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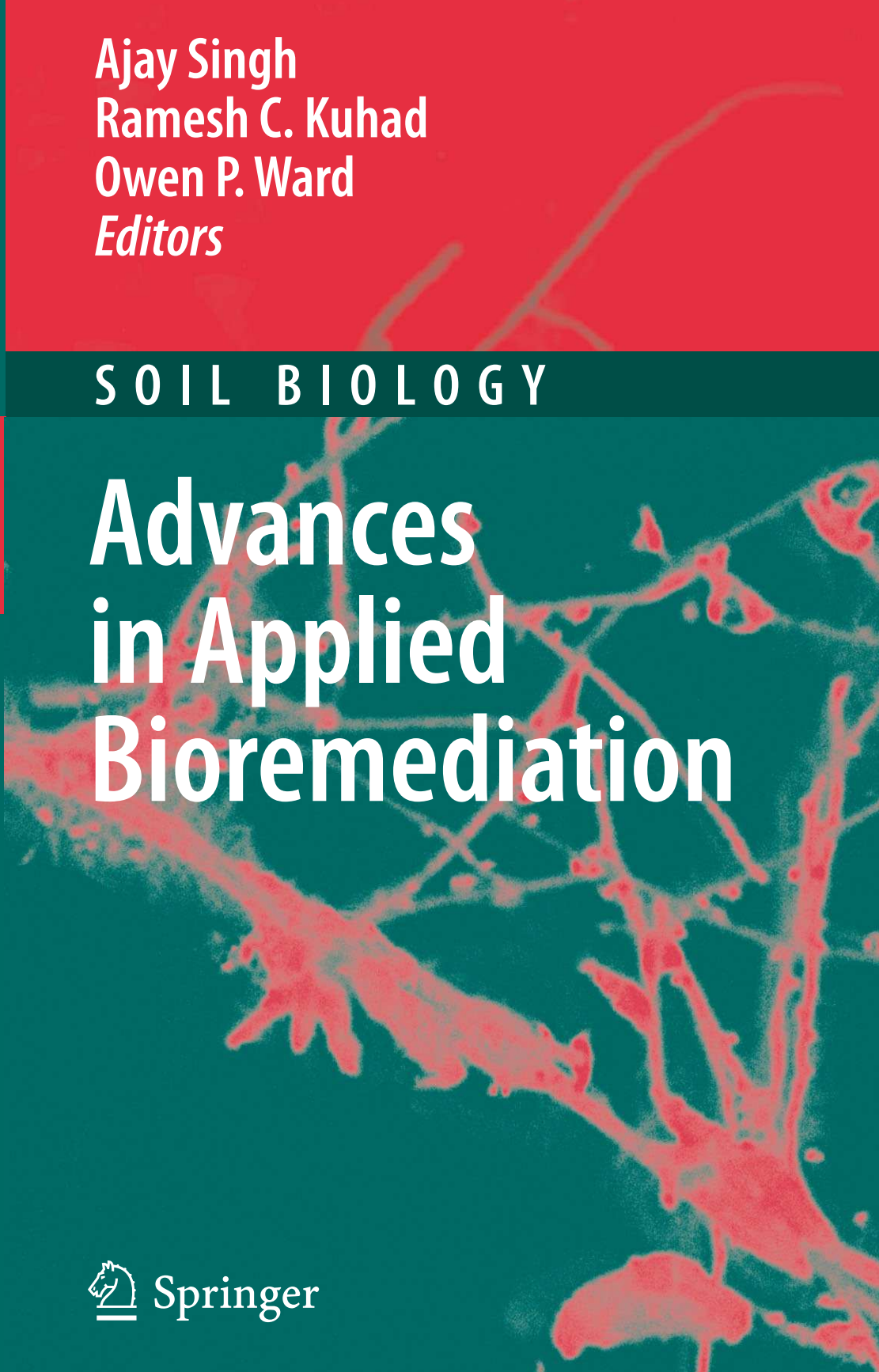
Editors

SOIL BIOLOGY

Advances in Applied Bioremediation



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Preface

The utilization of naturally occurring and mainly prokaryotic organisms in soil for detoxifying and rehabilitating polluted soils provides an effective, economical, versatile and eco-compatible means of reclaiming polluted land. Soil microbial communities are relatively evenly distributed in unpolluted environments. In the soil, microorganisms may develop various mechanisms to access sorbed compounds on soil particles and sediments, as well as to utilize water-insoluble pollutants, facilitating the development of new equilibrium states. These mechanisms may create concentration gradients, bring about micro-environmental pH shifts, and cause secretion of extracellular enzymes and production of surfactants, emulsifiers, solvents or chelators in order to partition chemicals from the non-aqueous phase liquid to the water phase, and to promote degradation of exposed substituents. The purpose of soil remediation is not only to enhance the degradation, transformation, or detoxification of pollutants, but also to protect the quality and capacity of the soil to function within ecosystem boundaries, to maintain environmental quality and sustain biological productivity.

It is difficult to evaluate this market with any specificity, but the international market for remediation is estimated to be around US $25–30 billion. It is challenging to establish such estimates, as many countries have not undertaken comprehensive identification of contaminated sites. Remediation markets usually develop after a country has considered and addressed its air, water and waste management priorities. The US, Canada, Western Europe, Japan and Australia are considered to be the dominant international markets for remediation, with an established presence of a large number of environmental companies, products and services. Emerging economies of some more developed Asian, Eastern European and Latin American countries will represent significant medium-term remedial market opportunities.

Soil remediation processes may be implemented using a variety of different engineered configurations applicable in situ, at the surface or subsurface, and to the excavated soils. Biological remediation technologies require knowledge of interdisciplinary sciences, involving microbiology, chemistry, hydrogeology, engineering, soil and plant sciences, geology and ecology. Biological processes are typically implemented at a relatively low cost, and biological remediation methods have been successfully used to treat polluted soils, oily sludges, and groundwater contaminated by petroleum hydrocarbons, solvents, pesticides and other chemicals.

This volume, "Advances in Applied Bioremediation", of the series Soil Biology is a selection of topics related to biological processes, with an emphasis on their use in remediation of soil pollutants. Topics include an overview of the global soil remediation market and available biotechnology solutions, the bioavailability of contaminants in soil, the role of biosurfactants in bioremediation, metabolism of nitroaromatics, bioremediation of explosive- contaminated soils, biodegradation of petroleum hydrocarbons, bioremediation of benzene-contaminated aquifers, microbial remediation of metals in soil, biotransformation of toxic metals and metalloids, biomining microorganisms and phytoremediation technologies, application of bacterial soluble di-iron monooxygenases and fungal enzymes, and advanced molecular tools for monitoring biological processes in soil remediation.

Experts in the area of environmental microbiology, biotechnology and bioremediation, from diverse institutions worldwide have contributed to this book. This book should prove to be useful to students, teachers and consulting professionals in the disciplines of biotechnology, microbiology, biochemistry, molecular biology, and soil and environmental sciences.

We gratefully acknowledge the cooperation and support of all the contributing authors, and the valuable advice and encouragement provided by Ajit Varma and Jutta Lindenborn throughout the preparation of this volume.

Canada — Ajay Singh
Canada — Owen P. Ward
India — Ramesh C. Kuhad
February 2009

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Chapter 1
Biological Remediation of Soil: An Overview of Global Market and Available Technologies

Ajay Singh, Ramesh C. Kuhad, and Owen P. Ward

1.1 Introduction

Due to a wide range of industrial and agricultural activities, a high number of chemical contaminants is released into the environment, causing a significant concern regarding potential toxicity, carcinogenicity, and potential for bioaccumulation in living systems of various chemicals in soil. Although microbial activity in soil accounts for most of the degradation of organic contaminants, chemical and physical mechanisms can also provide significant transformation pathways for these compounds. The specific remediation processes that have been applied to clean up contaminated sites include natural attenuation, landfarming, biopiling or composting, contained slurry bioreactor, bioventing, soil vapor extraction, thermal desorption, incineration, soil washing and land filling (USEPA 2004).

Biological remediation using microorganisms and plants is generally considered a safe and less expensive method for the removal of hazardous contaminants. The microorganisms have the primary catalytic role in degrading or mineralizing various contaminants and converting non-toxic by-products during soil bioremediation processes (Seshadri and Heidelberg 2005; Head et al. 2006; Gomez et al. 2007). Plants have an inherent ability to detoxify some xenobiotics in soil by direct uptake of the contaminants, followed by subsequent transformation using enzymes similar to detoxification enzymes in mammals, transport and product accumulation (Macek et al. 2008). Phytoremediation, with the associated role of rhizospheric microorganisms, is therefore an important tool in bioremediation processes. Various bioremediation configurations as options for treatment of different classes of chemicals have been evaluated (Hughes et al. 2000). Natural attenuation and electron

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donor delivery were considered as options for remediation of chlorinated solvents, biostimulation for treatment of chlorinated solvents and phenols, bioventing for polycyclic aromatic carbons (PAHs); landfarming or composting were options for nitroaromatics, phenols, monoaromatic hydrocarbon and PAHs (Prince 1998; Mishra et al. 2001). Slurry bioreactor processes were considered suitable for treatment of all of the above mentioned chemicals. Optimizing the environmental conditions in bioremediation processes ensures that the physiological and biochemical activities are directed towards biodegradation of the target contaminants. Environmental factors influencing biological activity include moisture, temperature, pH, oxygen, soil type and chemical nature of contaminant for aerobic degradation and redox potential for anaerobic degradation (Van Hamme et al. 2003).

Bioremediation of some recalcitrant xenobiotic chemicals may require a combination of chemical, physical and biological steps to increase the efficacy of contaminant destruction. Risk assessment is an emerging multi-disciplinary scientific practice used to evaluate health and ecological risks posed by chemical contaminants. Such evaluation helps in devising risk-based management plans to achieve target risk reduction. However, to develop a cost-effective remedial action plan, there is a need to introduce a systematic and scientifically sound methodology to assess the associated risks at a site and identify appropriate remediation technologies.

1.2 Global Remediation Market

In 2001, the global environmental market, including hazardous waste management and disposal, approaches to brownfield redevelopment and site remediation was reported to be of the order of $1 trillion (Masons Water Yearbook 2000–2001). Based on current literature, the international market for the remediation sector is estimated to be in the range US$30–35 billion. The application of bioremediation and phytoremediation cleanup technologies is rapidly expanding and according to an estimate, worldwide demand for these biological technologies is thought to be valued in the region of $1.5 billion per annum. The soil remediation sector has a ready market in countries such as the US, Canada, Western European countries, Japan and Australia. More developed Eastern European, Latin American and Asian countries represent emerging markets for the remediation sector. Understandably, it is not as easy to quantify the value of these emerging remediation markets, especially since comprehensive catalogues of contaminated sites in these countries have not been established. Remediation markets usually develop after a country has dealt with air, water and waste management priorities. The US is possibly the only country that has undergone such an extensive assessment of contamination for federal sites which contributes to solid market evaluation data. As a result, market figures for many jurisdictions are variable, limited and/or inexact.

Nevertheless, the global remediation business is undergoing a process of change and is exhibiting indications of attaining a state of market maturity. Many contaminated sites are in the post-remedial action phase, and have benefited from better and more

reliable technology and the availability of greater process performance information. Many other contaminated sites have been characterized as essentially ready for implementation of a preferred remediation process. Clearly, many other contaminated sites have yet to be formally identified, declared or characterized. There has also been a shift in the general factor(s) motivating remedial action. Up to the mid-1990s, implementation of cleanup of contaminated properties was driven by regulatory compliances, and guided by clean-up end points or residual limits which bore little relationship to the proposed use of the remediated land. More recently, great attention has been placed on relating remedial action to the intended use of the property, as well as remediation economics and risk assessment. Analysis of international environmental markets in the following subsections clearly shows that substantial growth will occur over the next decade in markets throughout the world. The discussion on marketing potential is assembled mainly from data cited by CTCS (2002), The Delphi group (2003), AEGIS (2003), USEPA (2004), Statistics Canada (2004), EcoLog Group (2005), JETRO (2007) and Industry Canada (2008).

1.2.1 North America

The current estimated hazardous remediation market in the United States is pegged at around $12 billion, which represents about 30% of global demand. Based on currently applicable regulatory standards in the United States, an estimated quarter of a million sites require some form of remediation, but the number of contaminated sites is larger than that if all brownfield sites are taken into account (see below). Most of these sites have one or combinations of the most common contaminants – solvents, petroleum products, VOCs and heavy metals, the nature and concentrations of which will influence technology choice. These contaminated sites can be divided into seven groups depending on which government agency/regulations have enforcement and/or decontamination responsibility: Superfund, Resource Conservation and Recovery Act (RCRA) Corrective Action, Underground Storage Tanks (UST), Department of Defense (DOD), Department of Energy (DOE), Civilian Federal Agencies, and States (USEPA 2004). The majority of these sites requires the collaboration of multiple stakeholders for successful cleanup, as well as the development and implementation of innovative remedial solutions. The United States Environmental Protection Agency (USEPA) enforcement of the Superfund program is still encouraging remediation by potentially responsible parties at the majority of highly contaminated sites. This is evident based on USEPA's precedent-setting order requiring General Electric to pay nearly half a billion dollars for the cleanup of polychlorinated biphenyls (PCBs) in the Hudson River.

EPA estimates that up to $100 billion will be spent during the next 30 years to meet new underground storage tank regulations. The USEPA brownfield development program promotes the remediation and redevelopment of industrial sites by enhancing the acceptance of cleanups based on the concept of risk-based standards and restricted future land use. The USEPA estimates that over half a million brownfield

sites exist across the United States. The brownfield market continues to be an area of growth for many remediation firms because of the opportunities to partner with property owners and developers.

The Canadian environment industry has annual sales of over $20 billion, and contributes 2.2% to Canada's GDP. Remediation is considered a part of the solid and hazardous waste management sector, comprising the second largest component (24%) of Canada's environment industry. Based on provincial programs such as Environment Canada's Green Plan, rising awareness of the need to clean-up public lands, and the expected positive image gained from establishing/enforcing regulations which mirror those of the USEPA, the Canadian market is expected to reach $1 billion for soil and groundwater remediation. Current Canadian demand for soil remediation services and products is estimated at $250–500 million. There are positive signs for further growth in Canada given the government's commitments for the next ten years of $3.5 billion for remediation of federally owned contaminated sites, $500 million for specific contaminated sites of concern across Canada for which it has shared responsibility, e.g., the Sydney Tar Ponds, and a budget of $150 million for redevelopment of municipal brownfields under the management of the Federation of Canadian Municipalities.

Canada has an estimated 30,000 contaminated sites, and approximately two-thirds of these sites can be economically cleaned up and redeveloped. Nevertheless, there is still great uncertainty with regard to the extent and number of contaminated sites in Canada. There is also no national legislation on contaminated land to coordinate approaches between provincial and territorial jurisdictions and create common approaches and standards. Awareness of the problem of contaminated sites is growing in Canada, as is effort to address them. According to Statistics Canada, Canadian revenues from the international environment market are in excess of $1.6 billion for exports of solid and hazardous waste management services. For large Canadian environmental consulting and engineering firms involved in remediation, approximately 10–30% of their business can come from export markets.

1.2.2 Europe

Although technologies for soil and groundwater remediation are at an advanced stage in Europe, the European Commission (EC) has recognized a need for strengthening innovation of environmental technologies in order to increase competitiveness in a global market and to achieve a more sustainable development. The European Co-ordination Action for Demonstration of Efficient Soil and Groundwater Remediation (EURODEMO), an EC-funded initiative, has been launched to promote investigations and application for innovative technologies for supporting these goals. EURODEMO is expected to bring together formerly isolated national-scale knowledge to build a greater confidence in remediation technologies while providing a platform for innovation potential (Spira et al. 2006).

Western Europe's estimated overall environmental market is around US$227 billion. Highest growth rates are forecast in areas such as waste management,

environmental consultancy, cleaner technologies and renewable energy sub-sectors. The number of contaminated sites in the Western Europe is estimated to be over 600,000, the remediation of which will cost an estimated ∈50 billion, over an extended clean-up duration. Potentially 0.5–1.5% of GDP is likely to be spent per annum to clean up the contaminated sites. Among the Western European countries, the United Kingdom (UK), France and the low-lying Netherlands have spent the most money to date for remediation. The UK environmental market is forecast to grow to £21 billion by 2010. Key contaminants are typical of other industrialized countries and include hydrocarbons, pesticides, radio-nuclides, localized contamination from abandoned industrial plants/land, past industrial spills or improper municipal and industrial waste management.

Environmental business opportunities in jurisdictions tend to parallel economic growth and prosperity and in the period 2000–2010, Central and Eastern Europe (CEE) is experiencing substantial economic development. Substantial investments opportunities are associated with waste management, water and wastewater treatment and contaminated land remediation. Overall, the environmental markets in CEE are forecast at US$15 billion by 2010 at a growth rate of 6.6%. Thus, the countries which have experienced the more successful economic transformations, for example the Czech Republic, Slovakia, Hungary, Poland and Slovenia, have likewise exhibited faster rates of environmental market growth than other Eastern European countries.

1.2.3 Australia and New Zealand

Regulatory and environmental policies are relatively well developed in Australia and New Zealand, and trends similar to European markets are emerging – including the need to improve resource use efficiency, minimize waste and reduce greenhouse gas emissions. The economies have performed well over the last few years, with a growth rate around 3% per annum. This growth is reflected in environmental market forecasts which are projected to be around US$13 billion in 2010 in the larger sectors of water and wastewater treatment, waste management and air pollution control. Higher growth is forecast in sectors such as water re-use technologies, environmental monitoring, renewable energy, energy management, cleaner technologies and environmental consulting over the next 10 years.

1.2.4 Asia

In Asia there has been high market demand for site assessment/soil testing services and for treatment technologies for oil decontamination, PCB destruction, bioremediation, and especially for in-situ technologies that permit the surface structure to remain intact, hazardous wastes from dry cleaners and gas filling stations.

The Japanese environmental market is relatively well developed and mature in areas such as water and wastewater treatment, waste management and air pollution

control. Overall, the environmental market is growing at around 2% per annum to an estimated US$113 billion by 2010. In Japan, the number of contaminated sites is estimated to be over 500,000. The Japanese remediation market is expected to grow to $3 billion by 2010. Site remediation and groundwater treatment are expected to grow substantially.

Over the past 30 years and dominated by their rich petrochemical resources and associated revenues, the Middle Eastern countries have experienced unprecedented economic growth. Traditionally, this region's health and environmental standards have substantially lagged behind those of North American and European jurisdictions. However, environmental pressures from the latter jurisdictions which import and consume large volumes of Middle Eastern oil are growing, based both on motivations related to achieving global environmental sustainability and also to ensure a level playing field with respect to competition among industries located in different jurisdictions, so that poor environmental practice does not afford a competitive advantage. The overall environmental markets in the region are forecast to approach US$8 billion in 2010, representing annual growth of greater than 4%. Significant developments in environmental regulations and cultural attitudes towards the environment are needed in order to achieve this growth.

East and South East Asian countries such as Malaysia, Indonesia, Singapore, the Philippines, Thailand, South Korea, Taiwan and Hong Kong require major investments in environmental infrastructure in order to alleviate significant public health impacts of pollution. Environmental markets in the East and South East Asia region are forecast to reach US$27 billion in 2010.

Perhaps the greatest environmental impact derives from human population growth and human population density and associated economic development. In this regard, the greatest environmental impact may be expected from the two countries with the largest populations; China with a population of about 1.25 billion, and India with a population exceeding 1 billion and expected to surpass that of China by 2050. Furthermore, these two countries are currently experiencing extraordinary annual economic growth rates (8–10%) through rapid industrialization. Both these economies will surpass the US as leading world economies within the next few years. As elsewhere, environmental market growth in these countries is driven by the growing environmental impacts associated with economic growth, rapid industrialization and indeed intensive agricultural practices, including widespread use of inorganic fertilizers and pesticides. Environmental challenges are exacerbated by the existence of very large urban populations and associated high levels of urban pollution. There is a limited infrastructure for mitigating the negative environmental impacts, which in turn leads to serious impacts on human and animal health and raises concerns regarding long-term environmental sustainability. The true potential size of the environmental sectors in these countries is not known.

The current environmental market in India is valued at approximately US$4 billion. China's environmental market approximately doubled from US$3 to 6 billion from 1995 to 2000, and is expected to double again by 2010, representing an annual growth of over 8%. In terms of size, the market is dominated by water and wastewater treatment, followed by waste management, site remediation and air pollution control.

1.2.5 Latin America and Africa

Overall, the environmental market in Latin America is forecast to grow from approximately US$9.7 billion in 2000 to US$15 billion by 2010, representing an annual growth of around 4.5%. The bulk of the market activity will relate to water and wastewater treatment and waste management activities, and related sectors such as environmental instrumentation and environmental consultancy. Sectors such as contaminated land remediation and marine pollution control are, at present, lower priorities, but opportunities in these fields are anticipated to emerge in the next 5 years plus. Although enforcement of environmental regulations is still limited, other market drivers are strong — notably the need to address the health impacts of environmental pollution, and increases in international donor aid for environmental improvement in the less developed countries.

Environmental degradation is one of the major factors constraining economic development in North and Sub-Saharan Africa. Substantial investments are required in basic water and waste management infrastructures — for example, an estimated US$80 billion to US$100 billion is needed just in basic water services in the next 10 years. International aid programs will help to provide a proportion of the funding requirements. Much of the environmental investment throughout Africa over the next decade will involve relatively 'low tech' equipment to address environmental problems and pressing human health needs.

1.3 Major Environmental Contaminants of Concern

The huge expansion of the chemical and petroleum industries in the twentieth century has resulted in the generation of a vast array of chemical products for daily use. According to an estimate, there are somewhere between 8 and 16 million molecular species of natural or man-made organic compounds present in the biosphere, of which as many as 40,000 are pre-dominant in our daily lives (Hou et al. 2003).

Since soil and groundwater are preferred sinks for complex contamination, various chemical and biological soil properties are profoundly altered, which affects biodiversity and soil function. The organic contaminants include the alkanes, monoaromatics, monocyclic and polycyclic aromatic compounds, chlorinated hydrocarbons, including the polychlorinated biphenyls, nitroaromatics and nitrogen heterocycles. Often the organic contaminants are present as complex mixtures of different chemical species, as are present in petroleum on sites including petroleum refineries, petrochemical plants, gas stations, leaking storage tanks, and exploration and production well-heads. Halogenated chemicals are potentially found in chemical manufacturing plants or disposal areas, pesticide/herbicide mixing areas, contaminated marine sediments, firefighting training areas, vehicle maintenance areas, landfills and burial pits, and oxidation ponds/lagoons. Explosive contaminants such as TNT, DNT, RDX and other nitroaromatics may be found on sites like artillery/impact areas, contaminated marine sediments, disposal wells, landfills, burial pits, and TNT washout lagoons.

Sites contaminated by heavy metal include battery disposal areas, burn pits, chemical disposal areas, contaminated marine sediments, electroplating/metal finishing shops, and firefighting training areas, as well as landfills and burial pits. Excessive levels of inorganic fertilizer-related chemicals introduced into soil, such as ammonia, nitrates, phosphates, and phosphonates, which accumulate there or lead to contamination of water courses and air, have resulted in significant environmental deterioration. Undesired contamination of soil with radionuclides represents an additional environmental hazard to all life forms. Radioactive and mixed waste disposal areas are the major sites for radionuclide contaminants.

The return of environmentally contaminated sites to pristine conditions is quite challenging, and often not achievable (Kostelnik and Clark 2008). Currently available remediation techniques do not completely eliminate hazardous waste, but rather only concentrate and contain the contaminants of concern (Table 1.1). Since most of the remedial decisions concerning these complex challenges often focus on mitigation actions to reduce risk to human health and the environment, the problem frequently remains with the residual waste at many sites even after regulatory-approved

Table 1.1 Soil remediation technologies

Soil remediation processes	Specific technologies	Comments
Biological processes	Landfarming	Involves excavation of soil and by placing on lined landfarms, and stimulation of natural microbial population by providing nutrients, water, bulking agents and tilling
	Ex situ bioremediation	
	Biopile, biocells, bioheaps Biomounds, compost cells Ex situ bioremediation	Involves excavation of soil and placing in heaps or aerated piles, and stimulating microbial activity by providing nutrients, water and oxygen
	Slurry bioreactor	Involves excavation of soil and treatment in a contained environment such as tanks/reactors by providing oxygen, water and nutrients under controlled conditions for accelerated biodegradation
	Ex situ bioremediation	
	Bioleaching	Clean up of heavy metal contaminated soil using acidophilic bacteria that oxidize reduced sulfur compounds to sulfuric acid. Performed either in slurry or by heap leaching system
	Enhanced bioremediation In situ bioremediation	Achieved by creating a favorable environment to stimulate the natural or inoculated population of microorganisms. Biodegradation rate is influenced by biostimulation, bioaugmentation or cometabolism
	Bioventing In situ bioremediation	Involves injection of air or water to supply oxygen and nutrients into the underground contaminated mass
	Biosparging	Addition of air/oxygen and nutrients to enhance biodegradation of groundwater contaminants. Also potentially improves biodegradation in the unsaturated zone

(continued)

Table 1.1 (continued)

Soil remediation processes	Specific technologies	Comments
	Anaerobic biodegradation	Anaerobic degradation of polychlorinated organic pollutants in sediments. Generally followed by an aerobic process for further dechlorination of the pollutants
	Phytoremediation	Higher plants are used either to degrade contaminants, to fix them in the ground, to accumulate them within plant tissue or to release them to the atmosphere
	Monitored natural attenuation	A strategy of allowing natural processes to reduce contaminant concentrations over time, involving physical, chemical and biological processes with continuous monitoring
Chemical processes	Oxidizing agents (oxygen, ozone, UV, H_2O_2, chlorine gas, etc.); reduction agents (Al, Na and Zn metals, alkaline polyethylene glycols, etc.)	Oxidation and reduction processes can treat a range of contaminants including organic compounds and heavy metals
	Dechlorination	Reduction reagents remove chlorine atoms from hazardous chlorinated molecules (PCBs, pesticides)
	Chemical extraction with inorganic acids (HCl, H_2SO_4, HNO_3), organic acids (acetic, tartaric, citric), chelating compounds (EDTA, DTPA, NTA)	Inorganic and organic acids are used to decrease the pH of contaminated soil to release the heavy metals; chelating compounds are used to form water-soluble metal-ion complexes
	Solvent extraction using organic solvents	An organic solvent that has a high solubility for the pollutants is intensively mixed with the soil. The solvent is recovered and reused and pollutant is concentrated
	Solidification/stabilization agents such as Portland cement, fly ash, silicates, lime, clays, and polymers	Achieved by solidifying contaminated soil, converting contaminants into a less mobile chemical form and/or by binding them within an insoluble matrix to reduce leaching
	Asphalt batching	An alternative stabilization/solidification method treats hydrocarbon-contaminated soils by incorporating petroleum-contaminated soils into hot asphalt mixtures as a partial substitute for stone aggregate and utilizing the mixture for paving
Physical processes	Soil washing	Uses water or surfactant solution and mechanical processes to scrub soils
	Soil flushing	Achieved by flooding contaminated soils with an extraction fluid that moves the contaminants to a particular area. Generally used in conjunction with activated carbon, biodegradation, or pump-and-treat

(continued)

Table 1.1 (continued)

Soil remediation processes	Specific technologies	Comments
	Soil vapor extraction	Removes gases and organic volatile or semi-volatile contaminants from soil atmosphere by vacuum pumping; also stimulates bioremediation process in the unsaturated zone
	Electrokinetic remediation	An in situ process induced by electrolysis, electro-osmosis and electrophoresis through an array of electrodes embedded in the soil to move contaminants in the pore water towards the electrodes
	Wet classification	Two steps involve an intensive mixing of sediment to disintegrate agglomerates of sediment particles, and a mechanical separation step
	Encapsulation	Physical isolation and containment of the contaminated soils by low permeability caps, slurry walls, grout curtains, or cutoff walls
Thermal processes	Thermal desorption	Heating to 600 °C results in evaporation of volatile contaminants and subsequent removal from the exhaust gases by condensation, scrubbing, filtration or destruction
	Incineration	Involves heating of excavated soil to temperatures of 880–1,200°C to destroy or detoxify contaminants
	Vitrification	Heating of excavated soil to temperature range of 1,000–1,700°C resulting in vitrification of the soil, forming a monolithic solid glassy product
	Wet oxidation	The oxidation process occurs in the water phase at high temperatures and high pressures ,but below the supercritical temperature and pressure of water
	Supercritical oxidation	Temperature and pressure above the critical point of water result in higher solubility of oxygen and toxic organic compounds in the water phase, and a higher oxidation rate

environmental remediation operations are complete. Chapter 2 in this volume provides a discussion on the holistic environmental merit of soil remediation to complement risk assessment, using two assessment software models, and tips and tools on how to improve remediation.

1.4 Biological Remediation of Contaminated Soils

The utilization of naturally occurring prokaryotic organisms in soil (around 4,600 distinct genomes in one gram of soil) for detoxifying and rehabilitating polluted soils provides an effective, economical, versatile and eco-compatible means of

reclaiming polluted land (Hunter-Cevera 1998; Van Hamme et al. 2003). Soil microbial communities are relatively evenly distributed in unpolluted environments. The general assumption stands that higher microbial diversity is proportional to an increased catabolic potential. This can be extrapolated to imply that high species diversity leads to more effective removal of metabolites and pollutants from a substrate. In the soil, microorganisms may develop various mechanisms to access sorbed compounds on soil particles and sediments, as well as to utilize water-insoluble pollutants by facilitating a new equilibrium state, creating concentration gradients, bringing about micro-environmental pH shifts, secreting extracellular enzymes, producing surfactants, emulsifiers, solvents, and chelators to partition chemicals from the non-aqueous phase liquid to the water phase, and degrading exposed substituents (Van Hamme et al 2006; Singh et al. 2007). Chapter 3 provides a detailed discussion on geochemical conditions, coupled biodegradation–sorption models, correlations between bio-resistant and desorption-resistant fractions of contaminant, and facilitated bioavailability. Chapter 4 outlines biosurfactant chemical characterization, physiological roles, applications in bioremediation, and both in situ and ex situ biosurfactant production.

Use of plants for transfer, accumulation and removal of pollutants from the environment is called phytoremediation. The approach can be used for removal of both inorganic and organic xenobiotics and pollutants present in the soil, water and air. The type of contaminants range from inorganic fertilizers to pesticides, from heavy metals and trace or radioactive elements to explosives, oil spills to PAHs and PCBs.

Biological remediation technology for restoration of a polluted site may be utilized in situ or ex situ. In situ treatment allows soil to be treated without being excavated and transported, but it requires longer time periods and extensive monitoring due to variability in soil and aquifer characteristics. Examples of in situ treatment methods are enhanced microbial bioremediation, bioventing and phytoremediation. Ex situ treatment generally requires shorter time periods with better process implementation and monitoring controls but requires excavation of soils, leading to increased engineering cost. Landfarming, biopiling, composting and slurry bioreactors are examples of ex situ technologies.

1.4.1 In Situ Biological Remediation

Enhanced microbial bioremediation is achieved by creating a favorable environment to stimulate the natural or inoculated population of microorganisms and exploit their catabolic potential to grow and consume the contaminants as a food and energy source. Among the most important of the enzymes used by bacteria in degradation of organic compounds are oxygenases. Recent advances in understanding of the diversity, distribution and physiology of monooxygenases are discussed in Chapter 5. Biodegradation or biotransformation rate is influenced by the type and concentration of specific contaminant present, oxygen supply, moisture, temperature, pH, nutrient supply or biostimulation, bioaugmentation with strains containing desired catabolic properties, and cometabolism (de Lorenzo 2006; Borden and Rodriguez 2006).

Chapters 6–10 outline a detailed account of different soil contaminants, and discuss biological methods for degradation of contaminants and remediation of surface and subsurface soil environments. The technology has been successfully applied to remediate soils, sludges, and groundwater contaminated with petroleum hydrocarbons, pesticides, wood preservatives, nitroaromatic compounds, explosives, solvents and other organic chemicals. The effectiveness of anaerobic microbial degradation of nitrotoluenes in soils contaminated with munition wastes has also been demonstrated at bench and pilot scales.

The development of enhanced microbial bioremediation processes for treatment of heavy metals and other inorganic compounds has lagged behind the use of bioremediation for degradation of organic contaminants (Lloyd and Lovley 2001). Microbial remediation methods for metal contaminants are discussed in detail in Chapters 11 and 12. The microbial solubilization of metals using chemolithoautotrophic microorganisms has been successfully used in industrial processes called biomining to extract metals such as copper, gold, uranium and others. Chapter 13 deals with molecular aspects and applications in biotechnology and bioremediation of biomining microorganisms. An understanding of microbial growth and activities in oxidizing metal ions may be useful to improve bioremediation of metals in acidic environments. However, the choice of which bioremediation option to use is determined by several economical and environmental factors. Although many microbes are known to transform metals from toxic to non-toxic species or to alter their solubility or availability, treatment of soils contaminated with a mixture of organic and inorganic contaminants could be quite challenging. In such cases, bioremediation can only be used effectively by using a treatment train comprising separate steps for organic destruction (or recovery) and for removal/separation of inorganic compounds.

Bioventing stimulates the natural in situ biodegradation of aerobically degradable compounds in soil by providing oxygen to existing soil microorganisms. In contrast to soil vapor vacuum extraction (SVE), bioventing uses low air flow rates to provide only enough oxygen to sustain microbial activity. Cleanup ranges from a few months to several years. Bioventing is receiving increased attention from environmental engineers, particularly for use in conjunction with SVE. Remediation time using bioventing is highly dependent upon the specific soil and chemical properties of the contaminated media. Bioventing techniques have been successfully used to remediate soils contaminated by petroleum hydrocarbons, non-chlorinated solvents, some pesticides, wood preservatives, and other organic chemicals. Low soil moistures, fine soil particle size, low temperature and high water table may limit the biodegradation process.

Phytoremediation methods use plants to extract, contain, immobilize or degrade contaminants from soil and water. Some plants can remove contaminants from soil by direct uptake, followed by subsequent transformation, transport and accumulation in a non-phytotoxic form (Rylott et al. 2006; Macek et al. 2008). The diverse approaches in phytoremediation include phytodegradation, phytoextraction, phytostabilization, phytovolatilization and rhizofiltration. Plant–microbial associations seem to be the key to enhancing removal of inorganic and organic pollutants. Genetic engineering methods have been used for creating transgenic plants with

improved detoxification capabilities under field conditions (Eapen et al. 2007; de Carcer 2007; Macek et al. 2008). Phytoextraction and rhizofiltration processes have shown promise for commercialization. Discussion on advances in phytoremediation and rhizoremediation methods is covered in Chapters 14–16. Phytoremediation technologies are most appropriate for large areas of low and moderately contaminated soils where the application of conventional remediation technologies would be prohibitively expensive.

Various groups of microorganisms are present in the soil (bacteria, actinomycetes, fungi, algae, and viruses). In the rhizosphere their numbers are higher than in the bulk soil, due to the presence of high amounts of available carbon released as exudates by roots. Plants respond to the presence of microorganisms by modifications of growth, e.g., symbiosis or interactions with free living organisms (Mackova et al. 2006). Positive effects are expected from inoculation of roots with selected microorganisms (suppliers of growth-promoting substances, nitrogen fixation, acquisition of phosphorus and especially those expressing degradative genes). Approximately 400 plant species have been classified as hyperaccumulators of heavy metals, and most of them accumulate Ni. Phytoremediation using conventional plants such as grasses, sunflower, corn, hemp, flax, alfalfa, tobacco, willow, Indian mustard, poplar, etc. shows good potential for the removal of pollutants from large areas with relatively low concentrations of unwanted compounds. The most important parameter for selection of suitable plants is not the tolerance of the plant to heavy metals, but effectiveness in the accumulation of heavy metals.

In general, the contaminant must be in a biologically accessible form, and root absorption must take place. Translocation of the contaminant from root to shoot makes harvesting easier. The harvested biomass could be reduced in volume and/or weight by composting, anaerobic digestion, low-temperature incineration, or leaching, leading to a decrease in the costs of handling and processing. After stabilization, potential subsequent landfilling can be considered.

1.4.2 Ex Situ Biological Remediation

In cases where soils cannot be treated in situ due to either regulatory reasons or the unavailability of sufficient land, risk to groundwater or air pollution, soils must be excavated and bioremediated using landtreatment units, composting, biopiles, or slurry bioreactor technologies.

Landfarming or land treatment is a bioremediation technology proven at full scale. The process requires excavation and placement of contaminated sludges, soils and sediments into lined beds which periodically tilled to aerate the soil. It usually incorporates liners and other methods to control leaching of contaminants from landfarms. The contaminated soil is augmented with nutrients and sometimes microorganisms, to improve processes for biodegradation of the contaminants. The landfarming process generally treats the top 30 cm layer of the soil and involves the addition of fertilizers and bulking agents, and includes periodic tilling and irrigation to stimulate

indigenous soil bacteria to degrade the contaminants. The soil environment condition is generally controlled for moisture by irrigation, near neutral pH by lime and aeration by frequent tilling. Landfarming is a cost-effective treatment if there is no risk of leaching hydrocarbons into the groundwater, or the volatilization of contaminants to create any health risk to workers and neighborhood communities. Land treatment units are designed with a leachate collection system to prevent the off-site migration of water-soluble hydrocarbons. Ex situ landfarming has been most successful in treating lighter petroleum hydrocarbons.

In the composting method, the contaminated soil is mixed with organic materials such as straw, wood chips, and sewage sludge, and is placed in piles or windrows. The addition of organic bulking agents improves soil aeration and provides easily metabolizable substrates to increase the soil temperature, which supports growth of mesophilic or thermophilic microorganisms. Pilot and full-scale projects have demonstrated that aerobic, thermophilic composting is able to reduce the concentration of explosives (TNT, RDX, and HMX), ammonium picrate, and associated toxicity to acceptable levels. The major drawback is the requirement of substantial space, and increase in the volume due to addition of bulking agents.

Biopile technology is an engineered composting system where aeration is provided through a network of sparger pipes and a leachate collection system is used for water-soluble hydrocarbons. Compared to landtreatment units, the biopiles require less space. The operation is more controlled, and suited for the treatment of more volatile hydrocarbons such as gasoline and jet fuels by covering the pile and applying a slight negative pressure to the system and pulling air through an exhaust prior to discharge. Biopile treatment has been successfully applied to treatment of non-halogenated VOCs and fuel hydrocarbons.

Contained bioreactor systems can overcome the deficiencies of landfarming, composting or biopiling methods. Accelerated bioremediation of contaminants can be achieved in bioreactors, where greater control over pH, temperature, moisture, mixing, bioavailability of nutrients, energy source (substrate) and oxygen promotes optimal microbial growth and activity (Ward et al. 2003). Continuous mixing results in dispersion of soil and desorption of contaminants from soil, and contact with the aqueous phase is promoted, resulting in increased biodegradation rate.

1.4.3 Nanotechnology and Site Remediation: An Emerging Field

Nanotechnology, creation and use of structures, devices, and systems that have novel properties because of their small size and very large surface area, is an emerging field of science, with broad applications in the fields of electronics, aerospace, chemical manufacturing, medical and environment (Tratnyek and Johnson 2006). Nanotechnology has potential applications for remediation sites contaminated with chlorinated hydrocarbons, pesticides and metals. In the early 1990s, the reducing capabilities of metallic substances, such as zerovalent iron (ZVI), began to be examined for their ability to treat a wide range of contaminants in hazardous waste/

water in the form of permeable reactive barriers (PRBs) designed to intercept plumes in the subsurface and subsequently remediate them (USEPA). Nanoscale zerovalent iron (nZVI) preparations (1–100 nm particles with a large surface area compared to their volume) rapidly destroy contaminants by oxidation–reduction reactions forming non-toxic byproducts. With time, iron particles partially dissolve or settle out, and reactivity declines.

There are other nanotechnology products such as bimetallic particles (Fe-Ni and Fe-Cu), semiconductor photocatalysts (TiO2 and ZnO), polymeric nanoparticles (amphiphilic polyurethane), single enzyme nanoparticles (peroxidases and polyphenol oxidases) and tunable biopolymers (elastin-like polypeptides) with potential application in remediation of some organic and metal pollutants (Watlington 2005). However, the application of these products for full scale is yet to be established.

Nanotechnology is an emerging area in the field of soil remediation. There is still need for more data on life cycle analysis (LCA) and effectiveness of techniques and assessment of potential exposure and toxicity of non-material. The National Science Foundation (NSF) of the United States estimates that by 2010–2015 approximately $1.1 trillion will be spent on nanotechnology research and development (USEPA 2007).

1.4.4 Designing Biological Remediation

Since the objective of bioremediation is to decrease the concentration of organic pollutants to levels which are undetectable or, if measurable, lower than the limits established as safe by the regulatory agencies, the following criteria may be established before seriously considering it as a practical remediation method:

- Microorganisms with the necessary catabolic activity and capacity to transform the compound at reasonable rates and bring the concentrations to levels that meet regulatory standards must exist.
- The target compound(s) must be bioavailable.
- Byproducts that are toxic at the concentrations likely to be achieved during remediation must not be produced.
- Site conditions must be made appropriate to sustain microbial growth or activity.
- The technology must be less expensive than other alternative technologies.

A careful bioremediation system design tailored to specific contaminants, and application of appropriate decontaminating organisms, requires knowledge of various environmental sciences and engineering disciplines. Furthermore, an economical evaluation of the overall bioremediation program is also required to assess the use of the remediation technique as a competitive and alternative decontamination strategy. Once a bioremediation program has been designed and implemented, it is important to assess that the toxicity at the contaminated site has been lowered and that the site has regained its initial biological activity and productivity (Andreoni and Gianfreda 2007).

Among the analytical methods, infra red, gas chromatography, mass spectroscopy and synchronous fluorescence spectroscopy are generally accepted as powerful and sensitive analytical tools to determine aromatic pollutants. Usually, an exhaustive chemical extraction of the remediated soil should be performed to evaluate the residual concentration of the pollutant. Indeed, the final contaminant concentration has to be lower than that established by the local regulatory agency.

Information about the factors controlling the growth and metabolism of microorganism in polluted environments is necessary, because several of the above criteria are highly empirical rather than knowledge-based. The continued generation of new information on microbial degradative pathways should expand opportunities for molecular environmental analysis. Examining the presence and the expression of the key genes involved in bioremediation could yield more information on microbial processes.

As the remediation industry matures, the importance of understanding contaminant distribution before and after implementing remedial programs is seriously realized. As a result, several novel methods have been developed in the recent past which enable quicker soil and groundwater remediation design and implementation (Simon 2008). Some of the examples include using tree cores and branches for VOC plume delineation, monitoring distribution of injected chemical distribution using electrical conductivity, and geophysical methods like hydraulic/electrical conductivity and tomography radar data to assess effectiveness of bioremediation. Chapters 17 and 18 cover a detailed account of environmental and molecular tools available for monitoring bio- and rhizoremediation processes.

Readers are also referred to Volumes 1 and 2 of this Soil Biology Series from Springer, which has also presented authoritative details on the fundamental and applied aspects of soil bioremediation.

1.5 Conclusions

The global remediation market is a dynamic market which has exhibited major changes over the past 10 years. As the market is maturing and refining, many contaminated sites are being identified, characterized, remediated and/or monitored using advanced technologies. The demand for application of bioremediation and phytoremediation cleanup technologies and services is rapidly growing. Since the majority of bioremediation processes rely on the activities of complex microbial communities, we need to have a better understanding of the following aspects:

- Develop strategies for improving the bioavailability of the hydrophobic contaminants which have extremely low water solubility and tend to adsorb to soil particles and persist there.
- Learn about the interactive and interdependent roles played by individual species in microbial communities employed in bioremediation.

- Unravel the complex aerobic and anaerobic metabolic pathways to better understand the nature of rate-limiting steps and underlying genetic/biochemical regulatory mechanisms.
- Characterize many of the key enzymatic reactions that participate in contaminant transformation in order to design and engineer biocatalysts with improved substrate specificities, reaction rates and other desired catabolic properties, and ultimately to engineer improved catabolic pathways for bioremediation.
- Extend our understanding of biodegradative pathways. Even after 50 years of extensive research on microbial biodegradation, detailed knowledge about biodegradative pathways is available for only about 900 chemical species (Urbance et al. 2003; Ellis et al. 2006).
- Develop strategies for combining chemical or physical methods with biological systems in order to achieve overall effective remediation, since some chemical species are inherently intractable to enzyme transformation.
- Continue to devise better monitoring and assessing methods to evaluate the progress and effectiveness of biodegradation processes.

Although accelerated bioremediation processes have been applied for sludges and soil slurries (Ward et al. 2003), most of the soil bioremediation processes are relatively slow processes, and frequently it either takes a long time or desired end points may not be achieved due to the lack of sub-optimal environmental conditions for selection and growth promotion of the biological system employed. However, there are certain other non-scientific reasons for the failure of remediation projects in the past. The United States Department of Energy (US DOE) appointed the Consortium for Risk Evaluation with Stakeholder Participation (CRESP) to find the root causes of remediation projects that fail to entirely achieve their goals, and then to offer suggestions to assist the DOE. In a report recently submitted by CRESP, five root causes identified for remediation project failure were: (1) complex science, engineering, and technology, (2) ambiguous economics, (3) project management shortcomings, (4) political processes and credibility, and (5) history and organizational culture (Greenberg et al. 2007). For example, in their excitement to apply bioremediation processes, industrial microbiologists have often ignored well-proven techno-economic and processing principles, related to imposing process control, optimization and reproducibility standards to bioremediation processes, principles that have served them well for a century in implementation of fermentation technology (Ward 2004). Thus it is important that systematic planning takes place, establishing clear goals, dynamic work strategies with contingency plans, and real-time measurement technologies which play an important role in remediation project planning (Simon 2004).

In the past ten years, emerging technologies such as phytoremediation, bioremediation, and nanotechnology (nZVI-based permeable reactive barriers) have become popular new tools. These novel treatments have begun to compete with more established technologies such as solidification/stabilization, soil vapor extraction, and thermal desorption for soil, and pump and treat systems for groundwater.

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Chapter 2
Local Gain, Global Loss: The Environmental Cost of Action

Pascal Suer, Yvonne Andersson-Sköld, and Jenny E. Andersson

2.1 Introduction

Lower toxicity and less pollution is the goal of all soil remediation. We are willing to spend money and time to achieve this. And our action of treating the soil causes new pollution in its turn.

The gain is local (cleaner soil), and the environmental cost is most often global or regional (global warming, particle and other air emissions, biodiversity, etc.). Balancing cost and gain is complicated by these different scales. Besides, everyone does not realise that the environmental costs are there. But if we are aware of the existence of such costs, there is also the possibility of minimizing them by choosing low-impact treatment options and low-impact materials. An example is the use of cement instead of steel for funnel walls (Bayer and Finkel 2006).

This chapter aims to show ways to improve the environmental impact of soil remediation. We will discuss the merits of various treatment techniques from this perspective, and point to areas where the environmental performance may be improved. Two simple evaluation models are applied to a petrol filling station as an example of environmental cost assessment. We conclude with aspects to consider which will help readers to improve their own soil treatment actions. The environmental cost is often calculated using various life cycle assessment methods (LCA). Many of our conclusions are based on LCA and LCA-related reasoning. The LCA method and problems encountered while applying the LCA method to contaminated soil are discussed in detail by Suer et al. (2004), and will not be addressed here.

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2.2 Better and Worse Treatment Choices

Unfortunately there is no general list of treatments in order of environmental cost. The costs depend on too many site-specific factors. Treatments can be ranked for a specific site, and this has been done for a number of sites (Table 2.1). Each study has used a consistent method for comparing treatments for the chosen site, while the methods themselves differ for the different sites. The exception is ScanRail Consult (2000a), which uses one method for comparing different technologies on different sites. Some general conclusions about better and worse treatments can be drawn from these site-specific studies.

A brief description of the impacts of the alternative remediation methods considered in the evaluations presented in Table 2.1 is given in the sections which follow. Some remediation methods have not been included in the reviewed studies, for example phytoremediation and monitored natural attenuation. These require

Table 2.1 Comparison of treatment techniques by various LCA methods

Techniques considered	Lowest environmental cost	Important factors	Reference
1. Isolation 2. Excavation and landfill 3. Soil washing 4. Vapour extraction 5. In situ bioremediation 6. No action	–	Land use Soil quality Discharge of chemicals	(Diamond et al. 1999)
1. Excavation and on site landfill 2. Surface sealing with asphalt 3. Excavation, soil washing, turning bed, thermal treatment and landfill	1	Energy consumption Soil quality	(Volkwein et al. 1999)
1. Covering and isolation 2. Ex situ thermal treatment 3. In situ anaerobic degradation 4. In situ aerobic degradation 5. No action	1, 3	Energy consumption Leakage of chemicals	(Vignes 1999)
1. Ex situ thermal treatment 2. Ex situ bioslurry 3. Ex situ soil washing 4. In situ electrodialysis	–	Energy consumption Transport of soil	(Ribbenhed et al. 2002)
1. Ex situ degradation 2. In situ biosparging 3. In situ bioventilation	2, 3, 5	Production of iron and concrete Transport of soil Use of backfill	(ScanRail Consult et al. 2000 a, b)

(continued)

Table 2.1 (continued)

Techniques considered	Lowest environmental cost	Important factors	Reference
4. Reactive wall 5. Biologically active wall			
1. Pump and air stripping 2. 1 with added activated carbon and in situ bioremediation with nitrate 3. 2 with hydrogen peroxide instead of nitrate	2	Energy consumption Production of electron acceptor	(Bender et al. 1998)
1. Pump and vacuum steam stripping 2. Pump and active carbon cleaning 3. No action	2	Energy consumption	(Vignes 1999)
1. Funnel and gate (steel wall) 2. Funnel and gate (bentonite/cement wall) 3. Pump and treat	2, 3	Steel amount Active carbon Pump energy	(Bayer and Finkel 2006)
1. Pump and treat 2. Bioslurping, bioventing and biosparging 3. Bioslurping, bioventing and chemical oxidation 4. Ex situ biopiles	2	Pavement treatment area	(Cadotte et al. 2007)
1.Pump and adsorption 2. In situ bioremediation	See Sect. 2.1.3	Energy use Groundwater use	(Andersson 2003)
1. On site biopiles 2. Ex situ biopiles	2	Site preparation and closure (site landfilling) Soil toxicity	(Toffoletto et al. 2005)

long timeframes that complicate the environmental evaluation. Many of the studies in Table 2.1 have been performed on sites where site development was imminent, which also excluded long-term remediation options.

2.2.1 Doing Nothing

Not acting is not necessarily best from a holistic environmental perspective. If the contamination remains in the soil, there is an impact on local human health and on the biosphere. The alternative of ‘no action’ was included in three cases in Table 2.1:

Diamond et al. (1999) and both cases described in Vignes (1999). In all these comparisons there were alternatives preferable to doing nothing, based on the environmental evaluation from a broader perspective than a local risk assessment. In other words: the environmental cost of the most benevolent treatment outweighed the cost of leaving the pollution in the soil. However, in none of the studies was 'no action' the worst alternative: when the entire environmental effect is taken into account, treating the contaminated soil can result in a net environmental cost, i.e., making things worse.

2.2.2 *In situ Bioremediation Can be Good or Bad*

In situ bioremediation can be both the best and the worst alternative for the environment. There is no need for excavation, transport and landfilling with in situ treatment, and consequently the environmental cost of these primary impact activities is avoided (see Sects. 2.2.4 and 2.2.5). On the other hand, there is an environmental cost through secondary impacts, such as producing wall materials, electron acceptors or other additives. These secondary impacts can add up to a considerable environmental cost. They are often excluded from traditional LCA since they are difficult to quantify within the LCA framework, but they are included in most studies dealing with soil remediation.

Another important aspect for bioremediation is the common need to pump down additives or air, or to pump up groundwater. Pumping uses energy, with concomitant consequences (see Sect. 2.4.1.1). The energy use can add up to a considerable impact when treatment times are long, as they often are for bioremediation.

The influence of energy use is illustrated in Sect. 2.3, where energy used for bioremediation or pump and treat remediation is a determining factor for choosing the best treatment option. The influence of chemical production is shown by Bender et al. (1998). The latter compared long-term groundwater extraction versus a combination of groundwater extraction and in situ bioremediation. Bioremediation was achieved by adding nutrients and an electron acceptor to the water before pumping it back into the soil. Bioremediation had the lowest impact on the environment when the electron acceptor was nitrate, but the impact was highest when the electron acceptor was hydrogen peroxide. Hydrogen peroxide use resulted in the highest energy demand, the highest waste production, and was disadvantageous from most other perspectives. Long-term groundwater extraction, without increased bioremediation, had an intermediate impact on the environment. In this case, pumping energy was a minor consideration.

2.2.3 *Other In Situ Methods: Manufacture of Materials*

The secondary impact can also be dominant for other in situ methods, such as funnel and gate systems, permeable reactive walls, or isolation through covering the site with low permeability materials (see also Sect. 2.4.1). The production of wall

and cover materials is an important source of environmental cost. Special attention should be paid to the use of iron and steel. Production of steel causes considerable secondary impact through the use of energy.

This is illustrated by ScanRail Consult (2000a) where two permeable reactive barriers for the treatment of chlorinated hydrocarbons on one site were compared. The barrier was either a continuous wall of iron filings (chemical degradation), or a series of wells where air mixed with methane was injected to increase biodegradation. The biodegradation barrier was the environmentally most beneficial, due to the environmental cost of steel production. The iron filings were made from scrap metal; otherwise the environmental cost would have been even higher (ScanRail Consult 2000a).

Another example is the use of steel or cement/bentonite for a funnel and gate system. In this case, a funnel of steel was created at the site of a former gas plant, to guide contaminated groundwater to the treatment at the gate. Environmental evaluation showed that the main environmental impact was due to steel production. When the steel walls were (hypothetically) replaced with cement/bentonite mixtures, environmental impact decreased to a level comparable with pump and treat (Bayer and Finkel 2006).

2.2.4 Excavation or Immobilisation: Surfaces and Transport

Ex situ treatments are generally intermediate alternatives from an environmental perspective. The impacts are even more site-dependent than for in situ techniques. In particular, the distance that the soil is transported to the treatment facility or landfill plays a major role. Generally a distance of 100–200 km is the limit at which other alternatives become extremely attractive. When an ex situ treatment is selected for a site, it is advantageous to minimise transport and choose an environmentally friendly transport option.

The importance of secondary processes (manufacture of materials, etc) is also prominent in the case of ex situ treatment and immobilisation. One of the major causes of environmental impact is the construction of a working surface for remediation. In particular, for on site remediation when the surface is only used once, the manufacture of the surface is important. The wear and tear on the surface in a permanent plant is also a cause of environmental impact through energy and material use for construction (Cadotte et al. 2007). The same applies to a low-permeability surface, constructed to decrease leaching from the soil and human exposure to the soil contaminants.

2.2.5 Landfilling

The disadvantages of surface construction and transport also apply to landfilling as a remediation option. But a third negative aspect of landfilling is the impact on

biodiversity. This is seldom included in evaluations, but when it is included the impact is considerable. The use of land for contaminated soil landfill entails that the land will not be available for other purposes or functions. Biodiversity is particularly threatened by lack of available land. If filling materials for the treated site are taken from another area, usually this area also suffers from a decrease in biodiversity. The comparative merit of landfilling as an option strongly depends on the value placed on surface use.

2.3 Case Study: Two Simple Models for a Petrol Filling Station

To further explain the concepts discussed above, we include the results from a case study. The case study had two aims: to compare two easy tools for holistic environmental assessment and to gain insight in the environmental cost from two treatment options. This was done by comparing two treatment techniques in two computer models, using approximately the same data set (Andersson 2003).

The treatment techniques that were compared were pump and treat with absorption (below called adsorption for short), and bioremediation. The computer models used are named REC and UvA, and have been developed for evaluation of environmental impacts caused by treatment of contaminated sites. Both models are based upon life cycle assessment thinking.

2.3.1 Site Description and Treatment Techniques

The data used is from the petrol filling station at Blackstad (Sweden) that was discontinued in 1980. A residential building and a car repair shop stand on the 2,000 m^2 estate. The soil was slightly contaminated with PAH (polycyclic aromatic hydrocarbons), benzene and aliphatics. The groundwater needed treatment, since it was used for irrigation and was contaminated with aliphatics, BTEX (benzene, toluene, ethyl benzene, xylene) and PAH.

Two alternatives were tested in a comparison of the environmental impact models. In one alternative the groundwater was pumped up, filtered, and released to a nearby ditch (the adsorption technique). The other alternative (the bioremediation technique) was enhanced biological degradation by adding bacteria, nutrients, and electron acceptors (air for a longer period, hydrogen peroxide for a short period) to the water and re-infiltrating. The techniques were simplified for the models, and cleanup times and results were assumed to be similar. This corresponds to evaluating the expected environmental performance prior to choosing a remediation technique.

2.3.2 The Case Models

The REC and UvA computer models were used to evaluate the environmental impact (Volkwein et al. 1999; Drunen et al. 2000). These are easy-to-use, West-European models. Unfortunately, no corresponding Swedish model was available. Complicated models would be preferable for an in-depth analysis of the environmental aspects of various treatments, but for everyday decisions simpler models are more suitable.

The REC model was used to assess environmental merit. The modules for Risk reduction and for (financial) Cost were not tested here. The UvA model (Umweltbilanz von Altlastensanierungsverfahren) has a more detailed LCA approach than the REC model, but does not include risk or financial assessment (Volkwein et al. 1999).

Realistic estimates of use of equipment and energy were available from the practical application of both techniques on the site. Detailed data is shown in Table 2.2 (REC) and Table 2.3 (UvA). This will also give you some idea of the data required to run the models. Input data is relatively simple since the models include average values for many processes, such as energy use and emissions from secondary processes like the production of machinery, pipes and hydrogen peroxide.

2.3.3 The Case Results

The REC and UvA models differed with respect to which treatment was the most advantageous. The REC model showed that the adsorption technique caused higher

Table 2.2 Data input for the REC model

Data category	Adsorption	Bioremediation
Current situation		
Quality objective (μg l^{-1})	100	100
Intervention value (μg l^{-1})[a]	100	100
Concentration of aliphates (μg l^{-1})	200	200
Volume of contaminated groundwater (m^3)	200	200
Treatment category		
Load (m^3a)	0.00001	0.00001
Consumed groundwater (m^3)	200	50
Volume of groundwater to pump (m^3)	200	50
Lifting height (m)	6	6
Volume groundwater to treat (m^3)	200	200
Waste (m^3)	0.0018	0
Land use (m^2)	5	5
Time requirement for remediation (a)	0.5	0.5

[a]Value to evaluate the risks of contaminated sites (Swedish guideline value)

Table 2.3 Data input for the UvA model

Data category	Adsorption	Bioremediation
Risk (before remediation/after remediation)[a]		
Relevant risk (Maßgebliches Risiko)[b]	5.3/4	5.3/4
Unsecured area	10.3/4	10.3/4
Area of the site (m^2)	2,000/0	2,000/0
Volume of contaminated groundwater (m^3)	200/0	200/0
The site is used as:	Residential area	Residential area
Demands		
Workdays to build the equipment (days)	3	3
Time requirement for the remediation (days)	180	180
Average density of the soil (t m^{-3})	1.8	1.8
Distance to settlement (m)	100	100
Land use (m^2)	5	5
Volume of soil to treat (m^3)	1	1
Volume of groundwater to treat (m^3)	200	200
Hydraulic pump		
Running time (days)	180	180
Pump rate (m^3/h)	0.05	0.01
Lifting height (m)	6	6
Reinfiltration (%)	0	100
Adsorption		
Total mass of hazardous substances (kg)	0.04	-
Concentration capacity of activated carbon (%)	5	-
The activated carbon after use is:	Disposed	-
Bioremediation		
Running time (days)	–	180
Sodium nitrate ($NaNO_3$) (kg)	–	0.5
Hydrogen peroxide (H_2O_2) (kg)	–	0.1

[a] Changes had negligible effect on the outcome of the model
[b] Acceptable risk level

environmental impact than the bioremediation. The categories in which the absorption technique caused more environmental costs were consumed groundwater, energy, air emissions and waste.

The category which had the most significant environmental costs, for both techniques, was land use. The land use in the case study was the area occupied by the equipment for the pump and treatment process (5 m^2). The reason that land use resulted in greatest environmental cost, even though 5 m^2 is not a big area, is that the techniques had a low impact generally, a consequence of the low contamination of the site.

The main reason for the higher environmental costs for the absorption technique in the REC model was that groundwater was released to a ditch after the adsorption treatment. In the bioremediation alternative the water was infiltrated back, and therefore no groundwater was consumed. Besides groundwater use, the adsorption treatment also consumed more energy. This was due to the fact that the total volume of contaminated groundwater was pumped up before treatment was complete,

while in the case of bioremediation only part of the groundwater was pumped up. Energy use leads to increased air emissions and waste as well, and the discarded filter material from the adsorption treatment also produced waste.

The UvA model showed on the contrary that bioremediation was inferior to adsorption, due to a much higher energy use. Detailed analysis showed that in the bioremediation treatment module, nitrate and hydrogen peroxide were added using a metering pump, and that the difference in energy use was due to the continuous functioning of this pump. The metering pump impact dominated any other differences, but groundwater loss was clearly visible as an important cost in the UvA model as well as in the REC model.

Activated carbon filter material and bioremediation additives were negligible in comparison with pump energy use and groundwater in the UvA model. This is contrary to the results from Bender et al. (1998), who found that production of additives was predominant in his similar comparison using the UvA model, though groundwater use also constituted a largely disadvantageous factor in their study.

The estimated energy use was four times lower in UvA then in REC for identical actions. The difference was probably caused by the different data sets, i.e., the activities and environmental impact parameters, as well as their values, that are included in the models.

2.3.4 Conclusions from the Case Study

The compared treatments had a low environmental cost generally. This was due to the low level of contamination of the site, and the low intensity of the remediation techniques that were used. The most significant difference between the techniques was due to energy use, but the models disagreed as to which technique was the most environmentally friendly.

The loss of groundwater was a notable factor in both models. This was not a surprise, since groundwater is a scarce resource in both Germany and the Netherlands. However, this is not the case in this Swedish region. The groundwater quality needed to be improved (through remediation), but the loss of 200 m^3 groundwater hardly constituted an important impact on the environment.

In summary, the models were helpful in identifying the important environmental effects from the treatment alternatives. Energy use, land use, and groundwater loss were important impacts, while additives and filter materials were not. The detailed results were influenced by the system boundaries, and the energy calculations in particular need to be adapted to the actual situation for a fair comparison of the treatment options.

In reality, the two treatments (adsorption and bioremediation) were used consecutively, starting with the adsorption technique. The contaminant concentrations did not decrease sufficiently using adsorption, and afterwards the bioremediation technique was initiated with successful results. The concentrations in the groundwater have now decreased to acceptable levels, and remediation activity has ceased.

The lower cleanup level resulting from the adsorption technique was ignored for the model and technique comparison, since it was not expected beforehand. Naturally, ineffective treatments should be avoided. Their environmental cost is not offset by a benefit, and thus they are only disadvantageous to the environment.

2.4 Improving Specific Remediations

Thus far we have discussed treatments in a general way (Sect. 2.2) and given a more detailed example of a comparison of treatment options (Sect. 2.3). Now we would like to provide direction for improving site-specific treatments. This need not be complicated or time-consuming. Sometimes a simpler checklist can be as relevant as a model. We include a checklist that can be a starting point when considering a treatment technique.

It is important to think through the entire chain of events and materials in order to do a holistic environmental impact assessment. Much improvement may be achieved simply through knowledge of the environmental effects, and awareness that remedial actions have an environmental impact. Therefore we describe the most significant environmental impacts below.

2.4.1 What to Consider

2.4.1.1 Energy

The use and source of energy is one of the environmental impacts of major importance in life cycle assessment. The source of the energy was also important in the case study. In this case it was possible to drive the pump through the Swedish electricity net (water power, nuclear power, and some fossil fuels). A solar cell may have been worth considering, and a fossil fuel aggregate as a power source may have increased the environmental cost considerably.

Energy use occurs in many activities and steps in soil treatment. Fossil fuel is commonly used as the energy source:

- It is the major source for the transport of soil, people and equipment to and from the site.
- The energy needed to drive pumps for in situ remediation is another common energy-demanding activity.
- The energy used to manufacture steel and hydrogen peroxide is a third activity where the energy demand is high and mostly based on fossil fuel.

2.4.1.2 Scarce Natural Resources

Evident scarce resources are soil and backfill, groundwater, fossil fuels and metals. The materials and additives discussed in Sect. 2.2 reoccur here. The manufacture of

the materials uses energy and scarce natural resources. This is an important factor, but for a really fair valuation it may need the application of LCA-like methods. It is difficult to assess the impact caused by manufacturing. For some products the reviews may have been done, or environmental assessments may have been done on some of the products which can be used as a basis. It would also be useful to have national and European guidelines, or guides summarising the environmental impacts available as a basis for simple but holistic environmental assessments, but for most products such information is unfortunately not yet available. Despite the lack of quantitative and supporting information, a qualitative discussion may be relevant regarding potential environmental impacts using different materials.

2.4.1.3 Land Use

Land surface is also a scarce resource, but its special nature has put it into a category by itself. Loss of ground surface is a major problem for maintaining biodiversity. Soil remediation may cause surface loss in various places: consider specifically the contaminated site itself, the area used for treatment or landfilling, and the area depleted by the production of backfill.

2.4.1.4 Emissions

Most forms of energy use cause emissions to air, and the result may be global warming, acidification, particle generation, photo-oxidant formation, eutrophication or human toxicity. Global warming is caused by carbon dioxide (CO_2) and other greenhouse gases, acidification is caused by emissions of sulphur oxides (SOx) and nitrogen oxides (NOx), photo-oxidants are caused by emissions of organic compounds (CO, VOC) and (NOx), eutrophication is caused by nitrate and phosphate emissions, and particulates are formed from non-efficient combustion in addition to dusting. The total and type of emissions depend on the emission source and the combustion efficiency.

Minimising the activities and steps using energy, and the energy need in each of the activities and steps involved, therefore is often the major step needed also to reduce many of the impacts on a regional to global scale which in general are due to air emissions. Most often, this is also profitable both from an economical and a general environmental perspective.

Emissions to soil and water may arise from the contaminants at the actual site and from additives used in the remediation process. Emissions from contaminated soil to the ground or water can also occur from the contaminated soil at a landfill or at a site for ex situ remediation. The extent of emissions release depends on the specific conditions. The more closed the system, the less uncontrolled the emissions. In controlled systems the contaminants are either trapped, destroyed or concentrated in processes, where on the other hand other environmentally cost-demanding steps, such as energy use, are involved.

The emissions to air, soil and water can also occur in different steps and activities involved in the production of additives or other products used in the remediation. As in the case of scarce materials, for some products environmental assessments may have been done that can be used, but for most products such information is unfortunately not yet available.

2.4.1.5 Human Exposure

Human toxicity may be due on the one hand to intake from the contaminated site, such as contaminated drinking water, but also due to emissions from the site, combustion and dust exposure caused by the remediation activities. The emissions from the site during remediation can be emissions of toxic gases released or the soil contaminants becoming more mobile and open for exposure during the remediation. Dusting increases while remediating, and contaminants may be carried by the dust. Treatment actions also cause increased noise and nuisance. Those should of course be included in an environmental risk assessment of remediation alternatives.

2.4.2 Tools to Use

Even simple efforts may lessen the environmental cost, despite the lack of information that would ensure a very fair environmental impact assessment. Simply by reading this chapter you may already have changed your next remediation. Taking a few hours to consider environmental impact can improve the result (but more time would be better). There are tools available to help with this, ranging from simple models to complete life cycle assessment.

If there is an opportunity to make a quantitative evaluation of remediation options, models should be chosen that were constructed for use in situations that resemble as closely as possible the current planned remediation. The source of the energy use is important, as is the value placed on land surface and groundwater resources. It should be checked that the models include these factors, since many traditional LCA models focus heavily on energy use. Other environmental costs may come unusually high in soil remediation, and need to be considered (Suer et al. 2004). Generally, local models are preferable, and the in data needs should correspond to the available in data to avoid excessive guessing. The reference list includes several options.

Otherwise a qualitative evaluation may be suitable. A structured qualitative approach is life cycle management (LCM) in four steps described by Diamond et al (1999). In the first step, goal and audience for the LCM are identified, and the processes are described for the entire remediation (including for example secondary materials, contaminant concentrations, and activities to close the site). In the second step, the processes are associated with potential impacts, for example energy use and waste production. Noise and other nuisance can also be included.

All these impacts are ranked as low, moderate or high. The impacts are evaluated in the third step to decide on actions to lessen the environmental impact, and applied in the fourth step.

2.5 Getting started, what to consider:

Energy

Transport
Pumping
Manufacture of additives
Manufacture of materials

Scarce natural resources

Soil and backfill
Groundwater
Fossil fuels
Metals

Land use

Landfill, temporary storage, working area
The time perspective is important

Emissions

To water (contaminants, chemicals, additives)
To air (mainly due to transport and energy use, dust)

Human exposure

Contaminated site
Working environment during remediation
Transport emissions
Noise and nuisance

2.6 Conclusion

There is great room for improvement in everyday remediation, since the holistic environmental aspects are often ignored completely today. Even a limited review of the environmental impact can indicate which techniques to avoid, and where there is potential for improvement. Such a review should consider the entire chain of the remediation. The list in Sect. 2.4.2 may be helpful in making it.

Research continues on environmental impacts, using more complete and complicated methods than what is possible in everyday remediation. The results of

these detailed studies will help to further determine the major areas where improvement is desirable/a priority. Use of energy, secondary materials (especially surfaces and electron acceptors), and land use have been identified so far as major impact parameters in a holistic environmental assessment of contaminated land.

Soil remediation measures may have an overall negative impact worse than doing nothing and leaving the contaminants in the soil. But usually there is a more beneficial alternative available. Experience and a good knowledge base are required to identify and exploit those possibilities. It is recommended when doing the first holistic environmental assessment to involve, or rely on someone experienced in life cycle assessments to ensure the robustness of the evaluation.

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Chapter 3
Bioavailability of Contaminants in Soil

Joseph J. Pignatello

3.1 Introduction

Bioremediation, whether carried out by stimulated indigenous organisms or added organisms, is potentially less costly and less environmentally disruptive than many physical or chemical methods of remediation. In some cases, natural attenuation may be the strategy of choice for site management; transformation by the natural microflora is usually an important, if not dominant mechanism of natural attenuation.

An assessment of the feasibility of bioremediation as an approach to the cleanup of contaminated soils and sediments (hereafter, collectively referred to as soils), or the choice of natural attenuation, requires a comprehensive understanding of both the biology and soil matrix effects. Biology refers to the intrinsic ability of the biota to assimilate and metabolize the contaminant. Matrix effects include the ways in which biodegradation is influenced by the interactions of the soil with the biota and the contaminants. Bacteria in soils are predominantly attached to soil particles, and so will be constrained by this attachment and by the physico-chemical properties of the surface. Contaminants interact with soils in complex ways through sorption and mass transfer resistance that generally impede their availability to organisms.

The focus of this chapter is on the processes and geochemical conditions that influence the *bioavailability* of organic contaminants in the soil matrix to microorganisms. To some degree, the principles discussed will apply to plants as contaminant removers in phytoremediation, but much less information is available on this subject. In the broader context, bioavailability is important in deciding "how clean is clean" — that is, in deciding what level of contaminant is protective of humans or other organisms determined to warrant protection (Linz and Nakles 1997). The chapter is meant to be a perspective of the author rather than an exhaustive

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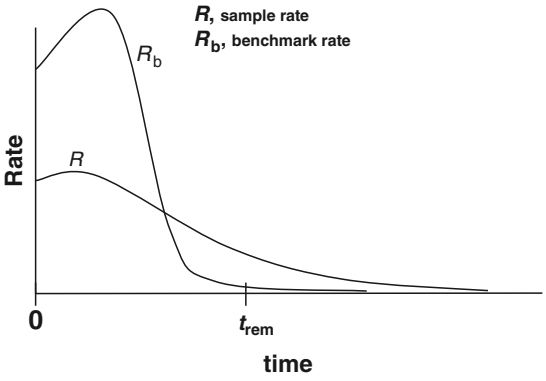
review of the topic. It will be concerned only with compounds, or their fractions, that undergo physisorption to soil components — interactions involving the weak forces of dispersion, dipolar interactions and hydrogen bonding. It thus excludes compounds or their fractions that chemisorb through covalent or coordination bonding.

The definition of bioavailability depends on the context and the reference organism (Harmsen 2007; NRC 2003; Semple et al. 2007). In the context of soil remediation, this chapter proffers the definition of *bioavailable fraction* simply as the ratio of mass removed after a given remediation time t_{rem} by an active population in a soil sample, relative to the mass removed in a benchmark system (water) having the same initial chemical mass, volume of water, and initial active population as the sample. It may be expressed as the ratio of areas under the respective curves of removal rate [M $M^{-1}T^{-1}$] vs time integrated over t_{rem} in (Fig. 3.1):

$$f_{\text{bioavail}} = \frac{\int_0^{t_{\text{rem}}} R(t)\mathrm{d}t}{\int_0^{t_{\text{rem}}} R_{\text{b}}(t)\mathrm{d}t}. \tag{3.1}$$

(Generic units used hereafter: M is mass, T is time, and L is length.) Integrated over infinity, the value of f_{bioavail} corresponds to the (ultimately) *bioaccessible fraction* defined by Semple et al (2004, 2007). No normalization is applied to account for differences in degrader population between sample and benchmark that may develop during the course of degradation; as such differences arguably can be regarded as part of the soil matrix effect. The value of f_{bioavail} may be practically difficult to obtain when



$$f_{\text{bioavail}} = \frac{\int_0^{t_{\text{rem}}} R(t)dt}{\int_0^{t_{\text{rem}}} R_{\text{b}}(t)dt} \quad (1)$$

Fig. 3.1 The bioavailable fraction is defined as the ratio of degraded masses for the sample and benchmark systems, which is equivalent to the ratio of areas under the respective degradation rate vs time curves over the remediation time t_{rem}. Rates may increase at intermediate times due to growth

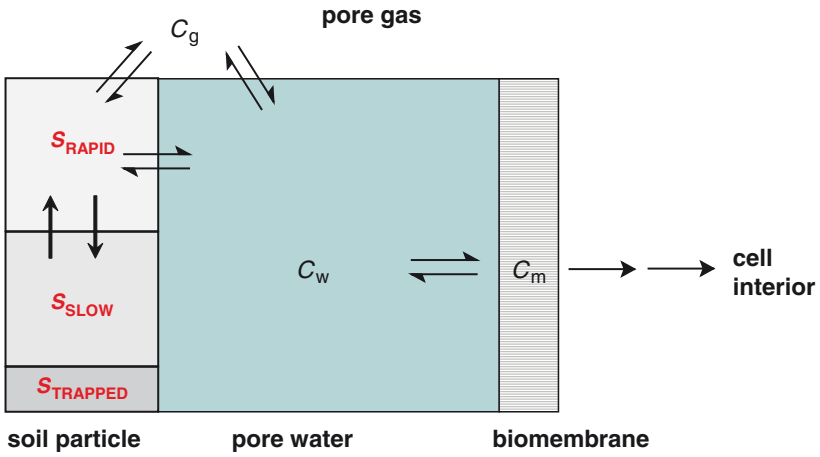


Fig. 3.2 Schematic of the distribution of a contaminant among soil particle, pore water, pore gas and biological membrane phases. Contaminant in the soil particle is arbitrarily partitioned into rapid, slow and trapped states. Realistically, there may be a continuum from instantly exchangeable to completely unexchangeable with the dissolved contaminant

the approach involves biostimulation of native organisms, because such organisms cannot quantitatively be isolated for use in a benchmark experiment. Nevertheless, the definition is simple, conceptually satisfying, and more quantitative than other definitions in the literature. We will see how both thermodynamic and kinetic behavior of the contaminant influences the value of $f_{bioavail}$.

The discussion starts from the twin premises, depicted in Fig. 3.2, that: (1) only molecules in the fluid phases of soil (i.e., the soil pore water and pore gas phases) are capable of interacting with a biological membrane, hence the cell, and (2) sorption is a key process regulating the fluid phase concentration surrounding the biological membrane. The first premise brings up the possibility of *facilitated bioavailability*, in which organisms may gain access to pools of contaminant that in the absence of the biota do not desorb into the fluid phases. That issue will be discussed in Sect. 3.3.7. We do not have to consider direct access from the gas phase if we assume either the condition of equilibrium between pore water and pore gas phases, or that biomembranes are coated with a film of water; both assumptions are reasonable for most soil environments.

Contaminant will be distributed between the sorbed and solution states (Fig. 3.2). In many cases, the contaminant will be largely sorbed and the concentration in the pore water relatively low. Uptake of contaminants by microbial cells is assumed to be passive for most compounds, and so the contaminant will distribute between the pore water and the cell membrane. The contaminant is ultimately transported within the cell to an enzyme active site where it is transformed. At relatively low pore water concentrations, and assuming passive uptake, the bulk rate of removal per unit mass of soil R will be proportional to the contaminant concentration within the membrane C_m [M L^{-3}]. This relationship may be expressed by

$$R = k_{int}\,\varphi C_m B, \tag{3.2}$$

where k_{int} [T^{-1}] is the rate constant for the limiting internal metabolic process, φ [L^3 M^{-1}] is the membrane content of cells, and B [M M^{-1}] is the microbial density in the soil, which may be time-dependent.

As the membrane is microscopic in width and always in contact with water, it is reasonable to assume local equilibrium between the membrane and the surrounding aqueous phase. Equation (3.2) may then be re-written in terms of the membrane–water partition coefficient K_m [L^3 L^{-3}] ($C_m = K_m C_w$) and the soil pore water concentration C_w [M L^{-3}]:

$$R = k_{int}\,\varphi(K_m C_m)B. \tag{3.3}$$

As the concentration in solution increases, biodegradation becomes subject to saturation kinetics expressed in the Monod model, which couples biodegradation to growth:

$$R = \frac{k_{int}\varphi K_M (K_m C_w)B}{K_M + C_w}, \tag{3.4}$$

$$\frac{\mathrm{d}B}{\mathrm{d}t} = Y_S R + Y_{UOM} r_{UOM} - \lambda B, \tag{3.5}$$

where K_M [M L^{-3}] is the Monod half-saturation constant (the solution concentration at half the maximum substrate utilization rate), $\mu_{max} = k_{int}\,\varphi(K_M K_m)$, Y_s [M M^{-1}] and Y_{UOM} [M M^{-1}] are the specific bioconversion factors for growth on the substrate and utilizable natural organic matter (UOM) respectively, r_{UOM} [M M^{-1} T^{-1}] is the rate of utilization of UOM, and λ [T^{-1}] is the first-order decay coefficient to account for predation and soil inactivation of degrader cells. Cell growth on UOM and cell decay in soils are poorly understood and not easy to parameterize.

An organism placed in contact with soil will initially experience the existing local pore water concentration. As degradation proceeds, the rate will depend on the opposing effects of active removal of contaminant by cells, driving the local pore water concentration down, and desorption from the soil, driving it up. At the extremes are the situations where: (1) the aqueous phase is continually in equilibrium with soil, so that intracellular transformation (or transport) is always rate-limiting, and (2) desorption from soil is always rate-limiting. These extremes reflect thermodynamic and kinetic control of bioavailability, respectively.

In Fig. 3.2 the sorbed concentration has been divided into rapid, slow, and ‘trapped’ fractions with respect to the facility of exchange of these fractions with fluid. Mathematically it does not matter whether the slow fraction exchanges with the fast fraction or directly with the fluid. Trapped implies a condition of unavailability such that these molecules could not leave the soil within the reference timeframe. In reality, soil sorption kinetics represents a continuum from instantaneous exchange to fix in the solid. Thus, it will be apparent that it is important to consider both thermodynamic and kinetic control of bioavailability.

3.2 Bioavailability Under Thermodynamic Control

For the case of soil–water equilibrium, we consider the time-invariant soil–water distribution ratio K_d [L^3 M^{-1}]:

$$K_d = \frac{S}{C_w}, \tag{3.6}$$

where S [M M^{-1}] is the sorbed concentration on a dry soil weight basis. The rate expression in (3.3) may then be re-written as:

$$R = k_{int}\, \varphi (K_{m\text{-}s} S) B, \tag{3.7}$$

where $K_{m\text{-}s}$ [M L^{-3}] is the membrane–soil distribution ratio equal to K_m/K_d or C_m/S.

For nonionic compounds which sorb predominantly to the soil organic matter (SOM) fraction of soils,

$$R = k_{int}\, \varphi (K_{m\text{-}OC}\, s_{OC}) B, \tag{3.8}$$

where $K_{m\text{-}oc}$ [M L^{-3}] and S_{oc} [M M^{-1}] are indexed to the mass fraction of organic carbon, f_{oc}, a surrogate for SOM fraction ($S_{oc} = S/f_{oc}$; $K_{oc} = K_d/f_{oc}$; $K_{m\text{-}oc} = K_{m\text{-}s}/f_{oc}$). One can see that $K_{m\text{-}s}S$ or $K_{m\text{-}oc}S_{oc}$ may similarly substitute for $K_m C_w$ in the Monod equations (3.4) and (3.5).

3.2.1 Structure Activity Relationships

An important question is how the rate of removal depends on compound structure. Obviously it depends on k_{int}, which is a function of the biochemical mechanisms associated with transport and transformation within the organism. But it also depends on $K_{m\text{-}(s,oc)}$. Thermodynamically controlled bioavailability is, in essence, a competition between soil and the biomembrane for partitioning. We can gain insight into this competition by examining linear free energy relationships (LFERs) developed to predict environmental partitioning in terms of molecular properties. The most commonly-used LFER for partitioning of nonionic compounds is the octanol LFER, which takes the form of a linear correlation between log K_x and log of the 1-octanol–water partition coefficient K_{ow} [L^3 L^{-3}]. The octanol LFER mainly reflects the influence of hydrophobic effects on partitioning — that is, expulsion from water due primarily to disruption of the H-bonded structure of water by the hydrophobic portion of the solute molecular surface. Values of K_{ow} are available for thousands of compounds, and may be calculated to reasonable accuracy for others using functional group and connectivity parameters (e.g., http://www.syrres.com/esc/kowdemo.htm). The single-parameter octanol LFER is often satisfactory for a closely-related series of nonionic compounds, although recently-developed polyparameter LFERs that take into account the multiplicity of forces possible in partitioning

are more accurate for groups of less closely-related compounds (Goss and Schwarzenbach 2001; Nguyen et al. 2005; Niederer et al. 2006a, b).

Numerous OC–octanol LFERs are available in the literature for sets of related compounds (Schwarzenbach et al. 2002). Since partitioning to real biomembranes is experimentally difficult to measure, most work has been done using biomembrane surrogates, especially bilayer phospholipid vesicles (liposomes) self-assembled from phosphatidylcholine monomers such as dimyristoylphosphatidylcholine (DMPC), dioleylphosphatidylcholine (DOPC), and dipalmitoylphosphatidylcholine (DPPC) (Escher and Schwarzenbach 1996) (Gobas et al. 1988; Kwon et al. 2006; Patel et al. 2002; Vaes et al. 1997). It is found that log K_m-log K_{ow} plots are essentially linear for related compounds in the range log K_{ow} 1–5.5. However, the LFER seems to break down for bulky compounds, e.g., polychlorinated biphenyls (PCBs) (Dulfer and Govers 1995; Gobas et al. 1988) and estrogenic compounds (Yamamoto and Liljestrand 2004) — which tend to sorb more weakly than predicted by K_{ow}. That is most likely because the energy penalty for forming an accommodation cavity in the membrane becomes appreciable as the molecule reaches a certain size (Gobas et al. 1988; Kwon et al. 2006).

Combining the OC–octanol LFER with the membrane–octanol LFER gives

$$\log K_{m\text{-}oc} = (a_m - a_{oc}) \log K_{ow} + (b_m - b_{oc}), \tag{3.9}$$

where a_m and b_m are the slope and intercept of the membrane–octanol LFER, and a_{oc} and b_{oc} are the slope and intercept of the OC–octanol LFER. Table 3.1 lists the slopes and intercepts of (3.9) for available data sets in which the correlation between K_{ow} and both K_{oc} and K_m is reasonably strong (i.e., below log K_{ow} ~5.5).

From these data the following can be said. (1) After multiplying the values of $K_{m\text{-}oc}$ in Table 3.1 by the presumed density of OC of ~ 2 g cm^{-3} to obtain a unitless partition coefficient, one can see that the solute tends to slightly favor the membrane over the OC phase. (2) $K_{m\text{-}oc}$ is similar for polar and apolar compounds. (3) The slopes of equation (3.8) are shallow (−0.269 to 0.25), indicating that membrane–OC partitioning is almost independent of hydrophobic character — the $K_{m\text{-}oc}$ changes by one order of magnitude or less over 4.5 orders of magnitude range in K_{ow}. This is contrary to conventional wisdom, which holds that bioavailability decreases with increasing hydrophobic character. If that statement is true, it must be largely for kinetic, not thermodynamic reasons.

Notwithstanding the correlations in Table 3.1, SOM is not a homogenous material that is uniform in its sorption properties. SOM contains macromolecular substances of different chemical and physical composition and in different stages of diagenesis. In addition, SOM contains micrographitic substances, referred to as black carbon, including soot and char that originate from pyrolysis of biomass and fuels. Figure 3.3 shows the $K_{m\text{-}oc}$ for naphthalene, using DMPC as the membrane surrogate and three types of soil organic substances spanning the full range of sorption behavior expected for nonionic compounds. The Amherst humic acid represents young (“soft”) macromolecular humic substances that typically give the weakest sorption. The lignite (a brown coal) represents geologically older, “harder”

Table 3.1 Correlation between $K_{m\text{-}oc}$ and K_{ow} (3.9) for several published data sets

Liposome system[a]	Soil system	a_m–a_{oc}	b_m–b_{oc}	$K_{m\text{-}oc}$ @ log K_{ow} = 1	$K_{m\text{-}oc}$ @ log K_{ow} = 5.5
20 neutral phenols in 8:2 DOPC/DPPC (log K_m = 0.815 log K_{ow} – 0.989; r^2 = 0.958) (Escher and Schwarzenbach 1996)	21 polar compounds in soil (log K_{oc} = 0.73 log K_{ow} – 0.52); (Nguyen et al. 2005)	0.085	–0.469	0.41	1.0
25 polar and apolar compounds, incl. 10 chlorinated benzenes in DMPC (log K_{mw} = 1.19 log K_{ow} – 0.645); (Gobas et al. 1988)	11 PAHs in soil (log K_{ocw} = 1.14 log K_{ow} – 1.02); (Nguyen et al. 2005)	0.05	0.375	2.7	4.5
25 polar and apolar compounds, incl. 10 chlorinated benzenes in DMPC (log K_{mw} = 1.19 log K_{ow} – 0.645); (Gobas et al. 1988)	33 halogenated hydrocarbons in soil (log K_{ocw} = 0.94 log K_{ow} – 0.43); Nguyen et al. (2005)	0.25	–0.215	1.1	14
19 polar compounds in DMPC (log K_{mw} = 0.904 log K_{ow} + 0.515); (Vaes et al. 1997)	21 polar compounds in soil (log K_{oc} = 0.73 log K_{ow} – 0.52); (Nguyen et al. 2005)	0.174	–0.005	1.5	9.0
47 polar and apolar compounds combined from published data sets in DMPC (log K_m = 0.761 log K_{ow} + 0.622, r^2 = 0.93); (Patel et al. 2002)	117 apolar hydrophobic compounds (log K_{OC} = 1.03 log K_{OW} - 0.61); (Seth et al. 1999)	–0.269	1.23	9.2	0.57

[a]Dimyristoylphosphatidylcholine (DMPC), dioleylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), polycyclic aromatic hydrocarbons (PAHs); K_{oc} is in units of L kg^{-1}; K_m is unitless and assumes the density of vesicles is 1.014 g cm^{-3}; K_{ow} is unitless; $K_{m\text{-}oc}$ is in units of kg L^{-1}

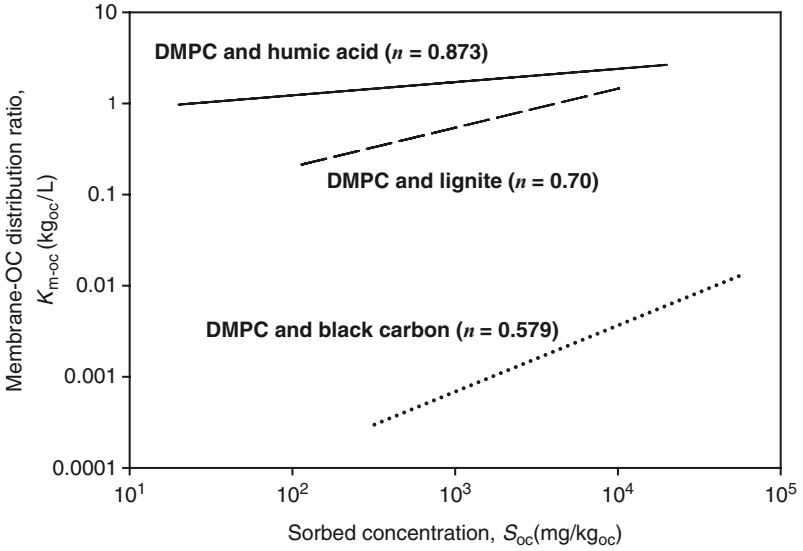


Fig. 3.3 The dimyristoylphosphatidyl choline (DMPC) vesicle membrane–organic carbon distribution ratio for naphthalene in different SOM as a function of sorbed concentration calculated over the experimental concentration range used in the soil-water sorption experiments. The value of K_m was calculated from the K_{ow} LFER of (Gobas et al. 1988) listed in Table 3.1. SOM sorption was represented by Freundlich fits. Humic acid was extracted from Amherst peaty soil (Lu and Pignatello 2004b). Lignite is represented by Beulah–Zap lignite (65.85% OC) (Zhu and Pignatello 2005b). Black carbon is represented by wood char (71.97% OC) (Zhu and Pignatello 2005a)

humic substances that tend to sorb organic compounds more strongly than young humic substances. The char is representative of black carbon, which typically gives the strongest sorption because it is highly microporous and contains less polar functionality than macromolecular forms of SOM. Figure 3.3 reveals that thermodynamically controlled bioavailability (as reflected in $K_{m\text{-}oc}$) decreases in the order humic acid > lignite > > black carbon, showing up to four orders-of-magnitude difference. Addition of strong carbonaceous adsorbents like activated carbon reduces the rate of mineralization of spiked chemicals (Rhodes et al. 2008). Indeed, addition of activated carbon to estuarine sediments has been suggested as a remediation tool to reduce the bioavailability of PCBs to benthic organisms (Cho et al. 2007; Sun and Ghosh 2007).

