequence of the amplicon, a higher or lower denaturant concentration or temperature is needed for a complete dissociation of the double strands into single strands, causing the amplicons to stop moving through the gel at different positions. To determine the

identities of bands separated on the gel, those bands can be excised from the gel and further analyzed via re-amplification, cloning, and sequencing, or by hybridization with molecular probes specific for particular taxonomic groups.

Although being valuable for the identification of changes in dominant species within a community, fingerprints generated from DGGE/TGGE can be very complex, especially when using universal bacterial primers. Those bands in which the fragments have similar melting points can be difficult to separate. Also, the rRNA operons of the same bacterium can show heterogeneity, leading to multiple bands and an overestimation of the microbial diversity; further, quantification of the extracted bands is not possible.

Compared to DGGE/TGGE, T-RFLP analysis has the advantage that fragments can be relatively quantified by the intensity of the fluorescent signal, and the method is quicker and less labor intensive. The inherent difficulty with T-RFLP, however, is that a collection or recovery of the fragments and thereby a subsequent analysis and identification of the microorganism via sequencing is not possible. To overcome this, fragments have been identified via comparison against databases of fragments produced by known gene sequences (Kent et al. 2003). Combining T-RFLP with clone library construction and sequencing, Huang et al. (2011) were able to find close associations of the four most dominant operational taxonomic units detected in T-RFLP to phylum or genus level, when analyzing spatial and temporal variations of the microbial community in a tailings basin of a Pb–Zn mine.

Different studies have used DGGE/TGGE and T-RFLP to assess microbial community composition in different contaminated environments. Spatial and temporal variations of microbial community composition were analyzed in different mining environments such as an acidic stream draining across a pyrite mine in China (Tan et al. 2009) or in a low-temperature, acidic, pyrite mine, where Kimura et al. (2011) were able to highlight the importance of bacterial species in iron transformation using T-RFLP and fluorescent in situ hybridization (FISH). Kim et al. (2009) used DGGE to examine the effects of mine tailings and waste rocks on the hydrogeochemistry and microbiology of a stream and groundwater near an abandoned copper mine. The effects of metal pollution on microbial community structure and composition in a salt marsh were analyzed by Cordova-Kreylos et al. (2006), who used T-RFLP to aid the development of bioindicators of toxicant-induced stress and bioavailability of contaminants for wetland biota. In another study, Gough and Stahl (2011) used T-RFLP to follow microbial community changes in lake sediments along a metal contamination gradient. In a recent study by Thavamani et al. (2012), the authors employed a holistic approach. To determine the soil microbial activity affected by a mix of polyaromatic hydrocarbons (PAHs) and heavy metals, they combined physicochemical, biological, and advanced molecular methods to analyze the activities of the soil microbial community in long-term mixed contaminated soils collected from a former manufactured gas plant (MGP) site. The study highlighted the difficulties of implementing remediation strategies when studying mixed contaminations, as well as the importance of combining different analysis methods.

Other studies used those fingerprinting techniques when monitoring the effects of different remediation techniques such as amending mine tailings with a mixture of organic carbon sources to treat pore water and drainage (Lindsay et al. 2011), incorporating compost into a heavy metal-contaminated acidic soil (Farrell et al. 2010), or testing the effects of phytoremediation approaches (Martinez Inigo et al. 2009) or land-farming on oil refinery sludge (Ros et al. 2010).

In laboratory-based studies, fingerprinting techniques have been used when testing the effect of different contaminants/elements on microbial communities. Jakobs-Schonwandt et al. (2010) investigated the shift of soil microbial communities when subjected to a biocide frequently found in wood preservatives. Brandt et al. (2010) compared a Cu-adapted and a corresponding nonadapted soil microbial community for the ability to resist experimental Cu pollution. Other studies investigated the abilities of indigenous bacteria on arsenic mobilization (Corsini et al. 2011) or the ability of specialized mixed communities to selectively precipitate transition metals from acidic mine waters (Nancucheo and Johnson 2011), or the acid tolerance response of a bioremediation system based on sulfate reduction (Lu et al. 2011a, b).

Fingerprinting techniques are extremely useful when starting to investigate an unknown microbial community; however, as mentioned previously, they have their limitations and can be time and labor extensive. On their own, both techniques can either quantify or identify fragments but not both.

5 Molecular Techniques for In Situ Monitoring of Microbial Communities, Bioremediation Processes, and Environmental Pollution

Traditional methods for characterizing microbial communities have been based on analysis of those bacteria that can be cultured. The overall structure of the community has been difficult to interpret as most of the bacteria are not culturable (Dokic et al. 2010). Modern methods focus towards molecular techniques, which do not require culturing the microorganisms but provide measures based on genetic diversity. The molecular-phylogenetic perspective is a reference framework within which microbial diversity is described; the sequences of genes can be used to identify organisms. A variety of approaches (Table 18.3) that have been developed to study molecular microbial diversity include DNA–DNA and mRNA–DNA hybridization, DNA re-association, DNA cloning and sequencing, and other PCR-based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and ribosomal intergenic spacer analysis (RISA). Other advanced techniques, such as DNA microarrays, have also improved specificity to a great extent.

Table 18.3 Advantages and disadvantages of molecular-based methods to study microbial diversity

Method	Advantage	Disadvantage
Mol% guanine plus cytosine (G + C)	Quantitative method, not affected by polymerase chain reaction (PCR) biases; includes all DNA extracted and also includes rare members of microbial community	Requires large quantities of DNA, highly dependent on lysing activities and extraction efficiency; level of resolution is low
Nucleic acid reassociation and hybridization	Can be studied in situ, not influenced by PCR biases; can be used to study both DNA and RNA	Sequences need to be in high copy number; lack of sensitivity; depends on lysing and extraction efficiency
qPCR and qRT-PCR	Use of gels eliminated; allows sample to be analyzed in real time	
Denaturing- and temperature-gradient gel electrophoresis (DGGE, TGGE)	Large number of samples can be analyzed simultaneously; reliable, reproducible, rapid	Only detects dominant species, PCR biases, dependent on lysing and extraction efficiency; one band can represent more than one species
Single-strand conformation polymorphism (SSCP)	Same as DGGE/TGGE; use of GC clamp and gradient eliminated	PCR biasing; some ssDNA can form more than one stable conformation
Restriction fragment length polymorphism (RFLP)	Detect structural changes in microbial community	PCR biasing, banding patterns often too complex
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP, can be automated, highly reproducible; ability to compare differences between microbial communities	Dependent on extraction and lysing efficiency; type of *Taq* can increase variability, choice of restriction enzymes will influence community fingerprint
Ribosomal intergenic spacer analysis (RISA)/amplified ribosomal DNA restriction analysis (ARDRA)	Highly reproducible community profiles	Requires large quantities of DNA (for RISA), PCR biases
DNA microarrays and DNA hybridization	Thousands of genes can be analyzed; increased specificity	Only detects the most abundant species; need to culture organisms; only accurate in low-diversity systems

