Soil Biology

Nagina Parmar
Ajay Singh *Editors*

Geomicrobiology and Biogeochemistry

Soil Biology

Volume 39

Series Editor
Ajit Varma, Amity Institute of Microbial Technology,
Amity University Uttar Pradesh, Noida, UP, India

For further volumes:
http://www.springer.com/series/5138

Nagina Parmar • Ajay Singh
Editors

Geomicrobiology and Biogeochemistry

Editors
Nagina Parmar
Department of Chemistry and Biology
Ryerson University
Toronto
Ontario
Canada

Ajay Singh
Lystek International Inc.
Cambridge
Ontario
Canada

ISSN 1613-3382 ISSN 2196-4831 (electronic)
ISBN 978-3-642-41836-5 ISBN 978-3-642-41837-2 (eBook)
DOI 10.1007/978-3-642-41837-2
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013956629

© Springer-Verlag Berlin Heidelberg 2014
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Earth sustains an immense diversity of prokaryotic and eukaryotic organisms. Microorganisms play an essential role in the functioning and sustaining of all natural ecosystems including biogeochemical cycling of nutrients. Biotechnology has become an important tool for manipulating and utilizing microorganisms and plants and providing new approaches in various industries including petroleum, food, feed, pharmaceutical, detergent, and pulp and paper. New strains are continuously being explored and genetic or enzymatic functions are often reconstructed through molecular recombination and protein engineering techniques to increase the gene expression and metabolic productivity of industrially important organisms. The unique characteristics of these microbes are widely utilized for industrial applications such as enzymes and chemical production, waste treatment and recycling, bioremediation of industrial pollutants in soils and aquifers, enhanced petroleum oil recovery, biomining, and soil fertility. Biochemical and molecular tools are continuously being developed in an attempt to evaluate community structures with ecosystem functions and to develop appropriate industrial approaches.

This volume of the Soil Biology series, *Geomicrobiology and Biogeochemistry*, is a selection of topics related to biological processes with an emphasis on their industrial applications. It gives an overview of various aspects in geomicrobiology and biotechnology including topics such as biomining, bioremediation, biotechnological applications of some extremophiles, subsurface petroleum microbiology, enhanced oil recovery using microbes and their products, metal extraction, and soil nutrient cycling and plant nutrition.

Experts in the area of geomicrobiology and environmental sciences from diverse institutions worldwide have contributed to this book, which should prove to be useful to students, teachers, and researchers in the disciplines of soil and geological sciences, microbiology, environmental engineering, and biotechnology.

We gratefully acknowledge the cooperation and support of all the contributing authors, the series editor Prof. Ajit Varma, and Dr. Jutta Lindenborn throughout the preparation of this volume.

Toronto, ON, Canada — Dr. Nagina Parmar
Cambridge, ON, Canada — Dr. Ajay Singh

Contents

1 **Geobiotechnology** 1
Nagina Parmar and Ajay Singh

2 **Novel Molecular Tools to Assess Microbial Activity in Contaminated Environments** 17
Nadine Loick and Christopher Weisener

3 **Advanced Molecular and Microspectroscopy Toolbox for Deciphering Soil Diazotroph Diversity** 37
Anu Kalia and Vir R. Parshad

4 **Haloalkaliphilic Bacteria: Molecular Diversity and Biotechnological Applications** 61
Megha K. Purohit, Vikram H. Raval, and Satya P. Singh

5 **Recent Trends in Bioremediation** 81
Asha A. Juwarkar, Rashmi R. Misra, and Jitendra K. Sharma

6 **Heavy Metal Bioremediation and Nanoparticle Synthesis by Metallophiles** 101
Arvind Sinha, Rajeshwari Sinha, and Sunil K. Khare

7 **Mineralogical and Geochemical Controls in Biomining and Bioremediation** 119
Bernhard Dold

8 **Microbial Recovery of Nickel from Lateritic (Oxidic) Nickel Ore: A Review** 137
Lala Behari Sukla, Sunil Kumar Behera, and Nilotpala Pradhan