3.2.2 Concentration Dependence

The distribution ratios K_m and K_d are not necessarily invarient with concentration. Partitioning of nonionic compounds (or the neutral form of ionizable compounds) to liposomes is typically linear with concentration, except at concentrations approaching

water solubility (Escher and Schwarzenbach 1996). This is not surprising, as the liposome represents a relatively fluid three-dimensional "phase." However, future research may reveal that sorption nonlinearity is more important for real membranes, which contain embedded or overlain proteins and lipoproteins that may favor more specific sorptive processes. In addition, active transport processes are likely to be concentration-sensitive.

Sorption to soils and SOM isolates is most often nonlinear, giving a trend of weaker interaction as concentration increases. In many cases nonlinearity is not great, and so the isotherm is fitted to a linear model for simplicity. Construction of detailed isotherms over many orders of magnitude in concentration on a high OC content soil for a variety of polar and apolar compounds revealed a tendency toward linearity at both very low and very high concentrations (Xia and Pignatello 2001). The sorption of benzene to char black carbon was highly nonlinear overall, but tended toward linearity at very low concentration (Braida et al. 2003).

The most generally applied nonlinear model for nonionic compounds in soils is the Freundlich model:

$$S_{oc} = K_{F,oc} C_w^n, \tag{3.10}$$

where K_F is the affinity parameter and n (usually, $0 \le n \le 1$) is the linearity parameter. Expressed in terms of either C_w or S_{oc}, the expression for $K_{m\text{-}oc}$ then becomes

$$K_{m\text{-}oc} = \frac{K_m}{K_{F,oc}} C_w^{1-n} = \frac{K_m}{K_{F,oc}^{1/n}} S_{oc}^{(1-n)/n}. \tag{3.11}$$

For compounds that undergo ion exchange, the Langmuir model,

$$S_{oc} = \frac{S_{oc}^0 K_L C_w}{1 + K_L C_w}, \tag{3.12}$$

where S^0 [M M^{-1}] and K_L [L^3 M] are the Langmuir maximum capacity and affinity parameters respectively, often works best, giving

$$K_{m\text{-}oc} = \frac{K_m (1 + K_L C_w)}{S_{oc}^0 K_L}. \tag{3.13}$$

Equations (3.11) and (3.13) predict thermodynamic bioavailability (as $K_{m\text{-}oc}$) to increase with initial contaminant concentration in the soil depending on the degree of nonlinearity in the soil–water isotherm. Hence, bioavailability will decrease as the contaminant is progressively removed during bioremediation. Figure 3.3 shows that, whereas the effect is relatively small for a membrane in proximity to a more linear-sorbing phase such as humic acid (factor of ~2.7 change in $K_{m\text{-}oc}$ over the experimental concentration range of 4 orders of magnitude; n= 0.873), the effect can be quite substantial near more nonlinear-sorbing phases such as lignite (factor of ~7; n= 0.70) or black carbon (factor of ~43; n= 0.579).

Other sorption models, and their combinations, have been used (Haws et al. 2006a; Hinz 2001) but the predicted outcome is quite analogous: an increase in bioavailability with concentration. It should be pointed out that sorption is always linear in the limit of infinite dilution. In this sense the Freundlich model is not realistic at very low concentration, because it predicts ever-increasing affinity as concentration approaches zero.

3.2.3 Competition by Co-Solutes

Nonlinearity ($n < 1$) in the isotherm implies a distribution of site energies; that is, a specificity of sorption. When additional solutes are present, they may compete with the principal solute for these sites and thereby suppress the K_d or K_{oc} of the principal solute. This follows from the realization that nonlinear sorption is a manifestation of self-competition. Competition between the principal solute and a co-solute at a given site requires mutual access to the site, and the degree of competition depends on the relative affinities of each solute for the site. Competitive effects are important, because contaminant mixtures occur more frequently in the environment than single contaminants. Competitive sorption is reported in soil and sediment systems containing multiple contaminants (McGinley et al. 1993, 1996; Pignatello 1991; White et al. 1999; Xing et al. 1996; Xing and Pignatello 1997, 1998) and can be predicted by established competitive models (Xing et al. 1996; Zhao et al. 2001, 2002). Competition seems to be greatest between compounds of similar size and functionality (Pignatello 1991; Xing et al. 1996). Competitive effects have also been observed between contaminants and naturally-occurring aromatic acids in soil (Xing and Pignatello 1998), and between contaminants and humic substances on black carbon surfaces (Pignatello et al. 2006).

Competitive sorption in liposomes may be expected to be weak or nonexistent, because the isotherms of the single solutes are typically linear. Competition has been reported between endocrine disruptor compounds and cholesterol (Kwon et al. 2006), but occurred at very high concentrations of cholesterol (56% mole fraction), where the liposome structure was likely altered. It may turn out that real membranes show compound-specific behavior in some cases due to specificity conferred by the presence of embedded proteinaceous molecules, or to active transport systems.

The model most successfully used for competitive sorption is Ideal Adsorbed Solution Theory (IAST) (Radke and Prausnitz 1972). IAST assumes that all solutes have access to sites, and that under dilute conditions the adsorbed phase forms an ideal two-dimensional "solution". Equations giving the sorbed concentration of one solute in the presence of others, based on the independently-obtained single-solute isotherm parameters (e.g., Freundlich, Langmuir) of each solute, are given in the literature (Crittenden et al. 1985; Radke and Prausnitz 1972).

Suppression of $K_{d(oc)}$ through competition will lead to an increase in the $K_{m\text{-}s(oc)}$ of the principal solute, and therefore enhanced bioavailability of the principal solute

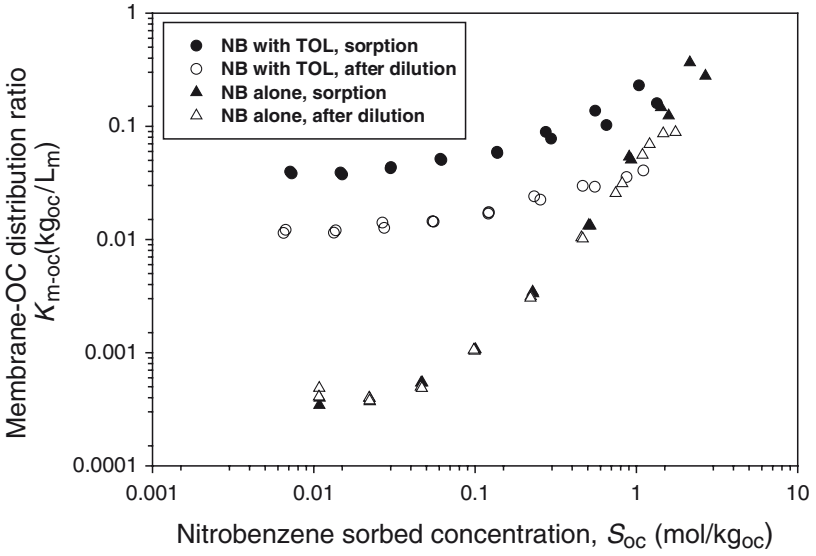


Fig. 3.4 Competitive effect and converse competitive effect on the thermodynamic bioavailability of nitrobenzene (NB) using toluene (TOL) co-solute. Sorbents are DMPC membrane (Vaes et al. 1997) and char black carbon (Sander and Pignatello 2005a, 2007). Sorption of nitrobenzene alone (*filled triangles*), followed by single-step desorption after diluting the supernatant by 10 (*open triangles*). Sorption in the presence of toluene at 4.23 x 10^{-3} mol L^{-1} initial concentration (*filled circles*), followed by single-step desorption after diluting the supernatant by 10 (*open circles*)

through (3.7)–(3.8) and related equations. Conversely, one can expect that if a co-solute were to be lost from the system — whether biotically or abiotically — the bioavailability of the principal solute would decline due to mitigation of competitive pressure for sorption sites. Competition also increases the linearity of the principal solute isotherm, which will make bioavailability of the principal solute less sensitive to concentration.

Figure 3.4 illustrates the effects of competitive sorption on thermodynamically-controlled bioavailability as reflected in the value of $K_{m\text{-}oc}$ for nitrobenzene with and without toluene. DMPC is the membrane and black carbon is the OC source. Analysis of the char–water sorption isotherms indicated that these compounds have fully-overlapping sorption domains on the char (Sander and Pignatello 2005a). The single-solute sorption isotherms of nitrobenzene showed only minor hysteresis; therefore, $K_{m\text{-}oc}$ is seen to be nearly the same in the sorption and desorption directions (*filled* and *open triangles*, respectively). Addition of a high concentration of toluene (*filled circles*) increases the $K_{m\text{-}oc}$ of nitrobenzene by as much as two orders of magnitude, depending on nitrobenzene sorbed concentration. Competition also reduces the concentration dependence of $K_{m\text{-}oc}$ for nitrobenzene by reducing the nonlinearity of its isotherm. Lastly, Fig. 3.4 illustrates the converse competitive effect brought about by diluting the supernatant with clean liquid (*open circles*): $K_{m\text{-}oc}$ decreases by a factor of 3–6 after a 10-fold dilution, due to relief of competitive pressure by toluene.

Recent studies have shown that humic substances (Kwon and Pignatello 2005; Pignatello et al. 2006) and metal ions (Chen et al. 2007) compete with organic compounds for sorption sites on black carbon. For example, flocculation of humic acid on surfaces of fresh char by addition of Al salts reduces the adsorption of phenanthrene to the char by up to two orders of magnitude depending on concentration (Pignatello et al. 2006). Therefore, it may be expected that black carbon contributes less to total sorption in environmental samples than predicted by experiments using soot and char reference materials, due to weathering effects (Cornelissen and Gustafsson 2004; Jonker et al. 2004).

Direct evidence of bioavailability enhancement in the presence of a competing solute has been reported (White et al. 1999). Mineralization of phenanthrene by a *Pseudomonas* sp. enrichment culture in two different soils was enhanced after adding pyrene, a nonbiodegradable substrate for this organism. Parallel experiments using sterilized soils showed that addition of pyrene partially displaced phenanthrene into solution, reducing its K_d by up to 83% (White et al. 1999). To the author's knowledge, no biological experiments have been performed to validate the converse competitive effect hypothesis — that bioavailability decreases after removing a competing co-solute.

Co-solute effects may also play important roles in the biology. Co-solute may affect degradation of the principal solute by: (a) dual-substrate enzyme inhibition mechanisms (for example, competition for enzyme active sites), (b) rate enhancement through co-metabolic effects, and (c) causing biomass decay as a result of toxic byproduct formation. Modifications of the Monod equation to take these roles into account have been reviewed (Haws et al. 2006a, b).

3.2.4 Effect of True Hysteresis

In most transport and bioavailability models, physisorption is assumed to be thermodynamically reversible. However, 'irreversible' sorption is possible if sorbate and sorbent interact to form a persistent metastable complex. In such cases, sorption and desorption follow different microscopic pathways, resulting in true hysteresis, or nonsingularity, manifested as a shift of the desorption branch of the isotherm towards greater sorption intensity. Irreversibility in this thermodynamic context does not necessarily imply irretrievability.

Two mechanisms of true hysteresis for physisorbing organic compounds are relevant to soils. One is capillary condensation hysteresis of vapors in mesopores (Rouquerol et al. 1999). The vapor initially condenses as a metastable film on pore walls. At a certain pressure, the film collapses to the thermodynamic plug state. The vapor pressures over the film and plug meniscus are different. As a result, the isotherm is different in the pressurization and de-pressurization directions, and shows a characteristic 'closed-loop' shape.

'Pore deformation hysteresis' is another mechanism of two hysteresis. It is characteristic of glassy organic solids. SOM shows properties characteristic of the

glassy state (DeLapp and Leboeuf 2004; DeLapp et al. 2005; LeBoeuf and Weber 2000; LeBoeuf and Zhang 2005; Schaumann and LeBoeuf 2005). Pore deformation hysteresis occurs by irreversible (inelastic) swelling of the solid by the penetrant over a sorption–desorption cycle. Inelastic swelling leaves the solid with a greater unrelaxed free volume (number and/or size of voids), which increases sorption affinity of sorbent for solute in a subsequent experiment, such as desorption or repeat sorption. Pore deformation hysteresis has been demonstrated for SOM (Lu and Pignatello 2002, 2004a; Sander et al. 2006; Sander and Pignatello 2005b). Swelling and pore deformation leading to enhanced sorption (up to 3-fold) has been shown both in sorption–desorption experiments and in 'conditioning effect' experiments in which the sorption isotherm is constructed before and after preconditioning the sample with the same or a similar compound.

Figure 3.5 shows how pore deformation hysteresis can lead to a decrease in the $K_{m\text{-}oc}$. Figure 3.5a plots $K_{m\text{-}oc}$ corresponding to the sorption and single-step desorption points in a soil of 1,4-dichlorobenzene, with the *arrows* directing the eye from the replicate sorption to the replicate desorption data clusters. Figure 3.5b shows 1,3-dichlorobenzene in solid humic acid before and after preconditioning the humic acid with a high concentration of chlorobenzene.

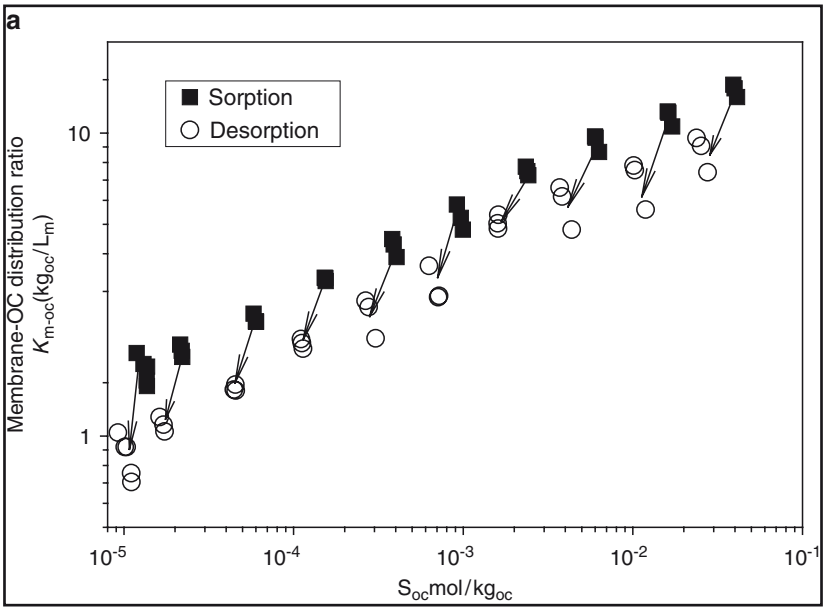


Fig. 3.5 Illustration of the effect of irreversible sorption on membrane–soil OC partitioning. Membrane partition coefficients are experimental values for DMPC vesicles (Gobas, 1988). **a** Sorption-desorption hysteresis of 1,4-dichlorobenzene in Pahokee peat adapted from (Pignatello 2006) with permission. *Arrows* indicate path between sorption and single-step desorption triplicate data clusters. **b** Sorption of 1,3-dichlorobenzene in Amherst humic acid before and after conditioning of humic acid with chlorobenzene. Sorption parameters are from (Sander et al. 2006). The *lines* are data fits to (3.11)

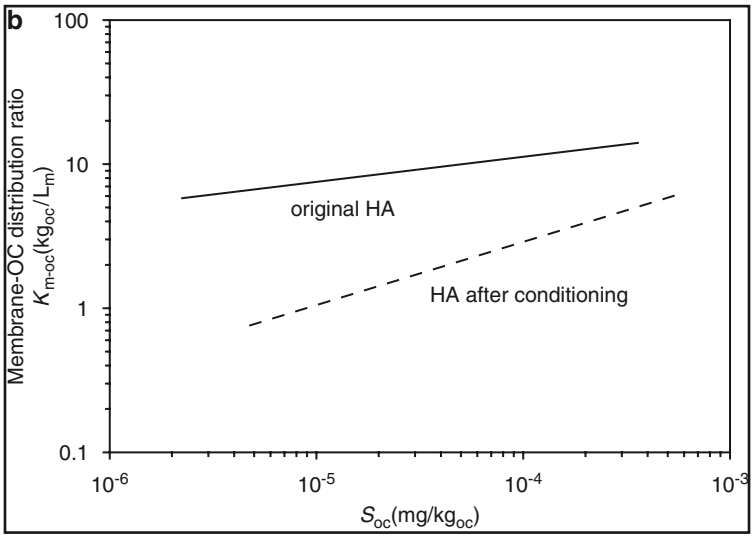


Fig. 3.5 (continued)

3.3 Bioavailability Under Kinetic Control

Next we consider the case where intracellular transformation (or transport) is fast relative to desorption from the soil. There are many studies showing biodegradation to be rate-limited by desorption [to name a few: (Bosma et al. 1997; Braida et al. 2004; Rijnaarts et al. 1990; White et al. 1999)].

Substrate is supplied to the solution by mass transfer from the solid phase (desorption), and is conveyed to the biomembranes by advection and/or diffusion. The simplest situation is one in which soil particles and cells are dispersed in a well-mixed aqueous medium, such as in a fluidized bioreactor; then, intraparticle diffusion governs the rate. More complex situations exist for stagnant soil columns, when water is flowing, and when an organism is acting on chemicals that migrate from distant locations in the soil column — for example, uptake by plant roots.

Mass transport of a physi-sorbing chemical within a soil particle and across the microscopic particle–bulk fluid interface is dictated by diffusion rate laws. Diffusion — the tendency of molecules to migrate in response to a gradient in chemical potential so as to achieve maximum entropy — is a function of molecular structure, nature and geometry of the diffusing medium, concentration gradients, interfacial boundary conditions, and temperature. Mathematical diffusion models for various types and shapes of diffusion media and various situations have been discussed (Crank 1975; Haws et al. 2006a; Kärger and Ruthven 1992; Pignatello 2000). All-encompassing rate laws for soil systems are not easy to write, given the heterogeneity of soils.

Diffusive equilibrium in well-mixed suspensions may require as short as a few hours or as long as several months, depending on the compound and the soil or soil isolate being tested (Pignatello and Xing 1996). Equilibrium after performing a dilution step can take even longer. Many studies have used physical stripping techniques to simulate desorption to an infinitely dilute fluid. Commonly, the stripping agent is a polymer resin, such as Tenax or XAD resin (Pignatello 1990a; Zhao and Pignatello 2004), added to the soil suspension in large excess to ensure quantitative thermodynamic mass transfer and aqueous-phase concentrations approaching zero. Another type operating on a similar principle is extraction by soluble inclusion agents such as cyclodextrins that contain a hydrophobic cavity (Rhodes et al. 2008; Semple et al. 2007). Gas streams have been used for the same purpose in vapor-phase desorption experiments in soil columns (Werth and Hansen 2002; Werth and Reinhard 1997a, b). Complete desorption of strongly sorbing contaminants in the presence of a stripping agent can require exceedingly long times. It will often be the case that contaminant removal for moderate to strongly sorbing compounds will become rate-limited by intra-particle diffusion at some point during degradation. Indices have been proposed to indicate the rate-limiting step, whether biodegradation or desorption. These indices are typically based on the Damköhler convention of a dimensionless number relating the biodegradation timescale to the desorption timescale — for example, the ratio of first-order biodegradation rate constant [T^{-1}] to first-order mass transfer rate coefficient [T^{-1}], or the "Best number" defined in Sect. 3.3.3. These indices, however, do not capture the changes in the respective rates that occur during the time course of degradation, and may not adequately reflect equilibrium sorption (Haws et al. 2006a). Simulations showing the transition between biodegradation and desorption rate-limitation, as degradation proceeds, are available (Haws et al. 2006b).

Braida et al. (2004) studied separately phenanthrene biodegradation (by a pseudomonad culture) and desorption in 15 different sterilized soils. Indices were proposed for bioavailability and biotransformation potential index over a specified time, t, to suggest the potential for success of bioremediation. The bioavailability index BA_t is the ratio of mass biotransformed to mass desorbed and trapped by an excess of Tenax, and reflects biotransformation rate relative to a desorption rate approximating the maximum possible desorption rate. The biotransformation potential index BTP_t is the ratio between mass biotransformed and mass remaining sorbed after maximal desorption, indicating the maximum biotransformation expected, assuming cells respond only to molecules in the aqueous phase. A plot of BTP_t as a function of BA_t for t= 30 days could be divided into four arbitrary quadrants (Fig. 3.6). A point appearing in Quadrant II, where biotransformation potential is high and desorption resistance is low, indicates a high likelihood of success. In Quadrant I, phenanthrene is physically labile, but biotransformation is intrinsically slow or inhibited. In Quadrant III, biotransformation is facile but desorption is limited. Quadrant IV includes outcomes with a low likelihood of success, where biodegradation is slow or inhibited and desorption is limited. One can see that control by mass transfer, or mass transfer plus microbial processes was applicable to most of the soils.

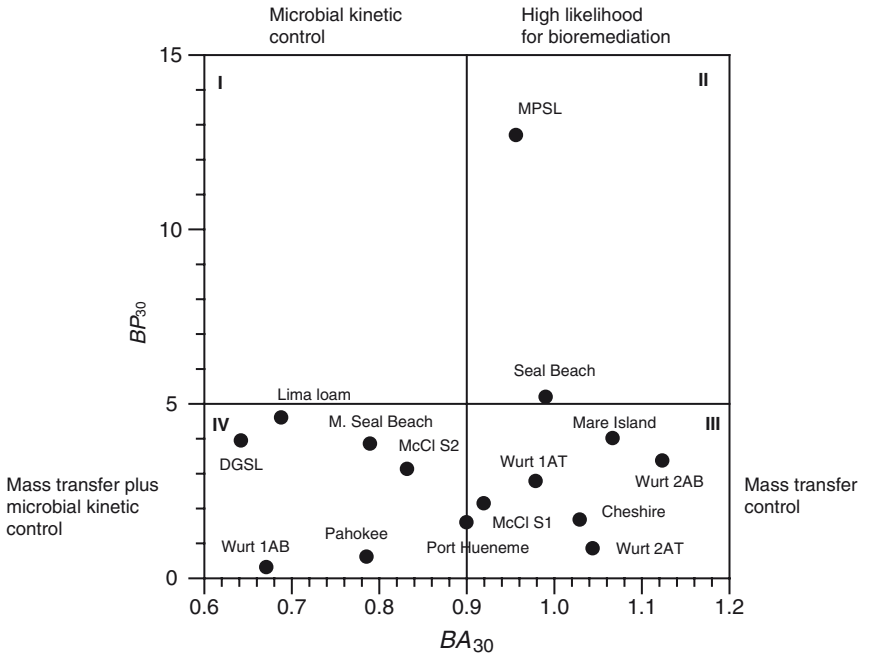


Fig. 3.6 Four-quadrant division of biotransformation potential index (BTP) and bioavailability index (BA) for phenanthrene in 30 soils corresponding to a 30 day period. From Braida et al. (2004) with permission from SETAC and Allen Press Publishing Services

3.3.1 Nature and Geometry of the Diffusing Medium

Soils may contain mineral grains with patches and/or coatings of humic substances on their surfaces. These gains may be cemented together in aggregates. The aggregates may include microscopic particles of organic matter in various stages of diagenesis, from young humic substances, to ancient humic substances found in soft and hard coal, to particles of charred carbon (black carbon).

Intraparticle mass transport may involve diffusion through pore fluids (pore diffusion), along pore walls (surface diffusion), or through the solid matrices of organic matter (solid-phase, or matrix diffusion). Pore and surface diffusion are conceptually difficult to distinguish in pores not much larger than the width of the diffusant, such as micropores (< 2 nm by the IUPAC definition). Diffusion of a molecule to or from the center of a soil particle requires multiple "jumps" and often involves the crossing of many grain–grain and grain–water interfaces. The length scale over which diffusion is rate-limiting may be much smaller than the macroscopic particle radius, and will depend on the micromorphology of the particle (Pignatello 2000). Cells cannot enter pores smaller than about 10^0 μm (IUPAC definition of a macropore is a pore aperture > 0.050 μm).

Diffusion through pores is retarded by the tortuosity of pore network pathways, sorption on pore walls, and steric hindrance. Steric hindrance begins to show when the minimum critical molecular diameter is about 10% of the pore diameter, and becomes severe as it approaches 100% (Kärger and Ruthven 1992). Steric effects for most molecules of interest will be important in micropores up to smaller mesopores. Molecular jumps during matrix diffusion in SOM require cooperative flexing or movement of humic macromolecules (Pignatello 2000). Soft ("rubbery") SOM represented by humic acid films impedes molecular diffusion by 3–4 orders of magnitude compared to water (Chang et al. 1997). Stiff-chain ("glassy") solids can impede molecular diffusion by many more orders of magnitude, depending on the molecular diameter and the glass transition temperature of the solid, a measure of its rigidity (Pignatello 2000). The conception of SOM as composed, in part, of glassy domains has been discussed elsewhere (Lu and Pignatello 2002, 2004a, b; Pignatello and Xing 1996; Sander et al. 2006; Sander and Pignatello 2005b; Xing and Pignatello 1997; Zhao et al. 2001).

Soil heterogeneity has an important influence on sorption/desorption kinetics. Theoretically, the uptake or release rate is inversely related to the square of the particle radius. In some studies, sorption/desorption rate is found to increase with decreasing nominal soil particle radius (Ball and Roberts 1991; Kleineidam et al. 1999; Wu and Gschwend 1986), while in other cases no dependence on particle size was observed (Carroll et al. 1994; Farrell and Reinhard 1994; Steinberg et al. 1987). However, it is usually true that pulverization of the soil increases sorption and desorption rates (Ball and Roberts 1991; Steinberg et al. 1987). Examining coaly sedimentary sands and gravels separated on the basis of size, color and porosity, Kleineidam et al. (1999) found that sorption rate decreases with increasing size, increasing OC content, and decreasing porosity.

Many compounds give nonlinear isotherms in soils, in the sense of decreasing affinity with increasing concentration, a result that reflects the energetic heterogeneity of sorption sites. Sorption nonlinearity has an effect on sorption and desorption rates (Braida et al. 2001, 2002). In general, the normalized sorption or desorption rate increases with absolute concentration, because the solid provides progressively lower resistance to diffusion. Whereas the normalized sorption and desorption rate curves for a linearly-sorbing compound will be coincident, the same will be true for a nonlinearly-sorbing compound only in the trivial case of sorption and desorption to/from an infinite source/sink. In all other cases, desorption will be slower than sorption at the same total chemical present. That follows directly from the concentration effect just mentioned, realizing that the strongest sites are filled from a relatively high-concentration source but emptied to a relatively low-concentration sink.

Competition sorption can also result in increased sorption and desorption rates of the principal solute, for the same reason as increasing concentration does: as the co-solute concentration increases, the principal solute occupies progressively weaker sorption sites, and becomes progressively more labile within the diffusive medium (White and Pignatello 1999; Zhao et al. 2001).

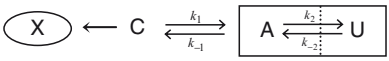
3.3.2 Influence of Molecular Structure

It is commonly observed that the characteristic rate parameter for sorption or desorption in soil decreases with increasing molecular size or hydrophobicity. Most studies showing this effect involve a limited number of compounds (usually two or three). Examples of more comprehensive studies include the one by Carroll et al. (1994) comparing the effective diffusion coefficient of PCBs in sediment, and those by Piatt and Brusseau (Piatt and Brusseau 1998) comparing the desorption mass transfer coefficient for the kinetically-controlled fraction of the "two-compartment" (instantaneous–slow) model for various hydrophobic compounds in packed soil columns. These results are consistent with all the mechanisms mentioned above — pore, surface and matrix diffusion — in light of fundamental studies on polymers (Berens 1989; Rogers 1965) and porous inorganic reference materials (Kärger and Ruthven 1992). No definitive studies contrasting polar with nonpolar molecules of comparable size have been reported for soils.

3.3.3 Coupled Sorption–Microbial Degradation Models

Substrate is supplied to the solution by desorption and removed from solution by biodegradation. First-order and Monod biodegradation kinetic models have been coupled to first order, linear driving force, and diffusion-type sorption kinetic models. An example of each is given below.

The model of Shelton and Doherty (1997a, b) for 2,4-dichloroacetic acid (2,4-D) degradation in unsaturated soils inoculated with an *Alcaligenes* species is an example of a model that couples Monod kinetics with first-order sorption/desorption kinetics. These researchers assumed 2,4-D is the primary growth substrate and limiting nutrient, and no natural decay or growth occurred. The model is depicted in (3.14):



(3.14)

where X represents intra-cellular chemical, C the external aqueous phase chemical, A the available sorbed chemical, and U the unavailable sorbed chemical.

The governing equations are Monod-type equations [similar to (3.4) and (3.5), with the last two terms of (3.5) cancelled] plus (3.15)–(3.17):

$$\frac{dC_w}{dt} = -k_1 C_w + \frac{1}{\theta} k_{-1} S_A - \frac{\mu_{max} C_w}{K_M + C_w} \frac{B}{\theta}, \tag{3.15}$$

$$\frac{dS_A}{dt} = k_1 \theta C - (k_{-1} + k_2) S_A + k_{-2} S_U, \tag{3.16}$$

$$\frac{dS_U}{dt} = k_2 S_A - k_{-2} S_U, \tag{3.17}$$

where k_1, k_{-1}, k_2, and k_{-2} [T^{-1}] are the first-order sorption and desorption rate constants, θ [L^3 M^{-1}] is the water content of the soil, and μ_{max} [M M^{-1} T^{-1}] is the Monod maximum utilization rate (in our notation, $\mu_{max} = k_{int}\,\varphi(K_M\,K_m)$). Mass transfer rate constants in sterile soils and Monod parameters in soil-absent systems were obtained in independent experiments performed over 3- and 48-h periods respectively. The parameters are thus specific to the timeframe of those experiments.

The linear driving force model for sorption and desorption processes is based on the idea that rate is proportional to the degree the system has reached equilibrium. The coupled biodegradation–linear driving-force model is exemplified by that of Bosma and co-workers (1997), who studied biodegradation of α-hexachlorocyclohexane residues in historically-contaminated soil. They postulated bacterial colonies harbored in vicinal pores (0.8–3 μm macropores) that receive mass from distal pores where cells cannot enter. They assumed that the rate of mass transfer q_d [M T^{-1}] is proportional to the difference in solute concentration between distal pores, C_d, and the cells, C_c [M L^{-3}]:

$$q_d = \alpha(C_d - C_c), \tag{3.18}$$

where α [L^3 T^{-1}] is the desorption rate parameter. Biodegradation rate q_c [M T^{-1}] was modeled with the Michaelis-Menton model:

$$q_c = \frac{q_{max} C_c}{K_{MM} + C_c}, \tag{3.19}$$

which is of the same form as the Monod model but without growth or decay; q_{max} [M T^{-1}] is the maximum biodegradation rate, and K_{MM} [M L^{-3}] is the Michaelis–Menton half-saturation constant. Assuming static degrader population is reasonable, considering the aged nature of the system and the low concentration of substrate.

Under conditions of steady-state $q_d = q_v$, and (3.18) and (3.19) can be combined to solve for q [M T^{-1}], the quantity transformed per unit time by the combined action of mass transfer and biodegradation. The 'bioavailability index' (the Best number) *Bn* is defined as the ratio of mass-transfer to biodegradation rate parameters:

$$Bn = \frac{\alpha}{q_{max} K_{MM}^{-1}}. \tag{3.20}$$

The reaction is rate-limited by cell metabolism when $Bn > 1$, and rate-limited by mass-transfer when $Bn < 1$. The *Bn* for α-hexachlorocyclohexane in soil slurry was 0.016–0.03, signifying — not surprisingly — mass-transfer limitation.

Other models couple biodegradation with intraparticle diffusion (Haws et al. 2006b; Rijnaarts et al. 1990; Scow and Alexander 1992; Scow and Hutson 1992). The basis for these models is Fick's second law, which, for diffusion in a sphere (a reasonable geometry for soil particles), is given by

$$\frac{\partial s}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2 D(T,s)\frac{\partial s}{\partial r}\right), \tag{3.21}$$

where r [L] is the radial coordinate, s [M L^{-3}] is the total local volumetric concentration in the diffusing medium (including sorbed chemical and chemical dissolved in internal pore water), and $D(T,s)$ [L^2 T^{-1}] is the diffusion coefficient, or diffusivity, which may be temperature- and concentration-dependent. The average sorbed concentration in a sphere of radius is given by

$$\overline{S}(t) = \frac{3}{r_0^3}\int_0^{r_0} s(r,t)r^2 \mathrm{d}r. \tag{3.22}$$

The boundary condition at the particle-external solution interface is given by

$$S(t)_{r=r} = \frac{\mathrm{d}S}{\mathrm{d}C}C(t), \tag{3.23}$$

where dS/dC is the distribution coefficient presumed equivalent to K_d from the isotherm. Isotherms are most often fitted to the Freundlich model so that $K_d = nK_F C^{n-1}$.

Analytical solutions to the diffusion equation when D is constant are available for uniform spherical particles applicable to various situations, such as finite external solution, infinite external solution of constant or variable concentration, and desorption to a "vacuum" (Crank 1975; Kärger and Ruthven 1992). Numerical solutions have been worked out for less homogeneous media and situations of greater complexity. Typically, the output parameter of the diffusion model is D/r_0^2 [T^{-1}], since the characteristic diffusion length scale r_0 is unknown.

The temperature dependence of D may be expressed by the Arrhenius equation:

$$D = D_0 \mathrm{e}^{-E/RT}, \tag{3.24}$$

where E is the diffusion activation energy, D_0 a constant, and R the universal gas constant.

The concentration dependence of D is dictated by the gradient in chemical potential with respect to concentration — i.e., the nonlinear characteristic of the equilibrium isotherm (Kärger and Ruthven 1992; Pignatello 2000). The D is constant when the isotherm is linear and as concentration approaches zero.

Haws et al. (2006b) computed degradation curves for a completely mixed system using a number of possible models representing biodegradation and sorption kinetics of a single substrate with and without a co-substrate. They tested scenarios spanning a range of biodegradation rates and desorption rates. The interested reader is referred to their paper for the details, but it is useful here to point out a few of their conclusions, focusing on the single-substrate case with Monod biodegradation kinetics when biodegradation was not rate-limiting.

In general, the aqueous-phase concentration declined to a low steady-state level. Meanwhile, total mass removal was initially fast, as mass in solution and mass sorbed to rapidly-equilibrating sites was biodegraded. But the fast phase was followed by a much slower phase as degradation became rate-limited by desorption. The discrepancy between the linear driving force and diffusion models was small initially, but increased with time. Choosing a linear driving force rate parameter

from a short-term desorption study would result in over-prediction of degradation at long times relative to the diffusion model. Assuming linear sorption, when nonlinear sorption was chosen as reality, overpredicts degradation at long times because sorption affinity in the real case becomes greater (and slower) as concentration declines with time. At initial mass high enough to promote growth, the value of the characteristic biodegradation rate coefficient [T^{-1}] initially increased due to biomass growth, but then declined as substrate became depleted and cell decay processes took over. This is apparent in the schematic depiction of Fig. 3.1.

3.3.4 High Desorption Resistance

Over the past two decades, many reports have appeared documenting the presence of chemical residues in soil that strongly resist desorption and largely resist biodegradation. Several reviews of this topic are available (Brusseau and Rao 1989; Luthy et al. 1997; Pignatello 1990b, 2000; Pignatello and Xing 1996). Resistant behavior has important implications for natural attenuation, bioavailability, and bioremediation (Alexander 1995, 2000; Loehr and Webster 1997; Pignatello and Xing 1996).

Desorption resistance is found in historically-contaminated samples from the field, and it can be induced in clean samples spiked with a test chemical in the laboratory. Desorption resistance may be exhibited even by small, weakly sorbing molecules like halogenated C_2 and C_3 hydrocarbons (Farrell et al. 1999; Kommalapati et al. 2002; Pavlostathis and Jaglal 1991; Pavlostathis and Mathavan 1992; Pignatello 1990a, b; Werth and Hansen 2002). The term "resistant" — and its converse "labile" — in this context are not rigorously defined and depend on the timeframe and methodology of the experiment. Nevertheless, the highly resistant fraction is non-trivial — in most cases making up several percent or more of added or found concentration. Historically-contaminated samples are often enriched in resistant fraction, due to the lengthy time that passes before sample collection, during which the more labile fractions become depleted by dissipation or degradation; for example, see Pignatello et al. (1993). Biodegradation of spiked chemical, allowed to pre-equilibrate with a sterilized soil before inoculation, often tails off to leave a bio-resistant fraction that correlates with a physically resistant fraction (Braida et al. 2004; White et al. 1999; also papers cited in Pignatello and Xing 1996). It has been shown that a highly resistant fraction can be generated after a contact time as short as a few hours (Cornelissen et al. 1997; Kan et al. 1997,1998; Pignatello 1990a, b; Ten Hulscher et al. 2005).

One of the earliest reports of desorption resistance — and a good example to illustrate the phenomenon — involved 1,2-dibromoethane, a volatile, biodegradable and moderately water-soluble soil fumigant. This compound was found to persist at microgram-per-kilogram levels in topsoil up to at least 19 years after its last known application (Pignatello et al. 1990; Steinberg et al. 1987). Compared to comparable concentrations of freshly-added ^{14}C-labelled dibromoethane, field residues exhibited much greater soil–water distribution ratios (K_d), far slower rates of

desorption, and greatly reduced microbial degradability. Some recent findings of highly sequestered residues in field samples include BTEX and PAHs in coal tar from manufactured gas plant soils (Hawthorne and Miller 2003) and BTEX and chlorinated solvents in low-OC soils (Pavlostathis and Jaglal 1991; Pavlostathis and Mathavan 1992). An example of extreme resistance in a spiked system is the work of Farrell and Reinhard (1994) who followed desorption of trichloroethene (TCE) from soil columns eluted with N_2 at 100% relative humidity; most TCE desorbed within 10 min, while a small fraction took months to years to desorb.

3.3.5 *Correlation of Desorption Resistance with Biodegradation Resistance*

Several studies have used physical stripping agents such as Tenax or XAD resin added in large excess to predict the bioavailable fraction. Since these materials are strong sorbents, it is implicitly assumed using this approach that cells are quickly faced with very low solute concentrations. This approach shows promise for compounds whose removal is not biologically rate-limited.

Cornelissen and co-workers (2000) have used an exponential desorption model that assumes "fast," "slow," and sometimes "very slow" compartments when desorption occurs in the presence of Tenax. The two-compartment model is given by

$$\frac{S_t}{S_0} = F_{\text{fast}} e^{-k_{\text{fast}} t} + F_{\text{slow}} e^{-k_{\text{slow}} t}, \tag{3.25}$$

where S_0 is the initial sorbed concentration, and F represents the mass fraction and k [T^{-1}] the rate constant for contaminant in the designated compartment. The Tenax is removed periodically and solvent-extracted to assay for the contaminant. An example is given in Fig. 3.7 for fit of (3.25) to the desorption of hexachlorobenzene at 20 and 60°C (Cornelissen et al. 2000).

Models like the one in (3.25) are to be regarded as empirical. Nevertheless, they have been useful for estimating bioavailability. Cornelissen et al. (Cornelissen et al. 2000) showed that the Tenax "fast" fraction of many PAHs in three contaminated sediments decreased after remediation by landfarming; of those PAHs, a correlation was found between the initial fast fraction and the fraction subsequently removed by landfarming (Fig. 3.8a), showing a general trend that landfarming — an activity that may involve abiotic as well as biotic loss processes — was more effective than Tenax. Lei et al. (2004) found an almost perfect correlation between the fraction of

Fig. 3.8 Desorption vs degradation of PCBs in sediments. **a** Correlation between percent removed by landfarming and percent of the Tenax "rapidly-desorbing" fraction (3.24) for three sediments. *Line* is perfect correlation. From Cornelissen et al. (1998) with permission. **b** Correlation between biodegradation in aerobic shake-flasks and XAD-resin desorption of 2–4 ring PAHs. From Lei et al. (2004) with permission

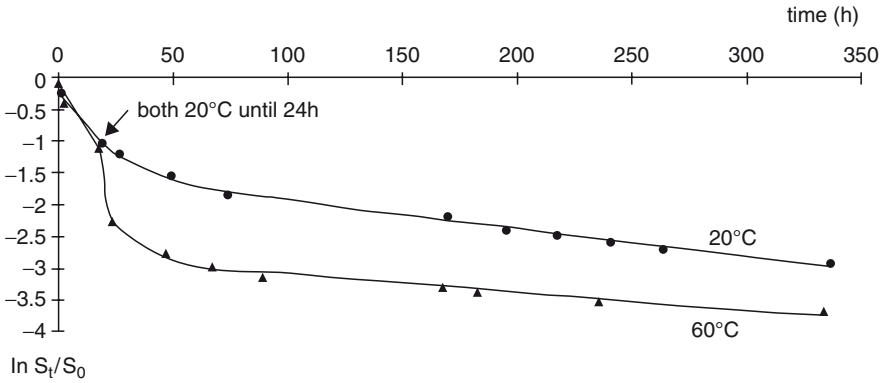
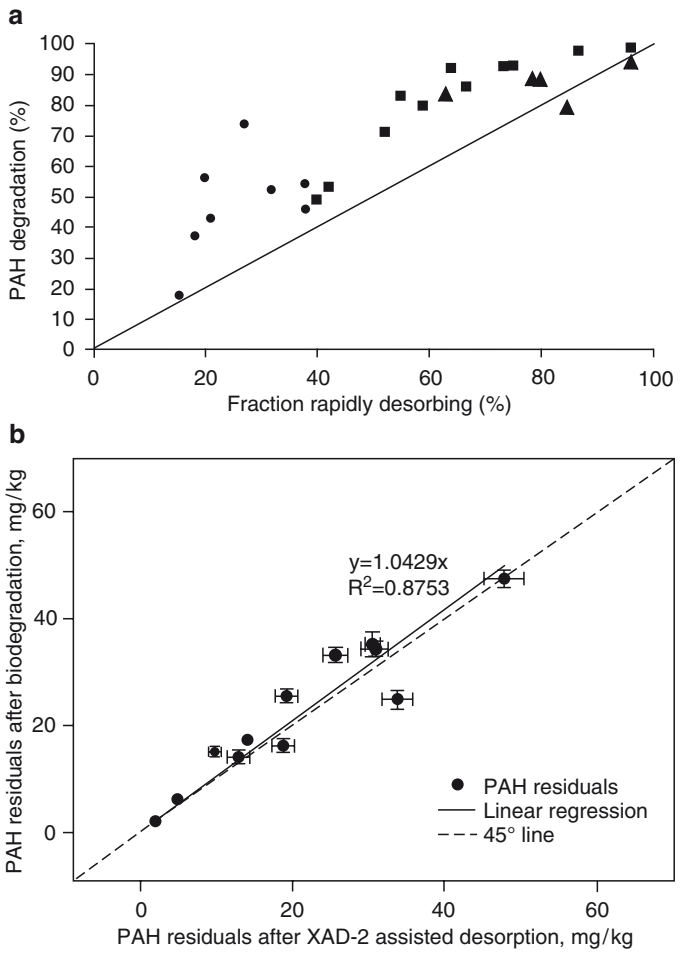


Fig. 3.7 Desorption of hexachlorobenzene from sediment in the presence of Tenax after a 14-day precontact time. The curves are fits to (3.24). From Cornelissen et al. (2000) with permission



2–4 ring PAHs removed by native sediment organisms in aerobic shake-flasks and the fraction desorbed to XAD-2 resin (Fig. 3.8b). (The 5- and 6-ring PAHs did not appreciably biodegrade, even though significant quantities desorbed.) A coal tar contaminated soil suspended in distilled water also gave a close to a 1:1 correlation between bio-resistant fraction (native organisms) and desorption-resistant fraction (Tenax) of 3–6 ring PAHs measured over the same timeframe (Li et al. 2005; Pignatello 2006). Likewise, Braida et al. (2001) found a correlation between bio-resistant and desorption-resistant fractions of phenanthrene in samples of 15 different sterilized soils contacted with phenanthrene for 6 months and then each split into portions that were either desorbed with Tenax or inocculated with a *Pseudomonad* degrader able to utilize phenanthrene for growth. The points for most of the soils were close to the 1:1 diagonal. Two soils showed a significantly greater biodegradation-resistant fraction than desorption-resistant fraction, indicating biological rate-limitation; whereas, one showed the opposite.

Bioavailability has also been found in several cases to correlate with extractability by hydroxypropyl-β-cyclodextrin solutions (Semple et al. 2007). However, in a study of phenanthrene mineralization by a *Pseudomonas* sp. inoculum in spiked soils containing up to 5% activated carbon, Rhodes (Rhodes et al. 2008) found that, while cyclodextrin extraction correlated with bioavailability in samples containing 0 and 0.1% activated carbon, it failed to correlate in samples at higher activated carbon contents; rather, mineralization became progressively more efficient than extraction as activated carbon content increased.

3.3.6 Causes of High Desorption Resistance

Possible mechanisms for the generation of strongly resistant fractions include:

1. The normal process of retarded diffusion in and out of remote domains
2. Occlusion, or trapping, of molecules in closed pores during particle synthesis and
3. Alteration of the soil matrix following sorption, which results in occlusion or highly hindered diffusion

Desorption resistance is usually attributed to retarded molecular diffusion through tortuous pore networks or highly viscous organic matter phases. The progress of diffusion through mineral aggregates may be retarded by the presence of tiny particles of organic matter occluded within the aggregates (Kleineidam et al. 1999). As mentioned, matrix diffusion requires cooperative motions between the diffusant and matrix strands. SOM phases may contain domains on the micro-scale that have high affinity for the contaminant but are dense and stiff, and so present great but not insurmountable barriers to diffusion. Sorption equilibrium is not easily reached in stagnant soil columns for highly hydrophobic compounds such as PAHs where the actual concentration in mobile phases (liquid, vapor) is very small. Sorption intensity increases and diffusivity decreases as soil moisture content decreases.

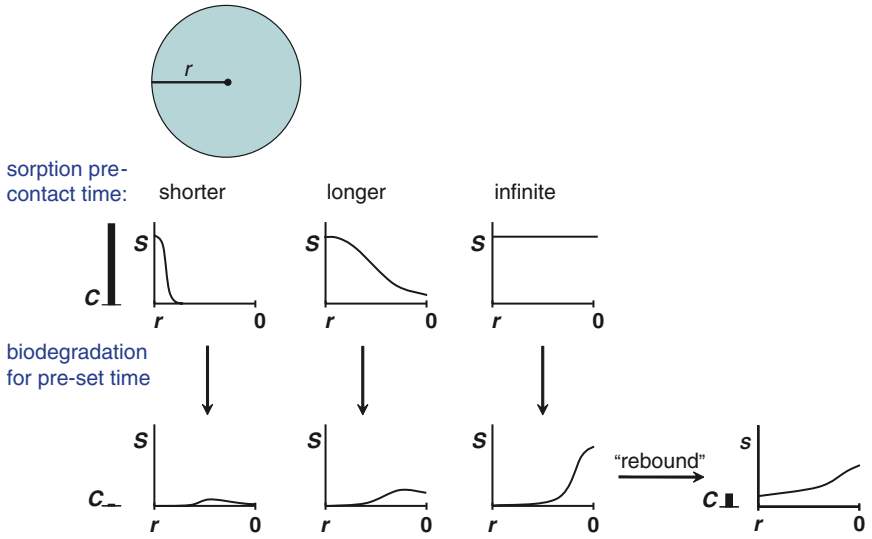


Fig. 3.9 Diffusion concept explaining the aging effect on contaminant bioavailability in a hypothetical uniform spherical soil particle first precontacted with solution at constant concentration *C*, and then biodegraded for a prescribed time, during which *C* is greatly reduced. After the biodegradation period the contaminant rebounds. Bar sizes and curves are intended to be illustrative only

Retarded diffusion can explain the often-observed "aging effect", in which bioavailability decreases with chemical precontact time (Alexander 2000). This can be illustrated for diffusion in a hypothetical uniform sphere shown in Fig. 3.9. Prior to biodegradation, the particle immersed in a solution at concentration *C* eventually reaches uniform sorbed concentration *S*. Contaminant then leaks out when *C* is lowered by biodegradation. The greater the degree of equilibrium reached in the precontact step, the less will leak out in the biodegradation step. Note, however, that due to the random nature of diffusion, contaminant will migrate both inward and outward of the particle during the biodegradation step. Even in the short precontact time case when only the "skin" of the particle was filled initially, some of the contaminant is driven close to the center of the particle, thus becoming 'bioresistant' to the observer. One will also recognize the likelihood of "rebound," in which the now "sequestered" fraction remaining after termination of a remediation event will redistribute over time, placing some of the contaminant in a more available state.

Occlusion during particle synthesis may apply to PAHs associated with fuel soot particles. Soot condenses out of a complex series of gas-phase free radical reactions, in which PAHs and other compounds are intermediates in the formation of the graphene platelets that make up soot (Smedley et al. 1992). It is possible that some unreacted PAH molecules become trapped in internal pores of the nascent condensate — pores that later may have no connection to external fluids. The concept of PAH occlusion during black carbon synthesis remains hypothetical. The evidence for it rests in part on the desorption-resistance of some fraction of PAH

content under extraordinary conditions, such as high temperatures (Harmon et al. 2001) or during supercritical fluid (CO_2) extraction (Jonker et al. 2005). Native PAHs in sediment thought to be associated primarily with soot particles poorly equilibrate with an isotope-labelled PAH spike (Jonker and Koelmans 2002). If some fraction of PAHs are truly occluded, their extractability and contribution to the total PAH is due ostensibly to the ability of solvents to swell the black carbon matrix (Akhter et al. 1985; Braida et al. 2003; Razouk et al. 1968).

Alteration of the soil matrix leading to sequestration has been little investigated but holds some credence. Kan et al. (Kan et al. 1997) suggested that a fraction of contaminant is transferred to "high-affinity" micro-environments "in equilibrium with the dissolved state" that are created by "conformational or physical rearrangement" of SOM. They provided no explanation for the underlying driving force for this rearrangement. Following that paper, studies suggested that inelastic swelling of SOM may lead to immobilization via an anti-plasticization mechanism, in which an abrupt decrease in sorbed concentration causes the matrix to collapse and stiffen around some molecules before they have a chance to escape (Braida et al. 2003; Sander and Pignatello 2008; Weber et al. 2002). This hypothesis has not been rigorously tested. It is possible also to imagine occlusion, or at least highly hindered diffusion, to be caused by changes in the physical structure of particles via interactions with other natural substances. For example, Farrell et al. (1999) found that the very slow desorbing fraction of TCE and PCE in silica did not conform to mesopore or micropore diffusion models. Instead, they proposed that mineral precipitation leads to blockage of intra-granular micropores.

3.3.7 Facilitated Bioavailability

Facilitated bioavailability refers to the ability of organisms to directly access sorbed molecules or to promote desorption by altering the properties of soil particles and/or the surrounding liquid.

The examples in Fig. 3.8 and the accompanying text correlating biodegradation resistance and desorption resistance are consistent with an assumption that microorganisms must await desorption into the aqueous phase. However, this assumption has been challenged by a number of findings that biodegradation rates for some microbial populations appear to exceed maximum desorption rates obtained by stripping techniques (Crocker et al. 1995; Guerin and Boyd 1992; Li et al. 2005; Park et al. 2001; Reid et al. 2001). A pertinent example is the study by Li et al. (2005), who found that PAH bioavailability in coal-tar contaminated soil with respect to native organisms was increased by the addition of a suite of macronutrients (NH_4^+, P, K) and micronutrients (Mg, Ca, Fe, Co, Zn, Cu, Ni, Mo, Se, W). Over a 100-day period the extent of biotransformation correlated approximately 1:1 with the Tenax-desorbable fraction when no nutrients were added, whereas the extent of biotransformation exceeded the Tenax-desorbable fraction when nutrients were added (Fig. 3.10). An important lesson from this experiment is that biodegradation

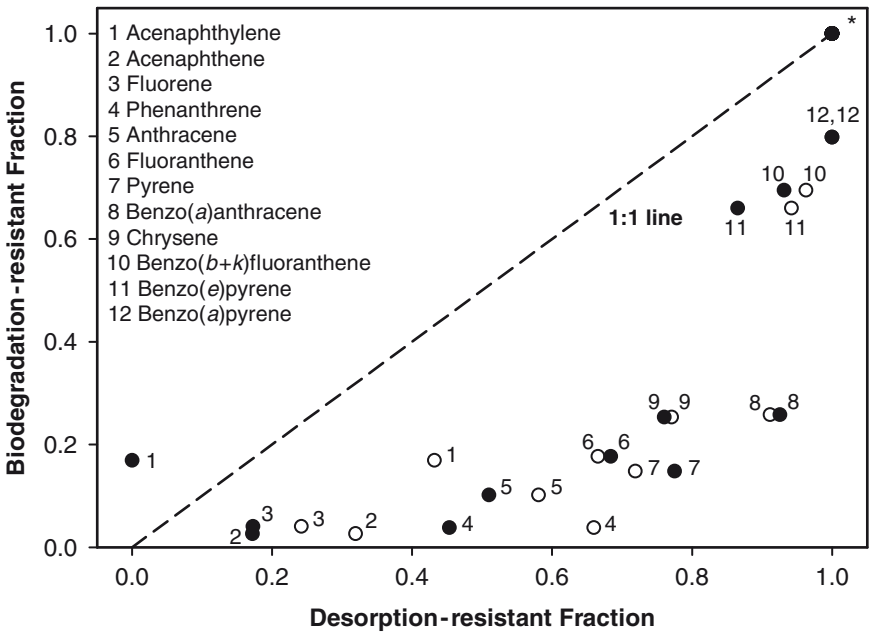


Fig. 3.10 Facilitated bioavailability of PAHs exhibited by native organism in a coal-tar contaminated soil amended with a mixture of inorganic nutrients. Degradation is compared to sterile desorption to Tenax. Desorption flasks contained either 0.01 M citrate (*filled symbols*) or 0.01 M pyrophosphate (*open symbols*) added to accelerate desorption. From Li et al. (2005) with permission

resistance by native soil colonies — at least relative to the benchmark (Tenax) desorption method — may be partly due to nutrient limitations. It should be noted, however, that an appreciable fraction of all PAHs remained biodegradation-resistant even when supplemented with nutrients (Li et al. 2005).

The causes of facilitated bioavailability are not completely understood. Most bacteria in soils live in attached biofilms, which are remote from most sorbed molecules. With diameters of about 10^{-6} m, cells cannot penetrate mesopores (2–50 nm) or micropores (< 2 nm), which together make up nearly all the surface area of soils. Nor can they penetrate the SOM phase, the predominant sorbent phase of nonionic compounds in soils. Thus, if cells can access sorbed molecules, they can access only those in close proximity to the cell. While an attached cell may experience, on an absolute scale, a far higher sorbed than dissolved concentration, the chemical potentials are equal if microscopic local equilibrium exists. Thus, the thermodynamic bioavailability from the accessible local surface and the local liquid phase should be the same. The only way direct mining from the surface would increase bioavailability is if the rate of uptake from the local surface was comparable to or exceeded the rate of uptake from the local solution. Apart from a direct mining mechanism, facilitated bioavailability has been attributed to:

- Alteration of soil interfacial chemistry in a way that disrupts contaminant-soil interactions
- Release of biosurfactants and
- Passive effects of attached biofilms on the interfacial concentration gradient ('surface depletion' effect)

Two plausible sources of chemical modification are a change in pH and chelation effects. A moderate change in pH can shift the speciation of acids and bases having a pK_a within range of the change, but is unlikely to appreciably affect sorption of neutral compounds (Zhu et al. 2004). Chelating agents may accelerate desorption by solubilizing polyvalent metal ions, which crosslink humic substances and tether humic molecules to mineral surfaces (Li et al. 2005; Subramaniam et al. 2004; Yang et al. 2001). However, the bioavailability of PAHs in a coal-tar soil to native or cultured bacteria was not significantly affected by adding citrate or pyrophosphate (Li et al. 2005), which are strong chelators. Exudation of chelating agents by plant roots may explain facilitated uptake of residual chlorinated hydrocarbon insecticides, but other explanations are possible (White et al. 2003). As mentioned, natural organic acids exuded by plants may increase bioavailability by competitively displacing contaminants from sorption sites (Xing and Pignatello 1998).

Bacteria may produce glycolipid, lipopeptide, phospholipid, fatty acid, and neutral lipid biosurfactants. Synthetic surfactants have been considered for use in bioremediation to increase bioavailability. Surfactants sometimes stimulate and sometimes inhibit biodegradation, depending on surfactant, surfactant concentration and other conditions (Seo and Bishop 2007; Singh et al. 2007).

Surfactants increase the total liquid-phase concentration of contaminant through formation of micelles, micro-emulsions, or colloids that serve as micropartition domains (Guha and Jaffé 1996; Guha et al. 1998; Shreve et al. 1995; Zhang and Miller 1992). The critical micelle concentration (cmc) of biosurfactants ranges from 1 to 200 mg l^{-1} and the molecular mass of biosurfactant molecules ranges from 500 to 1,500 Da (Guha et al. 1998). It can be shown using a thermodynamic cycle that thermodynamically-controlled bioavailability is independent of the presence of surfactant micropartition domain if bio-uptake occurs exclusively via the truly-dissolved state. Thus, surfactant can increase bioavailability only by accelerating desorption from soil or by facilitating entry into the biomembrane.

Below the cmc, surfactant appears to have little effect on dissolution mass transfer rate coefficients of NAPLs (Johnson et al. 1999) or crystals (Huang and Lee 2001; Johnsen and Karlson 2004). Above the cmc, the presence of surfactant actually *reduces* the water mass transfer coefficient of chemicals in NAPLs (Bernardez and Ghoshal 2008; Johnson et al. 1999) or crystals (Huang and Lee 2001) (although the rate increases due to enhanced equilibrium partitioning). Therefore, it is expected that surfactant will not accelerate desorption from soil surfaces. In regard to facilitated entry into the biomembrane, a direct transfer into the cell from hemi-micellular phases adsorbed on the cell surface has been suggested (Beal and Betts 2000; Kappeli and Fiechter 1977; Guha and Jaffé 1996; Guha et al. 1998).

Many bacterial strains produce exopolysaccharide (EPS) mucus that binds cells within a biofilm and assists biofilm attachment to surfaces (Sutherland 2001).

Partitioning of hydrophobic chemicals into EPS from water is favorable (Johnsen and Karlson 2004; Wicke et al. 2007). Again, it can be shown that equilibrium partitioning between cell membrane and the local soil matrix is independent of EPS. Under a nonequilibrium condition, which may be more typical of a pore or within a biofilm where the substrate is locally depleted, EPS may actually *lower* the membrane concentration due to competition for available chemical mass.

Biofilms of PAH degraders are known to grow directly on the surfaces of PAH crystals (Eriksson et al. 2002; Johnsen and Karlson 2004; Wick et al. 2002). Johnsen and Karlson (2004) showed for a number of isolated PAH degraders that such biofilms do not decrease the surface tension of the liquid, nor do added bacterial polysaccharide colloids increase mineralization rates despite boosting solubility. Their results point against solubility enhancement as a general strategy to increase bioavailability.

The surface depletion hypothesis holds that degrader biofilms reduce the chemical concentration in the diffusive boundary layer of water near the soil surface, which increases the driving force for molecular diffusion out of the solid. It is supported by circumstantial evidence. Dissolution rates of non-aqueous phase liquids (NAPLs) are increased when biodegradation takes place in the aqueous phase, as one might expect (Seagren et al. 2002; Yang and McCarty 2000). The results of Johnsen and Karlson (Johnsen and Karlson 2004) mentioned above on biofilm formation on PAH crystals, which point away from both solubility enhancement and dissolution rate enhancement as strategies for bioavailability enhancement, favor, by process of elimination, the surface depletion hypothesis. Once contaminant molecules leave the soil interface, the EPS-biofilm matrix provides a moderately effective sorptive medium and kinetic barrier for their release into bulk solution (Wicke et al. 2008). Disruption of biofilm attachment was invoked to explain the reduction in bioavailability by the addition of synthetic surfactants below their cmc (Seo and Bishop 2007). Stripping agents such as gas bubbles or polymer beads commonly used as benchmarks for bioavailability may underestimate bioavailability because they establish a shallower concentration gradient at the interface than the gradient established by actual biofilms, due to their inability to approach the surface as closely and to enter pores that can be colonized.

3.4 Conclusions

Bioavailability is a critical consideration in bioremediation and natural attenuation. Bioavailability may be defined simply as the ratio of contaminant mass removed in the presence and absence of soil after a given time. Bioavailability is a function of the thermodynamic and mass transfer behavior of the contaminant in the non-living and living part of the soil. Bacterial cells assimilate most, if not all, substrate through the aqueous phase. Sorption dictates the activity of the contaminant in the aqueous phase. What matters is the biomembrane–soil distribution ratio of the contaminant. That ratio is strongly dependent on the nature of the soil particles, but seems to be only weakly dependent on solute hydrophobicity and polarity. Sorption

to soils is commonly nonlinear, which tends to suppress bioavailability as concentration declines. Partitioning to model biomembranes is linear, and correlates with K_{ow} until a certain molecular size. However, further work is needed to characterize sorption to real cell membranes and to identify more specific pathways of uptake. Co-solutes affect bioavailability through competitive sorption and through certain roles in the biology. The degree of competitive sorption is governed by the degree of nonlinearity in the single-solute isotherms. Through competition, co-solutes increase or decrease bioavailability of the principal solute as their concentrations increase or decline respectively. The effect can be dramatic for some types of particles. The operation of processes leading to true sorption hysteresis may result in over-prediction of bioavailability based on the uptake isotherm.

Mass transfer via molecular diffusion and advective flow delivers contaminant molecules from distal to vicinal sites with respect to the cell. Mass transfer often is, or becomes rate-limiting during bioremediation or natural attenuation. Diffusion is a complex process controlled by molecular structure, nature and geometry of the diffusion medium, concentration gradients, interfacial boundary conditions, and temperature. Mass transfer rate laws have been coupled to biodegradation rate laws with some success, but such models are usually case-specific. Extremely strong resistance to desorption of a fraction of total contaminant is a commonly observed phenomenon. The simplest explanation is hindered diffusion from remote domains in the soil matrix, but the possibility of occlusion in closed intraparticle pores as a viable mechanism in certain cases deserves further examination.

Facilitated bioavailability appears to be real, but the underlying causes are poorly understood. The role played by released biosurfactants is murky. Promotion of contaminant diffusion through the influence of biofilms in the interfacial region is a likely cause, but further study is needed.

It is a desirable goal to be able to predict the bioavailability of a contaminant solely on the basis of the physical-chemical properties of contaminant and soil and the (aqueous phase) degradation parameters of the organism. We are not very close to that goal, and perhaps it is unattainable. Certain surrogate methods for predicting the bioavailable fraction are attractive, but a more systematic evaluation is required. Solid-phase stripping agents like Tenax may not mimic the concentration gradient at the soil-water interface that is established by biofilms. Liquid-phase extractants, such as cyclodextrin solutions or weak solvents, have an uncomfortable degree of arbitrariness in the way they are used.

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Chapter 4
Biosurfactants in Bioremediation

Jonathan D. Van Hamme and Joanna Urban

4.1 Introduction

At the cellular level, amphipathic molecules characterized by hydrophobic and hydrophilic, or non-polar and polar regions, are common and essential due to life's aqueous nature and aqueous environment. Single and multicellular lifeforms evolved amphipathic lipid bilayers to segregate external and internal water, and exploit this separation to generate energy, concentrate solutes, extrude or exclude waste and toxic materials, and to create a controlled environment for biomolecular synthesis which is, among other things, the basis of heredity. Threaded through these processes are mixed polarity molecules whose functions rely on amphipathy. Consider transmembrane sensory proteins, electron transport chain proton and sodium motive pumps, flagellar motors and internal membranes as examples. The very molecules that dictate individuality, nucleic acids, are built upon contrasting base pair and sugar phosphate polarities.

This molecular commonality extends to what may be described as helper molecules produced to modulate and facilitate diverse physical, chemical and behavioural activities within and without the cell in both single and multicellular lifeforms. These helpers are the biosurfactants and bioemulsifiers, now recognized as essential to many cellular processes, and increasingly considered for industrial and therapeutic applications as environmentally and biologically compatible alternatives to chemically synthesized surfactants.

This chapter will introduce the natural history of microbial biosurfactants, and extend into a discussion of their applications in bioremediation and biodegradation. Classes, properties and physiological roles will be described, followed by examples illustrating mass transfer effects on biodegradation, soil washing, and in situ and ex situ biosurfactant production.

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4.2 Natural History of Biosurfactants

4.2.1 Biosurfactant Properties and Classes

The defining characteristic of biosurfactants is the separation of hydrophobic and hydrophilic regions within single molecules, allowing for diverse interactions with interfaces at which so many life processes are concentrated. These molecular bridges span physiochemically incompatible strata, bringing together, via surface active properties, aqueous and non-aqueous environments. Biosurfactants tend to accumulate at air–water, oil–water, surface–air and surface–water interfaces, forming allegiances with compatible regions in the opposing environments.

A classic example is biosurfactant micelle and vesicle formation in aqueous environments, undoubtedly an illustration of ancient cell-forming processes. When a single amphipathic biosurfactant molecule is introduced into liquid water, the hydrophobic regions will orient above the water surface in order to minimize energetic interactions with polar water molecules. Of course, hydrophilic regions will tend to the polar aqueous environment in favour of the relatively hydrophobic gas phase above the surface. This partitioning will continue as additional biosurfactant molecules are added to the system, until the liquid surface is saturated. Throughout these additions, the hydrogen bonds providing the high surface tension at the water surface will be disrupted, and surface tension will drop. At surface saturation, further surface tension reductions will be minimal and, as biosurfactant is added, molecules will be forced into the aqueous milieu.

Once a number of biosurfactant molecules are in the aqueous environment, the lowest energy state between the water and biosurfactant molecules will be achieved via micelle formation, in which hydrophobic biosurfactant regions aggregate together, protected from water interactions by a hydrophilic shell. Micelles are dynamic, and continuously form, disperse, and reform by recruiting new biosurfactant molecules. Should purely non-polar molecules be present in the aqueous system, they will partition into the micellar core. If a sufficient quantity of non-polar molecules are present, perhaps as a second non-aqueous phase, the biosurfactant molecules may form a unit membrane around a bulk volume of hydrophobic molecules, resulting in the formation of an emulsion droplet. Should a bilayer of biosurfactant molecules form, then a vesicle will result with an aqueous interior. On solid surfaces, including cell surfaces, biosurfactant molecules may form hemimicelle monolayers or admicelle bilayers. Of course, in a bulk non-aqueous environment, the non-polar biosurfactant regions will be exposed, with hydrophilic regions aggregating together on the interior.

These inter- and intra-molecular interactions, and the environmental diversity which biosurfactant molecules encounter, lead to an equally diverse set of natural biological functions and bioremedial applications, as will be described. But first, microbial biosurfactant structural diversity will be explored.

4.2.2 Biosurfactant Chemical Characterization

Advances in chemical analysis, coupled with expanding application of proteomic and genomic tools, are providing deeper insights into biosurfactant structural and biosynthetic diversity. Biosurfactants from bacteria, cyanobacteria, fungi and yeast may be classed into low and high molecular weight groups, or segregated based on major structural features. These groups include fatty acids, glycolipids, lipopeptides, phospholipids, glycoproteins, polymeric and particulate biosurfactants (Desai and Banat 1997). Growing lists of unique biosurfactant molecules are available, although upon examination it becomes evident that biosurfactants share a commonality in that they are generally synthesized from the basic products of central metabolism: amino acids, sugars, fatty acids and lipids.

Among biosurfactants known to date, the rhamnolipids produced by *Pseudomonas* spp. are arguably the best understood with respect to biosynthesis, structure and function due to a long history of inquiry and application. The rhamnolipids are composed of one or two rhamnose sugars linked to a fatty acid generated from de novo fatty acid synthesis and glucose-6-phosphate via a number of anabolic steps. Final synthetic steps are catalyzed by RhlA, RhlB and RhlC, whose expression is induced by a quorum-sensing mechanism. RhlA produces 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) from 3-hydroxyl-ACP, RhlB combines dTDP-L-rhamnose and HAAs to form mono-rhamnolipids, and RhlC adds a second rhamnose to form di-rhamnolipids (Soberón-Chávez et al. 2005). Strains have been found to produce a range of rhamnolipid homologues (Perfumo et al. 2006) and, in recent years, precursor HAAs have been recognized as important biosurfactant molecules themselves. Using highly purified mono- and di-rhamnolipids as well as HAAs, Tremblay et al. (2007) found that the respective functions of HAAs and rhamnolipids may be confounded by incomplete purification. Specifically, in *Pseudomonas aeruginosa* strain PA14, di-rhamnolipids act as attractants to swarming cells, while HAAs act as repellants, and mono-rhamnolipids serve as wetting agents. They showed that previous experiments concluding that rhamnolipids act as repellants were probably misinformed by the fact that the purification methods used left significant quantities of HAAs in the rhamnolipid preparations. These observations, biosynthetic intermediates with biosurfactant properties and the difficulty in performing purifications from groups of structurally similar molecules, will undoubtedly result in renewed interest in other biosurfactant groups that have been characterized, and will hopefully serve as a guide to the study of newly discovered biosurfacants.

With that, some discussion of recently described biosurfactant molecules is warranted, as more widespread application of gas chromatography–mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nuclear magnetic resonance, along with other basic and advanced analytical techniques, is supporting the resolution of structurally unique biosurfactants. Often, novel structures are related to previously characterized biosurfactants, with differences being found in amino acid, lipid and sugar composition. Fukuoka et al. (2007) characterized mono-acylated mannosylerythritol lipids (MELs) containing

4-*O*-β-D-mannopyranosyl-D-erythritol and medium chain fatty acids (C_8 to C_{14}) as hydrophilic and hydrophobic moieties respectively, from the yeasts *Pseudozyma antarctica* and *P. parantarctica*. The mono-acyl homologues found are similar to previously described diacyl- and triacyl-MELs but with less hydrophobic nature (HLB 12–15), cmc values 100 times higher (3.6×10^{-4} M), while retaining good surface tension reduction abilities (33.8 mN m^{-1}). These compounds form stable oil-in-water emulsions and have not been found to disrupt membranes. Another new preparation, also with excellent oil-in-water emulsification abilities, is a high molecular weight (> 2,000 kDa) extracellular emulsifier isolated from *Antarctobacter* sp. strain TG2C grown into the death phase (Gutiérrez et al. 2007). The described glycoprotein exhibits limited surface tension reductions, and is made up of a variety of sugars, amino sugars, amino acids and uronic acids. No lipid has been detected, but this type of material is difficult to characterize due to chemical stability.

As previously noted, many biosurfactant-producing strains generate more than one type of biosurfactant, often depending on growth conditions. Lee et al. (2007) characterized bamylocin A, a novel lipopeptide containing ß-hydroxy-C13-fatty acid and seven amino acids, three of which are unique from surfactin, a similar biosurfactant produced by this strain along with fengycin and iturin. *Rhodococcus ruber* strain 3C-9, able to utilize C_5 to C_{36} *n*-alkanes for growth, produces free fatty acids and short fatty acid (C_{12}, C_{14} and C_{16}) containing glycolipids when growing on alkanes, but not water-soluble substrates such as glucose, glycerol and sucrose. With shorter fatty acid chains than previously described glycolipids in Rhodococci, the biosurfactants from 3C-9 increased dibenzothiophene, naphthalene and phenanthrene solubility, and enhanced alkane degradation by two other microbial species.

Two novel lipid biosurfactants recently described are from the marine bacterium *Myroides* sp. strain SM1 and the freshwater cyanobacterium *Aphanizomenon flos-aquae*. *Myroides* sp. strain SM1 produces a mixture of ornithine lipids capable of emulsifying oil over wide temperature (30–121°C) and pH (5–12) ranges, with no antimicrobial activity detected (Maneerat et al. 2006). *Aphanizomenon flos-aquae* was found to produce 2-acyloxyethylphosphonate containing mostly hexadecanoic acid and an interesting C-P bond linking the PO_3^- moiety to the head of the fatty acid (Kaya et al. 2006).

As in other fields, genomics is now proving useful for identifying biosurfactant biosynthesis operons when characterizing microbial genomes. Morita et al. (2006), when searching for novel MEL-producing microorganisms as part of the work mentioned previously, selected *Pseudozyma rugulosa* NBRC 10877 as a candidate based on sequence alignments of internal transcribed spacer regions 1 and 2, and the 5.8S rRNA gene, with other known producers. Of course, 16S genes are in no way related to biosurfactant biosynthesis, so having better information around known biosurfactant metabolic pathways would be a more direct approach. For example, the full genome sequence for *Alcanivorax borkumensis*, a marine hydrocarbonoclast able to emulsify oil thanks to excreted glycolipids, has recently been published. Two putative glycosyltransferase genes homologous to *rhlB* in *P. aeruginosa*, along with other genetic determinants that may play a role in biosurfactant

production, namely OprG/OprW, OmpH and OprF/OmpA, were identified (Schneiker et al. 2006). A proteomic exploration of hydrocarbon-grown cells revealed up regulation of lipoprotein-releasing protein (Lol), which may be associated with biosurfactant production for enhanced hydrocarbon accession and uptake (Sabirova et al. 2006).

Using a genomic approach to locate novel non-ribosomal petptide synthetases in *Pseudomonas syringae* pv. tomato DC3000, Berti et al. (2007) were able to discover a novel class of lipopeptide biosurfactants important in swarming motility. The six lipopeptides found are linear, as opposed to previously characterized cyclic peptides, and are produced from proteins coded in a single gene cluster. These lipooctapeptides named syringafactin A through F were detected and characterized thanks to the use of genomic, physiological and metabolic analyses within the study.

Obviously, having prior knowledge around biosurfactant biosynthesis gene sequences is essential for this type of study, and there remains a place for chemical and physiological screening of new isolates for novel biosynthetic pathways.

4.2.3 Physiological Roles of Biosurfactants

Biosurfactants and bioemulsifiers are exploited by a variety of life forms to facilitate diverse physical and chemical processes. In the microbial world, biosurfactants have been implicated as key elements in physical, chemical and behavioural activities, many holding potential value for enhanced biodegradation and bioremedation. This section will outline the physiological roles of biosurfactants while keeping an eye on bioremedial processes.

Given the focus, it is useful to envision the key steps preceeding pollutant biodegradation, biotransformation or metabolism by microorganisms. Simply put, a pollutant must normally be taken from an extracellular to an intracellular location prior to metabolism, which may require a microbe to sense, move towards, bind, and passively or actively transport molecules of interest. In some cases, extracellular enzymes may be deployed to oxidize a contaminant prior to uptake, or membrane-bound oxidases may serve to execute initial catabolic steps during the transport process (Van Hamme 2004). Many pollutants have limited solubility and, as such, often exist as a second organic phase, a major limiter of biodegradation. Thus, perhaps the most important roles for biosurfactants in bioremediation are the reduction of interfacial tension between aqueous and organic phases, and micellization or pseudosolubilization of pollutant so that a hydrophilic microbe may interact with a hydrophilic micelle in which hydrophobic substrate is found. The importance of mass-transfer effects on biodegradation will be described in more detail in the next section. In the meantime, other intra-, extra- and inter-cellular biosurfactant impacts will be described for whole-cell systems, in order to rationalize the diversity of effects observed when exogenous biological and chemical surfactants are included in bioremedation strategies.

From the inside out, biosurfactants function at many levels given their structure (Van Hamme et al. 2006) Rich in sugars, lipids and amino acids, it is possible that biosurfactants may serve as nutrient storage molecules, supported by the observation that limitations in nutrients such as nitrogen, phosphorous and iron may induce biosurfactant production under high carbon conditions (Amézcua-Vega et al. 2007; Soberón-Chávez et al. 2005; Teichmann et al. 2007). This has implications for biosurfactant applications in bioremediation, as biosurfactants may serve as competing carbon and energy sources.

Extracellular and intracellular interactions such as quorum sensing and biofilm formation may be facilitated by biosurfactants, thanks to physical effects directly influencing each process as well as by influencing gene expression. Once again, the biosurfactants produced by *P. aeruginosa* (HAAs and rhamnolipids) are an excellent example, due to the environmental and health impacts associated with this organism. These biosurfactants contribute to biofilm structural development, namely initial microcolony formation, migration-dependent cap formation, maintenance of open channels within maturing biofilms, and biofilm dispersal (Pamp and Tolker-Nielsen 2007). These are facilitated by biosurfactant surface-active effects and, given that biofilm formation is quorum-sensing dependent, on the interaction of biosurfactants with quorum-sensing autoinducer molecules. For example, biofilm formation and rhamnolipid production are dependent on autoinducer signalling molecules such as *Pseudomonas* quinolone signal (PQS or 2-heptyl-3-hydroxy-4-quinolone). Experiments with sparingly soluble PQS showed that rhamnolipid increased PQS solubility and bioactivity, thus enhancing further rhamnolipid production (Calfee et al. 2005). As with pollutant biodegradation, it appears that at high rhamnolipid concentrations PQS activity decreases, presmably due to micellar sequestration.

Similarly, by excluding access, biosurfactants may play a protective role for micoorganisms in the presence of toxic levels of organics or metals, leading to applications in metal sequestration. Much as biosurfactants may concentrate substrates via micellization to allow for increased uptake, they potentially reduce access to undesirable compounds, provided the microbial cell surface repels biosurfactant molecules. For example, a cell secreting biosurfactants may also display a hydrophobic cell surface to avoid interacting with polar micelles or bioemulsifier-coated non-aqueous phases.

An additional tool microbes use to minimize cell damage from potentially harmful molecules, such as polyaromatic hydrocarbons and antibiotics, is efflux. From a bioremedial standpoint, microbes with the potential for solvent efflux are important, as they are able to regulate intracellular levels of membrane-disrupting solvents during metabolism. Efflux pumps are being characterized at a rapid pace, but evidence for biosurfactant-mediated active efflux has yet been reported. Having said that, microorganisms producing biosurfactant as antagonistic membrane-disrupting agents, for example the cyclic lipopeptides of *Bacillus subtilis*, may express biosurfactant efflux pumps as self-resistance tools (Tsuge et al. 2001).

As alluded to above, microbe–biosurfactant–contaminant interactions are determined in part by relative hydrophobicities of each member of the triad. Microbes

show great plasticity with respect to their physiological form over short time frames, and biosurfactants play important roles in cellular differentiation. Fungal mycelia and bacterial aerial hyphae formation is often assisted by biosurfactants whose stimulating activities may be species-specific (Straight et al. 2006; Wessels 1997) In bacteria, cell-surface hydrophobicity may be modulated through changes in lipid composition, expression of hydrophobic surface proteins, incorporation of extracellular compounds such as alkanes, and intercalation, adsorption or action of biosurfactant molecules (Sokolovská et al. 2003; Soltani et al. 2004). Lipopolysaccharide, a key hydrophobic component in bacterial cell walls, can be an important cell-surface hydrophobicity determinant. Biosurfactant may strip lipopolysaccharide from cell walls when added exogenously, with subsequent impacts on overall cell hydrophobicity (Al-Tahhan et al. 2000)

This interesting phenomenon, along with membrane-disrupting abilities, may have roots in antagonistic (e.g., antibacterial, antifungal, antiviral) behaviours, two behaviours assisted at times by biosurfactant production (Rodrigues et al. 2006a). Teichmann et al. (2007) described the biosynthetic gene cluster for ustilagic acid biosynthesis in *Ustilago maydis*. Mutations in the gene cluster eliminated the ability of this phytopathogenic basidiomycetous fungus to prevent *Botrytis cinerea* infections of tomato leaves, indicating an antagonistic role for the secreted cellobiose lipid. Using mutational studies, Debode et al. (2007) showed that the antagonism of *Pseudomonas* spp. against the plant pathogen *Verticillium* is mediated by biosurfactant and that biosurfactant may enhance *Pseudomonas* colonization of microsclerota, disrupt cell membranes, release lipid storage molecules from the fungi, and enhance the toxicity of free radical forming, membrane soluble phenazine-1-carboxylic acids via micellization. From these specific examples, it is important to recognize the impacts which biosurfactants may have on community structure, as mixed communities are relied upon in real-world bioremediation efforts.

In subsurface environments, understanding pollutant transport is of utmost importance and, further, understanding microbial transport, with the aim of developing methods to enhance microbial movement through subsurface environments, is paramount. Linkages between chemotactic genes and biodegradation operons have been described (Parales and Harwood, 2002) highlighting, given the high energy costs associated with motility, the importance of movement in biodegradation processes. Motile and sensitive microorganisms have the ability to seek out novel nutrient sources, and are able to position themselves in an appropriate concentration of potentially toxic compounds. Biosurfactants are involved in various ambulatory modes associated with surface colonization and biofilm formation. Quorum-sensing mechanisms can be central to biosurfactant production, biofilm formation, and even expression of flagellin genes (Taguchi et al. 2006). For example, biosurfactant enhances *Bacillus subtilis* spreading via surface tension reductions and increased surface wettability (Leclère et al. 2006), swarming of *Pseudomonas syringae* pv. tomato DC3000 (Berti et al. 2007), type IV pili-mediated twitching motility (Pamp and Tolker-Nielsen 2007), and flagella-mediated swarming in *Pseudomonas aeruginosa* (Tremblay et al. 2007).

Thus, while it is often reported that the literature on biosurfactant and surfactant effects on biodegradation is confusing and lacks consensus, it can be argued that diversity is expected and inherent, given the diversity of biosurfactant roles and the diversity of the microbial world.

4.3 Biosurfactant Applications in Bioremediation

Various sources and applications of biosurfactants are shown in Table 4.1.

4.3.1 Mass Transfer Effects on Biodegradation

Pollutant mass transfer into a microbial cell is a significant determinant of biodegradation rates and extents. Depending on the physiology of the microbes involved, mass transfer may be affected positively, negatively, or not at all in the presence of a biosurfactant. As discussed above, these variable effects may originate with a number of physiological responses. In addition, it must be understood that, prior to uptake, microorganisms approach substrates via three general accession mechanisms. First, a microbe may encounter dissolved substrate that is readily available for passive or active uptake. Second, a microbe may access a poorly water-soluble substrate that has been pseudosolubilized in biosurfactant micelles. Third, a microbe may exhibit a hydrophobic cell surface in order to adhere directly to a non-aqueous phase liquid or solid and extract substrate from that phase (Van Hamme et al. 2003).

Generally, biosurfactant micelles can increase mass transfer thanks to increased pollutant pseudosolubility in aqueous environments (Das and Mukherjee 2007). Hickey et al. (2007) found that biosurfactant greatly increased polyaromatic hydrocarbon desorption from soil and enhanced biodegradation of four-ring PAHs by *Pseudomonas alcaligenes* PA-10, even though the biosurfactant reduced adherence of the microorganism to solid fluoranthene. The authors speculated that enhanced biodegradation just above the cmc could be due to a combination of increased solubility and increased biomass, given that the biosurfactant could serve as a carbon source.

It is possible that biosurfactant micelles may coalesce with microbial membranes and deliver a contaminant molecule directly to the outer membrane of a cell (Miller and Bartha 1989). However, should the micelle concentration be too high, pollutant may become effectively diluted across a large number of micelles, resulting in decreased mass transport due to stronger partitioning into the micellar phase. Brown (2007) has expanded on a mathematical model to incorporate direct uptake of hydrophobic substrates from the aqueous phase, transfer of micellar substrate to cell-surface adsorbed hemi-micelles that deliver substrate to the cell, and the possibility that hemi-micelle formation fails and thus sequesters substrate from the cell.

Table 4.1 Biosurfactant sources and their applications

Class	Surfactant	Microorganism	Application/activity	Reference
Glycolipid	Trehalose lipids	*Arthrobacter paraffineus*, *Corynebacterium* spp., *Mycobacterium* spp., *Rhodococcus erythropolis*, *Nocardia* sp.	Cosmetics, antifungal, antiviral Oil bioremediation and recovery	Singh et al. (2007), Peng et al. (2006)
	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp., *Serratia rubidea*	Oil recovery, metals removal, hydrocarbons removal, PAH removal	Bai et al. (1997), Mulligan et al. (2001), Rahman et al. (2003), Santa Anna et al. (2007), Whang et al. (2007)
	Sophorose lipids	*Candida apicola*, *Candida bombicola*, *Candida lipolytica*, *Candida bogoriensis*	Hydrocarbons removal Metals removal	Mulligan et al. (2001)
	Glycolipids	*Rhodococcus erythropolis*, *Serratia marcescens*, *Tsukamurella* sp.	Oil bioremediation	Peng et al. (2006)
	Lipopolysaccharides	*Acinetobacter calcoaceticus* (RAG1), *Pseudomonas* sp., *Candida lipolytica*	Bioavailability of surfaces	Van Hamme et al. (2006)
Lipopeptides	Mannosylerythritol lipids (MELs)	*Pseudozyma sp.*	Oil emulsifications	Fukuoka et al. (2007)
	Surfactin	*Bacillus subtilis*, *Bacillus pumilus*	Metal remediation	Mulligan et al. (2001)
	Arthrofactin	*Pseudomonas* sp. MIS38	Oil emulsification	Morikawa et al. (2000)
	Lichenysin A, Lichenysin B	*Bacillus licheniformis*	Metal remediation	Zouboulis et al. (2003)
	Bamylocin	*Bacillus amyloliquefaciens*	Oil-emulsification	Lee et al. (2007)
	Syringafactin A-F	*Pseudomonas syringae* pv. tomato DC3000	Swarming motility	Berti et al. (2006)
	Ornithine lipids	*Myroides* sp. strain SM1	Oil- emulsification	Maneerat et al. (2006)

(continued)

Table 4.1 (continued)

Class	Surfactant	Microorganism	Application/activity	Reference
Peptides	Ornithine, lysine peptides	*Thiobacillus thiooxidans*, *Streptomyces sioyaensis*, *Gluconobacter cerinus*	Oil emulsification	Maneerat et al. (2006)
	Lantibiotics	*Streptococcus mutans*, *Streptococcus salivarius*	Antibiotic activity	Willey and van der Donk (2007)
	Streptofactin	*Streptomyces* sp.	Aerial	Straight et al. (2006)
Lipids	Phospholipids	*Acinetobacter* sp.	De-emulsifying capability	Singh et al. (2007)
	Fatty acids (corynomycolic acids, spiculisporic acids, etc.)	*Capnocytophaga* sp., *Penicillium spiculisporum*, *Corynebacterium lepus*, *Arthrobacter paraffineus*, *Talaramyces trachyspermus*, *Nocardia erythropolis*	Enhanced oil recovery	Makkar and Cameotra (2002)
		Rhodococcus erythropolis strain 3C-9		Peng et al. (2006)
Glycoprotein	Alasan	*Acinetobacter radioresistens*	Phenanthrene, fluoranthrene and pyrene biodegradation	Mulligan (2005)
	Biosur PM	*Pseudomonas maltophilia*	Emulsifying capability	Bodour et al. (2003)
	Particulate surfactant (PM)	*Acinetobacter calcoaceticus*	Increase mass transfer	Rosenberg (1993)

From this, optimal surfactant concentration windows can be determined, taking into account the micellar surfactant concentration and the concentration of bioavailable substrate. Brown (2007) also points out that increased biodegradation kinetics may not be observed even if there is increased mass transfer due to surfactant addition if the cell is operating at a maximum biodegradation rate already. In a similar vein, it has been found that biosurfactant can not only have direct toxic effects on a cell, but that increased solubility of toxic compounds such as phenanthrene can have negative impacts on biodegrading microorganisms (Shin et al. 2006; Van Hamme et al. 2006).