5.1 *Mole Percentage Guanine + Cytosine (Mol% G + C)*

The base composition of DNA was used for taxonomic purposes as mole percentage guanine + cytosine (mol% G + C). Much diversity in base composition occurs within bacteria (25–75%) but certain microorganisms have constant GC values. Mol% G + C can be determined by thermal denaturation of DNA, and it has been reported that closely connected organisms have fairly similar GC profiles whereas

taxonomically connected groups only differ from 3% to 5% (Tiedje et al. 1999). However, similar base composition is not a confirmation of relationship, although it suggests that the difference can be evidence of a missing relationship.

5.2 Nucleic Acid Hybridization and Reassociation

Nucleic acid hybridization is an important qualitative and quantitative tool in molecular bacterial ecology (Clegg et al. 2000). Valuable spatial distribution information on microbial communities in natural environments is often provided by hybridization methods, mostly using extracted DNA or RNA, or in situ. Probes (oligonucleotide or polynucleotide) can be designed from well-known sequences, ranging in specificity from domain to species, and can be tagged with markers at the 5′-end (Goris et al. 2007). After lysing the sample to release all nucleic acids, dot blot hybridization with specific and universal oligonucleotide primers is used to quantify rRNA sequences of interest relative to total rRNA. The relative abundance may represent changes in the abundance in the population or changes in the activity and hence the amount of rRNA content (Theron and Cloete 2000). Another approch is in situ cellular level hybridization. The only limitation is the lack of sensitivity of hybridization of nucleic acids, which requires the sequences to be present in high copy number, such as those from dominant species; otherwise, the probability of detection is low.

The kinetics of DNA reassociation estimates diversity by measuring the genetic complexity of the microbial community (Torsvik et al. 1996). Total DNA is extracted from environmental samples, purified, denatured, and allowed to reanneal. The rate of reassociation depends on the similarity of sequences present, and as the complexity of DNA sequences increases, the reassociation rate decreases (Theron and Cloete 2000). Two parameters controlling the reassociation reaction are the concentration of DNA product (Co) and time of incubation (t), usually described as the half-association value, Cot1/2 (the time needed for half the DNA to reassociate). The value of Cot1/2 can be used as a diversity index in special conditions, as it takes into account both the amount and distribution of DNA reassociation (Torsvik et al. 1998). Thus, the similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics (Griffiths et al. 1999).

5.3 Quantitative PCR and RT-PCR

DNA amplification by PCR has been used in many studies to detect, characterize, and identify pollutant-degrading microbial populations. PCR detection of genes encoding for microbial monooxygenases and dioxygenases such as NAHA, PHNAc, NIDA, and NARB are useful for the detection of pollutant-degrading

microbial populations (Lu et al. 2011a, b). However, one of the most commonly used approaches for the detection and identification of microorganisms is the PCR amplification of microbial ribosomal RNA (rRNA) genes (e.g., 16S, 18S, 23S rRNA). The rRNA genes are the basis for microbial phylogenetic analyses, as several million sequences have been published in the GenBank database. During bioaugmentation treatments, rRNA of introduced microorganisms can be easily amplified by PCR and detected by gel electrophoresis. In most cases it is necessary to analyze the rRNA amplification products by additional techniques, such as terminal-restriction fragment length polymorphism (T-RFLP), or fully sequencing the amplified product, to increase the specificity of detection and identification. On the opposite end, quantitative PCR (qPCR) or real-time PCR (RT-PCR) has been also used to quantify microorganisms after introduction to different environmental matrices (Kikuchi et al. 2002). One of the most specific and popular ways to perform qPCR is with the use of Taqman probes. In this technology, Taq polymerase cleaves a fluorogenic Taqman probe that binds to an internal site within the sequence being amplified during the extension step, which releases a fluorescent molecule (fluorophore), resulting in fluorescence. The cycle threshold value (Ct) is determined at the point where a significant increase in the fluorescence emission occurs, as compared with a background baseline. A larger initial concentration of target DNA results in a lower Ct value. qPCR eliminates the use of gels and allows the sample to be analyzed in real time, in less time than conventional PCR. qPCR has been used in bioremediation studies to calculate the copy number of the benzyl succinate synthase gene (BSSA) and naphthalene dioxygenase (NAHAc) in hydrocarbon-contaminated soils, bioaugmentated with degrading microbial consortia (da Silva and Alvarez 2004; Nyyssonen et al. 2006). Cebron et al. (2008) reported the use of qPCR to detect and quantify PAH-ring hydroxylating dioxygenases (PAH-RHDα) in soil and sediment samples contaminated with PAHs. The results of these studies highlighted a positive correlation among the PAH-degrading gene copy levels and microbial biodegradation potential, as well as the contamination levels in the studied soils.

5.4 DGGE and TGGE

Molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism analysis (T-RFLP), and temperature gradient gel electrophoresis (TGGE) are the most popular today for analysis of microbial communities in environmental issues. In these methods, PCR-amplified fragments are separated by their differing mobility in a capillary or on a gel. The generated patterns based on each band or peak reflect the diversity of the microbial community. DNA fragments of each band can be separated and sequenced to give phylogenetic information for microbial strains. In nucleic acid hybridization techniques, short oligonucleotide probes specific to target microorganisms are used. The applied probe contacts the specific extracted nucleic acids.

The dot blotting technique frequently has been used in metabolic study of a microbial community by investigation of the changes in the gene expression level.

By the use of DGGE/TGGE, complex communities of bacteria and fungi can be analyzed after PCR amplification with the use of primers designed to specifically bind to conserved regions of bacterial 16S rRNA genes or fungal 18S rRNA. By the usage of group-particular primers, it is likewise possible to investigate bacterial communities, together with actinomycetes or Archaea. Although the 16S and 18S rRNA genes are most often used, other genes along with the β-subunit of bacterial RNA polymerase (rpoB) also can be used to analyze the microbial range and survival of inoculated microorganisms in soil (Dahllof et al. 2000). DGGE has been proven to be useful in monitoring the bioremediation of freshly and aged PAH-infected soils (Cunliffe and Kertesz 2006), allowing monitoring of the survival of inoculated *Sphingobium yanoikuyae* in addition to tracking adjustments within the local bacterial communities over time. In some studies (Zhou et al. 2009; Wang and Tam 2011; HuiJie et al. 2011), DGGE has also been used to observe microbial community dynamics and biodegradation throughout PAH biodegradation in soils and sediments.