9 **Subsurface Petroleum Microbiology** 153
Ajay Singh, Jonathan D. Van Hamme, Ramesh C. Kuhad, Nagina Parmar, and Owen P. Ward

10 Nutrient Cycling: Potassium Solubilization by Microorganisms and Improvement of Crop Growth 175
Satyavir S. Sindhu, Priyanka Parmar, and Manisha Phour

11 Phosphorus Cycling: Prospects of Using Rhizosphere Microorganisms for Improving Phosphorus Nutrition of Plants . . . 199
Satyavir S. Sindhu, Manisha Phour, Sita Ram Choudhary, and Deepika Chaudhary

12 Biotechnology in Enhanced Petroleum Oil Recovery 239
Ajay Singh, Nagina Parmar, and Owen Ward

13 The Chemistry of Removal of Inorganic Compounds from the Soil 247
Innocent Oseribho Oboh

14 Bioremediation of Contaminated Sites and Aquifers 261
Nagina Parmar, Ajay Singh, and Hammad Khan

Index 297

Chapter 1
Geobiotechnology

Nagina Parmar and Ajay Singh

1.1 Introduction

Earth has a large wealth of biodiversity with taxa including microorganisms, protozoa, microscopic and large invertebrates, vertebrates, vascular plants and lichens, algae, and mosses. Many of these smaller groups can be found in a handful of soil. Because of the abundance and diversity of the multiple taxonomic groups, identifying all species and their interactions in a single soil sample is not trivial. Instead, our understanding of soil biodiversity is largely based on trophic or functional classifications. A further and critical recognition of the dependence of humans on the benefits provided by soil biodiversity is the concept of ecosystem services including carbon sequestration, generation and renewal of soil structure and fertility, flood and erosion control, geochemical nutrient cycling, waste management, bioremediation, and biocontrol of pathogens and parasites. Application of molecular techniques to the study of microbial diversity has revealed the existence of an incredible variety of genotypes and species in all known habitats above or below the earth surface (Croal et al. 2004). Theoretical and empirical analyses of the microbial diversity of soils indicate that soils harbor in the order of 7,000 different taxa at an abundance of approximately 10^9 cells per cm^3.

Over the past 4 billion years, microorganisms have been helping to shape the earth by making it more habitable for higher forms of life. Geomicrobiology has become an important learning tool that integrates geology and microbiology studying interactions of subsurface extremophiles and their environments such as soils, rocks, springs, aquifers, petroleum reservoir, etc. Biogeochemical processes carried out by the microorganisms in near or deep subsurface sediments have been the

N. Parmar (✉)
Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada M5B 2K3
e-mail: naginap@ryerson.ca

A. Singh
Lystek International Inc., 1425 Bishop Street North, Unit 16, Cambridge, ON, Canada
N1R 6J9

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_1, © Springer-Verlag Berlin Heidelberg 2014

subject of much interest. Bacteria are remarkable in their metabolic diversity due to their ability to harvest energy from oxidation and reduction reactions. In some cases, their metabolisms involve redox transformations of metal(loid)s, which lead to the precipitation, transformation, or dissolution of minerals. Microorganism/mineral interactions not only affect the geochemistry of modern environments but may also have contributed to shaping the near surface environment of the early earth. How these microbial life influence the inhabitants of earth have led to an evolving and advancing field of geomicrobiology linking the field of geosphere, biosphere, and biotechnology.

1.2 Geomicrobiology and Biogeochemistry

The concept of a deep microbial biosphere has advanced over the past several decades due to the ability to culture phylogenetically diverse prokaryotes and detection via characterization of directly extracted nucleic acids from a wide range of deep surface and near surface environments. Recent advances have linked the metabolic potential of these microorganisms, determined directly or inferred from phylogeny, to biogeochemical reactions determined via geophysical, geochemical, and geobiological analysis and modeling (Madsen 2011). Microbial exploration of the deep terrestrial subsurface has been driven by a combination of keen interest in exploring planetary microbial biodiversity and practical aspects ranging from enhanced production of petroleum to exploitation of extremophiles for industrial applications in the new biotechnology era.