Below the critical micellization concentration, mass transfer can be improved due to interfacial tension reductions between aqueous and organic phases, and associated increases in solubility. Alternately, biosurfactant-mediated LPS stripping can increase cell hydrophobicity and improve hydrocarbon biodegradation, as described for rhamnolipid below the cmc (Al-Tahhan et al. 2000)

For microbes that employ hydrophobic cell surfaces for direct adherence to non-aqueous phases, exogenous biosurfactant addition can decrease mass transfer by disrupting cell–NAPL hydrophobic interactions. The classic example of this is the removal of *Acinetobacter calcoaceticus* RAG-1 from hydrocarbon droplets by emulsan (Rosenberg 1993). Here it is believed that hydrophobic cells adhered to hydrocarbon droplets release an emulsan coat to mark a used oil droplet with a hydrophilic repulsive layer. Zhong et al. (2007) found that rhamnolipid adsorption to cell surfaces modulated hydrophobicity depending on the species involved, the phase of growth, and the rhamnolipid composition and concentration (below the cmc).

Noordman and Janssen (2002) found that rhamnolipid increased hexadecane uptake by *Pseudomonas aeruginosa* UGS but not by RAG-1 or two *Rhodococcus* spp. In this case, the results did not indicate biosurfactant-mediated attachment to hydrophobic substrates, but rather that micellar-medicated accession occurred. Further, uptake of 1-naphthylphenylamine, a hydrophobic fluorescent probe, was enhanced by rhamnolipid for energized, but not de-energized cells, implying a biosurfactant-assisted active uptake mechanism.

4.3.2 Soil Washing

Adsorption of hydrophobic contaminants to soil particles may greatly reduce substrate accessibility for microbial uptake and metabolism. Biosurfactant production by some microorganisms increases the pseudosolubility of poorly soluble substrates, so the addition of biosurfactants during soil washing has been explored.

During soil washing, contaminants are extracted into a wash solution, frequently water, by process of desorption or solubilization (Lao et al. 2007). The wash solution can be augmented with a basic leaching agent, surfactants, acid or base, or chelating agents to help remove organics and heavy metals. Instead of synthetic surfactants such as SDS or Tween 80, biosurfactants may be used, as they are known for their less harmful environmental effects. Generally, soil-washing technologies can be

divided into in situ and ex situ processes. In situ processes allow soil to be treated at the place of contamination, reducing costs; however, treatments require longer time, and results are not always uniform due to site variability. Contrary to in situ procedures, ex situ processes require soil excavation and transport prior to treatment (Kosaric 2001).

For organic contaminants, Santa Anna et al. (2007) showed that sandy soil contaminated with aromatic or paraffinic hydrocarbons can be cleaned with a blend of four rhamnolipid types produced by *Pseudomonas aeruginosa* PA1. Organics were removed from the soil with 78–91% efficiency during laboratory testing. Enhanced hydrocarbon removal with rhamnolipids has been shown in a variety of other studies (Bai et al. 1997; Rahman et al. 2003; Whang et al. 2007). However, *P. aeruginosa* UG2 rhamnolipids were not as effective at removing the pesticides trifularin, caumpaphos and atrazine from soil as was Triton X−100 (Mata-Sadoval et al.2000).

Envrionmental contamination by toxic heavy metals such as Cd, Pb, Zn, Mg and Cr(III) is problematic (Juwarkar et al. 2007), as metals released may adsorb to soil, accumulate in food crops, and are not normally removed by municipal water treatment (Mulligan et al. 2001). In soils, sediments and sludges, adsorption begins as a reversible process, but over time sorption increases, limiting bioavailability for biotransformation reactions. One of the three main treatments for metal-contaminated soils, besides solidification and stabilization, is a soil-washing procedure augmented with specific biosurfactants that help in metal dispersion, solubilization and desorption.

Mulligan et al. (2001) showed that surfactin produced by *Bacillus subtilis* ATCC 21332, rhamnolipids produced by *Pseudomonas aeruginosa* ATCC 9027, and sophorolipids from *Torulopsis bombicola* ATCC 22214 improved copper (110mg kg^{-1}) and zinc (3,300 mg kg^{-1}) removal from soil. Rhamnolipid (0.5%) removed 65% of the copper and 18% of the zinc, while 4% sophorolipid removed 25% of copper and 60% of zinc. Surfactin (2%) was less effective than surfactant-free wash solutions. Stability constants for metal-rhamnolipid complexes determined using an ion-exchange resin technique revealed that copper does indeed complex more strongly than zinc: Al^{3+}> Cu^{2+} > Pb^{2+}> Cd^{2+}> Zn^{3+}> Fe^{3+}> Hg^{+3}> Ca^{2+}> Co^{2+}> Ni^{2+}> Mn^{2+}> Mg^{2+}>K^{+} (Ochoa-Loza et al.2001). Other studies have shown that rhamnolipids enhanced removal of nickel and cadmium from soil in lab (80–100%) and field (20–80%) trials (Neilson et al. 2003), removal of stable chromium(III) from kaolinite, and removal of carcinogenic chromium(VI) (Massara et al. 2007).

4.3.3 Biosurfactant Production

Microbial surfactants have found a range of applications in the oil, paint, food, cosmetic and agricultural industries, as well as in bioremediation of organic and heavy metal contaminated sites. However, there is a need to reduce large-scale production costs in order to make biosurfactants economically attractive and competitive over synthetic surfactants. There are two possible models that can be considered: in situ and ex situ biosurfactant production.

Biosurfactant production in natural environments, for example at remediation sites, aims to stimulate microbial growth by creating a favourable environment by altering parameters such as nutrient types and concentrations, moisture content and pH. Either autochthonous microorganisms or specific inocula tailored for biosurfactant production may be encouraged. To date, the best examples of in situ biosurfactant production have been shown during microbial-enhanced oil recovery (MEOR) operations. For example, *Bacillus* sp. strain RS-1 and *Bacillus subtilis* subspecies *spizizeni* NRRL B-23049 were injected with nutrients into limestone petroleum reservoirs in an effort to induce lipopeptide production to lower the interfacial tension between hydrocarbon and aqueous phases to enhance hydrocarbon recovery (Youssef et al. 2007). Biosurfactant production was confirmed using HPLC and it was estimated that in situ biosurfactant production can lower oil recovery costs to as low as $1.6 per barrel. Over 400 MEOR tests have been carried out in the US using microorganisms such as *Xanthomonas campestri, Bacillus licheniformis, Pseudomonas aeruginosa* and *Desulfovibrio desulfuricans.* By injection of biosurfactant-producing bacteria to oil wells, 30–200% increases in oil recovery have been obtained (Singh et al. 2007).

Unlike in situ production, it is easier to achieve high biosurfactant yields in bioreactors under controlled conditions. Biosurfactant producers endeavour to compete with lower cost synthetic surfactants by using inexpensive raw substrates, enhancing yields through media formulation optimization and overproducing mutant strains, and by employing efficient downstream processing techniques. Oils such as sunflower, soyabean, grapeseed, Turkish corn, Babassu, as well as waste frying oils and oil refinery wastes, can be used as substrates. Curd whey, distillery wastes, potato process effluents, cassava flour wastewater have also been tested for biosurfactant production. Employing these substrates can potentially lower production costs by 10–30% (Mukherjee et al. 2006). To obtain a value added product from waste, Kosaric (1992) used municipal waste water sludge contaminated with organic pollutants as a substrate to grow *Torulopsis bombicola* anaerobically for sophorose lipid production.

Process optimization considers the level and ratios of key elements such as carbon, iron, nitrogen, phosphorous and manganese. For example, elevated C/N ratios enhanced biosurfactant production by *Pseudomonas aeruginosa*, while increases in divalent cation concentrations had a limiting effect (Soberón-Chávez et al. 2005). Oxygen tension, pH and temperature are also very important, and to optimize all parameters, statistical optimization approaches based on response surface methodology (RSM) can be used. RSM is a three-factorial design method which provides the relationship between one or more measured dependent responses and a number of input (independent) factors (Popa et al. 2007). This method has been successfully used to optimize biosurfactant production for *Pseudomonas aeruginosa* EM1 (Wu et al. 2008), *Bacillus subtilis* (Sen and Swaminathan 2004), *Lactococcus lactis* 53 and *Streptococcus thermophilus* A (Rodrigues et al. 2006b).

The genetic make-up of the producer strain influences biosurfactant yields and is critical for industrial applications. Knowledge about the biosurfactant structural and regulatory genes, as well as secretion mechanisms is valuable information to

have and can direct genetic modifications. *Pseudomonas aeruginosa* and *Bacillus subtilis* are often used because their genetics is well known (Sullivan 1998). As an example, Wang et al. (2007) used the genetically engineered strains *Pseudomonas aeruginosa* PEER02 and *Escherichia coli* TnERAB, which were able to produce rhamnolipids with high yield from inexpensive substrates. Produced rhamnolipids were then used in oil-displacement experiments, showing high oil recovery rates from a sand pack.

Downstream processing may account for as much as 60% of total biosurfactant production costs, and classic precipitations, solvent extractions or centrifugation are frequently used with more recent technologies such as ultrafiltration, foam fractionation, exchange chromatography or adsorption-desorption on polystyrene resins (Mukherjee et al. 2006).

4.4 Conclusions

Biosurfactants play a variety of critical roles in microbial lifecycles and, as such, have potential for applications in a variety of industrial, medical and environmental technologies. The ability of biosurfactants to decrease interfacial and surface tensions, and to pseudosolubilize hydrophobic organic pollutants as well as metals, creates excellent opportunities for applications in bioremediation. Both in situ and ex situ biosurfactant production and bioremediation will benefit from current research into the molecular, biochemical and physiological basis of biosurfactant metabolism. As production methods are improved, biosurfactant production costs should decrease and find wider applications as natural products for environmental remediation.

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Chapter 5
The Diversity of Soluble Di-iron Monooxygenases with Bioremediation Applications

Andrew J. Holmes

5.1 Introduction

Pollution with organic compounds (especially xenobiotics) is a significant problem of industrial societies. Treatment of waste streams to remove potential pollutants or remediation of contaminated sites can be achieved by exploiting the degradative capacity of bacteria. Among the most important of the enzymes used by bacteria in degradation of organic compounds are oxygenases. These enzymes catalyse the addition of oxygen atoms into organic compounds to produce alcohols, epoxides, etc. whose greater reactivity makes them substrates for a wider range of enzymes (Urlacher and Schmid 2006). The substrate range accommodated by the known oxygenases is enormous and for many organic pollutants, degradation pathways are initiated by oxygenases. This makes these enzymes applicable as fundamentally important enzymes to many bioremediation projects. They encompass a number of different protein families, utilizing distinct chemistries (Park 2007; Urlacher and Eiben 2006; van Beilen and Funhoff 2005; van Berkel et al. 2006; Wackett 2002).

This chapter focuses on one family of monoxygenases that have been termed the soluble di-iron monooxygenases (SDIMO), also termed bacterial multicomponent monooxygenases (BMM), that have wide applications in bioremediation (Leahy et al. 2003; Notomista et al. 2003). Recent advances in understanding of diversity in the SDIMOs are creating new opportunities for their effective use in bioremediation.

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5.2 Soluble Di-iron Monooxygenases (SDIMOs)

5.2.1 Biochemistry

SDIMOs are multicomponent enzymes found in bacteria and archaea (Notomista et al. 2003) that typically consist of three components: the multisubunit H protein (hydroxylase), the R protein (reductase) and the B protein (after protein B of soluble methane monooxygenase). The active site for oxygen insertion lies within the alpha sub-unit of the H protein, and has a non-haem (carboxylate-bridged) di-iron centre. The SDIMOs can perform a range of chemistries including hydroxylation, dihydroxylation and epoxidation of hydrocarbons (Wackett 2002). In all characterized examples the substrate range is very broad, and within the family it extends from methane through small alkanes to complex substituted ring compounds (Colby et al. 1977). It is important to note that while all SDIMOs have broad substrate ranges, these are non-overlapping, and different SDIMOs can show considerable kinetic differences. As a consequence, different enzymes have different applications (Wackett 2002; Watanabe et al. 2002).

5.2.2 Physiological Roles

The physiological role of all known SDIMOs is aerobic catabolism of organic compounds. Experimentally, they have exhibited proven roles in the first step in the utilization of methane, ethene, propane, propene, butane, tetrahydrofuran, phenol, benzene, and toluene. Despite the broad substrate range of all SDIMOs, the different enzymes apparently have restricted physiological roles and are often only expressed when the physiological substrate is present. This has implications for their exploitation in bioremediation, since it is relatively easy to selectively enrich for bacteria expressing enzymes with narrow physiological roles.

The SDIMOs are best considered as accessory enzymes, with possible exceptions in the case of some methanotrophs (Dedysh et al. 2005; Theisen et al. 2005). The vast majority of SDIMO-containing organisms are facultative heterotrophs able to grow on a wide range of substrates. Where present, SDIMOs are not essential genes, and result in only a modest expansion of the growth substrate range. The methanotrophs are different, in that the vast majority of known methanotrophs are obligately methanotrophic. Even here the sMMO (methane monooxygenase) (group 3 SDIMO) can be considered an accessory enzyme, in that an alternative methane monooxygenase (the heterologous particulate methane monooxygenase) is apparently the more important enzyme to the cell (Dumont and Murrell 2005).

5.2.3 Genetics, Diversity and Classification

Presently known SDIMOs are a diverse group, yet distinctive and easily recognized by sequence relationships. The proteins are encoded in a single operon of 4–6 genes. If we only consider those enzymes for which complete operon sequence information is available, then at least six groups are easily distinguished (Coleman et al. 2006; Leahy et al. 2003; Notomista et al. 2003). Each of these groups is well-separated from the others in phylogenetic analysis and also shares a distinctive operon arrangement, further supporting the phylogenetic-based classification (Fig. 5.1). The quaternary structure of the enzymes is also consistent with this scheme, insofar that SDIMOs of the same subgroup share the same configuration. In the case of groups 1, 2, and 3 there are three H protein sub-units in an $\alpha 2\beta 2\gamma 2$ configuration, and groups 4, 5, and 6 apparently lack the gamma sub-unit and have an $\alpha 2\beta 2$ configuration. Despite the neat correlation between phylogenetic relationships and operon structure, it is inevitable that as databases grow the distinction between groups will at some point be lessened. Already there is one example of an SDIMO of unknown function that is phylogenetically affiliated to the group 3 SDIMOs, but has a distinct operon structure (Holmes and Coleman 2008). It is likely that further groups will be recognized. PCR-based surveys of alpha sub-unit genes have revealed high diversity of SDIMOs, with many that are not unambiguously classified into one of the existing six subgroups (Coleman et al. 2006).

Closely related enzymes do appear to share similar physiological roles. For example, the soluble methane monooxygenases all comprise a closely related subgroup within group 3 (sMMOs), and ethene and propene monooxygenases each comprise closely related subgroups within group 4 (Fig. 5.1). There also appears to be a general correlation between more distantly related SDIMO subgroups and physiological roles. The group 1 and 2 SDIMOs are phylogenetically distinct from the remainder of the family and are pre-dominantly involved in degradation of aromatic substrates (aro-SDIMOs). Virtually all group 3, 4, 5, and 6 SDIMOs target short chain alkanes or alkenes (alk-SDIMOs). A notable exception to this general pattern is that of the group 1 SDIMO of *Xanthobacter* Py2, which is an alkene monooxygenase (Champreda et al. 2004). As databases grow, it is likely that many more exceptions to the broad aromatic vs alkene/alkane SDIMO generalization will emerge.

The classification system for SDIMOs appears to be very robust, and has at least some useful predictive power. The propensity of SDIMOs to reside on mobile elements and undergo horizontal transfer means there is a very limited capacity to predict the identity of the host bacterium from SDIMO sequence relationships. However, there is sufficient correlation between SDIMO classification and the enzymes' physiological roles in the host organism that for closely related sequences prediction of the physiological substrate may be made. Nevertheless, we should be

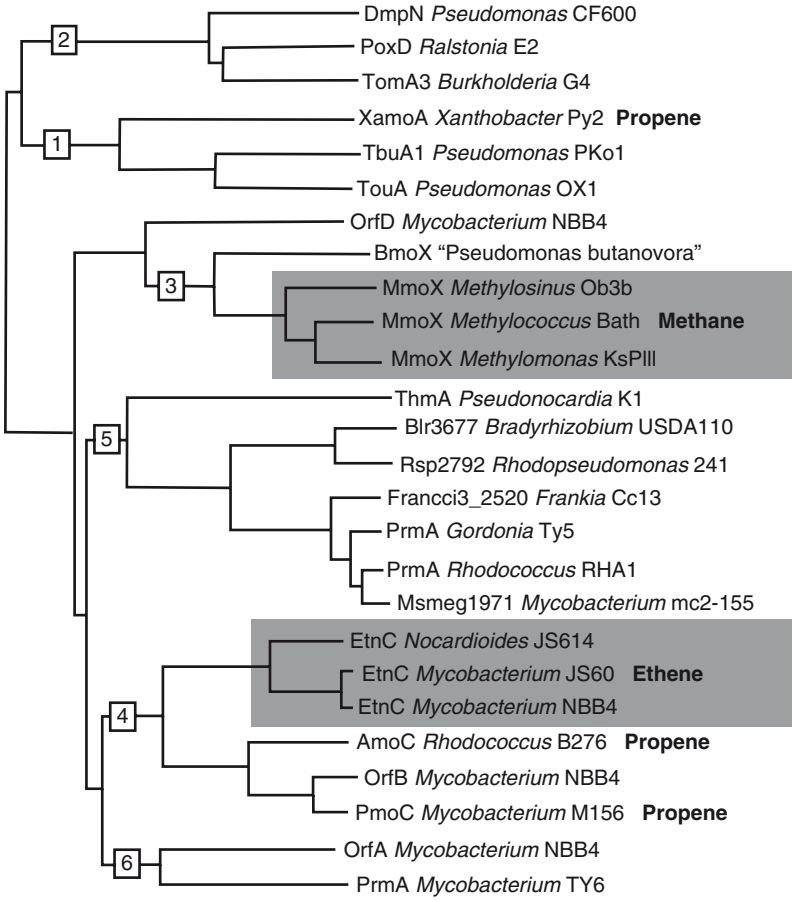

Fig. 5.1 Evolutionary relationships of SDIMOs. Evolutionary branches leading to each of the six groups are indicated on the dendrogram. In each case, all known members of the group share the same operon structure. Note that OrfD from *Mycobacterium* sp. NBB4 is phylogenetically affiliated to the group 3 SDIMOs but is part of a distinct operon arrangement. Subgroups in which all members share the same physiological role are indicated by shading. More detailed analyses of aro-SDIMOs (groups 1 and 2), sMMOs (group 3), and alk-SDIMOs (groups 4, 5, and 6) can be found in Coleman et al. 2006; Iwai et al. 2007; McDonald et al. 2006.

aware that the characterised SDIMOs are not representative of the naturally occurring diversity, and some strains are known to carry multiple different SDIMOs (Coleman et al. 2006). Consequently our view of the range of SDIMO-linked physiologies is potentially strongly biased, and caution must be exercised in inferring the physiological role of the enzymes.

5.3 Applications of SDIMOs in Bioremediation: Pollutants and Approaches

The conjunction of a narrow physiological role and a broad substrate range creates two distinct modes of application for organisms expressing SDIMOs in environmental biotechnology: growth-linked metabolism (exploiting the physiological role) and cometabolism (exploiting the substrate range).

5.3.1 Growth-Linked Metabolism

Where the substrate being attacked benefits the growth of the organism, the degradative process is considered growth-linked. In the case of the SDIMOs, any growth benefits are derived from subsequent metabolism of the reaction products, since the SDIMO-catalysed step is energy-consuming. They typically enable further degradation of the compound as a source of both carbon and energy. Aromatic compounds can be significant components of industrial waste streams, and are frequent environmental contaminants. Isolations of biochemically and phylogenetically diverse organisms capable of growth on aromatic substrates have been reported. The aro-SDIMOs include a number of very efficient enzymes for catalysis of these substrates, and organisms expressing these enzymes are frequently encountered. This is perhaps best illustrated in the case of phenol-degraders. In bioreactors, where phenol concentrations may be maintained at relatively low levels by microbial activity, organisms with low Ks (high affinity) tend to dominate. In numerous studies these organisms have been found to express multicomponent phenol hydroxylases (Group 2 SDIMOs) (Basile and Erijman 2008; Futamata et al. 2001; Watanabe et al. 1998). Growth-linked metabolism of organisms expressing Group 1 or Group 2 SDIMOs either currently, or potentially, find application in degradation of many aromatics, including phenol, benzene and toluene.

It is worth emphasizing that it is the metabolic context of SDIMO expression (presence of appropriate downstream steps of a degradative pathway) that determines whether their activities will have any benefit for the cell. Many SDIMOs from groups 3, 4, 5 and 6 can also attack aromatic compounds, but organisms possessing these enzymes typically lack the additional genes necessary to exploit this for growth. Perhaps the best illustration of this, however, is in the degradation of chlorinated ethenes. Aerobic utilization of vinyl chloride has been reported from a number of strains expressing a group 4 SDIMO (Coleman et al. 2002). However, not all strains expressing this enzyme are capable of growth on vinyl chloride. The key enzyme in this case is an ethene monooxygenase and only where an epoxyalkane (coenzyme M transferase) is also present can the cells grow on VC (Coleman and Spain 2003a). All ethene-degraders are capable of VC degradation, but only in

those with the additional enzyme is degradation growth-linked. In other cases, VC degradation occurs via co-metabolism.

5.3.2 Cometabolism

In cometabolism there is no benefit to the cell performing the reaction, and it can even retard growth by competitive inhibition of growth substrates. Nevertheless, for many recalcitrant pollutants this is a useful option to initiate degradation by other processes. Many oxygenases are very powerful catalysts, able to insert oxygen atoms into chemically stable compounds. The hydroxylation (or epoxidation) of the substrate either renders it less toxic, or more amenable to further degradation. The SDIMOs are good examples, and their broad substrate range means they can insert oxygen atoms into a wide range of organic compounds. Significantly this includes priority or emerging pollutants such as the chlorinated ethenes, methyl *tert*-butyl ether (MTBE), *N*-nitrosodimethylamine (NDMA) and dioxane. Both the alk- group and aro-group SDIMOs are potentially applicable to some or all of these compounds (Futamata et al. 2005; Haase et al. 2006; Lee et al. 2006; Mahendra et al. 2007; Mattes et al. 2005; Sharp et al. 2007). Successful exploitation of cometabolism for bioremediation requires more careful management of the process, and this is particularly the case with organisms using SDIMOs where the enzymes are typically not constitutively expressed and different forms vary in their ability to degrade substrates. Strategies are necessary to ensure that the cells are expressing the desired enzyme activity, and that the activity can be sustained.

For example, characterization of lab model strains suggests that the sMMOs (group 3 SDIMOs) have the most favourable kinetic properties for TCE degradation. However, in cometabolic transformations the target compound gives rise to competitive inhibition but can also exert toxic effects. SDIMOs with lower affinity for TCE are potentially more effective in cometabolic remediation, as they are less susceptible to competitive inhibition. The concept of transformation capacity was developed to describe these effects (Alvarez-Cohen and McCarty 1991a; 1991b). Recently, this concept has been taken further, and experimental evidence indicates that the cometabolic transformation performance of two kinetically distinct enzymes varies according to substrate concentration (Lee et al. 2006).

5.3.3 Strategies for Field Application

Not all organisms can degrade all pollutants, and even those that can will not do so under all conditions. Fundamentally, the goal of bioremediation is to have the right organisms, expressing the right genes, under the right conditions. In any contaminated site, it is highly unlikely that optimal contaminant removal conditions will exist naturally. The opportunity to improve the efficiency of the process will nearly

always exist, but one must also consider the practicality of this. The area of contamination is rarely homogeneous and can be quite large, particularly where contaminants are being transported by groundwater. The economic (and environmental) cost of large-scale manipulation of site conditions can be enormous. From a practical perspective, the aim of bioremediation is not to identify the perfect conditions for the process, but the more modest goal of identifying which, if any, of the parameters that are amenable to manipulation are likely to give useful outcomes. For in situ bioremediation, monitored natural attenuation is often the first step, and where site manipulation is found to be desirable there are two broad strategies: biostimulation and bioaugmentation. In the biostimulation approach, the activity of the natural microbiota is manipulated towards desirable activities by supply of substrates or manipulation of the physico-chemical environment. Organisms expressing SDIMOs are readily exploited in this fashion by virtue of their distinctive physiology. Since most SDIMOs have a narrow physiological role, often for unusual substrates, it is theoretically possible to selectively enrich for them. In bioaugmentation, the additional step of inoculation with desired organisms is undertaken. In all cases, there is a role for monitoring of the community to guide management decisions.

5.4 Monitoring Microbial Communities

The presence and activity of organisms in a contaminated site has the potential to yield much information relevant to site-management decisions (Head 1998). Broadly speaking, an ecosystem's community structure is both a historical record of the site (relative abundance of organisms reflects past ecological successes and failures) and an indicator of present biochemical potential. As such, community information can be used as a basis for choosing appropriate site management strategies, or for confirming the outcomes of existing management strategies. There are two approaches to assessing communities, mainly use of culture-dependent (isolation of organisms) or culture-independent (nucleic acid-based) methods. These are best viewed as complementary rather than competing alternatives, since each offers different advantages and suffers from different drawbacks (Gray and Head 2001).

5.4.1 Culture-Based Sampling for Degradative Organisms

The fundamental weakness of culture-based approaches is that they do not recover a representative sample of the in situ community, and are prone to completely fail to sample the ecologically relevant populations (Head et al. 1998). This issue has been widely discussed, and will be only briefly dealt with here. Organisms that exploit pollutants to enable growth are readily enriched and isolated. Theoretically, high-throughput culture approaches or hybrid molecular-enrichment culture strategies such as stable isotope probing could effectively sample all those organisms relevant

to growth-linked degradation. Such approaches may be effective for assessing the likely success of natural attenuation as a site management strategy, or for confirming the success of a biostimulation strategy in enriching a known target group. However, culture-based approaches cannot efficiently predict the cometabolic degradation potential within a community. There is no universal enrichment-dependent strategy to recover all organisms relevant to cometabolic applications. The SDIMO-expressing organisms represent a classical illustration of this limitation. Enzymes from subgroups 1, 2, 3, 4, and 5 have all been shown to cometabolically degrade chlorinated ethenes, but those of groups 1 and 2 predominantly function as aromatic monooxygenases (Futamata et al. 2003; Hopkins et al. 1993; Hopkins et al. 1997), those of group 3 as either methane (Lee et al. 2006) or butane monooxygenases (Halsey et al. 2005), those of subgroup 4 as ethene monooxygenases (Coleman and Spain 2003b), and those of subgroup 5 as propane monooxygenases (Connon et al. 2005). The narrow and distinct physiological roles of different SDIMOs means that enrichment on any one substrate (e.g., methane) will reveal the presence of some potential TCE-degraders, but not all potential degraders.

5.4.2 Culture-Independent Sampling for Degradative Organisms

A revolution in microbial ecology began with the application of molecular techniques to description of microbial communities. Amplification and sequencing of rRNA genes from total environmental DNA offers the most comprehensive view available of microbial community structure. The drawbacks of this approach are that one can be overwhelmed with the volume of data, and the data are often not easy to interpret. Arguably the two greatest problems are that classification based on sequence relationships is database-dependent, and using the sequence datasets is dependent on robust classification. As a consequence, predicting the physiology of uncultivated organisms from rRNA sequence data is notoriously difficult, and classification of highly divergent sequences can be ambiguous. The former problem is especially acute in the case of degradative organisms, since the relevant genes are frequently carried on plasmids or other mobile elements and show very poor correlation with rRNA phylogeny.

A potential solution to these issues is the use of metabolically diagnostic genes (frequently referred to in the literature as “functional gene probes”) rather than the 16S rRNA genes to explore community structure. Metabolically diagnostic marker genes are only present in a subset of the community and thus greatly reduce the complexity of sample sets. Applications of SDIMO-expressing organisms in bioremediation will potentially benefit from monitoring via this approach. The robust classification scheme for SDIMOs is expected to aid classification of sequences recovered from environmental communities. The apparently good correlation between physiological role and sequence relationship for closely related enzymes should facilitate interpretation of the data. However, a representative database is still a pre-requisite for culture-independent community monitoring.

Arguably the most successful strategy for surveying gene diversity presently available is use of broad-specificity primers to target the gene family of interest by PCR. In the case of SDIMOs, this approach to gaining a representative sample of all SDIMOs genes present in an environment is relatively difficult, owing to restrictions on primer design. The genetic context of SDIMO operons and of individual genes within the operons of different subfamilies varies. This means that any PCR-based strategy for sequence recovery is limited to using primer pairs within a single gene. In the case of SDIMOs, the sequence divergence of each gene is such that it is not possible to design a single set of 'universal' primers able to target all known SDIMOs by PCR. This constraint has meant that efforts at PCR-based recovery of SDIMO gene diversity have focussed on specific subgroups. Primer sets have been described for group 1 (Baldwin et al. 2003), group 2 (Baldwin et al. 2008; Basile and Erijman 2008), group 3 sMMOs (McDonald et al. 1995), and groups 4, 5, 6 (Coleman et al. 2006).

Application of these primers to community structure surveys can reveal useful insights. A good example is seen with the alkene monooxygenases. The broad specificity primers described by Coleman et al. (Coleman et al. 2006) were used to compare the diversity of SDIMO genes present in a range of natural environments and in ethene-enrichment cultures. Very high diversity was recovered in natural environments, but a strongly skewed distribution towards group 4 SDIMOs was seen in ethene enrichments and in sites contaminated with chlorinated ethenes. This was interpreted to reflect that ethene is highly selective for a subset of SDIMO-containing organisms (Coleman et al. 2006). Culture-based approaches have shown a similar bias towards recovery of a very narrow set of SDIMOs, as has previously been observed for phenol and methane. Observation of a comparable phenomenon using broad-specificity primers gives greater confidence that many SDIMOs are associated with predictable physiological roles.

The significance of these observations is that SDIMOs may be very useful as metabolically diagnostic probes for both growth-linked and cometabolic bioremediation processes. Recent advances in the development of microarray technology for environmental microbiology (Bodrossy et al. 2006) are widely anticipated to lead to many opportunities for enhanced bioremediation using SDIMOs. The expanded databases have enabled the recognition of new groups of SDIMOs (Coleman et al. 2006), and the construction of microarrays representative of diversity in certain groups (Bodrossy et al. 2003; Iwai et al. 2007).

5.5 Conclusion

It seems likely that when a limited set of carbon sources is supplied, either in vitro for enrichment attempts or in situ for biostimulation, the responsive organisms will encompass a limited set of SDIMOs. Available data suggest that substrates that may stimulate organisms containing particular SDIMO subgroups will include toluene (group 1), phenol (group 2), methane (group 3) and ethene/propene (group 4).

Other substrates worthy of further investigation include butane (group 3), and propane (group 5, group 6). The availability in lab culture of model organisms from each of these groups enables predictions regarding the spectrum of kinetic properties and substrate ranges within these groups. The combination of predictable enzyme properties, with the ability to monitor both the community of cells and expression levels of different SDIMO genes, is likely to lead to significant developments in cometabolic bioremediation in the near future.

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Chapter 6
Bioremediation of Polluted Soil

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6.1 Introduction

Global industry depends on fossil fuels as a primary energy source. Due to fossil fuel imports/exports and manufacturing, there is a high risk of environmental pollution, and consequently severe ecological disruption, as a result of fuel by-products and spills in areas where storage, transport, refining, distribution, consumption and fossil fuel-related industries exist.

Hydrocarbons have traditionally been considered to be of biological origin, since methane and other longer chain hydrocarbons appear to be exclusively the result of biological processes. However, it is now known that the largest supply of carbon in the planetary system is in the form of hydrocarbons. Petroleum and coal contain a class of molecules known as hopanoids commonly found in bacterial cell walls (Gold 1985): thus, it can be concluded that at some point all of these fuels originated, at least in part, from microbes. Based on this, the assumption can be made that biodegradation of these fuels has always been occurring to some extent. To extrapolate from this knowledge, the biological origins of these hydrocarbons could be the reason that the adaptation of microbes to degrade them so readily occurs upon technological industrialisation of the Earth, and why phytoremediation is such an applicable method for polluted soil reclamation. Microorganisms, natural or genetically engineered, can mineralise toxic polycyclic aromatic hydrocarbons (PAHs) into carbon dioxide and water (Fig. 6.1).

In petrol-polluted soil, benzene, toluene, ethylbenzene and xylene (BTEX) isomers are present in the water-soluble fraction, causing pollution (Prenafeta-Boldú et al. 2002). However, the most notorious class of hazardous compounds found in petrol, diesel, hydrocarbon-based oil, as well as coal-tar and its derivatives, are the polycyclic aromatic hydrocarbons (PAHs). PAHs are hydrophobic, chemical compounds consisting of fused aromatic rings, not containing heteroatoms (any atom other than carbon or hydrogen) or carrying substituents, e.g., naphthalene, anthracene,

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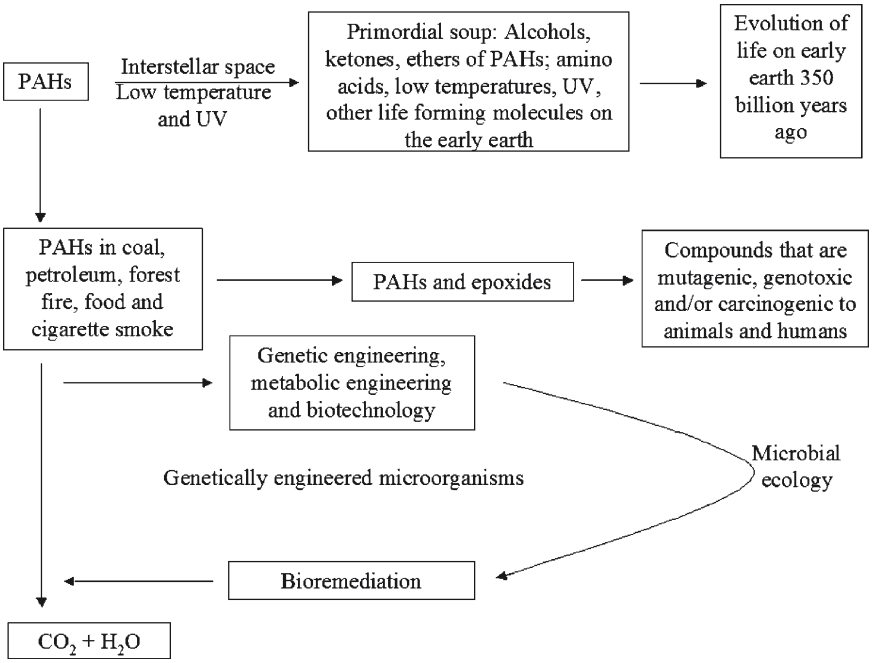

Fig. 6.1 Fate, toxicity and remediation of PAHs in the environment

phenanthrene, benzo(a)pyrene, coronene, pyrene, triphenylene, chrysene and benzo(ghi)pyrene (Wikipedia 2005a). Known to be carcinogenic, PAHs are formed by incomplete combustion of carbon-based fuels such as wood, coal, diesel, fat and tobacco. PAHs with up to four fused benzene rings are known as light PAHs, the simplest of these being benzocyclobutene (C_8H_6). Those containing more benzene rings are known as heavy PAHs, and are more stable and more toxic.

Naphthalene and toluene, found in petroleum and diesel products, are two of the most common PAHs that are subject to biodegradation. Naphthalene is a crystalline, aromatic, white, solid hydrocarbon which is volatile and forms a flammable vapour. The name is derived from the Latin *naphtha*, meaning liquid bitumen, and is of Semitic origin. It consists of two fused benzene rings (Fig. 6.2), is classified as a benzenoid PAH, and is manufactured from coal-tar. When converted to the phthalic anhydride, it is used in the manufacture of plastics, dyes and solvents, and as antiseptic and insecticide (Wikipedia 2005b).

Toluene, also referred to as methylbenzene or phenylmethane, is a clear, water-insoluble liquid. The name is derived from *toluol*, referring to tolu balsam, an aromatic extract from the tree *Myroxylon balsamum* (L.) Harms, from which it was first obtained (Wikipedia 2005c). It is an aromatic hydrocarbon with a methyl side-chain (Fig. 6.3), widely used as an industrial feedstock, octane booster in fuel, solvent in paints, rubber, printing, adhesives, lacquers, in leather tanning, disinfectants, and in the production of phenol, polyurethane foams and TNT (Wikipedia 2005c):

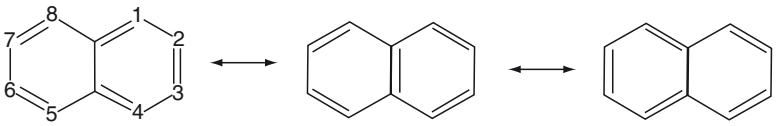

Fig. 6.2 The dynamic molecular stucture of naphthalene

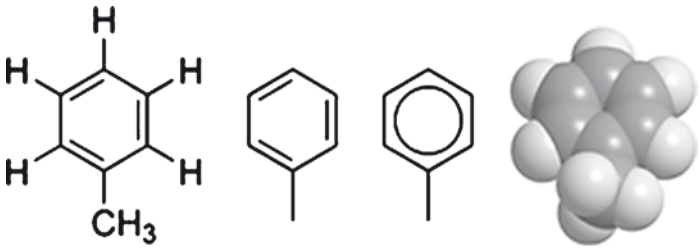

Fig. 6.3 The molecular structure of toluene

Hydrocarbon sources of pollution affect the environment and specifically the soil. Soil disruption caused by pollution with these compounds decreases biodiversity, and selects for microbial species better adapted to survive in the changed environment (Lindstrom et al. 1999; Kozdrój and Van Elsas 2001). Environmental changes due to this pollution affect the soil structure and fertility, and therefore the fauna and flora. Affected soils become relatively sterile to all but resistant microbial life forms. Certain indigenous microorganisms, including bacteria and fungi, are able to degrade PAHs in soil, leading to in situ rehabilitation of polluted soils. The utilisation of such microorganisms for detoxifying and rehabilitating PAH-polluted soils provides an effective, economical, versatile and eco-compatible means of reclaiming polluted land (Guerin 1999; Bogan et al. 2001; Margesin and Schinner 2001; Mishra et al. 2001; Tesar et al. 2002).

The bioremoval capacity of a soil can be improved by inoculation with specific strains and/or consortia of microorganisms (Halden et al. 1999; Dejonghe et al. 2001), particularly those from the rhizosphere of plants, since they are less readily destabilised due to the buffering in the presence of their host plant, but nevertheless are sustained in the biotic and abiotic environment they inhabit (Bahme et al. 1988). BTEX isomers are the most susceptible to elimination from the environment by indigenous microorganisms. However, degradation can be impeded by the micronutrient balance within the natural system (Koizumi et al. 2002).

Remediation is usually limited by the amount of free carbon, phosphorus or nitrogen available (Bogan et al. 2001; Margesin and Schinner 2001; Röling et al. 2002). Nitrogen is the most important of these elements required under limited nutrient conditions, as it is used in the synthesis of proteins, nucleic acids and other cellular components. Elemental nitrogen present as an atmospheric gas is almost inert, due to the stability of the triple bond between the two nitrogen atoms. Thus, elemental nitrogen

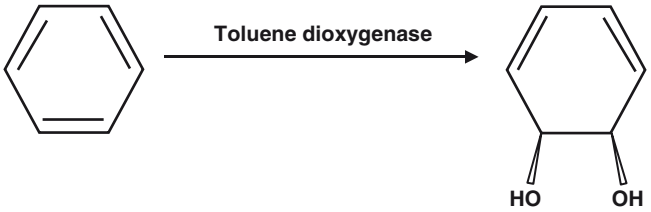

Fig. 6.4 Catabolism of two- to four-ring PAHs by aromatic hydrocarbon dioxygenases

must be "fixed" by bacteria in soil for plant, termite and protozoan growth (Deacon 2004). However, there are some exceptions to this synergistic nitrogen fixation relationship that exists between bacteria, plants, termites and protozoans. Struthers et al. (1998) reported that the herbicide atrazine is degraded by *Agrobacterium radiobacter* in soil without addition of extra carbon or nitrogen sources. Despite this, injecting soluble nutrients, like nitrogen sources, a few centimetres under the surface of the soil, can increase microbial community numbers. Gaseous nitrous oxide has been used to supply nitrogen to polluted soils in the process of bioremediation (Bogan et al. 2001). Addition of nutrients to soil, such as nitrogen fertilisers, has been proven to enhance biodegradation of PAHs (Kasai et al. 2002).

The first culture-independent estimate of prokaryotic organisms in soil indicated the presence of 4600 distinct genomes in 1 gram of soil (Kent and Triplett 2002). Extracted DNA or RNA can, via molecular genetic techniques, facilitate microbial community analysis to be coupled with phylogeny (Blackwood et al. 2003). The uncultured diversity will reflect species closely related to known cultured organisms and also species from virtually uncultured lineages (Blackwood et al. 2003). Characterisation of genes of microbes involved in the degradation of organic pollutants has led to the application of molecular techniques in microbial ecology of polluted areas (Milcic-Terzic et al. 2001).

Molecular methods usually involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene. These methods include denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP). Most of these methods do not reveal diversity unless the community is very simple. This is due to only a very low amount of species indicated in re-hybridization or sequence analysis being visualised on a gel (Linderman 1988; Blackwood et al. 2003). However, DGGE in particular is applicable to the present study, since diversity in PAH-polluted soils is expected to be low due to the high environmental selection pressure on the microbial species present. Furthermore, catabolic gene probes can be used in nucleic acid hybridisation analysis to characterise sequences (Nakatsu et al. 2000). DGGE also allows for the elucidation of major differences between communities, and for testing of hypotheses on the basis of sample comparison (Blackwood et al. 2003).

Fairly recently developed, DGGE is an ideal molecular technique for monitoring microbial ecology. It relies on variation in genetic sequence of a specific amplified

region to differentiate between species within microbial communities (Banks and Alleman 2002; Koizumi et al. 2002). DGGE allows a high number of samples to be screened simultaneously, thus facilitating more broad-spectrum analyses. PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The band pattern on the gel forms a genetic fingerprint of the entire community being examined (Gillan 2004). Resulting gel images can be digitally captured and used for species identification when samples are run against known standards (Temmerman et al. 2003). 16S rRNA genes are most commonly used to give an overall indication of the bacterial species composition of a sample. Partial sequence of this gene has been analysed from as complex environments as soil (Throbäck et al. 2004).

DGGE allows for determining total community as well as specific community or gene diversity without further analysis and without elucidating particular individuals. It has been used in the identification of sequence variations in multiple genes among several organisms simultaneously (Muyzer et al. 1993). However, functional genes, having more sequence variation, can be used to discriminate between closely related but ecologically different communities. Rosado et al. (1998) used *Paenibacillus azotofixans nifH* species-specific primers in DGGE analyses of soil samples. They found that *nifH* is probably a multicopy gene in *P. azotofixans*, and also identified intraspecific genetic diversity within this important functional gene. Following this, Milcic-Terzic et al. (2000) isolated diesel, toluene and naphthalene-degrading microbial consortia from diesel-polluted soils. Using PCR with gene-specific primers, they screened for the presence of the catabolic genes *xylE* and *ndoB*, responsible for toluene/xylene and naphthalene biodegradation respectively, from petroleum and diesel-polluted soils. These genes were targeted in order to assess the bioremediation potential of microbial consortia in petrol and diesel-polluted soils (Greer et al. 1993).

Some microorganisms, e.g., nitrogen-fixing microbes, are difficult to culture due to their specialised growth requirements and physiology, limiting simultaneous cultivation of several species (Widmer et al. 1999). Molecular methods for identifying nitrogen-fixing bacteria and Archaea are now available through the design of broad-spectrum highly degenerate primers. Widmer et al. (1999) designed a set of nested degenerate primers based on the amino acid sequence of the *nifH* gene. This is the general marker gene in nitrogen-fixing bacteria, and encodes the enzyme nitrogen reductase. Similarly, Zehr and McReynolds (1989), Simonet et al. (1991) and Yeager et al. (2005) successfully designed three more sets of degenerate primers for universal targeting of the *nifH* gene in microorganisms. The *nif* gene operon structure and regulation have been relatively conserved during evolution, making it a good candidate for focus in diversity studies (Gussin et al. 1986).

6.2 Soil Health

Soil health can be defined as “the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality and maintain plant and animal health” (Doran and Zeiss 2000). Productivity of

conventional agricultural systems largely depends on the functional process of soil microbial communities (Girvan et al. 2003). The structure and diversity of these communities are influenced by the soil structure and spatial distribution, as well as by the relationship between abiotic and biotic factors of microbial communities (Torsvik and Øvereås 2002). With the advent of various types of industries over the past 200 years, the ecology of earth's ecosystems has been severely disrupted. The commercialisation, extraction, refining, transportation, distribution and storage of petroleum products have led to oil, petrol and diesel pollution of soils. In petrol-polluted water that may seep into soil, benzene, toluene, ethylbenzene and xylene (BTEX) isomers are present in the water-soluble fraction (Prenafeta-Boldú et al. 2002). This disruption has decreased biodiversity, and selected for cosmopolitan microbial species better adapted to survive in the changed environment (Kozdrój and Van Elsas 2001). Not least impacted by these changes is the microbiota inhabiting the soil.

6.3 Pollution

Hydrocarbons are currently the main source of the world's energy resources, due to the energy they produce when combusted. This also makes them the world's main source of pollution in the case of spills and waste products. There are essentially three types of hydrocarbons, viz. (1) aromatic hydrocarbons that have at least one aromatic ring, (2) saturated hydrocarbons, including n-alkanes, branched alkanes and cycloalkanes that do not have double-, triple- or aromatic-bonds, and (3) unsaturated hydrocarbons with one or more double- or triple-bonds between carbon atoms, referred to as alkenes and alkynes respectively (Atlas 1981; Wikipedia 2006a). The most notorious class of hazardous compounds found in petrol, diesel, oil, as well as in coal-tar and its derivatives, are the PAHs. Polyphenols and PAHs are common industrial pollutants, and are found as co-contaminants in the environment. They are hydrophobic organic compounds consisting of two or more benzene rings fused into a single aromatic structure. They may form naturally from burning of organic matter or from production and partial combustion of fossil fuels (Joner et al. 2002). Hopanes, complex alicyclic compounds, are of the most environmentally persistent components of petroleum spillage (Atlas 1981). Mammalian liver enzymes (cytochrome P-450 and epoxide hydrolase) oxidise certain PAHs to fjord- and bay-region diol-epoxides which, in turn, form covalent adducts with DNA (Bogan et al. 2001). Due to this, many PAHs promote effects similar to other carcinogens, once taken up by the body (Guerin 1999; Bogan et al. 2001). Sixteen PAHs have been included in the United States Environment Protection Agency's priority pollutant list (Bogan et al. 2001).

6.4 Plants and Phytoremediation

The presence of plant rhizospheres in hydrocarbon-polluted soils facilitates an increase in microbial numbers and metabolic activity within the soil. Studies have shown that root length, surface area, volume and diameter play a role in the rehabilitative effect of plants in crude oil-polluted soil (Merkl et al. 2005). Roots can also improve the physical and chemical properties of pollutant-stressed soil, in addition to increasing contact between microbes associated with plant roots and pollutants in the soil (Aprill et al. 1990). This effect was first described by Hiltner (1904), who defined the rhizosphere as the zone of soil in which microbes are influenced by plant root systems, and where soil organisms have an impact on plants. Microbes isolated from the rhizosphere may have root growth-promoting or growth-inhibiting properties (Kuiper et al. 2004). Studies of plant species involved in phytoremediation have indicated that various grass species and leguminous plants are suitable for biodegradation. It is known that gram-negative rods such as *Pseudomonas* species dominate the rhizosphere (Kuiper et al. 2004). Some success in rehabilitation of hydrocarbon-polluted soils has been achieved by phytoremediation. It is defined as the use of plants to remove, destroy or sequester hazardous substances from the environment (Glick 2003). It has been documented that remediation of hydrocarbon-polluted sites is enhanced by cultivation of plants (Merkl et al. 2005).

Plants can reduce hydrocarbon levels in the soil, although the mechanism by which this happens is not yet entirely understood. Phytoremediation depends greatly on the stimulation of rhizosphere microorganisms by plant roots (Tesar et al. 2002). However, hydrocarbon uptake is limited by the lipophilicity of the hydrocarbons in question, which affects their passage through the cell membrane. This uptake is thought to be attributed to increased microbial activity in polluted soils, as supported by community levels of degrading bacteria increasing during phytoremediation (Wünsche et al. 1995; Siciliano et al. 2003). BTEX isomers are the most amenable to elimination from the environment by indigenous microorganisms, though degradation can be impeded by the natural ecological system (Koizumi et al. 2002). Most polluted environments are anoxic, and since aerobic degradation of hydrocarbons is faster than anaerobic processes, their removal can be less efficient in a polluted environment (Koizumi et al. 2002).

A variety of grass species, legumes and fast-growing trees such as poplar, alder and willow, with high transpiration rates, have been used in phytoremediation (Jordahl et al. 1997). Such plants have extensive root systems that provide large root surface areas available for soil contact. Merkl et al. (2005) proved that larger root surface areas are proportionately related to petroleum hydrocarbon degradation levels in the plant genera *Brachiaria*, *Cyperus* and *Eleusine*. Plant roots provide attachment sites for microbes and a source of nutrients, consisting mainly of organic acids, including amino acids, as well as sugars and complex carbohydrates, in the form of exudates (Mehmannavaz et al. 2002; Tesar et al. 2002). By way of example, Jordahl et al. (1997) reported that the numbers of microbes degrading

benzene, toluene and xylene are five times higher in the rhizosphere of poplar trees than in surrounding soil. Successful rhizoremediation by plants depends on factors such as primary and secondary metabolites, colonisation, survival and ecological interactions with other organisms. In addition, the mucigel secreted by root cells, lost root cap cells, starvation of root cells and the decay of complete roots also provides nutrients (Reilley et al. 1996). Thus, plant roots have been suggested as a substitute for tilling of soil to incorporate additives and to improve aeration as a method of remediation (Kuiper et al. 2004). A broad phylogenetic range of bacteria, including the genera *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Norcardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Xanthomonas* are involved in the breakdown of hydrocarbons (Tesar et al. 2002).

Soil microbial communities are influenced by plant roots in various ways, e.g., excretion of organic compounds and competition for nutrients and attachment surfaces. Kuiper et al. (2004) reported that *Cyperus esculentus* L., *Eleusine coracana* (L.) Geartn. and *Brantha serratia* L. rhizospheres accommodate a large variety of bacteria. This probably is due to their ability to harbour large numbers of microorganisms on their highly-branched root systems. Plants with extensive root systems provide larger root–soil surface areas for attachment of microbes (Tesar et al. 2002). Plants influence soil pH, moisture and oxygen content by secretion of substances into the surrounding rhizosphere (Schroth and Hilderbrand 1964). Root exudates are common to all higher plants, and are known to influence the abiotic and biotic environment of the rhizosphere (Schroth and Hilderbrand 1964). Studies characterising the culturable rhizosphere bacteria showed that plants have specific effects on communities. However, these bacteria represent only a very small component of those actually present in soil (Duineveld et al. 2001).

6.5 Biodegradation

Indigenous microorganisms, including bacteria and fungi, are able to degrade PAHs in soil, leading to in situ rehabilitation of the soils. Bioremediation of hydrocarbon-polluted soils using microbes for detoxification and rehabilitation is an efficient, economic and versatile environmental treatment. PAH-degrading microbes are pervasive in ecosystems where pollutants may serve as carbon sources, and seem to establish themselves soon after pollution occurs (Margesin et al. 2000). The reclamation of polluted land reduces the possibility that groundwater will become polluted, and enhances the rate of biodegradation (Gibson and Parales 2000; Mishra et al. 2001). It has been shown that hydrocarbon-degrading bacteria are ubiquitously distributed in natural pristine environments. Wünsche et al. (1995), for instance, reported a 3.6% baseline community of hydrocarbon-utilising bacteria that increased on addition of hydrocarbon pollutants. Thus, natural degradation of pollutants in low-risk oil-polluted sites is a cost-effective rehabilitation alternative to more traditional clean-up procedures (Gibson and Parales 2000; Margesin and

Schinner 2001). Microbes have also been shown to use BTEX compounds as electron-donors in their metabolism, thereby facilitating pollution remediation in affected sites (Stephen et al. 1999). Supporting this, Wünsche et al. (1995) reported that substrate utilisation patterns in the Biolog system changed upon addition of hydrocarbons. Previously pristine soil bacterial communities shifted to a predominantly *Pseudomonas* population with hydrocarbon-degradation capability, thus demonstrating a natural bioremediation adaptation potential. Similarly, Maila et al. (2005b), using a combination of Biolog and molecular methods, found that pollution removal by indigenous microbial communities at different soil levels was 48% in topsoil, 31% at 1m deep and 11% at 1.5m deep. Thus, PAHs and phenols have been shown to be biodegradable under appropriate conditions (Guerin 1999). However, the most readily degraded hydrocarbons are the n-alkanes with a relative molecular mass of up to n-C_{44} (Atlas 1981). Biodegradation of these n-alkanes commences via a mono-terminal attack, forming a primary alcohol, an aldehyde and a monocarboxylic acid. Further degradation is via b-oxidation, forming a two-carbon unit, shorter fatty acids, acetyl co-enzyme A and CO_2 (Atlas 1981). Various bacteria are known to catabolise two- to four-ring PAHs as sole source of carbon, thus rendering them good candidates for site remediation applications (Bogan et al. 2001). This catabolism takes place using aromatic hydrocarbon dioxygenases within multicomponent enzyme systems (Samanta et al. 2002). Dioxygen is added to the aromatic nucleus of the PAH in question, forming an arene cis-diol as follows:

It has been hypothesised that metabolic engineering may improve microbial capacity for degradation of toxic compounds (Samanta et al. 2002). However, the efficiency of naturally occurring organisms capable of this metabolism could be enhanced by optimising bioavailability, adsorption and mass transfer (see Fig. 6.1). Widada et al. (2002) isolated 19 PAH-degrading bacterial species belonging to the genera *Ralstonia*, *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Comamonas*, *Flavobacterium* and *Bacillus* from environmental samples in Kuwait, Indonesia, Thailand and Japan. Enrichment cultures from these samples were supplemented with either naphthalene or phenanthrene as sole carbon source, and multiple phenotypes, in terms of utilisation and degradation metabolism, were observed. Tesar et al. (2002) listed a broad range of bacterial genera capable of hydrocarbon breakdown, including *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Xanthomonas*. In addition to this, Riis et al. (2003) found certain bacteria capable of bioremediation of diesel-polluted soils under high salinities. Bacteria from the genera *Cellulomonas*, *Bacillus*, *Dietzia* and *Halomonas* rehabilitated soils with a salinity of up to 15% (Riis et al. 2003). Recently, Kleinsteuber et al. (2006) determined that salinity affects the dominant species in diesel-polluted soils differently, low salinity favouring *Sphingomonas* spp., higher salinities *Ralstonia* spp. and very high salinities the halophilic genera *Halomonas*, *Dietzia* and *Alcanivorax*. Some bacteria have been described as degrading specific PAHs in culture. Willison (2004), for instance, found a species designated *Sphingomonas* sp. CHY-1 capable of degrading chrysene

as sole carbon source in culture after enrichment. More specifically, members of the *Providencia* genus are known to completely break down hexahydro-1,3,5-trinitro-1,3,5-tiazine (RDX) and nitroso-RDX, and have been used for this purpose in bioremediation (Kitts et al.1994).

Ecto- and endomycorrhizal fungi are cosmopolitan, and form symbiotic associations with the roots of plants (Linderman 1988). These endophytic fungi, particularly the ectomycorrhizae, aid plants in the absorption of nutrients from soil, especially immobile elements such as zinc, copper, sulphur, calcium, potassium, iron, magnesium, manganese, chlorine, boron and nitrogen. Absorption of phosphorus is enhanced by both ecto- and endomycorrhizae (Linderman 1988). Mycorrhizal fungi have been reported to reduce plant responses to other stresses such as high salt levels and noxious compounds associated with mine pollution, landfills, heavy metals and micro-element toxicity (Linderman 1988).

Bioremediation, by virtue of biodegradation, depends primarily on overcoming any nutrient limitations in the soil to be rehabilitated. Remediation of hydrocarbon-polluted soils is usually limited by the amount of free carbon, phosphorus and/or nitrogen present (Bogan et al. 2001; Margesin and Schinner 2001; Röling et al. 2002). However, Struthers et al. (1998) found that the herbicide atrazine could be degraded by *Agrobacterium radiobacter* in soil without addition of extra carbon or nitrogen sources, although inoculated cell numbers did not increase, indicating a state of survival rather than growth. Microbial community numbers can be increased by the injection of soluble nutrients just below the surface of the soil. This can, however, lead to excessive localised microbial growth in nutrient-injected areas, resulting in "biofouling" (Bogan et al. 2001). The use of gaseous formulations has been demonstrated to better distribute nutrients throughout a system for bioremediation purposes (Bogan et al. 2001). Rather than injecting nutrients, nutrient supplementing, particularly with nitrogen and phosphorus fertilisers, is known to enhance biodegradation of oil released into a marine environment (Kasai et al. 2002). However, amendments to rectify nutrient deficiencies must be optimal, as too high amounts may lead to eutrophication, and too little may result in suboptimal biodegradation (Röling et al. 2002). Triethylphosphate (TEP) and tributylphosphate (TBP) are the safest phosphorus compounds that can readily be gasified and forced through deficient soil, whereas gaseous nitrous oxide has been used to supply nitrogen (Bogan et al. 2001). While not enhancing remediation of PAH-polluted soil, delivery of gaseous nutrients has been shown to expedite in situ remediation of soils polluted with chlorinated solvents, volatile organic compounds, C_4-C_{10} alkanes and monoaromatic hydrocarbons (Bogan et al. 2001). Lee et al. (2003) found that adding pyruvate at optimal levels as an additional carbon source to PAH-polluted soils, aided in the breakdown of PAHs (naphthalene used in model). They were able to determine the optimal concentrations of carbon sources for complete degradation of naphthalene by *Pseudomonas putida* G7.

Microorganisms intended for inoculation into polluted soils can be carried on various materials. Agricultural by-products are most commonly used to transfer microbes without affecting their degradative capacity (Mishra et al. 2001). In this respect, the rate and intensity of pollutant degradation in influenced by environmental

factors such as the original indigenous microbial community, nutrient availability, oxygen levels, pH, temperature, moisture content, quality, quantity and bioavailability of pollutants, and soil properties (Margesin et al. 2000). Although bioremediation is the primary mechanism involved in removal of soil pollutants, other processes such as dispersion, dilution, sorption, volatilisation and abiotic transformation are also instrumental in the rehabilitation process (Margesin and Schinner 2001).

6.6 Rhizosphere

The rhizosphere is a niche that maintains indigenous soil microbial communities involved in the plant–soil nutrient cycle. It also plays a vital part in the survival of plants under adverse chemical soil conditions (Izaguirre-Mayoral et al. 2002). Phytoremediation uses rhizosphere technology in biodegradation enhancement. Plant health can be influenced by the promotion of production of phytohormones, furnishing of nutrients, nitrogen fixation, and the suppression of microbes detrimental to plants through antagonism (Da Silva et al. 2003). Siciliano et al. (2003) demonstrated that effective TPH phytoremediation systems promote an increase in numbers of bacteria with hydrocarbon catabolic genes. PAHs may be removed by volatilisation, photo-oxidation, sorption and leaching. This is enhanced by the presence of plants (Joner et al. 2002).

6.6.1 Exudates

Rhizosphere soil is modified with respect to pH, O_2, CO_2 and nutrient availability. Plants exude readily degradable substances into the soil that augment microbial activity in the rhizosphere (Schroth and Hildebrand 1964; Joner et al. 2002). These substances are released via volatilisation, leaching, exudation or decomposition, and can influence the growth of other organisms in the soil, including that of nearby plants (Meissner et al. 1986).

The exact composition of root exudates in soil is unknown, mainly as a result of sloughing and autolysis of epidermal cells constantly affecting the environment (Schroth and Snyder 1961). However, three aspects of modified soil characteristics in the rhizosphere contribute to phytoremediation of organic pollutants, viz. higher microbial activity, higher oxidation potential, and a modified microbial community (Joner et al. 2002). Plant secondary compounds (exudates) found in rhizosphere soil can include polyphenols and flavanoids. Some of these compounds are suppressive to microbial growth, while others enhance it (D'Arcy Lameta and Jay 1987). Thus, microbial communities within the rhizosphere are definitely affected by the type of root exudates produced by plants. In combination with bacterial PAH-degradative ability, plant roots contain soluble and wall-bound oxidative enzymes that are directly

implicated in PAH-degradation (Joner et al. 2002). Phytoremediation systems, including the plant and its microbial rhizosphere community, can therefore be implemented as a means of increasing the hydrocarbon degradation potential of soil, but fertilisation is required for maximum results (Siciliano et al. 2003).

6.6.2 Microbial Communities

The "population concept" is central to the fields of ecology, evolutionary biology and conservation biology. Krebs (1994) defined a population as "a group of organisms of the same species occupying a particular space at a particular time". Waples and Gaggiotti (2006) recently reviewed the definition of a population when considered in the context of ecological and evolutionary paradigms, and suggested several criteria for determining when groups of individuals are different enough to be considered separate communities. A natural population is bounded by ecological or genetic barriers only, for example within a local population individuals interact ecologically and reproductively. Based on this interaction, Waples and Gaggiotti (2006) concluded that a cluster of individuals, without using locality sampling information, detects true communities only under moderate to low gene flow. Therefore, for the purposes of this chapter, studying a large number of different species interacting within an environment will be referred to as studying a community. Thus, due to gene flow between communities within a community, it follows that the fairly recent advent of DNA markers has led to a great interest in studying natural communities genetically.

Soil microbial communities are relatively evenly distributed in unpolluted environments. However, Smalla et al. (2001) proved that there is a reduced evenness in the rhizosphere compared to unplanted soil. Zhou et al. (2002) examined microbial communities in 29 different soil types. They found that in low-carbon soils the diversity pattern of the surface soil was evenly distributed, while subsurface samples exhibited a distinct pattern. High-carbon soils, by contrast, displayed uniform diversity throughout the soil layers examined, indicating that spatial isolation differences in community structure could be overcome when the carbon content of a soil is high.

The general assumption stands that higher microbial diversity is proportional to an increased catabolic potential (Dejonghe et al. 2001). This can be extrapolated to imply that high species diversity leads to more effective removal of metabolites and pollutants from a substrate. Improving the bioremoval capacity of the soil by inoculating specific strains or consortia of microorganisms is referred to as bioaugmentation (Halden et al. 1999; Dejonghe et al. 2001). Two components constitute diversity in an environment, viz. total number of species present (species richness/abundance) and species distribution (species equitability) (Dejonghe et al. 2001). To promote and increase the degradative potential of a microbial community, competence for certain reactions under the conditions is required, implying that genes within the system need to be activated to participate in the energy flux of the environment (Dejonghe et al. 2001).

6.6.3 Assessment of Species Richness and Diversity

Several methods are available to determine the richness of diversity in an environment, including different plating methods, light and fluorescence microscopy, and DNA and RNA analysis (Dejonghe et al. 2001; Duineveld et al. 2001; Torsvik and Øvereås 2002). There are some general limitations to be taken into account when studying microbial diversity. Spatial heterogeneity is noteworthy, since most environmental replicates consist of 1–5 g of sample material, which does not give a true reflection of the spatial distribution of microorganisms (Kirk et al. 2004). Culturing colony-forming units (CFUs) on different media was the most popular method for investigating microbial diversity. However, most bacteria targeted for isolation from environmental samples are difficult to culture due to constraints imposed by artificial media on which they are to be grown (Sekiguchi et al. 2002). Culture-based methods are tedious, and certain organisms, e.g., mycobacteria, can take a long time before starting to grow. Only 1–10% of global bacterial species are culturable, due to the selectivity of growth media and conditions (McCaig et al. 1999; Von Wintzingerode et al. 2002; Kirk et al. 2004). Less than 1% of microbes from soils in polluted environments are culturable (Stephen et al. 1999). Respiration analysis of individual cells within soil samples has indicated higher numbers of metabolically active bacteria than the number of culturable bacteria (McCaig et al. 1999). Thus, both microscopy and plating lack the capacity to discriminate between multiple bacterial communities and to assess their diversity (Duineveld et al. 2001). Furthermore, should an organism be cultured on an artificial medium, substances produced by the organism in culture can either inhibit or stimulate growth of other microbes. These substances may have a markedly reduced effect once introduced into soil as an ameliorant, due to pH, adsorption by clay and microbial utilisation, all of which can influence the rhizosphere (Schroth and Hilderbrand 1964).

Molecular methods have provided a more accurate view of species richness within diversity. Initially, random fragments of environmental genomic DNA were cloned, and those containing rRNA genes were selected for sequencing (Dejonghe et al. 2001). The next advance in molecular analysis came when PCR was used to selectively amplify these rRNA genes from total microbial community DNA, using different sets of primers to amplify the genes from all types of organisms (Archaea, Bacteria, Eukarya) (Dejonghe et al. 2001; Torsvik and Øvereås 2002). Ahn et al. (1999) probed DNA from PAH-polluted soil for naphthalene and other PAH metabolism. They found that most PAH-degrading bacteria had a NAH7-like genotype using the *nah*A probe, and only 15% were not detected using this probe. New gene probes were thus suggested for enumeration of PAH-degrades. The next logical step from this technology was that mixed PCR fragments could be cloned and sequenced or be separated and visualised by various fingerprinting techniques, e.g., DGGE (Dejonghe et al. 2001; Duineveld et al. 2001). However, these techniques are only as efficient as their methodologies, i.e., efficient cell lysis, maximum unsheared DNA extraction, unbiased PCR amplification and effective downstream analysis (Kirk et al. 2004).

6.6.4 Remediation

Several methods are available for determining the level of remediation in polluted soils. Screening for the disappearance of pollutants can be achieved by monitoring toxicity in a test organism for product or change. Classically, species used for toxicity response have been *Ceriodaphnia* (crustacean of the family Daphniidae) and *Pimephales promelas* Rafinesque (a fish, commonly known as "Fathead minnow", of the family Cyprinidae) in water, and several invertebrates in soils (White et al. 1998). However, analyses of microbial communities have since proved to be a far more comprehensive indicator of residual pollutants. Monitoring the return of a baseline community is used to indicate that the biological community of a soil is returning to normal (White et al. 1998). Li et al. (2006) found that species of tolerant bacteria in heavy metal-polluted soils increase in numbers with time and further pollution, and can consequently be indicative of the level of heavy metal pollution and thus of soil quality.

Rhizosphere microflora are not easily destabilised, due to the buffering effect of the biotic and abiotic surroundings they inhabit (Bahme et al. 1988). Research has shown, however, that the rhizosphere microflora can be altered by inoculation of plant roots with specific rhizobacteria. The capacity of the shift in microflora depends on several factors, e.g., the nature of the introduced strain, the effectiveness of its colonisation and its ability to persist on root systems for a prolonged period (Bahme et al. 1988). The inoculum size and mode of delivery affects the community dynamics within the soil, i.e., community density declines proportionately to the distance from the point/source of inoculation (Bahme et al. 1988). Two delivery systems for applying rhizobacteria to underground plant organs have been described by Bahme et al. (1988), namely bacteria-impregnated granules that are mechanically incorporated into soil before planting, and low-pressure drip-irrigation systems containing the desired bacterial strain.

Burkholderia species are regularly isolated from plant rhizospheres, thus making them good potential agents for rhizoremediation. O'Sullivan and Mahenthiralingam (2005) found *Burkholderia* to be the predominant genus isolated from PAH-polluted soils. Of the various *Burkholderia* strains, six (CSV90, EML1549, K712, RASC, TFD2 and TFD6) were also capable of 2,4-dichlorophenoxyacetate degradation. *B. xenovorans* strain LB400 is an aerobic degrader of polychlorinated biphenyls (PCBs) using the enzyme biphenyl–2,3-dioxygenase. This species can degrade up to hexachlorinated biphenyls when supplemented with maltotriose esters to increase water solubility and hence bioavailability (O'Sullivan and Mahenthiralingam 2005). *B. vietnamiensis* strain G4 is able to co-metabolise trichloroethylene (TCE), which is an organic pollutant in groundwater aquifers, and toluene or phenol, using the enzyme toluene *o*-monooxygenase. Strain G4 has been extensively studied, and is subject to two US patents, 4925802 and 5543317 (O'Sullivan and Mahenthiralingam 2005). Strain G4 preferentially degrades toluene in culture, and therefore toluene levels have to be maintained to achieve maximum (100%) TCE biodegradation. Since toluene and phenol cannot be used during in situ environmental rehabilitation, a mutant of the G4 strain, PR1, which does not

require additional nutrients, has been engineered to remove most TCE within a few weeks. Despite this, the G4 strain still proved to be a more efficient bioremediator. A mutant toluene *o*-mono-oxygenase gene was therefore spliced from G4 into *Escherichia coli* to yield an organism with a higher rate of TCE degradation, and with an enhanced PAH as well as naphthalene degradation capacity (O'Sullivan and Mahenthiralingam 2005).

Petrol and diesel, as well as crude oil spills in soils at fuel stations, have been found to be bioaugmented to a certain extent by members of the genera *Micrococcus, Corynebacterium, Flavobacterium, Bacillus* and *Pseudomonas* (Rahman et al. 2002). More specifically, pentachlorophenol was remediated with *Flavobacterium* and *Arthrobacter*, whereas augmentation of 2,4,5-trichlorophenooxyacetic acid with *Rhodococcus chlorophenolicus* and *Pseudomonas cepacia* accelerated its removal (Halden et al. 1999). Petroleum PAHs in a marine environment are known to be biodegraded by bacteria belonging to the genus *Cycloclasticus* (Kasai et al. 2002). Da Silva et al. (2003a) found a number of *Paenibacillus* species to have agricultural importance, due to their ability to degrade several PAHs.

There has been much focus on the use of bacteria for bioremediation purposes in recent research. However, fungi may also play an important role in the rehabilitation process. In general, fungi are capable of tolerating harsher environmental conditions than bacteria, and could well be involved in the degradation of petroleum hydrocarbons in soil (Prenafeta-Boldú et al. 2002). Da Silva et al. (2003b) isolated filamentous fungi from estuarine sediments in Brazil, and monitored their ability to degrade PAHs, particularly pyrene, in culture. They found a *Cyclothyrium* sp. to be the most efficient, simultaneously degrading 74, 70, 59 and 38% of pyrene, phenanthrene, anthracene and benzo[a]pyrene respectively. Additionally, toluene, ethylbenzene and xylene have been shown to be degraded by a *Cladophialophora* sp. (Prenafeta-Boldú et al. 2002).

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Chapter 7
Soil Bioremediation Strategies Based on the Use of Fungal Enzymes

Christian Mougin, Hassan Boukcim, and Claude Jolivalt

7.1 Introduction

The pollution of soils as a result of anthropogenic activities has received substantial attention in the past few decades, as compared with the previous two centuries of industrial activities. Soil contamination requiring clean-up exists at approximately 250,000 sites in the EEA member countries, according to recent estimates. And this number is expected to grow. The number of sites needing remediation will increase by 50% by 2025. In France, nearly 4,000 industrial contaminated sites have been listed, with more than 70% presenting pollution of the sediments and subsoil and/or surface water resources (BASOL 2008). In addition, agricultural soils are also contaminated with numerous chemicals resulting from atmospheric deposition (metals), direct contamination (e.g., use of pesticides) or amendments with contaminated residual organic products (wastewater, sludge and compost land filling). Because of pollution impacts on the environment (ecological diversity, ecosystem functioning) and human health (air quality and water resources), it is a great challenge to develop processes for soil rehabilitation.

In addition, the recent development of crops for green chemistry purposes, including the production of biomaterials and biofuels, limits worldwide the availability of soils for feed and food production. The reuse of decontaminated soils for agricultural production is generally to be excluded, as they are of a high risk for human health, but is expected to provide suitable soils for industrial crops. In the case of diffused pollution, in situ bioremediation techniques are better adapted for treatment of large surfaces of contaminated soils. Such treated land becomes available

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for less risky uses at an economically acceptable cost. Development of replanting programs on a large scale, using symbiotic fungi coupled with the bioremediation techniques based on the use of filamentous fungi and/or extra-cellular enzymes, is of great interest in the valorisation of polluted soils.

Among available processes allowing the reuse of treated soils, bioremediation is of priority interest. It exploits the capability of microorganisms to transform pollutants, thus offering permanent solutions such as immobilisation or degradation of the contaminants. Because of their powerful capabilities, filamentous fungi, and especially ligninolytic (white-rot) strains have been studied and used for at least 2 decades to target specific pollutants in wastes and soils (Aust 1989; Aitken 1993; Barr and Aust 1994).

The use of microorganisms, however, is fraught with problems (Whiteley and Lee 2006). The accumulation in the environment of highly toxic pollutants only emphasises the fact that microorganisms, by themselves, are insufficient to protect the biosphere from anthropogenic pollution. Furthermore, although microorganisms may enhance the transformation of the pollutants, making them more effective agents of biodegradation, they imply the use of a considerable amount of biomass. Any biostimulation approach has limited potential, since individual bacteria capable of remediation of a given pollutant may be inhibited by the presence of other pollutants. Another limiting factor in the bioremediation of polluted contaminated sites is the very slow rate of degradation, which further limits the practicality of using microorganisms in these processes.

Here, we would like to demonstrate that fungal enzymes appear to be promising tools for remediating moderately polluted soils, and that enzyme-based technology can be used even in the case of large-scale contaminations. We will describe in this chapter the main principles of soil bioremediation, discuss the relevance of fungal enzymes for soil bioremediation, and present some prospects for future research intended to improve the efficiency of these tools.

7.2 Principles of Soil Bioremediation

7.2.1 Definitions

The nature and the origin of pollution due to human activities are variable (industry, agriculture, transport, etc.). Continuous growth of organic and metal pollutants due to human activities causes deterioration in agricultural production, ecosystem functions and the quality of soils and subsoil waters. According to their extent, we can distinguish between two types of pollution:

- Diffused pollution which concerns significant soil surfaces, and which originated primarily from use of liquid or solid products (e.g., pesticides) or from atmospheric deposition,
- Punctual pollution, concerning limited surfaces and whose origin is generally accidental or chronic, generally due to industrial activity.

Contaminants can be mineral or organic compounds. Heavy metals and mineral oil are identified as the main soil contaminants, followed by organic contaminants including polycyclic aromatic hydrocarbons and aromatic hydrocarbons.

Two general methods can be developed for soil treatment: (1) in situ without excavation, and (2) ex situ with excavation. The first method is useful in the presence of deep contamination of the soil by pollutants, which are often volatile. It can also be adapted to large-surface contamination. Ex situ processes begin by the excavation or scraping off of the polluted soil, which can be moved into a treatment plant (off-site treatment) or treated on site. Only ex situ processes allow an efficient optimization of incubation parameters, including pH, aeration, agitation, moistening and addition of suitable electron acceptors, nutrients, solvents or surfactants. Ex situ processes enhance the rate of pollutant desorption, and increase the activity of native microorganisms by specific supply of nutrients or additives (biostimulation). Refinements to the process also include isolation and/or production of degradative organisms or enzymes, which are then introduced into the polluted material (bioaugmentation).

7.2.2 Bioremediation Techniques

Bioremediation techniques include a set of biological systems using microorganisms to clean various types of polluted media: air, water or soil. Bioremediation aims at decreasing pollutants amounts in soils by any natural process. Accelerated bioremediation involves increasing the rate of biodegradation or biotransformation of contaminants by bioaugmentation or biostimulation. In the case of biostimulation, soil properties such as pH, pedoclimate, and redox potential can be altered by the presence of the additives. Biostimulation and bioaugmentation are often used in conjunction with supply of nutrients, to enhance microbial growth and to improve environmental hazard waste degradation (Whiteley and Lee 2006).

Bioslurry reactor, biopile and landfarming are the main methods commonly used for bioremediation of polluted soils which are consistent with the use of fungal enzymes. In a bioslurry reactor, water is mixed with the sieved polluted soil to produce slurry treated in a bioreactor. The use of reactors provides rapid degradation of pollutants, due to enhanced mass transfer rates and increased contaminant-to-microorganism contact. The system can be supplemented with nutrients, electron acceptors, surfactants and degrading organisms (native or exogenous). The treatment units, static or mixed, make it possible to treat high concentrations of pollutants in the sludge. Soils with high clay content are easily treated by bioslurry. Other approaches involve combining advanced oxidation processes (used sequentially or in simultaneously) with biotransformation (e.g., addition of Fenton's reagent) (Mougin 2002).

Biopiles involve soil excavation, sifting and heaping into piles. The soil is packed on a protective layer formed by a bottom inert liner. Slotted or perforated piping placed throughout the pile collects leachates and forces air to move by

injection or extraction (static biopiles). The soil is periodically reversed in the dynamic biopile to ensure aeration. Nevertheless, the soil needs to be turned or tilled at certain times during the operational life of all biopiles to promote continued biodegradation. In addition, the watering system at the top of the pile distributes water, surfactants and nutrients throughout the soil. All of the material may be covered with a greenhouse or a gore-tex cover to regulate temperature and limit water evaporation. Volatile constituents tend to evaporate rather than biodegrade during treatment. Vapour generation during aeration can be controlled and treated. A related method is composting with addition of fertilizers such as manure (EPA 2008).

Landfarms are similar to biopiles in that they are aboveground, engineered systems that use oxygen from air to degrade pollutants. In contrast to biopiles, excavated soil is spread on the ground, and landfarms are periodically aerated by tilling or plowing to encourage microorganism growth. In some cases, polluted soil is incorporated in the top layer of an agricultural soil. Nutrients and moisture may be added, and collection of leachates may be necessary. Landfarming concerns all types of soil polluted by organics and heavy metals (Mougin 2002).

7.2.3 Interest of Bioremediation Vs. Physico-chemical Processes

Compared to physical or chemical remediation techniques, bioremediation is of major interest for a sustainable rehabilitation of contaminated sites, without strong modifications of soil properties. The bioremediation techniques are intended in priority for sites where there is no urgency for rehabilitation, and where the traditional methods of depollution are not adapted and/or ineffective. They could also be coupled with enhanced natural attenuation, in which involves the stimulation of faculties of the ecosystems to evolve and to regenerate. They allow also in most cases the subsequent reuse of cleaned soils.

Among the remediation methods available, several parameters indicate that bioremediation is an interesting technology in contrast to physico-chemical treatments. The first parameter is related to the pollutant. When bioavailable, common chemical compounds are generally well-degraded by microorganisms. On the other hand, ageing of the pollutant appears to limit biodegradation, as pollutants become less available for degradative enzymes. Bioremediation technologies can be applied to all types of soils, whatever their texture or permeability. They are partially governed by local constraints, such as space, noise, smell and dust. In other terms, off-site methods are useful in the case of urban areas. The advantages of bioremediation processes are that they are economically and environmentally acceptable solutions. They induce low costs, and the treated soil can be re-used if acceptable target pollutant levels are reached. Their disadvantages are that they require a long duration to achieve the required pollutant concentration thresholds.

7.2.4 Biotransformation Pathways of Organic Pollutants

The biotransformation of organic pollutants can be due to direct metabolism or to an indirect effect of organisms on the environment (Mueller et al. 1996). Three processes are involved in direct metabolism, namely biodegradation, cometabolism and synthesis.

During biodegradation, one or several interacting organisms metabolize a given xenobiotic into carbon dioxide and other inorganic components. In this way, the organisms obtain their requirements for growth and energy from the molecule. From an environmental point of view, biodegradation is the most interesting and valuable process, because it leads to the complete breakdown of a molecule without the generation of accumulating intermediates.

The prevalent form of xenobiotic metabolism in the environment is cometabolism, in which organisms grow at the expense of a cosubstrate to transform the xenobiotic without deriving any nutrient or energy for growth from the process. Cometabolism is a partial and fortuitous metabolism, and enzymes involved in the initial reaction lack substrate specificity. Generally, cometabolism results in only minor modifications of the structure of the xenobiotic, but different organisms can transform a molecule by sequential cometabolic attacks, or another can use cometabolic products of one organism as a growth substrate. Intermediate products with their own bio- and physico-chemical properties can accumulate, thus causing some adverse effects on the environment.

Synthesis includes conjugation and oligomerization. Xenobiotics are transformed into compounds with chemical structures more complex than those of the parent compounds. During conjugation, a xenobiotic (or one of its transformation products) is linked to hydrophilic endogenous substrates, resulting in the formation of methylated, acetylated, or alkylated compounds, glycosides, or amino acid conjugates. These compounds can be excreted from the living cells, or stored. During oligomerization (or oxidative coupling), a xenobiotic combines with itself, or with other xenobiotic residues (proteins, soil organic residues). Consequently, they produce high molecular weight compounds, which are stable and often incorporated into cellular components (cell wall) or soil constituents (soil organic matter). This biochemical process not only affects the activity and the biodegradability of a compound in limiting its bioavailability, but also raises concern about the environmental impact of the bound residues.

7.2.5 Bioremediation of Metal-polluted Soils

Currently, the techniques most used for the stabilization of soils contaminated by heavy metals are containment and solidification/stabilisation, or settling and discharge. Some plant species have a natural capacity to fix, degrade or eliminate the toxic chemicals and the pollutants from soils. The establishment of a vegetable cover on contaminated soils constitutes a bioremediation solution viable economi-

cally and complementary to the already existing techniques of depollution. As more than 90% of plant species are concerned with the mycorrhizal symbiosis which is established between roots of photosynthetic plants and mycelia of higher fungi, this symbiotic partnership plays an evident role in the attenuation of metal mobility and toxicity (Smith and Read 1997).

Much research has shown that the ectomycorrhizal fungi have extracellular and intracellular mechanisms which confer to them a tolerance to the presence of metal pollutants higher than that of the non-mycorrhized host plant. The identified mechanisms combine reduction of the absorption of metals in the cytoplasm and immobilization of metallic pollutants outside the cells by secretion of ligands into the medium or by their retention on the fungal cell wall (Bellion et al. 2006). Some fungal species from the basidiomycota phylum are able to produce metalothionein in great quantity, which enables them to detoxify their cytoplasm against metallic stress (Courbot et al. 2004).

Great differences have been observed between fungal species and isolates in their capacity to fix pollutants and to confer to the host plant a tolerance to toxicity. Isolated fungi from industrial contaminated sites exhibit a higher tolerance to high heavy metal levels, when compared to fungal isolates from non-polluted sites. They are also able to transfer their tolerance to host plants with which they have an association (Adrianson et al. 2004, 2005; Colpaert and Van Aasche 1987, 1992).

Knowledge of the mechanisms involved in the tolerance of some symbiotic fungi to metal pollutants makes possible their potential to contribute to the remediation of soils by supporting the accumulation and the immobilization of the pollutants in the roots of selected plants and the associated symbiotic fungi. This objective requires selection of adapted and effective fungal species and the optimisation of their use under site conditions.

7.3 Relevance of Fungal Enzymes for Soil Bioremediation

7.3.1 Filamentous Fungi

The degradation of organic compounds (natural or xenobiotic) through microbial metabolic processes is considered to be the primary mechanism of biological transformation. The different groups of microorganisms can mediate an almost infinite number of biochemical transformations. The most abundant organisms in soil are bacteria, whereas fungi form the largest biomass. They are involved in numerous functions, such as mineralization and humification of soil organic matter, biogeochemical cycles, production of toxins and compounds of industrial interest (antibiotics), and degradation of pollutants. The eukaryotic fungi comprise molds, mildews, rusts and mushrooms (all aerobic), as well as yeasts (fermenting organisms). Filamentous fungi are characterized by extensive branching and mycelial growth, as well as by the production of sexual (for asco- and basidiomycetes) and asexual spores. Deuteromycetes (*fungi imperfectii*) lack sexual reproduction capabilities,

but a sexual stage is quite often discovered, in which case these organisms are reclassified into other groups. Fungi are more tolerant to acidic soils and low moisture than bacteria. They can be pathogenic to plants and animals, or associated with plants in forming mycorrhyzae.

7.3.2 Fungal Oxidases

Filamentous fungi such as white-rot basidiomycetes, which are among the major decomposers of biopolymers, have developed non-specific and radical-based degradation mechanisms in their extracellular environment. Many studies have identified the role of this enzymatic machinery (e.g., laccase, lignin peroxidase and Mn-dependent peroxidase) in the transformation capacity of ligninolytic fungi towards a wide range of organic pollutants in contaminated soils (Pointing 2001; Riva 2006; Anke 2006; Gianfreda and Rao 2004; Baldrian 2006).

7.3.2.1 Peroxidases

Lignin peroxidase (LiP) and manganese peroxidase (MnP) were discovered in the mid-1980s in *P. chrysosporium*, and described as true ligninases because of their high redox potential (Martínez 2002). LiP and MnP catalyse the oxidation of lignin units by H_20_2. LiP degrades non-phenolic lignin units (up to 90% of the polymer), whereas MnP generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions (Jensen et al. 1996). More recently, versatile peroxidase (VP) has been described in *Pleurotus* (Camarero et al. 1999) and other fungi (Pogni et al. 2005) as a third type of ligninolytic peroxidase that combines the catalytic properties of LiP and MnP (Heinfling et al. 1998), being able to oxidize typical LiP and MnP substrates.

Peroxidases share common structural and catalytic features: they are glycosylated proteins with an iron protoporphyrin IX (heme) prosthetic group located at the active site. Their catalytic mechanism involves a two-electron oxidation of the heme moiety to a high redox potential oxo-ferryl intermediate known as compound I. This two-electron reaction allows the activated enzyme to oxidize two substrate units. Two successive one-electron reductions return the enzyme to its resting state using a second intermediate, compound II (one-electron oxidized form) (Veitch 2004). The primary reducing substrate in the MnP catalytic cycle is Mn^{2+}, which efficiently reduces compound I and II, generating Mn^{3+}, which is stabilized by chelators such as oxalic acid, itself also excreted by the fungi. Chelated Mn^{3+} acts as a highly reactive, low molecular weight, diffusible redox-mediator. Its redox potential, up to 1.5 V, in turn oxidizes the organic substrate. Therefore, MnP enzymes are able to oxidize and depolymerise their natural substrate, i.e., lignin, as well as recalcitrant xenobiotics such as nitroaminotoluenes and texile dyes (Knutson et al. 2005; Wesenberg et al. 2003). Phenol cleanup by commercial horse radish

peroxidase (Wanger and Nicell 2002), or alternatively soybean peroxidase (Ryan et al. 2006) has been reported, but several drawbacks limit its widespread application, including intolerance of high concentrations of the primary substrate H_2O_2, low enzymatic reusability and financial costs (Nicell and Wright 1997). Bodalo et al. (2005) noted that the choice of peroxidase for wastewater treatment also depends on effluent characteristics, operational requirements and costs. The use of peroxidases for soil cleaning has been studied, specifically for soils historically contaminated with aromatic hydrocarbons and detoxified by autochtonous fungi producing peroxidases (D'Annibale et al. 2006).

7.3.2.2 Laccases

Laccases belong to a large group of enzymes termed multicopper oxidases, which includes among others ascorbate oxidases and ceruloplasmin. Their name originates from plant lacquer; they were first described in *Rhus vernicifera* by Yoshida in 1883, where they were ranked among the oldest enzymes ever described. They are produced by plants, insects (*Bombyx sp*.), bacteria (*A. lipoferum*) and they also occur widely in lignin degrading filamentous fungi, including the white-rot basidiomycete *Trametes versicolor*. They perform the reduction of dioxygen to water while oxidizing organic substrates by a one-electron redox process. Laccases can oxidize a wide range of aromatic substrates, mainly phenolic and anilines.

Laccases contain four copper ions distributed into three sites, defined according to spectroscopic properties. The different copper centres can be identified on the basis of their spectroscopic properties. The T1 copper is characterized as having a strong absorption around 600 nm, whereas the T2 copper exhibits only weak absorption in the visible region. The T2 site is electron paramagnetic resonance (EPR)-active, whereas the two copper ions of the T3 site are EPR-silent, due to an antiferromagnetic coupling mediated by a bridging ligand (Messerschmidt 1997). The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where reduction of molecular oxygen to water takes place (Fig. 7.1). Despite the substantial amount of information available for laccases as well as other related blue copper oxidases, mechanistic details of dioxygen reduction in these enzymes are not fully understood (Garavaglia et al. 2004).

Recently it was shown that white-rot fungi cultivated on natural solid lignin-containing substrates produce another form of laccases, lacking the T1 copper and named "yellow" laccases because these enzymes do not show the characteristic absorption band around 600 nm (Leontievsky et al. 1997). One interesting feature regarding these enzymes is that they seem to show a relatively high activity in the degradation of some polycyclic aromatic hydrocarbons (Pozdnyakova et al. 2004).