DGGE/TGGE is a preferred method although the desired information is no longer as phylogenetically exhaustive as that furnished by 16S rRNA gene clone libraries; however, these methods can determine the dominant participants of microbial communities with average phylogenetic definition (Sanz and Kochling 2007). For environmental or contaminated supply samples wherein microbial diversity is basically unknown, the DGGE/TGGE method may provide identification of the microbial population through the excision of selected bands followed through their reamplification, cloning, and sequencing, which can indicate the phylogenetic affiliation of the ribotypes (Evans et al. 2004; Forney et al. 2004; Van Elsas et al. 2007). DGGE specifically has been widely used for the evaluation of microbial community shape in infected soil and water (Chang et al. 2000; Ralebitso et al. 2000; Watanabe et al. 2000; Kleikemper et al. 2002; Cummings et al. 2003; El-Latif Hesham et al. 2006; Mahmoud et al. 2009). Apart from microbial network profiling, the DGGE technique has been used to study gene clusters such as dissimilar sulfite reductase β-subunit (dsrB) genes in sulfate-decreasing bacterial communities (Geets et al. 2006) and benzene, toluene, ethylbenzene, and xylene (BTEX) monooxygenase genes from bacterial traces obtained from hydrocarbon-polluted aquifers (Hendrickx et al. 2006). Coulon et al. (2012) used DGGE evaluation of opposite-transcribed bacterial 16S rRNA from the upper 1.5 cm of a hydrocarbon-polluted sediment in coastal mudflats to examine the essential function of dynamic tidal biofilms by using aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons. Their research found phylotypes related to straight chain and polycyclic hydrocarbon degradation including *Cycloclasticus*, *Alcanivorax*, *Oleibacter*, and *Oceanospirillales* strain ME113.

5.5 Single-Strand Conformation Polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP), which is also dependent on electrophoretic separation based on differences in DNA sequences, allows differentiation of DNA molecules having the same length but different nucleotide sequences. This technique was originally developed to detect known or novel polymorphisms or point mutations in DNA (Peters et al. 2000). In this method, single-stranded DNA separation on polyacrylamide gel was based on differences in mobility resulting from their folded secondary structure (heteroduplex). As formation of folded secondary structure or heteroduplex and hence mobility is dependent on the DNA sequences, this method reproduces genetic diversity in a microbial community. All the limitations of DGGE are equally applicable for SSCP. Again, some single-stranded DNA can exist in more than one stable conformation, so that the same DNA sequence can produce multiple bands on the gel (Tiedje et al. 1999). However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity (Stach et al. 2001). SSCP has been used to measure the succession of bacterial communities (Peters et al. 2000), rhizosphere communities (Schmalenberger et al. 2001), bacterial population changes in an anaerobic bioreactor (Zumstein et al. 2000), and arbuscular mycorrhizal fungi (AMF) species in roots (Kjoller and Rosendahl 2000).

5.6 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP), another method for reading microbial range, depends on DNA polymorphisms. During previous years RFLP packages have also been implemented to estimate diversity and network structure in extraordinary microbial groups (Moyer et al. 1996). In this approach, electrophoresed digests are blotted from agarose gels onto nitrocellulose or nylon membranes and hybridized with suitable probes prepared from cloned DNA segments of associated organisms. RFLP can be very useful, especially in a mixture with DNA–DNA hybridization and enzyme electrophoresis, for the differentiation of carefully associated lines (Palleroni 1993); the technique also appears to be useful for detection of intraspecies variants (Kauppinen et al. 1994). RFLPs can also provide a easy and effective device for the identity of bacterial lines at and below species level. This method is helpful for detecting structural modifications in microbial groups, although not for degree of diversity or detection of precise phylogenetic agencies (Liu et al. 1997). Banding patterns in various communities are too complicated to analyze by RFLP because a single species may have four to six limit fragments (Tiedje et al. 1999). However, one must be aware that a similar banding pattern does not necessarily indicate a very close connection among the organisms compared.

5.7 *Terminal Restriction Fragment Length Polymorphism (T-RFLP)*

Terminal restriction fragment duration polymorphism (T-RFLP) overcomes a number of the limitations of RFLP (Thies 2007). T-RFLP is an extension of RFLP analysis that offers an alternative approach for rapid analysis of microbial network range in diverse environments. It follows the same precept as RFLP except that one PCR primer is categorized with a fluorescent dye, together with TET (4,7,2′,7-′-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). PCR carried out on sample DNA uses regularly occurring l6S rDNA primers, one of which is fluorescently categorized. Fluorescently labeled terminal restriction fragment duration polymorphism (FLT-RFLP) patterns can then be created by digestion of labeled amplicons using restriction enzymes. Fragments are then separated by gel electrophoresis on an automated collection analyzer. Each precise fragment length can be counted as an operational taxonomic unit (OTU), and the frequency of each OTU may be calculated. The banding sample may be used to measure species richness and evenness in addition to similarities between samples (Liu et al. 1997). T-RFLP, as every totally PCR-based approach, may underestimate authentic range because numerically dominant species are detected by the massive amount of template DNA (Liu et al. 1997). Incomplete digestion by means of restriction enzymes can also cause an overestimation of diversity (Osborn et al. 2000). Despite these obstacles, some researchers believe that when standardized, T-RFLP can be a useful tool to observe microbial variety in the environment (Tiedje et al. 1999), although others consider it to be inadequate (Dunbar et al. 2000).

T-RFLP has been used to measure spatial and temporal modifications in bacterial groups (Lukow et al. 2000), to study complex bacterial groups (Moeseneder et al. 1999), to screen populations (Tiedje et al. 1999), and to evaluate the diversity of arbuscular mycorrhizal fungi (AMF) within the rhizosphere of *Viola calaminaria* in a metal-contaminated soil (Tonin et al. 2001). Tiedje et al. (1999) reported five instances of greater success at detecting and tracking specific ribotypes by using T-RFLP rather than DGGE.