So far, more than 100 laccases have been purified from fungal cultures and characterized in terms of biochemical and catalytic functions (Xu et al. 1996). Their occurrence, characterization, functions and applications have been reviewed in recent years (Mayer and Staples 2002; Mougin et al. 2003; Baldrian 2006, Riva

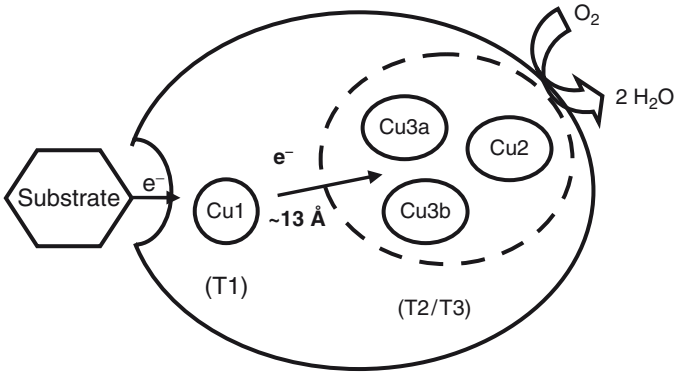

Fig. 7.1 Principles of the oxido-reduction reaction catalyzed by laccases

2006). Biological functions of laccases reflect their diversity, and clearly depend on the producing microorganisms. They are involved in many in vivo processes such as pigmentation, plant cell wall biosynthesis (which proceeds via oxidative polymerisation of monolignols in the cell wall matrix), phytopathogenesis or insect sclerotisation. Fungal laccases are involved in the process of wood delignification but also play a role in fungal morphogenesis, and could influence fungal virulence.

Being able to oxidize various aromatic compounds, laccases have excellent potential as industrial biocatalysts for many applications such as wood fibre modification in the paper pulp industry (Lignozym®-process, Call and Mücke 1997), or green organic chemistry and water/soil remediation, in order to protect the environment from damage caused by industrial or urban effluents (Xu 1999; Torres et al. 2003; Wesenberg et al. 2003; Dubroca et al. 2005). Commercially, laccases have been used to bleach textiles (Denilite® from Novozyme) or as a biosensor to distinguish between morphine and codeine.

One crucial point explaining such intense research and development activity in recent years is that laccases exhibit interesting properties: in addition to their broad specificity (which allows them to transform a wide range of substrates) and to their wide diversity, most fungal laccases are very stable, especially at pH values near neutrality, their organic substrate oxidation site exhibits a high redox potential [around 0.78V/normal hydrogen electrode (NHE)], and finally they use dioxygen, a harmless and abundant compound, as a co-substrate instead of oxygen peroxide which other oxidases (like peroxidases) use.

Laccases are therefore involved in the transformation of a wide range of phenolic compounds, including natural substrates such as lignin and humic substances. They can also transform xenobiotics such as trichlorophenols, pesticides, polynitrated aromatic compounds (Ramos et al. 2005), azo dyes and PAHs, which are a major source of contamination in soil; therefore, their degradation is of great importance for the environment. The potential use of oxidative enzymes from white-rot fungi for detoxification of these organic pollutants has been extensively reviewed (Torres et al. 2003; Pointing 2001; Gianfreda et al. 2004; Mougin et al. 2003; Couto and Herrera 2006).

7.3.3 *Examples of Xenobiotic Biotransformation Mediated by Fungal Enzymes*

Here, we would like to focus on three families of these compounds, i.e., polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds and phenolic estrogenic chemicals, because of their present or future importance in soil contamination.

7.3.3.1 Polycyclic Aromatic Hydrocarbons (PAH)

PAHs are persistent organic pollutants widely distributed in terrestrial and aquatic environments (Samanta et al. 2002) as diffuse pollutants (Johnsen and Karlson 2007), where they result from fuel combustion of engines or as localized contaminants of old gas plants for example. They are composed of two or more fused benzene rings, and are classified as highly toxic (16 HAP have been listed as priority pollutants by the US Environmental Protection Agency (Mougin 2002) owing to their mutagenic and carcinogenic potential. Microbial bioremediation of PAHs has become popular since November 2002, when bioremediation was used to remove the pollution from the Prestige ship spill on the north coast of Spain (Alcalde et al. 2006). Numerous microorganisms, including bacteria, yeasts or fungi are known to be able to degrade PAHs (Mougin 2002; Aitken and Long 2004). Bacteria exhibit the advantage of being able to use PAHs as a sole source of carbon but are unable to mineralise them entirely, in contrast to soil fungi. In order to combine the advantages of both types of microorganisms, the use of consortia emerges as a promising method (Canet et al. 2001; Johnsen et al. 2005). White-rot fungi (Anastasi et al. 2008; Mollea et al. 2005; Cohen et al. 2002) were reported to be efficient in PAH degradation and the role of laccases in their degradation has been established through numerous studies. In 1996, Johannes et al. reported that a laccase from *T. versicolor* oxidized about 35% of acenaphtylene in solution after 72 h of enzymatic treatment. Since this pioneering work, many results, sometimes controversial, have been published. According to Collins et al. (1996) or more recently to Han et al. (2004), *T. versicolor* laccase does not oxidize phenanthrene in accordance with its high ionisation potential, whereas Pickard et al. (1999) and Wu et al. (2008) concluded the opposite result. It is generally reported that the transformation of PAH is significantly enhanced in the presence of mediators, i.e., chemical compounds that reduce substrates for laccases and are enzymatically transformed into radicals. These radicals in turn oxidize PAH: 80% of oxidation was reported for anthracene (Mougin et al. 2002), phenanthrene (Han et al. 2004) or benzo[a]pyrene (Mougin 2002). Some “natural” compounds such as tyrosine or cysteine have been demonstrated as potential mediators in these reactions (Johannes and Majcherczyk 2000).

Several microorganisms producing laccases, including *P. ostreatus* (Bogan et al. 1999) and *T. versicolor* (Rama et al. 2001) have been shown to efficiently transform PAH in soils. A positive correlation was found between PAH degradation

and ligninolytic enzymatic activity (Novotny et al. 1999). However, remedial strategies based on inoculation of PAH-degrading fungi seem to be difficult to apply under field conditions, due to sub-optimal growth conditions, high toxicity of PAH or potential interactions between microorganisms that limit laccase activity (Canet et al. 2001). However, recent research by Wu et al. (2008) on the direct application of free laccase in PAH-contaminated soil reported very promising results: a mixture of 15 PAHs was significantly degraded, anthracene and benzo[a]pyrene being the most efficiently transformed, to 8% and 60% respectively.

7.3.3.2 Nitro-Aromatic Compounds

Nitro-aromatic compounds are produced by incomplete combustion of fossil fuel or nitration reactions, and are used as chemical intermediates for synthesis of explosives (2,4,6-trinitrotoluene-TNT), pesticides (parathion), dyes or pharmaceuticals, with an estimated annual production of 10^8 tons (Ye et al. 2004). Therefore, large areas of soil and ground water have been highly contaminated by these xenobiotics. Nitro-aromatics are readily reduced by mammalian non-specific nitroreductase systems (Nishino et al. 2000). However, this enzymatic conversion of nitro groups leads to reactive carcinogenic derivatives such as nitroso and hydroxylamino groups. Nitro-aromatics are therefore now recognized as recalcitrant and given Hazardous Rating-3, where 3 denotes the worst level of hazard and/or toxicity (Sax and Lewis 1999). During the last few decades, extensive research has resulted in isolation of a number of microorganisms with the potential to degrade nitroaromatic compounds (see the review from Kulkarni and Chaudhari 2007) following aerobic or anaerobic pathways. Anaerobic processes generally lead to the formation of aromatic amines through a six-electron transfer mechanism, while aerobic pathways exploit mono- or dioxygenases to eliminate the nitro groups from mono-nitrophenols, as exemplified more than 50 years ago in *Pseudomonas sp*., which is capable of converting 4-nitrophenol to hydroquinone, with the release of nitrite (Simpson and Evans 1953). However, bacteria utilizing nitro-aromatics as a sole source of C and/or N are very rare (Bennet et al. 1995). Because they possess suitable oxidative enzyme systems, white-rot fungi are capable of TNT degradation and mineralization to CO_2 (Pointing 2001). *Phanaerochaete chrysosporium* has been the organism of choice in such studies (Hodgson et al. 2000; Jackson et al. 1999; Bayman and Radkar 1997), and the involvement of the ligninolytic enzymatic system has been confirmed by studies using purified MnP (Van Acken et al. 1999). Addition of the surfactant Tween-80 to cultures of *P. chrysosporium* enhanced TNT mineralization 2-fold (up to 29.3% over 24 days) and reduced mutagenicity of aqueous TNT wastes by up to 94%, as measured using the *Salmonella*/microsome bioassay (Donnelly et al. 1997). Mineralization by *P. chrysosporium* has been demonstrated also for nitroglycerin in mixed culture with bacteria; however, anaerobic mineralization by bacteria was shown to occur at a faster rate (Bhaumik et al. 1997). Laccases have been shown to be involved in the degradation of TNT by catalysing the coupling of reduced TNT metabolites to the organic soil

matrix, which resulted in detoxification of the munition residue (reviewed by Duran et al. 2000). Recently, Nyanhongo et al. (2006) showed that a laccase from *Trametes modesta* was involved in immobilisation of TNT degradation products.

Soil remediation attempts for nitro-aromatic removal has mainly concerned bench-scale assays and has mostly used soil slurry technologies (Kulkarni and Chaudary 2007). Slurry processes consist of reactors filled with a mixture of soil and water to which co-substrates and nutrients can be added as necessary. There are two different approaches to TNT bioremediation in slurry reactors: mineralization of the explosive as the main target, and irreversible binding of TNT metabolites to the soil matrix (Esteve-Numez et al. 2001). A process designated as SABRE (sequential anaerobic biological remediation ex situ), developed and patented at the University of Idaho, consists of a consortium of facultative anaerobic organisms including strains of the genus *Clostridium* that transform explosives such as TNT to nontoxic, nonaromatic, and aerobically mineralizable products (Funk et al. 1995). *P. chrysosporium* was also reported to mineralize TNT present in soil at levels of up to 10,000 ppm (Fernando et al. 1990). However, the accumulation of starchy material in treated soil produces a high oxygen demand, which may be detrimental in agricultural soils because of the rapid development of anaerobic conditions when the soil is wetted, such as after irrigation or rainfall. Moreover, whole bacteria/fungi or their consortia used for degradation suffer from several drawbacks: (1) survival of inoculum gets difficult because of the chemicals toxicity, (2) reduction of chemical load is limited, and (3) presence of heavy metals inhibits treatment. To overcome these limitations, immobilization of degradative microorganisms or enzymes has been successfully used (Alexander 1999; Kulkarni and Chaudary 2007).

7.3.3.3 Endocrine-Disrupting Phenolic Compounds

Scientific and public attention has recently focused on the potential effects of certain environmental hormone-like chemicals on wildlife and human health. These chemicals, for the most part of anthropogenic origin, are known as endocrine disruptor chemicals (EDCs) because they modulate the endocrine system producing various pathologies, particularly during reproduction and development. In 2001, the Stockholm Convention under the auspices of United Nation Environmental Program specified a list of potential endocrine-disrupting chemicals in the environment, including certain pesticides, phthalates, phytoestrogenes, and several phenolic compounds (UNEP 2001).

Such concerns have heightened the need for novel and advanced bioremediation techniques to effectively remove these compounds from a variety of contaminated environmental media including water, sediments, sludge used to fertilize agricultural soils and soils (Duran and Esposito 2000; Romantschuk et al. 2000).

Enzymatic transformation of EDCs by the oxidative enzymes of ligninolytic fungi has mainly focused on two families: (1) alkylphenols such as nonylphenol and octylphenol, and (2) biphenyls such as biphenyl methane, known also as Bisphenol

A, usually used as a model compound for endocrine disruptors. Bisphenol A is a ubiquitous substance used mainly in the production of epoxy resins and polycarbonate plastics. The latter are used in food and drink packaging applications, while the former are commonly used as lacquers coating metal products such as food cans, or in water supply pipes. Because they are phenolic derivatives, these compounds are readily transformed by fungal laccases, as reported in several papers (Tanaka et al. 2001; Tsustumi et al. 2001; Saito et al. 2004). As an example, it has been reported that nonylphenol, octylphenol, bisphenol A and ethynylestradiol (synthetic estrogen) adsorbed on sea sand (2 pmol g^{-1}) was transformed by a laccase from *T. versicolor* at an optimum pH of 5. The authors suggest that the phenolic EDCs might have polymerized via enzymatic conversion to their phenoxy radicals. Our group (Dubroca et al. 2005) recently showed that the ligninolytic basidiomycete *T. versicolor* was able to catalyze partly the conversion of nonylphenol into carbon dioxide, and that laccases purified from *T. versicolor* cultures are involved in nonylphenol oxidative coupling, leading to oligomerization of nonyphenol via C-C bounds formation.

Very recently, Diano et al. (2007) showed that a laccase from *T. versicolor* immobilized on nylon membranes is able to transform efficiently Bisphenol A, and that the values of the percentage activity increases of immobilized enzymes proved to be higher at low substrate concentrations, i.e., at concentrations that really exist in polluted waters, considering the low aqueous solubility of these compounds.

7.3.4 Engineering of Fungal Oxidases

Numerous works cited above show that fungal laccases can be efficient tools for bioprocesses, leading to the cleanup of polluted water (Jolivalt et al. 2000) and soil bioremediation (Rama et al. 2001). Nevertheless, laccase-mediated biotransformation of xenobiotics in natural media suffers from several limitations of the enzyme: (1) a redox potential (so far, in the range 0.4–0.8 V) lower than that of the targeted organic compound for transformation, (2) an acidic optimal pH for activity which is too low compared to pHs of effluents or soils, and (3) the need for a redox mediator when the reducing substrate is too large to be accommodated into the active T1 site.

Engineering of laccases appears as a promising approach to overcome such limitations, and several attempts are reported to have improved laccase (or other enzymes potentially used in bioremediation processes) properties using biomolecular technologies (Lui et al. 2005). Two different and complementary approaches have been reported: a rationale approach based on structural knowledge of the protein, leading to targeted site-directed mutagenesis experiments, or more random-based directed evolution techniques.

Based on sequence alignments, without precise information concerning either the substrate cavity geometry or the interactions between amino acids and substrate, the pioneering work of Xu et al. (1996) and Xu (1999) suggested that a non-ligating

tripeptide in the vicinity of the active site was involved in the redox potential value. Although the redox potentials were not significantly altered, the triple mutants had a phenoloxidase activity, the pH optimum shifted 1 unit lower or higher while the kinetic parameters were greatly changed. The results were interpreted as possible mutation-induced structural perturbations of the molecular recognition between the reducing substrate and the enzyme. In 2002 for the first time, the three-dimensional structures of laccases from *T. versicolor* (Bertrand et al. 2002; Piontek et al. 2002), *P. cinnabarinus* (Antorini et al. 2002), *M. albomyces* (Hakulinen et al. 2002) with a full complement of copper ions was elucidated. In addition, two of these laccase structures have been obtained in the presence of a reducing substrate (Bertrand et al. 2002; Enguita et al. 2003). Our group showed that the presence of an arylamine (2,5-dimethylbenzeneamine or 2,5-xylidine) at the T1 active site of the enzyme revealed two important residues for the interaction between the amino group of the reducing substrate and the enzyme. In particular, aspartate 206 is hydrogen-bonded via the terminal oxygen of its side chain to the amino group of 2,5-xylidine. Moreover, the analysis of the dependence of kinetic parameters on pH suggests that an acidic residue may be involved in the binding of phenolic compounds. Site-directed mutagenesis experiments were performed towards Asp206 using the yeast *Yarrowia lipolytica* (Madzak et al. 2006). It was shown that the transformation rates remain within the same range whatever the mutation of the Asp206 and the type of substrate: at most a 3-fold factor increase was obtained for k_{cat} between the wild-type and the most efficient mutant Asp206Ala with ABTS as a substrate. Nevertheless, the Asp mutation led to a significant shift of the pH (pH = 1.4) for optimal activity against 2,6-dimethoxyphenol.

Engineering of laccase by laboratory evolution also showed interesting results as reported by the group of Ballesteros involved in the functional expression of a thermophilic laccase in *Saccharomyces cerevisiae* (Bulter et al. 2003). As the low aqueous solubility of some xenobiotics such as PAHs may require the addition of organic solvents to minimize mass transfer limitations, whereas laccases are known to be fairly unstable in such conditions, a thermophilic laccase was engineered by in vitro evolution to be highly active and stable in the presence of increasing concentrations of acetonitrile and ethanol. After only one generation of directed evolution, one mutant displayed about 3.5-fold higher activity than the parent type in the presence of 20% acetonitrile or 30% ethanol (Alcade et al. 2005). Mutant laccases were also tested for the oxidation of anthracene in the presence of 20% (v/v) acetonitrile (Zumarraga et al. 2007).

Another interesting attempt in laccase engineering aims at enhancing the expression of the enzyme in recombinant systems, which is an important bottleneck to overcome when aiming to use “optimized” enzymes in bioremediation processes. Directed evolution of a laccase from *Myceliophthora thermophila* (MtL) expressed in functional form in *Saccharomyces cerevisiae* improved expression 8-fold, to the highest level reported for a laccase in yeast (18 mg l^{-1}) at that time. Specific activities of MtL mutants toward ABTS and syringaldazine indicate that substrate specificity was not changed by the introduced mutations (Bulter et al. 2003).

Recently, random mutagenesis was performed, leading to an improved expression of a *T. versicolor* laccase in *Pichia pastoris* by 3.7-fold to 144 mg l^{-1} of enzyme, together with a 1.4-fold increase in k_{cat}. In comparison with the wild type, the best mutant enzymatic properties (K_M for ABTS and guaiacol, thermal- and pH-stability, optimal pH) were not changed (Hu et al. 2007).

7.3.5 Advantages of the use of Enzymes for Soil Bioremediation

The above examples show the potential of extracellular oxidases from white-rot fungi for the bioremediation of some aromatic pollutants. Even if the complete removal of these compounds, i.e., their mineralization, relies on the action of additional intracellular enzymes present in their originating fungi or in other soil-endogenous microorganisms and requires the presence of whole cells and their metabolic pathways, the biodegradation of the pollutant is efficiently started by these oxidases. The use of cell-free enzymes could therefore facilitate overcoming a drawback of bioremediation (the low degradation rate) by accelerating the initial degradation phase. The pollutant, being transformed into a very reactive radical by the enzymatic reaction, is then likely to react with other nucleophilic species in soil, leading to the formation of bound residues via coupling reactions to soil humic substances, a process analogous to humic acid synthesis in soils. In this case, the degradation of the pollutant is incomplete because no mineralization occurs, but the immobilized product is less bioavailable and thus has reduced its toxicity (Bollag 1992). As pointed out by several authors (Gianfreda and Rao 2004; Pointing 2001; Alcade et al. 2005; Ruggaber and Talley 2006; Kulkarni and Chaudary 2007), the use of enzymes instead of microorganisms undoubtedly presents some advantages, from environmental, engineering or economic points of view. Enzyme use overcomes some limitations of microorganisms.

- Enzyme sensitivity to the pollutant concentration changes is low: high pollutant concentration may be toxic for the cell, thus reducing the degradation efficiency, but low concentration may have a negative impact on the expression of the enzymatic system, especially when it is related to secondary metabolism of the microorganisms.
- Enzymes are active over a rather wide range of physicochemical (temperature, pH and salinity) gradients in the environmental matrix, often unfavourable to active microbial cells, as well as the presence of toxic substances or inhibitors of microbial metabolism.
- The biotransformation reaction can be selected not to generate toxic products, as is often the case with chemical and some microbiological processes.
- The requirement to enhance bio-availability by the introduction of organic co-solvents or surfactants is much more feasible for enzymes than for whole cells.
- It is easy to control the ecological impact in the field: to be efficient in the degradation of the targeted pollutant, cells must stay alive, which may cause an imbalance of the ecological equilibrium of the ecosystem, preventing any sustainable further

use of the soil for agricultural purpose for example. By comparison, the future of free enzymatic systems is more under control, since the enzymes are digested, in situ, by the indigenous microorganisms after the treatment.

In addition to the advantages of using enzymes to overcome the drawbacks of microorganisms, enzymes offer a series of intrinsic advantages, mainly focused on the capability of biomolecular engineering to improve the efficiency of the enzymes in bioremediation systems. The use of recombinant-DNA technology is likely to produce optimized biocatalysts, with high reaction activity towards recalcitrant pollutants, enhanced specificity and stability, at a higher scale and at a lower cost. Of course, genetically engineered microorganisms (GEMs) with enhanced capabilities can also be produced using the same technology, but the use of GEMs still faces significant constraints regarding their application. Release of GEMs into the environment is strictly regulated to avoid the spreading of undesirable mobile genetic elements such as recombinant plasmid containing antibiotic resistance markers. By comparison, pure enzymatic systems have a low ecological impact in soil because of their low life time: the enzymes are rapidly digested in situ by the indigenous microorganisms after their application (Ahn et al. 2002).

However, the number of published reports dealing with enzymatic remediation of soil is limited, owing to difficulties in the purification and cost of enzymes. A rare example is the remediation of 2,4-dichlorophenol-contaminated soil by laccase (Ahn et al. 2002). The authors compared the performances of both free and immobilized laccase, and concluded that taking into account the cost of immobilization and the activity loss during the immobilization procedure, the advantage of immobilized enzyme is minimal. Using free *T. villosa* laccase for soil remediation thus appears to be the more practical option. If such a conclusion could be extended to other enzymes and further applications, it would render the use of enzymes for the remediation process even more attractive.

7.3.6 Limitations of the Use of Enzymes for Soil Bioremediation

The use of fungal enzymes in the bioremediation of contaminated soils necessitates an accurate assessment of the activity of enzymes in soil. The diversity of physicochemical proprieties of soil and surface properties of enzymes make it difficult to understand the mechanisms involved in the interactions between these two interfaces. Soil medium is a physical environment organized in aggregates (Brewer 1964), which hosts the biological events and fluxes of water, air and matter, at various levels of structure. It is necessary to recognize and take into account these complexities in order to optimise bioremediation techniques of soils, in particular by understanding and managing the various local processes occurring in soils (fixation of pollutants, enzymes adsorption, etc.).

Regarding the complexity and the heterogeneity of soils, modelling of the thermodynamic status of the soil medium is a relevant tool for the monitoring of the

efficiency of bioremediation enzyme-based techniques. This modelling cannot be possible without a precise characterisation of the soil structure and the thermodynamic state of the soil–water interactions within this structure. A new paradigm has recently been developed, based on a fine characterisation of the pedostructure and pedoclimate, which makes it possible to bridge the gap between pedology and soil physics with a view to understanding of enzyme–soil interaction and for optimizing the use of extracellular enzymes in bioremediation programs (Braudeau and Mohtar 2004, 2009).

Thus, the use of enzymes for the remediation of polluted soils necessitates more knowledge about the effects of environmental conditions on the fungal survival and dissemination and on enzyme behaviour and activity. Many problems are identified as limiting factors to performing bioremediation of contaminated soils by organic pollutants.

7.3.6.1 Heterogeneity and Availability of Pollutants in the Soil Medium

Soil pollution with organic contaminants is often accompanied by high concentrations of heavy metals like lead or mercury. This pollutant mixture has multiple negative effects on the survival of fungi and soil microflora (Baldrian and Gabriel 1997; Baldrian et al. 1996; Bogan and Lamar 1996), on the catalytic activities of the enzymes, and by consequence on the effectiveness of bioremediation of soils.

A large variety of ionic and nonionic surfactants or emulsifiers may facilitate the partitioning of pollutants from the solid phase of soil to the water phase. Numerous studies on this topic have been performed for many years (Reid et al. 2000). Synthetic classical surfactants and biosurfactants have been extensively investigated. Thus, a number of hydrocarbon-degrading microorganisms produce extracellular emulsifying agents, which enhance contact between them and hydrocarbons. However, inhibition of pollutant transformation has often been reported in the presence of surfactants. Proposed mechanisms for inhibition of microbial degradation, mostly at supra-cmc levels, include surfactant toxicity, or preferential use of the surfactant as a growth substrate (Mougin 2002).

7.3.6.2 Behaviour of Enzymes in the Soil Medium

Soil is a porous medium which is characterised by high interfacial areas between solid, liquid and gaseous phases. The thermodynamic state of the soil water medium, which constitutes the local physical conditions, namely the pedoclimate, affects the bio-geochemical processes in soil and by consequence the interactions between enzymes and soil medium (Braudeau and Mohtar 2009). On the other hand, soil clay minerals have high adsorptive proprieties that affect directly the interaction of enzymes with physical surfaces (Gianfreda et al. 1991; Ramirez-Martinez and McLaren 1966). The strong affinity of enzymes for the solid–liquid and liquid–gas interfaces results in frequent interactions of these proteins with surfaces in

soils. The great variety of enzyme physicochemical properties and monomeric structures makes their adsorption capacities rather higher than those of sugars or nucleic acids. The physicochemical proprieties of soil have a direct effect on the adsorption intensity of enzymes and on the quantity of adsorbed proteins. Many studies have highlighted the role of protein conformation on their adsorption properties. Indeed, the structural conformation of proteins at soil surfaces has the opposite effect, as they increase the entropy of the system and at the same time lead to an increase of the specific interfacial area in the contact surface between enzymes and the soil interface (Quiquampois 1987; Sandwich and Schray 1988). Adsorption of enzymes on soil surfaces involves both electrostatic and hydrophobic interactions (Norde 1986; Staunton and Quiquampois 1994). This affects directly the activities of enzymes, and by consequence the degradation of xenobiotics, and the biogeochemical cycles of major elements like carbon, nitrogen or phosphate, in soils. Extracellular ligninolytic enzymes are both catalysts and important modules/elements (N source) in the soil nitrogen cycle, and consequently are subjected to biodegradation (Quiquampois 2000; Quiquampois et al. 1995). Indeed, interactions of enzymes with solid surfaces have not only had a significant effect on their activity, but also on their degradation.

The interaction of enzymes with soil surfaces, especially clay minerals, can affect enzyme activity. The main consequence of this interaction is a pH shift of the optimum catalytic activity of the enzymes adsorbed on electrically charged surfaces (Mc Laren 1954; Mc Laren et al. 1958). On the other hand, the adsorption of enzymes often induces a decrease in the velocity of the enzymatic reactions and catalytic activity (Gianfreda et al. 1991; Ramirez-Martinez and McLaren 1966). Irreversible negative effects of the adsorption of enzymes on their catalytic activity has been observed and supposed to be related to the variation in the pH of the activity of the adsorbed protein (Quiquampois 1987). Mechanisms were supposed to involve the interaction of enzymes with mineral surfaces. This mechanism may include variations of the microenvironment of the enzyme, such as local pH or ion concentration, and the modification of the conformation of the protein (Quiquampois 2000).

The effects of pH of the soil on the interaction of proteins with soil surfaces had been widely studied. Many observations indicate that the maximum adsorption of a protein on an electrically charged surface occurs often around the pI of the protein (Haynes and Norde 1994; Mc Laren et al. 1958; Norde 1986). The presence of organic matter on soils also has a protective effect on the catalytic activity of enzymes, by reducing the adsorption of enzymes on clay surfaces. Experimental studies have shown that the destruction of organic matter increases the quantities of adsorbed enzymes, and as a result reduces the activity of the protein (Quiquampois et al. 1995). Thus, the organic composition of polluted soils will have to be evaluated and managed to ensure the optimum activities of fungal enzymes used in bioremediation programs. The hydrophobic/hydrophilic properties of soils also contribute to the interactions of proteins and soils and on enzyme conformation. These properties vary according to the mineral composition of soils.

7.3.6.3 Production of Fungal Oxidases

Another important limitation to the use of enzymes in environmental treatment processes is enzyme availability, which depends on the quantity and cost of the enzyme. Numerous studies have been done to determine favourable conditions for laccase production by fungi (Tavares et al. 2005; Ikehata et al. 2004; Kahraman and Gurdal 2002; Pointing et al. 2000).

Filamentous fungi are able to produce laccase levels of about 20–50 mg l^{-1}; however, an efficient production system at bioreactor scale is still lacking, and several limitations must be overcome, such as uncontrolled fungal growth, the formation of polysaccharides around mycelia, and secretion of proteases that inactivate laccases (Rodriguez-Couto and Toca-Herrera 2007). The addition of inducers such as xylidine (Minussi et al. 2007) or its metabolites (Couto et al. 2002; Mougin et al. 2002; Kollmann et al. 2005), guaiacol (Ryan et al. 2007) for *T. versicolor* and copper for the white-rot fungi, *Pleurotus ostreatus* (Palmieri et al. 2000), *Trametes trogii* (Lenin et al. 2002) and *T. versicolor* (Tavares et al. 2005) have been found to significantly increase laccase production (by a factor of ten) compared to production without any inducer.

Another means of enhancing the enzyme availability is to overproduce it by recombinant organisms in which high production yields are achieved, making their production processes economically attractive. Unfortunately, the expression of oxidases from filamentous fungi is rather difficult in heterologous systems (Jolivalt et al. 2005) and over-expression of these enzymes has yet to be achieved. However, some work is in progress in this field.

Peroxidases suffer from multiple post-translational modifications, including disulfide bonds, *O*- and *N*-glycosilations as well as signal-peptide removal (Conesa et al. 2002), so that their expression in *E. coli* proceeds through inclusion body formation (Ryan et al. 2006). So far, efficient expression of peroxidases in heterologous systems has not been achieved, and they still have to be obtained from natural sources.

Fungal laccases undergo post-translational modification similar to those of peroxidases; their expression in a heterologous organism requires the use of a eukaryotic microbe, since glycosylation seems to be implicated in the stability of fungal laccases, impairing their production in *E. coli* (Yoshitake et al. 1993). *Aspergillus species* are capable of performing posttranslational modifications and show no extensive hyperglycosylation, and therefore have been used as an expression system to produce laccases. However, they generally show low production levels in comparison to other proteins (Sigoillot et al. 2003; Valkonen et al. 2003). Nevertheless, the commercial production of a laccase from *Myceliophthora thermophilia* expressed in *Aspergillus oryzae* has been undertaken by Novozymes. The manufacturing process for the enzyme production is done by submerged, fed-batch pure culture fermentation, and the laccase was generally recognized as safe (GRAS) by the US administration (USFDA 2003).

Yeasts are also favourable hosts, because of their ability to grow rapidly on simple media with high cell densities at low cost, together with the ease of manipulation of

this eukaryotic organism, enabling post-translational modifications. Historically, baker's yeast (*Saccharomyces cerevisiae)* is the most popular host, and several laccases have been produced at low expression level (Kiiskinen and Saloheimo 2004; Klonowska et al. 2005). Another yeast, *Yarrowia lipolytica*, has been used for production of *T. versicolor* laccase (Jolivalt et al. 2005). Combined with the knowledge of substrate–enzyme interactions derived from *T. versicolor* laccase structure, it made it possible to engineer the enzyme by site-directed mutagenesis. However, the expression level of laccases remains low at 2 mg l^{-1}. The expression level in *Y. lipolytica* is expected to be increased 10-fold by the use of multi-copy vectors and the strong hp4d promoter (Madzak et al. 2004).

7.4 Prospects for Future Research

7.4.1 Improving the Ability of Natural Enzymes to Transform Pollutants

The development of the capabilities of given strains by genetic construction, leading to genetically modified organisms (GMOs), offers promising opportunities for obtaining enzymes with improved catalytic capabilities. These strategies require knowledge of the structural and catalytic properties of the key enzymes involved in pollutant metabolism, as a basis of their directed evolution, to obtain the most effective isoforms. The fact that laccase can use atmospheric oxygen as a final electron acceptor represents a considerable advantage for industrial and environmental applications compared with peroxidases, which require a continuous supply of H_2O_2.

Taking into account the fact that the advantage of peroxidases is their high redox potential, engineering the active site of laccases to obtain high redox potential variants would be of considerable biotechnological interest. A complete knowledge of the molecular environment of laccase type 1 copper, which seems to regulate the redox potential of the enzyme, may offer exciting opportunities (Piontek et al. 2002). That approach could allow suppression of the requirements for redox mediators.

7.4.2 Discovering Enzymes with New or Increased Potential

The majority of enzymes utilized in biotechnology are still derived from well-characterized non-extremophilic microorganisms. Because extremophilic microorganisms are adapted to survive to adverse environmental conditions, they are expected to express enzyme activities, even under harsh conditions. Both terrestrial and aquatic environments may be extreme with respect to pH, temperature, salinity, or water activity. Utilizing natural biodiversity can speed up the process to find

performing enzymes, as compared to more sophisticated and more expensive engineering approaches. To our knowledge, no fungal peroxidases or laccases have been identified by this approach. By contrast, novel cellulases have been produced and characterized from the extremophilic filamentous fungi *Penicillium citrinum* (Dutta et al. 2008).

7.5 Conclusion

Biological processes remain of great interest for the remediation of contaminated soils. In that context, enzymes appear as potent tools, because they provide solutions to some limitations encountered with whole organisms. Fungal oxidases, such as peroxidases and laccases, have been extensively studied. They exhibit great potential for the transformation (degradation or coupling) of numerous types of pollutants. Nevertheless, their capabilities have been demonstrated in liquid axenic cultures and remain, in most cases, to be demonstrated in soils. Approaches to research, including the screening of enzymatic systems produced by extremophilic microorganisms, appear to provide a good opportunity to discover other powerful bio-catalysers.

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Chapter 8
Anaerobic Metabolism and Bioremediation of Explosives-Contaminated Soil

Raj Boopathy

8.1 Introduction

Many xenobiotic chemicals introduced into the environment for agricultural and industrial use are nitro-substituted aromatics. Nitro groups in the aromatic ring are often implicated as the cause of the persistence and toxicity of such compounds. Nitroaromatic compounds enter soil, water, and food by several routes, such as use of pesticides, plastics, pharmaceuticals, landfill dumping of industrial wastes, and the military use of explosives. The nitroaromatic compound trinitrotoluene trinitrotoluene (TNT) is introduced into soil and water ecosystems mainly by military activities such as the manufacture, loading, and disposal of explosives and propellants. This contamination problem may increase in the future because of demilitarization and disposal of unwanted weapons systems.

Biotransformation of TNT and other nitroaromatics by aerobic bacteria in the laboratory has been reported frequently (Boopathy et al. 1994a, b; Dickel and Knackmuss 1991; Duque et al. 1993; Funk et al. 1993; McCormick et al. 1976, 1981; Nishino and Spain 1993; Spain and Gibson 1991; Zeyer and Kearney 1984). Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. has been reported to occur via 4-methyl-5-nitrocatechol in a dioxygenase-mediated reaction dioxygenase-mediated reaction (Spanggord et al. 1991). Duque et al. (1993) successfully constructed a *Pseudomonas* hybrid strain that mineralized TNT. White-rot fungus has been shown to mineralize radiolabeled TNT (Fernando et al. 1990). The work of Spiker et al. (1992) showed that *Phanerochaete chrysosporium* is not a good candidate for bioremediation of TNT contaminated sites containing high concentration of explosives, because of its high sensitivity to contaminants. Michels and Gottschalk (1994) showed that the lignin peroxidase activity of *P. chrysosporium* is inhibited by the TNT intermediate hydroxylaminodinitrotoluene. Valli et al. (1992) found that 2,4-dinitrotoluene is degraded completely by the white-rot fungus.

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Ecological observations suggest that sulfate-reducing and methanogenic bacteria might metabolize nitroaromatic compounds under anaerobic conditions if appropriate electron donors and electron acceptors are present in the environment, but this ability had not been demonstrated until recently. Under anaerobic conditions, the sulfate-reducing bacterium, *Desulfovibrio* sp. (B strain) transformed TNT to toluene (Boopathy and Kulpa 1992; Boopathy et al. 1993a) by reduction. Gorontzy et al. (1993) reported that under anaerobic conditions, methanogenic bacteria reduced nitrophenols and nitrobenzoic acids. Preuss et al. (1993) demonstrated conversion of TNT to triaminotoluene by a *Desulfovibrio* sp.

8.2 Anaerobic Biotransformation of Nitroaromatic Compounds

The anaerobic bacterial metabolism of nitroaromatics has not been studied as extensively as that of aerobic pathways, perhaps because of the difficulty in working with anaerobic cultures, and perhaps due to the slow growth of anaerobes. Earlier studies on anaerobic metabolism of nitroaromatic compounds by McCormick et al. (1976) laid the foundations for such study, and established the usefulness of anaerobic organisms. Successful demonstration of the degradation of hexahydro-1,2,3-trinitro-1,3,5-triazine (RDX) by sewage sludge (McCormick et al. 1981; Carpenter et al. 1978) under anaerobic conditions further demonstrated the usefulness of anaerobes in waste treatment. RDX RDX was reduced sequentially by the anaerobes to the nitroso-derivatives, which were further converted to formaldehyde and methanol. Hallas and Alexander (1983) showed successful transformation of nitrobenzene, nitrobenzoic acid, nitrotoluene, and nitroaniline by sewage sludge under anaerobic conditions.

Methanogens are obligate anaerobes that grow in an environment with an oxidation-reduction potential of less than −300 mV. They transform various substrates to C1 products such as CH_4 and HCOOH. The role of some novel compounds and the mechanism of single carbon flow in these bacteria remain to be formally proved, along with the arrangement of the electron transport chain. Because of the limited substrate capabilities, the metabolism of more complex molecules to methane depends on the activity of non-methanogens in association with the methanogens. Under pure culture conditions, methanogens have not been reported to degrade aromatic compounds. The studies of Gorontzy et al. (1993) on microbial transformation of nitroaromatic compounds by methanogenic bacteria revealed that methanogens can transform nitroaromatic compounds to corresponding amino compounds. Boopathy and Kulpa (1994) isolated a methanogen, *Methanococcus* sp. Methanococcus sp. from a lake sediment which transformed TNT to 2,4-diaminonitrotoluene. This organism also transformed nitrobenzene and nitrophenol. The intermediates observed were amino derivatives of the parent compounds. According to some reports, the reductive transformation of nitroaromatic compounds leads to detoxification of the substance (Boyd et al. 1983; Battersby and Wilson 1989). The specific enzymes responsible for the reduction process in methanogens are not yet

characterized. Angermeier and Simon (1983) suggested that the reduction of aromatic compounds may be catalyzed by hydrogenase and ferredoxin. The observation of sulfate reducers and methanogenic bacteria by many workers (Boopathy et al. 1993a, b; Boopathy and Kulpa 1994; Boopathy 1994; Gorontzy et al. 1993; Preuss et al. 1993) suggests that these organisms could be exploited for bioremediation under anaerobic conditions by supplying proper electron donors and electron acceptors.

Boopathy et al. (1993b) showed that TNT can be transformed under anaerobic conditions by using different electron acceptors. A soil sample collected from the Joliet Army Ammunition Plant, Joliet, IL, USA was incubated under sulfate-reducing, nitrate-reducing and methanogenic conditions. The results showed that TNT was transformed under all three conditions. However, when no electron acceptor was supplied, no TNT was transformed. The intermediates observed during the study were 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. This study showed that if the appropriate electron acceptor is present in the system, anaerobic bacteria will reduce TNT to amino compounds.

8.3 Sulfate-Reducing Bacteria

Although oxygen is the most widely used electron acceptor in energy metabolism, a number of different kinds of bacteria are able to reduce other compounds and hence use them as electron acceptors. This process of anaerobic respiration is less energy-efficient, but it allows these bacteria to live in environments where oxygen is absent.

Sulfate-reducing bacteria are obligate anaerobes that are conveniently considered together because of their shared ability to perform dissimilatory sulfate reduction, a process analogous to aerobic respiration in that the sulfate ion acts as an electron acceptor, like oxygen in the aerobic process. The genera of sulfate reducers are defined on the basis of morphology rather than physiology. All sulfate reducers are Gram-negative, except *Desulfotomaculum*. The most frequently encountered genus is *Desulfovibrio* sp. Desulfovibrio sp.

The use of various non-fermentable aromatic compounds in the absence of oxygen or nitrate is apparently one of the natural roles of sulfate-reducing bacteria. Aromatic compounds with more than two hydroxyl groups are readily degraded by fermenting bacteria (Widdel and Hansen 1992). Several new types of sulfate-reducing bacteria have been isolated directly with aromatic compounds (Bak and Widdel 1986; Widdel 1988; Schnell et al. 1989). Most of these isolates are extremely versatile sulfate reducers that use many aliphatic compounds. Aromatic compounds oxidized by sulfate-reducing bacteria include benzoate, phenol, p-cresol, aniline, and the n-heterocyclic compounds like nicotinate, indole, and quinoline. All the known degraders of aromatic compounds are complete oxidizers. The sulfate-reducers employ reactions like those detected in denitrifying bacteria, phototrophic bacteria, and methanogenic co-cultures using aromatic compounds (Berry et al. 1987; Evans and Fuchs 1987; Harwood and Gibson 1986; Tschech 1989). The sulfate-reducing

bacteria are capable of carrying out the following reactions: activation of benzoate to benzoyl CoA (Geissler et al. 1988; Holland et al. 1987), carboxylation of phenol to p-hydroxybenzoate (Knoll and Winter 1989; Tschech and Fuchs 1989) or the reductive removal of hydroxyl groups (Tschech and Schink 1986).

8.3.1 Metabolism of TNT Metabolism of TNT and Other Nitroaromatic Compounds by Sulfate-Reducing Bacteria

Boopathy and coworkers (1993a) showed that a sulfate-reducing bacterium, *Desulfovibrio* sp. (B strain) can convert TNT to toluene. This organism, isolated from an anaerobic digester treating furfural-containing wastewater (Boopathy and Daniels 1991), used nitrate as electron acceptor apart from using sulfate as electron acceptor. It also used nitrate as a nitrogen source. Further experiments showed that this bacterium could use the nitro group present in TNT molecules either as an electron acceptor or as a nitrogen source.

Some sulfate-reducing bacteria can use nitrate in addition to sulfate as their terminal electron acceptor terminal electron acceptor (Keith and Herbert 1983). The reaction is coupled to electron transfer phosphorylation (LeGall and Fauque 1988; Steenkamp and Peck 1981) and is catalyzed by a respiratory nitrite reductase nitrite reductase that has a molecular mass of 65 KDa and contains six c-type haems. This nitrite reductase, known as the hexahaem cytochrome C3, is widely distributed in strict and facultative aerobes (Liu and Peck 1981; 1988). This nitrite reductase is unrelated to the regulated nitrite reductase (nonhaem iron sirohaem containing) found in many plants and bacteria (Vega and Kamin 1977), where its function is nitrogen assimilation. According to Steenkamp and Peck (1981), nitrite reductase is closely associated with a hydrogenase, and is probably a transmembrane protein. This conclusion is based on the presence of proton-releasing and nitrite-binding sites on the periplasmic aspect of the cytoplasmic membrane, and a benzyl viologen-binding site on the cytoplasmic side of the membrane.

TNT (100 mg L^{-1}) was metabolized by *Desulfovibrio* sp. (B strain) within 10 days (Boopathy et al. 1993a), with pyruvate as the main substrate, sulfate as the electron acceptor and TNT as the sole nitrogen source. Boopathy et al. (1993a) showed under different growth conditions that this bacterium used TNT as its sole source of nitrogen. This result indicates that the isolate has the necessary enzymes to use the nitro groups present in TNT molecules as a nitrogen source.

Apart from pyruvate, lactate served as the best substrate for TNT metabolism, followed by H_2+ CO_2, ethanol, and formate. Comparison of the rate of TNT biotransformation by *Desulfovibrio* sp. with that of other sulfate-reducing bacteria showed that this new isolate has a unique metabolic ability to degrade TNT. *Desulfovibrio* sp. transformed 100% of TNT present in a relatively short period of time (7 days). Other *Desulfovibrio* spp. (ATCC cultures) converted 59–72% TNT within 21–23 days, whereas *Desulfobacterium indolicum* transformed 82% of TNT in 36 days of incubation (Boopathy et al. 1993a).

Mass spectral analyses showed that various intermediates were produced depending upon the culture conditions of the isolate. When ammonium was the main nitrogen source, 2,4-diamino-6-nitrotoluene was the major intermediate. When TNT was the sole source of carbon and energy, it was first reduced to 4-amino-2,6-dinitrotoluene and then to 2,4-diamino-6-nitrotoluene. When TNT was the sole source of nitrogen, all the TNT in the medium was converted to 2,4-diamino-6-nitrotoluene within 10 days of incubation, and traces of 2- and 4-amino compounds were identified. Later, these intermediates were converted to toluene. The quantitative analysis of the aqueous and gas phases of the culture bottle by gas chromatograph showed a good mass balance of TNT to toluene (Boopathy et al. 1993a).

Nitroaromatic compounds are considered resistant to microbial attack (Fewson 1981; Haigler and Spain 1993), partly because the reduction of electron density in the aromatic ring by the nitro groups can hinder electrophilic attack by oxygenases and thus prevent aerobic degradation of nitroaromatic compounds (Bruhn et al. 1987). Under anaerobic conditions, the sulfate-reducing bacteria metabolized TNT. Of all the metabolites produced, the formation of toluene from TNT seems to be very novel and significant.

TNT was reduced to diamino-nitrotoluene by the isolate through the 2-amino- and 4-amino-dinitrotoluenes when pyruvate served as the main substrate in the presence of sulfate and ammonia, in a simple reduction process carried out by the enzyme nitrite reductase. The cell-free extract showed high activity of nitrite reductase. The nitroreductase activity was monitored photometrically at 325 nm by the consumption of diaminonitrotoluene. Most *Desulfovibrio* spp. have nitrite reductase enzymes that reduce nitrate to ammonia (Widdel, 1988). This isolate reduced the nitro groups present in TNT to amino groups. When TNT served as the sole source of nitrogen, toluene was formed from the TNT. McCormick et al. (1976) showed that TNT was reduced by H_2 in the presence of enzyme preparations of *Veillonella alkalescens* to triaminotoluene: 3 mol H_2 is required to reduce each nitro group to the amino group. Preuss et al. (1993) observed the formation of triaminotoluene from TNT by a sulfate-reducing bacterium isolated from sewage sludge.

Boopathy et al. (1993a) showed the formation of toluene from triaminotoluene, and in the process, the isolate used the ammonium released from the original TNT molecule as a nitrogen source for growth. This is achieved by reduction of nitro groups followed by reductive deamination. A significant quantity of toluene concentration was observed in the culture sample (Boopathy et al. 1993a), and virtually no nitrite ions were detected during TNT metabolism. The aromatic ring structure was not cleaved, and no metabolites other than toluene appeared even after 6 months of incubation. Reductive deamination is catalyzed by a deaminase enzyme in *Pseudomonas* sp. (Naumova et al. 1986). Reductive deamination reactions were postulated first for 2-aminobenzoate degradation by methanogenic enrichment cultures (Tschech and Schink, 1988). Reductive dehydroxylation of gentisate to benzoate and acetate was demonstrated in the fermenting bacterium HQGO1 (Szewyk and Schink 1989).

Beller et al. (1992) and Edwards et al. (1992) demonstrated the complete mineralization of toluene under sulfate-reducing conditions. These toluene-degrading sulfate reducers could be used in combination with the *Desulfovibrio* sp. described by Boopathy et al (1993a) to degrade TNT completely to CO_2.

Desulfovibrio sp. (B strain) also metabolized other nitroaromatics such as 2,4-dinitrophenol (2,4-DNP), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and aniline (Boopathy et al. 1993a). As shown by Boopathy and Kulpa (1993), *Desulfovibrio* sp. used all the nitroaromatics studied as a sole source of nitrogen. It also used 2,4-DNT, 2,6-DNT and 2,4-DNP as electron acceptors in the absence of sulfate. The GC/MS analyses of the culture samples showed the presence of phenol from 2,4-DNP and benzene from aniline as intermediates. Gorontzy et al. (1993) showed transformation of nitrophenols and nitrobenzoic acids by the sulfate reducers *Desulfovibrio desulfuricans*, *D. gigas*, *Desulfococcus multivorans*, and *Desulfotomaculum orientis*. All of the nitroaromatics were transformed to corresponding amino compounds.

Schnell et al. (1989) isolated a new sulfate-reducing bacterium, *Desulfobacter anilini* Desulfobacter anilini, which degraded aniline aniline completely to carbon dioxide and ammonia, with stoichiometric reduction of sulfate to sulfide. This is the first obligate anaerobic bacterium observed to grow in pure culture with aniline as its sole electron donor and carbon source. The organism oxidizes aniline completely to carbon dioxide and releases the amino nitrogen quantitatively as ammonia. Two metabolic pathways were suggested. First, aniline could be carboxylated to 2-aminobenzoate or 4-aminobenzoate, with the aminobenzoate then reductively deaminated to benzoate and metabolized further (Zeigler et al. 1987). Alternatively, aniline could be deaminated hydrolytically to phenol, which is subsequently degraded either by carboxylation to 4-hydroxybenzoate or by reductive transformation to cyclohexanol or cyclohexanone. Both pathways appear possible, because the bacterial strain used each of these intermediates as a sole source of carbon.

Schnell and Schink (1991) reported that *Desulfobacterium anilini* degraded aniline via reductive deamination of 4-aminobenzoyl CoA. The first step, the carboxylation of aniline to 4-aminobenzoate, is followed by activation of 4-aminobenzoate to 4-aminobenzoyl CoA, which is reductively deaminated to benzoyl CoA. This product enters the normal benzoate pathway leading to three acetyl CoA. Carbon monoxide dehydrogenase and formate dehydrogenase are present in *Desulfobacterium anilini*, indicating that acetyl residues are oxidized via the carbon monoxide dehydrogenase pathway (Schnell et al. 1989).

Schnell and Schink (1992) isolated a sulfate-reducing bacterium that oxidized 3-aminobenzoate to carbon dioxide, with concomitant reduction of sulfate to sulfide and release of ammonium. High activity of carbon monoxide dehydrogenase carbon monoxide dehydrogenase indicated that acetyl CoA is oxidized via the carbon monoxide dehydrogenase pathway, although 2-oxoglutarate synthase activity was found as well. Similar activity was found with pyruvate as substrate. Perhaps both synthase activities can be attributed to an enzyme needed in assimilatory metabolism. Carbon monoxide dehydrogenase and pyruvate synthase are probably

also key enzymes during autotrophic growth with hydrogen and sulfate. The complete oxidation of 3-aminobenzoate yields −186 kJ per mole according to the following equation:

$$2C_7H_6NO_2^- + 7SO_4^{2-} + 11H^+ --> 14CO_2 + 2NH_4^+ + 7HS^- + 4H_2O \Delta G_{o'} = -180\,kJ$$

The first step in degradation of 3-aminobenzoate by this new sulfate-reducing bacterium was found to be activation to 3-aminobenzoyl CoA (Schnell and Schink 1992). Further reduction of 3-aminobenzoyl Co A did not yield benzoyl CoA, but rather a product tentatively described as a reduced Co A-ester. The activation of benzoyl CoA depends on the presence of the cofactors, ATP and Mg^{2+}. Acyl-CoA synthetase reactions were identified as the initial step in the degradation of benzoate by anaerobic bacteria.

8.3.2 *Bioremediation of TNT Under Sulfate-Reducing Conditions*

Soil and water in most U.S. Military facilities are contaminated with explosive chemicals, mainly because of the manufacture, loading, and disposal of explosives and propellants. This contamination problem may increase in future because of demilitarization and the disposal of unwanted weapon systems. Disposal of obsolete explosives is a problem for the military and the associated industries because of the polluting effect of explosives in the environment (Wyman et al. 1979). TNT is the major contaminant in many U.S Army Ammunition facilities. TNT represents an environmental hazard because it has toxicological effects on number of organisms (Fernando et al. 1990; Won et al. 1974) and it is mutagenic (Kaplan and Kaplan 1982). The disposal of large quantities of TNT in an environmentally acceptable manner poses serious difficulties. The present approach to the remediation of TNT contamination is incineration of soil, a very costly and destructive process. Bioremediation would be a safe and cost-effective method for treating TNT contamination. Biological removal of explosives from soil has been demonstrated using aerobic/anoxic soil slurry rectors (Boopathy et al. 1998; Boopathy 2000, 2001, 2002). In our lab, we isolated a well-defined sulfate-reducing consortium consisting of *Desulfovibrio* spp., namely, *D. desulfuricans* strain A, *D. desulfuricans*, strain B, *D. gigas*, and *D. vulgaris* from a creek sediment (Boopathy and Manning, 1996). The ability of this consortium to degrade and remediate TNT was explored.

The consortium was grown in anaerobic serum bottles under various growth conditions, including TNT as the sole carbon source, co-metabolic condition with pyruvate (30 mM) as co-substrate, and heat-inactivated control as shown in Boopathy and Manning (1996). Figure 8.1 shows the results of bacterial growth.

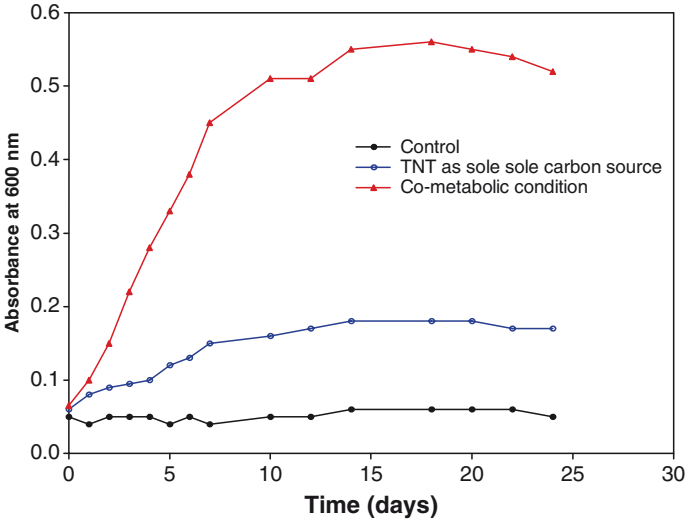

Fig. 8.1 Growth of *Desulfovibrio* spp., under various conditions

Growth was observed in all conditions except in the killed control. The maximum growth was observed under co-metabolic conditions, and bacteria also grew under the conditions where TNT served as the sole carbon source. Figure 8.2 shows the removal of TNT under various culture conditions. In all the cultures, the initial TNT concentration was 100 mg l^{-1}. In the killed control, the TNT concentration remained constant throughout the experiment, indicating that no physical or chemical removal of TNT occurred. TNT removal was fastest in the co-metabolic condition, where 100% of TNT was removed within 10 days of incubation. TNT removal in the culture condition where TNT served as the sole carbon source was very slow, but 100% of the TNT was still removed within 25 days. The results show that the consortium can remove TNT faster in the presence of an additional carbon source like pyruvate. This could be due to an increase in the bacterial cell numbers in the pyruvate-containing cultures.

The GC-MS analysis of culture samples with and without pyruvate revealed the presence of various intermediates, which were identified by comparison of their GC retention times and their mass spectra with authentic standards. The first intermediates observed were 4-amino-2,6-dinitrotolune (4-ADNT) and 2-Amino-4,6-dinitrotoluene (2-ADNT). The ratio of 4-ADNT and 2-ADNT formed from the TNT metabolism were approximately 80:20. These products were further reduced to 2,4-diamino-6-nitrotoluene (2,4-DANT). Other compounds appearing in the culture medium in order were nitobenzoic acid (NB), cyclohexanone, 2-methyl pentanoic acid, butyric acie, and acetic acid. All of these compounds were identified in cultures with both TNT and pyruvate as carbon sources, as well as in the cultures that received TNT alone as a carbon source. These intermediates were not present in the control.

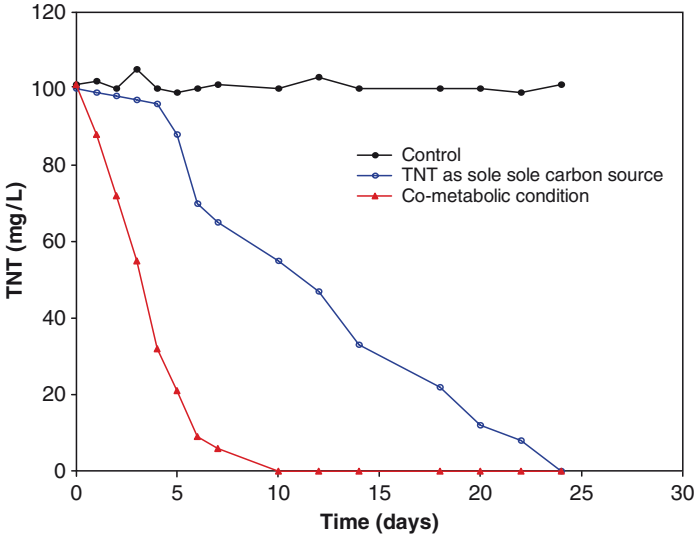

Fig. 8.2 Concentration of TNT under various growth conditions

Radiolabeled study was conducted with uniformly ring labeled [^{14}C] TNT in the culture condition, where TNT served as the sole carbon source. The experimental procedure used by Boopathy and Manning (1996) was used in this study. The results of radiolabeled study are presented in Table 8.1. The data showed the production of various metabolites and biomass at the end of the experiment on day 30. TNT was not mineralized, as there was no production of CO_2. Most of the TNT was converted to acetic acid (49%), and 27% of TNT was assimilated into cell biomass. Apart from acetate, the other major intermediates present in the culture medium were nitrobenzoic acid (6%) and butyric acid (9.5%). In killed control, TNT was reduced to a smaller extent to 4-ADNT (3%), yet nearly 95% of the original TNT was recovered unaltered. Traces of cyclohexanone were observed, which accounted for 0.01% of the original [^{14}C] TNT. This radiolabeled study showed a reasonable mass balance, with a recovery of 95% of [^{14}C] TNT. Since the ring carbons of TNT were uniformly labeled, conversion of TNT to acetic acid and butyric acid clearly denotes ring cleavage.

The production of various intermediates in both culture conditions (with TNT as the sole carbon source and co-metabolic condition with pyruvate) suggested that the bacterial consortium has all the necessary enzymes to degrade TNT. The anaerobic metabolic pathway as shown in Fig. 8.3 was proposed for TNT metabolism by sulfate-reducing bacteria. TNT was reduced to 4-ADNT and 2-ADNT, which were further reduced to 2,4-DANT. These reductions may have been accomplished by the production of sulfide from sulfate by the *Desulfovibrio* spp., as demonstrated by Preuss et al. (1993) and Gorontzy et al. (1993). The sulfide analysis showed 10.6 and 3.1 mM of sulfide on day 20 in the cultures with and without pyruvate respectively. The large difference in sulfide production in the cultures with

Table 8.1 Results of radiolabeled TNT study: mass balance for TNT metabolism by *Desulfovibrio* consortium

[^{14}C] TNT recovered	Active culture (%)	Killed control (%)
CO_2	0	0
Biomass	27.4	0
Acetic acid	49.5	0
Nitrobenzoic acid	6.2	0
Cyclohexanone	0.01	0
Butyric acid	9.5	0
2-Methyl pentanoic acid	0.2	0
4-ADNT	0.5	3.2
2-ADNT	0.9	0
2,4-DANT	0.7	0
TNT	0	94.5
Unrecovered	5.09	2.3

and without pyruvate may be due to the availability of higher electron donor in pyruvate-containing cultures compared to cultures with only TNT. The next metabolite identified was NB. There may be two or three intermediates between 2,4-DANT and NB, which were not identified. These compounds might be transient and thus not detected in the GC analysis. The NB was converted to cyclohexanone. This step was accomplished by ring cleavage, which under anaerobic conditions would generally be accomplished by a series of hydrogenation and dehydrogenation reactions (Harwood and Gibson 1986), converting NB to cyclohexanone. Harwood and Gibson (1986) reported that under anaerobic conditions *Rhodopseudomonas palustris* produced pimelic acid from benzoic acidy by dehydrogenation and hydration reactions. The major intermediate observed in the study by Harwood and Gibson (1986) was cyclohexanoic acid. Cyclohexanone was further converted to 2-methyl pentanoic acid. From 2-methyl pentanoic acid, butyric acid was formed, which was further converted to acetic acid. The radiolabeled study showed no production of CO_2 from TNT metabolism, and the final end product is acetic acid. This fatty acid can be easily removed under anaerobic conditions by various acetate utilizing sulfate-reducing and methanogenic bacteria.

The application of this consortium to the treatment of TNT-contaminated soil was evaluated using a TNT-contaminated soil collected from the Joliet Army Ammunition Plant (JAAP), Joliet, IL, USA. The soil contained a very high concentration of TNT of 6,000 mg/kg of soil. An anaerobic soil slurry reactor was designed based on the previous study by Boopathy et al. (1998). The anaerobic condition in the reactor was maintained by bubbling helium gas in the headspace of the reactor. The contaminated soil was sterilized using an autoclave. Soil slurry (10%) was made using sterile tap water containing 20 mM sodium sulfate as electron acceptor, 15 mM pyruvate as co-substrate, and 5 mM ammonium chloride as nitrogen source. A 5% pre-grown inoculum of the sulfate-reducing consortium was added to the soil slurry reactor to start the bioremediation experiment. A control soil slurry reactor was maintained with similar conditions as described above, except bacterial inoculum. The experiment was

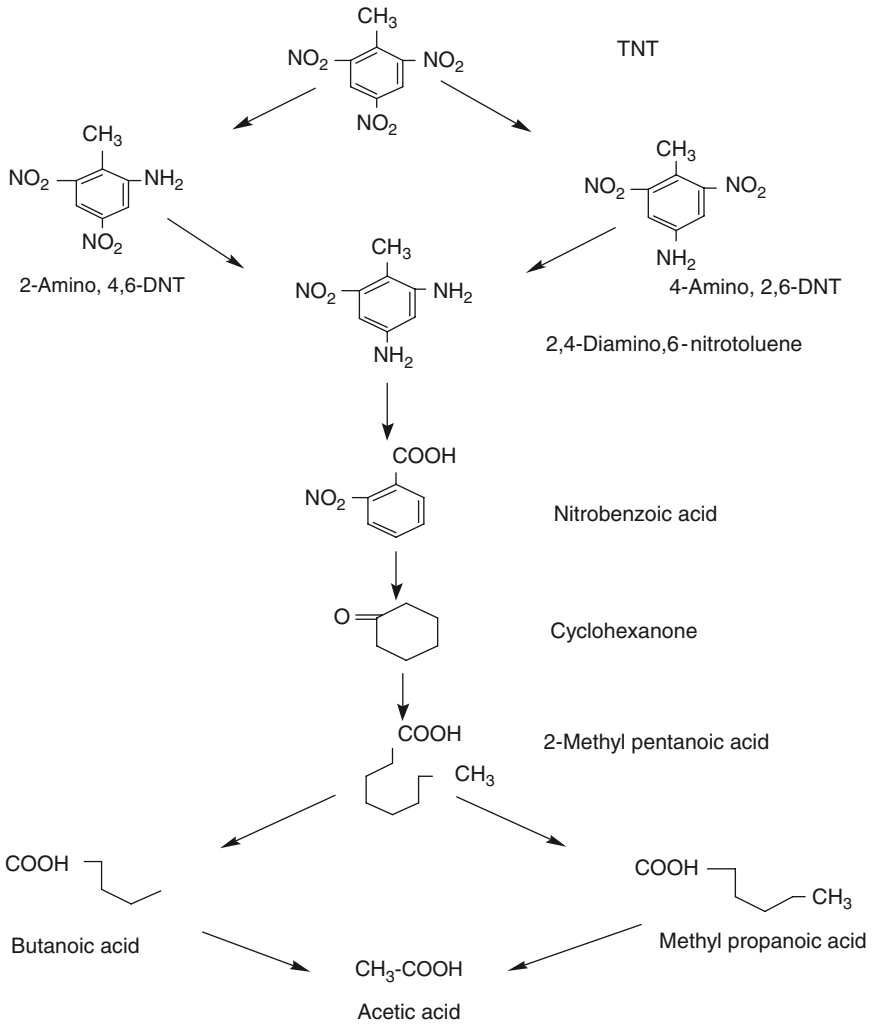

Fig. 8.3 Proposed TNT metabolic pathway by *Desulfovibrio* spp

run for 125 days. The results shown in Fig. 8.4 indicated that the sulfate-reducing bacterial consortium effectively removed TNT compared to the control reactor. The TNT removal in the reactor with bacterial inoculum was almost 100%, and in the control there was no TNT removal. This study showed that the sulfate-reducing bacteria can remove TNT under anaerobic conditions. This was the first report on sulfate-reducing bacteria that can remove TNT in a soil slurry condition. This report on the removal of TNT in soil by the sulfate-reducing bacterial consortium in a soil slurry reactor may have significant implications for the decontamination of TNT-contaminated soil. Most munitions contamination is in the surface layer of soil, which can be excavated and treated in an anaerobic soil slurry reactor.

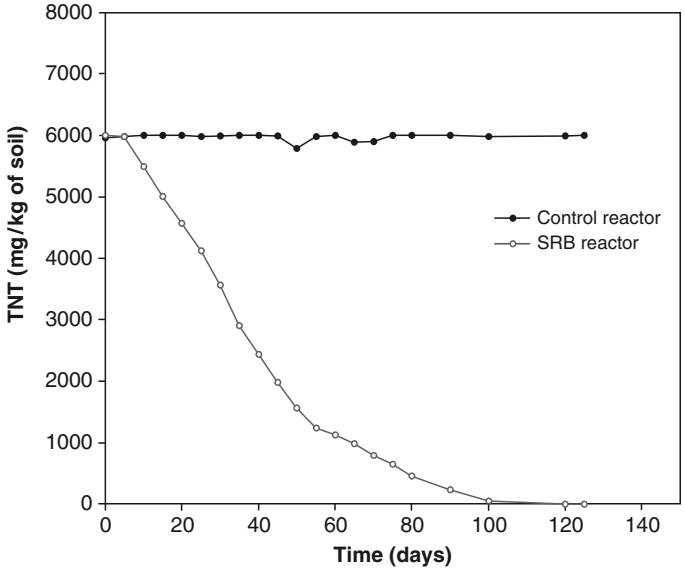

Fig. 8.4 Concentration of TNT in the soil slurry reactor operated with *Desulfovibrio* spp

8.4 Bioremediation of Explosives-Contaminated Soil: A Case Study

Two bioremediation methods, namely, soil slurry reactor and land farming technique for the treatment of explosives-contaminated soil explosives-contaminated soil in Louisiana Army Ammunition Plant (LAAP) in Minden, Louisiana were studied. The contaminated soil was collected from the LAAP in Minden, Louisiana, USA. The contaminant concentrations in the soil are given in Table 8.2. The TNT concentration in the soil ranged from 4,000 to 10,000 mg kg^{-1}. The rapid detention explosive (RDX) concentration in the soil ranged from 800 to 1,900 mg kg^{-1}. The high melting explosive (HMX) concentration ranged from 600 to 900 mg kg^{-1}. The soil had a total organic matter content of 4–5%, which included the contaminants. The soil had an average pH of 6.5.

8.4.1 Soil Slurry Reactor and Landfarming Methods

Four 2-L laboratory-scale soil slurry reactors soil slurry reactors were set up and were operated at ambient temperature (20–22°C). The batch reactors were operated starting with 20% (w/v) slurry of explosive contaminated soil obtained from the Louisiana Army Ammunition Plant (Minden, LA, USA) in water. The first two reactors were the control, which received no molasses as a co-substrate. The second

Table 8.2 Explosive concentrations in the contaminated soil

Explosive	Concentration range (mg/kg of soil)
TNT	4,000–10,000
RDX	800–1,900
HMX	600–900

two reactors received 0.3% (v/v) molasses (Grandma's Molasses, Mott's LLP, Cadbury Beverages Inc., Stanford, CT, USA) as a co-substrate once every week. Air was supplied through a diffuser once a day for 10 min using a timer. The soil slurry was mixed continuously at an average rate of 100 r min^{-1} by using a stirring motor (model RW 20 DZM, Tekmar Company, Cincinnati, OH, USA). The explosive concentrations, bacterial growth, pH, and dissolved oxygen were monitored weekly in the reactors, and the results were averaged for the two sets of reactors.

Four sets of pans were used for this landfarming experiment. A set consisted of a small aluminum pan (8 in. × 3.75 in. × 2.375 in.) placed in a larger steel pan (9.5 in. × 5.2 in.× 2.7 in). The bottoms of the smaller pans were perforated with 2-mm-diameter holes spaced 4 cm apart to allow for the drainage of fluids during and after flooding cycles. Each small pan contained 300 g of contaminated soil obtained from the LAAP (Minden, LA, USA), which was placed in a larger pan. Water (500 ml) was added every 2 weeks during the wet cycle. After 2 weeks of flooding, the water was emptied from the larger pans to allow the soil to dry for a 2-week dry cycle. The first two sets of pans served as the control in which no molasses was added as a co-substrate. The second sets of pans received 0.3% (w/v) molasses (Grandma's Molasses, Mott's LLP, Cadbury Beverages Inc., Stanford, CT) as a co-substrate once a week for 2 weeks during the flooded cycle. Each pan received tillage once a week. The explosive concentrations and bacterial growth were monitored weekly in the pans, and the results were averaged for the control and treatment sets of pans.

8.4.2 Analyses

The explosives in the soil were extracted by the method recommended by the US Army environmental Research Center. The soil slurry was dried in an oven at 100°C. Then 1 g of soil was transferred into a 15 ml serum vial and extracted with 9 ml of acetonitrile. A Teflon-lined septum cap was placed on the vial, and the suspension was subjected to vortex mixing for 1 min. The mixture was sonicated for 18 h. After sonication, the sample was allowed to settle for 30 min, and then 1.0 ml of the supernatant was removed and combined with 1.0 ml of aqueous $CaCl_2$ solution (5 g l^{-1}) in a glass scintillation vial. The vial was hand-shaken, allowed to stand for 15 min, and then centrifuged in a microcentrifuge at 10,000 rpm for 5 min. The supernatant was removed and stored in a vial to be analyzed by high-performance liquid chromatography (HPLC) using a liquid chromatograph equipped with two

Model 210 solvent pumps, a Model 320 programmable multi-wavelength ultraviolet (UV) detector set at 254 nm, a Model 410 system auto sampler (Varian, Walnut Creek, CA, USA), and an LC-CN 4.6-mm-i.d. × 25-cm HPLC column (C-18 Supelco column) with a particle size of 5–6 μm. The mobile phase was methanol:water (50:50, v/v) at a flow rate of 1.5 ml min^{-1}, with an injection volume of 50 μl.

Dissolved oxygen in the soil slurry was monitored weekly using an oxygen analyzer before the aeration event (YSI 5000, Yellow Springs, OH, USA). The probe of the analyzer was placed directly in the reactor, and the dissolved oxygen concentration in the reactor was measured and expressed as mg l^{-1}. The pH of the slurry was also measured weekly with a pH meter (UltraBasic UB-10, Denver Instrument) by placing the probe directly in the reactor.

Bacterial activity in the reactors was monitored weekly. Slurry samples (1 ml from each reactor) were serial-diluted with a phosphate buffer solution. Standard methods for total plate counts were followed with tryptic soy agar plates. [^{14}C]TNT [14C]TNT mineralization studies were also carried out. After 49 days of operation, 20 ml of slurry was taken from each soil slurry reactor, and 10 g of dry soil was taken from each land-farming pan. The samples were incubated with uniformly labeled TNT to provide mass balance and determine metabolite production, including [^{14}C]CO_2. The [^{14}C]TNT was added to the soil slurry at the level of 20,000 disintegrations per minute (dpm)/ml in a respirometer flask. Samples were withdrawn periodically, and the quantity of TNT converted to biomass was determined as trichloroacetic acid (TCA) precipitable material by using a Beckman (Palo Alto, CA, USA) model LS5000TD liquid scintillation spectrometer.

Respirometer flasks Respirometer flasks containing [^{14}C]TNT were used to monitor the carbon dioxide evolved by the soil bacteria. This experiment was conducted anaerobically with a modified respirometer. Potassium hydroxide (KOH) (0.5 N) was added to the side arms. The flasks were incubated at ambient temperature in a shaker at 50 r min^{-1}. The respirometer flasks were sampled periodically by withdrawing the potassium hydroxide, measuring the radioactivity with a liquid scintillation spectrometer, and replacing the potassium hydroxide. The percentage of [^{14}C]TNT mineralized as [^{14}C]CO_2 was calculated.

The TNT metabolites TNT metabolites were analyzed by collecting the fractions every 30 s after passage through the HPLC column. The radioactivity in each fraction was measured using a liquid scintillation counter. Soil-bound radioactive TNT was analyzed using the soil extraction procedure described above, and the radioactivity in the soil was measured using a liquid scintillation spectrometer.

8.4.3 Results

The concentrations of TNT in the slurry reactors are given in Fig. 8.5. The soil–TNT concentration in the no-carbon control reactor remained high, around 7,500 mg/kg of soil throughout the experiment. This observation suggests that the indigenous

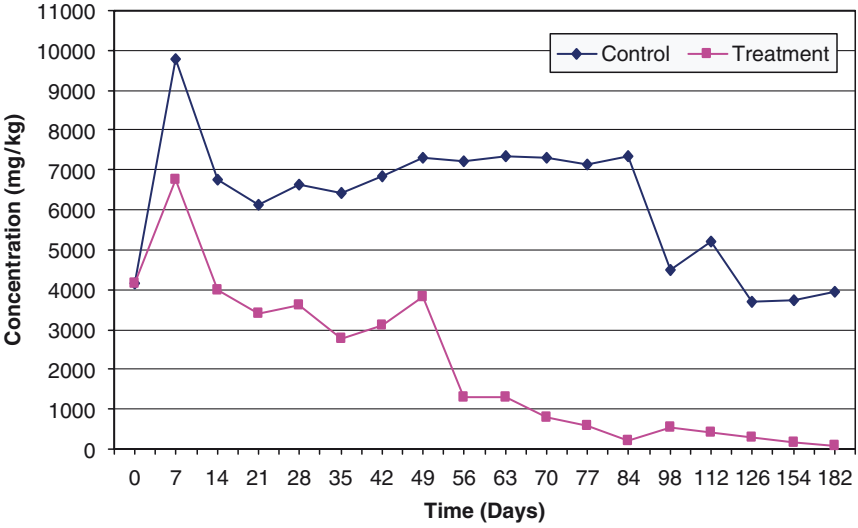

Fig. 8.5 Concentrations of TNT in the soil slurry reactors

microflora from the contaminated site would not degrade TNT without the addition of nutrients or co-substrates. The soil–TNT concentration in the reactor that received molasses as co-substrate dropped gradually, and fell below 50 mg/kg of soil on day 182 of the study.

Our previous study demonstrated that TNT-removal in the soil slurry system was accomplished by a co-metabolic process that required an additional carbon source such as molasses or succinate (Boopathy et al. 1994b). Molasses is a very effective carbon source that enhances the TNT degradation rate significantly over other carbon sources (Boopathy et al. 1994b). This study showed that the soil slurry reactors can effectively remediate TNT in the contaminated soil. The operation of laboratory-scale soil slurry reactors over 182 days showed that 99% removal of TNT can be achieved. The soil slurry reactor also removed other explosives, namely, HMX and RDX (Figs. 8.6 and 8.7). However, the removal efficiency was not as high as that for TNT. This could be due to the complexity of molecules. HMX and RDX degradation can be achieved, but it will take a longer period of time, as indicated by many other studies (Williams et al. 1992; Boopathy 2001).

The addition of radiolabeled TNT to the reactor biomass provided evidence for the mineralization of TNT. Of the original radiolabeled TNT (20,000 dpm ml^{-1}), 23% was converted to CO_2 and 24% was used in making cellular materials. Radiotracer studies Radiotracer studies with the reactor biomass also revealed various intermediates, including 4-amino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, fatty acids, and an unidentified metabolite 3 weeks after the start of the radiolabeled studies. Extraction of soil with acetonitrile showed that 2% of the TNT was adsorbed on to the soil. The rest of the TNT was accounted for as intermediates. The mass balance was reasonable, with 98% recovery of radiolabeled TNT.

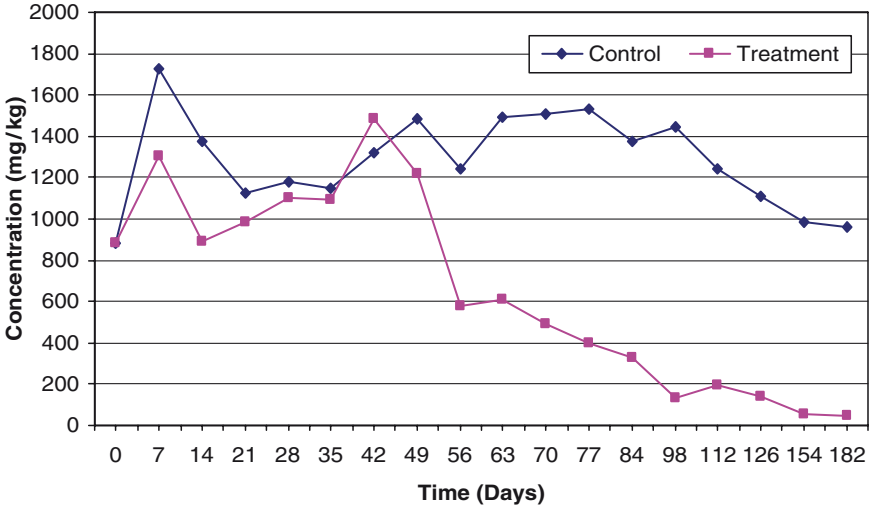

Fig. 8.6 Concentrations of HMX in the soil slurry reactors

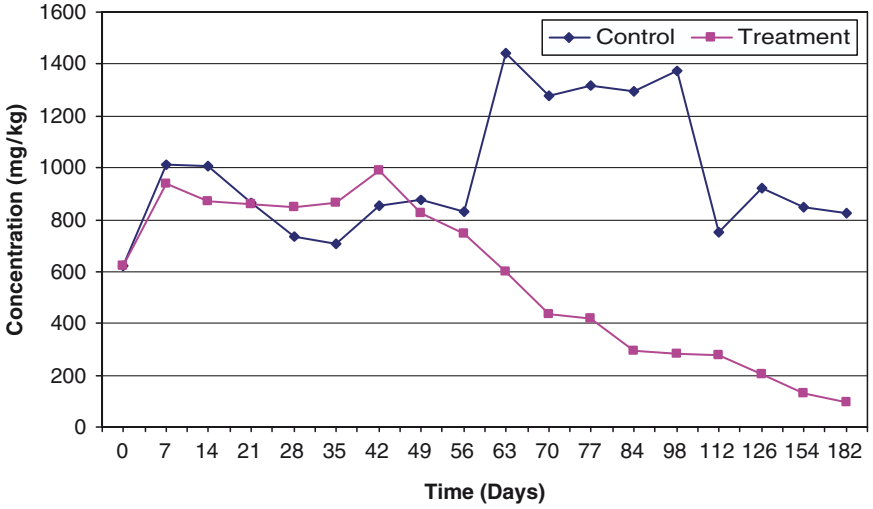

Fig. 8.7 Concentrations of RDX in the soil slurry reactors

In the no-carbon control, 78% of radiolabeled TNT was recovered as TNT, which did not undergo degradation. This radiolabeling study showed that the natural soil bacteria present in the contaminated soil can cause extensive degradation of TNT in a reasonable time under optimum conditions. Degradation was demonstrated by mineralization of radiolabeled TNT, metabolite formation, and the presence of radioactivity in the cell biomass as TCA-precipitable material.

The pH in the reactors was monitored throughout the experiment. The pH remained approximately neutral in the no-carbon control reactor. However, the molasses-containing reactor tended to be acidic, with a pH value of 5. Dissolved oxygen (DO) concentrations were monitored weekly in the soil slurry reactors. The DO concentration remained around 6.5 mg l^{-1} in the no-carbon control, and in the reactors with molasses the DO was less than 1 mg l^{-1}.

Bacterial plate counts were performed several times over the course of the experiment. The bacterial plate counts in the reactor receiving molasses were consistently higher than those in the no-carbon control reactor (Table 8.3). This result also shows the value of molasses addition, which helps to increase the population of soil bacteria in the reactor. Molasses is the best among various substrates studied, such as succinate, glucose, acetate, and citrate (Boopathy et al. 1994b), it is well-balanced with nutrients including carbon, nitrogen, phosphorous, vitamins, and minerals for bacterial activity.

In the landfarming method, the TNT concentration in soil samples taken from pans 1–4 during the 182-day study is shown in Fig. 8.8. For each sampling date, three soil samples were collected from each pan, analyzed, and the mean concentrations were plotted. The TNT concentrations in the control pan remained at high levels over the course of the experiment. The treatment that included molasses solution was biologically active, and showed removal of TNT. Starting from a high concentration of approximately 7,000 mg/kg of soil, the average concentration of TNT after 182 days of treatment was less than 1,250 mg/kg of soil, which was equal to 82% removal of TNT. Very little RDX and HMX HMX were removed in soil in both the control and treatment pans (data not shown). The degradation rates of RDX and HMX are extremely slow, and continuation of the experiment over a 300-day period might show a significant reduction in HMX and RDX (Boopathy 2001).

The radiolabeled study used biomass taken from the pans, and provided evidence for the mineralization of TNT. In pan biomass samples that received molasses, the proportion of the initial radiolabeled spike that was transformed to radiolabeled CO_2 was 6.5%. In the control pans, the radiolabeled CO_2 was 1.2%.

Table 8.3 Bacterial counts in the soil slurry reactors[a]

Days of treatment	Control (CFU ml^{-1})	Treatment (CFU ml^{-1})
0	72×10^2	81×10^2
14	33×10^7	53×10^7
28	46×10^5	167×10^6
42	60×10^5	235×10^6
56	47×10^5	55×10^6
70	123×10^4	36×10^6
84	187×10^4	103×10^6
98	93×10^4	59×10^6
112	173×10^4	105×10^6
126	88×10^4	78×10^6
154	67×10^4	104×10^6
182	72×10^4	121×10^6

[a]The data represent an average of two plates

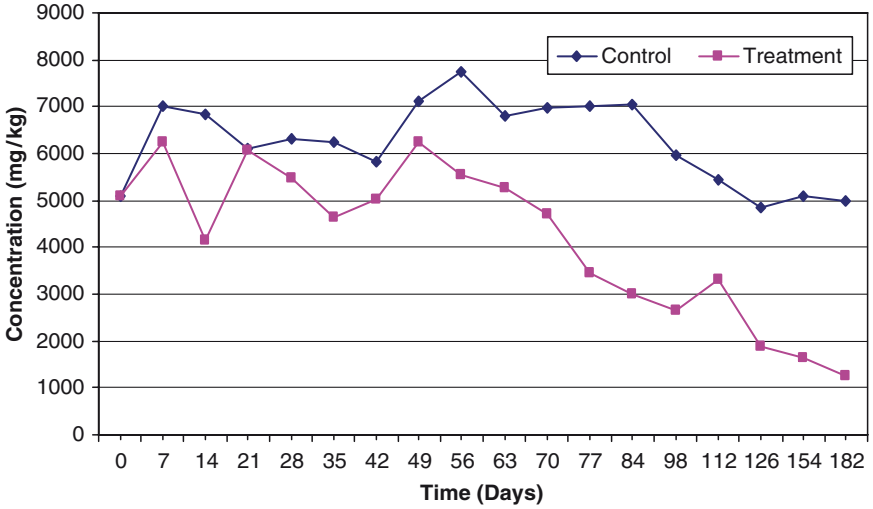

Fig. 8.8 Concentrations of TNT in the land farming pans

This result clearly demonstrates that TNT was mineralized by the soil bacteria in the treatment that received molasses. The analysis of TCA-precipitable material showed that a significant amount of radiolabeled TNT was converted to cell biomass. Various TNT metabolites were present in varying degrees. The calculated mass balance was very reasonable, with recovery of 98% of radiolabeled TNT in this study. This experiment showed that the control pans did not have an active biomass to convert TNT to CO_2, due to the lack of molasses as co-substrate.

Bacterial plate counts were performed several times over the course of the experiment on soil samples taken from each pan. The bacterial population densities in the soils receiving molasses solutions were consistently higher than those in the control pans (Table 8.4). This result also shows that the control pans consistently exhibited plate counts of the order of 10^4 colony forming units/g of soil, and thus were not strictly abiotic controls; however, negligible biodegradation of TNT occurred in the control, as TNT concentration in control remained high throughout the study.

Between the two different bioremediation methods, the soil slurry reactor system showed efficient removal of TNT for the LAAP soil in Minden, LA, USA. The landfarming method also removed TNT, but the removal rate was very slow. The land farming in the field should be done in a constructed cell with liner to prevent any leachate migrating to ground water. Both methods showed that the native soil bacteria present at the contaminated site are capable of mineralizing TNT as demonstrated in the radiolabeled study. The advantage of the soil slurry reactor is its simple operating conditions. The method needs only mixing, supply of air and a carbon source. Molasses is an inexpensive carbon source that could be used in a large-scale operation at low cost. Based on this study, the soil slurry reactor can be used for effective and fast remediation of TNT at LAAP, Minden, LA, USA. The removal of HMX and RDX in the soil can also be achieved with prolonged incubation.

Table 8.4 Bacterial counts in the land farming pans[a]

Days of treatment	Control (CFU/g of soil)	Treatment (CFU/g of soil)
0	63×10^2	74×10^2
14	58×10^6	46×10^6
28	41×10^5	44×10^6
42	40×10^5	88×10^6
56	32×10^4	158×10^5
70	63×10^4	60×10^6
84	273×10^4	129×10^6
98	73×10^4	112×10^6
112	220×10^4	162×10^6
126	188×10^4	119×10^6
154	112×10^4	67×10^6
182	23×10^4	87×10^6

[a]The data represents an average of two plates

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Chapter 9
Biological Remediation of Petroleum Contaminants

Ramesh Chander Kuhad and Rishi Gupta

9.1 Introduction

All the operations in the petroleum industries, such as exploration and production of oil, transportation, refining, refined product handling and oily waste management activities are potential sources of water, soil and air pollution. Crude oil spills that occur during transportation, and refinery wastes, pose serious threats to the environment. The increase in environmental contamination through infiltration of petroleum products, both in water and on land, has led to a progressive deterioration of environmental quality. There are various alternatives available for the treatment and containment of oil wastes. Use of conventional techniques includes adsorption of spilled oil from water bodies, excavation and treatment using physical, chemical or biological methods. However, more effective measures of remediation and restoration of the environmental condition are continually being sought. Of the various technologies available for cleaning of hydrocarbon contaminations, biological remediation methods are considered least expensive.

Bioremediation utilizes application of microorganisms or plants to clean up contaminated sites. Hydrogeological and engineering principles are applied to design a bioremediation technology. Many microorganisms can adapt their catabolic activities to make use of toxic organic pollutants as food sources, and thereby mineralize complex organic compounds to simpler compounds, carbon dioxide and water. Non-biological methods have been effectively combined with biological methods to enhance the biodegradation of recalcitrant organic compounds (Van Hamme et al. 2003; Brar et al. 2006). Microbial degradation of crude oil is cost effective and environmentally sound, and is therefore often the best means of removing oil from the polluted environment (de Lorenzo 2006; de Carcer 2007). Indigenous microorganisms present at the contaminated site are often inhibited by

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the toxic level of these contaminants, or often do not possess the required catabolic capabilities to completely degrade the petroleum contaminants (Forsyth et al. 1995; Mishra et al. 2001a). In such cases, biodegradation of contaminating oil by the introduction of microorganisms after enrichment or genetic modification increases the efficiency and rate of degradation.

9.2 Fate of Hydrocarbons in Soil

Crude oil constitutes of four broad fractions, namely the alkanes, the aromatics, the nitrogen-sulfur-oxygen compounds (NSO) and the asphaltene fraction. The alkane fraction mainly contains saturated hydrocarbons, which can range from methane to compounds with carbon chain lengths of 40 or more which occur as straight-chain or branched-chain compounds (Scullion 2006). The aromatic fraction contains ringed hydrocarbons, ranging from benzene to multi-ring polycyclic aromatic structures composed of nitrogen, oxygen and sulfur derivatives of hydrocarbons generated via geochemical reactions of carbon compounds with inorganic sulfur or nitrogen compounds. The most recalcitrant fraction in crude oil is the asphaltene fraction, which contains higher hydrocarbons formed primarily by cross-linking of NSO units (Salanitro 2001). Light oils typically contain high levels of saturated and aromatic hydrocarbons, and a smaller proportion of resins and asphaltenes. Heavy oils, resulting from the biodegradation crude oil under anoxic conditions in situ in petroleum reservoirs, have a much lower content of saturated and aromatic hydrocarbons and a higher proportion of the more polar chemicals, the resins and asphaltenes.

Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, chrysene, phenanthrene, benzo(a)pyrene and others, and BTEX compounds (benzene, toluene, ethyl benzene and xylene) have been listed as priority pollutants by the US EPA. PAH and BTEX compounds enter the soil and air through processing associated with gasoline and petroleum fuels at petroleum wells and refineries, industrial effluents, wood-processing activities, and manufacturing of pesticides, detergents, chemicals, paints and varnishes. They are also transferred through leakage of underground storage tanks, and spills during transportation. Since the majority of these substances are highly persistent within ecosystems because of their low water solubility, their intrinsic chemical stability, and high recalcitrance to degradation, they form the main soil and groundwater contaminant groups (Husain 2008). The fate of hydrocarbons in soil is governed by various factors related to the soil environment, as well as properties and chemical characteristics of the compound (Alexander 2000). Bioavailability and biodegradation of PAHs and BTEX compounds is affected by the distribution and partitioning of the contaminants in soil particles, and ageing of the contaminated soil. Further interactions occurring at the interface of organic and inorganic particles through sorption and desorption mechanisms may strongly

affect the movement of the contaminants and their availability for biological or non-biological transformations.

A closed landfarming mesocosm mesocosm prepared from contaminated soil provided evidence that the extent of hydrocarbon biodegradation is largely affected by the molecular composition of the soil contaminant (Huesemann 1995). Based on the results, a predictive algorithm was developed to estimate the extent of biodegradation, based on the average reduction of 86 individual hydrocarbon classes and their initial concentrations. Forsyth et al. (1995) demonstrated that significant bioremediation occurred when the population of the hydrocarbon-degrading microorganism in the soil is more than 10^5 CFU per g of soil. In another study, a bioremediation treatment that consisted of liming, nutrient fertilization, and tilling was evaluated on the laboratory scale for its effectiveness in cleaning up a sand, a loam, and a clay loam soil contaminated with gasoline, jet fuel, heating oil, diesel oil, or bunker C oil, using an indigenous microbial population (Song et al. 1990). Bunker C oil was found to be structurally recalcitrant, with about 80% residual hydrocarbons persisting even after 1 year of incubation.

9.3 Microbial Diversity and Biodegradation

Biodegradation of crude oil is a natural process. The changes in the composition of polluting hydrocarbons, induced by the various physical, chemical and biological factors, are collectively known as weathering. A large number of microorganisms belonging to a variety of genera are able to utilize hydrocarbons as the sole source of carbon and energy, and these microorganisms are widely distributed in nature (Van Hamme et al. 2003). Hydrocarbons in the natural environment are biodegraded by a diverse group of bacteria, yeast and filamentous fungi, algae, cyanobacteria and some protozoan organisms (Paul et al. 2005). Most of the hydrocarbon-degrading bacteria belong to a Gram-negative group. Bartha and Bossert (1984) have listed 22 genera of hydrocarbon-degrading bacteria and 31 genera of fungi isolated from soil. In a comparative study of hydrocarbon degradation by bacteria and fungi, Song et al. (1986) found that 82% of *n*-hexadecane mineralization n-hexadecane mineralization in a sandy plume was attributed to bacteria, while only 14% was due to fungi.

Based on published reports in the literature, among the most common and efficient hydrocarbon-degrading bacteria in both soil and marine environments are species of *Acinetobacter, Pseudomonas, Alcaligenes, Arthrobacter, Achromobacter, Bacillus, Flavobacterium* and *Nocardia*; among the yeasts are *Aureobasidium, Candida, Rhodotorula and Sporobolomgers.* Among other fungi the dominant organisms are species of *Trichoderma*, *Mortiecerella, Penicillium* and *Aspergillus* (Leahy and Colwell 1990). Species of *Aspergillus*, *Penicillium* and *Phanerochaete,* capable of degrading hydrocarbons, have been frequently isolated from both types of environment. In a successful biopile remediation test, the soil microbial

characterization showed one *Bacillus*, two *Corynebacterium*, and three *Mycrococcus* species among the existing Gram-positive bacteria, and *Enterobacter amnigenus*, *Enterobacter cloacae*, and *Pseudomonas sp*. were detected as the main Gram-negative bacteria present in the soils (Iturbe et al. 2007).

During the last 10 years, many interesting marine bacteria have been isolated and characterized to use hydrocarbons as a carbon source (Head et al. 2006). Some of the examples include species of *Alcanivorax*, *Cycloclasticus*, *Oleiphilus*, *Oleispira*, *Thalassolituus* and some members of the genus *Planomicrobium* (previously known as *Planococcus*). *Alcanivorax* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp. and *Planomicrobium* spp. use a variety of branched- and/or straight-chain saturated hydrocarbons, whereas *Cycloclasticus* spp. can use a range of polycyclic aromatic hydrocarbons.

The selection of the microbial population is determined by the characteristics of the pollutant to be treated. The challenge of characterizing the roles of a range of organisms in degrading contaminants present in natural media is clearly substantial. It is important to analyze the microbial community structure and its changes during biodegradation processes. Such studies can provide major insights into important biochemical and physiological aspects of biodegradation. Culture-dependent and culture-independent methods are commonly applied to characterize the microbial community.

Since the majority of the biological treatment processes rely on the activities of complex microbial communities, we still have much to learn about the interactive and interdependent roles played by individual species in these communities (Ram 2005). Community dynamics are mainly followed using cultivation-independent, 16S-rRNA-gene-based methods or functional-gene-based methods (Spiegelman et al. 2005). The temporal and spatial changes in bacterial populations, and the diversity of the microbial community during biodegradation processes, can be determined using a combination of sophisticated molecular, chemical and instrumental methods. Recent advances in molecular techniques, combined with genomic information, are greatly assisting microbiologists in unraveling some the diverse roles of microbes in these communities, as well as determining the importance of lateral gene transfer in complex microbial communities. Careful monitoring of microbial community dynamics can lead to the discovery of common biodegradation patterns, which can help in the development of new tools for rapid evaluation of ongoing bioremediation processes.

Among saturated hydrocarbons, *n*-alkanes are generally considered to be readily degradable components of crude oil (Setti et al. 1993). Based on the chain length, *n*-alkanes are divided into three degradation groups: lower alkanes (C_8–C_{16}), medium alkanes (C_{17}–C_{28}), and higher alkanes, (>C_{28}). Most of the microorganisms have been reported to show satisfactory growth and faster degradation of *n*-alkanes up to C_{16} carbon chain length. Biodegradation of n-alkanes with carbon chain length from C_8–C_{16} is generally favoured by increased solubility and reduced surface tension. Biodegradation of *n*-alkanes up to C_{44} has been demonstrated (Sakai et al. 1994). In most instances, enhanced solubilization was a result of surface-active

compounds produced by the hydrocarbon-degrading microorganisms. Emulsification of the substrate enhanced the contaminant surface area, and this facilitated higher dissolution rates and overcame mass-transfer limitations (Herman et al. 1997; Yuste et al. 2000; Koma et al.2001; Singh et al. 2006).

The metabolism of branched-chain alkanes has received relatively little attention, in contrast to metabolism of *n*-alkanes. Most hydrocarbon-degrading microorganisms cannot effect the complete degradation of branched-chain alkanes. Transformation of cyclohexane by mixed or pure culture has been reported (de Klerk and van der Linden 1974; Uribe et al. 1990). Earlier transformation of cyclohexanes was demonstrated using pure cultures (Ooyama and Foster 1965) in the presence of co-substrates only.

The degradation of aromatic hydrocarbon has been extensively reviewed (Gibson and Parales 2000; Gibson and Harwood 2002; Andreoni and Gianfreda 2007). Light aromatic hydrocarbons are subjected to evaporation and to microbial degradation in a dissolved state, but extensive methyl substitution does inhibit initial oxidation. A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes, but those compounds are transformed to a limited number of common metabolites such as protocatechuate and catechols (Reineke and Knackmuss 1988; Commandeur and Parsons 1990; Chaudhary and Chapalamadugu 1991). These key intermediates are subjected to one of the two ring-cleavage pathways, either to an extra-diol (meta) or to an intra-diol (ortho) cleavage-type pathway (Harayama et al. 1999). In the majority of the cases, ortho-fission of catechol by catechol 1,2 dioxygenase and of prototechuate by protocatechuate 3,4 dioxygenase has been demonstrated (Fewson 1991).

The resins and asphaltenes are generally considered to be recalcitrant to biodegradation, and the metabolic pathways for the degradation of these fractions are less understood (Van Hamme et al. 2003). Bacterial strains capable of utilizing resins as the sole source of carbon and energy have also been isolated (Venkateswaran et al. 1995). These strains, isolated from mixed populations, were clustered into 5 phenotypic groups: *Pseudomonas, Vibrionaceae, Enterobacteriaceae, Moraxella* and *Neisseria*-like rods. Among them, one *Pseudomonas* strain, when grown on resins as the sole source of carbon, showed significant adaptation towards these high molecular weight classes of crude oil. Microorganisms that biodegrade resins and asphaltenes by cometabolism have been identified (Raymond et al. 1975). Mixed populations of marine microorganisms *Pseudomonas, Brevibacterium, Alcaligenes, Arthrobacter and Bacillus* were found to cometabolize resins and asphaltenes. The presence of saturated hydrocarbons (C_{12}–C_{18}) appeared to be a pre-requisite for the degradation of resins and asphaltene. The *n*- alkanes probably served as carbon and energy source for the bacteria, or might be useful in solubilization and emulsification of resins and asphaltene by their fatty acid degradation products. Characterization studies of crude oil resins by FTIR spectroscopy have shown that resins are more oxidized, more aliphatic and less aromatic than asphaltenes (Boukir et al. 2001).

9.4 Biological Remediation

Petroleum refinery effluents contain floating or emulsified oil, which can be physically separated and recovered, but some of this oil associates with inorganic and organic particulates to form sludges which need appropriate disposal as solid wastes. Oily sludges from the separators in the wastewater treatment plants and 'tank bottoms', where sediments accumulate in the storage tanks, cannot be economically reprocessed. Most of these materials are classified as hazardous wastes, and therefore disposal of these sludges is tightly regulated and monitored. Bioremediation technology is considered an effective and economically viable method for cleanup of hydrocarbon-contaminated soils and sludges, either in-situ or ex situ after excavation or transportation. In cases where soils cannot be treated in-situ, either due to regulatory reasons or the lack of availability of sufficient land, or due to risks to groundwater or air pollution, these soils must be excavated and bioremediated using landtreatment landtreatment units, composting composting methods, or use of biopiles biopiles or slurry bioreactor slurry bioreactor technologies.

Bioremediation of petroleum hydrocarbon-contaminated sites in developing countries typically involves landfarming processes in which contaminated soil is augmented with nutrients, and periodically tilled and irrigated to stimulate the natural microbial population that degrades the contaminants over a long period of time, usually 6–24 months. Landtreatment units can be designed with a leachate collection system to prevent the off-site migration of water-soluble hydrocarbons. Landfarming is a cost-effective method of treating biodegradable petroleum products in the soil, if the treatment system is designed in such a way that the transfer of pollutants to other environmental media is minimized or prevented. One of the disadvantages of landfarming is the difficulty in reducing hydrocarbon concentration by more than 80–85%, which may not be adequate to meet regulatory standards in some countries. Landfarming has been used to treat volatile and readily biodegradable pollutants with relative success. However, the technology could not be successfully used to treat persistent hydrocarbons like high molecular weight polycyclic aromatic hydrocarbons. There is also evidence that significant amount of hydrocarbons are volatilized instead of being biodegraded (Ramzi and Husain 2004).

Composting involves mixing of contaminated soil with organic materials such as straw, wood chips, and sewage sludge to improve soil aeration, and placing the mixture in piles or windrows to support growth of hydrocarbon-degrading microorganisms. An engineered composting system is called biopiles, where aeration is provided through a network of sparger pipes, and a leachate collection system is used for water-soluble hydrocarbons (Von Fahnestock et al. 1998). Long treatment time in landfarming or composting methods is generally due to the lack of control of parameters affecting microbial activity, such as moisture, oxygen, temperature, pH and mixing. Bioreactors designed for accelerated hydrocarbon degradation provides greater control of operational parameters (Van Hamme et al. 2003). Continuous aeration and mixing facilitates desorption of hydrocarbons from soil to the liquid phase, and provides sufficient oxygen for microbes to enhance the

biodegradation rate. In contained bioreactors, optimized operational parameters such as pH, temperature, oxygen, moisture, mixing, and bioavailability of nutrients promote desirable microbial growth and hydrocarbon-degrading activity (Ward et al. 2003).

Phytoremediation methods involve some specific plants and their rhizospheric microorganisms by either providing favorable conditions for contaminant degradation by plant root colonizing microbes, or accessing contaminants through the plant roots (Monetsinos 2003; Krämer 2005; Macek et al. 2008). Decaying biomass and plant root exudates provide nutrients, and stimulate co-metabolic transformations of some organic contaminants. Soil phytoremediation methods include:

- Phytostabilization. Plants suitable for phytostabilization should be able to develop an extensive root system, provide good soil cover to prevent wind and water erosion, possess tolerance to the contaminant metals, and immobilize the contaminants in the rhizosphere.
- Phytovolatilization. In phytovolatilization processes, the contaminant, after uptake by the plant, volatilizes to the atmosphere in its original or modified form after its uptake. This method is not considered suitable for commercial applications.
- Phytostimulation. Root exudates from the plant support the growth and activity of rhizospheric bacteria to stimulate contaminant degradation in the phytostimulation process.
- Phytotransformation. In the phytotransformation process, the contaminants are generally transformed into less toxic forms within the plant.
- Phytoextraction. Phytoextraction involves suitable plants to concentrating soil contaminants in their above-ground tissues. Plant biomass is harvested, dried or incinerated, and the contaminant-enriched material is either disposed of in a landfill or sent to a smelter for burning.

9.5 Microbial and Nutrient Amendments

The influence of microbial cultures and inorganic and organic amendments on the degradation of various soil contaminants has been examined in both surface and sub-surface soil environments (Smindoll et al. 1988; Cho et al. 1997; Juhasz 2005; Singh et al. 2006). Abiotic factors such as soil texture, pH, temperature, moisture content, organic matter content and substrate availability should be carefully considered as part of the nutrient or microbial augmentation strategy in bioremediation processes (Thompson et al. 2005). Addition of soil amendments such as coconut, charcoal, cellulose, straw, soybean hulls, humic acid fraction of the composted soil, saw dust, wood ash, oats, root exudates and root debris have been found to enhance the biodegradation process (Pometto et al. 1998; Mishra et al. 2001a, b; Miya and Firestone 2001; Udosen et al. 2001).

Microbial commercial products are also used to degrade crude oil. Vecchioli et al. (1990) found that soil amended with commercially available hydrocarbon degraders was decontaminated more rapidly than the control with fertilizers only. Natural inoculums, either adapted or non-adapted, were the most effective in reducing the time period of bioremediation. However, in a field study in Delaware undertaken by deliberately contaminating the shoreline and comparing the efficiency of bioremediation using nutrients and/or microbial inoculum, it was concluded that microbial inoculation didn't increase the bioremediation significantly as compared to nutrient addition (Venosa et al. 1996).

Bioremediation of oily sludge-contaminated soil in the presence of a bacterial consortium, inorganic nutrients, compost and a bulking agent (wheat bran) showed oil degradation in the range of 60–80% (Vasudevan and Rajaram 2001). Amendment with poultry litter, coir pith and rhamnolipid surfactant in bioremediation of gasoline contaminated soil by a bacterial consortium increased hydrocarbon degradation by 67–78% (Rahman et al. 2002). Supplementation of biodiesel (rapeseed oil methyl ester) significantly improved mobility, and increased the degradation rate of crude oil in an artificial sand column study (Miller and Mudge 1997).

Enhanced in-situ anaerobic bioremediation using organic substrates such as acetate, propionate, butyrate, benzoate, glucose, lactate, methanol, and toluene has been employed for the treatment of chlorinated solvents (Ellis et al. 2000). Inexpensive, complex substrates such as molasses, cheese whey, corn steep liquor, vegetable oils, food shortening and beef tallow can also support reductive dechlorination (He et al. 2002). Any organic material that can be fermented to hydrogen and acetate is expected to stimulate reductive dechlorination (Sung et al. 2003). A variety of food-grade organic substrates such as vegetable oil and sucrose esters of fatty acids have been found to support long-term anaerobic bioremediation processes in the subsurface (Borden and Rodriguez 2006).

Effective methods for selection, preparation and preservation of the vitality of the oil-degrading microorganisms are required for field bioaugmentation (Ladousse and Tramier 1991; Barbeau et al. 1997). The inoculum for the bioremediation studies can be developed using the hydrocarbons as the source of carbon and energy using fermentation methods. The development of the inoculum should consider a shortened life cycle to full-scale production, competitive advantage, and cost savings. Enriched microbial consortia have been found useful to treat diesel-contaminated loam soil (Rubin and Narkis 2001). In a large-scale contaminated soil study carried out by our group, bioaugmentation of an inoculum consisting of a consortium of enriched microorganisms along with nutrients achieved degradation of 89–92% of TPH (Mishra et al. 2001a). In another study, by the same authors, a carrier-based bacterial consortium was applied for in-situ bioremediation. We also confirmed the feasibility of large-scale application of a carrier-based microbial consortium for treatment of oily sludge-contaminated soil (Mishra et al. 2001b).

Microbial inoculation of oil-contaminated soils requires the inoculum to be produced prior to its transportation to the field. When the inoculants added to soil do not meet ecological selectivity requirements to maintain the required number of cell density, various inoculant formulations involving carrier materials are a better

option. A wide range of carriers prepared from natural and plant-derived compounds have been tested and used. Carrier materials are generally intended to provide a protective environment for the microorganism (van Elsas and Heijnen 1990). Polyurethane- or alginate-immobilized bacteria have also been used for degradation of PCP in soil (O'Reilly and Crawford 1989; Weir et al. 1996). Encapsulated microbial cells have been used for applications in contaminated aquifers (Stormo and Crawford 1992; Manohar and Karegoudar 1998).

9.6 Factors Affecting Hydrocarbon Bioremediation

A range of environmental parameters that are physical, chemical or biological in nature affect bioremediation of petroleum contaminants. Weathering, sorption, evaporation or volatilization, leaching and photo-oxidation processes may cause the removal of certain hydrocarbon compounds during bioremediation, resulting in overestimation of the extent of biodegradation. Around 15–60% of fuel hydrocarbons, including semi-volatile 4- and 5-ring PAHs, can be lost during soil bioremediation due to volatilization only (Huesemann 1995; Hawthorne and Grabanski 2000; Salanitro 2001; Mphekgo et al. 2004). Sludge loadings with high oil concentration may reduce the biodegradation activity due to oxygen limitation and/or inhibitory levels of hydrocarbons to the microorganisms. Since sludge loading rates of 10–15% considerably slow down the biodegradation rate, sludge loading with around 5% oil has been recommended (Dibble and Bartha 1979).

Hydrocarbon biodegradation can occur over a wide pH and temperature range. The optimum pH for petroleum bioremediation in soil ranges from 6.0 to 8.0. Generally, lime is added to increase the pH of soil if it is in the acidic range ($pH < 6$), and elemental sulfur or ammonium sulfate is added to lower the pH if the soil is too alkaline ($pH > 8$). The biodegradation rate generally increases from the psychrophilic to mesophilic temperatures. The optimum temperature for biodegradation has been reported in the range of 25–40°C (Van Hamme et al. 2003). Temperature influences hydrocarbon biodegradation by affecting the physical state and chemical composition of oil as well as the metabolic activities and composition of the microbial community (Crawford and Zhou 1995; Walworth and Reynolds 1995). *Bacillus sp.* was reported to be capable of growing on petroleum hydrocarbons in the range of 45–70°C (Klug and Markovetz 1971; Sorkhoh et al. 1993). A psychrophilic strain of *Rhodococcus* sp. Q15 was found to mineralize short-chain alkanes to a greater extent than long-chain alkanes at low temperatures of 0 and 5°C (Whyte et al. 2002). Low temperatures also reduce the rates of volatilization of low molecular weight hydrocarbons. Studies on the feasibility of bioremediation as a treatment option for a chronically diesel-oil-polluted soil in an alpine glacier area at an altitude of 2,875 m above sea level for three summer seasons indicated that at moderate temperatures (20–40°C), there are both greater abiotic losses and higher rates of hydrocarbon metabolism (Margesin and Schinner 2001).

Nutrient availability plays an important role in adaptation of microbes and their growth on hydrocarbons. Two major nutrients, nitrogen and phosphorus, are considered to be the most important, as they are required for incorporation of carbon into the biomass. Nitrogen and phosphorus often become limiting factors in hydrocarbon biodegradation, as was found to be the case in Prince William Sound after the spill from Exxon Valdez (Pritchard and Costa 1991), and the high C:N and/or C:P ratio became unfavourable for microbial growth (Toccalino et al. 1993). Oil-sludge biodegradation was found optimal at C:N and C:P ratios of 60:1 and 800:1 respectively (Dibble and Bartha 1979).

The importance of oxygen for hydrocarbon degradation is realized by the fact that the major degradative pathways in saturated, cyclic and aromatic hydrocarbons in bacteria and fungi involve oxygenases for which molecular oxygen is required (Cerniglia 1984; Atlas 1995). To maintain metabolic activities of microbial cells, the oxygen supply rate must match the overall oxygen consumption rate under equilibrium conditions (Anderson and Helder 1987; Huesemann and Truex 1996). Oxygen diffusion in soil can be severely restricted in wet or flooded soils. Addition of bulking agents and periodic tilling can increase soil porosity, and the rate of oxygen consumption due to bacterial metabolism can be improved (Devinny and Islander 1989). Appropriate aeration is provided by a network of spargers in biopiles, and optimum mixing and aeration in bioslurry reactors (Ward et al. 2003).

Anaerobic degradation of petroleum hydrocarbons has also been reported, but the degradation rates were found to be very slow for a practical bioremediation system (Ward and Brock 1978). It has been demonstrated that sulphate-reducing bacteria can utilize aliphatic and aromatic hydrocarbons directly under anoxic/anaerobic conditions (So and Young 1999). Bolliger et al. (1999) have shown that petroleum hydrocarbon mineralization in anaerobic aquifers was linked to the consumption of oxidants such as O_2, NO^{3-} or others, with subsequent production of corresponding reduced species. Denitrifying microorganisms also carry out degradation of alkane and alkylbenzenes under anaerobic conditions (Ehrenreich et al. 2000; Rabus et al. 1999).

Reduced bioavailability of hydrocarbons can limit biodegradation, particularly in aged soils that have been contaminated for many years and during the final stages of a soil bioremediation treatment processes (Alexander 2000). Mass transfer of hydrocarbons into microbial cells is a significant determinant of biodegradation rates and extents. Hydrocarbon bioavailability and subsequent degradation can be improved by addition of chemical surfactants and biosurfactants biosurfactants. However, depending on the physiology of the microbes involved, mass transfer may be affected positively, negatively, or not at all in the presence of a (bio)surfactant (Das and Mukherjee 2007). In a recent study, a biosurfactant was found to greatly increase desorption of four-ring PAHs from soil and enhance biodegradation by *Pseudomonas alcaligenes* PA-10, possibly due to a combination of increased solubility and increased biomass, given that the biosurfactant could also serve as a carbon source (Hickey et al. 2007). Thus, microorganisms that overproduce biosurfactants may have an important role in the hydrocarbon degradation process.

9.7 Conclusion

A successful soil bioremediation depends on numerous environmental, nutritional and operational factors. Since it is unlikely that all contaminants would be removed from a contaminated soil, even under optimal conditions, the effectiveness of a biological process depends on the success in identifying the rate-limiting factors and optimizing them in order to achieve maximum treatment benefits. Inadequately designed or engineered systems are likely to fail to achieve the required local regulatory treatment criteria. Knowledge regarding the biodegradation of petroleum hydrocarbons has been advanced in the last 2 decades. A number of hydrocarbon-degrading strains have been isolated and characterized using advanced molecular techniques. Increase in our understanding of the ecology of hydrocarbon-degrading microbial communities and the mechanisms by which biodegradation occur will help in developing practical soil bioremediation strategies.

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Chapter 10
Bioremediation of Benzene-contaminated Underground Aquifers

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10.1 Introduction

Contamination of groundwater with gasoline is a serious environmental problem, since it may affect drinking water resources. Benzene, toluene, and xylenes (BTX) are the major components of gasoline-derived contaminants and are of great concern, since they are toxic and soluble in water (Mehlman 1992). Among them, benzene is of particular health concern due to its carcinogenicity (ATSDR 1997; US EPA 2002). Conventional remediation techniques, such as pump-and-treat and air sparging, have applied to the treatment of groundwater polluted with high concentrations of BTX. However, tailing and rebounding of the contaminants have, in many cases, been observed during such treatments, resulting in longer operation periods than those predicted (US EPA 1996).

Natural attenuation (NA) has been considered as an attractive option for the treatment of contaminated groundwater, which relies on natural processes, rather than artificial treatments, to clean up or attenuate pollution. In natural attenuation, a variety of processes, including sorption, dispersion and dilution, chemical reaction, volatilization, and biodegradation contribute to the clean-up (US EPA 1999), while biodegradation was suggested to be particularly important. Benzene is known to be biodegraded readily under aerobic conditions. However, contamination of subsurface aquifers with gasoline often results in the development of anaerobic zones, where benzene is much more persistent than toluene and xylenes (Anderson and Lovely 1997; Christensen et al. 1994; Lovely 1997). Although studies have shown that

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benzene biodegradation occurs under nitrate-reducing (Burland and Edwards 1999; Ulrich and Edwards 2003), sulfate-reducing (Edwards and Grbic-Galic 1992; Lovely et al. 1995), iron-reducing (Lovely et al. 1994), perchlorate-reducing (Chakraborty et al. 2005), and methanogenic conditions (Grbic-Galic and Vogel 1987; Kasai et al. 2005), in situ activities of anaerobic benzene degradation are generally very low, suggesting that bioaugmentation, rather than biostimulation, may be useful to accelerate it.

In this chapter, we summarize our efforts to identify anaerobic benzene-degrading bacteria in a gasoline-contaminated underground aquifer, isolate an anaerobic benzene-degrading bacterium (*Azoarcus* sp. strain DN11), and evaluate it for its application to bioaugmentation of benzene-contaminated groundwater. Although strain DN11 has not yet been practically applied to bioaugmentation, our data strongly suggest that it will be useful for that purpose.

10.2 Analyses of a Gasoline-Contaminated Underground Aquifer

The BTX-contaminated site examined in this study is located at Higashino in Kumamoto, Japan (Fig. 10.1). Detailed geochemical characteristics of this site have been reported elsewhere (Takahata et al. 2006). In January 1991, groundwater at this site was found to be contaminated with gasoline that leaked from an underground

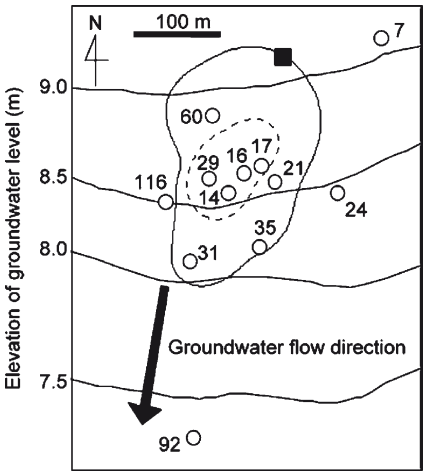

Fig. 10.1 Map of the study area. The area contaminated with BTX immediately after the leakage of gasoline (in March 1992) is surrounded by the *continuous line*, while BTX was detected in the area surrounded by the *dashed line* just before MNA was started (in April 2002). The elevation contours in this map (0.1 mg l^{-1} total BTX concentration) were determined by SURFER version 7 software (Golden Software, Golden, CO, USA). The contaminant leakage point (gas station) is marked with the *filled square*, and the sampling wells are marked with *open circles*

fuel storage tank of a gas station. The underground tank and heavily contaminated soil were first excavated and removed, and the downstream-contaminated aquifer was treated by a pumping and aeration system installed at the center of the contaminated zone from February 1992. This system greatly reduced contaminant concentrations, although they were persistent at low concentrations (Takahata et al. 2006). A particular problem was that even after the 10-year treatment, benzene concentrations in groundwater obtained from some sampling wells situated at the center of the contaminated zone were over 0.1 mg l^{-1}, concentrations more than 10 times higher than those in the environmental regulations (e.g., 10 mg l^{-1} in Japan). In April 2002, the treatment strategy was changed from this costly system to monitored natural attenuation (MNA). Chemical analyses of BTX in groundwater revealed that at some sites benzene concentrations decreased during the MNA period; for example, the 1-year treatment resulted in its reduction at wells 14 and 17 from over 100 mg l^{-1} to below the environmental regulation value (Fig. 10.2). Further analyses of chemical parameters relevant to intrinsic biodegradation (such as oxygen, nitrate, sulfate, ferrous ion, and bicarbonate concentrations, oxidation/reduction potential and microbial-cell count) indicated that sites where benzene concentrations reduced (including wells 14 and 17) were anaerobic.

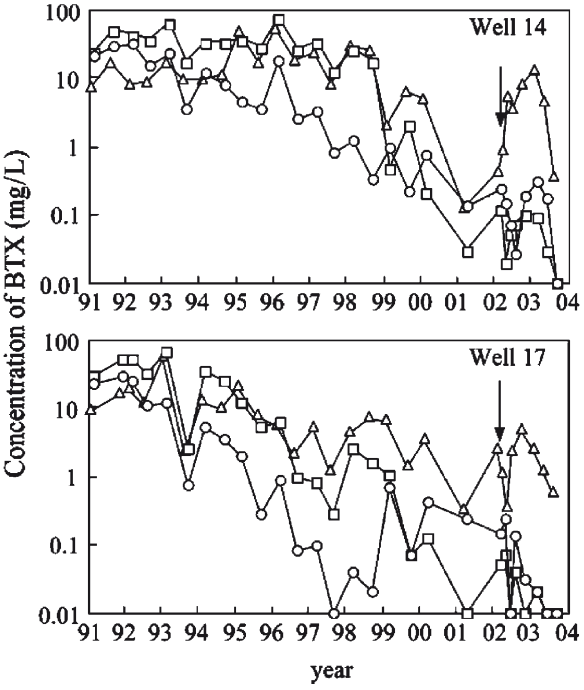

Fig. 10.2 Changes in concentrations of benzene (*open circle*), toluene (*open square*), and xylenes (*open triangle*) in groundwater samples obtained from some wells in the contaminated and boundary zones. The *arrow* indicates the starting point for MNA

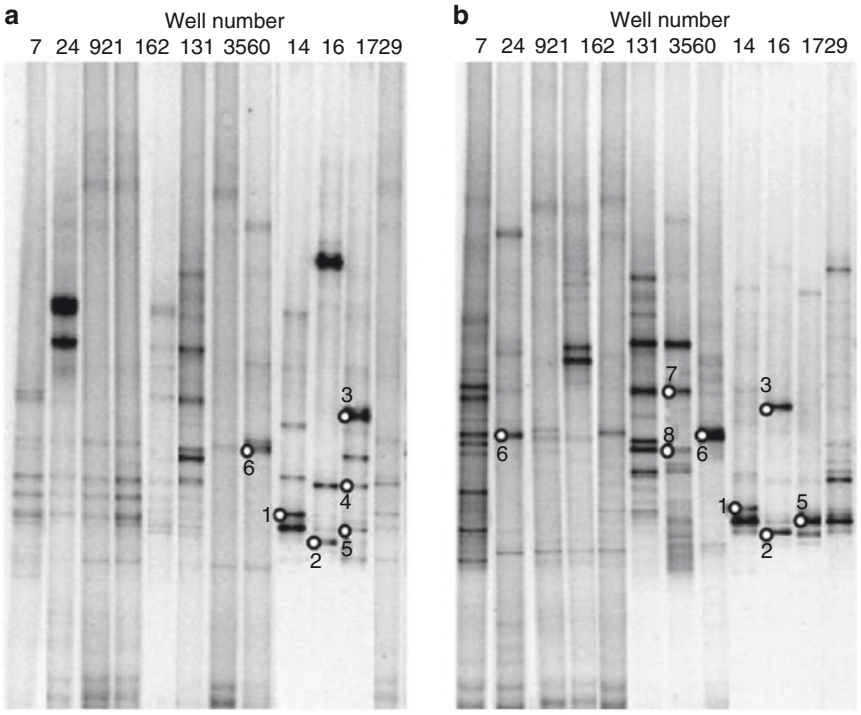

Fig. 10.3 DGGE profiles for the bacterial populations in groundwater samples obtained in November 2002 (**a**) and March 2003 (**b**). The *bands marked with circles* were excised and sequenced

We were interested in analyzing microbial populations that occurred at several different points of the contaminated aquifer, and denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified bacterial 16S rRNA gene fragments was performed (Takahata et al. 2006). Figure 10.3 presents DGGE profiles for groundwater obtained from the 12 wells in November 2002 and March 2003. We found that two bands (1 and 4) derived from the contaminated zone were affiliated with the *Rhodocyclaceae* family; this family is known to include the *Azoarcus*, *Dechloromonas*, and *Thauera* genera and many denitrifying aromatic hydrocarbon-degrading bacteria (Hess et al. 1997; Zhou et al. 1997). On the other hand, two bands (7 and 8) derived from the boundary zone were closely related to the *Sphingomonas* genus that is well-known for its abilities to aerobically degrade a variety of hydrocarbons (Fredrickson et al. 1995). These analyses suggest that at some points of this gasoline-contaminated aquifer, anaerobic biodegradation of benzene may have occurred during the MNA period, although no direct evidence was provided from these analyses on the presence of anaerobic benzene-degrading bacteria at these sites.

10.3 Identification and Isolation of Anaerobic Benzene-Degrading Bacteria

Although, as described above, many studies have shown that anaerobic benzene biodegradation occurs at contaminated sites, only one study has succeeded in isolation of anaerobic benzene-degrading bacteria from such a site (Kasai et al. 2006). In that study, we used stable isotope probing (SIP), a culture-independent approach for linking microbial community function to the phylogenetic identities of key organisms (Boschker et al. 1998; Manefield et al. 2002; Orphan et al. 2001). This method enables the identification of members in a microbial community responsible for specific activities based on the incorporation of stable isotopes (e.g., ^{13}C) into cellular components. We used RNA-based SIP (RNA-SIP) to label and identify [$^{13}C_6$] benzene-degrading organisms (Kasai et al. 2006). RNA-SIP exploits the relatively efficient (compared to that into DNA) ^{13}C incorporation into RNA (Manefield et al. 2002; Gallagher et al. 2005), which is particularly useful when substrate degradation and growth rates are likely to be slow, as in the context of anaerobic benzene degradation. Furthermore, we used phylogenetic information to isolate RNA-SIP identified benzene-degrading denitrifying bacteria (Kasai et al. 2006).

In that study (Kasai et al. 2006), a groundwater sample obtained from the above-described BTX-contaminated aquifer was analyzed. For SIP, bottles were filled with the groundwater, which was supplemented with an electron acceptor (oxygen, nitrate, or sulfate), inorganic nutrients, and [$^{13}C_6$] benzene at 200 mM, and groundwater samples were obtained from these bottles at appropriate time intervals for analyzing benzene concentrations (Fig. 10.4a) and extracting microbial RNA. The extracted RNA was subjected to ultracentrifugation to separate $^{13}C_6$-containing heavy RNA (RNA from [$^{13}C_6$] benzene-eating microbes) from normal RNA. The heavy fraction was collected and used as a template to amplify bacterial 16S rRNA fragments by reverse transcription-PCR. The amplicons were analyzed by DGGE (Fig. 10.4b) to show the occurrence of specific bacterial populations with different electron acceptors. Sequence analyses of major bands revealed that a bacterial population affiliated with the genus *Azoarcus* (band 4 in Fig. 10.4b) specifically incorporated ^{13}C when nitrate was supplemented as the electron acceptor. This result suggested that the *Azoarcus* population grew on benzene under the nitrate-reducing condition. Recently, Ulrich and Edwards (2003) demonstrated that the dominant microbial population in a benzene-degrading denitrifying enrichment culture was closely related to *Azoarcus* species. It is also noteworthy that the sequence of this *Azoarcus* population was 100% identical to that of band 1 in Fig. 10.3 retrieved from the gasoline-contaminated groundwater, suggesting that the *Azoarcus* population may have been important in the contaminated plume.

In order to examine if bacteria represented by the *Azoarcus* phylotype were actually capable of degrading benzene under denitrifying conditions, we attempted to isolate them for axenic benzene degradation tests (Kasai et al. 2006). The groundwater incubated for SIP under denitrifying conditions for 28 days was

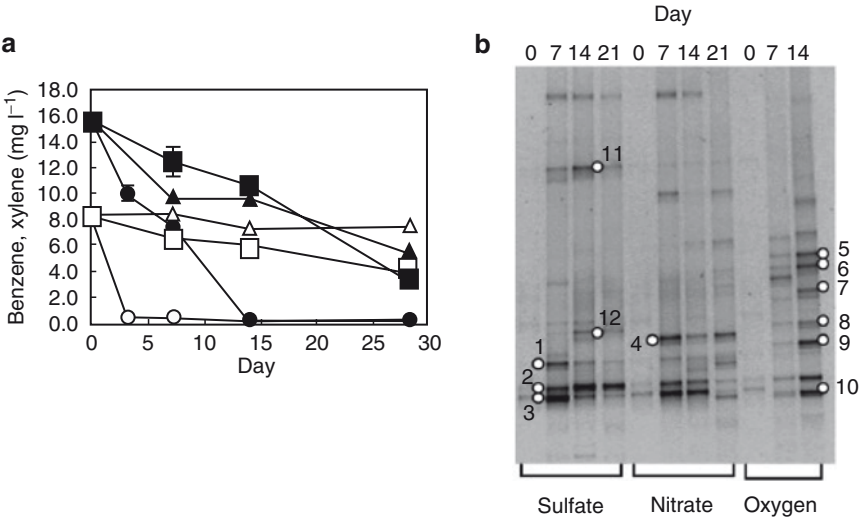

Fig. 10.4 **a** Degradation of benzene and xylene during incubation of groundwater for SIP. *Closed squares*, benzene under denitrifying conditions; *open squares*, xylene under denitrifying conditions; *closed triangles*, benzene under sulfidogenic conditions; *open triangles*, xylene under sulfidogenic conditions; *closed circles*, benzene under aerobic conditions; *open circles*, xylene under aerobic conditions. Data points are means of triplicate experiments, and error bars represent standard deviations. **b** Time courses of DGGE profiles for ^{13}C-labeled community RNAs obtained from groundwater incubated under sulfidogenic (sulfate), denitrifying (nitrate), and aerobic (oxygen) conditions. The numbered bands were excised for DNA sequencing analysis

spread onto dCGY plates and incubated under aerobic and denitrifying conditions. We used dCGY medium because previous studies demonstrated that this medium facilitated the isolation of bacteria affiliated with the *Betaproteobacteria* from activated sludge (Watanabe et al. 1998; Watanabe et al. 1999). A total of 60 colonies were picked and restreaked on the same medium for purification, and cells on the new plates were subjected to DGGE analysis of PCR-amplified 16S rRNA gene fragments. In the DGGE analysis, band positions were compared with that of band 4 in Fig. 10.4b on the same gels, and nucleotide sequences of bands migrating to the same position as band 4 were determined to confirm their identity. As a result, we obtained five colonies whose 16S rRNA gene fragments were 100% identical in nucleotide sequence to that in band 4. Sequence analysis of their 16S rRNA gene fragments (over 1,450 bp) found that their sequences were almost identical (> 99% identity) and very similar to *Azoarcus evansii*, which metabolizes such aromatic compounds as benzoate, toluene, and phenol under denitrifying conditions (Anders et al. 1995), and *Azoarcus* sp. strain ToN1, which degrades toluene (Rabus and Widdel 1995).

We have examined five isolated strains for their ability to degrade BTX under denitrifying conditions (Kasai et al. 2006), and found that only two strains (DN11 and AN9) were capable of anaerobic benzene degradation (Fig. 10.5). Furthermore, radiorespirometry experiments were conducted to investigate if benzene was

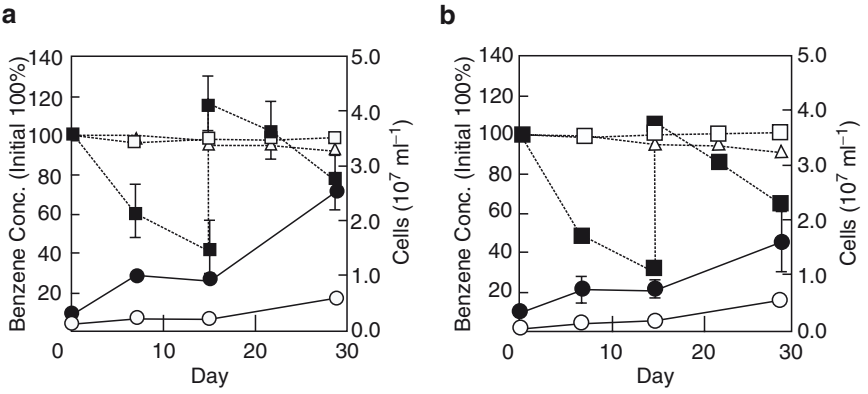

Fig. 10.5 Growth and benzene degradation by strains DN11 (**a**) and AN9 (**b**). The cultures were re-fed benzene at the time point indicated with *vertical lines*. Residual benzene was measured in the nitrate-amended (*closed squares*), unamended (*open squares*), and uninoculated (*open triangles*) cultures. Cell numbers are given for the nitrate-amended (*closed circles*) and unamended (*open circles*) cultures. Data points are means of triplicate experiments, and error bars represent standard deviations

anaerobically mineralized (converted to CO_2) by these strains, and found that large fractions of radioactivity initially added as [^{14}C] benzene were recovered in a CO_2 trap. In that experiment, significant amounts of radioactivity were not recovered in the CO_2 trap in the absence of nitrate, showing that benzene mineralization was coupled to denitrification (Kasai et al. 2006).

Successful application of the RNA-SIP technique demonstrated that the *Azoarcus* population is involved in benzene degradation in the aquifer. However, among the five isolated strains affiliated with the *Azoarcus* phylotype, only two strains were capable of anaerobic benzene degradation. These data indicate that functional heterogeneity exists among strains with the *Azoarcus* phylotype, and more specific molecular markers than the 16S rRNA gene will be necessary for discriminating benzene-degrading *Azoarcus* populations from other closely related *Azoarcus* populations in the gasoline-contaminated aquifer.

Among the two anaerobic benzene-degrading strains, we selected *Azoarcus* sp. strain DN11 for further characterization in terms of its higher activities than those of strain AN9. Substrate-range tests indicated that this strain could grow on benzene, toluene, *m*-xylene, and benzoate as the sole carbon and energy sources under nitrate-reducing conditions, although *o*- and *p*-xylenes were transformed in the presence of toluene (Kasai et al. 2007). Phenol was not utilized under anaerobic conditions. Transformation of *o*-xylene to (2-methylbenzyl)succinate by toluene-growing cells was also observed for several nitrate-reducing strains (Beller and Spormann 1997; Evans et al. 1992). In the case of strain DN11, however, the loss of *o*- and *p*-xylene in the presence toluene was not accompanied by the accumulation of any detectable metabolites. The result that DN11 is capable of degrading all xylene isomers in addition to benzene and toluene indicates that this strain is useful for

bioremediation of BTX-contaminated aquifers. In addition, since DN11 could not grow on phenol, this strain seems to employ a different anaerobic benzene degradative pathway from the pathway of *Dechloromonas* strain RCB, in which phenol was produced by hydroxylation of benzene (Chakraborty and Coates 2005).

Anaerobic benzene degradation was observed from pH 6 to 8 and from 15 to 42°C; the highest activity was observed at 30°C and pH 7 (Kasai et al. 2007). Activity was also observed at 10°C, albeit low. At pH 5, benzene degradation was observed during the initial 20 days, after which it stopped. For estimating kinetic constants for anaerobic benzene degradation (at 25°C, pH 7.0), we obtained benzene-degradation curves at different concentrations. It was found that benzene disappearance rates increased in accordance with the increase in the benzene concentration at lower concentrations (up to 3 mM) and decreased at higher concentrations. We therefore used the Haldane equation (Kasai et al. 2007) for the kinetic analysis, and estimated kinetic constants (K_s [affinity constant], V_{max} [theoretical maximum activity], and K_{SI} [inhibition constant]) as 0.82 mM, 24 nmol h^{-1} mg^{-1} of dry cell, and 11 mM respectively. We conclude that the affinity constant of DN11 for benzene is sufficiently low for this stain to remove benzene down to concentrations below the environmental-regulation value.

In order to evaluate bioaugmentation potential of DN11 for treating benzene-contaminated groundwater, we used groundwater sampled from a former coal distillation plant site, in which benzene was the major contaminant (Kasai et al. 2007). The benzene-contaminated groundwater was anaerobically incubated in laboratory bottles and supplemented with inorganic nutrient (nitrogen, phosphorus, and nitrate) alone, or the nutrients plus strain DN11. Changes in benzene concentrations in the groundwater samples are presented in Fig. 10.6. This figure shows that

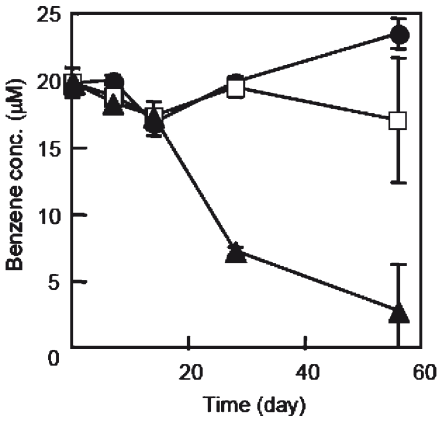

Fig. 10.6 Changes in benzene concentrations (the initial concentration of 42 mM) during anaerobic bioremediation of the benzene-contaminated groundwater; *closed circle*, the control (without any supplementation); *open square*, biostimulation (supplementation with inorganic nutrients and nitrate); *closed triangle*, bioaugmentation (supplementation with inorganic nutrients, nitrate and DN11). Data points are means of triplicate experiments, and error bars represent standard errors

benzene was significantly degraded only in the DN11-augmentated bottles. In these bottles, benzene degradation was initiated on day 7 and continued up to day 56. During this period, 3.88 mmol of benzene was degraded concomitant with the consumption of 22.5 mmol of nitrate. The nitrate/benzene molar ratio of 5.80 was in good agreement with the theoretical ratio of anaerobic benzene degradation coupled to nitrate reduction ($C_6H_6 + 6\ NO_3- = 6\ HCO_3^- + 3\ N_2$ [Spormann and Widdel 2001]). Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments, and quantitative PCR revealed that DN11 decreased after benzene was degraded. Following the decrease in DN11, 16S rRNA gene fragments corresponding to bacteria related to *Owenweeksia hongkongensis* and *Pelotomaculum isophthalicum*, appeared as strong bands. *P. isophthalicum* has been known to utilize benzoate and hydroxybenzoate for growth in coculture with hydrogen-consuming microbes, such as methanogens (Qiu et al. 2006). We therefore assume that this bacterium may have utilized some intermediate metabolites of anaerobic benzene degradation. In such a case, an organism represented by band 6 may have been a syntrophic partner of *P. isophthalicum*. It is likely that the DN11 population did not increase because these two organisms consumed intermediate metabolites produced from benzene by DN11.

The above results demonstrate that strain DN11 is potentially useful for removing benzene from underground aquifers, at least from the groundwater of the former coal distillation plant site. Considering the benzene-degradation kinetics, DN11 bioaugmentation will be applied to removing relatively low concentrations of benzene. Although further technical developments (e.g., optimization of supplementing nutrient concentrations, development of a mass-cultivation method and risk assessments) should be performed prior to a field trial of bioaugmentation with DN11, we consider that the results of the present study have opened up a new possibility for bioremediation of benzene-contaminated underground aquifers.

10.4 Conclusion

Our study to develop the benzene-bioremediation strategy was initiated by analyzing the gasoline-contaminated aquifer, followed by identification and isolation of the anaerobic benzene-degrading bacteria from the groundwater. The isolated strain (strain DN11) was evaluated by the laboratory bioaugmentation experiment using benzene-contaminated groundwater obtained from the former coal distillation plant site, suggesting that DN11 is potentially useful for degrading benzene that contaminates underground aquifers at relatively low concentrations. We have evaluated the pathogenicity of DN11 using model organisms, including mice, rabbits, and carp, showing that there were no harmful effects on these organisms (unpublished results). Based on these results, we are planning to conduct a pilot-scale test of DN11 bioaugmentation using the former coal distillation plant site.

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Chapter 11
Microbial Remediation of Metals in Soils

K.A. Hietala and T.M. Roane

11.1 Introduction

Of metal-contaminated systems, metal-contaminated soils present the greatest challenge to remediation efforts because of the structural, physical, chemical, and biological heterogeneities encountered in soils. One of the confounding issues surrounding metal remediation is that metals can be readily re-mobilized, requiring constant monitoring of metal toxicity in sites where metals are not removed. Excessive metal content in soils can impact air, surface water, and groundwater quality. However, our understanding of how metals affect organisms, from bacteria to plants and animals, and our ability to negate the toxicity of metals are in their infancies. The ubiquity of metal contamination in developing and industrialized areas of the world make remediation of soils via removal, containment, and/or detoxification of metals a primary concern. Recent examples of the health and environmental consequences of metal contamination include arsenic in drinking water (Wang and Wai 2004), mercury levels in fish (Jewett and Duffy 2007), and metal uptake by agricultural crops (Howe et al. 2005). The goal of this chapter is to summarize the traditional approaches and recent developments using microorganisms and microbial products to address metal toxicity and remediation.

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11.2 Concern About Metals in Soils

Metal contamination results primarily from industrial and mining activities (North American Commission for Environmental Cooperation 2007). The top five released metals of concern are Cu, Cr, Ni, Pb, and Zn. The current number of contaminated sites on the USA National Priorities List (NPL) was 1,256 as of April 2007, with an additional 60 sites in the proposal stage (updates available at www.epa.gov/superfund). Of these sites, 604 are metal-contaminated soils, and 98% of these are co-contaminated with organic chemicals, such as 1,4-dioxane, trichloroethylene and BTEX (benzene, toluene, ethylbenzene and xylene), adding to the remediation challenge. Metals in the environment directly impact quality of life. For example, in the Minimata Bay, Japan, incident an estimated 27 tons of methylmercury were released into the Bay by the Chisso Corporation chemical company. The consumption of mercury-contaminated fish led to thousands of people suffering from mercury poisoning. Efforts are being made to better understand metal interactions with biological systems, and metal transformations in the environment, and to identify methods for metal mitigation.

Because of their environmental recalcitrance, metals present unique remediation challenges. Metals cannot be degraded, and consequently metal remediation of soils often relies on the reduction of metal toxicity versus removal. The presence of clays and organic matter in soils, for example, results in metal binding and complexation-reducing metal reactivity and, thus, toxicity. However, with changing soil conditions, including pH and redox, metals can become chemically active and biologically toxic. The dynamic nature of metal interactions in soils makes remediation difficult, and unless the metal content is substantially reduced, metal-impacted soils require long-term monitoring.

11.3 Metal Interactions in Soil

The toxicity of a metal can be defined in a number of ways, including (1) how a metal influences biological processes at the cellular level, (2) what effect a metal has on multicellular organisms, and (3) how a metal changes ecosystem processes. While the presence of metals in a soil may indicate potential toxicity, the total amount of metal present does not necessarily reflect actual toxicity. In soils, there are two ways to measure metals: the total metal amount and the biologically reactive metal or bioavailable metal amount. Bioavailability can be defined as the “extent to which a chemical can be absorbed by a living organism” (Kelley et al. 2002). Taken into consideration in risk assessments, bioavailable metals are directly toxic to the cell. In soil risk assessment, bioavailability includes the extent to which a substance can “desorb, dissolve, or otherwise dissociate from the environmental medium in which it occurs to become available for absorption” (Kelley et al. 2002). Thus, when assessing the extent of contamination at a site, the determination of the bioavailable metal is important (Alvarenga et al. 2008).

Influenced by metal speciation, metal bioavailability is affected by a number of environmental factors, including soil cation exchange capacity, pH, and redox potential. One of the most important factors influencing metal bioavailability in soil is cation exchange capacity (CEC), the capacity of a soil to exchange positively charged ions at the soil–soil solution interface, which is dependent on both the organic matter and clay content of the soil. Generally speaking, soils dominated by clays exhibit higher CECs. In a study by Jarecki and Lal (2005), a southwestern Ohio silt loam with 112 g/Kg clay had a CEC of 30.7 cmol/Kg, while a northwestern Ohio clay loam with 376 g/Kg clay had a CEC of 49.4 cmol/Kg. Soils with higher CECs tend to have a decreased level of bioavailable metals because they are tightly sorbed by the soil matrix. Additionally, organic matter readily sorbs metals, reducing metal bioavailability through the binding to anionic functional groups such as carboxyl, carbonyl, phenolic, hydroxyl and ester groups; some of these form soluble metal complexes and others insoluble complexes, such as those seen with high molecular weight humic acids (Bradl 2004). Simple aliphatic acids can also form soluble metal chelates. The soluble complexes are of concern, as the metal remains mobile even though less bioavailable.

Under acidic conditions, cationic metals are generally found as free ionic species or soluble organo-metals. Sandrin and Maier (2002) showed that cadmium solubility and speciation varied with a changing pH. At pH 4, ionic cadmium (Cd^{2+}) measured 44 mg l and at pH 7 measured 4 mg l, producing insoluble cadmium phosphate ($Cd_3(PO_4)_2$) and small amounts of monovalent hydroxylated cadmium ($CdOH^+$). Table 11.1 summarizes the predominant metal species in soils as a function of pH.

Table 11.1 Speciation of metals in soils as a function of pH

Metal	Soil pH <7	Soil pH >7
Silver (Ag)	Ag^+, AgCl	Ag^+, organic-Ag
Arsenic (As)	$As(OH)_3$, $H_2AsO_4^-$	$HAsO_4^{2-}$, AsO_3^{3-}
Beryllium (Be)	Be^{2+}	$Be(OH)_3^-$, $Be(OH)_4^{2-}$
Cadmium (Cd)	Cd^{2+}, $CdSO_4$, $CdCl^-$	Cd^{2+}, $CdCl^-$, $CdSO_4$, $CdHCO_3^+$
Cobalt (Co)	Co^{2+}, $CoSO_4$	$Co(OH)_2$
Chromium (Cr)	$Cr(OH)^{2+}$, CrO_4^{2-}	$Cr(OH)_4^-$, CrO_4^{2-}
Copper (Cu)	Cu^{2+}, $CuCl^-$, organic-Cu	$CuCO_3$, $CuHCO_3^+$, organic-Cu
Mercury (Hg)	Hg^{2+}, $Hg(Cl)_2$, CH_3Hg^+, organic-Hg	$Hg(OH)_2$, organic-Hg
Manganese (Mn)	Mn^{2+}, $MnSO_4$, organic-Mn	Mn^{2+}, $MnSO_4$, $MnCO_3$, $MnHCO_3^+$
Molybdenum (Mo)	H_2MoO_4, $HMoO_4^-$	$HMoO_4^-$, MoO_4^{2-}
Nickel (Ni)	Ni^{2+}, $NiSO_4$, $NiHCO_3^+$, organic-Ni	$NiCO_3$, $NiHCO_3^+$, Ni^{2+}
Lead (Pb)	Pb^{2+}, $PbSO_4$, $PbHCO_3^+$, organic-Pb	$PbCO_3$, $PbHCO_3^+$, $PbOH^+$, organic-Pb
Tin (Sb)	$Sb(OH)_2^+$, $Sb(OH)_3$, $Sb(OH)_5$, $Sb(OH)_6^-$	$Sb(OH)_4^-$, $Sb(OH)_6^-$
Selenium (Se)	$HSeO_3^-$, SeO_4^{2-}	SeO_3^{2-}, SeO_4^{2-}
Thallium (Tl)	Tl^+, $Tl(OH)_3$	Tl^+, $Tl(OH)_4^-$
Vanadium (V)	VO^{2+}, VO_2^+, polyvandates	$VO_2(OH)_2^-$, $VO_3(OH)^{2-}$
Zinc (Zn)	Zn^{2+}, $ZnSO_4$, organic-Zn	$ZnHCO_3^+$, $ZnCO_3$, Zn^{2+}, $ZnSO_4$, organic-Zn

Adapted from Hayes and Traina (1998)

The metal species vary in their solubility and chemical reactivity, and, thus, bioavailability. The pH of a soil can also influence metal sorption to soil particles and organic matter, such that as pH increases the electrostatic attraction between a metal and soil constituents also increases, due to the increased CEC. With increased binding to surfaces and decreased solubility, a higher pH results in less bioavailable metal. Acidic systems tend to enhance metal solubility while decreasing binding, thus increasing the bioavailable metal concentration. On the other hand, anionic metal species, such as chromate (CrO_4^{2-}), vanadate (VO_4^{3-}), and selenate (SeO_4^{2-}), tend to sorb at lower pHs. For example, anionic metal species were shown to sorb to chitin most efficiently from pH 2–3 (Niu and Volesky 2003).

The redox potential of a soil also influences metal bioavailability. Under aerobic oxidizing conditions (+ 800 to 0 mV), metals are often found as soluble salts and cationic forms, e.g., $CdCl_2$ and Cd^{2+}. In comparison, anaerobic saturated soils with low redox potentials (0 to −400 mV) tend to accumulate precipitated metal species as metal sulfides, e.g., CdS and PbS, and metal carbonates including $CdCO_3$. In a recent study by Cornu et al. (2007), CdS formation occurred within a metal-contaminated soil as the soil conditions became increasingly reducing (from 9.2 to −3.6 pe).

Soil microbial activities can change soil properties, altering metal speciation (Majewska et al. 2007; Carrillo-González et al. 2006; Amezcua-Allieri et al. 2005). Microbial degradation of organic matter can increase available sites for metal binding. Microbial waste products such as organic acids and alcohols can alter pH, increasing or decreasing metal solubility. Majewska et al. (2007) found that increases in microbially-produced citric acid, acetic acid, catechol siderophores, and Fe-chelators may have contributed to cadmium mobilization within soils, decreasing the pH from 6.5 to 5 after 48 hours. In addition to these biotic influences, abiotic factors such as rainfall can alter metal speciation.

Consequently, much work is being done to accurately assess environmental metal bioavailability. Current assessment methods include water extraction (Cook et al. 2002), acid extraction (Larner et al. 2007), charge neutralization extraction (Kassab and Roane 2006), and three-stage sequential extractions (Alvarenga 2008; Zhu et al. 2006). Though not easily quantifiable, toxicity bioassays can also assess the ecological risks associated with a metal (Alvarenga et al. 2008; Cook et al. 2002). Alvarenga et al. (2008) used biological and chemical assays to determine metal bioavailability within a mining soil, and results indicated that most metal remained in an immobile, non-reducible, non-oxidizable form, with less than 10% in the water-soluble/exchangeable phase. As of yet, the accuracy of these methods to determine the concentration of metal influencing biological systems is not clear. Newer work proposes the use of microorganisms, such as bacteria, as biosensors for metal toxicity (Yagi 2007; Horswell et al. 2006; Weitz et al. 2002). For example, *Escherichia coli* HB101 pUCD607, which contains the *lux* operon from *Vibrio fischeri*, was used as a biosensor for detecting water-soluble and total Zn, Cu, and Ni; a reduction in bioluminescence revealed a response to metal toxicity (Horswell et al. 2006). A naturally bioluminescent fungal biosensor, *Armillaria mellea*, was suitable for detecting Cu and Zn, in addition to toxic organics (Weitz et al. 2002). Other studies are using microbial activity as a reflection of metal biological toxicity,

seen as determinations of metal ecological dose (ED_{50}) (Renella et al. 2003; Moreno et al. 2001) and specific enzymatic activities (Belyaeva et al. 2005; McLaughlin et al. 2000; Dick 1997). Regardless of the applied method, total metal concentrations will always exceed bioavailable metal concentrations (Table 11.2).

11.4 Physical and Chemical Approaches for Metal Remediation

There are two main approaches for traditional remediation of metal-contaminated soils: removal of the metal from the soil or immobilization of the metal species within the soil. As metals cannot be degraded, excavation of contaminated soil is one method of removal; however, the large volumes of contaminated soil found at many sites make this approach costly and not feasible.

11.4.1 Metal Removal

Soil washing solubilizes metals so they may be flushed from the soil system and recovered in an aqueous phase. Common leaching agents used to desorb metals from organic matter and soil particulates include acids and chelators such as EDTA (ethylene diamine tetraacetic acid) and DTPA (diethylene trinitrilo pentaacetic acid). With increased metal solubility, complexing agents bind metals for removal. Jang et al. (2007) described a sequential washing technique using hydrochloric acid and sodium hydroxide to reduce arsenic in mining waste in Korea. Unfortunately, soil washing with harsh acids can lead to physico-chemical changes in soil structure, impacting soil productivity and biological diversity. Reed et al. (1996) found a final

Table 11.2 Variations in bioavailable versus total metal concentrations in two distinct metal-contaminated soils

Metal	Soil leachate[a] (mg l)	Total metal (mg/kg dry weight)
As[b]	0.22	25
Cd[c]	0.01	3
Cr[c]	0.09	22
Cu[c]	0.11	362
Mn[b]	2.03	152
Mo[b]	0.05	5
Ni[c]	0.04	15
Pb[c]	0.10	1250
Zn[c]	0.04	254

[a] Water used as extractant
[b] Adapted from Cook et al. (2002), after 24 h
[c] Adapted from Alvarenga et al. (2008)

pH of 1 in a soil after flushing with 0.1N HCl. Additionally, chelators can be recalcitrant and difficult to remove from soil effluent (Hong et al. 2002).

Surfactants, also used in soil washing, are amphipathic molecules that can reduce the surface tension of water. They can also complex and increase soluble concentrations of bound metals. Individually and in combination, surfactants and ligands, e.g., Triton X and saponin, have been shown to help desorb metals from soil particles (Chang et al. 2005; Shin et al. 2005; Hong et al. 2002). Shin et al. (2005) showed that up to 61% of cadmium was desorbed from a sandy loam soil when flushed with Triton X and the ligand sodium iodide. Physical washing or scrubbing of soils without chemical additives can also help decrease soil metal content through the removal of clay particles with bound metals (Petruzzelli et al. 2004).

11.4.2 Metal Immobilization

Chemical treatment of soils with oxidizing and reducing agents involves the conversion of metals to inert species (US EPA 2004). Reagents used include ozone, hydrogen peroxide, hypochlorites, potassium permanganate, Fenton's reagent (hydrogen peroxide and iron), chlorine, and chlorine dioxide. A benzene-contaminated soil co-contaminated with lead and cadmium was treated with Fenton's reagent, decreasing cadmium in the leachate by 50% (Bragato and Tenorio 2007). Liming of soils (addition of calcium carbonate) is another method to increase soil pH to decrease metal mobility and toxicity. Kukier and Chaney (2001) showed that liming soils in situ alleviated nickel toxicity, allowing plant growth that was otherwise prevented. The addition of organic matter to soils, which increases the cation exchange capacity (CEC) of the soil, also helps sorb free metal species (Bradl 2004).

Physical methods of immobilization involve capping, solidification/stabilization (S/S) and vitrification. Soil capping is a method of covering contaminated soils in order to contain contaminants. For example, vegetation caps have been successfully used on lead-contaminated soils; native shrubs, grasses and wildflowers were able to stabilize an industrially-contaminated soil containing 3,000–10,000 mg/Kg Pb (Fetzer et al. 2006). Contaminated sediments are commonly capped as an alternative to dredging, as dredging replaces anoxic zones with oxidizing metal solubilizing conditions (Zoumis et al. 2001). Solidification/stabilization (S/S) uses cement, anhydrite ($CaSO_4$) or bitumen (similar to tar) to physically stabilize contaminated soil. The physical containment decreases metal leaching and bioavailability. A review of S/S technologies in remediation is provided by Paria and Yuet (2006). Finally, vitrification uses heat (1600-2000°C) to melt contaminated soil into a glass-like, leach-resistant material. In a study by Meegoda et al. (1996; 1999), soils containing a TCLP (toxicity characteristic leaching procedure) leachate containing above 30 mg l Cr were reduced to below 5 mg l following vitrification. Jou (2006) showed that microwave radiation could be used to vitrify soils within 30 min and retained a TCLP of less than 1.0 mg l Pb after 6 years. Because of the stability of vitrified soils, their re-use in construction materials is possible.

11.4.3 New Preventative Methods of Metal Contamination

Mine backfilling is the practice of refilling already mined cavities or stopes with mine tailings (Scoble et al. 2003). Tailing waste is re-deposited into mining cavities as a portion of the backfill media. The mine tailings are stabilized with a binder such as cement. Environmentally, refilling mined stopes helps decrease oxidation of metal sulfides and therefore acid mine drainage. In an in situ study of a paste-backfilled underground stope using more than 50% sulfidic tailings, the oxidation rate decreased after 14 days and stabilized after 80 days, reducing the formation of acid mine drainage (Ouellet et al. 2006). Nehdi and Tariq (2007) provide a review of additional mine tailings stabilization techniques.

The treatment of metal wastes prior to release into the environment is the ideal treatment alternative. The PELLU Chemicals Company has designed a one-step metal removal system for wastewater (www.pelluchemicals.com). The system removes metals from industrial laundry, electroplating, aircraft maintenance shops, mine tailings and metal refinery wastewaters that include Cd, Pb, Cu, and Cr, as contaminated water is pumped into a reactor where a proprietary chemical is injected into the tank to the desired pH. A polymer is then added to bind the metals as the resulting precipitate is collected for disposal. The proprietary chemical used can change depending on the type of wastewater being treated. Another commercially available product, called LeadX, is a granular chemical additive that can be tilled into soils to decrease bioavailable levels of metal (www.leadx.org); metals such as Ag, Cd, Cr, Hg, and Pb were reduced by more than 86% upon addition of LeadX as determined by TCLP.

11.5 Microbial Interactions with Metals

Metals are toxic to all biological systems from microbial to plant and animal, with microorganisms affected more so than other systems, due, in part, to their small size and direct involvement with their environment (Patel et al. 2007; Sarret et al. 2005; Giller et al. 1999). Metal toxicity negatively impacts all cellular processes, influencing metabolism, genetic fidelity and growth. Loss of bacterial populations in metal-contaminated soils impacts elemental cycling, organic remediation efforts, plant growth, and soil structure. Renella et al. (2003; 2005) found that certain metal combinations, e.g., Cu + Zn and Ni + Cd, in soils resulted in decreased soil microbial activities due to bacterial diversity changes. Palmroth et al. (2007) similarly found decreased hydrocarbon degrading abilities associated with soil microbial communities impacted by lead. Stefanowicz et al. (2008) found that metals influence fungal and bacterial communities differently. In their study, lead and zinc soil contamination from mining activities resulted in decreased bacterial functional diversity, whereas fungal functional diversity increased amongst the culturable organisms. Computational analyses performed by Gans et al. (2005) showed a 99.9% reduction in bacterial diversity in soils impacted by highly toxic metals, with preferential loss of rare taxa. While these studies and others (Weber et al. 2008;

Niklińska et al. 2006; Bååth et al. 2005) find that metal toxicity can change respiration rates, carbon source utilization, and degradation patterns, the long-term effects of such changes in functional microbial diversity remain unclear. Some evidence indicates that microbial community activities recover upon metal remediation. de Mora et al. (2005) found restoration of microbial function, including biomass, carbon mineralization and dehydrogenase activities, following the in situ organic treatment of metal-contaminated soils in Spain.

11.6 Microbial Transformations of Metals

Numerous microbially-mediated transformations of metals have been identified (Roane and Pepper 2000; Nies 1999), many of which may immobilize or mobilize metals in the environment (Table 11.3). Microbial transformations of metals are often the result of metal resistance mechanisms that include complexation and precipitation mechanisms as well as solubilization mechanisms that offer bioremediation strategies.

11.6.1 Complexation/Precipitation Mechanisms

Exopolymers, e.g., exopolysaccharides (EPS), and cell walls made up of lipopolysaccharides, proteins and carbohydrates with various functional groups, create sorption sites for metal binding and metal immobilization. The exopolysaccharide produced by the bacterium *Paenibacillus jamilae* complexed up to 230 mg Pb per g EPS (Morillo et al. 2006). Similarly, the EPS from the cyanobacterium *Nostoc spongiaeforme* is a highly effective sorbent of zinc (Singh et al. 2003). In general, microbial biomass, live or dead, can be used to sorb metals. For example, a study by Mohamed (2001) found that wet and dry mass of the cyanobacterium *Gloeothece magna* was able to bind cadmium (38–43%) and manganese (18–47%). An actinomycete, *Streptomyces* sp., highly resistant to chromate, was found to have 94.7% of the chromium associated with the cell wall; accumulation was thought to be due to reduction of CrO_4^{2-} to Cr^{3+} (Amoroso et al. 2001). Depending on soil type, pH, metal type, the surface properties of the microorganism, and other environmental factors, sorption rates for various metals will differ.

Metal reduction can result in decreased metal mobility and/or toxicity, as in the reduction of Cr^{6+} to Cr^{3+} (McLean and Beveridge 2001) and reduction of U^{6+} to U^{4+} (Finneran et al. 2002). Useful in remediation, anaerobic sulfate-reducing and metal-reducing bacteria can produce less soluble metal species, such as metal sulfides and phosphates (CdS, PbS), elemental metals (Hg^0, Se^0), and reduced metals. For example, Hg^{2+} and mercuric salts can be reduced to elemental mercury by the well-studied mercury resistance (*mer*) operon, found in both Gram-positive and Gram-negative

Table 11.3 Microbial metal resistance mechanisms

Metal	Mechanism of resistance	Microorganism	Examples
Ag	Intracellular sequestration	*Lactobacillus* spp.	Nair and Pradeep (2002)
As	Reduction, efflux, intracellular sequestration	*Pseudomonas* sp. As-1	Patel et al. (2007)
Au	Intracellular and extracellular sequestration	*Lactobacillus* spp., *Thermomonaspora* sp.	Nair and Pradeep (2002); Ahmad et al. (2003b)
Cd	Intracellular and extracellular sequestration, efflux, reduction	*Gloeothece magna, Staphylococcus aureus, Fusarium oxysporum, Pseudomonas aeruginosa* BS-2, *Pseudomonas* sp. H1	Mohamed (2001); Nies and Silver (1989); Ahmad et al. (2002); Juwarkar et al. (2007); Roane et al. (2001)
Cr	Extracellular sequestration, reduction	*Streptomyces* spp.	Amoroso et al. (2001); Lloyd (2003)
Cu	Intracellular and extracellular sequestration	*Enterobacter* sp. J1, *Saccharomyces cerevisiae*	Lu et al. (2007); Culotta et al. (1994)
Hg	Volatilization	*Clostridium glycolicum* ASI-1	Meyer et al. (2007)
Mn	Oxidation	*Proteobacteria* SD-21	Francis et al. (2001)
Pb	Biosurfactant, extracellular sequestration	*Pseudomonas aeruginosa* BS2, Enterobacter sp. J1	Juwarkar et al. (2007); Lu et al. (2007)
Se	Reduction	*Desulfomicrobium norvegicum, Pseudomonas stuzeri*	Hockin and Gadd (2003); Lortie et al. (1992)
U	Extracellular sequestration	*Bacillus sphaericus* JG-A12	Pollman et al. (2006)
Zn	Efflux	*Ralstonia metallidurans* CH34	Nies (2003)

bacteria (Nies 1999). A review of microbial dissimilatory metal reduction is provided by Lloyd (2003). In a study by Hockin and Gadd (2003), the sulfate-reducing bacterium *Desulfomicrobium norvegicum* removed 35 mg l sodium selenite ($NaSeO_3$) from solution upon precipitation with bacterially produced sulfide. In the presence of up to 25 g l Cu, the bacterium *Acidithiobacillus ferrooxidans* used cellular polyphosphate to bind copper and transport the metal–phosphate complex out of the cell (Alvarez and Jerez 2004).

Intracellular accumulation of metals can occur with metallothionein complexation. Produced by animal, plant, and microbial cells in response to metal toxicity, metallothioneins (MT) and MT-related proteins are cysteine-rich, low molecular weight (ranging from 6,000 to 10,000 kDa) proteins involved in metal binding. Clearly studied in yeast (Culotta et al. 1994), recent work is identifying MT-related proteins in bacteria. Figueria et al. (2005) described an increase in glutathione (GSH), a MT-related protein, in the bacterium *Rhizobium leguminosarum* bv. *viciae* in response to cadmium exposure. Up to 75% of the cadmium was complexed by GSH in a cadmium-tolerant *Rhizobium leguminosarum*, with 23% of the cadmium complexed in a cadmium-sensitive strain (Lima et al. 2006).

11.6.2 Metal Solubilization Mechanisms

As opposed to metal immobilization, metal mobilization occurs through processes resulting in the production of organic acids or H_2SO_4. A result of growth, many microorganisms release H^+ into their surrounding environment, lowering the pH. Additionally, organic acids produced through the degradation of organic substrates in soils, e.g., sugars, cellulose, etc., lower pH and complex metals. For example, citric and oxalic acids can form soluble metal complexes with Fe, Al, and Zn (Strasser et al. 1994; Franz et al. 1993). This ability of some microorganisms is used extensively in bioleaching. Bioleaching generally involves the oxidation of iron (e.g., Fe^{2+} to Fe^{3+}) or sulfur-containing minerals (e.g., S^{2-} to SO_4^{2-}) to produce sulfuric acid. The resulting acidic pH solubilizes metals, such as copper and zinc, making them available for recovery. Microorganisms involved in bioleaching processes are both metal-resistant and acidotolerant. Bacteria such as *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* are commonly associated with bioleaching (Watling 2008).

Containing functional groups such as hydroxamates and catecholates, siderophores can also be observed in mobilizing metal complexation (Nair et al. 2007). Although they serve a specific purpose for the organism in obtaining iron, they are nonspecific for the range of metals that they can bind. Siderophores have been shown to bind Al, As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sn, and Zn, in addition to Fe (Nair et al. 2007), increasing their desorption from soil particles (Neubauer et al. 2000). Siderophore pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas stutzeri* KC precipitated As, Cd, Hg, and Pb, conferring resistance to the bacterium

(Zawadzka et al. 2007). Similarly, microbially-produced biosurfactants including rhamnolipid by *Pseudomonas aeruginosa*, surfactin by *Bacillus subtilis*, and sophorolipid from the yeast *Torulopsis bombicola* have been implicated in metal-resistance (Mulligan et al. 2001; Sandrin et al. 2000). While biosurfactants have been found to bind a variety of metals, including lead and zinc (Maier and Soberon-Chavez 2000), surfactant production specifically in response to metal toxicity is not clear. However, biosurfactant-producing microorganisms can be isolated in greater diversity from metal-contaminated soils than from uncontaminated ones, as many sites are co-contaminated with organics (Bodour et al. 2003). It is important to note that while siderophore and surfactant–metal complexes are less toxic, biosurfactant complexation can increase metal solubility. Consequently, the use of these microorganisms in metal remediation has limited application, and has been proposed in soil-washing technologies.

Biomethylation is a widespread phenomenon among microorganisms for metals such as As, Hg, Se, Sn, Te, and Pb (Gadd 2004). Methylation of metals by bacteria transforms a metal into a gaseous state via the addition of methyl groups, resulting in increased toxicity due to the resulting lipophilic nature. Methylmercury, for example, is 10–100 times more toxic than inorganic forms of mercury. Natural mercury methylation tends to be mediated by sulfate-reducing bacteria, with HgS as the substrate (Barkay et al. 2003; King et al. 2000; Compeau and Bartha 1985). On the other hand, the volatile nature of the metals promotes diffusion away from the bacterial cell, resulting in a less toxic environment for the bacterium. Volatilization on a large scale can result in decreased metal concentration. Remediation of high selenium soils in the San Joaquin Valley, California, occurred when soils were amended with readily degradable carbon, such as manure and casein. The addition of carbon stimulated bacterial volatilization of the selenium, removing approximately 68–88% of the total selenium within the top 15 cm of soil (Flury et al. 1997). Other bacterially-methylated metals in soils include Se, Sn, As, and Pb (Meyer et al. 2007). An extensive review by Thayer (2002) is available on biomethylation of metals and metalloids.

Metal efflux systems are used by a number of microorganisms to actively remove intracellular metals. These systems make use of ATPase or chemiosmotic ion/proton pumps. Arsenite efflux by the ArsA/B transporter follows the enzymatic reduction of arsenate (AsO_4^{3-}) to arsenite (As^{3+}) by arsenate reductase ArsC (Mukhopadhyay et al. 2002). Interestingly, arsenite is still toxic to the cell. Cadmium can also be actively transported out of the cell by the pumps CadA or ZntA in *Bacillus subtilis* and *Pseudomonas putida* (Silver and Phung 2005). These pumps can show non-specificity and pump other metals out of the cell, such as Pb or Zn. The Czc system, for example, involves active transport of Cd, Co and Zn from the cytoplasm in *Cupriavidus metallidurans* CH34 (Nies 2003). While for individual cells active transport keeps intracellular metal concentrations low, this mechanism is generally not considered for remediation purposes, given its limited ability to detoxify and immobilize the metal.

11.7 Approaches to Microbial-Based Remediation of Metal-Contaminated Soils

The primary goal of soil metal remediation is to remove the metal from the soil or to decrease metal mobility and toxicity within the soil. Numerous microbially-mediated reactions can achieve these goals, including metal methylation, oxidation–reduction reactions and metal complexation (Fig. 11.1). The diverse nature of microbial metabolic activities has long been exploited for human purposes, for example in extraction of precious metals from ores in bioleaching. Understanding metal–microbe relationships has led to advances in bioremediation (Malik 2004; Bruins et al. 2000).

11.7.1 Indirect Use of Microbial Activities

Microbial transformations of metals can be encouraged through alteration of soil conditions. As discussed in section 11.3, metal interactions in soil are dynamic and susceptible to environmental change. The addition of organic matter, for example, will decrease metal solubility and reduce toxicity. The initial abiotic oxidation of Fe^{2+} increases acidity, and later encourages microbial acid production and enhanced metal bioavailability. Likewise, changing soil chemical and physical parameters will directly influence the activities of indigenous microbial populations. Saturation of a soil for reducing conditions will facilitate the growth of sulfate-reducing bacteria and the formation of metal sulfides. Such facilitated intrinsic bioremediation of metals in soils through modification of soil conditions can be achieved, but may be short-lived given the temporary nature of the environmental change.

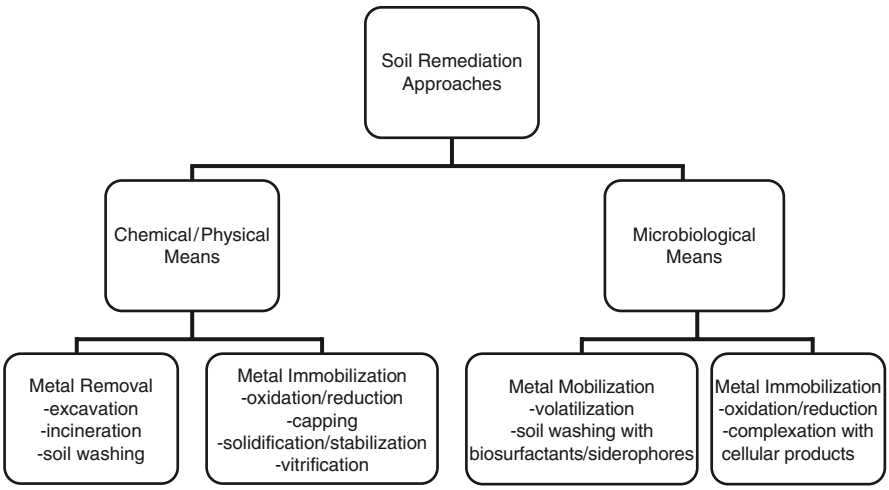

Fig. 11.1 Summary of traditional and microbiological approaches to soil metal remediation

11.7.2 Augmentation with Microorganisms

The goal of microbial bioaugmentation, the addition of specific metal-transforming microbial populations, is to add to the biological potential of a system. While different metal microbial activities can be introduced into a system, bioaugmentation often involves the introduction of a metal-immobilizing population to stabilize metal movement and availability in a soil, as seen with exopolymer-producing and metal-reducing bacteria. An interesting new use of bioaugmentation and metal-reducing microorganisms is an intersection between nanotechnology and bioremediation. Several microorganisms are capable of producing inorganic nanoparticles from soluble environmental metal (Rajendran and Gunasekaran 2007). Bacteria and fungi have been found to produce nanoparticles of silver (Nair and Pradeep 2002), gold (Ahmad et al. 2003a, b; Nair and Pradeep 2002), and cadmium (Ahmad et al. 2002). The actinomycete *Rhodococcus* sp. accumulated gold particles near the cytoplasmic membrane and cell wall, without any observed toxic effects (Ahmad et al. 2003a). *Lactobacillus* strains produced intracellular gold and silver granules without effect (Nair and Pradeep 2002). Extracellular production of nanoparticles has been documented with fungi and actinomycetes (Ahmad et al. 2002; 2003 a,b). Recovery of these nanoparticles is of interest, while in situ stabilization of metals can occur for use in bioremediation.

Bioaugmentation is challenging, because introduced populations may not necessarily establish themselves among existing populations long enough to carry out the desired activity or, although they become established, introduced populations may not disperse throughout the contaminated environment, minimizing their remediation potential. Baxter and Cummings (2006) showed that the bacterium *Rhodococcus* spp. could become an established member of the microbial community in a contaminated coke works soil. In a study by Roane et al. (2001), a Cd-resistant bacterium *Pseudomonas* sp. H1 and a known 2,4-D (2,4-dichlorophenoxyacetic acid) degrader *Ralstonia eutropha* JMP134 were co-introduced into a cadmium-contaminated (60 mg/kg) and 2,4-D-contaminated (500 mg/kg) soil to enhance 2,4-D degradation. *Pseudomonas* sp. H1 reduced the solubility of the cadmium, allowing the cadmium-sensitive degrader to survive. Both organisms became active members of the community within the 70-day study period. Pepper et al. (2002) demonstrated the need for bioaugmentation for the remediation of mixed-contaminated soils. In this case, one bacterial population was needed to address cadmium toxicity, while a metal-sensitive second bacterial species degraded either 2,4-D or 3-chlorobenzene.

11.7.3 Soil Washing Using Microorganisms or Their By-products

An ex situ process, washing soils with microbial products relies on the resulting increase in metal solubility for enhanced metal removal. The use of bacterially-

produced di-rhamnolipid surfactant has recently been shown to alleviate metal stress in cadmium- and lead-contaminated soils by removing 92% Cd and 88% Pb (Juwarkar et al. 2007); soil microbial diversity was also shown to increase once the metals were removed. In a study by Mulligan et al. (2001), single washes with 0.5% rhamnolipid removed 65% Cu and 18% Zn; with 4% sophorolipid removed 25% Cu and 60% Zn; and with 0.25% surfactin removed 15% Cu and 6% Zn from contaminated soil sediments. Siderophore produced by a *Pseudomonas azotoformans* isolated from oil sludge was able to remove 92.8% of the arsenic from an arsenic contaminated soil after five washings (Nair et al. 2007). Nair et al. (2007) also indicated that siderophores can desorb metals from soil surfaces, aiding in removal. Metal solubilization via acid production is also a viable approach to release bound metals in soils. As a soil becomes more acidic, cationic metals are increasingly soluble and available for removal. Bioleaching is a primary example of this type of microbial process.

11.7.4 Gene Transfer and Genetic Engineering of Metal-Resistance Genes

Genes associated with metal resistance/detoxification mechanisms have long been studied in microorganisms (Silver and Phung 2005). The *cad* operon responsible for cadmium efflux is well-characterized in *Staphylococcus aureus* (Nies and Silver 1989). The *mer* operon in mercury resistance is well-understood in a variety of microorganisms (Nies 1999). The *czc* operon has been elucidated in numerous bacteria (Abou-Shanab et al. 2007). Such metal-resistance genes have been introduced into other bacteria and plants to help establish metal-tolerant populations within contaminated systems. For example, numerous tree species have been genetically altered to produce the MT-related glutathione for metal sequestration (Merkle 2006).

Gene bioaugmentation is defined as the process of obtaining enhanced biological activity after gene transfer from an introduced donor organism into a member of the indigenous soil population (Maier 2000). In a metal-contaminated soil, enhanced metal detoxification activity could be achieved if metal-resistant/detoxifying genes were transferred to transconjugant bacterial populations within the soil (Pepper et al. 2002). Gene bioaugmentation has primarily been studied for mitigation of organic pollutants (Jussila et al. 2007; Urgun-Demirtas et al. 2006), and while not widely used gene bioaugmentation can potentially establish large, diverse metal-resistant populations within a soil.

11.7.5 Microbial Influence on Phytoremediation in the Rhizosphere

Phytoremediation, the use of plants for the cleanup of polluted sites, is another attractive method for metal remediation. Mechanisms include phytoextraction, phytoaccumulation, phytostabilization, and phytovolatilization and rhizofiltration (Manousaki et al. 2008; Mendez and Maier 2008; Pilon-Smits 2005). In addition to understanding these mechanisms, researchers are also focused on the relationships between plants and their microbial symbionts in the rhizopshere. Microbial diversity, density and activity are much more abundant within the rhizosphere than in bulk soil, which can promote increased phytoremediation activity. Vivas et al. (2003a) showed that the bacterium *Brevibacillus* sp. was able to increase nitrogen and phosphorous uptake, nodule formation, plant growth and mycorrhizal infection of *Trifolium pratense* L. (red clover) in a lead-polluted soil. A similar situation was observed in a cadmium-polluted soil (Vivas et al. 2003b). Furthermore, dual-inoculation of *Brevibacillus* sp. and the arbuscular mycorrhiza (AM) fungi *Glomus mosseae* at either site decreased lead and cadmium uptake by *Trifolium*, indicative of a possible phytostabilization mechanism involving metal sorption by the microorganisms and the metal chelator indole acetic acid (IAA) produced by *Brevibacillus* sp. The bacterium *Bacillus subtilis* strain SJ-101 was also able to increase Ni uptake in the shoots of *Brassica juncea* (mustard plant), probably through the production of IAA (Zaidi et al. 2006). Microbially-produced siderophores also help increase plant uptake of heavy metals by making the metal more bioavailable to the plant, enhancing phytoextraction of the metal from the surrounding soil (Neubauer et al. 2000).

11.8 New Frontiers in Microbial Metal Remediation

The biological treatment of metal-contaminated systems is a continuing field of research. While much need for elucidation remains, the field of microbial metal remediation is making great strides towards applicable technologies. There are many new and exciting proposed uses of microorganisms and their products in soil metal remediation. With our increased understanding of multiple microbial metal-resistance mechanisms, treatment of sites with fluctuating metal concentrations and multiple metal and organic contaminants may be possible. The use of microbial products, the microbial enhancement of phytoremediation technologies, in combination with the genetic engineering of plants and microorganisms for enhanced metal uptake, represent continuing, exciting directions in the use of microorganisms in soil metal remediation.

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Chapter 12
Transformations of Toxic Metals and Metalloids by *Pseudomonas stutzeri* Strain KC and its Siderophore Pyridine-2,6-bis(thiocarboxylic acid)

Anna M. Zawadzka, Andrzej J. Paszczynski, and Ronald L. Crawford

12.1 Introduction

Microbial metabolism strongly influences metal speciation in the biosphere through the diverse responses of microorganisms to metal deficiencies or surpluses. The responses exhibited depend on both metal chemical properties and microbe species. Microorganisms have coexisted with metals throughout evolutionary history, and have recruited metals for physiological functions through development of intricate homeostatic mechanisms for metal acquisition and protection against metal toxicity (Valls and de Lorenzo 2002). Redox-active metal cations can form complex structures with organic molecules. Such complexes in turn can play important roles in many biochemical reactions by acting in active enzymatic centers to foster electron-transfer reactions. Bacteria require the metals used in such complexes in micronutrient amounts; however, they often are toxic at higher concentrations. Some heavy metals (e.g., Hg, Cd, and Ag) form strong nonspecific complexes inside cells, which lead to toxic effects and are therefore not compatible with physiological functions or metabolic pathways. Heavy metal toxicity may also occur if the metals enter cells via unspecific uptake systems. Once inside the cell, heavy metals tend to bind to thiol groups, inhibiting many essential enzyme activities. Their binding to glutathione causes oxidative stress. Some heavy metal ions can interfere with other physiologically important divalent cations, and heavy metal oxyanions may interfere with the metabolism of structurally related anions, the reduction of which

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leads to free radical formation and mutagenesis (Nies 1999). Bacterial resistance to heavy metal toxicity is common, and in general involves one or a combination of three systems: extrusion of the metal ion from the cell by an efflux mechanism, reduction of a metal to a less toxic oxidation state, or complexation and segregation of the metal from sensitive biomolecules by thiol-containing molecules (Nies 1999). As a consequence of these different interactions, microbes alter metal chemistry and often cause changes in metal spatial distribution, mobility, and toxicity in the natural environment.

Siderophores are iron-specific chelators that microorganisms produce and secrete under iron-limiting conditions as a part of an iron homeostasis system (Andrews et al. 2003). Some siderophores chelate metals other than iron, forming soluble or insoluble metal compounds and affecting mobility and toxicity of the metals (Cortese et al. 2002; Hu and Boyer 1996; Visca et al. 1992). In some cases, siderophores protect microbes against metal toxicity (Cortese et al. 2002; Fekete and Barton 1992). Pyridine-2,6-bis(thiocarboxylic acid) (pdtc), a siderophore produced by *Pseudomonas stutzeri* KC, *Pseudomonas putida* DSM 3601, and *Pseudomonas putida* DSM 3602, has fortuitous carbon tetrachloride degradation activity (Criddle et al. 1990; Lee et al. 1999; Ockels et al. 1978). Recent research has shown that pdtc promotes iron transport into the cell (Lewis et al. 2004); its production is regulated by iron, and also by zinc (Leach et al. 2007). The latter finding indicates that pdtc may assist in acquisition of transition metals other than iron. *P. stutzeri* KC, its spontaneous pdtc negative mutant CTN1, and other *P. stutzeri* strains also produce proferrioxamines (pFOs), which are most likely their primary siderophores (Essén et al. 2007; Zawadzka et al. 2006b). Pdtc is a broad-range metal chelator; it chelates many transition metals, some heavy metals, lanthanides, and actinides. In general, complexes of pdtc with micronutrient metals are soluble, while complexes with toxic metals form insoluble precipitates (Cortese et al. 2002; Stolworthy et al. 2001). Our latest research has shown that selenium and tellurium oxyanions are among the toxic metalloids precipitated by pdtc and pdtc-producing *Pseudomonas stutzeri* KC cultures (Zawadzka et al. 2006a). Pdtc also reduces chromium(VI) to chromium(III), which is then chelated by pdtc. The oxidation products of pdtc eventually hydrolyze, releasing a chromium precipitate that is of low solubility (Zawadzka et al. 2007). In addition, studies of the genes involved in pdtc synthesis and regulation are adding to our knowledge about pdtc chemistry and genetics (Leach et al. 2007; Lewis et al. 2004). Because we have significant knowledge about pdtc, and because it can be easily chemically synthesized (Hildebrand et al. 1983), it serves as an excellent model microbial metabolite among other bacterial siderophores. Pdtc's properties also make it a good candidate for heavy metal remediation through metal reduction and complexation, selective precipitation of heavy metals from solution, and also solubilization in the case of microelement metals. Pdtc also has potential for application in remediation of metal and mixed metal-carbon tetrachloride-contaminated wastes. The interactions of pdtc with toxic metals and metalloids that are the focus of recent research are reviewed in this chapter.