Dojka et al. (1999) monitored microbial variety in a hydrocarbon- and chlorinated solvent-infected aquifer undergoing intrinsic bioremediation with T-RFLP, determining sequence types characteristic of *Syntrophus* spp. and *Methanosaeta* spp. They hypothesized from their findings that the terminal step of hydrocarbon degradation inside the methanogenic zone of the aquifer became aceticlastic methanogenesis, with these organisms existing in a syntrophic relationship. Bordenave et al. (2004) studied bacterial network modifications in microbial mats following crude oil pollution using T-RFLP. Their results indicated a clean succession of various bacterial populations with operational taxonomic units that were associated with the genera *Chloroflexus, Burkholderia, Desulfovibrio*, and *Cytophaga*. T-RFLP has also been used to characterize microbial groups recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer (Reardon et al. 2004) and dechlorinating microorganisms from a basalt aquifer

(Macbeth et al. 2004; Fahy et al. 2005). The use of T-RFLP showed that the chronic presence of benzene in groundwater reduced bacterial range and network composition in comparison with that of available groundwater resources. The use of automatic detection systems and capillary electrophoresis in T-RFLP analysis permits high throughput and greater correct quantitative analysis of microbial community samples than with any of the opposite genetic fingerprinting strategies.

5.8 Ribosomal Intergenic Spacer Analysis (RISA) and Amplified Ribosomal DNA Restriction Analysis (ARDRA)

RISA makes use of the length and sequence heterogeneities present within the intergenic spacer (IGS) among the small (SSU) and large subunit (LSU) rRNA genes inside the rRNA operon (Van Elsas et al. 2007). Similar to RFLP and T-RFLP, RISA and ARDRA provide ribosomal-based fingerprinting of the microbial network. In RISA the IGS location between the 16S and 23S ribosomal subunits is amplified through PCR, denatured, and separated on a polyacrylamide gel under denaturing conditions. This location may encode tRNAs and can differentiate between bacterial lines and closely related species by the heterogeneity of the IGS duration and series (Fisher and Triplett 1999). Sequence polymorphisms are detected through silver staining in RISA. RISA has been used to evaluate microbial range in soil (Borneman and Triplett 1997), inside the rhizosphere of plant life (Borneman and Triplett 1997), in contaminated soil (Ranjard et al. 2000), and in response to inoculation (Yu and Mohn 2001). RISA is a very rapid and easy fingerprinting approach but its application in microbial community evaluation from contaminated assets is restricted, partly because the database for ribosomal intergenic spacer sequences is not as large or as complete as the 16S collection database (Spiegelman et al. 2005). Limitations of RISA include requirement of massive portions of DNA, the longer time requirement, insensitivity of silver staining in some instances, and acrylamide choice (Fisher and Triplett 1999). Banding patterns in ARDRA may be used to display clones or the degree of bacterial network shape (Kirk et al. 2004). ARDRA is easy, rapid, and cost-efficient and thus has been utilized in microbial identification (Vaneechoutte et al. 1995; Kita-Tsukamoto et al. 2006; Krizova et al. 2006) and microbial network research (Weidner et al. 1996; Bai et al. 2006; Babalola et al. 2009). Microbial network composition and succession in an aquifer exposed to phenol, toluene, and chlorinated aliphatic hydrocarbons have been assessed by means of ARDRA to identify the dominant microbial community involved in the biodegradation of trichloroethylene (TCE) following biostimulation (Fries et al. 1997). In another study, (Gich et al. 2000) used ARDRA to examine the microbial differences in activated sludge from remedial plants consumed at home or from industrial wastewater. Bacterial groups in activated sludge could be distinguished between commercial and domestic wastewater treatment plant life. Hohnstock-Ashe

et al. (2001), using ARDRA as a fingerprinting method, also discovered that microbial community composition in waters contaminated with TCE had shifted towards a fairly diverse community dominated by *Dehalococcoides ethenogenes*-like microorganisms. Babalola et al. (2009) used ARDRA to study the phylogenetic relationships of actinobacterial populations associated with Antarctic valley mineral soils. Further sequencing of the amplicons restricted singly with endonucleases *Rsa*I, *Bsu*RI, or *Alu*I determined that the phylotypes were most closely related to uncultured *Pseudonocardia* and *Nocardioides* spp. In contrast, complementary traditionally established research had isolated more species of *Streptomyces* that were detected at a low frequency in metagenomic analysis. ARDRA is useful in detecting structural adjustments in microbial groups but cannot display microbial variety or detect unique phylogenetic groups within a network fingerprinting profile (Liu et al. 1997). Optimization with restriction enzymes is required, which frequently is difficult if sequences are unknown. Thus, further optimization can be required to produce fingerprinting styles characteristic of the microbial community (Vaneechoutte et al. 1995; Spiegelman et al. 2005). In addition, banding styles in diverse groups appear to be too complicated to use ARDRA (Kirk et al. 2004). In recent research, ARDRA has been blended with different molecular techniques including T-RFLP and DGGE to signify microbial groups from contaminated sources (Watts et al. 2001; Haack et al. 2004). An important assignment in the usage of ARDRA lies within the interpretation of the fingerprints obtained from complicated microbial communities.

5.9 Fluorescent In Situ Hybridization (FISH)

A critical step towards determining the variety of microbes in environmental samples is to harness the statistics received from the direct sequencing of rRNA genes extracted from such samples. The full-cycle rRNA method essentially makes use of the series statistics of cloned, rRNA-encoding genes from environmental habitats to broaden phylogenetic oligonucleotide probes that permit specific hybridization to the goal region of the ribosomal RNA in constant permeabilized cells. Referred to as fluorescent in situ hybridization (FISH) (Van Elsas et al. 2007), this is a speedy and sensitive approach that allows the direct visualization and identity of environmental microorganisms without culturing (Bakermans and Madsen 2002). Microbial cells are penetrated with fixatives, hybridized with precise probes (generally 15- to 25-bp oligonucleotide fluorescently labelled probes) on a tumbler slide, then visualized with epifluorescence or confocal laser microscopy (Malik et al. 2008). Hybridization with rRNA-targeted probes enhances the characterization of uncultured microorganisms and also enables the description of complicated microbial communities (Edgcomb et al. 1999). FISH is a taxonomic approach used more often than not to determine whether participants of a particular phylogenetic affiliation are present; it offers direct visualization of uncultured microorganisms and also facilitates the quantification of precise microbial organizations (Sanz and Kochling 2007). FISH

use alone does not provide any perception of the metabolic function of microorganisms. However, it may be combined with different techniques such as micro-autoradiography to describe the practical properties of microorganisms in their natural surroundings (Wagner et al. 2006). Two kinds of FISH probes based on conserved or precise areas of 16S rRNA genes may be advanced: domain- or organization-specific probes and stress-unique probes. Domain- or institution-specific probes discriminate or indicate participants of larger phylogenetic assemblages, even as stress-particular probes quantify or investigate the abundance of a specific species or presence inside a microbial network (Dubey et al. 2006). Richardson et al. (2002) blended institution-unique FISH and T-RFLP in the characterization of microbial groups involved in TCE biodegradation. From the FISH analysis, the authors found that a wide variety of organisms consisting of *Cytophaga*, *Flavobacterium*, and *Bacteroides* had greater abundance than the TCE degrader *Dehalococcoides ethenogenes* in the microbial consortium. However, the lack of useful gene evaluation meant that the relative abundance of these organisms and their ecological importance for TCE biodegradation could not be established. FISH has been used to analyze microbial community composition in PAH-infected soils, in particular detecting the 16S and 23S rDNA genes (Van Herwijnen et al. 2006; Hesham et al. 2012; Chang et al. 2014). However, unique care must be taken using FISH on soil or sediment samples.

FISH techniques are frequently used with different genetic fingerprinting strategies including DGGE (Onda et al. 2002; Collins et al. 2006) and T-RFLP (Richardson et al. 2002; Kotsyurbenko et al. 2004; Jardillier et al. 2005; Collins et al. 2006) for the enumeration and characterization of microbial populations from contaminated resources. The downside of FISH is that a confined variety of probes may be utilized in a single hybridization experiment and the fluorescence history thus can be complex in some samples (Dubey et al. 2006; Sanz and Kochling 2007). An earlier examination of the sample and the maximum microorganisms that may be detected is important (i.e., rRNA sequence) for the selection of specific probes. A predominant obstacle of the same FISH technique is its restricted sensitivity because bacterial cells with decreased ribosome content that frequently appear in oligotrophic environments, such as maximum soil habitats, are not satisfactorily stained for microscopic analyses. Modifications of FISH, such as the catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) and fluorescence in situ hybridization micro-autoradiography (FISH-MAR) are being used as alternatives to circumvent these issues and measure the potential for PAH degradation in soils (Teira et al. 2007; Lekunberri et al. 2010).

In CARD-FISH, signal intensities of hybridized cells are multiplied by enzymatic signal amplification using horseradish peroxidase (HRP)-categorized oligonucleotide probes in combination with tyramide signal amplification (TSA). TSA is based on the patented catalyzed reporter deposition (CARD) method using derivatized tyramide. In the presence of small quantities of hydrogen peroxide, immobilized HRP converts the categorized substrate (tyramide) into a short-lived, extremely reactive intermediate. The activated substrate molecules then very swiftly react with, and covalently bind to, electron-rich areas of adjacent proteins. This binding

of the activated tyramide molecules occurs best without delay adjacent to the websites at which the activating HRP enzyme is certain. Multiple deposition of the categorized tyramide takes place in a brief time (normally less than 3–10 min). Subsequent detection of the label yields a successfully massive amplification of sign (Van Elsas et al. 2007). The mixture of FISH with micro-autoradiography allows the identification of bacteria and concomitantly illustrates their particular in situ interest for the usage of appropriate isotope-categorized substrates (in particular, β-emitters such as ^{14}C and ^{3}H) (Van Elsas et al. 2007). The substrate uptake patterns in FISH-classified bacteria can be investigated in situ in combined natural groups at an unmerged cellular stage although the bacteria are not yet culturable.

5.10 DNA Microarray and Reverse Sample Genome Probing (RSGP)

DNA–DNA hybridization has been used collectively with DNA microarrays to assess bacterial species (Cho and Tiedje 2001) or to evaluate microbial range (Greene and Voordouw 2003; DeSantis et al. 2007) with great specificity. This is a chip technology containing nucleic acids as probes that are ideal for the high-throughput analysis of the collection range of 16S rRNA genes as well as of other useful genes in environmental samples (Van Elsas et al. 2007; Malik et al. 2008). In contrast to FISH, it offers a method for simultaneous evaluation of many genes (Cho and Tiedje 2002). The DNA microarray is a miniaturized array of complementary DNA probes (~500–5000 nucleotides in length) or oligonucleotides (15–70 bp) connected to a strong support, which allows simultaneous hybridization against a very large set of probes complementary to their corresponding DNA/RNA targets in a pattern.

The application of microarrays in environmental microbiology, especially in the examination of microbial populations engaged in biodegradation, can identify organisms in addition to defining their ecological position (Wu et al. 2001). However, extra-rigorous and systematic evaluation and improvement are needed to comprehend the overall ability of microarrays for microbial detection and community analysis (Zhou 2003). Microarrays detect the simplest dominant populations in many environments (Rhee et al. 2004). In addition, probes designed to be precise to acknowledge sequences can move or hybridize to similar or unknown sequences and might produce deceptive alerts (Gentry et al. 2006). Moreover, soil, water, and sediments regularly contain humic acids and different organic substances that may also inhibit DNA hybridization on microarrays (Saleh-Lakha et al. 2005). Finally, limits in RNA extraction from many environmental samples imply that advances in RNA extraction and purification and amplification techniques are needed to make microarray gene expression evaluation possible for a broader variety of samples (Gentry et al. 2006).

Another DNA microarray-based approach for reading a microbial community is reverse sample genome probing (RSGP). This technique uses genome microarrays to research the microbial network composition of the most dominant culturable species in an environment. RSGP has three steps: isolation of genomic DNA from pure cultures, hybridization checking to attain DNA fragments with much less than 70% cross-hybridization and preparation of genome arrays on a strong support, and random labelling of a defined mixture of general network DNA and general inner DNA (Greene and Voordouw 2003). This approach has been used to investigate microbial groups in oil fields and in polluted soils (Greene et al. 2000). As in DNA–DNA hybridization, RSGP and microarrays have the advantage of not being confounded via PCR biases. Microarrays can incorporate many target gene sequences, but are handiest in detecting the maximum plentiful species. In this fashion, species need to be cultured; however, in precept cloned DNA fragments of uncultured bacteria may also be used. The variety must be minimum, or enriched cultures will be needed for this approach. Otherwise, pass-hybridization can be intricate. Using genes or DNA fragments rather than genomes in the microarray removes the need to maintain cultures of live organisms, because genes can be cloned into plasmids or PCR can be consistently used to amplify the DNA fragments (Gentry et al. 2006). In addition, fragments would increase the specificity of hybridization over using genomes, and useful genes within the community might be assessed (Greene and Voordouw 2003).