12.2 Overview of Pdtc Interactions with Metals

Pdtc is one of the most versatile bacterial siderophores because it interacts with numerous metals and because its copper complex promotes reaction and subsequent degradation of carbon tetrachloride. Pdtc was found to have affinity not only for iron but also for other metals. A collection of studies has reported on the structures of the complexes which pdtc forms with Fe, Co, Ni, and Pd, and on the redox cycling properties of Fe and Ni complexes (Budzikiewicz 1993; Espinet et al. 1994; Hildebrand et al. 1984; Hildebrand et al. 1985; Hildebrand and Lex 1989) (Fig. 12.1). Pdtc is the only known siderophore that uses two sulfur atoms of

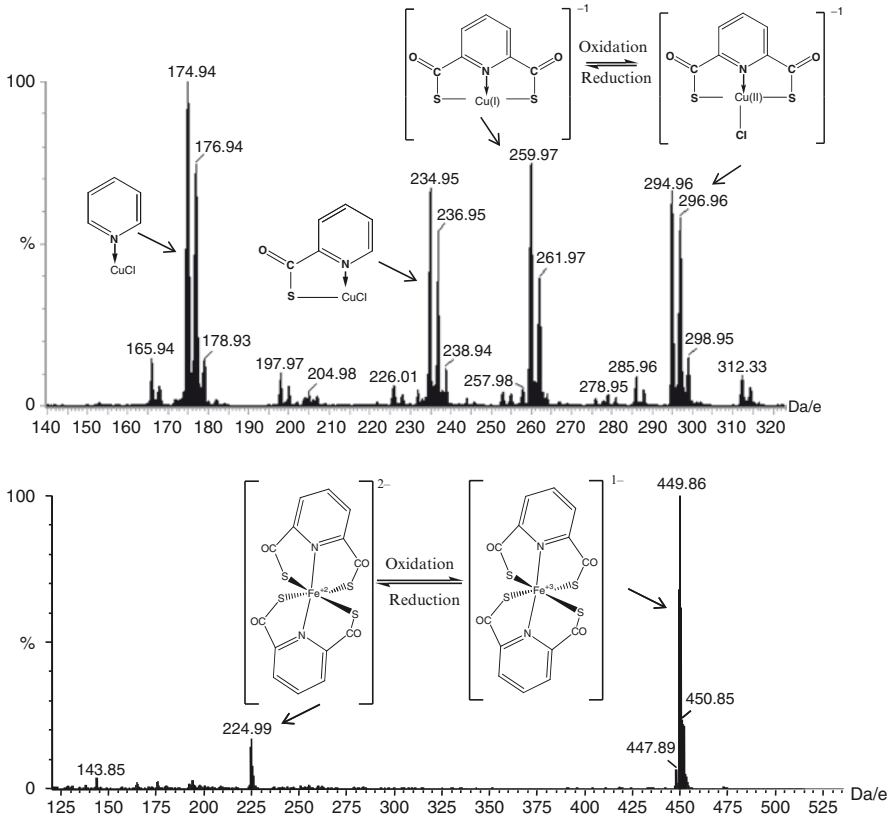

Fig. 12.1 Examples of electrospray mass spectra (ES-MS) of tridentate planar complexes and hexacoordinate octahedral complexes formed by pyridine-2,6-bis(thiocarboxylic acid) (pdtc). The ES-MS spectrum of copper:pdtc complexes shows characteristic +2 peaks related to copper and chlorine natural isotope distribution. The iron:pdtc and cooper:pdtc complexes can redox cycle between the two different oxidation states of the metals (cupric/cuprous and ferric/ferrous) resulting in overall complex charge number change without the complex decomposing. Some metals were found to promote pdtc hydrolysis. The hydrolysis products of pdtc chelate the metals as well. For structures of Cr(III):pdtc and pdtc hydrolysis products of Cr(III) complexes see Fig. 12.8

thiocarboxylate groups and pyridine nitrogen to bind metals. Neu et al. documented the only exception thus far, where oxygen rather than the sulfur of the thiocarboxylate groups was used as the pdtc ligand for binding UO_2(II) (Neu et al. 2001). Sulfur and nitrogen are soft Lewis bases that have lower affinity for the hard Lewis acid Fe(III) than the oxygen ligands (hard Lewis base) used by other siderophores (Dhungana and Crumbliss 2005). Therefore, because of its binding ligands, pdtc has affinity for soft Lewis metals like cadmium, mercury, gold, or copper. However, pdtc also has high affinity for hard Lewis metals, probably due to the orientation of ligand atoms that form a tridentate 'binding pocket'. The stability constants (log K) for pdtc complexes with Fe(III), Ni(II), Co(III), and Fe(II) were determined to be 33.36 for Fe(III):$(pdtc)_2$, 33.93 for Co(III):$(pdtc)_2$, 33.28 for Ni(II):$(pdtc)_2$ (Stolworthy et al. 2001), and 12 for Fe(II):$(pdtc)_2$ (Brandon et al. 2003). The effective stability constant (K_{eff}) for the ferric iron:pdtc complex is one of the highest among known bacterial chelators, even exceeding the K_{eff} of enterobactin at acidic pH (Stolworthy et al. 2001). Cortese et al. (Cortese et al. 2002) studied pdtc interactions with 19 metals and three metalloids, and the biological activities of the formed complexes. Pdtc was found to form complexes with 14 of the metals, and complexes of three metals, Fe:$(pdtc)_2$, Co:$(pdtc)_2$, and Cu:pdtc, were able to cycle between redox states. The metal-binding stoichiometry of pdtc depends on the metal orbital structure. One pdtc molecule can bind one metal ion, forming tridentate planar complexes, or two pdtc molecules can bind a metal ion forming hexacoordinate octahedral complexes (Fig. 12.1). Some metals were found to promote hydrolysis of pdtc; pdtc hydrolysis products chelated those metals as well (see Figs. 12.3, 12.4, and 12.8 below). Pdtc was found to form soluble complexes with numerous metals (Au, Bi, Co, Cr, Cu, Fe, Mn, Nd, Ni, Pd, and Zn), but caused toxic heavy metals and metalloids (As, Cd, Hg, Pb, Se, Sn, Te, and Tl) to precipitate from solution. Additionally, some bacterial strains were protected against Cd, Hg, and Te toxicity by the presence of pdtc. Pdtc was also shown to have antimicrobial properties, most likely through a metal sequestration mechanism (Sebat et al. 2001).

12.3 Nature of Pdtc Interactions with Heavy Metals and Metalloids

Pdtc was found to form poorly soluble precipitates with mercury, cadmium, lead, and arsenic(III) that were whitish, white, orange, and white-colored, respectively. The precipitates were identified as complexes of metals with pdtc, using electrospray ionization-mass spectrometry (ESI-MS). The precipitates were partially soluble in N,N-dimethylformamide (DMF), and we confirmed that pdtc formed complexes of known structures with Cd(II) and Pb(II) (Cortese et al. 2002). After the addition of pdtc in excess to a mercury solution, we observed a Hg(II):$(pdtc)_2$ complex with one sulfide ligand protonated (Zawadzka et al. 2007). Mercury is known to trigger pdtc hydrolysis, and complexes of Hg with pdtc hydrolysis

products are also observed. It is possible that the Hg(II):(pdtc) complex also forms as in the case of other divalent metals, but because the complex is insoluble in water or organic solvents, it is not detectable using ESI-MS. A complex of pdtc with arsenic was not detected, probably also due to its low solubility. The colors of the precipitates changed gradually to the colors corresponding to the respective metal sulfides, and their elemental compositions were confirmed by scanning electron microscopy-energy dispersive X-ray spectrometry (SEM-EDS) analysis (Fig. 12.2). Since all metals were accompanied by sulfur, we concluded that the following sulfides, HgS, CdS, PbS, and As_2S_3, were formed as a result of metal:pdtc complex hydrolysis. In the case of As, the atom percentage of As and S was 2:3, suggesting the presence of As_2S_3 precipitate (orpiment), similar to another microbiologically formed arsenic trisulfide, although the latter is formed by a different mechanism (Newman et al. 1997). In other metal precipitates, the sulfur content was nearly double that of metals, indicating the possibility of elemental sulfur co-precipitation during complex hydrolysis reactions where two sulfur ligands complex one metal cation. We concluded that pdtc forms complexes with Hg, Cd, Pb, and As as with other metals, but these complexes are poorly soluble in water, resulting in precipitate formation. Heavy metal:pdtc complexes are also less stable than transition metal complexes and hydrolyze,

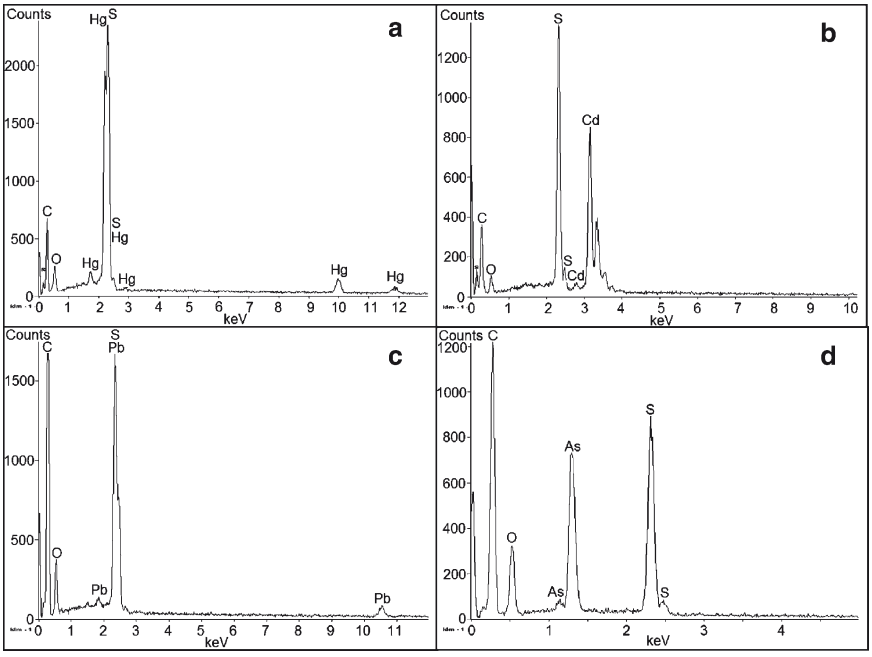

Fig. 12.2 EDS spectra of pdtc:metal precipitates. *Panels* are: (**a**) mercury, (**b**) cadmium, (**c**) lead, and (**d**) arsenic (III). Peak intensities are related to the concentration of the given element in the sample

releasing metal sulfides. In a related study, complexes of Cd:thiocarboxylate were shown to release CdS as a result of thermal decomposition at temperatures as low as 25°C (Nyman et al. 1997).

The secretion of pdtc which then binds heavy metals into insoluble complexes can foster the release of even more insoluble sulfides as these complexes hydrolyze. This can serve as a detoxification mechanism for *P. stutzeri* KC. This process renders metals insoluble and thus not bioavailable (e.g., the solubility product K_{sp} of HgS is 6.38×10^{-29}; the K_{sp} of CdS is 1.4×10^{-29}). The minimum inhibitory concentrations (MIC) of Hg, Pb, and As(III) were twice as high for strain KC as compared to pdtc-negative mutant CTN1, confirming that KC benefits from a pdtc-mediated heavy-metal detoxification mechanism. Only cadmium exhibited similar toxicity in both strains. Since only the fates of pdtc complexes with iron (Lewis et al. 2004) and zinc (Leach et al. 2007) are known, we can speculate that some of the pdtc complexes with other metals are transported into the cells as a means of micronutrient acquisition. Traces of solubilized cadmium complex may be following this route, causing toxicity rather than preventing it. Cortese et al. (Cortese et al. 2002) showed that supplementation with pdtc protected several bacterial strains, including *P. stutzeri* KC and CTN1, from both Hg and Cd toxicity. Our experimental conditions were different, however, and a toxic effect of Cd may have occurred before the bacterial cells from the inoculum had a chance to grow in the iron-limited media containing Cd and thus produce enough pdtc for detoxification.

12.4 Reduction and Precipitation of Selenium and Tellurium Oxyanions

Selenium and tellurium oxyanions were among the toxic metalloids that were precipitated by pdtc and pdtc-producing *Pseudomonas stutzeri* KC cultures, and these precipitates were subsequently converted to elemental selenium and tellurium (Zawadzka et al. 2006a). We proposed a mechanism for pdtc's detoxification of tellurite and selenite (Fig. 12.3) based on determination of the chemical structures of compounds formed during initial reactions of tellurite and selenite with pdtc, using mass spectrometry and SEM-EDS (Figs. 12.4–12.6). Selenite and tellurite are reduced by pdtc or its hydrolysis product H_2S, forming zero-valent pdtc:selenides and pdtc:tellurides that precipitate from solution. These insoluble compounds then hydrolyze, releasing nanometer-sized particles of elemental selenium or tellurium. ESI-MS analysis identified several types of pyridine-2,6-bis(thiocarboxyl) tellurides, tellurides containing two or three pdtc molecules, and the pdtc hydrolysis product pyridine-2-carboxylic-6-thiocarboxylic acid (pctc). Polymerized pdtc and pctc molecules were also observed (Fig. 12.4). Pdtc binds tellurium atoms through its sulfur moieties, forming intra- or intermolecular tellurotrisulfides analogous to selenodiglutathione (Kessi and Hanselmann 2004), and H_2S abiotically reduces Te(IV) via a mechanism similar to that described for sulfate-reducing bacteria

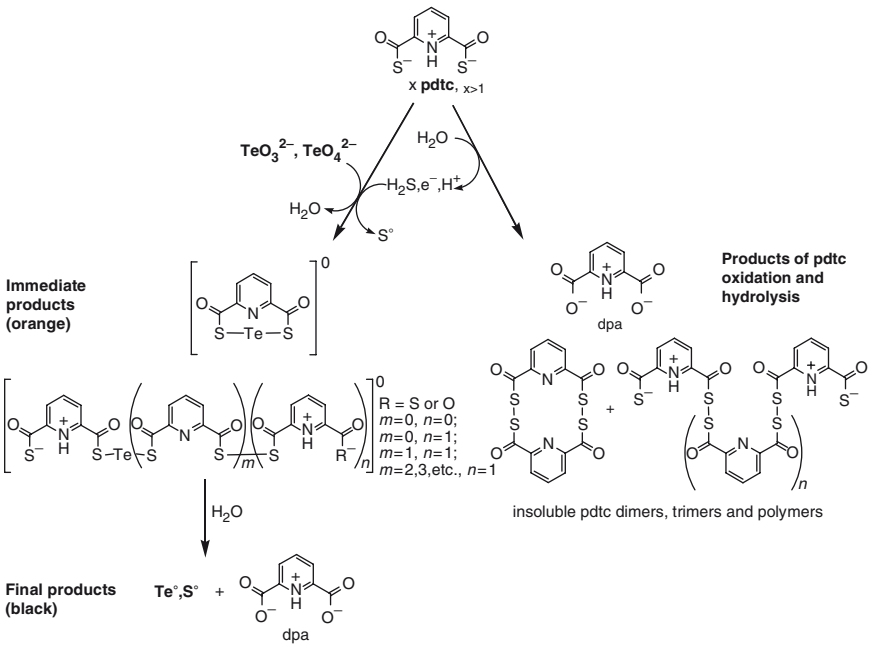

Fig. 12.3 Proposed pathway showing nonspecific oxidation and hydrolysis of pdtc and interactions of pdtc and its decomposition products with tellurium compounds. Tellurite and tellurate are reduced by pdtc or its hydrolysis product H_2S, and subsequently bound by pdtc and pdtc hydrolysis products, including pyridine-2-carboxy-6-thiocarboxylic acid and dipicolinic acid (dpa). Initially formed zero-valent tellurides release elemental Te and Se. All of the products were detected using ESI-MS as charged molecules; their structures were deducted from ESI-MS/MS daughter analysis

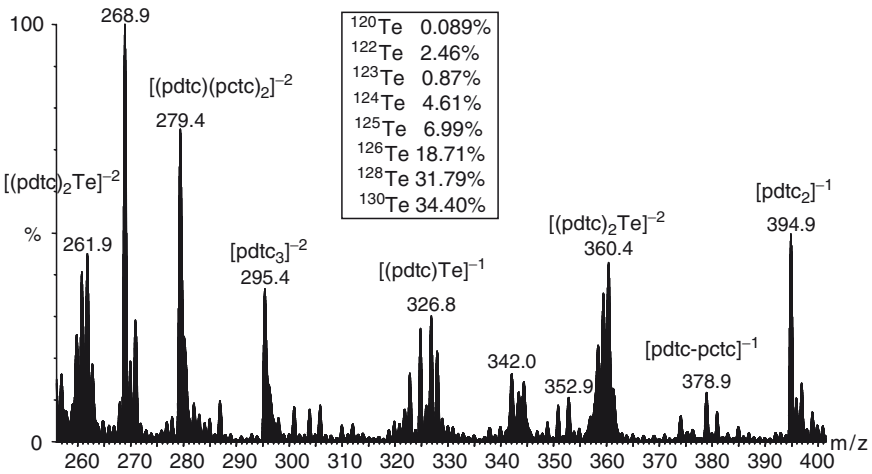

Fig. 12.4 ESI-MS spectrum showing peaks corresponding to charged pdtc tellurides and products of pdtc hydrolysis and polymerization; the natural abundances of tellurium isotopes (shown in the *box*) were used to solve for structures of m/z anions

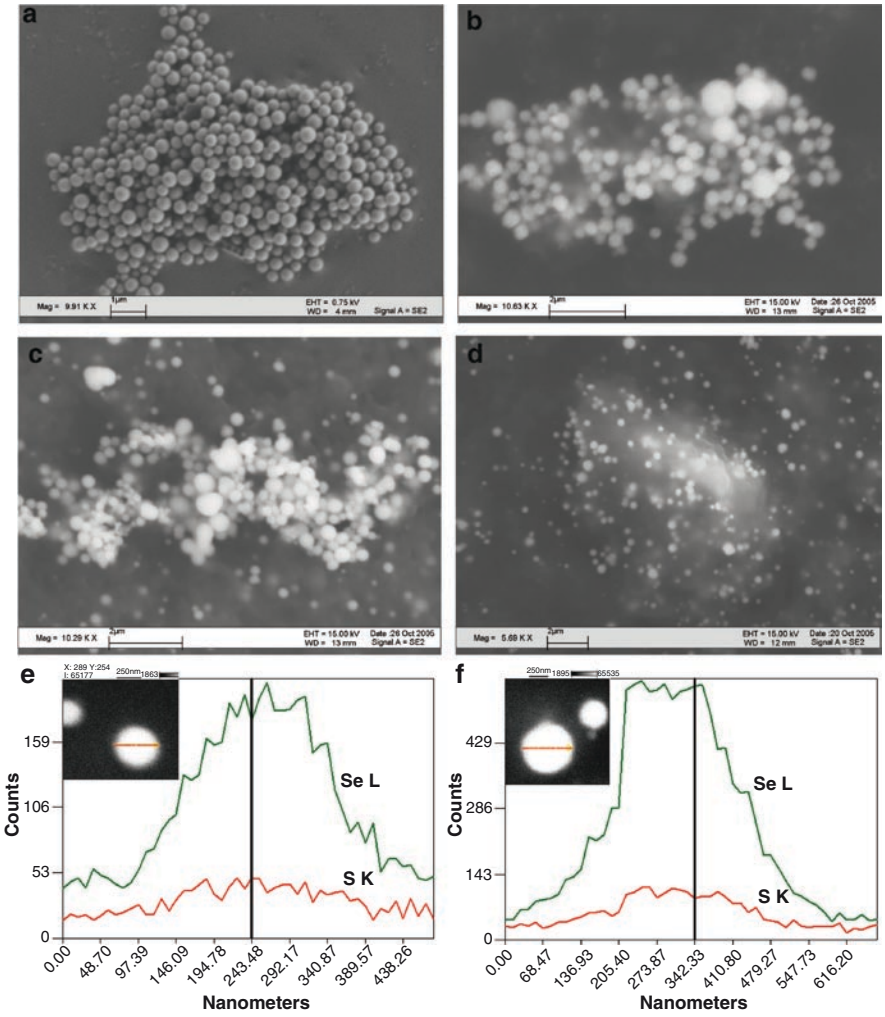

Fig. 12.5 SEM images of selenium precipitates formed: (**a**) in a *P. stutzeri* KC culture filtrate containing nearly 0.1 mM pdtc produced by the bacterium, (**b**) abiotically by chemically synthesized pdtc, (**c**) in a bacterial culture of *P. stutzeri* KC, and (**d**) in a bacterial culture of *P. stutzeri* CTN1. EDS line scans of selenium spheres formed: (**e**) in a *P. stutzeri* KC culture filtrate and (**f**) abiotically by chemically synthesized pdtc; accelerating voltage = 15.0 kV

(Hockin and Gadd 2003). Although the pdtc selenides were not detectable using ESI-MS, the initial color change indicated formation of pdtc:selenide compounds analogous to those identified for tellurium. The detection of polymerized pdtc and pctc molecule peaks in pdtc-treated selenite solutions suggests that the same or a similar mechanism is possible for selenium interactions with pdtc. We also expect selenium to demonstrate chemistry similar to that of tellurium, since both elements

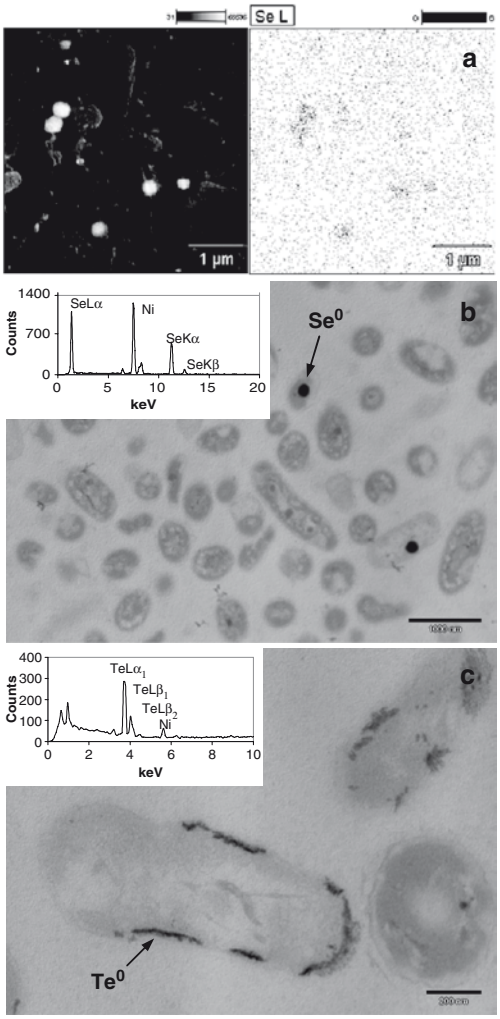

Fig. 12.6 (**a**) Scanning electron micrographs of whole mounts of *P. stutzeri* KC cells grown in iron-limited medium supplemented with 0.5 mM sodium selenite. The *right panel* shows the selenium EDS density signal correlating with extracellular white granules visualized in *left panel*. (**b**) TEM images of bacteria grown in the presence of 0.5mM selenite (*P. stutzeri* KC) and (**c**) 0.05 mM tellurite (*P. stutzeri* CTN1). *Arrows* indicate Se^0 and Te^0 deposits localized in the electron-dense particles inside the cells. Composition of the deposits was confirmed by EDS analysis (spectra shown is *insets*); the Ni signal comes from the SEM nickel grid

belong to the same group 16 of the periodic table. Finally, hydrolysis of pdtc tellurides and selenides leads to the formation of black and orange elemental tellurium and selenium, respectively, accompanied by co-precipitation of sulfur that originates from pdtc's carbonyl sulfides. Pdtc-mediated reactions would be analogous to

selenium interactions with glutathione (GSH) in the Painter reaction (Painter 1941). The complete removal of selenite was observed when the molar ratio of pdtc to selenite was 4:1 in solution. The selenite concentration decrease was accompanied by the change of precipitate color to orange-red. A synergistic effect was observed when proferrioxamine B was present together with pdtc, where selenite was completely removed at a molar ratio of 2:1 siderophores to selenite.

The appearance and composition of metalloid precipitates formed abiotically upon reaction of chemically synthesized pdtc with selenite and tellurite were examined using SEM-EDS. Results were compared to results from analysis of the precipitates formed in bacterial cultures of *P. stutzeri* strain KC and CTN1 and culture filtrates of strain KC (Fig. 12.5 and 12.6). Only in cell-free spent culture filtrate of *P. stutzeri* KC containing pdtc did the addition of selenite or tellurite result in a, respectively, red or dark brownish precipitate, which indicated pdtc-induced reduction. The appearance and elemental makeup of these precipitates were more similar to precipitates formed in an abiotic reaction with pdtc rather than in bacterial cultures of strain CTN1, suggesting that it is mainly pdtc that is responsible for extracellular reduction and precipitation of these metalloids. Pdtc reactivity toward selenite resulted in a precipitate of similar properties to those of extracellular selenium precipitated by sulfate-reducing bacteria (Hockin and Gadd 2003). The presence of sulfur in selenium and tellurium precipitates formed by pdtc is indicative of pdtc thiol group involvement in metalloid oxyanion reduction. Elemental selenium and tellurium precipitation was a result of pdtc and pdtc:selenide and :telluride hydrolysis. In contrast, EDS analysis of intracellular selenium and tellurium precipitate formed by strain KC and CTN1 did not show the presence of significant amounts of sulfur (Fig. 12.6b, c). This indicates that there are different mechanisms involved in intracellular Se and Te deposit formation than those occurring extracellularly.

Electron microscopic and EDS analyses also allowed for localization of elemental selenium and tellurium deposition sites in bacterial cultures. SEM images of strain KC cultures clearly showed the abundance of extracellular spherical deposits of selenium (Fig. 12.6a) similar to those found in SRB biofilms (Hockin and Gadd 2003). TEM analysis showed the presence of spherical electron-dense selenium particles present inside the cells of both strain KC and CTN1, and intracellular Te^0 deposits associated with cell membranes of both strains (Fig. 12.6b, c). However, intracellular deposits were observed to be more abundant in strain CTN1. Clearly, pdtc was able to reduce and precipitate large amounts of selenium outside the cells of strain KC, but in the culture conditions used here, it did not completely prevent the entrance of selenite into the cells. We believe, however, that extracellular precipitation plays a significant role in preventing selenite toxicity, as was shown by a six-fold higher minimum inhibitory concentration (MIC) for selenite in stationary cultures of strain KC. Tellurium precipitates were more difficult to observe with SEM; EDS analysis indicated tellurium presence in association with either cells or extracellular matter. Although cells of strain KC contained less deposited tellurium, pdtc production did not prevent the entrance of tellurite into cells under our experimental conditions. In addition, the tellurite MIC for strain KC was not drastically

higher, as was the case for selenite, demonstrating a higher toxic effect of tellurite and reduced effectiveness of pdtc-mediated detoxification. Also tellurite was shown to be ~1,000-fold more toxic to *E. coli* than selenite (Turner et al. 2001). However, pdtc was previously shown to protect four other bacterial strains that do not produce pdtc from Te toxicity (Cortese et al. 2002), suggesting that pdtc decreases the bioavailability of tellurium.

We concluded that in addition to pdtc's other functions, production and secretin of pdtc results in extracellular metalloid reduction and serves as an environmental detoxification mechanism for *P. stutzeri* KC. We observed selenite removal in abiotic reactions as well as in spent culture filtrate of pdtc-producing strain KC, confirming that pdtc mediates toxic selenite reduction and Se^0 precipitation. The composition and appearance of selenium and tellurium precipitates formed in *P. stutzeri* KC cultures and in abiotic reactions of pure pdtc were similar, indicating a common route for precipitate formation. Pdtc can be considered as a preventive measure against selenite and tellurite toxicity before these metalloid oxyanions enter bacterial cells. Once secreted into the environment, thiol-containing molecules like pdtc can perform functions analogous to intracellular thiols, but without causing oxidative stress inside the cells. Unlike many other reductive detoxification mechanisms, pdtc-mediated selenite and tellurite reduction can take place in aerobic conditions.

12.5 Chromium(VI) Reduction Mediated by Pdtc

Cr(VI) occurring as chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) is a dominant form of chromium in aerobic conditions, and is considered more toxic and more mobile than Cr(III). Evaluation of chromium(VI) toxicity on *P. stutzeri* KC and CTN1 showed that Cr(VI) inhibited growth of *P. stutzeri* strain KC at concentrations higher than 25 mM, while strain CTN1, which lacks the ability to synthesize pdtc, was not able to grow in any of the dichromate concentrations tested (Fig. 12.7) (Zawadzka et al. 2007). Additionally, the concentration of dichromate was observed to decrease in actively growing cultures of strain KC. Both strains produced desferrioxamine siderophores, but only KC produced pdtc. These observations suggest a correlation between pdtc production and chromium resistance. Chromium toxicity in *P. stutzeri* KC results in a lengthened logarithmic growth phase and decreased growth rates, which is similar to the toxic effects of chromium on algae (Cervantes et al. 2001). When strain CTN1 was grown for 72 h in the presence of 10 mM Cr(VI), some growth was recorded, accompanied by a slight decrease in dichromate concentration. This result may be indicative of an alternative mechanism of chromium resistance, based for example on an inducible efflux mechanism (Aguilera et al. 2004) or enzymatic reaction (Park et al. 2000), but with these being less effective than pdtc activity.

Pdtc was found to reduce Cr(VI) to Cr(III) both in bacterial cultures and in abiotic reactions with chemically synthesized pdtc. Cr(III) subsequently formed

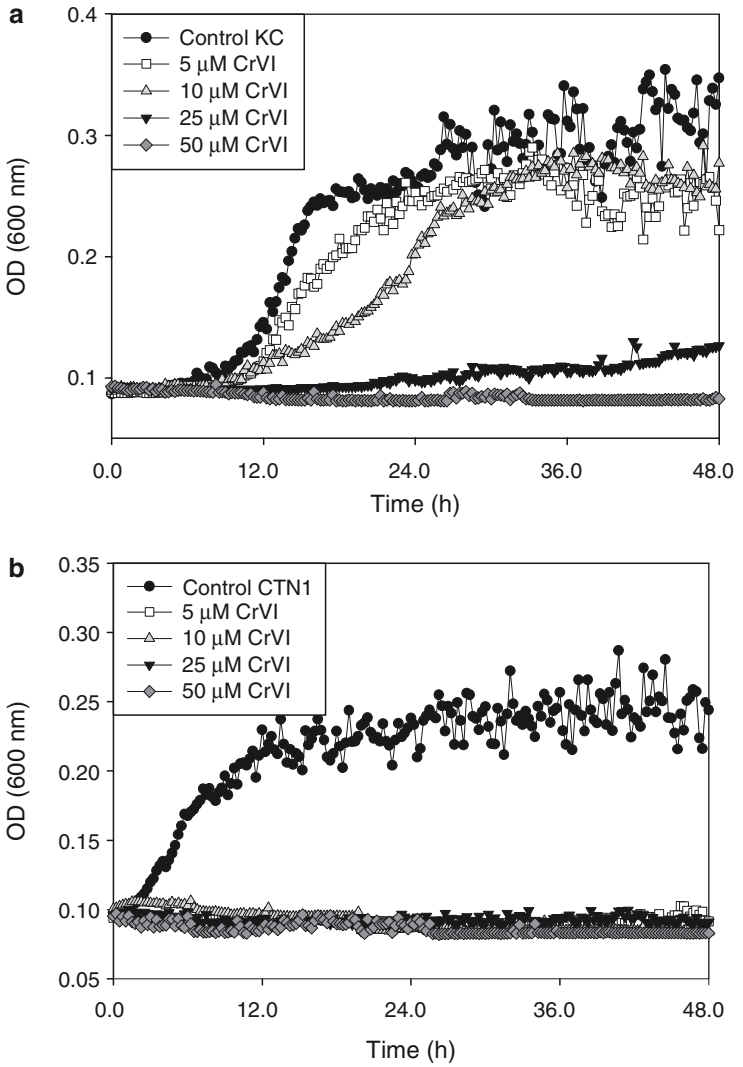

Fig. 12.7 Growth curves of (**a**) *P. stutzeri* KC and (**b**) CTN1 cultured in iron-limited media with Cr(VI) added as potassium dichromate in microtiter plate format. The 5 mM concentration of Cr(VI) inhibited growth of the CTN1 mutant that lacks a pdtc biosynthesis pathway. A concentration of 50 mM Cr(VI) was required to completely inhibit growth of the wild-type strain KC growth

complexes with pdtc and pdtc hydrolysis products, and their presence was confirmed using ESI-MS analysis (Fig. 12.8). We postulate that Cr(VI) is reduced to Cr(III) by H_2S released from pdtc hydrolysis, similarly to sulfide produced by sulfate-reducing bacteria (Chardin et al. 2003). The reduced chromium is then complexed by remaining pdtc and its hydrolysis products, pyridine-2-thiocarboxylic-

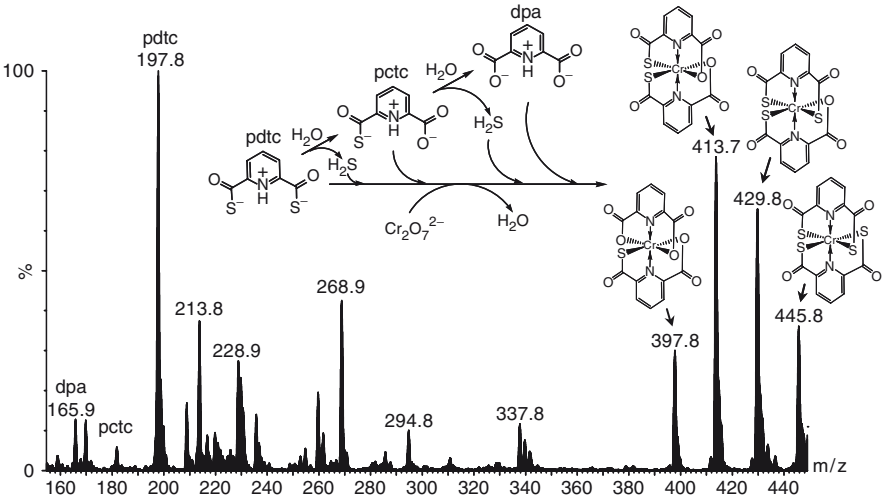

Fig. 12.8 ESI-MS spectrum and structures of the complexes of Cr(III) with pdtc and pdtc hydrolysis products in DMF formed upon interaction of pdtc with Cr(VI). The schematic shows possible interactions of Cr(VI) (as dichromate) and pdtc that lead to Cr(III):$(pdtc)_2$ and Cr(III):$(pdtc\ hydrolysis\ product)_2$ complexes, including hybrid complexes containing pdtc, pyridine-2-carboxy-6-thiocarboxylic acid (pctc), and dipicolinic acid (dpa)

6-carboxylic acid (pctc) and dipicolinic acid (dpa). The presence of the Cr(III) complexes and free pctc and dpa in solution provides evidence for these reactions. The complexes were fairly stable up to 90 days; however, their concentration decreased significantly with time, indicating slow hydrolysis of these complexes, accompanied by a black precipitate formation (Zawadzka et al. 2006a). The Cr(III) precipitate is expected to contain chromium(III) sulfides or chromium(III) hydroxides (Cervantes et al. 2001; Evanko and Dzombak 1997). The precipitate formed at high reagent concentrations was harvested and analyzed using SEM-EDS (Fig. 12.9). Elemental analysis revealed the presence of chromium and sulfur in the black precipitate, which possibly corresponds to water-insoluble Cr_2S_3. Consequently, we concluded that the reduction of dichromate by pdtc leads to the release of insoluble chromium(III) sulfides, oxides, and hydroxides. Also, culture filtrates of *P. stutzeri* KC containing biologically produced pdtc were effective at dichromate removal to the same extent as cell-containing cultures. Finally, abiotic reactions of chemically synthesized pdtc showed that pdtc was effective at dichromate reduction, but this process was more efficient in the presence of proferrioxamine B and transition metals (Zawadzka et al. 2007). Pdtc was most effective at Cr(VI) reduction at a pH of 7. At this pH, however, the metals themselves catalyzed dichromate reduction, a process well-recognized and used in Cr(VI) remediation by using zero-valent iron in permeable barriers (Evanko and Dzombak 1997). At a pH of 8, pdtc mixed with metals was more efficient at Cr(VI) reduction than pdtc itself,

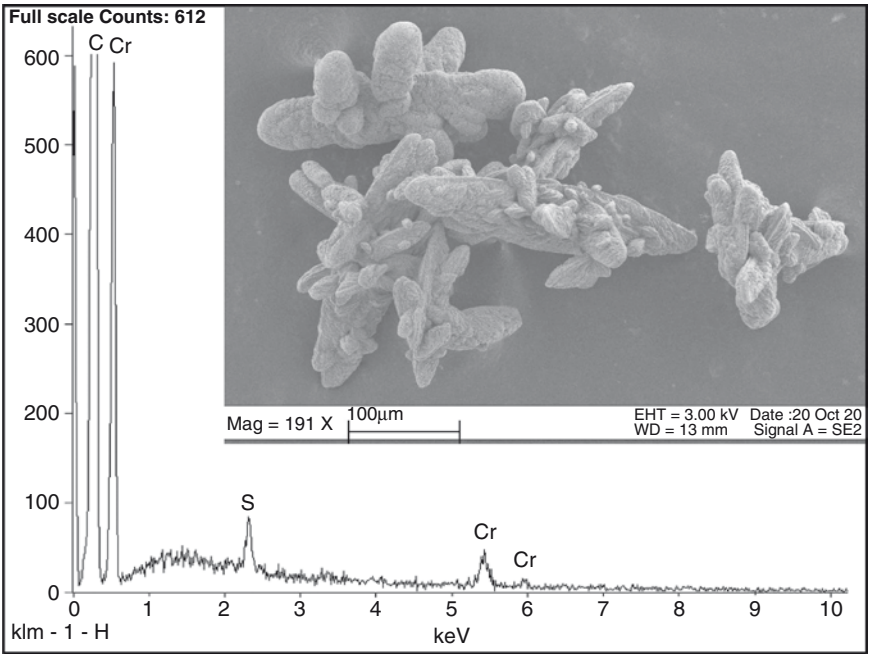

Fig. 12.9 EDS spectrum of black precipitate (picture) formed in the mixture of dichromate (10 mM) and pdtc (10 mM) in water; possibly Cr_2S_3

especially when present together with proferrioxamine B. This process cannot be attributed to metals alone, since in the controls containing metals without siderophores, the dichromate level remained constant. Because we have confirmed metal:pdtc complex formation in these conditions using ESI-MS, it appears that siderophore complexes with metals were even more effective at Cr(VI) reduction than pdtc-derived hydrogen sulfide.

12.6 Biotechnology Perspective of Microbial Interactions with Metals

Microbially-mediated processes affecting metal mobility are gaining more interest among researchers in environmental biotechnology. Microbial metabolism can significantly influence metal speciation and thus metal mobility and toxicity. The nature of interactions depends on availability of biologically important metal ions and a need for protection against toxicity of excessive amounts of both essential and toxic heavy metals. Bacteria can mobilize metals by leaching, through siderophore complexation, and through methylation or redox transformations. Bacteria also may immobilize metals by sorption, uptake and sequestration, precipitation

as organic or inorganic compounds (e.g., sulfides), or redox processes. From a bioremediation perspective, solubilization of metals provides a means of metal removal from solid waste, while immobilization of metals may be useful for *in situ* treatments of soils or sediments as well as metal removal from aqueous solutions (Gadd 2004). For example, siderophore-mediated metal solubilization by *Alcaligenes eutrophus* has been used for the treatment of metal-contaminated soil (Diels et al. 1999). Microorganisms use siderophores to solubilize Fe(III) for iron assimilation, but siderophores very often can bind other metals and thus act as metal solubilizing agents. For example, the siderophore pyridine-2,6-bis(thiocarboxylic acid) solubilizes ferric iron and several other metals, but also forms insoluble precipitates with many toxic metals and metalloids and can potentially be used for removal of those metals from solution (Cortese et al. 2002).

Another example of employing bacteria in treatment of metal contamination is the use of SRB for metal precipitation from water and soil, and for metal reduction (Diels et al. 2002; Gadd 2004; Valls and de Lorenzo 2002). SRB reduce sulfate to hydrogen sulfide that transforms metals into insoluble metal sulfides under anaerobic conditions. SRB can also mediate reductive precipitation of toxic metals and metalloids, including U(VI), Cr(VI), Tc(VI), As(V), Se(IV), and Te(IV). The reduction process can be enzymatic and involve cytochromes, or can be catalyzed indirectly by ferrous iron or biogenic sulfide. Dissimilatory metal reduction is widespread in microorganisms as a means of reducing metal toxicity, and has been used for bioremediation (Gadd 2004; Valls and de Lorenzo 2002). For instance, many organisms efficiently reduce Cr(VI) to Cr(III) by NAD(P)H-dependent reductases or cytochromes or microbially-produced hydrogen sulfide (Lloyd 2003; Lovley and Coates 1997). Also, bacteria use a variety of mechanisms to reduce metalloids like selenium, and this phenomenon has been employed for bioremediation efforts (Long et al. 1990; Oremland et al. 1991). Pdtc is a unique natural molecule that has numerous properties that might be exploited for use in bioremediation processes.

12.7 Conclusion

Pdtc is one of the most versatile among known siderophores, and it clearly has a role in bacterial environment conditioning (Budzikiewicz 2003). Here we reviewed newly recognized activities of pdtc, finding that precipitates formed by pdtc with heavy metals are unstable. The heavy metal:pdtc complexes decompose, releasing metal sulfides in the form of precipitates. We also described chemical interactions of pdtc with toxic selenium and tellurium oxyanions. Pdtc and pdtc-derived sulfides reduce Se(IV) and Te(IV), and the formed pdtc selenides and tellurides precipitate from solution. These insoluble compounds hydrolyze, releasing elemental selenium and tellurium, which are also insoluble and therefore non-bioavailable and non-toxic. The Se^0 and Te^0 precipitates formed in *P. stutzeri* KC cultures producing pdtc had properties similar to those of precipitates formed in abiotic reactions mediated

by chemically synthesized pdtc of high purity. Furthermore, the extracellular location of Se^0 supported a pdtc-mediated detoxification mechanism hypothesis. Moreover, we found that pdtc can reduce mobile and toxic chromium(VI) to less mobile and less toxic chromium(III) under aerobic conditions, and will chelate Cr(III) and subsequently release it in the form of chromium sulfides and possibly chromium oxides and hydroxides. Pdtc-producing *P. stutzeri* KC conferred higher tolerance to toxic metals than pdtc-negative mutant CTN1. Our results clearly suggest a function for pdtc in heavy metal and metalloid detoxification. The pdtc molecule can act as a reducer, supplying reactive hydrogen sulfide from hydrolysis, and redox active metal:pdtc complexes can transfer electrons to a substrate such as Cr(VI) and reduce it to Cr(III). Pdtc-mediated detoxification utilizes the chemistry of heavy metals (their extremely high affinity to S) to remove them from solution outside the cells by mediating precipitation of metals as their insoluble sulfides (Nies 1999; Raab and Feldmann 2003; Valls and de Lorenzo 2002). Pdtc can also be viewed as a provider of an extracellular pool of thiols that mediate reactions outside the cells, thus preventing intracellular metal toxicity by preventing entrance into the cells. The energetic expense of pdtc synthesis may be in the end less costly for *P. stutzeri* KC than the metabolic expense needed to handle toxic metals inside cells.

Pdtc is a member of a novel class of microbial metabolites with a uniquely broad array of properties. Our research lends support to the hypothesis that pdtc plays a role in bacterial environment conditioning in the cases of several metals and metalloids. The work expands the list of established functions of pdtc, which include its role as a siderophore, activity in carbon tetrachloride degradation, and antimicrobial properties. As a mechanism of pdtc-mediated metal detoxification, we can add to pdtc's function that of metal and metalloid reduction which results in metalloid precipitation in elemental form or as metal sulfides. It is unique for a siderophore ligand to function as a carrier of thiol groups to the outside of the bacterial cell, where they can catalyze different mechanisms that decrease metal and metalloid toxicity. These mechanisms include reduction of the metal to a less toxic form, formation of insoluble complexes, and precipitation of metals as insoluble sulfides. The great versatility of pdtc interactions with environmental contaminants including metalloids, metals, and chlorinated solvents like carbon tetrachloride make *P. stutzeri* KC an excellent candidate for the use in bioremediation processes (Cortese et al. 2002; Dybas et al. 1998).

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Chapter 13
Biomining Microorganisms: Molecular Aspects and Applications in Biotechnology and Bioremediation

Carlos A. Jerez

13.1 Introduction

The microbial solubilization of metals is widely and successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium and others (Rawlings 2002; Watling 2006). This process is done by using chemolithoautotrophic microorganisms. These microorganisms belong to those groups known as extremophiles, since they live in extremely acidic conditions (pH 1-3.0) and in the presence of very high toxic heavy-metal concentrations. A great variety of microorganisms is capable of growth in situations that simulate biomining commercial operations, and many different species of microorganisms live at acid mine drainage (AMD) sites (Hallberg and Johnson 2001; Schippers 2007). The most studied leaching bacteria are from the genus *Acidithiobacillus*. *Acidithiobacillus ferrooxidans* and *A. thiooxidans* are acidophilic mesophiles, and together with the moderate thermophile *A. caldus*, they belong to the Gram-negative γ-proteobacteria. *Acidithiobacillus ferrooxidans* is a chemolithoautotrophic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds (Lundgren 1980; Suzuki 2001; Rawlings 2002; Olson et al. 2003).

Members of the genus *Leptospirillum* are other important biomining bacteria that belong to a new bacterial division Nitrospora. Some Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium, Ferrimicrobium* and *Sulfobacillus* have also been described (Hallberg and Johnson 2001; Schippers 2007). Biomining extremely thermophilic archaeons capable of oxidizing sulfur and iron (II) have been known for many years, and they are mainly from the genera *Sulfolobus*, *Acidianus*, *Metallosphaera* and *Sulfurisphaera.* Recently, some mesophilic iron (II)-oxidizing

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archaeons belonging to the *Thermoplasmales* have been isolated and described: *Ferroplasma acidiphilium* and *F. acidarmanus*. In fact, a consortium of different microorganisms is responsible for the oxidative reactions which result in the extraction of dissolved metal values from ores (Hallberg and Johnson 2001; Schippers 2007).

13.2 Metal Mobilization and Generation of Acid Mine Drainage (AMD)

Some of the general oxidation reactions that acidophiles are able to catalyze are:

$$\text{Ferrous iron } 2Fe^{2+} + 0.5\ O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

$$\text{General metal sulfide } MS + 2O_2 \rightarrow M^{2+} + SO_4 + H^+$$

$$\text{Pyrite } 4FeS_2 + 15\ O_2 + 2\ H_2O \rightarrow 2Fe_2(SO_4)_3 + 2SO_4 + 2H^+$$

$$\text{Chalcopyrite } CuFeS_2 + 4Fe^{3+} \rightarrow 5\ Fe^{2+} + Cu^{2+} + 2\ S^0$$

$$\text{Sulfur } S^0 + 1\ ½\ O_2 + H_2O \rightarrow SO_4 + H^+$$

These reactions not only solubilize the metals present in the minerals but also generate sulfuric acid. The acidophilic microorganisms, therefore, are able to withstand not only low pH values but also very high metal concentrations (Dopson et al. 2003), since they possess heavy metal resistance or detoxification mechanisms (Das et al. 1998; Butcher et al. 2000; Dopson et al. 2003; Alvarez and Jerez 2004; Remonsellez et al. 2006). Neutrophilic bacteria have metal resistance mechanisms involving either an active efflux or a detoxification of metal ions by different transformations. In the case of copper, for example, these include intracellular complexation, decreased accumulation, extracellular complexation or sequestration in the periplasm. Neutrophilic bacteria are able to grow in a range of copper concentrations between 1 and 8 mM depending on the species (Outten et al. 2001; Puig et al. 2002). However, acidophiles such as *A. ferrooxidans* or archaeons such as *Sulfolobus metallicus*, can resist concentrations of copper up to 800 mM (Das et al. 1998) and 200 mM (Dopson et al. 2003) respectively. Most likely, they possess additional mechanisms to those present in neutrophiles, which allow them to have such dramatic metal resistance. It has been proposed that in microorganisms accumulating large amounts of inorganic polyphosphate (polyP), such as some chemolithoautotrophic acidophilic bacteria and archaea, these polymers may be actively involved in the elimination of toxic heavy metals such as Cu (Alvarez and Jerez 2004; Remonsellez et al. 2006). This detoxification would take place through the enzymatic hydrolysis of polyP that generates free phosphate, which would bind the excess of cytoplasmic metal to form a metal–phosphate complex which is transported outside the cell through phosphate transporters.

The properties already described make these microorganisms very appropriate for their use in biomining. Also, they are used to remove pyrite and sulfur compounds from coal, and for the bioremediation or removal of heavy metals from polluted places.

13.3 Molecular Aspects of Acidophilic Microorganisms-Mineral Interactions

It is known that most leaching bacteria grow attached to the surface of the solid substrates such as elemental sulfur and metal sulfides. This attachment is predominantly mediated by extracellular polymeric substances (EPS) surrounding the cells, and whose composition is adjusted according to the growth substrate. Bacteria attach to the surface of the mineral carrying Fe (III) bound to its exopolysaccharides, and when the microorganism forms a biofilm, this metal would chemically attack the metal sulfide generating, in the case of pyrite, ferrous iron that is reoxidized to Fe (III) and thiosulfate which can be further oxidized to sulfuric acid (Rohwerder et al. 2003). This close contact of the bacterium with the mineral makes for more efficient and specific sulfide oxidation.

The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the reoxidation of the generated ferrous iron to obtain additional ferric iron. Considerable effort has been spent in the last years to understand the biochemistry of oxidation of iron and sulfur compounds (Rohwerder and Sand 2003; Ramirez et al. 2004; Rawlings 2005; Valenzuela et al. 2006), bacteria–mineral interactions (chemotaxis, quorum sensing, adhesion, biofilm formation) (reviewed in Jerez 2008 and Ruiz et al. 2008) and several adaptive responses allowing the microorganisms to survive in a bioleaching environment (Das et al. 1998; Butcher et al. 2000; Dopson et al. 2003; Alvarez and Jerez 2004; Remonsellez et al. 2006; Chi et al. 2007; Quatrini et al. 2007; Vera et al. 2008). All of these are considered key phenomena for understanding the process of biomining.

The reactions involved in ferrous iron oxidation by *A. ferrooxidans* have been studied in detail. In the electron transfer pathway from ferrous iron to oxygen, the terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin, and cytochrome c552. A high molecular weight c-type cytochrome, Cyc2, has been implicated as the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (Yarzábal et al. 2004; Rawlings 2005). This pathway is represented by: Cyc2 → rusticyanin → Cyc1(c552) → aa3 cytochrome oxidase. In addition, there is an apparent redundancy of electron transfer pathways via bc(1) complexes and terminal oxidases in *A. ferrooxidans* (Bruscella et al. 2007) .

The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (Silver and Lundgren 1968;

Sugio et al. 1987; Kelly et al. 1997; Suzuki 1999; Rohwerder and Sand 2003; Müller et al. 2004). Thiosulfate has been postulated as a key compound in the oxidation of the sulfur moiety of pyrite (Schippers and Sand 1999). Iron (III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental sulfur being a side product. Some of the reactions to oxidize ferrous iron and some sulfur compounds that take place at the surface of *A. ferrooxidans* are illustrated in Fig. 13.1.

Effective tools for the study of *A. ferrooxidans* genetics and physiology are not in widespread use and, despite considerable effort, an understanding of its unusual physiology remains at a rudimentary level. An efficient and reproducible technique for DNA transfer is still missing (Rawlings 2005; Valenzuela et al. 2006). *A. ferrooxidans* was the first biomining microorganism to have its genome entirely sequenced, and the annotation of all its genes is now available (http://www.tigr.org). This information has been very useful to many researchers, to look for the genome-wide candidate genes for important metabolic pathways and several important physiological functions, and to predict for the functions of many new genes. The main focus of research has been the energetic metabolism which is directly responsible for bioleaching. Some researchers have used "chromosome walking" to find genes involved in sulfur and iron metabolisms (Rawlings 2005; Valenzuela et al. 2007). The genomics (Quatrini et al. 2006) and high throughput proteomics studies of the global regulatory responses that the biomining microorganisms use to adapt to their changing environment are just beginning to emerge (Valenzuela et al. 2006). As an example, the entire set of periplasmic proteins (around 130) present in *A. ferrooxidans* grown in thiosulfate has been determined experimentally by using high throughput proteomics. About 30% of these proteins are without homologues in databases ("unknown proteins" in Fig. 13.1), indicating that they are most likely characteristic of this bacterium and may have important roles yet to be assigned (Chi et al. 2007).

Metagenomics is the culture-independent genomic analysis of microbial communities (Handelsman 2004; Streit and Schmitz 2005). In conventional shotgun sequencing of microbial isolates, all the shotgun DNA fragments are derived from clones of the same genome. To analyze the genomes of an environmental microbial community, the ideal situation is to have a low-diversity environment. Such systems were found when analysing the microbial communities inhabiting a biofilm developed in a site of extreme AMD production. Still, variation within each species might complicate assembly of the DNA fragments. Nevertheless, random shotgun sequencing of DNA from this natural acidophilic biofilm was used (Tyson et al. 2004). These authors were able to reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II, and partially recover three other genomes. The extremely acidic conditions of the biofilm (pH about 0.5) and relatively restricted energy source combine to select for a small number of species, a situation ideal for testing these new culture-independent genomic approaches in the environment (Tyson et al. 2004).

The analysis of the gene complement for each organism has revealed the metabolic pathways for carbon and nitrogen fixation and energy generation. For example, genes for biosynthesis of isoprenoid-based lipids and for a variety of

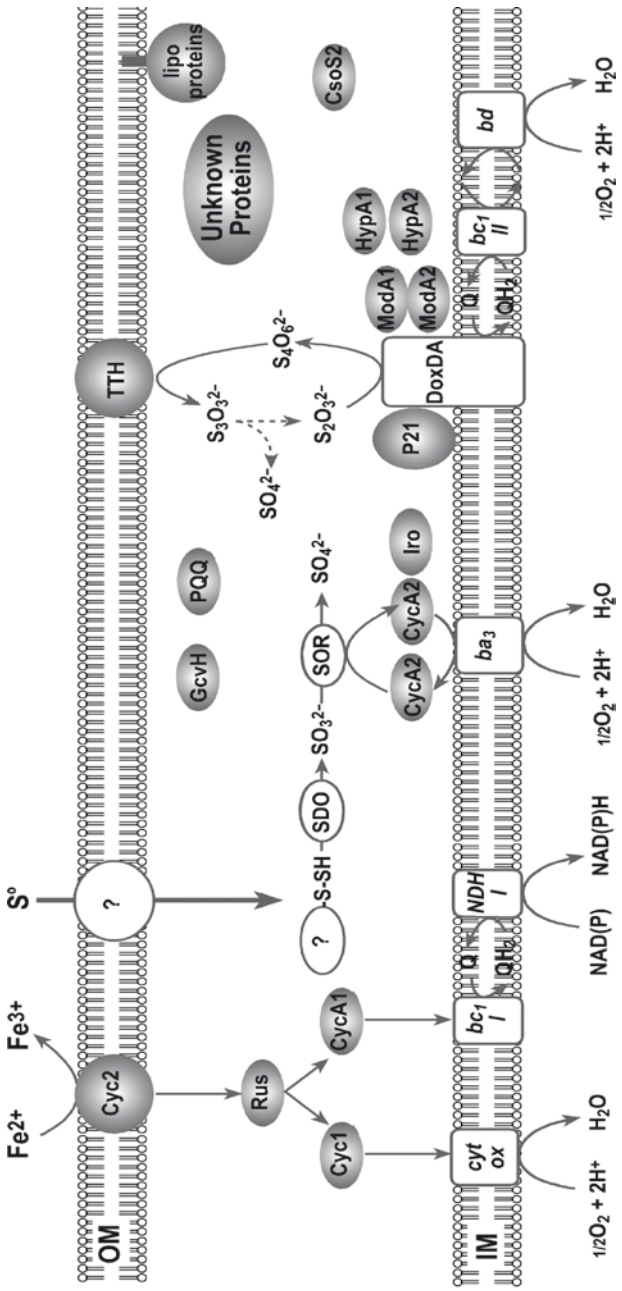

Fig. 13.1 General scheme showing how an acidophilic chemolithoautotroph such as *A. ferrooxidans* oxidizes ferrous iron and sulfur and reduced sulfur compounds to generate sulfuric acid. Surface proteins from *A. ferrooxidans*, most of which are periplasmic proteins involved in energetic metabolism, are shown. The proteins shaded in *gray* were identified experimentally by proteomic analysis of the periplasmic fraction. The unknown periplasmic proteins indicated are most likely unique of *A. ferrooxidans*, and may have several different unknown functions (Chi et al. 2007). Adapted from Rawlings 2005; Chi et al. 2007 and Jerez 2008

proton efflux systems were identified, providing insights into survival strategies in the extreme acidic environment. However, this information will have to be confirmed by biochemical and physiological approaches. Clearly, the metagenomic approach to the study of microbial communities is a real advancement in fully understanding how complex microbial communities function, and how their component members interact within their niches.

A metaproteomic approach has been used to study the community in a natural AMD microbial biofilm (Ram et al. 2005; Lo et al. 2007). These authors were able to detect 2,033 proteins from the five most abundant species in the biofilm, including 48% of the predicted proteins from the dominant biofilm organism, *Leptospirillum* group II. The authors also determined that one abundant novel protein was a cytochrome which is central to iron oxidation and acid mine drainage formation in the natural biofilm (Ram et al. 2005). Lo et al. (2007) used community genomic data sets to identify, with strain specificity, expressed proteins from the dominant member of a genomically uncharacterized natural acidophilic biofilm. Proteomics revealed inter-population genetic exchange, which may be crucial for adaptation to specific ecological niches within the very acidic metal-rich environment studied. All this knowledge, together with that obtained in other bioleaching microorganisms, will allow future improvements in industrial bioleaching and bioremediation processes.

Recently, the description in theoretical and practical terms of how to develop and optimize a mineral-oxidizing microbial consortium (naturally isolated or constructed) depending on the mineral being used has been described (Rawlings and Johnson 2007). It is possible that in the future, similar consortia could also be designed to control the generation of AMD or to bioremediate contaminated sites.

13.4 Biomining Microorganisms and Their Industrial Applications

Industrial biomining operations are of several kinds depending on the ore type and its geographical location, the metal content and the specific minerals present (metal oxides, metal sulfides of different kinds). One of the most used setups for the recovery of gold or copper is the irrigation type of processes. These involve the percolation of leaching solutions through crushed ore that can be contained in a column, a heap or a dump (Watling 2006). In Fig. 13.2 , a scheme is presented in which the crushed ore to bioleach is transported to an agglomeration tank or drum where it is acidified. This process is a key one, since the bigger ore particles are surrounded by the very fine particles that stick to them, thus preventing all the particles, especially the fine material, from sedimenting to the bottom of the heap. In this way, irrigation and aeration of the heap takes place from the top to the bottom, allowing a much more homogeneous growth of the microorganisms and therefore a better metal solubilization.

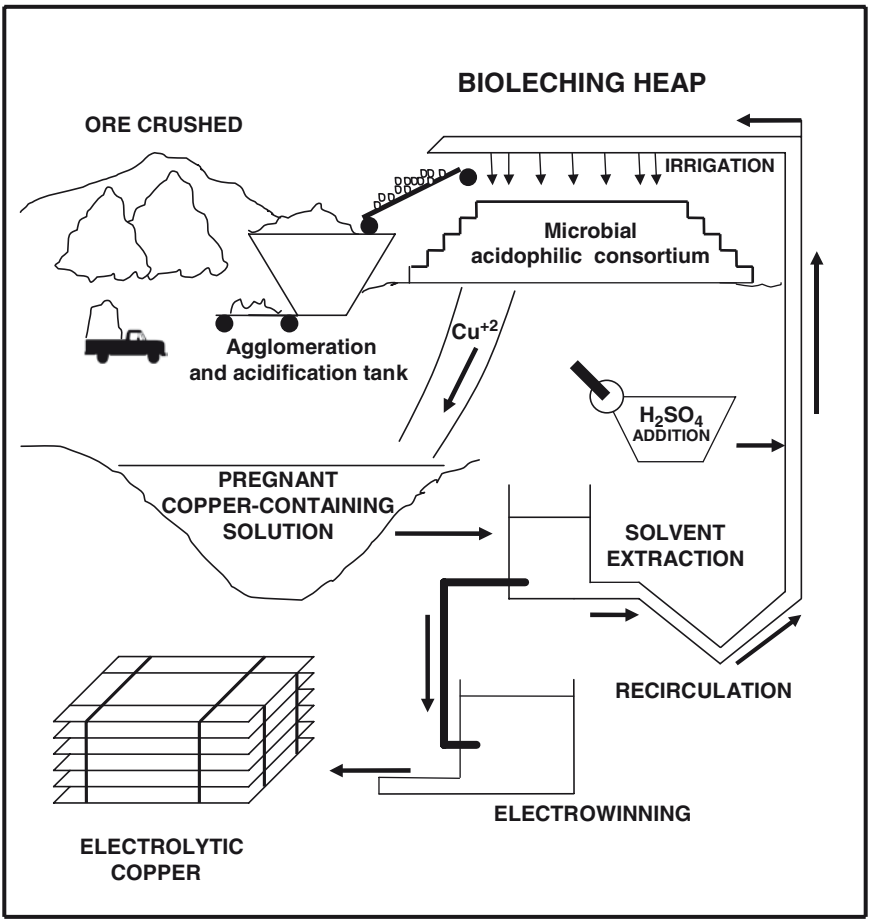

Fig. 13.2 A scheme showing heap bioleaching of minerals, one of the most common industrial biomining set-ups

The heap can be 6–10 m tall and 100 or more meters long and wide, and is constructed over irrigation pads lined with high-density polyethylene to avoid losses of the pregnant copper-containing solution. This solution, containing copper sulfate generated by the microbial solubilization of the insoluble copper sulfides present in the ore, is subjected to solvent extraction, to have a highly concentrated copper sulfate solution from which the metal is recovered in an electro-winning plant to generate electrolytic copper of high purity (Fig. 13.2). Since most mining operations are located in areas where water is scarce, the spent leach liquors or raffinates are recirculated to the heap for further irrigation.

Bioleaching bacteria can also be used for gold recovery (Rawlings 2002). Gold is usually found in nature associated with minerals containing arsenic and pyrites

(arsenopyrites). During gold bioleaching, the iron- and sulfur-oxidizing microorganisms attack and solubilize the arsenopyrite, releasing the trapped gold particles. Followed this release, the gold is complexed with cyanide according to standard gold-mining procedures. Instead of using big leaching heaps or dumps as in the case of bioleaching of copper ores, gold bioleaching is implemented by using highly aerated stirred tank bioreactors connected in series. Since these reactors are expensive to build, they are used with high-grade ores or with mineral concentrates. The advantage of tank reactors over heaps and dumps, which are "open bioreactors", is that conditions in the tanks can be controlled, thus facilitating a much faster and efficient metal-extraction process.

Currently, there are operations using both mesophilic and thermophilic microorganisms (Watling 2006). Biomining has distinctive advantages over the traditional mining procedures. For example, it does not require the high amounts of energy used during roasting and smelting, and does not generate harmful gaseous emissions such as sulfur dioxide. Nevertheless, AMD can be generated, which if not properly controlled pollutes the environment with acid and toxic metals. Biomining is also of great advantage, since not only discarded low-grade ores from standard mining procedures can be leached in an economically feasible way but also some high-grade ores. In countries like Chile, which is currently the first world copper producer, many mining operations process from 10,000 to 40,000 tons of ore per day, and produce between 10,000 and 200,000 tons of copper per year by using heap or dump bioleaching of minerals such as oxides, chalcocite, covellite, chalcopyrite and others. Similar situations exist in the United States, Australia, and other countries. The most successful ones have been those processing copper oxides and secondary copper sulfides. However, chalcopyrite is the most abundant copper sulfide in the world. Since it is the most difficult to solubilize by microorganisms, there is actually great interest in developing processes mainly using thermophilic biomining microorganisms (Watling 2006).

13.5 Environmental Bioremediation of AMD and Metals Using Biomining Microorganisms

13.5.1 General Methods for AMD Bioremediation

As discussed before, when acidophiles oxidize elemental sulfur and reduced sulfur species such as sulfides, sulfite, polysulfides and several others, they generate sulfuric acid that acidifies their environment to pH values between 1 and 2 (Suzuki 1999; Rohwerder and Sand 2007). As a consequence, these microorganisms have an important impact in the environment, generating a serious contamination with acid and toxic metals that can get into water sources as AMD. This environmental pollution can be generated in many active biomining industrial operations or in abandoned mining sites, as well during normal biogeochemical processes. Therefore, there is an important need to control and optimize sulfur oxidation (Rohwerder and Sand 2007).

The main factors influencing the generation of acid include pH, oxygen availability, chemical oxidizing activity of Fe(III), exposed surface area of the mineral and bacterial activity among others. Its environmental impact can be minimized by preventing the acid-generating process, containment of acid drainages and by collection and treatment of the effluents (Akcil and Koldas 2006). AMD should be remediated or abated. Often there is a sealing of the contaminated sites or the location of barriers to contain the acidic fluids. Many approaches use prevention techniques to avoid further spillage of acidic effluents in the contaminated area. It can be controlled by chemical treatments such as the use of calcium oxide that neutralizes the acid pH. It is also possible to inhibit the acidophilic microorganisms responsible for the acid generation. This can be done by using certain organic acids, sodium benzoate, sodium lauryl sulfate or quarternary ammonium compounds that will affect the growth of bacteria such as *A. ferrooxidans*. The choice of which remediation option to use is determined by several economical and environmental factors. Reviews on most of the passive and active abiotic or biological methods for AMD remediation are available (Johnson and Hallberg 2005; Akcil and Koldas 2006). Therefore, only some recent developments in bioremediation by using acidophilic microorganisms will be reviewed here.

13.5.2 Bioshrouding to Prevent AMD Generation

To minimize the exposed surface area of the minerals to be attacked by acidophilic chemolithoautotrophs, the novel technique called "bioshrouding" for safeguarding highly reactive sulfidic mineral tailings has been proposed (Johnson et al. 2008). Freshly-milled wastes are colonized with heterotrophic acidophilic bacteria capable of reducing Fe(III). These microorganisms form biofilms on the reactive mineral surfaces, covering the mineral with EPS. In this way, the iron-oxidizers are prevented from attaching to the mineral. Figure 13.3 shows a scheme illustrating this idea.

Initial experiments have shown that the dissolution of pyrite could be reduced by 57–75% by "bioshrouding" the mineral with species of *Acidiphilium, Acidocella* and *Acidobacterium* spp., under conditions allowing the microbial oxidative dissolution of pyrite (Johnson et al. 2008). Bioshrouding of sulfide minerals could in theory be readily achieved in the field by adding low-cost substrates and using the most effective heterotrophic acidophilic bacteria.

13.5.3 Bioremediation of Heavy Metals

Bioremediation or removal of the toxic metals from contaminated soils can be achieved by a very interesting combination of two opposite biological activities: that of sulfur-oxidizing bacteria with the one of the sulfate-reducing microorganisms. In a first step, the sulfur-oxidizing bacteria generate sulfuric acid which bioleaches

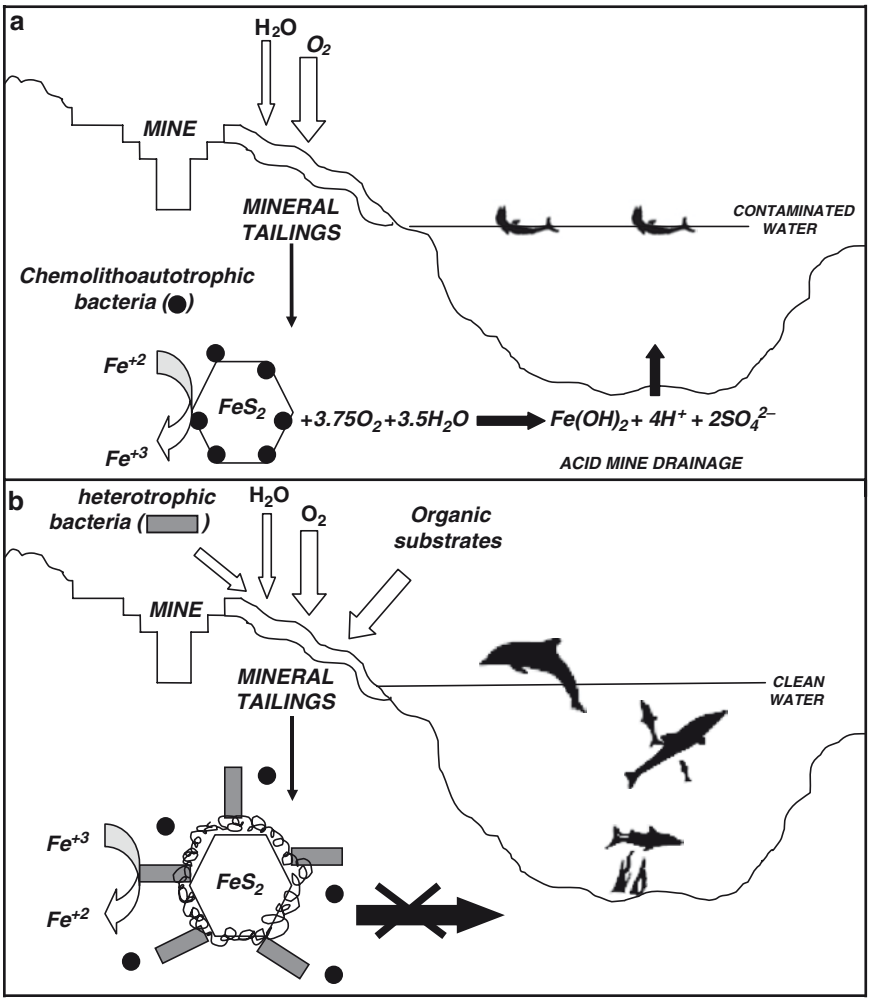

Fig. 13.3 Use of "bioshrouding" for securing reactive mineral tailings to prevent contamination by AMD. **a** A setting where mineral tailings containing pyrite and other metal sulfides are being solubilized by iron-oxidizing acidophilic chemolithoautotrophs, generating acid and toxic metals that contaminate close sources of water. **b** Inoculation of the mineral with heterotrophic acidophiles, together with their appropriate organic substrates, would avoid the attachment of iron oxidizers to the minerals, preventing or greatly reducing AMD that contaminates the water. The scheme was based on the work of Johnson et al. 2008

or solubilizes the metals in the solid phase of the soil. The leachate metals are then precipitated in a second step by using a bioreactor in which the hydrogen sulfide generated by the sulfate-reducing bacteria under neutral and anaerobic conditions

forms insoluble metal sulfides (Barnes et al. 1991; Lloyd et al. 2005) (Fig. 13.4). This process has been patented under the registered trademark Thiopaq. Metal contaminants such as Cu, Cd, Ni and others can be efficiently leached from contaminated soils. The effluents obtained from such a process are sufficiently cleansed of the metals that they can be reused in the environment (White et al. 1998).

A. ferrooxidans has been used for the removal of different metals from various contaminated sources at laboratory scale. These studies offer promising ways to treat different wastes in the industry. Some examples are the use of this microorganism for bioremediation of AMD. The addition of *A. ferrooxidans* to a real AMD increased metal precipitation kinetics by approximately 70%, thus accelerating metal removal (Darkwah et al. 2005). Mercury was volatilized and recovered from mercury-polluted soils and wastewaters by using a mercury-resistant *A. ferrooxidans* strain (Takeuchi and Sugio 2006). On the other hand, bacterial removal of chromium (VI) and (III) from real electroplating waste has been reported (Cabrera et al. 2007).

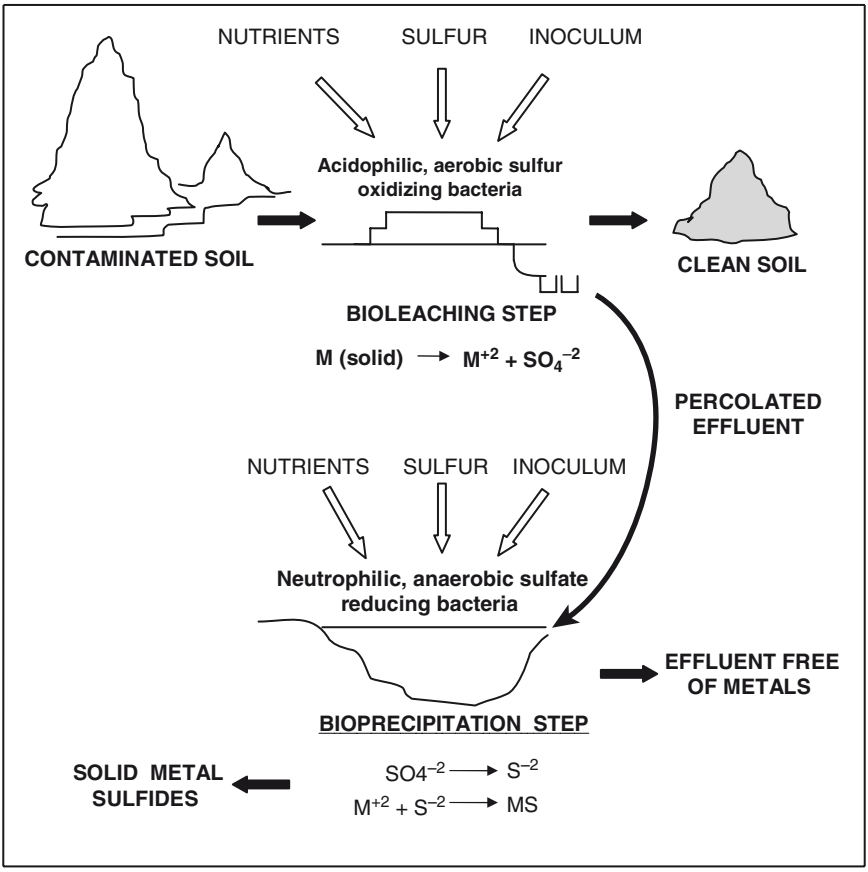

Fig. 13.4 Schematic industrial application of a process to remediate metal-contaminated soils

These authors removed about 93% of the hexavalent chromium, representing a promising way to treat this kind of industrial waste.

13.5.4 Bioremediation of Arsenic

Arsenic is a very important pollutant in many places around the world. It is an extremely toxic metalloid that can affect not only the environment but human health. The World Health Organization has classified it as a strong carcinogen. Its toxicity is due to arsenate being capable of substituting phosphate in the cells, and arsenite for its action on protein thiol groups and damage caused in both proteins and DNA (Norman 1998).

Arsenic contaminates water sources, mainly due to mining of arsenopyrites to extract gold or other metals. Also, anthropomorphic sources such as pesticides and herbicides containing arsenic contribute to the toxic matter entering water supplies. Due to its great toxicity and the much more demanding regulatory limits allowed for arsenic in aquatic sources, there is a greater need for efficient methods to remove this toxic metal.

Several methods are used to remove arsenic from contaminated waters. They include conventional sorption, coagulation and others. Several biological methods have also been implemented for bioremediation, due to their lower costs for metals bioremediation. Several species of different heterotrophic bacteria are used for this purpose, as can be seen in other chapters from this book. However, only a few approaches with acidophilic biomining microorganisms will be described here.

Bioleaching of arsenopyrite has been described for *A. ferrooxidans,* (Ehrlich 1964; Collinet and Morin 1990), since it is a bacterium capable of tolerating high concentrations of arsenic due to the presence of arsenic resistance genes on its chromosome (Butcher et al. 2000). Duquesne et al. (2003) have shown that *A. ferrooxidans* is not only resistant to arsenic but is also involved in arsenic precipitation. An *A. ferrooxidans* strain CC1 was isolated from the effluents of As-rich pyrite-rich tailings of an abandoned mining site. This strain was able to remove arsenic from a defined synthetic medium only when grown on ferrous iron, and it did not oxidize arsenite to arsenate. The isolated microorganism unexpectedly precipitated arsenic as arsenite but not arsenate, with the ferric iron generated by its energetic metabolism. Removal of arsenite by coprecipitation with ferric iron appears to be a common property of *A. ferrooxidans* species, and therefore could be used to efficiently remove arsenite from heavily contaminated waters (Duquesne et al. 2003).

Northern Chile is an active mining region with high concentrations of both natural and anthropogenic arsenic. Therefore, there is great interest in controlling and remediating this contamination. To control AMD containing high levels of As, microorganisms such as *A.ferrooxidans* can be used. When arsenopyrites are treated in the presence of Fe (III), scorodite is generated:

$$FeAsS + 14Fe(III) + 10H_20 \rightarrow 14Fe(II) + SO^{=}_4 + FeAsO_4 \cdot 2\,H_2O + 16\,H^+$$

This reaction is catalyzed by *A. ferrooxidans* since it oxidizes Fe (II) to Fe (III). The formation of scorodite involves the oxidation of arsenopyrite at low pH to give

locally high arsenate and iron activities which lead to scorodite precipitation (Dove and Rimstidt 1985). One example of the use of this reaction is an invention which provides a process for removing and immobilizing arsenic from arsenic-containing waste (Ruitenberg and Buisman 2000). This comprises oxidizing the arsenic to pentavalent arsenic in an aqueous medium by contacting Ars(V) with Fe(III) to form an insoluble scorodite, and the separation of the precipitated iron–arsenic compound from the aqueous medium. The oxidation of arsenic is implemented by using different mesophilic and thermophilic chemolithoautotrophic bacteria and archaea capable of oxidizing sulfur and Fe(II) at pH values between 0.5 and 4 and at temperatures between 20 and 90°C in the presence of a mineral catalyst such as pyrite (Ruitenberg and Buisman 2000). This process involves different reactors and tanks, and is claimed to be much less expensive than the conventional chemical oxidation using oxygenated autoclaves at about 90°C.

13.5.5 Biosensors to Monitor Arsenic and other Metals Bioremediation: Use of Biomining Microorganisms-derived Genetic Constructions

Biosensors are devices that use a biological component to detect a signal such as a small amount of a metal in solution. In general, they offer several advantages such as specificity, sensitivity, portability and measurement of the bioavailable fraction (Yagi 2007). However, several disadvantages also exist, such as a short lifetime, toxicity of samples and other factors which still have to be resolved in order to have a practical tool to be used in the field (Harms et al. 2006).

The most common design is a whole-cell bacterial biosensor in which a recombinant genetic construction, including a response element to which the chemical to be detected (extracellular signal), binds and as a consequence induces the expression of a reporter gene which will emit a signal proportional to concentration of the external stimulus. Several of these biosensors to detect arsenic have been constructed by using genetically modified whole *E. coli* cells. These biosensors have variable sensitivities under 50 μg As/l (Guiliani et al. 2001; Stocker et al. 2003; Yagi 2007). In *E. coli* the arsenic resistance system is composed of the ars operon containing the regulatory genes *arsR* and *arsD* and the structural genes *arsA, arsB* and *arsC* (Wu and Rosen 1991; Kaur and Rosen 1992). Figure 13.5 shows an example of the use of some of these genes to construct an *E. coli* biosensor (Guiliani et al. 2001). The repressor ArsR binds to the promoter/operator region (P/O) of the *ars* operon, inhibiting the transcription of the genes related with arsenite resistance (in this case arsB and arsC in the constructions shown in the figure). When As (V) and As (III) are present, they bind to ArsR, causing its dissociation from the DNA and allowing the expression of the downstream gene(s). If these genes are replaced by a reporter gene such as *luc* from the luciferase reaction of the firefly, in the presence of As, a light signal can be measured by means of a luminometer. This signal in turn will be proportional to the concentration of As present in the sample. When the construction was done using the genetic elements of the *ars* operon from *A. ferrooxidans*

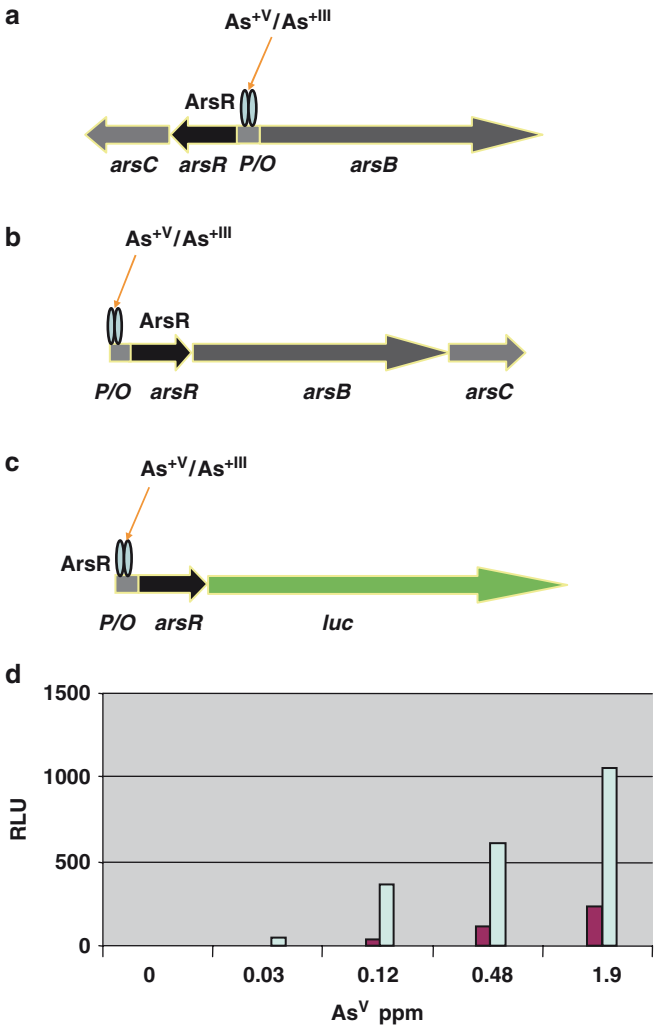

Fig. 13.5 Construction of a biosensor to detect and monitor arsenic in contaminated drinking water. **a** genomic context of some of the genes from *A. ferrooxidans* ATCC 33,020 involved in arsenic resistance (Butcher et al. 2000). **b** Part of the *ars* operon from *E. coli* (Xu and Rosen 1997). **c** A genetic construction in which the reporter gene *luc* is under the control of *A. ferrooxidans ars* promoter. **d** Relative light emission (RLU) units of a whole-cell *E. coli* biosensor, with the genetic construction shown in c (*empty bars*), or with a similar construction in which arsenic resistance genetic elements from *E. coli* were used (*filled bars*). A control *E. coli* containing only the plasmid used to carry the genetic construction was also measured, resulting in undetectable light

(Fig. 13.5 d), a several-fold increase in the signal intensity was obtained (Guiliani and Jerez, unpublished results). These results are promising, and can be improved further by controlling variables such as the use of phosphate-starved cells to make the biosensor sensitive enough to determine As in potable water.

13.5.6 Recycling Waste Metals to Avoid Environmental Pollution

Bioleaching microorganisms such as *A. ferrooxidans* can also have other uses to help avoiding metal contamination in modern societies. For example, this bacterium has been successfully used to recover metals such as cadmium from spent batteries. By using bioreactors, *A. ferrooxidans* is grown attached on elemental sulfur. The bacteria generate sulfuric acid through the oxidation of sulfur, which is then used for the indirect dissolution of spent nickel–cadmium batteries, recovering after 93 days 90–100% of cadmium, nickel and iron (Cerruti et al. 1998). Bioleaching of spent lithium ion secondary batteries, containing lithium and cobalt, has also been explored by using *A. ferrooxidans* (Mishra et al. 2008). These authors concluded that it is possible to use the acid generated during growth of the bacterium in the presence of sulfur and iron to recycle the valuable metals. A similar approach has been implemented for the treatment of a spent nickel catalyst using *A. thiooxidans*, and the nickel was recovered from the leachates by precipitating the metal with sulfide generated by *Desulfovibrio* cells (Bosio et al. 2007). Also the microbial recovery of copper from printed circuit boards of waste computers by *A. ferrooxidans* has been achieved (Choi et al. 2004). These approaches are not only economically valuable but may be effective methods for recycling spent and discarded abundant metal-containing materials, thus preventing many problems of environmental pollution.

13.6 Conclusions

The microbial solubilization of metals in acid environments is successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium and others. On the other hand, the acidophilic microorganisms mobilize metals and generate AMD, causing serious environmental problems. However, bioremediation or removal of the toxic metals from contaminated soils can be achieved by using the specific properties of acidophilic and other microorganisms interacting with metals. The choice of bioremediation option to use is determined by several economic and environmental factors.

Current approaches to studying microorganisms consider the microorganism or the consortia as a whole, integrating fundamental biological knowledge with genomics, proteomics, metabolomics, and other data to obtain a global picture of how microbial cells or communities function. This new knowledge will not only enhance our understanding of microbiological phenomena, but will also lead to improved applied microbial biotechnologies such as biomining and bioremediation of metals in acidic environments.

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Chapter 14
Advances in Phytoremediation and Rhizoremediation

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14.1 Introduction

With an ever increasing level of importance being accorded to biological remediation systems, the role that plants play in this process cannot be excluded from any comprehensive book. Most of the problems connected with environmental contamination can also have a phyto-solution and be addressed with the help of plants (McCutcheon and Schnoor 2003; Mackova et al. 2006). Phytoremediation does not solely rely on plant functions, and must always be considered in combination with the effect of rhizospheric microorganisms (Rittmann 2006; Mackova et al. 2006). Although plants have an inherent ability to detoxify some xenobiotics (i.e., to make them non-phytotoxic), in contrast to microorganisms plants generally lack the mechanisms necessary for the complete degradation of toxic compounds (Eapen et al. 2007). Plants often use pathways and enzymes similar to those present in mammals, which led to the 'green liver' concept (Sandermann 1994). However, being autotrophic organisms, plants do not utilize organic compounds for their energy and carbon metabolism (Van Aken 2008). As a consequence, plants usually lack the catabolic enzymes necessary to achieve full mineralization of organic molecules, which results in the accumulation of toxic metabolites (Eapen et al. 2007) that could be later released into the environment.

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Plants remove compounds from soil by direct uptake of the contaminants, as summarised by Schnoor et al. (1995), followed by subsequent transformation, transport and contaminant accumulation in a form non-toxic to plants, which does not necessarily mean non-toxic to humans. If the biotransformation reactions lead to a decrease of the toxicity of a given xenobiotic compound, the reaction is called detoxification. In some cases, the biotransformation reactions can lead also to an increase in the toxicity of the compounds. This is mostly the case with organic compounds. It can be the outcome of transforming a recalcitrant compound into a more easily degradable one. Thus, a more toxic or more soluble compound which has been formed can be rendered harmless in further steps. The use of plants for transfer, accumulation and removal of pollutants from the environment, or at least reduction of their mobilities, has been discussed for more than a decade (Schnoor et al. 1995; Cunningham et al. 1995; Macek et al. 2000, 2004). The approach can be used for removal of both inorganic and organic xenobiotics and pollutants present in the soil, water and air. One of the main aims is to prevent migration of pollutants to a site which would cause greater risk to human health (Salt et al. 1995). The type of contaminants range from inorganic fertilisers to pesticides, from heavy metals and trace or radioactive elements to explosives (French et al. 1999), oil spills to chemical weapons (Macek et al. 1998, 1999). Endocrine-disrupting chemicals like tributyl tin, bisphenol A or nonylphenols (Bock et al. 2002) are a focus of interest, as well as recalcitrant organic compounds like PCBs or PAHs. Remediation of chloroacetanilide herbicides and explosive compound removal has been studied among others by Mezzari et al (2005). A large-scale comparison of the activity of glutathione S-transferase in plants from various treatment sites in Europe (Schroeder et al. 2008) addresses the second phase in plant detoxification: namely, the conjugation of the activated compound. This aspect has recently been summarised in books (McCutcheon and Schnoor 2003; Mackova et al. 2006).