6 Omics in Bioremediation

Various ‘omics’ strategies have opened the way for environmental probing at the molecular level and have also created a new paradigm in bioremediation design and control. Ecogenomics – the utility of genomics to ecological and environmental sciences – defines phylogenetic and functional biodiversity on the DNA, RNA, and protein levels, with emphasis on the capabilities and interactions of organisms in the surroundings relative to ecological and evolutionary methods. For effective bioremediation of halo-organic pollution in anaerobic environments, we need the information from catabolic ability and in situ dynamics of organohalide-consuming and cometabolizing microbes. Current methods of the OMICS pathways are outlined in Fig. 18.1.

6.1 Genomics

Genomics is a powerful computer-based technology used to understand the structure and feature of all genes in an organism. Study of pure cultures have become very easy using the software of genomics to bioremediation. Next-era genome sequencing strategies will be critical in elaborating the physiological and genomic features of

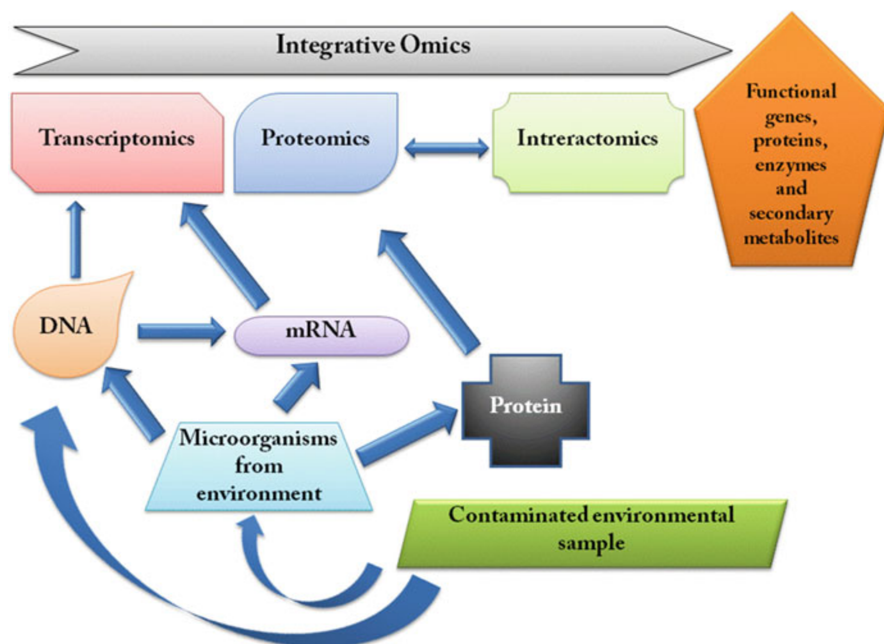

Fig. 18.1 Bioremediation monitoring and "OMICS"

microorganisms applicable to bioremediation. Complete genome sequences should be known for such microbes, which can be important in bioremediation (Table 18.4).

The views of researchers changed after observing the utility of bioremediation to the advanced sciences such as genomics, which have given exclusive results to many questions. For instance, molecular analyses have indicated that *Geobacter* species are crucial for the bioremediation of organic and metal contaminants in subsurface environments. This realization led to the sequencing of several species of the genus *Geobacter*, in addition to other related organisms, which has altered the concept of how *Geobacter* species help in reducing the contaminants in subsurface environments. Earlier it was thought that *Geobacter* species were nonmotile, but genes encoding flagella were subsequently determined in the *Geobacter* genomes (Childers et al. 2002). Later it was found that *Geobacter metallireducens* in particular produces flagella only when the microbe is growing on insoluble ferrous and manganese oxides. Pili genes were also found to be expressed during culture on insoluble oxides (Childers et al. 2002).

Similarly, other microbes with bioremediation capability have been screened for complete genome sequencing. With the completed genome sequences, it is better to design whole-genome DNA microarrays to analyse the expression of all the genes under various environmental situations. When bioremediation approaches are researched in detail, trials are commonly made to isolate the concerned microorganisms (Rogers and McClure 2003). The isolation and characterization of such

Table 18.4 Genomes of microorganisms pertinent to bioremediation

Microorganism	Relevance to bioremediation	Website for genome documentation
Dehalococcoides ethanogenes	Dechlorination of solvents to ethylene in reduced conditions	http://www.tigr.org
Geobacter sulfurreducens	Oxidation of aromatic hydrocarbons anaerobically and precipitation of uranium in reduced conditions	http://www.jgi.doe.gov
Geobacter metallireducens		http://www.tigr.org
Rhodopseudomonas palustris	Anaerobic metabolism of aromatic compounds	http://www.jgi.doe.gov
Pseudomonas putida	Aerobic degradation of organic contaminants and capabilities for genetic engineering	http://www.tigr.org
Dechloromonas aromatica	Perchlorate-reducing microbes capable of anaerobic oxidation of benzene	http://www.jgi.doe.gov
Desulfitobacterium hafniense	Dechlorination of chlorinated solvents and phenols under reductive conditions	http://www.jgi.doe.gov
Desulfovibrio vulgaris	Reductive precipitatation of uranium and chromium	http://www.tigr.org
Shewanella oneidensis	Reduction of uranium in vitro	http://www.tigr.org
Deinococcus radiodurans	Resistant to radiation and thus can be used in genetic engineering for bioremediation of highly radioactive environments	http://www.tigr.org

microbes has been and will remain critical for the development and interpretation of molecular analyses in microbial ecology. However, before the utility of molecular techniques to bioremediation, it was not sure whether the isolated microbes had been critical in bioremediation under natural conditions, or whether they grew as "weeds" unexpectedly in the laboratory, but were now not the expected organisms responsible for bioremediation.

6.2 Transcriptomics

The transcriptome is the subset of genes transcribed in any given organism, a dynamic link among genome, proteome, and cellular phenotype. The control of gene expression is the key in modifying changes in environmental situations and hence for survival. Transcriptomics describes this expression procedure in a genome-extensive manner. DNA microarrays allow detection of RNA expression of each gene of an organism (Diaz 2004; Golyshin et al. 2003; Gao et al. 2004; Muffler et al. 2002; Schut et al. 2003). One important challenge in this method is elucidation of the huge amounts of data recorded (Dharmadi and Gonzalez 2004). A global gene expression pattern analysis revealed the numerous to-date-unknown

genes at some stage in the degradation of alkyl benzenes (Kuhner et al. 2005). Besides this, DNA microarrays were used to decide bacterial species, in quantitative programs of stress gene analysis of microbial genomes and in genome-wide entranscriptional profiles (Greene and Voordouw 2003; Muffler et al. 2002).

6.3 Proteomics

Proteomics is the study of all the proteins in an organism. Among many functions these proteins serve as catalytic enzymes in metabolic pathways and in signal transduction of regulatory pathways of cells (Graham et al. 2007; Zhang et al. 2010). Environmental conditions can lead to differences in their cellular expression in an organism, and the toxicity present in the environment acts as a trigger in adjusting to the physiological response. Proteomics has made it possible to acquire an in-depth overview of global modifications and the abundance of proteins, apart from identification of major proteins involved in bioremediation at a given physiological state (Vasseur et al. 1999; Wilkins et al. 2001). Various reports have suggested that proteins are up- or downregulated in response to the contamination (Vasseur et al. 1999; Wilkins et al. 2001; Kim et al. 2002). The downregulated proteins were found to be a part of nucleotide biosynthesis and cellular motility (Santos et al. 2004). A proteomic evaluation also revealed the participation of energy- and stress-related proteins when *Pseudomonas putida* DOTT1E was exposed to toluene (Segura et al. 2005). Such detailed facts are important for the development of bacteria with greater solvent or contaminant tolerance that can be used for bioremediation.

7 Metabolomics

Metabolomics is a young and vibrant discipline of technological advancement in its exponential increase. Metabolome evaluation has become very popular currently, and novel strategies for obtaining and reading metabolomics statistics that are useful for different biological studies are increasing. Bioremediation is one such field which gains from the advances in this emerging field. Various bioremediation research options such as finding strategies for elucidation of biodegradation pathways, using isotope distribution analysis and molecular connectivity analysis, assessing the mineralization system using metabolic footprinting evaluation, and improving the biodegradation system through metabolic engineering can be analysed in a more comprehensive manner using metabolomics.

7.1 Metabolomics, Metametabolomics, and Fluxomics

Metabolomics is analysis of all the cellular metabolites in a cell and their interactions in the microbial community. Analysis of cellular metabolites within a cell and community over a time period in real time is known as fluxomics (Wiechert et al. 2007). Information on factors regulating growth and metabolism of microbial communities can be accessed by metabolomics, and fluxomics can provide the missing links in the regulatory pathways involved in metabolism of environmental pollutants. A microbial cell releases a number of low molecular weight primary and secondary metabolites in response to an environment challenge or stress. The influence of the local environment on the metabolome of an organism or community can be exploited in bioremediation to characterize the effects of xenobiotic compounds. Metabolites can be characterized by mass spectrometry and various spectroscopic techniques. Villas-Boas and Bruheim (2007) have discussed the scenario of metabolome analysis in bioremediation. The application of metabolomics can significantly extend and enhance the power of existing bioremediation approaches by providing a better understanding of the biodegradation process (Villas-Boas and Bruheim 2007). Keum et al. (2008) studied the metabolic profiles of *Sinorhizobium* sp. C4 using mass spectrometry and gas chromatography during the degradation of phenanthrene in comparison to natural carbon sources. Tang et al. (2009) also performed a fluxomics analysis on *Shewanella* sp. known to have co-metabolic pathways for bioremediation of halogenated organic compounds, toxic metals, and radionuclides. In addition, they analyzed that a mixed bacterial consortium can degrade benzene and its derivatives and other aromatic ring organic members more than 97%. Maphosa et al. (2010) studied organ halide-catabolizing bacteria and its molecular diagnostics with mass-balancing and kinetic modeling in an in situ dechlorinating bioreactor and showed its application in monitoring bioremediation.

7.2 Improvement of the Biodegradation Process via Metabolic Engineering

Recombinant DNA technology can drastically improve metabolic engineering (ME) and thus improve cellular properties or introduce new ones (Stephanopoulas et al. 1998). The metabolic engineering (ME) approach has been successfully used for the improvement of industrial microorganisms. However, the use of ME tools and principles is also relevant for bioremediation, as potential natural microbial populations to degrade contaminations are found in fewer numbers (Urgun-Demitras et al. 2006; Pieper and Reineke 2000; Rui et al. 2004). Accumulation of contaminants in the environment represents a potential pollution problem because many such are highly toxic, mutagenic, or carcinogenic. Metabolic engineering can lead to both the introduction of novel biodegradation pathways and to modification and extension of a substrate range of enzymes in existing degradation pathways in the

development of microorganisms with improved bioremediation properties (Dua et al. 2002). A psychrotolerant nonpathogenic *Pseudomonas fluorescens* strain that was genetically engineered for degradation of 2,4-dinitrotoluene was studied by transferring the *Burkholderia* sp. strain DNT pJS1 mega plasmid containing the dnt genes (Monti et al. 2005). Thus, a stable 2,4-dinitrotoluene-degrading phenotype was first achieved by integrating the dnt genes into the *P. fluorescens* chromosome.

Haro and Lorenzo (2001) combined two catabolic segments from the toluene (TOL) and toluene dioxygenase (TOD) pathways of *Pseudomonas putida* to create an upper hybrid pathway for bioconversion of 2-chlorotoluene into 2-chlorobenzoate. This pathway was integrated into the pathway of two 2-chlorobenzoate-degrading *Pseudomonas* strains, expecting to provide complete degradation of 2-chlorotoluene. The two strains were able to co-metabolize 2-chlorotoluene to 2-chlorobenzoate with citrate as co-substrate but failed to grow on 2-chlorotoluene alone. Analysis of the cultures with gas chromatography–mass spectroscopy (GC-MS) showed that citrate-grown cells accumulated 2-chlorobenzoate and other intermediates in the upper pathway during exposure of 2-chlorotoluene. However, no reason was found for the lack of degradation of 2-chlorobenzoate. This study clearly emphasizes that an unbiased and nontarget analysis of the performance and physiology of the biological model system via metabolomics is necessary.

Metabolomics and other genome-wide methodologies as transcriptomics, proteomics, interactomics, and fluxomics have became important tools for describing how a phenotype is generated from its genotype and the environmental conditions. These technologies became important for both metabolic engineering and system biology because the focus is on integrated networks of metabolic pathways and not on individual isolated reactions (Friboulet and Thomas 2005). The strategy of inverse metabolic engineering is through comparisons between mutated strains and reference strains to gain insight into the metabolism (Bro and Nielsen 2004). In bioremediation, this approach is also relevant (e.g., comparison of reference strains with recombinant strains modified with a new degradation pathway). Also important is the exploration of the response of the microorganisms to pollutant exposure. A metabolome analysis can supply information regarding which pathways are activated under expression of the new heterologous pathway and under pollutant-exposure conditions. The strength of metabolome analysis is that it points out the pathways that need to be targeted by metabolic engineering (Trethewey 2001). Because the other OMICS-related approaches provide invaluable complementary information, the future of metabolic engineering is to integrate the different sets of data to develop optimal strains for use in both bioprocessing and bioremediation (Park et al. 2005). Lee et al. (2006) used a quantitative proteomics approach to provide important insights into the metabolic and physiological changes that occur upon *cis*-dichloroethylene degradation by engineered *Escherichia coli* strains. Because the number of identified central proteins in the degradation pathways was less than the number of genes, a transcriptome analysis may complement the proteomics approach as would the metabolomics and fluxomics analyses on the physiological characterization.