Harvesting plants is a familiar agricultural technology, but most of the highly productive plants accumulate usually amounts of pollutants too low to represent an economically feasible approach for cleaning of contaminated soil. Finding or developing plants that acquire high levels of metal contaminants in harvestable tissue was thought impossible until the (re)discovery of a small group of remarkable plants called hyperaccumulators. These plants are relatively uncommon, but present throughout the plant kingdom, and can accumulate surprising amounts of some metals (Brown et al. 1995), probably by mechanisms similar to the uptake of metals essential for their enzymatic activities. Plants have developed their own systems for binding of heavy metals based largely on the synthesis of phytochelatins, described by Grill et al. (1989). Heavy-metal binding in plants is normally achieved (Kotrba et al. 1999; Mejare and Bulow 2001) by different peptides, phytochelatins and phytosiderophores. The latter are known to be ubiquitous in plants since studies on nicotianamine by Rudolph et al. (1985).

Approximately 400 plant species have been classified as hyperaccumulators of heavy metals, and most of them accumulate Ni (Baker et al. 1994). Most hyperaccumulators are unfortunately characterised by a relatively low biomass formation, and that is why they are not well-suited for phytoremediation purposes. The mechanisms

involved in hyperaccumulation are not yet fully understood, but attempts have already been made to increase the accumulation of heavy metals in plants forming high amounts of biomass (Macek et al. 1996; Krämer and Chardonnens 2001; Clemens et al. 2002).

During the accumulation of heavy metals, and also during removal of organic contaminants, the main limiting factor in large-scale exploitation of plants is the long time necessary for soil decontamination. Nevertheless, phytoextraction of metals presents large economic opportunities because of the size and scope of environmental problems associated with metal-contaminated soils and the competitive advantages offered by a plant-based technology (Chaney et al. 1997). Especially useful are plants able to remove more than one pollutant, because contamination is usually caused by a combination of more toxic compounds. Many elemental pollutants enter plants by basic transport systems designated for nutrient uptake. A number of xenobiotics are then stored in vacuoles as a plant protection mechanism against their toxic effects.

To convert contaminants to inoffensive harmful compounds, we need also to consider what products are formed, and also what is their toxicity towards plants, animals or man, and how these compounds will be further metabolised by soil microorganisms (Macek et al. 2002), because these can be released during leaf fall, wood decay, etc. PCBs can serve as an example, as some of the transformation products of these ubiquitous contaminants formed by plants have been identified (Rezek et al. 2007, 2008), and can be further metabolised by soil microorganisms (Francova et al. 2001, 2004).

Phytoremediation using conventional plants (grasses, sunflower, corn, hemp, flax, alfalfa, tobacco, willow, Indian mustard, poplar, etc.) shows good potential, especially for the removal of pollutants from large areas with relatively low concentrations of unwanted compounds: areas for which it is not cost-effective to use traditional physical or chemical methods. The most important parameter for selection of suitable plants is not the tolerance of the plant to heavy metals, but effectiveness in the accumulation of heavy metals. In addition to accumulation capacity, biomass production must be considered in order to determine the total metal uptake (Tlustos et al. 2006). In the case of contaminated water, a wider range of organisms seems to be promising, from water plants to microalgae, and from root filters to immobilized bacteria. The use of plants in heavy-metal-containing sludge decontamination was discussed by Tomaszewski et al. (2006). Laboratory activities in the field which were presented in the Phytoremediation Inventory (Vanek and Schwitzguebel 2003) indicate how much effort has recently been devoted to exploitation of plant biotechnologies for the benefit of the environment.

In general, the contaminant must be in a biologically accessible form, and root absorption must take place. Translocation of the contaminant from root to shoot makes harvesting easier. The harvested biomass could be reduced in volume and/or weight by composting, anaerobic digestion, low-temperature incineration, and leaching, leading to a decrease in the costs of handling and processing (Salt et al. 1995). After stabilisation, potential subsequent landfilling can be considered.

With some metals, after leaching or smelting in a kiln (e.g., Ni, Zn, and Cu), the value of the reclaimed metal may provide an additional incentive for remediation. This step reduces generation of hazardous waste and generates recycling revenues (Nedelkoska and Doran 2000, 2002).

More information has appeared in literature about possibilities for increasing the expression of the genes already present, or introduction of bacterial or mammalian genes into plants, in order to increase the natural ability of plants to cope with xenobiotics (Raskin 1996; Pilon-Smits and Pilon 2002; Macek et al. 2006). Transgenic plants of environmental benefit typically consist of plants that either reduce the input of agrochemicals into the environment or make the biological remediation of contaminated areas more efficient (Macek et al. 2008). Examples include the construction of species that result in reduced pesticide use, and of species that contain genes for either the degradation of organics or the increased accumulation of inorganics.

At the moment, the greatest promises for improvement in the effectiveness of phyto/rhizoremediation systems are seen in high-throughput methodologies like microarrays, understanding and manipulation of rhizosphere consortia using newly introduced methods like DNA-SIP (stable isotope probing), exploitation of metagenomic studies (Leigh 2006; Leigh et al. 2007; Uhlik et al. 2008) and improving root colonisation by beneficial microorganisms (Villacieros et al. 2005; Aguirre de Carcer et al. 2007).

14.2 Role of the Rhizosphere

Rhizoremediation is a term inseparable from phytoremediation. Living plant roots exert strong changes on the physical, chemical and biological properties of the soil. The soil–root interface, rhizoplane, and the narrow volume surrounding roots called rhizosphere, is characterised by several processes such as exudation of organic compounds, root respiration (absorption of oxygen and release of carbon dioxide), release of protons and other mineral ions, and uptake of water and solutes (Morel et al. 1999).

Various groups of microorganisms are present in the soil (bacteria, actinomycetes, fungi, algae, and viruses). In the rhizosphere, their numbers are higher than in the bulk soil. Qualitative changes in the microbial population have been observed, until recently based on evaluation of culturable microorganisms, while nowadays DNA-based molecular techniques permit the comprehensive determination of microbial diversity (Leigh 2006). The composition of the microbial population is controlled both by soil factors and by plant factors, including compounds with allelopathic effects. The intensive microbial activity in the rhizosphere is due to the presence of high amounts of available carbon released as exudates by roots. Plants respond to the presence of microorganisms by modifications of growth, e.g., symbiosis or interactions with free living organisms. Rhizobium-legumes or endo- and ecto-mycorhizas cause direct and positive actions. The case for the use of arbuscular

mycorrhizal fungi (AM) in soil remediation was discussed by Vosatka (2001). AM symbiosis affects many aspects of plant physiology, rooting, nutrient cycles, nutrient acquisition and plant protection (Janouskova et al. 2005; Sudova et al. 2007).

Indirect actions involve growth-promoting substances (Glick 2006), antibiotics or siderophores (Rudolph et al. 1985) released both by plants and bacteria. Positive effects are expected from inoculation of roots by selected microorganisms (suppliers of growth-promoting substances, nitrogen fixation, acquisition of phosphorus and especially those expressing degradative genes).

14.2.1 Exudates and Enzymes Released

Root exudation is the major process associated with the rhizosphere. In addition to uptake and direct phytodegradation, plants support bioremediation by release of exudates and enzymes that stimulate both microbial and biochemical activity in the surrounding soil. The composition of exudates, sites of exudation and various factors affecting root exudation were discussed by Morel et al. (1999). These include nutritional stress, excess of metals or presence of some microorganisms. Exudates include compounds of low molecular weight, like phenolics or sugars and amino acids and other compounds, secretions, lysates, and also compounds of high molecular weight, i.e., mucilages. All these compounds play important roles in the formation of consortia of microorganisms in rhizosphere.

14.2.2 Methods Used in Phytoremediation

The diverse approaches include phytoextraction, direct phytodegradation, rhizofiltration, and formation of artificial wetlands and lagoon systems, co-operation with microorganisms in the process of rhizoremediation, or development of plants tailored for specific phytoremediation needs, and using genetic engineering, and can be summarised as follows (Cunningham et al. 1995; Macek et al. 2000; McCutcheon and Schnoor 2003):

Phytostabilisation serves to reduce the mobility and availability of the pollutants, and helps to stabilise the soil surface by covering the contaminated soils with adapted plants, reducing the risk of transport of pollutants adsorbed on fine solid phase.

Phytoextraction (phytoaccumulation) is based on the ability of plants to take up compounds from soil, translocate and concentrate some substances, preferably in the harvestable parts.

Rhizofiltration is the exploitation of plant roots for absorption or precipitation of pollutants, especially metals from solution surrounding the root zone.

*Phytodegradation (*phytotransformation) is the conversion or breakdown of organic pollutants by plants through metabolic processes within the plant, or the breakdown of contaminants external to the plants through the effect of enzymes released by plants.

Phytovolatilization is uptake and transfer of some pollutants into gas phase by plants. It includes transpiration of a contaminant by a plant, with release of the contaminant or a modified form of it from the plant to the atmosphere.

Use of plants for the removal of pollutants from the air represents the ability of plants to accumulate and metabolise toxic compounds from the air (Burken and Xingmao 2006).

14.2.2.1 Artificial Wetlands

The use of artificial wetlands for treatment of contaminated water is well-established and cost-effective. The wetland systems have to be mentioned when discussing phytoremediation, because the wetland plants are an inherent component of engineered wetland systems, often using common reed, cattail or sweetgrass. The majority of treatment wetlands rely on a combination of moderately reducing substrate conditions and the oxidising conditions surrounding plant roots (Otte and Jacob 2006; Wiessner et al. 2006; Czako et al. 2006).

14.2.2.2 Perspectives of Plants in Detoxification in CWD

Plants can be exploited in chemical weapon demilitarisation (CWD), for detoxification of some chemical warfare compounds as such, and also for the detoxification of some products formed during breakdown or neutralisation of chemical warfare agents in demilitarisation processes. Plants are often expected to be used only for final polishing of sites decontaminated in other ways, but it was shown that plants and their enzymes might play an active role in degradation of many toxic compounds directly as part of the technological set-up (Macek et al. 1998, 1999).

14.3 Basic Research Aspects

14.3.1 Plant in vitro Cultures in Phytoremediation Studies

In addition to soil-grown plants or hydropony, for understanding of some phytoremediation processes, homogeneous non-differentiated callus and suspension cultures, differentiated embryogenic cultures, shooty teratomas and hairy root cultures grown aseptically *in vitro* have also been proven to be useful (Macek et al. 2004). All the systems mentioned can be grown under standard laboratory conditions, growth is independent of the weather or climate, plant *in vitro* cultures grow more rapidly than normal plants and the results can be obtained with less analytical expense, allowing separation of the effects of plants from those of microorganisms.

14.3.1.1 Callus and Cell Suspension Cultures

Plant *in vitro* cultivated cells have been used in studies of herbicide resistance and metabolism for many years (Harms and Kottutz 1990), or of pesticide metabolism (Wimmer et al. 1987). The results obtained under *in vitro* conditions have to be carefully evaluated, especially in the case of non-differentiated cultures, because the variability within more cultures of the same species is high (Mackova et al. 1997a). Testing 12 *in vitro* cultures of the same species, *Solanum aviculare* (Macek 1989), some strains were found which were not able to convert PCBs at all, while others exhibited very high conversion rates under the same conditions. The degradation activity depends much on the level of morphological and biochemical differentiation of the strain or clone being tested. Independently of the "negative" clones where some enzyme may be missing, those that detoxify, convert or degrade represent very important tools in establishing the fate of the xenobiotic in the plant cell.

14.3.1.2 Hairy Root Cultures

Hairy root cultures are formed by genetic transformation of a single plant cell by the soil bacterium *Agrobacterium rhizogenes*. Due to fast growth, unlimited propagation in culture media, genetic and biochemical stability and growth in hormone-free media, these tissues have proven to be very good model systems. Exploitation of hairy root models was summarised in reviews by Shanks and Morgan (1999) and Pletsch et al. (1999). The limitation of the *in vitro* cultivated hairy root surface is the altered properties compared to the roots grown *in vivo* with full production of cuticle, waxes and other compounds that influence the sorption and uptake of xenobiotics. The excellent monograph of Doran (1997) covers all aspects of studies and exploitation of hairy roots. In the last few years more reviews have followed, but the application of phytoremediation studies has been discussed only in some of them (Pletsch et al. 1999; Gleba et al. 1999). The exploitation of hairy root cultures in phytoremediation studies has been investigated for more than a decade; first for cadmium uptake studies (Macek et al. 1994, 1997) and for PCB degradation studies (Mackova et al. 1997b). Soudek et al. (1998) compared the uptake of different metals by horseradish hairy roots. Using long-term hairy root cultures of *Alyssum*, it was shown that hyperaccumulation does not necessarily depend on the presence of shoots or root–shoot transfer (Nedelkoska and Doran 2002).

14.4 Genetic Engineering Approach

The potential of genetic engineering to enhance the biodegradation of xenobiotics has been recognised since the early 1980s, with initial attempts being focused on microorganisms. However, the major problems associated with the introduction of genetically modified organisms (GMO) are the legislative barriers blocking their

release into the environment, and the poor survival rate of those engineered strains that were introduced into real contaminated soil. The latter problem reflects the inadequate level of knowledge that currently exists about the consortia of microorganisms present in real soil, and the ways in which they interact. The survival rate of introduced bacterial species might be improved by the use of strains that have a selective advantage over others, such as strains supported by plants — for example, root colonisers (Villacieros et al. 2005).

The use of plants, rather than microorganisms, as genetically engineered environmental cleanup biosystems might also help to overcome the legislative barriers. For remediation purposes, in addition to their ability to take up, accumulate or metabolise the xenobiotics, one of the most important criteria is the ability of the plant to selectively support the metabolism and survival of degrading bacteria in the rhizosphere (Leigh et al. 2006, 2007).

Enhancement of metabolic abilities of plants can be achieved by direct insertion of novel genes. The methods of genetic engineering are widely used for the improvement of different crop plants. A similar approach is expected to largely improve the abilities of plants in the field of environmental detoxification (Macek et al. 2000, 2008).

The generation of transgenic plants for environmental protection involves two quite separate fields of pollution prevention and pollution removal, with specifically tailored plants already existing for both purposes. Pollution-preventing GM plants can significantly reduce the amount of agrochemicals needed for crops, thus reducing environmental pollution. Recently, a new approach to pest management has been developed, based on the construction of plants that produce and emit insect pheromones (Nesnerova et al. 2004; Macek et al. 2008). Pollution-removing GM plants, which deal with contamination caused by explosives, chlorinated solvents, mercury, selenium, phenolics, etc., have been developed (Meagher 2000; McCutcheon and Schnoor 2003; Macek et al. 2006; Mackova et al. 2006; Eapen et al. 2007). These plants contain either transgenes responsible for the metabolism of organic compounds (thereby leading to the accumulation of less toxic or less recalcitrant compounds) or transgenes that result in the increased accumulation of inorganic compounds. Once optimised, this approach should lead to the accumulation of pollutants in the harvestable parts (Macek et al. 1996), and thus either enable their removal or prevent their migration.

Complex interactions of transport and chelating activities control the rates of metal uptake and storage. Clemens et al. (2002) in an excellent review summarised the determinants of metal accumulation, mobilisation, uptake, sequestration, and xylem transport, and discussed the role of different families of transporter proteins.

14.4.1 Pollution Prevention

The first generation of commercially available transgenic plants (e.g., plants expressing the Bt toxin) were able to reduce the loss of crop yield caused by insect damage, at the same time as reducing the amount of pesticide required. Other

examples include soya, which enables the use of more environmentally-friendly herbicides. Both these and herbicide-resistant plants have been the subject of numerous reviews (Montagu 2005; Vain 2006), but plants emitting insect pheromones are new (Nesnerova et al. 2004). Grown close to or around, for example, a field of food crops requiring protection, this type of GM plant emits a pheromone that attracts male moth pests, thereby reducing their ability to mate effectively. In such cases, the protected crop does not itself need to be transgenic. Applying this method, neither the transgene itself nor its products would be able to enter the human food chain, thus eliminating the possible health risks associated with genetically modified plant consumption. Moreover, such an approach means that non-targeted insect life remains unaffected, and no resistant insect strains will develop, while pesticide use can be significantly lowered (Macek et al. 2008).

14.4.2 Genetically Modified Organisms for Phytoremediation

There have been many attempts to breed willow, poplar and other plants with properties useful for phytoremediation. The aim is the formation of plants with a high ability to accumulate, detoxify or degrade xenobiotics and pollutants, with resistance towards the toxic compounds present and with suitable agronomic characteristics (Macek et al. 2002). The importance of improvement of metal uptake by breeding or genetic modification can be illustrated by the fact that more important and interesting reviews on engineering of GM plants suitable for metal accumulation appeared simultaneously (Clemens et al. 2002; Pilon-Smits and Pilon 2002; Lasat 2000).

Thus, through GM plant research we are developing not only a better understanding of possibilities for modifications and improvements of normal plant mechanisms, but also ways to improve the overall yield of the remediation process. Improved bioavailability of metals caused by changes of exudate, increased excretion of organic acids, or cooperation with rhizospheric microorganisms represent prospective targets of genetic engineering, as well as increased performance of transgenic plants. Our very early screening of different plant species for production of phytosiderophores (Rudolph et al. 1985), based on the example of nicotianamine, demonstrated that their presence was ubiquitous in higher plants. Their formation in plants might be increased, or alternatively such compounds can be produced by appropriate bacteria.

14.4.2.1 Methods for Preparation of Transgenic Plants

The main vectors used for routine plant genetic transformations are different plasmids derived from *Agrobacterium* plasmids Ti or Ri, e.g., by methods described already by Marton et al. (1979), now with many modifications. Other tools used are ballistic methods, allowing transfer of genes into cells and into whole intact plants.

These methods are based on use of accelerated heavy-metal particles (gold or wolfram) covered by genetic material.

14.4.3 Examples of GM Plants Tailored for Phytoremediation

14.4.3.1 Increased Accumulation of Heavy Metals

Plants exploit their natural metabolic mechanisms to take up essential trace metals. Cations or oxyanions must either be accumulated in harvestable parts or transformed into less-toxic forms. Although hyperaccumulators, such as *Thlaspi caerulescens*, can uptake sufficient levels of metals to make harvesting and metal recovery economic, they are often limited by their low biomass (Meagher 2000; Nedelkoska and Doran 2002), since the amount of pollutant they can remove from soil is a function of their tissue concentration multiplied by the quantity of biomass formed.

With the aim of improving metal uptake, transgenic plants have been prepared with increased formation of glutathione synthase or phytochelatin synthase, which in both cases led to increased Cd accumulation (Kärenlampi et al. 2000). Transgenic plants bearing foreign genes for proteins transporting metal across membranes have also been prepared, as summarised by Krämer and Chardonnens (2001). In some laboratories, genes encoding different types of metallothionein (mammalian, yeast, insect and human) were introduced into plants which led mostly to increase of the resistance towards some heavy metals (Kärenlampi et al. 2000; Macek et al. 1996), but not yet to an increase of accumulation.

Another promising approach to enhancing metal uptake employed the nicotianamine synthase gene involved in the formation of phytosiderophore, the metal-binding amino acid (Rudolph et al. 1985) that increases the bioavailability of metals to plants. Interesting examples are plants bearing bacterial mercuric reductase (reducing mercury ions into volatile metal), and merB — bacterial organomercurial lyase. The genes, separately or both together, were cloned into Arabidopsis or tobacco (Heaton et al. 1998; Rugh et al. 1996), yielding a mercury-resistant transgenic plant, that volatilises mercury into the atmosphere. To test the effectiveness of using transgenic plants for phytoremediation of Hg-contaminated soil, the yellow poplar has been chosen due to its promising biological and structural characteristics (Rugh et al. 1998). Further plants have been prepared that are able to accumulate the mercury instead of releasing it (Bizily et al. 2000).

However, the most common strategy targeted the proteins involved in metal homeostasis (metallothioneins, phytochelatins, glutathione) for genetic manipulations (Kotrba et al. 1999; Clemens et al. 2002). Although such approaches typically involve the manipulation of plant enzymes responsible for the formation of phytochelatins and related compounds, e.g., overexpression of glutathione synthetase, gamma-glutamylcysteine synthetase and phytochelatin synthase, manipulations

with other enzymes have also been successful. Expression of metallothioneins in plants have been dealt with before (Pan et al. 1994, Hasegawa et al. 1997), but work in our laboratory has focused on improving a plant's ability to accumulate metals by introducing (into the targeted protein) additional metal-binding domains with a high affinity to heavy metals (Macek et al. 2002). Such a fusion product with a histidine anchor (Macek et al. 1996; Pavlikova et al. 2004a) has been tested in real contaminated soil, and transfer factors have been estimated for cadmium, zinc and nickel (Pavlikova et al. 2004b; Macek et al. 2005). In our trials, transgenic tobacco accumulated 190% of the amount of cadmium in above-ground biomass as compared with the controls.

A possible enhancement to this approach, involving the cloning of short (cysteine-rich) metal-binding sequences into plants to improve their metal-binding properties, is currently being tested (Kotrba et al. 1999). This approach was followed up by quantum chemical studies of the interactions of metal ions with biologically relevant functional groups, studies that suggested further possible developments in the metal binding capacities of fusion proteins. Subsequently, theoretical combinatorial chemistry was applied to the complexation and selectivity of metal ions in model sites (Rulisek and Havlas 2000, 2003), resulting in the design of highly selective combinations of metal-binding sites that might be merged into one polypeptide chain.

14.4.3.2 Plants with an Enhanced Ability to Detoxify Persistent Organic Compounds

To cope with organic xenobiotics, plants use a mechanism developed to fight allelochemicals, which are toxic compounds produced by other species competing for their resources (Sandermann 1994; Meagher 2000; Bais et al. 2004). The removal of organic compounds by plants has been widely discussed in reviews (Eapen et al. 2007; Macek et al. 2000; Raskin 1996) and books (McCutcheon and Schnoor 2003, Mackova et al. 2006).

To increase their natural abilities, different P450 cytochromes have been introduced into plants. These enzymes are considered to be responsible for the first phase in plant detoxification, the activation reaction of recalcitrant compounds in plants. A recent publication by Doty et al. (2007) described the development of transgenic poplars overexpressing a mammalian cytochrome P450. The engineered plants showed enhanced performance for the removal and metabolism of a range of toxic volatile organic pollutants, including trichloroethylene, vinyl chloride, carbon tetrachloride, chloroform and benzene.

Intriguingly, however, overexpression of a basic peroxidase in tomato (Wevar Oller et al. 2005) resulted in increased phenol phytoremediation, thereby supporting the hypothesis that, apart from P450 cytochromes, peroxidases are also involved in this first phase (Chroma et al. 2003).

The biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase is the classical example of the exploitation of a bacterial

gene for phytoremediation (French et al. 1999). More recently, plants have been constructed that express bacterial enzymes capable of transformation of TNT and degradation of RDX (hexahydro-1,3,5-trinitro-1,3,5 triazine, an explosive nitroamine widely used in military and industrial applications) (Bruce 2007). To achieve the aerobic degradation of ubiquitous persistent PCBs, they must first be activated by hydroxylation. The vital missing step in the efficient degradation of PCBs by plant cells is the opening of the biphenyl ring by the bacterial enzyme bphC, which is responsible for the cleavage of hydroxylated PCB derivatives, even those formed by plants (Francova et al. 2004). Therefore, we have thoroughly studied the cooperation of plants and bacteria in PCB degradation (Villacieros et al. 2005, Leigh et al. 2006, Leigh et al. 2007, Mackova et al. 2007, Aguirre de Carcer et al. 2007). The generation of tobacco plants carrying the bacterial *bphC* gene, and their abilities to germinate in high concentrations of PCB, has been summarised by Macek et al. (2005). Improved substrate specificity has since been achieved by the expression of bacterial biphenyl–chlorophenyl dioxygenase genes in tobacco by Mohammadi et al. (2007) and Novakova et al. (2009).

14.5 Other Approaches to Improve the Effectiveness of the Phytoremediation Process

14.5.1 Secondary Plant Metabolites and their Role in Phytoremediation

The importance of such compounds has been discussed (Singer et al. 2000, 2003; Singer, 2006). With regard to the metabolism of organic xenobiotic compounds by plants, the whole process is based on a mechanism originally developed to cope with different allelopathic compounds of natural origin. Through exudate and root turnover, many plant products enter the root zone. Some of these compounds supply soil microorganisms with energy, some act as a carbon source and some can even serve as inducers of degradative pathways. The content of root exudate and compounds released from dying and decaying roots (Fletcher et al. 1995) represents a vast array of molecules that might be involved directly in metal uptake, and stimulation of bacteria. Biosynthesis of such compounds could also be a suitable target for genetic manipulation, as discussed by Gleba et al. (1999).

Growing plant roots explore the surrounding soil particles and take up water, nutrients, trace elements and other compounds, thus playing an important role in biological remediation. Plant roots can extract contaminants from soil and accumulate, transform and transport them into the parts of the plant that are above-ground. In leaves, fruits or stems, many compounds are stored, transformed or volatilised. Such processes are known as phytoremediation. Growing roots also help to spread microorganisms within the soil, thereby supporting rhizoremediation.

14.5.2 Effect of Symbiotic Bacteria

An example is the finding of a plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Burd et al. (1998) described *Kluyvera ascorbata*, which produced a siderophore, and displayed 1-aminocyclopropane-1-carboxylic acid deaminase activity. The presence of the bacterium did not reduce the nickel uptake by seedlings in contaminated soil, thus probably promoting the plant growth by lowering the level of the plant stress hormone ethylene that is induced by nickel (Glick 2006).

14.5.2.1 Genetically Modified Symbiotic Bacteria

Genetically modified symbiotic bacteria represent a further development of mutual cooperation between plants and bacteria. Studies on GM organisms have recently also involved other approaches to genetic manipulation of the properties of phytoremediation system, e.g., growth promotion for better biomass formation. For this reason, Glick and co-workers (Ma et al. 2002; Glick 2006) manipulated plant growth-promoting bacteria, testing factors like production of indoleacetic acid, antibiotics, 1-aminocyclopropane-1-carboxylic acid deaminase activity or siderophore. Overproduction of the genes in bacteria inoculated into the rhizosphere of canola resulted in a significant increase in root and shoot elongation, thus increasing the potential for exploitation in phytoremediation. Grichko et al. (2000) described increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn.

The genetic modification of microorganisms to improve their performance in the rhizosphere represents a challenging opportunity that should not be abandoned simply because their release into the environment is currently restricted. The ability of degrading bacteria to colonise roots may be manipulated by improving symbiotic microorganisms. One such example is the rhizoremediation of PCBs by *Pseudomonas fluorescens*, in which biphenyl degradation is regulated using a system that responds to signals from alfalfa roots (Villacieros et al. 2005). The introduction of such GM microorganisms ensures that any changes are limited to the consortia of native bacteria in the rhizosphere and are not introduced into the surrounding soil (Aguirre de Carcer et al. 2007).

The development of engineered endophytic bacteria that improve the phytoremediation of water-soluble, volatile organic compounds appears to be a rather promising approach (Barac et al. 2004). Trichloroethylene (TCE)-degrading bacteria have been proven to protect host plants against the phytotoxicity of TCE, and to contribute to a significant decrease in TCE evapotranspiration.

14.5.2.2 Mycorrhizal Symbiosis

Arbuscular mycorrhizal fungi represent another very important system for influencing the behaviour of plants in contaminated soil. Such a symbiosis has an important effect on nutrient uptake by plants. Its effect on the phytoremediation

process has been evaluated by Donnelly et al. (1994), Fletcher et al. (1995) and Vosatka (2001).

14.5.2.3 Metagenomics and Molecular Methods

The genomic-driven strategies based on culture-independent molecular approaches have provided new insights in our understanding of the contaminated environment (Leigh 2006). Recently developed methods of detection, including RT-PCR and stable isotope probing, have lead to a deeper understanding of the effect of pollutants and plants on microorganisms (Leigh et al. 2006; Mackova et al. 2007). Metagenomics, analysis of the collective genomes of the studied habitat, has revealed the presence and activity of degrading microorganisms within rhizosphere consortia, enabling the tracking of responses to compounds released by plants (Singer et al. 2003). Understanding the function of microbial populations within complex communities is a major challenge in microbial ecology and bioremediation research (Leigh et al. 2007). Cultivation studies underestimate the microbial diversity of a site, since only less than 1% of all microbes are cultivable. DNA-based molecular techniques permit the comprehensive determination of microbial diversity, but generally do not reveal the relationship between the identity and the function of microorganisms. The first direct molecular technique for linking phylogeny with function is DNA-based stable isotope probing (DNA-SIP). The principle of the method lies in providing ^{13}C-labeled substrate to a microbial community and subsequent analysis of the ^{13}C-DNA isolated from the community (Uhlik et al. 2008).

14.6 Conclusions

Researchers are finding that the use of trees (Komives and Gullner 2006) rather than smaller plants allows them to treat deeper contamination because tree roots penetrate more deeply into the ground. Specifically, two subsets of phytoremediation are nearing commercialisation. The first is phytoextraction, in which high biomass metal-accumulating plants and appropriate soil amendments are used to transport and concentrate metals from the soil into the harvestable part of roots and above-ground shoots, which are harvested with conventional agricultural methods. The second is rhizofiltration, in which plant roots grown in water absorb, concentrate and precipitate toxic metals and organics from polluted effluents. In both cases, the cost for remediation of organic contaminants can be expected to be at the lower end of the expected price ranges, and the cost for remediation of heavy metals to be at the higher end (Boyajian and Carreira, 1997).

The main advantages of phytoremediation in comparison with classical remediation methods are (Salt et al. 1995, Schnoor et al. 1995):

- It is far less disruptive to the environment
- There is much less need for soil disposal sites

- It has better public acceptance
- It mostly avoids excavation and heavy traffic and
- It has potential versatility to treat a diverse range of hazardous materials

Considering these factors and the much lower cost expected for phytoremediation, it appears that it will allow for clean-up operations on a much larger scale than possible by other methods. The process is relatively inexpensive, using the same equipment and supplies as agriculture.

Phytoremediation has a number of inherent technical limitations. The contaminant must be within, or drawn toward, the root zones of plants. This implies water, depth and nutrient, atmospheric, physical and chemical limitations. The site must be large enough to make farming techniques appropriate. There may be also a considerable delay in time needed for obtaining satisfactory clean-up results between phytoremediation and "dig and dump" techniques. In addition, formation of vegetation may be limited by extremes of environmental toxicity. Contaminants collected in leaves can be released again to the environment during litter fall. Accumulation of pollutants and their metabolites in energy crops can be considered as an advantage in the case of planned use of proper incineration and metal removal from exhaust. In some cases the solubility of contaminants may be increased, resulting in danger of greater environmental damage and/or pollutant migration.

Growing knowledge about the factors that are important in phytoremediation will provide a basis for genetic modification of plants directed to improved performance (Raskin 1996; Macek et al. 2000, 2008). These changes will include transforming of the plants to add specific proteins or peptides for binding and transporting xenobiotics, increasing the quantity and activity of plant-biodegrading enzymes (Francova et al. 2003), including those that are exported into the rhizosphere and the surrounding soil in order to improve the performance of soil bacteria.

Other approaches which have been discussed might increase the effectiveness of the process, and the probably concerted action of more methods together will permit the exploitation of the real potential of plants. Only a decrease in the amount of environmental contamination can prevent the toxic compounds entering the food chain, help to maintain human health and conserve biological diversity (which is surely more endangered by the contaminated environment than by the use of genetically modified plants) (Macek et al. 2002, 2008), and in this way contribute to sustainable development.

It is expected that in the near future GM plants will be widely used, not only to significantly reduce pesticide use in agriculture, but also to actively remove the residues of agrochemical, industrial and accidental contaminations of the environment. In the great environmental cleanup required, the future lies in tailored phytoremediation-specific plants able to support microbial activities in the rhizosphere. However, to exploit these possibilities on a large scale, it will first be necessary to achieve changes in the existing legislation, overcome regulatory barriers and educate the public into improving their opinion of GM plants (Macek et al. 2008). Metagenomic studies might in the near future lead to design of conditions, plants and microorganisms supporting the proper consortia of organisms with high required efficiency.

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Chapter 15
Phytoremediation for Oily Desert Soils

Samir Radwan

15.1 Introduction

Desert and semi-desert regions cover relatively vast areas of our planet, representing about one third to one fourth of the total land mass of earth. Such regions occupy considerable surface areas of all continents, with the exception of Europe. Globally, deserts keep spreading instead of diminishing, and this is a serious problem from an economical viewpoint. Day after day, the earth becomes more threatened with environmental pollution and deserts receive a share of that threat. Rehabilitation of the earth's various environments, including the deserts, will represent one of the environmentalist's urgent duties in the twenty-first century.

Since the beginning of extensive production and use of crude oil as an energy source early in the twentieth century, this valuable raw material and its products and derivatives have become major environmental pollutants. This may not be true in the majority of the vast pristine desert areas, but it is certainly valid for the limited regions in which oil has been discovered and is being produced and transported.

The major objective of this chapter is to shed light on biotechnologies that could potentially be applied for remediation of oily desert areas. Therefore, two relevant topics need to be addressed in the introduction to this subject; the pollution of deserts with oil, and the composition of crude oil.

15.2 Desert Soils and Oil Pollution

Desert soils are characterized as having low organic substance and water contents, and are usually subjected to rather extreme environmental conditions, viz. temperature and illumination. Apparently, the primary producers in such poor soils are mainly the phototrophic micro-organisms, i.e., micro-algae and cyanobacteria. These organisms

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occur most of the time as surviving units, and become active only temporarily, especially after precipitation, and when the temperature becomes suitable for their growth. Desert soils are also mostly free of conventional pollutants, e.g., pesticides. However, the restricted regions in which crude oil is produced and transported are routinely subjected to varying degrees of pollution by this raw material. Extensive oil-pollution may occur in such desert regions in association with accidents or even with military conflicts, as will be described later.

15.2.1 Normal Desert Microflora

It is a difficult task to give a comprehensive review of the desert microflora on earth, simply because deserts are not all alike. Furthermore, a single desert normally comprises heterogeneous locations, with different conditions and consequently different microflora. A recent report has just appeared on the microorganisms in the Atacama Desert (Gomez-Silva et al. 2008), an ancient temperate desert along the Pacific coast of South America. Information in that report will be summarized to present this desert just as a model. Some areas of that desert were found to be almost free of bacteria, whereas some other areas contained up to 10^7 cells g^{-1} soil. This huge difference depended on differences in the prevailing environmental factors, e.g., precipitation levels, chemical soil-composition, site elevation, pH and others. The methods adopted for counting included both culture-dependent and -independent techniques (Navarro-Gonzalez et al. 2003; Maier et al. 2004; Glavin et al. 2004; Drees et al. 2006; Lester et al. 2007). Most of the bacteria isolated from soils of that desert belonged to the Actinobacteria and Firmicutes; the Proteobacteria were much less frequent. In certain areas the Actinobacteria were members of the family Geo-dermatophilaceae, as well as the genera *Sphingomonas, Bacillus, Arthrobacter, Brevibacillus, Kocuria, Cellulomonas* and *Hymenobacter.* As will be seen later, many of these genera have the potential for oil and hydrocarbon utilization. In the above studies, microbial diversities in the surface were similar to those in the subsurface soils.

Desert pavement (surface soils mantled by gravels) was colonized by hypolithic and endolithic bacteria growing in biofilms (Warren-Rhodes et al. 2006; Wierzchos et al. 2006). In this context, similar bacterial consortia had also been found in other cold and hot deserts (Friedmann 1992; Schlesinger et al. 2003). Cyanobacteria inhabiting porous and loose stones were the major primary producers in the above consortia.

15.2.2 Crude Oil

Although discovered and extensively used only in modern times, oil has existed in the ground for many centuries. It originated from biomass of marine organisms that were buried over geologic time under anaerobic conditions. With time, most organic constituents had been biodegraded leaving resistant molecules, mainly hydrocarbons in geological reservoirs.

Crude oil is composed of four major constituents (Leahy and Colwell 1990); saturated hydrocarbons, aromatic hydrocarbons, asphaltenes and resins. Although there are considerable variations of the proportions of those constituents in crude samples from different sources, saturated hydrocarbons usually represent the predominant fraction. This fraction is composed mainly of normal alkanes, with chains up to more than 40 carbon atoms, and naphthenes which are cyclic alkanes. This fraction may make up to 60% of light oils. The fraction of aromatic hydrocarbons comprises benzene and substituted benzene derivatives, and may make up to 20% of light oils. The molecules in this fraction contain between one and six or more rings. The asphaltene fraction consists of very high molecular weight hydrocarbons. The resin fraction represents oil constituents that contain sulfur and oxygen, in addition to carbon and hydrogen. Obviously, asphaltenes and resins are rather complex, and therefore their chemical composition has not yet been precisely elucidated. These two fractions may constitute between 1 and 5% of light oils. Heavy oils, on the other hand, may contain up to 25% asphaltenes and resins.

15.2.3 Desert Soil Pollution with Oil

Organic residues in soil normally contain low proportions of hydrocarbons, which are natural constituents of the inhabiting micro- and macro-flora and -fauna. Since desert soils are usually low in organic matter, their hydrocarbon content should expectedly be minimal, and this is a normal situation. However, desert soil areas in oil-producing countries are obviously characterized by higher levels of hydrocarbon vapors that have volatilized from deep oil reservoirs. Yet oil-pollution problems in such desert areas only started to arise in association with the modern interests of man in this raw material as an energy source. Oil production and transport on a wide scale resulted in oil-spills of varying magnitudes. Accidents and military conflicts in such regions have made the situation even worse. As a representative example, the oil-pollution catastrophe in the Kuwaiti desert in association with the Iraqi invasion and occupation of Kuwait (August 1990– February 1991) is described. Shortly, before they had to withdraw, the Iraqi forces blew up and ignited more than 700 wells distributed all over the Kuwait desert. According to reliable estimates about 22 million barrels of crude oil kept gushing throughout 7 months, the time it took the authorities to kill the last fire. The gushing crude formed about 300 oil lakes of varying dimensions that cover a total surface area of 49 km^2 of the desert. This is a huge area given the rather small size of the country. The oil lake beds are heavily contaminated (10–20%), and the so-called fall-out areas around the oil lakes are lightly contaminated (<10%). According to reports from Kuwait Oil Company, about 19×10^6 barrels were recovered up to 1994 from the lakes and exported, but about 3×10^6 barrels remained as soil pollutants, reaching depths between 40 and 60 cm.

15.2.4 Oil-Utilizing Microorganisms

Since the 1970s many reviews and reports have been published on oil-utilising microbes (Klug and Markovetz ; Levi et al. 1979; Einsele 1983; Radwan and Sorkhoh 1993; Van Hamme et al. 2003; Rosenberg 2006; Widdel et al. 2006; Radwan 2008) According to these and other reports, oil-(or hydrocarbon)-utilization potential is widely distributed among prokaryotic and eukaryotic conventional microorganisms. The defining biochemical characteristics of such microorganisms are their possession of oxygenase systems which catalyze the introduction of oxygen atoms from oxygen molecules into aliphatic and/or aromatic hydrocarbon molecules, producing the corresponding alcohols. This step paves the way for further oxidation of the alcohols via aldehydes to acids, which are then metabolized in the conventional way to produce energy and cell materials (for relevant early reviews see Rehm and Reiff 1981; Fukui and Tanaka 1981; Boulton and Ratledge 1984). Bacterial genera most commonly recorded as hydrocarbon degraders include *Acinetobacter, Aeromonas, Alkaligenes, Arthrobacter, Azospirillum, Bacillus, Brevibacterium, Chromobacterium, Corynebacterium, Flavobacterium, Klebsiella,Micrococcus, Mycobacterium, Nocardia* (and other nocardioforms), *Pseudomonas, Rhodococcus, Vibrio,* and *Streptomyces.* Most commonly recorded yeasts include *Candida, Dabaromyces, Endomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Saccharomycopsis, Schwanniomyces, Selenotila, Sporidiobalus, Sporoboloyces, Torulopsis, Trichosporon* and *Wingea.* Most commonly recorded molds include *Absidia, Aspergillus, Aureobasidium, Beauveria, Botrytis, Cephalosporium, Cladosporium, Corellospora, Cunnninghamella, Dendyphiella, Fusarium, Hormodendrum, Lulworthia, Mortierella, Mucor, Penicillium, Phiolophora, Phma, Scedosporium, Scoleobasidium, Sporotrichum, Varicosporina* and *Verticillium.* In addition to the above organotrophic microorganisms, the literature includes reports on phototrophic microorganisms that were claimed to have the potential for hydrocarbon oxidation. Those include the phototrophic bacterial genera *Rhodospirillum* and *Rhodopseudomonas* (Cerniglia et al. 1980b), the cyanobacteria *Oscillatoria* (Cerniglia et al. 1980a), *Microcoleus* and *Phormedium* (Al-Hasan et al. 1994; Radwan et al. 1998b), the microalgae *Chlamydomonas* and *Chlorella* (Ellis 1977), and the phytoflagellate *Euglena* (Ellis 1977).

Some hydrocarbon-utilizing micro-organisms may exhibit specific morphological and cytological features during their growth on these compounds as substrates. Most frequent is the appearance in scanning electron micrographs of cytoplasmic less electron-dense inclusion bodies, where hydrocarbons have accumulated (Scott and Finnerty 1966; Atlas and Heintz 1973; Kennedy and Finnerty 1975; Cundell et al. 1976; Koval and Redchitz 1978; Redchitz 1980; Barabas et al. 1995). In some microorganisms, growth on hydrocarbons is associated with the appearance in the cell cytoplasm of dense intraplasmic membranes (Kennedy and Finnerty 1975; Ivshina et al. 1982) as well as volutin inclusions (Redchitz and Koval 1979;

Ivshina et al. 1982). In shaken liquid cultures with hydrocarbons as substrates, *Penicillium* grows as hollow mycelial balls enclosing hydrocarbon droplets; in hydrocarbon-free media on the other hand, the balls are solid (Cundell et al. 1976).

Information regarding desert hydrocarbon-utilizing microorganisms on the global scale is limited. However, for certain desert regions this information is available. An example of such latter regions is the Kuwaiti (probably the whole Arabian Gulf) desert environment, whose hydrocarbon-utilizing microflora merits some consideration, here. The indigenous oil-utilizing bacterial and fungal genera in the Kuwait desert soils are listed in Table 15.1. In pristine (clean) desert samples, numbers of such organisms are low, normally in the range of only hundreds to thousands per g soil. In oil-polluted soil samples, on the other hand, such numbers may reach up to tens or hundreds of millions per g soil (Radwan et al. 1995c). Rhizospheres of wild desert plants growing in pristine and oily desert soils contain as a rule more oil-utilizing microorganisms than the soil samples distant from the roots (Radwan et al. 1995b, 1998a). This is also true for desert soil samples supporting legume crops, e.g., *Vicia faba* and *Lupinus albus* (Radwan et al. 2000). The rhizospheres of Kuwaiti desert plants contain as predominant oil-utilizing bacteria the genera *Arthrobacter, Cellulomonas* and *Rhodococcus.* In view of the fact that the Gulf region has a very long hot summer, desert soil samples may be expected to accommodate thermophilic oil-utilizing microorganisms. The analysis of numerous soil samples revealed between tens of thousands and tens of millions of such thermophiles (with an optimum of 55°C) per g soil, that all belonged to *Bacillus stearothermophilus* (now

Table 15.1 Predominant oil-utilizing microorganisms in Kuwaiti desert soil samples

Microbial group	Genera	References
Bacteria	*Arthrobacter*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, and other nocardioforms	Sorkhoh et al. (1990, 1995); Radwan et al. (1997)
Actinomycetes	*Streptomyces*	Barabas et al. (1995); Radwan et al. (1998b)
Molds	*Aspergillus*, *Fusarium*, *Mucor*, *Penicillum*	Sorkhoh et al. (1990)

Table 15.2 Alkaliphilic and halophilic oil-utilizing bacteria genera in Kuwaiti coastal desert areas (from Al-Awadhi et al. 2007)

Alkaliphilic bacteria	Halophilic bacteria
Marinobacter	*Marinobacter*
Micrococcus	*Microbacterium*
Oceanobacillus	*Georgenia*
Bacillus	*Stappia*
Dietzia	*Bacillus*
Citricoccus	*Cellulomonas*

Note: Bacterial genera were arranged in decreasing order of frequency

Geobacillus stearothermophilus) (Sorkhoh et al. 1993). Another thermophilic hydrocarbon-utilizing bacterium that had been isolated from other environments was identified as *Thermoleophilum album* (Zarilla and Perry 1984). In this context, Kuwaiti coastal desert sand was found to contain low numbers of alkaliphilic and halophilic hydrocarbon-utilizing bacteria (Al-Awadhi et al. 2007); see Table 15.2.

15.2.5 Cleaning of Oily Desert Soil

It is obvious that cleaning of desert soil subjected to a huge oil spill is a rather difficult job. Physical means of oil removal becomes complicated by crude penetration to depths that may reach 60 cm, as it is the case in the Kuwaiti desert after the 1991 war. In that spill, the amount of the spilled crude was so large that the so-called oil lakes formed, as mentioned above. Mechanical removal through pumping was only in part successful, an amount of about 19 million barrels had been recovered and exported, but about 3 million barrels remained in the lake beds, in addition to oil that had penetrated into sand (Radwan et al. 1995a). The total oil-polluted desert soil is about 20×10^6 m^3. The only way to rid the desert of that pollutant is to depend on microbial activities, either through self-cleaning or by bioremediation technology. The second approach will be discussed in the next section. Self-cleaning is a natural process in which pollutants are left to the indigenous microflora to digest them without human intervention. However, given the harsh nature of most desert environments, especially the Gulf desert environment, which is naturally reflected in slow microbial activities, it should be expected that the self-cleaning process would occur very slowly and/or only during a rather short period of the year. Thus, desert soils are characterized by very low moisture contents through most of the year. Due to drought, microbial activities, including oil biodegradation, occur at a satisfactory rate only during the short rainy periods (Radwan et al. 1995c). It is also known that hydrocarbons are consumed as carbon and energy sources, but for protein and nucleic acid synthesis excessive amounts of nitrogen are needed (Atlas and Bartha 1972; Gibbs 1975; Gibbs et al. 1975). Desert soils are poor in nitrogen; therefore, without nitrogen fertilization oil degradation there would be very limited. Furthermore, the desert temperature in summer is usually too high for the mesophilic microflora, and this is probably reflected in slower rates of oil-mineralization. In winter nights on the other hand, the chilling temperature arrests or slows down such activities. Recent findings indicate that simple organic carbon compounds in the soil enhance the oil-utilizing microflora, which becomes reflected in enhancing oil-biodegradation (Radwan et al. 2000b). Desert soils are poor in such organic carbon compounds. In other words, the self-cleaning of oily desert areas normally occurs at a rather slow rate. The objectives of bioremediation protocols for such soils should therefore involve simple and economical management that may improve these conditions and thus enhance the indigenous microflora.

15.3 Bioremediation

By definition, bioremediation is the biotechnology in which microorganisms are applied to mineralize and thus remove xenobiotic pollutants (Atlas and Pramer 1990). The theoretical basis of bioremediation has been repeatedly discussed and reviewed since the beginning of the 1990s (Hinchee and Olfenbuttel 1991a, b; Riser-Roberts 1992; Rosenberg 1993; Alexander 1994; Stoner 1994; Atlas 1995; Radwan et al. 1995c; Radwan 2008). There are also reports on bioremediation of oily environments (Mueller et al. 1989; Song et al. 1990; Hinchee and Olfenbuttel 1991a, b).

Bioremediation involves two approaches; promoting the activities of the indigenous oil-degrading microflora (biostimulation), and inoculating the oily environment with exogenous oil-utilizing microorganisms (seeding). To fulfill these two objectives in practice is by no means an easy job, especially as far as the second objective is concerned. Biostimulation of hydrocarbon utilizing microorganisms in oily desert could be achieved through proper agricultural management. Thus, aeration could be improved by occasional soil turning. Moisture levels could be elevated by irrigation eventually with treated sewage effluent. Fertilization, especially with nitrogen fertilizers and simple organic compounds, could be done by conventional means. Excessive acidity could be neutralized by liming. In spite of that, such approaches are not exclusively problem-free, given that oil-polluted desert areas are usually isolated and difficult to reach.

Technically, oily soils could be inoculated with hydrocarbon utilizing micro-organisms rather easily; however, it is not always guaranteed that the seeded strains will establish themselves in the polluted sites. Commercial mixtures of oil-utilizing micro-organisms can be purchased for in situ application (Applied Biotreatment Association 1989, 1990). However, it is commonly accepted among microbiologist that inoculated strains usually have only a weak competition potential with the prevailing microbial population in the soil.

Experience with the contaminated Kuwaiti desert areas showed that the two objectives, biostimulation and seeding, could in practice be easily fulfilled by cultivating contaminated desert areas with suitable plants, depending on rhizosphere technology, i.e., through phytoremediation (Radwan 1990).

15.4 Phytoremediation by Rhizosphere Technology

As the name indicates, phytoremediation involves the use of plants for cleaning soil pollutants. In many cases, e.g., in the phytoremediation of heavy-metal contaminated soils, the plants normally take up and accumulate and/or volatilize these pollutants. Oil and oil products on the other hand are water insoluble, and there is no evidence that plant roots may absorb these compounds. In fact, phytoremediation for oily soils makes use of the activities of the rhizospheric microflora rather than

those of the plant itself. Since plants have a direct effect on their rhizospheric soils, as will be summarized in this section, their role in phytoremediation of oily soils, albeit indirect, is quite important.

15.4.1 The Rhizosphere Environment

The rhizosphere is defined as the thin soil layer directly coating the root system and adhering to it. The magnitude of the rhizosphere varies according to the type of the roots; fibrous roots obviously provide much larger contact areas with soil than tap roots (Paul and Clark 1996).

Being under the direct influence of the plant, the rhizosphere environment is quite different from the environment of the non-rhizospheric soil. Generally, rhizospheric environments are more suitable for microbial life than the environment of the non-rhizospheric soil. It is known that higher plants pump down oxygen from the aerial organs, through roots into the soil. Therefore, the rhizospheric environment is usually better aerated than the rest of the soil (Curl and Truelove 1986). The moisture content of the rhizosphere is also higher than elsewhere in the soil, since plant roots keep these thin microenvironments almost as moist as the plant itself. This factor is particularly important for the arid and semi-arid desert soil microflora. Rhizospheres are also richer in organic matter than the soil far away from the root influence. As much as 20% of materials produced by photosynthesis may leak as exudates from roots into the rhizosphere (Vevrek and Campbell 2002). Plant root exudates, which obviously enhance the rhizospheric microflora, include simple carbohydrates, amino acids, vitamins, tannins, alkaloids, phosphatides and other unidentified organic materials (Curl and Truelove 1986; Knaebel and Vestal 1994; Sato 1994; Paul and Clark 1996; Atlas and Bartha 1998). Furthermore, it is known that during growth root cells secrete mucigel, as a lubricant for root penetration through the rough soil particles. This occurs particularly in young plants. As plants approach maturity, autolysis of some root materials occurs, resulting in additional enrichment of the rhizosphere with simple sugars and amino acids (Atlas and Bartha 1998). Interestingly, some rhizospheric bacteria, e.g., *Pseudomonas putida* (a typical hydrocarbon utilizer) and *Azospirillum* spp. (asymbiotic nitrogen fixers), enhance the release of simple organic compounds by plant roots (Curl and Truelove 1986). Other groups of rhizospheric micro-organisms also contribute to enriching this microenvironment with nutrients. Roots surrounded with living micro-organisms have been found to produce more organic exudates than sterile roots (Shann and Boyle 1994). Rhizospheric microorganisms may contribute directly to the production of critical nutrients. Typical examples are nodule bacteria associated with roots of legumes, and the asymbiotic diazotrophs which enrich the rhizospheres with fixed nitrogen. Rhizospheres also accommodate the group of the "plant growth-promoting rhizobacteria", which as their name indicates, enhance plant growth through the production of nutrients and auxins, and by facilitating nutrient transport across membranes (Zhang et al. 1996, 1997; Glick 2003; Radwan et al. 2005b).

15.4.2 The Rhizosphere Microflora

The favorable environmental conditions in the rhizosphere are reflected in enhanced microbial numbers and bioactivities as compared with non-rhizosphere soil areas (Curl and Truelove 1986). The numbers of many rhizosphere micro-organisms steadily decrease with increasing distance from the root. The so-called "rhizosphere effect", which is the ratio of microbial numbers in the rhizosphere to the numbers in the non-rhizosphere soil, is commonly between 5 and 20, but may reach as high as 100 or more (Atlas and Bartha 1998).

Rhizospheres of different plants are colonized primarily by Gram–negative, rod-like bacteria (see Atlas and Bartha 1998). The motile, rapidly growing pseudomonads are usually are more frequently present in rhizosphere than in non-rhizosphere soils. Apparently, this difference is attributed to the direct effect of plant root exudates which enhance microbial growth. The plant growth-promoting bacteria referred to above also colonize the rhizospheres, and are predominantly pseudomonads (Polonenko et al. 1987; Zhang et al. 1996, 1997).

Rhizospheric microorganisms have the potential for metabolizing many xenobiotic compounds (Walton and Anderson 1990; Boyle and Shann 1995). In addition, vegetation of contaminated soil probably reduces off-site movement of pollutants by wind and water erosion. On the other hand, some problems could be associated with vegetation as a means for soil stabilization and bioremediation. Thus, many pollutants are phytotoxic; and they, or specific metabolites accumulated by the plants in their presence, may reach the food web (Pfender 1996; Radwan et al. 2000a). Nevertheless, it is assumed that herbicide-decomposing rhizospheric bacteria may protect plants raised in soils contaminated with such herbicides (Anderson et al. 1994). Also, lead-resistant strains of *Pseudomonas*, when applied to wheat grains, were reported to reduce, by an unknown mechanism, the seedling uptake of this heavy metal from solution (Hasnain et al. 1993).

15.4.3 Phytoremediation for Xenobiotic Compounds

It may be useful here to refer to phytoremediation studies of conventional xenobiotic compounds before specifically considering studies of phytoremediation for oily desert soils. However, it is to be noted that many of these xenobiotic pollutants are in fact hydrocarbon derivatives.

A relatively large amount of work has been published on the biodegradation of pesticides in rhizospheres of many plant species (for earlier reviews see Anderson et al. 1993, 1994; Atlas and Bartha 1998). In most of the reviewed studies, enhanced biodegradation of these contaminants in the rhizospheres was attributed to enhanced microbial activities compared to those in the unvegetated soil. Furthermore, the increased biodegradation potential in the rhizosphere was correlated in many cases with increased numbers of pesticide-degrading microorganisms. Thus, for example, the rhizospheres of temik [2-methyl-2(methylthiol) propionaldehyde

O-(methylcarbamoyl) oxime]-treated corn, bean and cotton plants contained more temik-degrading microorganisms than the untreated soil (Abdel-Nasser et al. 1979). A higher atrazine (Z-chloro-4-ethylamino-6-isopropylamino-S-triazine) biodegradation was measured in the rhizosphere of corn than in the non-rhizosphere soil, even after harvesting the plant aerial organs (Seibert et al. 1981). Meanwhile, more of the pesticide-degrading microorganisms were counted in the rhizosphere than in the non-rhizosphere soil. Similar results were recorded for rice plants treated with parathion (O, O-diethyl-O-P-nitrophenyl phosphorothioate) (Reddy and Sethunathan 1983). The rhizospheres of diazinon [O, O-diethyl O (2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate]- treated wheat, corn and pea accommodated 100-fold higher numbers of diazinon-degrading microorganisms than the pesticide-treated unvegetated soil (Gavrilova et al. 1983). These microorganisms comprised bacteria, fungi and actinomycetes. Similar observations have been made on 2,4-dichlorophenoxyacetic acid-treated sugarcane plants (Sandmann and Loos 1984), and mecoprop herbicide [2-(2-methyl-4-chlorophenoxy) propionic acid]-treated wheat plants (Lappin et al. 1985).

In addition to studies on pesticide-contaminated soils, much work has also been done on soils contaminated with hazardous nonagricultural wastes, many of which are derivatives of aromatic hydrocarbons. It was also found that the biodegradation of these hazardous wastes was enhanced in plant-root zones. Thus, for example, the polyaromatic hydrocarbons, benzo[a]anthracene, chrysene, benzo[a]pyrene and dibenzo[a,h] anthracene were biodegraded more actively in the rhizospheres of eight tested prairie grasses than in the non-vegetated soil (Aprill and Sims 1990). Slurries of rhizosphere soils of four different plant species that had grown in a former solvent disposal site were found to biodegrade 1,1,2-trichlorethylene actively (Walton and Anderson 1990). The tested plants had different root types, namely fibrous, tap, leguminous and mycorrhizal roots. Root ectomycorrhizae have been found to have a potential for biodegradation of polychlorinated biphenyls (Donnelly and Fletcher 1992). Also, the rhizosphere-competent fungus, *Trichoderma harzianum*, was able to biodegrade the organochlorine compounds, pentachlorophenol, endosulfan and dichlorodiphenyl trichloroethane (Katayama and Matsumura 1993). Several authors reported the biodegradation of the volatile monoaromatic hydrocarbons (benzene, toluene, ethyl benzene and xylenes) by microorganisms in the rhizospheres of poplars, eastern cottonwoods, willows and others (Hong et al. 2001; Franzman et al. 2002; Spriggs et al. 2003).

15.5 Phytoremediation Strategies for Oily Desert Soils

This section will start with describing the effect of oil pollution on the plant community. Vegetation as means of seeding desert soils with oil-utilizing microorganisms and fertilization of such soils with chemicals especially nitrogen fertilization known to enhance hydrocarbon utilizing microorganisms will then be discussed.

15.5.1 Oil Plant Interaction

Soil pollution with crude oil and/or its products may dramatically affect plants growing in such soils. The effects are rather complex, and involve both direct contact toxicity and indirect deleterious influences through interaction of hydrocarbons with abiotic and microbial constituents of the soil (Bossert and Bartha 1984). Most contact toxicity arises from low boiling point hydrocarbons which may dissolve and damage cell membranes of delicate portions of plant roots and shoots (McGill et al. 1981). Indirect deleterious effects include oxygen deprivation of plant roots as a result of competitive oxygen exhaustion by the rhizospheric microflora, particularly the hydrocarbon-utilizing microorganisms (Bossert and Bartha 1984). Furthermore, crude oil is known to affect the physical soil structure, thus reducing its water-holding capacity and its degree of aeration (Dejong 1980). Large-scale oil spills apparently have more deleterious effects on plants than the mere application of moderate amounts (1–5% oil) of oily wastes (Kinako 1981). Crude oil levels less than about 1%, on the other hand, may enhance plant growth and productivity (Pal and Overcash 1978), probably because some constituent aromatic hydrocarbons may act as plant growth hormones (Fattah and Wort 1970). Intermediate products arising during microbial hydrocarbon degradation may also exhibit phytotoxicity, e.g., fatty alcohols, fatty aldehydes, fatty acids, terpenoids and others (Stevenson 1966).

There are reports in the literature indicating that some plants are more tolerant of oil pollution than others (Kinako 1981). Crop plants of a Nigerian agricultural soil polluted by an oil-well blowout responded differently during recovery (Odu 1972). Thus, tuberous crops, e.g., yams and cassava, were most adversely affected, whereas mango and banana were most adaptable. In a sub-arctic community contaminated with jet fuel, herbs and conifers failed to reappear, willow and dogwood seedlings exhibited moderate ability to become reestablished, and spruce and larch were inhibited most (Hunt et al. 1973). Most sensitive to oil and hydrocarbons are seedlings, annuals, and plants with large surface areas and those with shallow roots, mosses and lichens (McGill and Nyborg 1975; McGill et al. 1981).

15.5.2 Vegetation for Seeding

Application of phytoremediation technologies has several advantages. The process is rather simple and economical, it counteracts the erosional transport of contaminated soil, it does not involve environmental disturbance, and it is an aesthetically pleasing technology. Therefore, phytoremediation has good public acceptance (Komives and Gullner 2000) and it may be viewed as a solar-driven cleaning system. The potential use of plants as detoxifiers, filters or traps in cleaning up polluted soils has been discussed above. Here, the discussion is limited to the potential use of the rhizospheric microflora for cleaning up of oily desert areas.

In practice, it is not easy to successfully inoculate oily desert areas with microbial cocktails for the reasons mentioned above. Furthermore, there is experimental

Table 15.3 Numbers of oil-utilizing bacteria in rhizospheres of plants in Kuwaiti desert soils

	Numbers × 10^6 g^{-1}		
Plant	Soil	Rhizosphere	Reference
Cyperus conglomeratus	90	200	Radwan et al. (1998)
Lunea mucronata	90	340	
Picris babylonica	90	108	
Vicia faba	40	900	
Lupinus albus	40	3,100	
Phaseolus vulgaris	1	10	Radwan et al. (unpublished)
Bermuda grass	8	12	
American grass	14	17	

evidence that oil-utilizing bacteria which are introduced normally fail to establish themselves in the new environment (Radwan 1990; Radwan et al. 1997). Therefore, vegetation appears to be a practical approach for seeding, particularly after it has been discovered that rhizospheres of many plants harbour a rather rich oil-utilizing mciroflora (Table 15.3). Although oil pollution is associated with enriching desert soil with hydrocarbon-degrading micro-organisms (Radwan et al. 1990, 1997), the wild Gulf desert plant rhizospheres are microenvironments with richer oil-utilizing microflora (Radwan et al. 1995b, 1998a). These desert plants included *Senecio glaucus, Cyperus cenglomeratus, Launaea mucronata, Picris babylonica* and *Salsola* imbricate. Also rhizospheres of legume crops, e.g., broad beans and lupin cultivated in pristine and oily desert soil samples, were richer in hydrocarbon-utilizing bacteria than non-rhizosphere soil samples (Radwan et al. 1995b). The predominant oil-utilizing bacteria were *Cellulomonas flavigena, Rhodococcus erythropolis* and *Arthrobacter* spp. It has been found that the rhizosphere of popular trees contained five times more microbes capable of degrading benzene, toluene and xylene than the surrounding soil (Jordahl et al. 1997).

There is experimental evidence that spilled oil disappears more rapidly in vegetated than unvegetated soils. Direct uptake of hydrocarbons by plant roots does not appear to occur, although small amounts of low molecular weight polyaromatic hydrocarbons have been detected in root peels of carrot (Vevrek and Campbell 2002). On the other hand, parameters such as organic carbon, pH, solubility of inorganic constituents and others are so significantly altered in the rhizosphere (Reilley et al. 1996) that the microflora there is enhanced as compared with that in the bulk soil. Raising broad beans in oily sand soil samples enhanced the cleaning-up of the sand via the rhizospheric microflora (Radwan et al. 2000a). This legume is capable of tolerating up to 10% crude oil in soil, and in addition enriches the rhizosphere with the symbiotically fixed nitrogen. Factors enhancing plant growth and activities were found to also enhance the hydrocarbon removal in soil. Thus, inoculation of broad beans roots with plant growth-promoting rhizobacteria and/or nodule bacteria increased the phytoremediation potential of this crop for oily desert soil (Radwan et al. 2005b). Interestingly, nodule bacteria, e.g., *Rhizobium, Bradyrhizobium* and others, and plant growth promoting rhizobacteria, e.g., *Pseudomonas,*

Table 15.4 Hydrocarbon attenuation by phytoremediation

Plant	Hydrocarbon	% HC consumed		References
		Unvegetated	Vegetated	
Vicia faba	Crude oil	30.9	50.1–71.3	Radwan et al. (2005)
Bermuda grass	Crude oil	59	74–80	Radwan et al. (unpublished)

Flavobacterium, Serratia and others, proved to have hydrocarbon-degradation potential (Radwan et al. 2007b). Unpublished results in our laboratory indicate that established turf (Bermuda Grass and American Grass) covers could attenuate crude oil polluting desert soil samples through rhizosphere biotechnology. The most dominant hydrocarbon-utilizing bacteria in the rhizospheres of both grasses were identified as *Arthrobacter dextranalyticus, Pseudomonas fluorescens* and *Gordonia polyisoprenivorans*.

The above discussion indicates that vegetation could be a feasible approach for seeding oily desert areas with oil-degrading microorganisms, thus enhancing oil attenuation (Table 15.4). In this context, conventional farming involves practices, e.g. regular irrigation, liming, physical management and others, which obviously enhance microbial activities.

15.5.3 Vegetation for Fertilization

Not only does the vegetation practice enrich oily soils with oil-utilizing microorganisms, but it also fertilizes this environment with materials that enhance the growth of these micro-organisms and their activities. It has already been mentioned above that plant root exudates into the rhizosphere include simple sugars, amino acids, vitamins and others. These sources of organic material are valuable for improving poor environments like the desert soil. Organic substances enhance the physical properties of sand, e.g., its water-holding capacity and ion-exchange capacity. This is certainly reflected in favoring oil-utilizing microorganisms surrounding plant roots. Furthermore, since most oil-utilizing microorganisms also have the potential for utilizing conventional organic carbon sources, these substances would lead to active propagation and thus, amplification of the oil-utilizing microflora in the desert soil, which would in turn be reflected in enhanced oil degradation. The results of one of our studies (Radwan et al. 2000b) confirmed this assumption. Thus, fertilizing an oily desert soil sample with a mixture of glucose and peptone enhanced hydrocarbon biodegradation in that sample. The magnitude of that effect was too great to be attributed to nitrogen fertilization by peptone. Fertilization with amounts of KNO_3 containing amounts of nitrogen equivalent to those in peptone resulted in considerably lower oil-biodegradation rates. Fertilization of oily desert samples with glucose/peptone was found to increase the total numbers of oil-utilizing microorganisms in that sample. After 2 weeks, the indigenous micro-organisms had consumed the added glucose and peptone, but their numbers remained high,

apparently because they continued to grow on hydrocarbons as substrates. These results allow us to conclude that organic root exudates, e.g., simple sugars and amino acids, favor the oil-utilizing rhizospheric microflora, and consequently phytoremediation potential. The same is probably true for vitamin exudates. Vitamins have been found to enhance growth and activities of oil-utilizing microorganisms in vitro (Radwan and Al-Muteirie 2001).

Vegetation also appears to be an efficient and feasible approach for fertilizing oily desert soil environments with nitrogenous compounds. Nitrogen fertilizers have frequently been reported as limiting factors for microbial degradation of hydrocarbons (Atlas 1981; Leahy and Colwell 1990; Radwan et al. 1995a). That is why raising legume crops in oily desert samples enhances oil biodegradation there (Banks et al. 2000; Radwan et al. 2001). Legume roots carry nodules that fix molecular nitrogen, thus providing a natural and economical route for nitrogen fertilization (Radwan 2008). Interestingly, diazotrophic nodule bacteria have also been shown to have potential for hydrocarbon utilization (Prantera et al. 2002; Radwan et al. 2007b). In this context, excised legume nodules suspended and shaken in oily water in batch-cultures brought about hydrocarbon attenuation (Dashti et al. 2005, 2007b). Factors enhancing legume root nodulation, e.g., root inoculation with nodule bacteria, and plant growth and activities, e.g., inoculation with plant growth-promoting rhizobacteria, simultaneously enhanced oil attenuation in soils supporting these plants (2005b). Obviously this effect is not only due to the nitrogen fertilization effect, but also indicates that both nodule bacteria and plant growth-promoting rhizobacteria are themselves hydrocarbon degraders. Most of the latter bacteria are pseudomonads, which have long been known as hydrocarbon degraders (Khig and Markovets 1971; Levi et al. 1979; Levi et al. 1979; Einsele 1983).

It may be believed that only legume crops can enrich oily soils with the fixed nitrogen needed for phytoremediation. Unpublished studies in our laboratory showed that nonlegume plants, e.g., grasses (Bermuda Grass and American Grass), cultivated as established turf cover harbour in their rhizospheres a rich bacteriaflora with the combined activities of hydrocarbon-utilization and asymbiotic nitrogen

Table 15.5 Rhizospheric bacterial genera with the combined activities of hydrocarbon-utilization and nitrogen fixation (unpublished results from our laboratory)

Oil utilizers[a] with nitrogen-fixation potential	Diazotrophs[b] with oil-utilization potential
Bacillus	*Rhodococcus*
Ochrobacterum	*Leifsonia*
Nocardia	*Bacillus*
Pseudoxanthomonas	*Rhizobium*
Rhodococcus	*Brevibacillus*
Pantoea	*Cellulosimicrobium*
Arthrobacte	*Stenotrophomonas*
Enterobacter	*Kocuria*
Micrococcus	*Arthrobacter*

Note: All isolates were nitrogenase-positive and are arranged in decreasing frequencies
[a]Initially isolated on an inorganic medium with oil vapour as a sole source of carbon and energy
[b]Initially isolated on a nitrogen-free medium

fixation. Similar organisms were also found to colonize periphytically the surfaces of nodules on roots of legumes. Table 15.5 lists those bacteria which are simultaneously hydrocarbon-degraders and diazotrophic. Obviously such microorganisms are valuable tools for cleaning oily environments poor in nitrogen such as desert soils. We have reason to believe that many of the common oil-utilizing bacteria are also nitrogen fixers, and vice versa.

15.6 Conclusions

Phytoremediation appears to be a feasible and an economical biotechnology for the oil-contaminated desert areas. Costs would be limited here mainly to those of conventional vegetation practices. Although such costs would be higher than managing a conventional agricultural soil for obvious reasons, they would undoubtedly be less than adopting alternative cleaning techniques, e.g., removing oil pollutants microbiologically in bioreactors. Furthermore, phytoremediation provides the advantages of being an aesthetically pleasing approach, environmentally friendly and active against erosional transport of contaminated soil. The two major problems to be solved in this technology are to select crops tolerant of desert conditions and oil, and to find a suitable water supply. There are several crops, especially legumes such as broad beans, that can tolerate up to 10% crude oil in soil. Such crops may be cultivated throughout the year in temperate deserts and during mild seasons in hot arid or semiarid deserts. Where the oil concentration is too high, the soil could be diluted using the nearby pristine sand prior to vegetation. Water, on the other hand, would have to be transported by suitable means.

Crops harbour in their surroundings rich consortia of oil-utilizing microorganisms, asymbiotic, and in legume crops symbiotic diazotrophic bacteria. Many of the rhizospheric bacteria exhibit the combined activities of hydrocarbon utilization and nitrogen fixation. Desert soil areas subjected to phytoremediation would probably turn into fertile and agriculturally productive soil areas, which is a further advantage of this biotechnology.

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Chapter 16
Heavy Metal Phytoremediation: Microbial Indicators of Soil Health for the Assessment of Remediation Efficiency

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16.1 Microbial Indicators of Soil Health

In the last few years, there has been a growing interest in the definition and evaluation of soil health, in part stimulated by an increasing awareness that soil is a critical component of the Earth's biosphere, functioning not only in the production of food and fibre but also in the maintenance of local, regional, and worldwide environmental quality (Doran and Parkin 1994; Alkorta et al. 2003a). Although its definition is currently a topic of much debate and confusion, a commonly used definition of soil quality/health that appears in many bibliographic references reads as follows: "the continued capacity of a specific kind of soil to function as a vital living system, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, to maintain and enhance the quality of air and water environments, and to support human health and habitation" (Doran and Parkin 1996; Doran and Safley 1997). The terms "soil quality" and "soil health" are often used interchangeably, but the former focuses more on the capacity of the soil to meet defined human needs, such as the growth of a particular crop, while, by contrast, the latter (1) relates more to the soil's continued capacity to sustain plant growth and maintain its functions (Coleman et al. 1998), and (2) captures the ecological attributes of the soil, mainly, those associated with its biota, such as biodiversity, food-web structure, activity, range of function, etc. (Pankhurst et al. 1997). Furthermore, the term "soil health"

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conveys the idea of soil as a living system, a concept of the utmost importance since, after all, the soil contains vast assemblages of organisms that are responsible for many of its vital functions, such as decomposition and recycling of nutrients from dead plant and animal tissues, nitrogen fixation, maintenance of soil structure, detoxification of pollutants, and so on (Alkorta et al. 2003b).

Unfortunately, our soils are presently being degraded through salinization, erosion, sealing, pollution, loss of organic matter and biodiversity etc., leading to the deterioration of the soil's physical, chemical and biological properties worldwide. Actually, the quantity and quality of the soil's ecosystem services and functions are nowadays being diminished at an alarming rate, making soil degradation an environmental issue that demands immediate attention and response. In this respect, history has repeatedly shown that mismanagement of the soil resource base can lead to poverty, malnutrition and economic disaster (Bezdicek et al. 1996). Within a community, a strong link can be found between soil quality/health, food quantity and quality, and the health, well-being, and prosperity of its citizens (Janke and Papendick 1994). Indeed, the quality of life on Earth is inextricably linked to the health of our soils.

In particular, the release of contaminants into our soils by human activities has increased enormously over the past several decades, overwhelming the self-cleaning capacity of the soil ecosystem and, as a consequence, resulting in the accumulation of dangerous toxic substances. Accordingly, in our time, soil pollution attracts considerable public attention, since the magnitude of the problem calls for immediate action (Garbisu and Alkorta 2003).

In this context, it is imperative to have reliable indicators for the assessment and monitoring of soil health. To date, emphasis has mostly been placed on physical and chemical soil properties as indicators of soil health, but biological parameters are becoming increasingly used due to their being more sensitive to changes in the soil, as well as to their capacity to provide information that integrates many environmental factors (Alkorta et al. 2003b; Hernández-Allica et al. 2006a; Mijangos et al. 2006). Many biological parameters have been proposed as bioindicators of soil health, such as microbial biomass, basal and substrate-induced respiration, mineralizable nitrogen, soil enzyme activities, abundance of soil microflora and fauna, root pathogens, structural and functional biodiversity, food-web structure, plant growth and diversity, and so on (Pankhurst et al. 1997). Microbial parameters, particularly those related to the size, activity and biodiversity of the soil microbial communities, are most relevant as indicators of soil health. After all, microbially mediated processes are central to the functions that soil performs and, what's more, microorganisms are responsible for 70–85% of the soil biological activity (Reichle 1977).

In any event, and because the pedosphere, hydrosphere, atmosphere and biosphere are overlapping, intimately associated in the environmental compartments of the ecosystem, whatever occurs in the soil has a profound effect not only on soil health but also on ecosystem health (Huang et al. 1998). The concept of ecosystem health has been elaborated as a comprehensive, multiscale, dynamic, hierarchical measure of: (1) system *vigor*, which may be quantified in terms of productivity, throughput of material and energy in the system etc., (2) system *resilience*, which may be determined

in terms of the system's ability to maintain its structure and pattern of behaviour in the presence of stress, and (3) system *organization*, which may be assessed in terms of both the diversity of components and their degree of mutual dependence (Mageau et al. 1995; Costanza et al. 1998; Rapport 1998; Alkorta et al. 2004a). Moreover, a healthy ecosystem must have the following attributes: (1) it should be free of the "ecosystem distress syndrome", which comprises a group of signs (e.g., leaching of soil nutrients, reduced species diversity, shifts in species composition to opportunistic species, reduced productivity, increased pest and disease loads) by which ecosystem breakdown is recognized, (2) it should be self-sustaining, and (3) it should not adversely affect or degrade surrounding systems (Costanza et al. 1992; Hildén and Rapport 1993; Rapport et al. 1997; Alkorta et al. 2004a). Although soil health must not be equated with ecosystem health, the application of the ecosystem health approach to studies of soil status and condition can indeed provide useful information to, for instance, assess the impact of pollution on soil functioning, and to establish the efficiency of a certain remediation procedure.

As mentioned above, *vigor* can be measured in terms of productivity, throughput of material and energy in the system etc. In the soil, these processes might be investigated by methods that focus either on broad physiological property, such as soil respiration or nitrogen mineralization, or on specific enzyme reactions carried out by soil microorganisms (Kandeler 2007). Soil respiration is related to ecosystem productivity, soil fertility, and regional and global carbon cycles (Luo and Zhou 2006). Soil enzyme activities, which control the rates of soil nutrient cycling, provide a unique integrative biological assessment of soil function, especially those catalyzing a wide range of soil biological processes, such as dehydrogenase, urease, phosphatase and others (Nannipieri et al. 2002).

Resilience is included in the concept of *stability*, which comprises both *resilience,* the property of the system to recover after disturbance, and *resistance*, the inherent capacity of the system to withstand disturbance. This concept can be applied to those studies dealing with the impact of pollution on soil health. Interestingly, two theories diverge with respect to the stability of ecological processes: (1) according to the first theory, non-stressed systems are more stable, thanks to the large resources they dispose to maintain function in case of stress (Loreau 2000), and (2) the second theory predicts that stressed systems are more stable because, due to first stress, they have gained abilities to cope with stress and thus maintain function (Odum 1981).

Finally, *organization* refers to ecosystem complexity, and is affected by both the diversity of species and the number of pathways of material exchange between each component (Costanza et al. 1998). From an ecological perspective, in the soil ecosystem, functional diversity, as opposed to structural diversity, provides more relevant information (Torsvik and Øvreås 2007). There are different methods for determining the functional diversity of soil microbial communities such as community-level physiological profiles (Preston-Mafham et al. 2002), soil enzyme activities (Larson et al. 2002), and a variety of culture-independent molecular techniques (Malik et al. 2008).

In this Chapter, the possibility of using microbial indicators of soil health to assess the efficiency of metal phytoremediation processes is discussed.

16.2 Heavy Metal Phytoremediation

Heavy metals are present in soil as natural components or as a result of human activity, with the primary sources of metal pollution being the burning of fossil fuels, mining and smelting of metalliferous ores, electroplating, downwash from power lines, municipal wastes, fertilizers, pesticides and sewage (Garbisu and Alkorta 2001; Alkorta et al. 2004b). Actually, metal pollution has become one of the most serious environmental problems today (Alkorta et al. 2004b). For instance, arsenic, a nonessential metalloid, is an environmental pollutant of prime concern which is causing a global epidemic of poisoning, with tens of thousands of people having developed skin lesions, cancers and other symptoms (Pearce 2003; Alkorta et al. 2004c; Rozas et al. 2006).

Some metals are essential for life (e.g., they provide essential cofactors for metalloproteins and enzymes) but, at high concentrations, metals are toxic for both higher organisms and microorganisms (Garbisu and Alkorta 1997). Indeed, at high concentrations, metals can act in a deleterious manner by blocking essential functional groups, displacing other metal ions, or modifying the active conformation of biological molecules (Collins and Stotzky 1989). Besides, due to their immutable nature (metals are unique in that they do not undergo either chemically or biologically induced degradation that could reduce their toxicity, but rather are transformed from one oxidation state or organic complex to another) (Alkorta et al. 2006) and persistence in soil (with residence times in the order of thousands of years) (McGrath 1987), metals are a group of pollutants of much concern (Garbisu and Alkorta 1997, 2003). In soil, rather than total metal concentration, a major factor governing metal toxicity is biovailability (Alkorta et al. 2006).

Figure 16.1 is included as an example of the impact of metal pollution on soil microbial communities (in particular, on the cultivable portion of the soil heterotrophic microbial community). In this figure, the average well colour development (AWCD) curves, obtained from the carbon substrate utilization patterns (i.e., community-level physiological profiles - CLPP) of the Biolog EcoPlates™, are presented. The data correspond to a soil artificially polluted with 250, 500, 100, 2,000 and 4,000 mg Zn kg^{-1} DW soil as $ZnCl_2$, and show that 250 and 500 mg Zn kg^{-1} did not cause a negative impact on the functional microbial diversity of the soil. In contrast, 1,000 mg Zn kg^{-1} and especially 2,000 and 4,000 mg Zn kg^{-1} clearly affected the capacity of the cultivable portion of the soil heterotrophic microbial community to utilize carbon substrates.

Traditional physicochemical methods for the remediation of metal-polluted soils are in general very expensive, which has stimulated the development of innovative biological technologies to economically remediate these soils (Hernández-Allica et al. 2006a). Bioremediation, "a managed treatment process that uses microorganisms to degrade and transform chemicals in contaminated soil, aquifer material, sludges and residues" (Dasappa and Loehr 1991), offers an effective, non-destructive, economical clean-up technique for the remediation of polluted sites, that must be considered an important tool in our attempts to mitigate environmental contamination

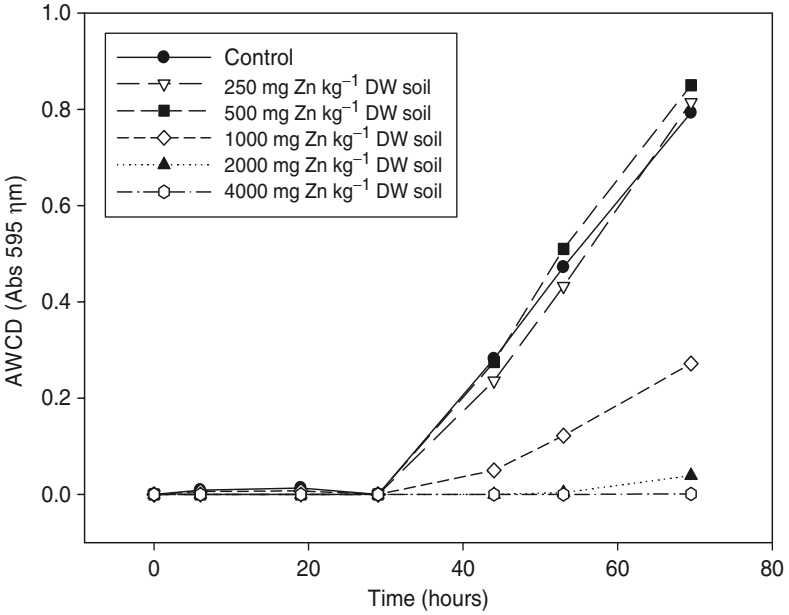

Fig. 16.1 Effect of Zn pollution on average well colour development (AWCD) curves obtained with Biolog EcoPlates. Soils were polluted with 250, 500, 1,000, 2,000 and 4,000 mg Zn kg^{-1} DW soil as $ZnCl_2$

(Garbisu and Alkorta 1997, 1999, 2003). But although many studies have been carried out to investigate the possibility of using microorganisms to aid in the remediation of metal polluted environments, microorganisms do not solve the critical problem of the removal of metals from the polluted soil. As a matter of fact, bacteria can only transform metals from one oxidation state or organic complex to another, but not extract them from the polluted soil (Garbisu et al. 2002).

Luckily, to overcome this limitation of bacterial metal soil remediation, the possibility of using plants that can literally extract the metals from the polluted soil was raised. In this respect, phytoremediation, "the use of green plants to remove pollutants from the environment or to render them harmless" (Cunningham and Berti 1993; Raskin et al. 1994), is currently viewed as the ecologically responsible alternative to the environmentally destructive physicochemical remediation methods (Meagher 2000). This phytotechnology has been reported as an effective, non-intrusive, inexpensive, aesthetically pleasing, socially accepted remediation process (Garbisu et al. 2002). The technical aspects of phytoremediation, together with the advantages and limitations of this technology, have been extensively reviewed elsewhere (Chaney et al. 1997; Raskin et al. 1997; Salt et al. 1998; Alkorta and Garbisu 2001; Garbisu and Alkorta 2001; Garbisu et al. 2002; McGrath et al. 2002; Alkorta et al. 2004b, d; Pilon-Smits 2005).

Within the field of phytoremediation, several categories have been defined: phytoextraction, phytofiltration, phytostabilization, phytovolatilization, phytodegradation, phytotransformation, etc. (Garbisu et al. 2002). With regard to soil metal remediation, two of these categories are most relevant: phytoextraction and phytostabilization.

Finally, it is most important to emphasize that, from an ecocentric point of view, the ultimate goal of any soil remediation process must be not only to remove the pollutant(s) from the soil but to restore soil health (Hernández-Allica et al. 2006a; Epelde et al. 2008).

16.2.1 Continuous Metal Phytoextraction

The term "phytoextraction" refers to the utilization of plants to remove pollutants (mostly, metals) from soils. In particular, "continuous phytoextraction" is based on the utilization of metal-hyperaccumulating plants (hyperaccumulators) that have the capacity to accumulate, translocate and tolerate high amounts of metals over the complete growth cycle (Salt et al. 1995; Baker et al., 2000). In this respect, *Thlaspi caerulescens* (i.e., alpine pennycress or alpine pennygrass), a hyperaccumulating plant extensively studied due to its remarkable capacity to phytoextract Zn and Cd from polluted soils (Hernández-Allica et al. 2006a, b; Epelde et al. 2008), has been suggested as a model species for research on metal phytoextraction (Assunção et al. 2003).

With a local ecotype of *T. caerulescens*, termed "Lanestosa", from the Basque Country (northern Spain), we carried out a microcosm study to: (1) evaluate the potential of such ecotype for Zn phytoextraction, and (2) assess the effect of the phytoextraction process, which includes both plant growth and metal phytoextraction, on microbial indicators of soil health. To this end, *T. caerulescens* seedlings were transplanted to 2.5-kg pots that had been artificially polluted with 1,000 mg Zn kg^{-1} DW soil and fertilized with 120 mg kg^{-1} DW soil of N, P and K. After 3 months of growth, on average, *T. caerulescens* plants accumulated 5,654 mg Zn kg^{-1} DW shoot and extracted 28.9 mg of Zn pot^{-1}. In addition, CLPPs were obtained from Biolog EcoPlates™ at the end of the experiment. Table 16.1 shows the values of AWCD, richness (S) and Shannon's diversity (H') calculated from Biolog EcoPlates data at an incubation time of 52 h. As observed in this Table 16.1, in the absence of plants, Zn pollution led to lower values of all these parameters, as compared to values of control non-polluted soils. Nevertheless, as a result of *T. caerulescens* growth and metal phytoextraction, all these values were recovered, indicating that the cultivable portion of the soil heterotrophic microbial community had recovered its capacity to use carbon substrates (its functional diversity). For all parameters of microbial functional diversity, the presence of plants proved more important than the amount of metal phytoextracted from the soil (actually, in this experiment, this amount was very low). As compared to bare soil, vegetated

Table 16.1 Values of average well colour development (AWCD) and diversity indexes calculated from Biolog EcoPlates absorbance data at 52 h incubation time, in soils from a Zn phytoextraction experiment with *Thlaspi caerulescens* Lanestosa plants. *S* = richness; *H'* = Shannon's diversity. Mean values ($n = 3$) ± standard error. Values followed with different letters ($^{a, b}$) are significantly different ($P < 0.05$ or lower) according to Fisher's PLSD-test

Soil	AWCD	S	H′
Control, non-polluted	0.91 ± 0.01^{ab}	24 ± 1^{a}	3.0 ± 0.1^{a}
Metal-polluted, unplanted	0.78 ± 0.03^{a}	18 ± 0^{b}	2.8 ± 0.0^{a}
Metal-polluted, planted	1.01 ± 0.05^{b}	24 ± 2^{a}	3.1 ± 0.1^{a}

soils are commonly described as having higher rates of microbial activity, due to the presence of additional surfaces for microbial colonization and organic compounds released by the plant roots (Tate 1995; Grayston et al. 1997; Delorme et al. 2001). In similar phytoextraction experiments with *T. caerulescens* plants, higher values of biological activity were found in rhizosphere vs non-rhizosphere soil (Gremion et al. 2004; Keller and Hammer 2004; Hernández-Allica et al. 2006a; Wang et al. 2006; Epelde et al. 2008). Finally, with regard to the recovery of soil health derived from a phytoextraction process, as reflected by the values of soil microbial parameters, an ideal target should be to return to the conditions of a valid control soil, i.e., a vegetated, unpolluted soil of similar physicochemical properties and subjected to the same edaphoclimatic conditions.

From a remediation point of view, phytoextraction demands a sufficient harvestable biomass. Unfortunately, most metal hyperaccumulators (e.g., *T. caerulescens*) are, in general, relatively small, have slow rates of biomass production and lack any established cultivation, pest management or harvesting practices (Wenzel et al. 1999). Consequently, nowadays, fast-growing, high biomass crop plant species that accumulate moderate levels of metals in their shoots are actively being tested for phytoextraction (Hernández-Allica et al. 2008). After all, in some cases, a greater shoot biomass has been reported to more than compensate for a lower shoot metal concentration (Ebbs and Kochian 1997).

16.2.2 Chelate-Induced Phytoextraction

The discovery that the application of chelating agents to the soil increases plant metal uptake and translocation opened a wide range of possibilities for phytoextraction (Blaylock et al. 1997), most importantly that of using high biomass plants for the remediation of metal polluted soils, particularly with low bioavailable metals such as Pb (Hernández-Allica et al. 2007). However, side-effects related to the addition of chelating agents to the soil, such as metal leaching and toxic effects on soil microbial communities, have usually been neglected (Römkens et al. 2002). In this respect, most studies on chelate-induced phytoextraction have focused on EDTA

(ethylenediaminetetracetic acid)-mediated Pb phytoextraction (McGrath et al. 2002). Nevertheless, EDTA and the formed EDTA-Pb complexes present low biodegradability and a high solubility, resulting in an elevated risk of adverse environmental effects due to metal mobilization and long persistence (Alkorta et al. 2004d). EDDS (ethylenediaminedisuccinate) has been proposed as an alternative for chelate-induced metal phytoextraction (Grčman et al. 2003; Santos et al. 2006). EDDS has been shown to be easily biodegradable (Jaworska et al. 1999), to form strong complexes with transition metals and radionuclides (Jones and Williams 2001), to cause a much lower leaching of Pb down the soil profile than EDTA (Grčman et al. 2003), and to be less toxic to soil microorganisms (Grčman et al. 2003). In any case, environmentally safe methods of chelate-induced phytoextraction must clearly be developed before steps towards further development and commercialization of this remediation technology are taken (Alkorta et al. 2004d).

As an example, Fig. 16.2 shows the response of several soil microbial parameters to EDTA and EDDS addition (1 g kg^{-1} DW soil) in a microcosm chelate-induced

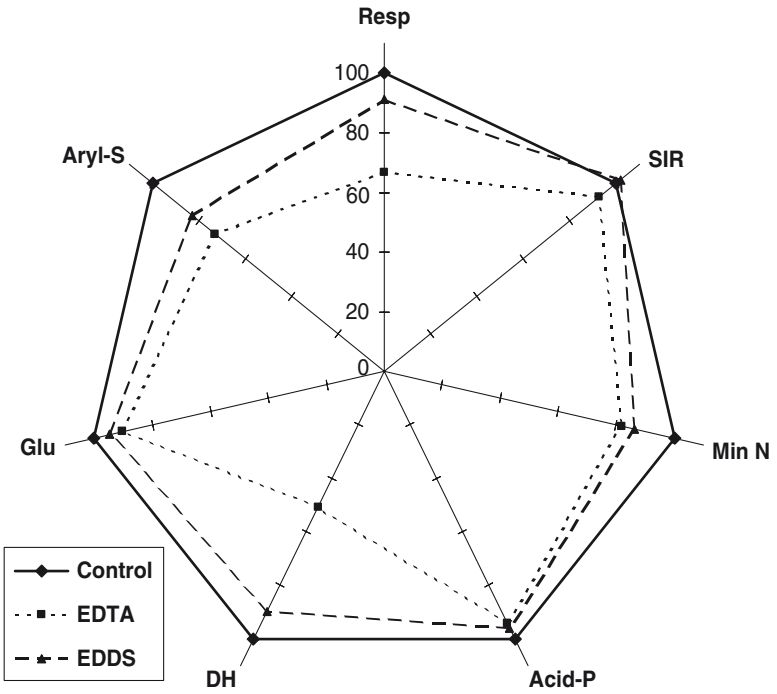

Fig. 16.2 Effect of chelating agents (EDTA, EDDS) on soil biological parameters in a chelate-induced Pb phytoextraction experiment with *Cynara cardunculus* plants. Controls: no chelating agents added. Values of control soils are used as reference points (values found in these control soils = 100%). *Resp*: basal respiration (indicator of soil microbial activity), *SIR*: substrate-induced respiration (indicator of potentially active microbial biomass), *Min N*: potentially mineralizable N (indicator of potential rate of N mineralization), *DH*: dehydrogenase (indicator of overall

phytoextraction experiment, with *Cynara cardunculus* plants, carried out in a soil artificially polluted with 1,000 mg Pb kg^{-1} DW soil. EDTA was much more effective (428.4 mg Pb kg^{-1} DW shoot) than EDDS (20.8 mg Pb kg^{-1} DW shoot) for the induction of Pb shoot accumulation (6.3 mg Pb kg^{-1} DW shoot in control pots). However, soil microbial parameters, especially dehydrogenase activity (which, on average, was halved) and basal respiration, were more negatively affected by EDTA than EDDS (Fig. 16.2). Dehydrogenase activity, which occurs in every viable microbial cell, and basal respiration are both used as indicators of overall microbiological activity in the soil (Nannipieri et al. 2002).

Then, apart from being effective for the induction of metal phytoextraction, chelating agents for enhanced phytoextraction must be as innocuous as possible for the soil biota. After all, the addition of chelating agents to the soil may increase, for instance, metal availability for soil microorganisms (Welp and Brümmer 1997) and, since soil microorganisms depend directly or indirectly on the soil solution for uptake of food and water, elevated metal concentrations in the soil solution might lead to toxic effects on the soil microbiota (Römkens et al. 2002).

16.2.3 Phytostabilization

The remediation of metal-polluted soils (particularly those presenting high levels of metal pollution) using phytoextraction procedures usually takes many years, and most probably decades. In fact, to overcome this often considered Achilles heel of phytoextraction, i.e., the long time needed for effective remediation, it has been suggested that this phytotechnology should be combined with a profit-making operation such as forestry or bioenergy production (Robinson et al. 2003). In any case, an alternative phytotechnology for the remediation of metal-polluted soils is phytostabilization or the use of plants to reduce the bioavailability of pollutants in the environment. More specifically, phytostabilization refers to the immobilization of a contaminant in the rhizosphere through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants, so that contaminant migration via wind and water erosion, leaching and soil dispersion are prevented (EPA 2000). Thus, although metals are not removed from the soil, their adverse environmental effects are reduced. The choice of metal phytoremediation strategy (phytoextraction vs phytostabilization) will depend on a variety of factors: type of metal(s) present in the soil, level of metal pollution, future use of the site etc. Interestingly, the combination of both strategies, so that the limitations of one strategy might be overcome by the advantages of the other strategy, appears a most promising approach.

Fig. 16.2 (continued) microbiological activity of soil), *Glu*: β-Glucosidase (an enzyme that plays a central role in the hydrolysis of polymers of plant residues, i.e., cellobiose), *Aryl-S*: arylsulfatase (an enzyme that catalyses the hydrolysis of organic sulphate ester releasing sulphate), *Acid-P*: acid phosphatase (an enzyme that releases phosphate from organic phosphorus)

Chemophytostabilization, the combination of a chemical method (such as the addition of organic or inorganic amendments to the soil) with phytostabilization (Knox et al. 2000), is most promising for the remediation of metal-polluted soils. Amendments, such as different sources of organic matter, are added to the soil in an attempt to reduce metal bioavailability by formation of insoluble metal organic complexes with humic acids, thereby lessening the risk of metal toxicity to plants and microbes (Stevenson et al. 1972; Kirkham 1977). The combination of both approaches is very interesting, as the chemicals bind the excess of metals, control pH and provide plant nutrients, and the plants prevent wind erosion, reduce leaching, and accumulate metals in their roots.

We have previously reported the phytostimulatory effect of plants on soil microbial communities within the context of the phytoremediation of metal-polluted soils (Hernández-Allica et al. 2006a; Epelde et al. 2008). However, for a chemo-phytostabilization procedure, it is essential to also take into account the effects of amendments on the soil microbial community. In this respect, we carried out a microcosm study with a moderately polluted mine soil (1,000 mg Zn kg^{-1} DW soil, 340 mg Pb kg^{-1} DW soil, 2.6 mg Cd kg^{-1} DW soil) amended with cow slurry [i.e., cow slurry, having 12% dry matter (DM), 3.25% DM nitrogen, 0.9% DM phosphorus, and 3% DM potassium, was applied at a dose of 0.1 l kg^{-1} DW soil] or a chemical fertilizer (i.e., urea plus PK14% fertilizer at similar nutrient doses to those applied as cow slurry). Twenty weeks after amendment addition, bioavailable ($CaCl_2$ extractable) soil metal concentrations had decreased considerably: a 45, 62 and 38% reduction in bioavailable Zn, Pb and Cd, respectively, was observed for the chemically fertilized soils; a 34, 50 and 33% reduction in bioavailable Zn, Pb and Cd, respectively, was found in those soils fertilized with cow slurry. At the same time, values of dehydrogenase activity increased in both amended soils (i.e., control, no amendment added, soil: 0.2 mg INTF kg^{-1} DW soil 20 h^{-1}; soil with chemical fertilizer: 12.0 mg INTF kg^{-1} DW soil 20 h^{-1}; soil with cow slurry: 77.4 mg INTF kg^{-1} DW soil 20 h^{-1}), indicating the stimulatory effect of both amendments on soil microbiological activity.

Since soil microbial biodiversity has a key role in the maintenance of soil fertility, functioning and resilience, in the same chemophytostabilization study we also determined the microbial functional diversity, through CLPPs obtained with the Biolog EcoPlates, of control and amended soils. Figure 16.3 shows the metabolic fingerprints of the CLPPs displayed by control and amended (cow slurry or chemical fertilizer) soils. For clarity purposes, only those substrates showing significant differences among treatments are presented. As observed in this figure, the addition of urea plus PK14% led to a different pattern of carbon substrates utilization by the cultivable portion of the soil heterotrophic microbial community. The addition of cow slurry resulted in higher values of functional diversity in the polluted mine soil (Fig. 16.3). In this case, the easily mineralizable organic matter might have favoured the microbial functional diversity of the soil. The addition of manure and compost to the soil has previously been reported to significantly increase values of microbial functional diversity according to Biolog data (Gómez et al. 2006; Toyota and Kuninaga 2006).

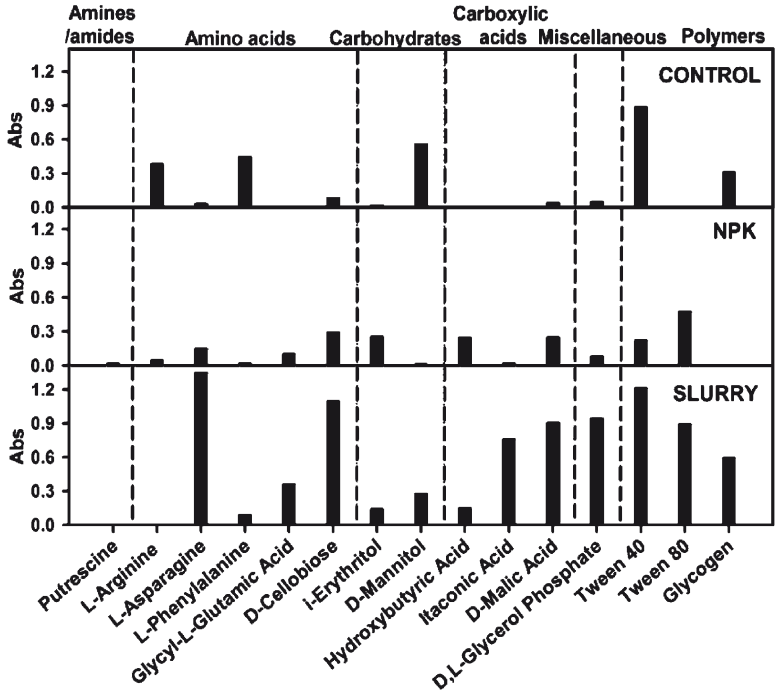

Fig. 16.3 Metabolic fingerprints of substrate utilization patterns obtained with the Biolog EcoPlates at an incubation time of 44 h, from control (no amendment), chemical fertilizer-amended (NPK = urea plus PK14%) and cow slurry-amended mine soil. For clarity purposes, only those substrates showing significant differences ($P < 0.05$) among treatments, according to ANOVA, are presented

16.3 Conclusions

Despite the logical interest in improving the metal extraction capacity of metal phytoremediating plants (phytoextraction) or reducing metal bioavailability, leaching and dispersion using plants (phytostabilization), it should never be forgotten that the ultimate goal of any soil remediation process must be not only to remove the contaminant(s) from the polluted soil but, most importantly, to restore the continued capacity of the soil to perform or function according to its potential (i.e., to recover soil health). In fact, in some cases it might be possible to recover soil health without decreasing soil metal concentrations to levels indicated in the different regulations (most current regulations governing metal toxicity in soils are still based on total metal concentration in the soil, whose validity as a basis for metal limits in soil is certainly questionable).

In this respect, although to date, emphasis has been placed on physical and chemical soil properties as indicators of soil health, biological parameters are becoming increasingly used due to their being more sensitive to changes in the soil, as well as to their capacity to provide information that integrates many environmental

factors. In particular, those biological indicators related to the size, activity and diversity of the soil microbial communities are most promising since microorganisms are, to a large extent, responsible for soil functioning. Microbial indicators of soil health are valid tools for evaluating the success of phytoextraction and phytostabilization processes.

Finally, it is important to point out that metal-polluted and phytoremediated soils are interesting scenarios for delving deeper into the still poorly understood plant–microbe interactions that occur below ground, and which fulfil vital roles in the functioning of terrestrial ecosystems. After all, disturbed environments usually provide a better insight into the workings of the system.

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Chapter 17
The Environment and the Tools in Rhizo- and Bioremediation of Contaminated Soil

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17.1 Techniques for Culture-Independent Assessment of Microbial Communities

Culturable proportions of bacterial communities from the environment are negligible compared with the number of species that are present. Thus, culture techniques for environmental bacterial community diversity analysis are becoming obsolete. Øvereås and Torsvik (1998) compared culturable bacterial diversity of agricultural soil communities with diversity obtained by molecular means. They found that molecular methods revealed a much higher bacterial diversity than classical isolation techniques, and concluded that bacterial diversity studies should embrace entire communities, not only the culturable portion.

Several molecular techniques have been developed to identify and determine species diversity of microorganisms without isolation (Kawai et al. 2002). PCR-based techniques are becoming increasingly popular for research ranging from diagnostic work to genome fingerprinting and probing (Torsvik and Øvereås 2002). PCR is regularly applied to assay environmental samples, due to the ability of the technique to detect relatively small numbers of target organisms without requiring cell culture (Volossiouk et al. 1995). Thus, PCR can be used to target certain types of genes expected within specific communities and performing specialised functions. Sei et al. (2003) developed a set of primers for detecting and monitoring alkane-degrading bacteria. The primers were designed to target the homologous regions of alkane hydroxylase genes (*alk* genes) and thus assess the alkane-degrading potential of a particular environment. These primers were tested on communities capable of degrading n-alkanes, the major component of crude oil. Sei et al. (2003) found that shorter n-alkane chains were degraded first by Group I alkane-degrading bacteria, whereas Group III alkane-degrading bacteria degraded longer chains later. However, as with most techniques there are some drawbacks to using PCR, e.g., preferential

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amplification of certain types of sequences, chimeric sequence generation and false results due to pollution (Osborne et al. 2005). Despite this, PCR remains reliable, and forms the base-technique for most biological molecular work.

Ribosomal RNA (rRNA) molecules are used as molecular chronometers, due to their high degree of structural and functional conservation. Consequently, domains within rRNA molecules harbour independent rates of sequence change (Kent and Triplett 2002). Phylogenetic relationships can be determined by examining these changes over time (Kent and Triplett 2002).

Initial assessment of soils, using culture-independent methodologies, revealed the presence of three main bacterial divisions, viz. *Proteobacteria*, *Fibrobacter* and low-GC Gram-positive bacteria (Kent and Triplett 2002). Specific gene coding for enzymes that are known to be involved in hydrocarbon catabolism has been identified. Widmer et al. (1998), realising the potential of environmental microorganisms, specifically *Pseudomonas* species, developed a PCR protocol for selective detection of *Pseudomonas* (*sensu stricto*) in the environment. They designed a highly-selective primer pair for the 16 rRNA genes of *Pseudomonas* species that was used with 91.7% efficacy for bacterial identification from the environment, based on sequence phylogeny. Following this, Milcic-Terzic et al. (2001) and Whyte et al. (2001) combined culture-dependent methods and molecular analysis, using hydrocarbon catabolic gene probes *alkB* (C_6-C_{32} n-paraffin degradation), *xylE* (toluene and xylene degradation) and *ndoB* (naphthalene degradation), to demonstrate the presence of hydrocarbon-degrading microbes in polluted soils.

Nitrogen-fixing microorganisms can be instrumental in hydrocarbon pollution bioremediation (see 2.1.4). However, they are difficult to culture, due to their different growth requirements and physiology, which limits simultaneous cultivation of separate species (Widmer et al. 1999). Molecular methods for identifying the presence of nitrogen-fixing bacteria and archaea are now available through the design of broad-spectrum highly degenerate primers. *nifH* is the general marker gene in nitrogen-fixing bacteria, and encodes the enzyme nitrogen reductase. It has an extensive database of sequences available for comparative purposes. Rosado et al. (1998) studied the diversity of *nifH* gene sequences in *Paenibacillus azotofixans*, and found sequence divergence at DNA level, but more conserved sequence at protein level — hence the design of degenerate primers. Widmer et al. (1999) followed suit and designed two universal sets of degenerate primers for nested PCR, based on the amino acid sequence of the conserved *nifH* gene.

17.1.1 Microbial Community Analysis

Microbial community analysis, independent of culturing the organisms, involves the extraction of signature biochemicals from the environmental samples (Blackwood et al. 2003). The first culture-independent estimate of prokaryotic organisms in soil indicated 4,600 distinct genomes in 1 gram of soil (Torsvik et al. 1990a). Extracted

DNA or RNA can, via molecular genetic techniques, facilitate the coupling of microbial community analysis with phylogeny. The uncultured diversity will reflect species closely related to known cultured organisms, and also species from virtually uncultured lineages (Blackwood et al. 2003).

Characterisation of genes of microbes involved in the degradation of organic pollutants has led to the application of molecular techniques in microbial ecology of polluted areas (Milcic-Terzic et al. 2001). Molecular methods usually involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene. However, taxonomic resolution of 16S rDNA sequences can be insufficient for discriminating between closely-related organisms in, e.g., cyanobacteria, where the rRNA 16S to 23S internal transcribed spacer (rRNA-ITS) provided better distinction between species (Janse et al. 2003). Molecular methods include DGGE, ribosomal intergenic spacer analysis (RISA), single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP). Several of these methods, such as SSCP, ARDRA and T-RFLP, do not reveal diversity unless the community is very simple, due to only a very small number of species indicated in rehybridisation or sequence analysis being visualised on a gel (Nakatsu et al. 2000; Blackwood et al. 2003). However, catabolic gene probes can be used in nucleic acid hybridisations to characterise sequences (Milcic-Terzic et al. 2001). Laurie and Lloyd-Jones (1999) probed a set of genes isolated from *Burkholderia* sp. RP007 involved in PAH catabolism. They found that the *phn* locus, containing nine open-reading-frames, codes for enzymes degrading naphthalene and phenanthrene.

A rapid means of determining the relative abundance of common species present in a given sample, which do not need to be culturable, can be provided by molecular techniques. Gelsomino et al. (1999) found after extensive molecular fingerprinting that similar soil types (clay, sand, loam, etc.) tend to contain similar dominating bacteria. Thus, it is evident that soil type affects the microbial community present, and not only the type of pollution to which they are exposed. Bundy et al. (2002) found that comparative bioremediation experiments on different soil types, all polluted with diesel, did not lead to the eventual development of a similar microbial community. They concluded that different soils have different inherent microbial potentials to degrade hydrocarbons. Molecular methods also allow for the elucidation of major differences between communities for testing of hypotheses on the basis of sample comparison (Blackwood et al. 2003). However, they do not always reveal the organisms primarily involved in the main energy flux of the system. Soil microbial ecologists suggest that only a few organisms are directly significant at a particular site (Dejonghe et al. 2001). If these organisms are targeted for non-culture analysis, more information could be revealed. For example, Leys et al. (2005) characterised fast-growing mycobacteria in PAH-polluted soils by means of PCR primers that targeted 16S regions of the *Mycobacterium* genome. PCR-DGGE was then used to distinguish between different species, and ultimately in elucidating the phylogeny (genetic relatedness) of the PAH-degrading species.

17.1.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a most appropriate molecular method for monitoring microbial community ecology. Wamberg et al. (2003) utilized DGGE to map the bacterial component in the pea (*Pisum sativum* L.) rhizosphere community, and observed that chemical changes in the rhizosphere during plant growth were mirrored by concomitant changes within the bacterial community present. MacNaughton et al. (1999) used DGGE to identify community members responsible for bioremediation of a crude oil spill, and to monitor community changes and pollution-level reduction over time. DGGE relies on variation in the genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks and Alleman 2002; Koizumi et al. 2002). PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The band pattern on the gel forms a genetic fingerprint of the entire community being examined (Gillan 2004). Most commonly, 16S rRNA genes are used to give an overall indication of the species composition of a sample. Partial sequence of this gene has been analysed from environments as complex as soil (Throbäck et al. 2004). Bodelier et al. (2005) screened the methane-oxidising bacteria from freshwater marshlands, using combinations of existing 16S primers. They found that, when combined, direct PCR of universal and specific primers yielded community profiles identical to those obtained from nested amplification.

Although 16S gene analyses presently are the most informative for broad community analyses, other genes can also be examined for community diversity. Functional genes have more sequence variation, and can be used to discriminate between closely-related but ecologically different communities. Throbäck et al. (2004) exploited the *nirS*, *nirK* and *nosZ* genes involved in denitrification as more discerning community biomarkers. DGGE has even been extrapolated to applications in plant protection research, including analysis of gut flora of several insect pest species, phylloplane and rhizosphere communities associated with different plant varieties, and the impact of biopesticides on natural microflora (O'Callaghan et al. 2003).

17.1.3 Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) of DNA is used in mutation detection and analysis. It involves the separation of single-stranded PCR rDNA products with the same number of base-pairs but a different conformational structure, on a polyacrylamide gel (Dejonghe et al. 2001). This technique has been adapted for the analysis of, and differentiation between, cultivated pure-culture soil microorganisms and non-cultivated rhizosphere microbial communities (Schwieger and Tebbe 1998). Under non-denaturing conditions, single-stranded DNA folds into sequence-dependent secondary conformations. These structures render different electrophoretic motilities to the molecules that can then be separated on a non-denaturing polyacrylamide gel. SSCP can be used in conjunction with an automated DNA

sequencer to differentiate between species using PCR products of 16S rRNA (Schwieger and Tebbe 1998). A limitation of using this technique for community DNA analysis is the high rate of re-annealing after denaturation, especially at high DNA concentrations. Another constraint of SSCP is the appearance of two bands on electrophoresis gels as a result of only double-stranded PCR product being obtained. Characteristically, three bands are observed on gels, one of a double-stranded product and two of the single-stranded DNA molecules from PCR. In some instances, there may be four or more bands visible on the gel due to differing structural conformations, e.g., hairpin folding due to palindromic sequences. Likewise, physical conformation of products may be similar, causing them to overlap in the gel, resulting in fewer bands being visualised on a gel. Finally, heteroduplex DNA strands with a similar sequence adhere together, forming breathing heteroduplexes of two or more PCR products (Schwieger and Tebbe 1998).

17.1.4 Amplified Ribosomal DNA Restriction Analysis

Another PCR-based DNA-fingerprinting technique, which makes use of restriction of amplified fragments, is amplified ribosomal DNA restriction analysis (ARDRA). This technique yields a high number of bands per species, and therefore cannot provide reliable genotypic characterisation at community level (Dejonghe et al. 2001). It is, however, particularly suitable for monitoring communities and assessing microbial diversity, and can focus on specific sub-groups within a community (Dejonghe et al. 2001). Lagacé et al. (2004) made use of 16S rDNA sequencing of ARDRA fragments for identifying bacterial communities in maple sap. The ARDRA profiles yielded a dendogram illustrating relationships between bacterial strains, and γ-proteobacteria were found to be dominant throughout the year.

17.1.5 Reverse Transcription-PCR

Reverse transcription-PCR (RT-PCR) involves the extraction of RNA instead of DNA, and profiles the metabolically active microorganisms in a system (Dejonghe *et al.*2001). It is a dual-step process. The first step entails the production of complementary DNA (cDNA) from a messenger RNA (mRNA) template using dNTPs and an RNA-dependent reverse transcriptase at 37°C. The second step involves the use of a thermostable transcriptase and a set of upstream and downstream DNA primers. Temperatures fluctuating between 38% and 95°C facilitate sequence-specific binding of the primers to the cDNA and allow transcriptase to produce double-stranded DNA. After approximately 30 cycles, the original RNA template is degraded by RNAse H, leaving pure cDNA in solution. It is now possible to simplify this process into a single step by using wax beads, containing the required enzymes, that melt at the higher temperatures releasing their contents.

Exponential amplification via RT-PCR provides a highly sensitive technique that can detect very low copy number RNAs. This technique is widely used in the diagnosis

of genetic diseases, and in the quantitative determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression, e.g., Northern blot.

17.1.6 Base-Specific Fragmentation and Mass Spectrometry

Base-specific fragmentation of PCR-amplified 16S rDNA, followed by mass spectrometry of the fragment pattern, is being used for rapid identification of bacteria (Von Wintzingerode et al. 2002). This method is inherently accurate and rapid, making it attractive as a tool for high-throughput microbe identification in pharmaceutical and industrial applications.

17.1.7 Signature Lipid Biomarker Analysis/Environmental Nucleic Acid Probes

Signature lipid biomarkers can be used in biomass shift monitoring. Signature lipid biomarker analysis/environmental nucleic acid probes (SLB/ENAP) are relatively inexpensive molecular fingerprinting techniques used to ascertain a quantitative measurement of the microcosm. Chemical extraction of phospholipid fatty acids from the soil can be useful in determining the diversity within the soil and in estimation of the microbial biomass (Banks and Alleman 2002). It determines when community ecology becomes analogous to a known community that is considered to be safe (White et al. 1998). Total cellular phospholipid fatty acids (PLFAs) are not stored in cells, and thus have a rapid turnover in communities. These make ideal markers for monitoring viable biomass within a community, viz. an increase in cis/trans monoenic PLFAs in cells is indicative of toxic stress within bacterial communities, and thus results in a change in their growth phase (Stephen et al. 1999). Specific PLFA biomarkers can be used to indicate broad microbial community diversity encompassing bacteria, fungi, algae, Gram-negative and -positive organisms, sphingomonads, actinomycetes and sulphate-reducing bacteria. Limitations of PFLA analysis include shortcomings in analysis of Gram-negative communities. These profiles are dominated by monoenoic, saturated and cyclopropane fatty acids that are broadly distributed, and thus fairly uninformative with regard to Gram-negative population structure. This method has been combined with nucleic acid-based analysis such as DGGE, to allow for better community elucidation (Stephen et al. 1999).

17.1.8 Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent method used to obtain a genetic fingerprint of a microbial community, and has been shown to be effective in discriminating between microbial communities in various

environments (Blackwood et al. 2003). Automation increases sample throughput and accelerates analysis of bacterial communities (Kent and Triplett 2002). PCR product of 16S rDNA is used for analysis (Dejonghe et al. 2001). One end of the PCR product is tagged with a primer carrying a fluorescent dye. It is then cut with a restriction enzyme to form terminal restriction fragments (T-RFs) that are separated by gel electrophoresis and visualised by excitation of the fluor (Dejonghe et al. 2001; Blackwood et al. 2003). A banding pattern is obtained, each band corresponding to one species or "ribotype" (Dejonghe et al. 2001). This provides quantitative data on each of the T-RFs in the form of size of base-pairs and intensity of fluorescence (peak height) (Blackwood et al. 2003). T-RF sizes can then be compared with a theoretical database obtained from sequence information (Blackwood et al. 2003), thus providing the species richness as well as community structure of the ecosystem (Dejonghe et al. 2001).

17.1.9 Other Techniques

A method for detecting extracellular DNA in environmental samples has been developed by England et al. (2004). This method circumvents disruption of cell membranes by not employing the use of harsh chemicals or physical disruption of whole cells within samples. England et al. (2004) hypothesised that the persistence of extracellular DNA in the environment is partially due to the formation of soil–DNA complexes, whereby the naked DNA released upon cell death and lysis is protected from nuclease degradation by the soil particles to which it adheres. Extracellular DNA serves two purposes in the environment, that of a nutrient source and of a gene pool. This DNA was extracted by using a gentle relatively fast extraction method involving suspension and shaking of a 0.5 g sample of leaf litter in 4 ml of sodium pyrophosphate (pH 8), followed by several filtrations and cleaning steps, resulting in application-ready extracellular DNA.

Other techniques such as ribosomal intergenic spacer analysis (RISA), ITS-restriction fragment length polymorphism (ITS-RFLP) and random amplified polymorphic DNA (RAPD) provide complex community profiles that can be analysed for community composition studies (Kent and Triplett 2002). Detection and resolution of fragment analysis can be approached with a number of methods, including automated ribosomal intergenic spacer analysis (ARISA) and length heterogeneity PCR (LH-PCR) (Kent and Triplett 2002). Most probable number (MPN) is a specialised enrichment technique using relevant substrates to estimate the number of organisms in an environment capable of degrading specific pollutants (Banks and Alleman 2002).

A widely used approach to studying bacterial diversity is using clone libraries of 16S rRNA genes. The genes are collected from naturally occurring bacteria through PCR with universal 16S rRNA gene primers (Cottrell and Kirchman 2000). Cottrell and Kirchman (2000) studied *in situ* marine microbial communities and found that data from a PCR-based clone library indicate that novel, uncultivated species are widespread in global oceans. However, clone libraries are effected by biases at each

step of the method (including sample collection, cell lysis, nucleic acid extraction, PCR amplification, and cloning) and can deviate from the compositions of actual communities (Cottrell and Kirchman 2000). During PCR, using controlled mixtures of 16S ribosomal DNA, the relative abundance of targeted DNA molecules in the final PCR product can be affected by biases. Several precautions have been proposed for minimizing these biases during PCR; however, the amount of bias is not known for natural habitats.

17.1.10 Possible Molecular Pitfalls

Due to the low number of cultured microorganisms compared to the large numbers of unculturable microbes, microbial diversity cannot be implied by cultured diversity. Therefore, PCR-based molecular techniques are favoured to give a better understanding of microbial communities in mixed samples. However, a review by Von Wintzingerode et al. (1997) indicated pitfalls of PCR-based genomic analyses. Briefly, they concluded that after initial sample collection several difficulties could be encountered during cell lysis, DNA/RNA extraction, PCR, separation of genes and sequence data analysis. These difficulties include the following:

- Insufficient cell lysis will skew an analysis if not all microbial DNA is released from cells in the sample.
- DNA/RNA can shear into fragments after release from cells during cleaning steps, and may impact on post-extraction steps thereafter.
- PCR can be inhibited by co-extracted contaminants such as humic acids from soil that hamper the reaction of template and enzyme. Amplification efficiencies should be the same across molecules; thus, assumptions must be made that:
    - All molecules are equally accessible to primer hybridisation, the primer–template hybrids form with equal efficacy,
    - The extension efficiency of the DNA polymerase is the same across templates.
    - Exhaustion of reaction components affects all templates equally.

Furthermore, the formation of PCR artefacts can occur due to the creation of chimeras between two homologous molecules, deletion mutants as a result of stable secondary structures, and point mutants because of misincorporation of bases by the DNA polymerase. In addition to this, the possibility of contamination as a result of foreign DNA introduced into the reaction due to experimental error must be negated; this is monitored by the incorporation of negative control reactions containing no template DNA.

Sequence analysis of 16S rDNA is usually done by comparison with previously identified sequences deposited on global databases. However, whether environmental sequences represent uncultured or novel organisms, or remain unassigned to known taxa, is yet to be determined. Many sequences on the database may be of low quality due to their length (only partial) or taxonomic ambiguity (Kirk et al. 2004).

In order to prevent these possible inaccuracies during molecular sample analysis, Von Wintzingerode et al. (1997) suggested that results of different extraction methods, PCR and cloning techniques be explored simultaneously to provide the most accurate results possible.

17.2 DGGE Technique and Application

Muyzer et al. (1993) introduced DGGE as a new genetic fingerprinting technique. This method is often preferred due to its capacity to provide rapid visual indications of community changes within a sample (Anderson et al. 2003). Bands can then be excised and sequenced. Sequence variation in rRNA has been used for elucidating phylogenetic relationships between organisms and in designing probes for detecting microbial taxa (Muyzer et al. 1993). DGGE is used to determine microbial genetic diversity, and particularly the predominant communities in a sample (Muyzer 1999; Coclin et al. 2001; Stamper et al. 2003). Janse et al. (2003) concluded that it can also be used to determine the purity and uniqueness of isolated strains.

Denaturing gradient gels are used for the detection of non-RFLP polymorphisms (Helms 1990). Double-stranded fragments (200–700 base pairs), the products of PCR of rRNA genes (rDNA) with the same length but differing in base-pair sequences, are separated on an increasing denaturant gradient gel (Ferris et al. 1996; Nakatsu et al. 2000; Dejonghe et al. 2001; Kawai et al. 2002). A portion of DNA can be deemed suitable for DGGE analysis if it can be specifically amplified from the target organism, has adequate heterogeneity for good resolution and is part of a gene that has a large database of sequences already available (Janse et al. 2003). A factor that limits DGGE efficacy is the primer design. Sequences targeted should not yield a fragment much longer than 500 base-pairs (bp) for successful analysis (Throbäck et al. 2004). At present, 16S rDNA sequences form the ever-increasing, largest gene-specific data set available on internationally accessible databases (>30,000), making tentative identification of unknown bacteria possible (Von Wintzingerode et al. 2002). Øvereås et al. (1997) were the first to analyse archaeal rDNA with DGGE. Using domain-specific sets of primers on samples from a meromictic lake in Norway, they found an increase in archaea and a decrease in bacteria the deeper they sampled.

Double-stranded DNA products that undergo electrophoresis through a DGGE gel are halted when they split into single strands due to a linearly increasing gradient of denaturants (Muyzer et al.1993; Curtis and Craine 1998). The denaturants most commonly used are heat (constant 60°C), formamide (0–40%) and urea (0–7 M) (Helms 1990). Initially, fragments move according to relative molecular mass. However, as the denaturation gradient increases, the fragments start separating as the hydrogen bonds between the double helix begin to break; this is known as melting (Helms 1990). This partial melting retards the progress of the DNA molecule through the gel, the resultant mobility shift differing for different sequences

(Muyzer et al. 1993). The sequence of the PCR product separation on the gel determines the denaturant concentration at which this occurs (Ferris et al. 1996; Curtis and Craine 1998; Nakatsu et al. 2000). As denaturant concentrations increase, the DNA will dissociate completely into two separate strands (Helms 1990). Fragments do not partially melt in a zipper-like fashion, and specific portions of DNA fragment become single-stranded suddenly within a narrow denaturant range (Helms 1990; Muyzer et al. 1993). After double-stranded DNA dissociation, the gel is stained with a DNA-intercalating dye that fluoresces under ultra-violet light. For the purposes of this review and work, SYBR gold nucleic acid gel stain was used. This stain is an asymmetrical cyanine dye with two fluorescence excitation maxima, ca. 300 and 495nm, when bound to DNA (Tuma et al. 1999). When used with 300nm transillumination and Polaroid black and white photography, SYBR gold is more sensitive during intercalation than ethidium bromide, forms dye-nucleic acid complexes ca. 70% higher than current counterpart dyes, produces up to a 1,0001999-fold fluorescence enhancement, and is as sensitive as silver staining, but requires only one step, and does not influence subsequent molecular biology protocols (Tuma et al. 1999).

Narrowing the denaturant range can increase the sensitivity of DGGE, hence yielding fast, reliable and reproducible results (Fromin et al. 2002; Temmerman et al. 2003). Mobility rate in the polyacrylamide gel is determined by the physical shape of the fragment, which in turn depends on the denaturant gradient and fragment sequence, with partially melted fragments moving more slowly than those that are still double-stranded (Helms 1990). During analysis of a complex microbial community, a ladder of bands forms on the gel, each corresponding to an individual PCR-product of a specific sequence (Curtis and Craine 1998; Fromin et al. 2002). This allows for simultaneous detection of multiple 16S rRNA sequences (Ferris and Ward 1997; Sekiguchi et al. 2002). The resulting gels can be probed with diagnostic oligonucleotides to identify specific sequences or bands, and may be excised, reamplified and sequenced (Ferris et al. 1996). The technique is sufficiently sensitive to detect as little as one base-pair difference in a sequence (Helms 1990). However, Gillan (2004) found that changes to the DGGE protocol can result in less robust results, and thus should be standardised across particular sets of experiments. Alternatively, "markers" can be constructed from known species sequences and run alongside test samples to determine the identity of bands within the sample. Theunissen et al. (2005) demonstrated this when analysing probiotic microorganisms from yoghurt and lyophilised capsule and tablet preparations. Two markers with known lactobacilli and *Bifidobacterium* PCR-product were run adjacent to test samples, and band patterns were then used for accurate and rapid species identification. Similarly, but more complex, Keyser et al. (2006) used a marker composed of five known methanogenic bacterial species to determine DGGE bands from an upflow anaerobic sludge blanket bioreactor that did not match the marker. These bands were then excised and sequenced, and a DGGE marker to monitor archeal members of the microbial consortium was developed based on the sequence results.

Resolution of DGGE can be enhanced by incorporation of a GC-rich sequence into one of the primers to modify the melting behaviour of the fragment and to

allow for the majority of sequence variation to be detected in the denaturing gel (Ferris et al. 1996; Curtis and Craine 1998). A GC-clamp attached to the 5′ end of a PCR product prevents complete melting during fragment separation in a denaturing gradient, and sensitises the technique enough to detect all single base changes in PCR fragments of 500bp (Heuer et al. 1997). Sheffield et al. (1989) found that attaching a GC-clamp of 40–45bp to primers allowed for the determination of single-base mutations, previously only 40% distinguishable in DGGE analysis, to increase to 100%. Furthermore, Boon et al. (2002) included a GC-clamp to stabilise large fragments in all final reactions during nested PCR intended for DGGE analysis. However, despite the advocation of the inclusion of a GC-clamp for melting stability during PCR-DGGE analysis, under certain conditions the clamp can be disregarded. In this case, if no GC-clamp is added, it is recommended that the PCR product must have at least two melting domains (Chang Bioscience 2004). Wu et al. (1997) found that GC-clamped products with a perfect melting curve yielded distorted smeared results when subjected to DGGE. They found that fragments containing a "high melting domain" provided better DGGE results when run without a GC-clamp, and concluded that if melting analysis of a PCR product predicts a high melting domain of <40bp, and differs by not more than 5°C melting temperature, then the fragment is suitable for DGGE analysis without a 5′ GC-clamp.

Lanes of bands can be analysed utilising gel image software for more accurate results, using known pure culture isolates as standards for well-characterised environmental samples. Thus, gel images resulting from DGGE analysis can be digitally captured and used for species identification when samples are run against these known standards (Temmerman et al. 2003). These images can also be compared when samples are collected and analysed over a period of time, hence permitting monitoring of community structural changes with time (Van Hannen et al. 1999). Manual fine-tuning of the gel image completes the initial analysis, and dendograms can be drawn to relate band pattern parallels (Fromin et al. 2002; Stamper et al. 2003). Software also calculates band densities necessary for determining the Shannon diversity index, where each band represents one species and the band intensity is proportional to the species abundance (Fromin et al. 2002; Stamper et al. 2003; Andreoni et al. 2004). Nübel et al. (1999) quantified diversity of oxygenic phototrophs within hypersaline microbial mats. The amount of bands per sample indicated species richness, whereas species abundance/"evenness" was determined by band intensity.

Limitations of DGGE include similar electrophoretic mobilities of phylogenetically related species sharing analogous sequences in the amplified area, and similar melting behaviour between phylogenetically unrelated species (Smalla et al. 2001). Consequently, there may be more than one species represented by a single band on the DGGE gel. This has been demonstrated by Jackson et al. (2000) making use of site-directed mutagenesis to create E. coli 16S rDNA fragments differing by 1–4 base-pairs. Migration on DGGE gels consistently determined single base-pair changes, but multiple base differences proved to be more difficult to distinguish. Two of the sequences tested, differing by two base-pairs only, showed identical migration patterns and could not be separated when run in a mixed sample.

Furthermore, Vallaeys et al. (1997) reported that DGGE analysis of a 200bp fragment of 16S rDNA from rhizobia and methantrophs was difficult to elucidate, due to low and high sequence polymorphism respectively.

One also needs to take into account the method used for DNA extraction and purification when screening DGGE samples. Niemi et al. (2001) tested five different DNA extraction methods and three purification methods on rhizosphere soil samples destined for DGGE analysis. They found that the isolation and purification methods both had an effect on the final bacterial DGGE community structures of the samples. In addition to this, O'Callaghan et al. (2003) concluded that extracted DNA should be representative of the habitat, PCR bias must be taken into account, as preferential amplification may occur due to inefficient primer annealing, and species determination should not be based on 16S rDNA sequences alone, although this is becoming increasingly more efficient as databases expand continually. There are, however, means of incorporating internal standards into the DNA extraction and PCR-DGGE process. Petersen and Dahllöf (2005) developed a protocol known as "Internal Standards in Molecular Analysis of Diversity" (ISMAD) that can monitor, and thus account for, experimental variability. A fluorescent 510bp PCR product is included in each sample prior to DNA extraction, and recovered afterwards. PCR is monitored by adding non-competitive primers coding for a 140bp section of *Drosophila melanogaster* DNA to the same PCR as the sample. Together, these internal controls reduced variation between replicate samples during DGGE analysis. Despite these minor pitfalls, DGGE is still considered to be a reliable, reproducible, rapid and relatively inexpensive method for the simultaneous analysis of multiple samples and for mapping community changes over time (Muyzer 1999; Fromin et al. 2002).

17.2.1 Community Diversity Analysis

Most microbial diversity indices are based on plant and animal models, e.g., the Shannon and Simpson indices. As such, there is some difficulty in applying these indices to microbial models, since they need a clear definition of species and unambiguous individual identification. This level of identification is difficult in bacteriology. An ideal bacterial index should encompass the following (Watve and Gangal 1996):

- Have three important diversity dimensions, viz. species diversity, species richness/abundance and taxonomic distance between biotypes
- Be based on a statistically justified parameter
- Be insensitive to possible errors and variability of test results
- Not be too sensitive to sample size

According to this, the use of Shannon algorithms to calculate microbial diversity according to DGGE gel fingerprints is acceptable. Dimensions such as diversity and richness/abundance can be determined from the number of bands and their

intensity on the gel, respectively. Sequencing of each band on the gel can indicate taxonomic distance between biotypes. Diversity within the 16S rDNA is statistically well-documented, and does account for possible errors and variability within the region that can be guarded by incorporating internal control standards. DGGE can be used for assessing anything from one sample individually to a large number of samples simultaneously.

DGGE allows for determining community as well as specific population diversity without further analysis, and without elucidating particular individuals (Muyzer 1999). It has also been used for the simultaneous identification of sequence variations in multiple genes among several organisms (Muyzer et al. 1993). Identity of community members can be further resolved by hybridisation of the gel with species/taxon-specific oligonucleotide probes to hypervariable regions of the sequence or by cloning and sequencing (Muyzer 1999). The gel can be used for direct analysis of genomic DNA by transferring separation patterns to hybridisation membranes, using capillary- or electro-blotting, and analysis with DNA-probes (Muyzer et al. 1993). PCR, with GC-clamp primers, can also be selectively employed to amplify sequences of interest, e.g., 16S, before DGGE is performed (Muyzer et al. 1993). Essentially, DGGE allows a high number of samples to be screened simultaneously, thus facilitating more broad-spectrum analysis. Kowalchuk et al. (1997) used DGGE to assess variation between different pathogenic fungal species within a taxon attacking the roots of *Ammophila arenaria* L. (marram grass). They amplified a 569bp region of the 18S rDNA gene by means of nested PCR with a GC-clamp on the final PCR. Upon assessing experimental and field/wild plants, they were able to distinguish between species of fungi and detect a much higher level of diversity than in previous culture-based surveys.

17.2.2 Community Dynamics Studies

Due to multiple sample screening, DGGE allows for monitoring of the dynamics that microbial communities undergo during seasonal and environmental fluctuations in their habitat (Muyzer 1999). Ward et al. (1998) made use of 16S rDNA fragments in DGGE to study seasonal community changes of microbial communities within hot spring microbial mats. Subsequently, PCR-DGGE has been used to monitor seasonal changes in communities of bacterioplankton, the rhizosphere of chrysanthemum, post-viral bacterial lysis communities, and diurnal behaviour of sulphate-reducing and phenol-degrading bacteria in activated sludge, as well as the impact of pesticide and herbicide applications on microbial communities (Muyzer 1999). DGGE has even been applied in the mapping of communities of bacteria utilising organic-wastewater/sludge as fuel for a bial communities within the cell electrode differed from those in the sludge.

17.2.3 Molecular Community Mapping Across Varied Environments

Culture techniques are important for the understanding of the physiology and function of microbes isolated from their natural environment. However, molecular tools can be used for monitoring enrichment cultures and facilitating the isolation of target communities from the environment (Muyzer 1999). Smalla et al. (1998) made use of DGGE and TGGE (temperature gradient gel electrophoresis) in the analysis of BIOLOG substrate utilisation patterns of two bacterial communities from potato rhizosphere and activated sludge. Both DGGE and TGGE showed enrichment of specific bacterial communities not evident from BIOLOG results. Prokaryotic communities are not the only type to have been mapped. Foucher et al. (2004) determined nematode diversity in soil samples using 18S rDNA PCR-DGGE, and found a significant relationship between morphological and DGGE estimates of species richness. Marshall et al. (2003) tested PCR-DGGE primers for compost fungi, finding an α-elongation factor primer set targeting a portion of the 18S rDNA to be best for fungal community amplification. Similarly, Zuccaro et al. (2003) demonstrated the use of four sets of 18S primers in DGGE analysis for the identification of ascomycetes associated with algae in lichens on ferns.

17.2.4 Niche Differentiation

Molecular microbial ecology is becoming more specialised, thus allowing analysis of specific functional communities within communities. Enzyme-coding genes are now being targeted for ecological studies. They tend to display a higher level of sequence variation than the conserved 16S rDNA genes, which makes them more efficient molecular markers for phylogenetically similar but ecologically distinct communities (Muyzer 1999). In addition, targeting functional genes facilitates the study of specific activities within microbial communities. Milcic-Terzic et al. (2001) used genes of microbes involved in the degradation of organic pollutants for the application of molecular techniques in the microbial ecology of polluted areas. As more sequences of functional genes become available on databases worldwide, PCR-DGGE would undoubtedly deliver considerably more information regarding community structure and function.

17.2.5 Determining Species Diversity

Banding patterns on DGGE gels give an indication of species diversity when analysed using a visual gel analysis software package. For the purposes of the studies included in this thesis, DGGE gel image analysis was performed using the Gel2K

program, and fingerprints were analysed in a cluster investigation using CLUST (Norland 2004).

Bands excised from DGGE gels can be sequenced. The resulting sequences can then be used for comparative phylogenetic analysis to determine the evolutionary relationships between organisms in the community being analysed. Anderson et al. (2003) investigated a soil fungal community by DGGE of the ITS region (ITS1-F with a GC-clamp and ITS2 yielding a 300bp fragment), sequencing of bands, and BLAST result phylogeny of the resulting sequences. Phylogeny gives an indication of species diversity and not richness, since only one band is produced and picked from the gel per species (Van Hannen et al. 1999). By determining the closest relatives of unknown organisms, the known characteristics can be inferred upon them (Ueda et al. 1995). The sequence data can also be used in the design of primers and probes for *in situ* identification of selected organisms.

17.3 Alternatives to PCR-Based Analyses

Microscopy and plate counts are traditional methods that are quick and inexpensive. Selective plating and direct viable counts can be used for providing information on the active heterotrophic portion of a community (Kirk et al. 2004). Methods are available that focus on physiological/metabolic characteristics of microbial communities, e.g., fatty acid methyl ester (FAME) profiles and phospholipid fatty acid analysis (Kent and Triplett 2002). Fluorescent in situ hybridisation (FISH) utilises fluorescent oligonucleotides to target rRNA sequences (Dejonghe et al. 2001). FISH can be used in conjunction with DAPI (4′,6′-diamidino-2-phenylindole), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)-formazan, or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining for determining the contribution made by communities of interest to the total abundance or active cell count (Kent and Triplett 2002). However, FISH has a low throughput, and this limits its application for comparison of high numbers of samples (Kent and Triplett 2002).

Various tests are also available for bacterial identification based on physiological reactions. Among these are the catalase reaction test, the oxidative–fermentative Hugh–Leifson test, Biolog and API, a standardised, miniaturised version of existing biochemical test techniques that is simple, rapid and reliable when used in conjunction with numerical identification, with or without computer software programmes.

17.3.1 Morphology

Prudent morphological analysis of bacterial cells can yield important information about diversity, microbial abundance and two-dimensional spatial distribution of microbial community members. Computer-aided systems such as CMEIAS (Centre for Microbial Ecology Image Analysis System), is a semi-automated analytic tool

that uses processing and pattern recognition techniques (with microscopy) to gather information on size and shape of digital images of organisms and classify them into their morphotypes (Kent and Triplett 2002).

17.3.2 Catalase Reaction

This is a test for production of the enzyme catalase by bacterial species. Hydrogen peroxide is a harmful by-product of metabolic processes; catalase catalyses its breakdown to water and oxygen. The enzyme has one of the highest turnover rates, since one molecule of catalase can convert 83,000 molecules of hydrogen peroxide to water and oxygen per second (Wikipedia 2007). Although the catalase test alone cannot identify bacteria, combined with other tests it can aid in identification (Krieg et al. 1984). The test is performed by picking bacterial cells from pure cultures on agar plates, using sterile wooden toothpicks, and placing them on clean microscope slides. One or two drops of 3% hydrogen peroxide are added to the bacteria and the formation of bubbles within 1 min is regarded as a positive reaction.

17.3.3 Aerobic and Anaerobic Bacteria

The fermentative or oxidative nature of bacteria is determined using the Hugh–Leifson test (Hugh and Leifson 1953). Colonies from pure culture on agar plates are stab-inoculated in duplicate into sterile test tubes containing oxidative fermentative base medium (OFBM) with added glucose. The medium in one tube of each duplicate is covered with 1 cm sterile liquid paraffin. Tubes are incubated at 37°C for 48h, and a colour change from green to yellow is deemed a positive test result. Bacteria can be considered fermentative when the colour changes from green to yellow in both test tubes. Oxidative bacteria induce a colour change only in the test tube containing no liquid paraffin.

17.3.4 Identification Using API and Biolog

API is a series of miniaturised metabolic tests deemed instrumental in bacterial species identification. Pure isolates from agar plates are subcultured on fresh agar medium for 48h. A sterile inoculation loop is then used to suspend cells in test tubes containing 0.85% NaCl. API strips are loaded with this suspension according to the manufacturer's instructions (OMNIMED). Several different tests are available for use, e.g., API 50CH, API 20NE, API 20E etc., based on different characteristics of bacterial species.

Garland and Mills (1991) developed a technique to assess the potential functional diversity of bacterial communities through sole carbon utilisation (SSCU) metabolic patterns. From this arose the Gram-negative and Gram-positive Biolog plate system that contained 95 different carbon sources and a control well for metabolic bacterial identification (Kirk et al. 2004). Biolog EcoPlate is specifically tailored for microbial community and ecological studies. Its development was initially prompted when Biolog GN microplates were inoculated with a mixture of microbes in culture, and the community fingerprint characteristics were measured over time. Known as community-level physiological profiling, this method proved to be effective in distinguishing spatial and temporal microbial community changes. The plates proved to be useful in assays of the normal community, and to detect changes based on an introduced variable. These studies have been conducted with communities from soil, wastewater, activated sludge, compost and industrial waste. The Biolog EcoPlate contains the 31 most utilised carbon sources for soil community analysis, each of which is repeated in triplicate for data purposes. Communities of organisms yield a characteristic reaction pattern or "metabolic fingerprint". These patterns can be statistically analysed by computer software at defined intervals over 2–5 days, hence providing data about microbial community changes over time. This method has also been compared with other methods such as PLFA, and proved to be more sensitive to important factors for instance temperature and water.

17.3.5 DNA Reassociation

A non-PCR-based molecular technique has also been established on the basis of DNA melting and reassociation measurements. Comparative chemistry of genomes between species gives an indication of species diversity during DNA–DNA and DNA–RNA reassociation (Sanderson 1976). Purified DNA is split into fragments and thermally denatured so that the double-helix strands separate or "melt" and, by slowly cooling the DNA, reassociate or reanneal again. Following this, the rate at which the double-helix renatures is measured spectrophotometrically (Curtis and Sloan 2005). This rate is affected by the size and complexity of DNA, with large complex DNA reannealing the slowest. Originally, this method was used to estimate the size and complexity of genomes from individual organisms. However, Torsvik et al. (1990a) reasoned that pooled genomic DNA from a microbial community might reanneal like the DNA from a large genome. They placed sheared total soil DNA in a French press to yield fragments with an average molecular mass of 420,000 daltons. It was then hypothesised that the heterogeneity of the DNA was a measure of genetic diversity of bacteria within the soil. Indeed, they showed that DNA extracted from soil reassociated so slowly that it resembled a genome 7,000 times as large as the genome of a single bacterium (Curtis and Sloan 2005). It follows that there could have been at least 7,000 different prokaryotic taxa in the sample of soil analysed.

Renaturation of the homologous single-stranded DNA follows second-order reaction kinetics (Torsvik et al. 1990a). The renatured DNA fraction is expressed as a product

of the nucleotide concentration in moles per litre ($C_o t$), and time is measured in seconds. $C_o t_{1/2}$ under defined conditions is directly proportional to the complexity or genome size of the DNA, complexity being defined as the number of nucleotides in the genome of a haploid cell, excluding repetitive DNA. Based on this, $C_o t_{1/2}$ can be considered to be a diversity index measurement of bacterial communities, which would equate to indices based on phenotypic analysis or species diversities.

DNA-reassociation has been used in combination with other molecular techniques such as DGGE to give a more complete idea of bacterial diversity within specific communities. Torsvik et al. (1998) investigated the community structure of natural, polluted and agriculturally perturbed environments. They compared DGGE diversity analysis of rRNA genes with total DNA reassociation to draw parallels between community diversity techniques. Their study indicated that total soil microbial diversity was 200 times higher than bacterial isolate diversity from the same samples, and that farming and pollution played a significant role in reducing bacterial diversity.

17.4 Use of 16S rDNA Sequences for Parsimony and Distance Analysis

Certain regions of rDNA sequences are highly conserved across all organisms, whereas other regions may vary. The variability within these regions increases proportionately to the increase in the evolutionary distance between organisms, thus allowing for the determination of phylogenetic relationships between microorganisms (Nakatsu et al. 2000). Due to their usefulness as markers in phylogenetic studies, 16S rRNA genes have been the main targets for prokaryotic ecological molecular surveys (Osborne et al. 2005).

Ribosomal RNA (rRNA) molecules are used as molecular chronometers because of their high degree of structural and functional conservation. As a result of this, domains within rRNA molecules harbour independent rates of sequence change. Phylogenetic relationships can be determined by examining these changes over time (Kent and Triplett 2002).

A large number of genes are available for phylogenetic studies on databases worldwide. Selected sequences should be appropriate, and can be affected by the following:

- Structural regions in the small and large subunit rRNA genes evolve at differing rates.
- Non-synonymous substitution rates at codon positions 1 and 2 are often slower than synonymous substitutions at position 3.
- Transitions occur more frequently than transversions.

Different substitution rates result in different levels of phylogenetic resolution in different areas of DNA. This should be taken into account when examining phylogenetic relationships at different taxonomic levels.

Patterns in sequence affect the suitability of data to be used in various phylogenetic tests:

- Phylogenetic signal: the level of conservation of sequence data
- Saturation: multiple changes at the same site due to lineage splitting. Over time two sequences saturate due to multiple changes at certain sites. Increasing substitutions will have a diminishing effect on the sequences in question. A non-linear relationship develops between sequence divergence and time, leading to information loss to the phylogeny being examined
- Base/codon composition

At present, 16S rDNA sequences form the ever-increasing, largest gene-specific data set available on internationally accessible databases (>30,000), making tentative identification of unknown bacteria more possible (Von Wintzingerode et al. 2002). However, they are not always the most informative genes to select for study. Dauga (2002) investigated 16S and *gyrB* phylogenetic gene trees showing relatedness between *Enterobacteriaceae*. *gyrB* is a single-copy gene present in all bacteria. It has been proposed as a suitable genetic marker for identification of bacteria, and encodes ATPase within the DNA-gyrase domain. Dauga (2002) found that *gyrB* trees proved to be more reliable determinants between closely-related species than the 16S trees. 16S has nevertheless been used in the comparison and resolution of closely-related species. Anzai et al. (1997) found a 93.9% homology in 16S rRNA sequence homology between *Chryseomonas*, *Flavimonas* and *Pseudomonas*, and on this basis proposed that they were synonymous. Similarly, Warwick et al. (1994) proposed that *Amycolata* and *Pseudonocardia* be classified in an emended *Pseudonocardia* genus on the basis of mixed clades emerging continuously from analysis of 16S data. *Pseudonocardia* has also, based on 16S sequence data, been observed to form a monophyletic unit with *Actinobispora*, and it has been suggested that the latter genus be also incorporated into *Pseudonocardia* (Lee et al. 2000).

17.4.1 Characterisation of 16S Region

The 16S gene of the bacterial genome holds the rDNA genetic code for the 16S subunit of the ribosome. Ribosomes are organelles in which translation of the genetic code (RNA to protein) takes place, and consist of two subunits of RNA and proteins (Tamarin 1996). Ribosome size is measured on the basis of its sedimentation rate during centrifugation in a sucrose density gradient. The unit of sedimentation is S, so designated after T. Svedberg, the developer of the method in the 1920s1996 (Tamarin). The 30S subunit of an *E. coli* ribosome consists of a 16S molecule of rRNA and 21 proteins (Tamarin 1996). This subunit of rRNA is encoded on the DNA of the bacterial cell and contains sequences that are highly conserved, thus allowing for sufficient resolution to distinguish between genera and species.

Advantages of using 16S rRNA gene sequences for analysis of microbial communities include the following:

- Essential component of ribosomes.
- Universal to all cell types.
- Universally conserved and variable taxon-specific sequences where the primary structure consists of conserved and variable sequences, allowing for comparison of homologous positions of different species
- Horizontal gene transfer not likely
- Extensive databases (e.g., GenBank) of rRNA gene sequences exist
- rRNA sequence-based "Tree of Life" provides a scaffold for comparison of unknown sequences from natural samples
- Acts as a molecular chronometer
- Allows for culture-independent analysis of unknown communities

17.4.2 Characteristic Base-Pairs

There are two types of sequence data generated, viz. genomic DNA and expressed sequence tags (ESTs). Genomic DNA represents the genetic material of entire organisms in the form of genomes. The genomes are constructed from multiple experiments of high accuracy. However, ESTs are short pieces of DNA, usually 400–800bp, which are transcribed into mRNA and later translated into proteins. ESTs comprise 62% of the 38.9 million genetic sequences on GenBank; they are fairly easy to sequence, and can be used to locate genes and their splice sites (Wu et al. 2005). Mapping of ESTs to known genomes has become more important in recent years for finding genes, EST clustering, alternative splice-sites and gene function. Wu et al. (2005) developed new computer software (EST mapper) which is 3–1,000 times faster than current market software for aligning and clustering DNA sequences, and produces alignments of better quality.

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Chapter 18
Molecular Tools for Monitoring and Validating Bioremediation

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18.1 Introduction

Hazardous waste sites around the world result from the manufacturing, storage, use or disposal of compounds such as petroleum hydrocarbons, nitroaromatics, organohalogens, pesticides, and metals. Most of these contaminants are synthetic compounds or "xenobiotics". They tend to accumulate in the environment because of structures or substituents (e.g., the nitro-group in nitroaromatics) that typically do not occur in nature and are therefore not easily recognized by existing degradative enzymes (Stenuit et al. 2005). To address the challenge of cleaning up contaminated sites in a sustainable and "green" manner, bioremediation technologies take advantage of the astonishing catabolic versatility of microorganisms to detoxify and/or convert wastes. However, the implementation of bioremediation techniques in situ is not always successful, because of the difficulty to control and scale up key biodegradative processes from the laboratory to full-scale (Paerl and Steppe 2003), as illustrated by the rather mixed record of bioaugmentation trials (El Fantroussi and Agathos 2005). In that perspective, bioreactors and other confined environments offer conditions far more controllable and manageable than most open large-scale ecosystems, in terms of predictability, dynamics of catabolic microbial populations and process monitoring. Nonetheless, in situ and on-site bioremediation can be steered with increasing confidence, to the extent that, like bioreactors, functional parameters linked to the evolution of pollutant degradation can be followed and controlled.

Bioremediation, be it natural attenuation, bioaugmentation or biostimulation, requires a good understanding of the physicochemical characteristics of the contaminated environment, as well as a detailed description of the microbial communities involved in key physiological processes. The site slated for bioremediation must be comprehensively characterized in hydrogeological and geochemical terms, from the point of view of the chemical nature of the contaminants and from the

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dimensions and dynamics of the polluting plume. The evaluation of the microbial communities, their abilities to biodegrade the target contaminants, and the durability of these abilities can often be the most decisive consideration in the design and implementation of a bioremediation application. For instance, it is crucial to assess the microbial population response when the contaminated site is bioaugmented with active microbial inocula. As a general approach, the monitoring of the microbial communities participating in the cleanup of a contaminated site is an essential element of process description, prediction and control and, ideally, must be performed at all scales, i.e., the laboratory, the pilot or demonstration stage and the field scale.

In order for such assessments to be as complete and meaningful as possible, microbial communities need to be characterized in terms of structure, phenotypic potential, function and interactions with the environment (Rittmann et al. 2006). As 90–99% of microbes living in the environment resist conventional cultivation in the laboratory on artificial solid or liquid media (Amann et al. 1995), a major methodological and conceptual revolution in microbial ecology occurred in the 1990s which made possible the application of culture-independent molecular tools to study the diversity and dynamics of microbial communities in fine detail.

Over the last few years, such powerful tools are enabling the qualitative (e.g., fingerprinting techniques) and quantitative (e.g., dot blot and fluorescence in situ hybridization, or real-time PCR) description of environmental microbial communities and are instrumental in identifying new catabolic operons of xenobiotics in environmental bacteria. However, their application is often time-consuming and limited to a small number of samples, which does not permit the comprehensive characterization of an ecosystem. To address this problem head-on, high-throughput approaches are emerging. They offer the advantage of miniaturization, automation and massive parallelization of time-consuming steps, allowing the simultaneous "real-time" analysis of numerous samples. In parallel, thanks to expanding sequencing infrastructure (e.g., the novel pyrosequencing "454" technology) and rapidly dropping genome-sequencing costs, there has been a dramatic increase in the number of sequenced bacterial genomes including those of bacteria involved in the elimination of recalcitrant compounds (Golyshin et al. 2003; Kube et al. 2005) and even of entire community genomes (Deutschbauer et al. 2006). It is expected that in the near future the combined application of such genomic approaches with post-genomic techniques such as high-throughput functional analysis of metaproteomes (Ram et al. 2005) will provide a comprehensive understanding of the composition and functioning of environmental microbial communities, including those in operation in sites undergoing bioremediation.

18.2 High-Throughput Techniques for Characterization of Contaminated Sites

A number of culture-independent molecular techniques currently used to study complex microbial communities are compatible with a high-throughput setup, such as fingerprinting techniques, real-time PCR, microarrays, metagenomics,

metatranscriptomics, metaproteomics or metabolomics. These techniques are discussed below. Their potential as essential analytical tools in the context of bioremediation of contaminated sites is summarized in Fig. 18.1. Detailed information on the polluted site at different time points in the course of a bioremediation treatment can be obtained regarding the local microbial community. Specifically, the complexity (*A*), structure (*B*) and diversity (*C*) of the community can be assessed on the basis of nucleic acid extraction from the environmental sample and subsequent assays (Fig. 18.1). Moreover, these molecular (DNA- or RNA-based) approaches, in combination with labeling of the active population of the sample using stable isotope probing (SIP) or bromodeoxyuridine (BrdU) incorporation (*D*, Fig. 18.1) can correlate the above information ("who is there") with community function ("who is doing what").

18.2.1 Fingerprinting Techniques

Genetic fingerprinting techniques provide a specific pattern or profile of a given microbial community. They are based on the separation of amplicons after PCR amplification of phylogenetic (e.g., 16S rRNA) or functional genes using universal or specific primers. Some of these fingerprinting techniques have the potential for high-throughput design, such as terminal restriction fragment length polymorphism

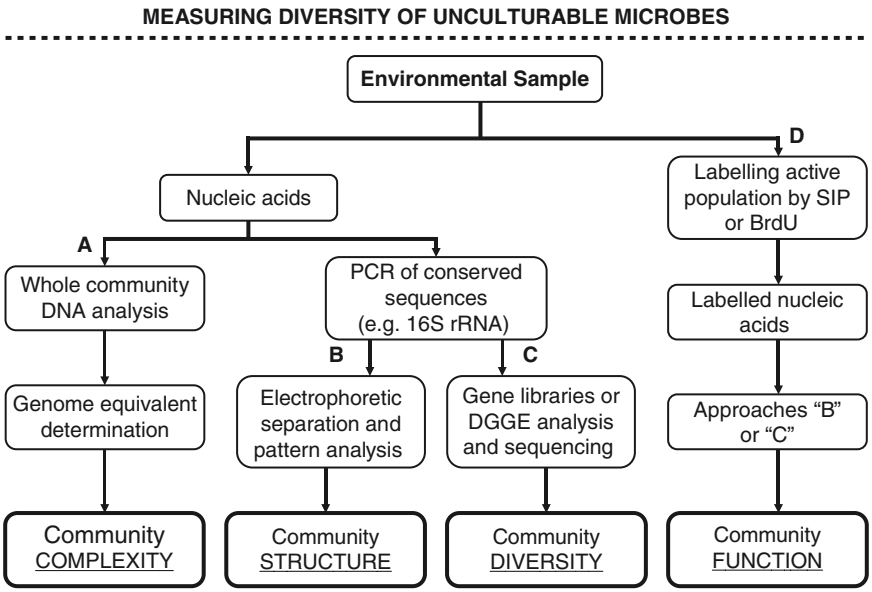

Fig. 18.1 Investigation strategies based on the analysis of total nucleic acids of a microbial community in order to explore the complexity, structure, diversity and function of environmental microbial populations (adapted from Daffonchio, 2005)

(T-RFLP), length heterogeneity analysis by PCR (LH-PCR), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), or ribosomal intergenic spacer analysis (RISA).

T-RFLP separates fragments obtained by enzymatic restriction of PCR amplicons according to their size. The use of labeled primers allows a rapid, automated and high-throughput detection of polymorphic terminal fragments. The separated fragments are visualized by an automated DNA sequencer as a pattern of peaks on an electropherogram. In addition, the identification of T-RFLP peaks can be directly obtained by comparing them to databases (Marsh et al. 2000). Denaturing gradient gel electrophoresis (DGGE) is another technique relying on the separation of PCR amplicons according to their sequence. It provides on a gel a pattern of bands that can be further excised and sequenced for taxonomic assignment (Eyers et al. 2004a). The high-throughput version of DGGE, denaturing high performance liquid chromatography (D-HPLC), separates DNA fragments within minutes using fast and repeatable reverse-phase ion-pair chromatography (Barlaan et al. 2005). Next, the fragments can be collected at the end of the column for further automated sequencing. RISA targets the intergenic transcribed spacer (ITS) regions that are located between the 16S and 23S ribosomal genes. RISA allows higher resolution in community profiling than 16S- or 23S-based techniques, because ITS regions display higher heterogeneity in both length and nucleotide sequence than their flanking genes. Similarly to T-RFLP or SSCP, an automated approach has been proposed (Fisher and Triplett 1999), in which (1) a fluorescence tagged primer is used for PCR, and (2) the electrophoretic step is performed with an automated system.

In addition to these fingerprinting techniques, two tagging methods for high-throughput profiling of complex microbial communities have been recently developed: serial analysis of ribosomal sequence tags/ribosomal DNA (SARST or SARD) (Ashby et al. 2007) and single-point genome signature tags (SP-GSTs) (van der Lelie et al. 2006). These techniques provide a fingerprint of microbial communities in the form of concatemers of PCR-amplified tag sequences. In the SARST/SARD method, PCR amplification targets short information-rich sequences of hypervariable regions of bacterial 16S rRNA genes. Then, the amplicons are ligated to yield concatemers with multiple, serially arranged PCR products that are further cloned and sequenced to characterize microbial community composition. In this way, multiple ribosomal sequence tags (RSTs) from many different organisms are obtained simultaneously from a single sequencing reaction. Phylogenetic assignment of individual RSTs is possible by comparing the tag sequences to a comprehensive database of full-length rRNA sequences of known phylotypes. The SARST technique may enable the qualitative and quantitative exploration of the ecological roles of “rare” bacteria, and holds great promise when combined with microarray technology, as shown in the case of a microarray designed with probes specific for the most abundant phylotypes in hexachlorocyclohexane-contaminated sites (Neufeld et al. 2006). Finally, the SP-GSTs method is similar to the SARST method, but offers broader possibilities to study microbial community composition, as the distribution of specific functional (e.g., biodegradative) genes and not only phylogenetic marker genes can be examined.

18.2.2 Real-Time PCR

Real-time PCR monitors the progress of a PCR reaction based on the detection and quantification of a fluorescent reporter molecule that binds to the target PCR template. From the amount of fluorescence emitted at each cycle in the exponential phase, it is possible to calculate the initial amount of target template. Real-time PCR is highly sensitive, down to a detection limit of 1–2 genome copies (Inglis and Kalischuk 2004), in contrast to microarrays (see below), which may be 100- to 10,000-fold less sensitive than PCR, a potential problem for sequences of poor abundance (Eyers et al. 2004b). Real-time PCR does not require any tedious post-PCR steps for the quantification of amplicons, as their amount is monitored in real time. Therefore, this is a high-throughput technique with superior analytical sensitivity for the detection and quantification of specific genes in environmental samples (Harms et al. 2003). A quantitative fingerprinting method combining real-time PCR and T-RFLP enabled simultaneous determination of microbial abundance and diversity within a complex wastewater community (Yu et al. 2005). Combined with stable isotope probing (SIP), this integrated approach allowed the concomitant identification and quantification of active naphthalene-degrading microorganisms in soil microcosms (Yu and Chu 2005).

18.2.3 DNA Microarrays

Microarrays (or microchips) are based on the property of a single-stranded DNA or RNA molecule ("target molecule") to hybridize to a complementary molecule ("probe") attached to a solid support (Zhou 2003). Compared to traditional nucleic acid membrane hybridization, microarrays offer the advantage of miniaturization (thousands of probes can be spotted on a slide), high sensitivity and rapid ("real-time") detection (Eyers et al. 2004b). Moreover, probe–target specificity is ensured by including single mismatch probes on the array that help distinguish sequence-specific signals from non-specific ones. Fluorescent dyes can be enzymatically or chemically incorporated in the sample to be hybridized, therefore readout of the microarray is based on the detection of a fluorescence signal. In addition, environmental samples can be incubated in the presence of a radioactively labeled substrate prior to hybridization, in order to identify microorganisms involved in the metabolism of a specific substrate, "isotope arrays" (Wagner et al. 2006).

Within environmental genomics, three major classes of microarrays have been developed: (1) phylogenetic oligonucleotide arrays (POAs), which contain oligonucleotide probes targeting taxonomic genes (e.g., 16S rRNA gene), (2) functional gene arrays (FGAs), where probes target genes encoding key enzymes involved in specific processes, and (3) community genome arrays (CGAs), which are constructed from whole genomic DNA of many different strains or species (Zhou 2003). The design of probes for POA and FGA formats is based on sequences retrieved from databases. For this reason, such microarrays do not give access to

unknown phylogenetic affiliations and functional activities. Considering the tremendous reservoir of unknown sequences in natural environments, this is a major drawback of "traditional" microarray analysis (Gentry et al. 2006). To circumvent this limitation, clone libraries of environmental bacteria whose genome sequence is still unknown can be spotted on microarrays. For instance, a clone library of a specific microorganism can be printed on a microarray and hybridized with mRNA isolated before and after exposure of this microorganism to a specific treatment. Differences in hybridization patterns will allow the identification of clones harboring genes expressed (or repressed) in response to this treatment.

A further step would be to use probes made directly from environmental DNA without any cultivation step, i.e., combining metagenomics (direct extraction and cloning of collective genomes from a given biotope, see next section) with microarrays. This metagenomic array (MGA) technology is still in its infancy, but is highly promising for high-throughput screening of environments, including contaminated sites in various stages of treatment, as it does not require prior sequence knowledge of the microbial communities being analyzed (Gentry et al. 2006). Another significant advance towards the use of microarrays for studying the dynamics of microbial communities in situ lies in the recent development of a comprehensive FGA by Zhou and colleagues (He et al. 2007b). This microarray, termed Geochip, seeks to provide direct linkages between biogeochemical processes and functional activities of microbial communities in various habitats. It contains 24,243 oligonucleotide (50mer) probes covering more than 10,000 genes involved in nitrogen, carbon, sulfur and phosphorus cycling, metal reduction and resistance, and organic contaminant degradation. A new generation of this microarray, called Geochip 3.0, has several additional features, including phylogenetic markers such as *gyrB* (He et al., 2007a).

Environmental application of array technology needs to overcome major challenges in specificity, sensitivity and quantification (Gentry et al. 2006). The specificity issue is especially critical for POAs hybridized with rRNA, because some regions of the rRNA molecule are highly conserved, and the stability of the secondary structure of the small-subunit rRNA can hamper proper hybridization specificity and sensitivity. The specificity issue can be resolved by thermal dissociation tests that help discriminate between perfect-match and single-mismatch probe–target duplexes (El Fantroussi et al. 2003).

The sensitivity of microarrays applied to environmental samples can be problematic, because potential contaminants (e.g., humic acids) in these samples can inhibit enzymatic reactions and generate a high signal background on the microarray. There exist protocols to extract RNA and DNA of sufficient purity from complex environments like soils or sediments (El Fantroussi et al. 2003). Usually, a sufficient amount of environmental rRNA can be extracted without the need for an amplification step prior to hybridization (El Fantroussi et al. 2003). When DNA or mRNA is the target molecule, an amplification step is necessary to reach the required level of sensitivity. PCR amplification must be used with caution because of its associated bias and artifacts (Suzuki and Giovannoni 1996; Polz and Cavanaugh 1998). Alternatively, whole genome amplification can be carried out

with phi29 DNA polymerase and random exonuclease-resistant primers without thermal cycling. Such amplification is surprisingly uniform across the genomic target compared to PCR-based whole-genome amplification (Dean et al. 2002). Microbial community DNA amplified using the phi29 DNA polymerase was successfully hybridized to a community genome microarray to analyze the structure of environmental microbial communities, and its application to groundwater samples containing sub-nanogram quantities of microbial DNA was demonstrated (Wu et al. 2006). When the goal is to monitor not just existing but transcribed, i.e., functioning genes (mRNA based analysis), a T7 polymerase-based linear amplification approach using fusion primers provides sufficient and representative amounts of mRNAs for functional analysis of microbial communities (Gao et al. 2007).

Finally, quantification is the third challenge. With FGAs and CGAs, Wu et al. (2001) measured a linear relationship between signal intensity and DNA concentration over a four-fold concentration of DNA isolated from pure cultures and mixed populations. On the other hand, quantifying microbial populations with POAs is difficult because of cross-hybridizations that may occur when dealing with complex environmental samples containing perfect and mismatch targets in unknown abundance (Chandler et al. 2006). Again, the use of thermal dissociation curves is promising for correct quantification of environmental samples (Wick et al. 2006).

18.2.4 Metagenomics

Metagenomics, i.e., the analysis of the collective genomes of a given environment, provides direct access to the entire pool of environmental genomes without the limitations of lab-based cultivation of microbial species (Eyers et al. 2004b). Usually, the term 'metagenomics' refers to the construction of metagenomic libraries: (1) generation of DNA fragments of appropriate size, (2) ligation of the fragments into an appropriate cloning vector [cosmid, fosmid or bacterial artificial chromosome (BAC) vectors], (3) introduction of the recombinant vectors into a suitable bacterial cloning host, and (4) screening of clones harboring particular activities, or containing specific sequences (Daniel 2005). Metagenomics enables the retrieval of unknown sequences or functions from the environment, whereas methods relying on PCR amplification, or microarrays (POA or FGA) pre-suppose knowledge of gene sequences. The isolation of genomic DNA from complex environmental matrices, such as sludge, wastewater, sediment or soil, presents major challenges in terms of quality (inhibitory contaminants are frequently co-extracted), molecular size (DNA is often sheared to low-size fragments) and representation of all microbial genomes (size fractionation or experimental normalization are often necessary) (Cowan et al. 2005). Moreover, in some heavily contaminated environments harbouring very low cell densities, direct extraction of metagenomic DNA does not provide enough genomic material for subsequent library construction. New PCR-independent amplification techniques are then necessary, like multiple

displacement amplification (MDA) using phi29 DNA polymerase (Abulencia et al. 2006; Binga et al. 2008). While introducing an amplification bias, such a technique of whole-genome amplification of metagenomic DNA from very minute microbial sources gives genomic information that would otherwise remain inaccessible (Abulencia et al. 2006).

Screening strategies for metagenomic libraries can be designed to explore functional and/or genetic diversity. In the function-driven analysis, clones of interest are screened or selected through the detection of heterologous expression of a desired trait. However, function-driven analysis is typically realized with low-throughput screens based on the visual detection of: (1) growth inhibition of indicator bacteria, (2) metabolic activity (clearing zone or coloration around colonies on specific solid media), or (3) growth on selective media (e.g., containing a xenobiotic as sole carbon source) (Handelsman 2005). Fortunately, library screening can be facilitated by automated colony picking, pipetting robotics, use of microtiter plates, informatics-assisted data management and sensitive assays targeting a broad range of biomolecules. Automation of the screening is particularly welcome as screening "hits" are typically low (less than two out of a total of 10,000 clones have industrially relevant enzymes) (Lorenz and Eck 2005). However, the screening hit rate can be improved by: (1) the use of different expression hosts, (2) the use of diverse strategies to enrich/select for community genomes with desired traits before metagenomic library construction, (3) the use of liquid enrichment cultures for the screening of multiple clones simultaneously, and (4) the development of novel high-throughput screening strategies compatible with fluorescence-activated cell sorting of the clones (throughput of 10^9 events per day) (Robertson and Steer 2004). In the sequence-driven analysis, clones of interest are identified based on the presence of conserved regions in phylogenetic marker genes (e.g., 16S rRNA genes) or in functional genes coding for key processes, such as oxygenases (Erwin et al. 2005). PCR and hybridization are commonly used for screening, using suitable primers or target-specific probes which are designed with information from databases. Therefore, the sequence-driven analysis can only be applied for the identification of members of known gene families or novel variants of known functional classes of proteins (Daniel 2005). Large DNA-insert libraries offer more possibilities for retrieving entire clusters of functional genes and linking potential metabolic functions to specific microorganisms, in the case that phylogenetic and functional genes are present on the same insert (DeLong et al. 2006). In contrast, the entire metagenome of a given environment, including that of a site undergoing bioremediation treatment, can be accessed through large-scale shotgun sequencing of small DNA insert libraries (Deutschbauer et al. 2006). In this case, the purpose is to get a global picture of the microbial ecosystem and not to focus on particular genes, pathways or organisms. The method involves the construction and random end-sequencing of small-DNA-insert libraries (~3–5 kbp), followed by the assembly of the subsequent sequences into contigs and scaffolds *in silico*. Genome assembly is particularly challenging, because of the massive amount of data accumulated by the shotgun sequencing approach, and also because environmental populations are highly dynamic in terms of microbial species richness, evenness and intraspecific genetic heterogeneity (DeLong 2005). It is worth

noting that Tringe et al (2005) have successfully characterized and compared the metabolic capabilities of marine and terrestrial microbial communities, using largely unassembled sequence data. In this approach, each environment is characterized by a particular 'fingerprint' of environmental gene tags (short DNA sequences that contain fragments of functional genes). Although the shotgun sequencing approach may be valuable in the quest to compare different environments, it remains unclear whether such sequencing will bring insights on particular genes and associated functions, especially in the context of bioremediated sites. In fact, both large- and small-DNA-insert libraries offer advantages and disadvantages (Whitaker and Banfield 2006), and a combination of these approaches (Hallam et al. 2004) is probably the best solution for enhancing the accuracy, coverage and reliability of genomics-based efforts to understand complex microbial communities (DeLong 2005).

18.3 Application of Molecular Techniques in Contaminated Sites for Characterization of Microbial Communities and Assessment of Biodegradation

The performance of a bioremediation process and the prediction of its outcome require a thorough and reliable monitoring of biodegradation activities. Ideally, it is necessary to use appropriate tools that link, in real time, the physicochemical conditions of the site undergoing reclamation with the dynamical characteristics (resilience, stability, etc.) of the microbial communities involved in these degradation activities. The molecular tools described in the previous sections are clearly capable of monitoring and quantifying the biotransformation of the pollutants in conjunction with the corresponding organisms. Moreover, most of these tools meet the criterion of massive parallel and real-time monitoring capabilities, in other words they possess "high-throughput" characteristics. A flow diagram showing the functional connection between bioremediation strategies at sites contaminated with hazardous pollutants, and the potential of corresponding molecular high-throughput technologies for the characterization and monitoring of such sites is given in Fig. 18.2.

Pilot-scale and field studies making use of these tools reflect the rapid pace of technological advances that are being incorporated into standard protocols. Although many of these techniques are still at the developmental stage and in need of extensive cross-validation, high-throughput molecular approaches have great potential for monitoring the efficiency and reliability of in situ bioremediation schemes.

Without being exhaustive, we are presenting a few characteristic examples illustrating applications of these molecular methodologies for the monitoring of contaminated sites undergoing remediation and for the discovery of new degrading microorganisms or new catabolic genes. Among these techniques,

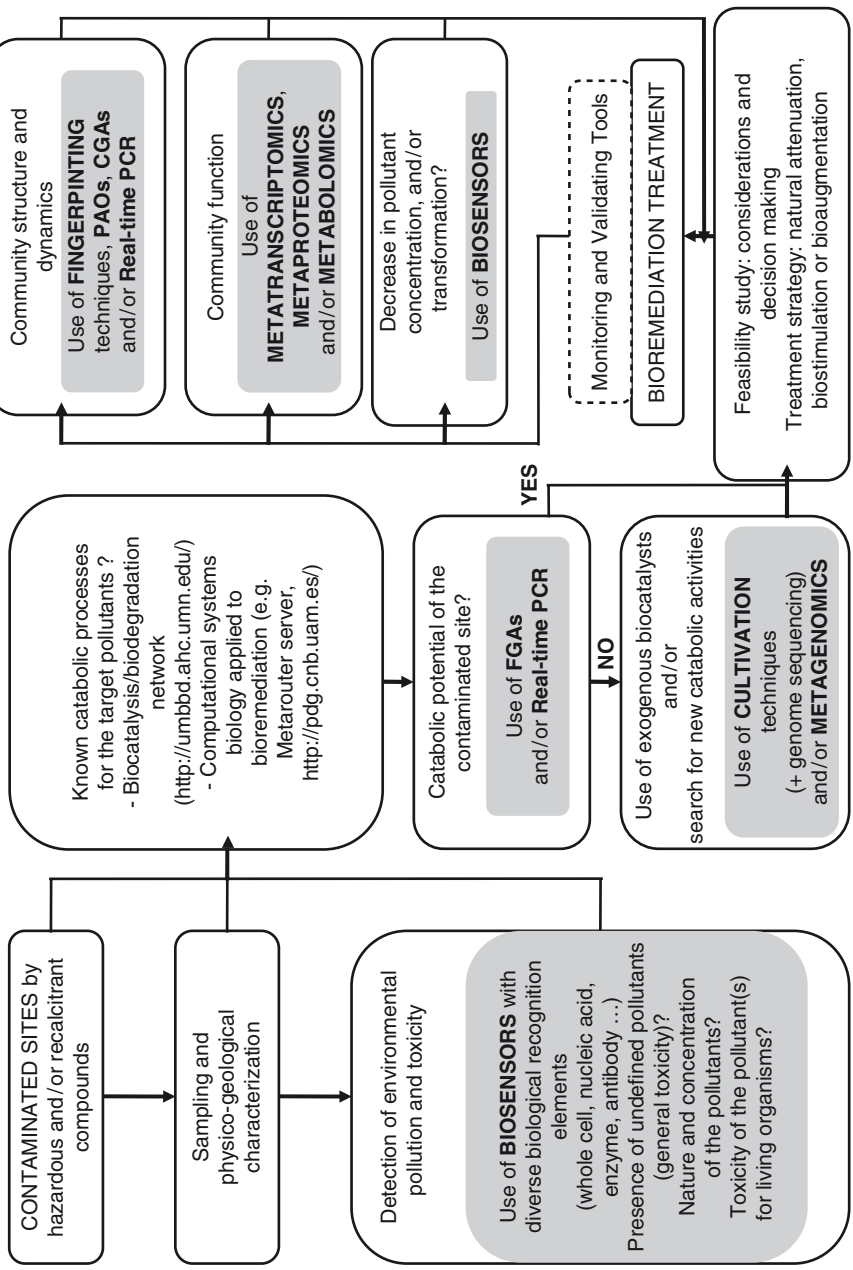

Fig. 18.2 Bioremediation strategies in sites contaminated with hazardous and/or recalcitrant waste, with special emphasis on the potential of molecular high-throughput technologies (in **bold**) for the characterization and monitoring of such sites. *FGAs* functional gene arrays; *PAOs* phylogenetic oligonucleotide arrays; *CGAs* community genome arrays

fingerprinting approaches like DGGE, RISA and T-RFLP are used with increasing success to characterize the status of bioremediation projects (Scow and Hicks 2005).

Microarrays (phylogenetic oligonucleotide- (POAs), functional gene- (FGAs) or community genome- (CGAs) arrays) are used ever more extensively to characterize the phylogenetic and catabolic diversity of contaminated environments. In addition, they have the potential of "dynamically" monitoring changes in the phylogenetic composition of microbial communities or changes in catabolic gene expression levels during biodegradation processes. In this latter case, environmental RNA (or, more accurately, cDNA) is hybridized to the microarray instead of DNA. An example of phylogenetic array-targeting bacterial groups potentially involved in biodegradative activity is the use of a POA to which soil RNA was directly hybridized (El Fantroussi et al. 2003). Neufeld et al. (2006) developed a "Ribosomal Sequence Tag" (RST) array for the characterization and comparison of various hexachlorocyclohexane (HCH)-contaminated soils. The RST array was designed to target the most abundant PCR-amplified phylotypes in the soil samples, and thus avoid sensitivity problems. The 100 most abundant RSTs (i.e., short information-rich sequences of the hypervariable regions of bacterial 16S rRNA genes) in a composite library of 2,290 RSTs from contaminated and uncontaminated soils were chosen for probe design. The analysis of soil samples by DGGE in parallel with the RST array confirmed the habitat-specific array design and validated the bulk of the probe signals. A strong correlation was observed between total HCH concentration and probe signals corresponding to unknown *Proteobacteria*, and between α-HCH concentration and probes targeting *Sphingomonas*. Regarding the specificity issue, it can be more easily evaluated thanks to the potential of dissociation curves. For instance, Eyers et al. (2006) successfully used this approach to discriminate shifts in a soil microbial community associated with 2,4,6-trinitrotoluene (TNT) contamination using a rRNA-targeted POA. In a remarkable recent development of microarray technology with potential for detecting catabolic genes, Gao et al. (2007) coupled whole-community RNA amplification (WCRA) with community genome microarrays to monitor the functions of microbial communities in a denitrifying reactor and in low-biomass groundwater samples contaminated with organic solvents, hydrocarbons, nitrate and uranium. Before testing their protocol on environmental samples, they examined whether representative amplification could be obtained using WCRA with mixed mRNAs from four known species. A good representative detection was obtained with mixed RNA templates, but the authors stressed that further tests with artificial communities having more complex structures may be needed. Finally, the genes expressed in the bioreactor and groundwater system were consistent with the expected functions of such settings. These results suggest that this methodology can lead towards reliable and comprehensive characterization of microbial community activities in situ using high-throughput approaches.

The implementation of innovative remediation strategies for the mineralization of recalcitrant pollutants is hampered by our relatively poor understanding of the degradation pathways of these pollutants. Therefore, metagenomic and whole genome sequencing approaches are progressively used to discover and characterize new microorganisms and catabolic operons involved in the degradation of hazardous contaminants. For instance, a metagenomic approach was taken by Suenaga et al. (2007) to retrieve catabolic operons for aromatic compounds from activated sludge from a coke wastewater treatment plant. The metagenomic library was screened in search of extradiol dioxygenases (EDO) using catechol as a substrate. A total of 43 EDO genes were identified in the positive clones, among which more than half belonged to new EDO subfamilies. Moreover, gene sequence analysis allowed the reconstruction of the evolutionary tree of EDOs. To illustrate the potential of metagenomes for providing new catabolic genes, Boubakri et al (2006) developed a methodology called "metagenomic DNA shuffling process". Their aim was to assess the potential of environmental metagenomes to furnish appropriate gene fragments for the creation of novel genes under recent environmental pressure like contamination with xenobiotics. Mechanisms thought to be involved in genomic evolution of bacteria in nature are lateral transfer of gene fragments and shuffling in the microbial genomes. The authors reproduced those mechanisms in vitro and successfully constructed a pesticide-degrading gene, *linA*, from the metagenome of a lindane-free soil in which analogous or homologous sequences of *linA* had, however, been detected. This study illustrates the possibility that DNA fragments from an entire microbial community might be available for the creation of novel genes capable of degrading pollutants.

Occasionally, even negative results of screening a metagenomic library because of an ill-adapted host organism can be valuable, as illustrated by our recent discovery of strong TNT denitration capacity in *Escherichia coli*, the host par excellence, while screening for TNT denitration activity a metagenomic library which we established using DNA from soil historically contaminated with this recalcitrant explosive (Stenuit et al. 2006). In this same context, valuable insights about shifts in microbial community structure as a result of TNT contamination were recently obtained by us using microarray probing as pointed out above (Eyers et al. 2006) and fingerprinting techniques (George et al. 2008).

18.4 Conclusion

In conclusion, although molecular tools have already brought about a revolution in the field of microbial ecology, as they have provided direct access to environmental microbes independently of their culturability, their true practical potential is just emerging in the field of bioremediation. Nowadays, these technologies are expected to boost the discovery of new catabolic activities, and to provide quantitative and timely information for the management and cleanup of contaminated sites and effluents in a perspective of sustainable development.

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