8 Bioinformatics

Bioinformatics is an important part of modern biotechnology that combines biology and information technology. Bioinformatics seek information and keep all the biological data, helping to investigate and decide relationships among organic molecules, such as macromolecular sequences, structures, biochemical pathways, and expression profiles. Biology and computer systems are running parallel, which might be collectively respecting, supporting, and influencing each other and synergistically merging as greater than ever (Fulekar 2008). Enormous amounts of data and statistics from biology, especially within the form of DNA, RNA, and protein sequences, are slowly accumulating, thus placing heavy demand on computers and scientists. Bioinformatics has also led to finding possible cures for detoxification of the environment. Scientists expert in analyzing biological data can use the computational tools to solve the problems of bioremediation (Westhead et al. 2003). The important branches of bioinformatics are genomics, transcriptomics, proteomics, organic databases, molecular phylogenetics, and microarray informatics, which are crucial in understanding bioinformatics. These bioinformatics-associated tools are very imporant for bioremediation of hazardous wastes.

8.1 Bioinformatics in Bioremediation-MetaRouter

MetaRouter is an application for laboratories working in bioremediation that need to maintain public and private data, linked internally and with external databases, and to extract new information from it. The system has a very versatile open and modular architecture adaptable to different customers. It is a multiplatform program, implemented in Postgre SQL (standard language for relational databases) and using SRS as an indexing system (used to connect and query Molecular Biology databases). MetaRouter uses a client/server architecture that allows the program to run on the user station or on the company server, so it can be accessed from any place in a secure way just by having a web browser.

Bioinformatics will facilitate and quicken the analysis of cellular processes and also the investigation of the cellular mechanisms used to treat wastes. Coming decades will yield greater understanding of molecular mechanisms and cellular metabolism coupled with bioinformatics.

8.2 Bioinformatics Resources

Many bioinformatic resources are available today exclusively for bioremediation studies and analyses. In 1955 University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD, http://umbbd.msi.umn.edu/) was developed and has been

regularly updated since its inception. It has a collection of databases related to organic compounds, enzymes, reactions, biotransformation rules, and different microorganisms. Further, it contains a biochemical periodic table (UM-BPT) and a pathway prediction system (UM-PPS) that predicts hypothetical pathways for microbial degradation. Other scientists are accessing UM-BBD data for their research purposes. Also, UM-BBD compound data are now contributed to PubChem and ChemSpider, which are other public chemical databases. Another database is being developed at ETH Zürich to improve the speed and reliability of online access from anywhere in the world (Gao et al. 2010). Urbance et al. (2003) have developed a database to provide detailed information on degradative bacteria and the hazardous substances they degrade (Biodegradative Strain Database, BSD) within the phylogenetic framework of the Ribosomal Database Project II (RDPII: http://rdp.cme.msu.edu/html). Pazos et al. (2003, 2005) developed a unique system, MetaRouter, for maintaining the variety of information related to bioremediation in a way that allows its query and mining. Arora et al. (2009) compiled a database of biodegradative enzyme oxygenases (OxDBase), taken from primary literature and converted in the form of a web-accessible database, that consists of two separate search engines for mono- and dioxygenases, respectively. For each enzyme queried the database shows its common name and synonym, family and subfamily, reaction in which enzyme is involved, structure and gene link, and literature citation. It can be also linked to several other external databases including ENZYME, BRENDA, KEGG, and UM-BBD, providing more background information. OxDBase, the first database specially dedicated to oxygenases, consists of more than 200 databases that provide comprehensive information about them. Moriya et al. (2010) developed PathPred, a web-based server focused on predicting pathways for microbial biodegradation. The server provides a platform to study the transformation patterns and reference transformation patterns in each predicted reaction. These patterns are then displayed in a tree-shaped graph. The transformation of xenobiotic compounds by microorganisms is essential for the bioremediation, and there is no single resource available that provides information about environmental contaminants as well as microorganisms with biodegradative capabilities. Thus, a database that consolidates the detailed information about xenobiotics, biotransformation methods, and hypothetical pathways would be a handy tool for academic and industrial researchers (Urbance et al. 2003).

9 Future Challenges

Advances in molecular biology techniques have opened a new era in bioremediation processes. Further, the development of various OMICs approaches offers efficient research avenues for analyzing biological systems at different levels. However, the utility of genome sequence facts and associated numerous OMIC approaches to link microbial genes and their products with bioremediation potential is an incredible mission. The goal of this chapter was to review a number of the most current

advances in molecular techniques and OMICs methods and point out some future challenges.

1. Can these metagenomics approaches be powerful in tracking microbial diversity and community dynamics at contaminated sites? If so, what are the essential goals to enforce metagenomics methods to bioremediation?
2. Can proteomic techniques be used as a quantitative tool for tracking in situ microbial metabolism? What are the technologically demanding situations in making efficient use of such equipment in the field?
3. Can these microarrays be used as a excessive throughput, particular, sensitive, and quantitative device for tracking microbial populations and activities?
4. Can OMICs tactics be used to clarify relationships among biomarker estimations and in situ activities?
5. What is the level of correlation between the abundance and expression of the practical genes and the rates of degradation or transformation of contaminants? Can methods primarily based on OMICs offer insights into the costs of kinetic predictions?
6. Can the collected data and information be scaled from molecules to subsurface ecosystems for improving our predicative capability of field bioremediation?

10 Conclusions

Molecular biology approaches have shown an exponential rise in the past few decades and, together with the latest developments in molecular nanotechnology, will lead to further insight into bioremediation processes. Scientists are trying to develop new bioremediation approaches by designing artificial pathways, developing databases for specific microbes or enzymes, and other inter- and intradisciplinary metabolic pathways related to bioremediation. During this entire chapter, one common point is that in spite of the disadvantages of all the molecular-based approaches, the idea that such cellular systems work has been clarified at some stage. In précis, the OMICs tactics should be developed in a manner to assist finding solutions to ecological questions in the context of biochemistry, physiology, microbiology, geochemistry, and ecology.

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