Microorganisms have remarkable capability to utilize a wide range of energy sources, including light, organic matter, inorganic materials, and their broad distribution across the surface of the planet. Many such environments contain all of the requirements for prokaryotic life including water-filled space in pores and fractures, energy in the form of buried organic matter (kerogen), gases (methane or hydrogen), and various other essential organic and inorganic elements (sulfur, iron, manganese, carbon, nitrogen, and phosphorous). One of the major unresolved questions in geomicrobiology is the depth limit to microbial life in the terrestrial subsurface. It has been suggested that temperature may ultimately limit the depth of the biosphere as it increases with depth on average at a rate of 3 °C per 100 m.

Bacterial genetics holds the key to understanding these metabolisms at different surface levels. Once the genes and gene products that catalyze biogeochemically relevant reactions are understood, as well as the conditions that trigger their expression, we may begin to predict when and to what extent these metabolisms influence modern geochemical cycles, as well as develop a basis for deciphering their origins and how organisms that utilized them may have altered the chemical and physical features of our planet (Banfield et al. 2005). Functional gene arrays (FGA) contain probes for genes encoding proteins or enzymes involved in functions of interest and allow for the study of thousands of genes at one time. The most comprehensive FGA to date is the GeoChip, which contains ~24,000 probes for

~10,000 genes involved in the geochemical cycling of C, N, P, and S, as well as genes involved in metal resistance and reduction and contaminant degradation (Van Nostrand et al. 2010).

The major types of analytical, microbiological, and/or molecular tools routinely used to study advance environmental microbiology are site geochemistry, cultivation, incubations, biomarkers, and microscopy. First step is to discover new microbiological process and prove that the microorganisms are capable of catalyzing the process of interest. Validation of the discovery by finding representative microbiological agents is followed by characterization of agents and the physiological, biochemical, and genomic mechanisms of the biogeochemical processes they catalyze. Field verification of ecological relevance of agents, their biogeochemical impact, and further exploitation of the innovation for commercial applications is the ultimate goal in the geobiotechnological context.

1.3 Bio-Mediated Improvement of Soil Engineering Properties

For improvement of soil structure and engineering properties, conventional approaches include injecting synthetic materials, such as microfine cement, epoxy, acrylmide, phenoplasts, silicates, and polyurethane into the pore space to bind soil particles together. This is accomplished using a variety of chemical, jetting, and permeation grouting techniques. However, these approaches create environmental concerns as all chemical grouts except sodium silicate may be toxic and/or hazardous.

Bio-mediated improvement of soil properties includes permeability, stiffness, compressibility, shear strength, and volumetric behavior (Ivanov and Chu 2008). Properties of permeability, stiffness, compressibility, shear strength, and volumetric behavior may realize a tenfold change. One of the most studied processes is the precipitation of calcite uniformly within soils through biological activity to elevate the pH that creates supersaturated conditions (DeJong et al. 2010). *Sporosarcina pasteurii*, an alkalophilic soil bacterium with a highly active urease enzyme consumes urea and decomposes it into carbon dioxide (CO_2) and ammonia (NH_3). In the presence of water, NH_3 is converted to NH_4^+ and CO_2 equilibrates in a pH-dependent manner with carbonic acid, carbonate, and bicarbonate ions. The increase in pH provides the alkaline environment and carbonate required for the reaction with Ca^{2+} and precipitation of calcite ($CaCO_3$).

In the area of geotechnical engineering, two biological processes, bioclogging and biocementation, are gaining interest (DeJong et al. 2010). Bioclogging is the production of pore-filling materials through microbial means so that the porosity and hydraulic conductivity of soil can be reduced. Biocementation is the generation of particle-binding materials through microbial processes in situ so that the shear strength of soil can be increased. The most suitable microorganisms for soil

bioclogging or biocementation are facultative anaerobic and microaerophilic bacteria, although anaerobic fermenting or respiring bacteria may also be suitable. The majority of the studies on microbial geotechnology at present are at the laboratory stage. Potential applications include liquefaction and erosion prevention, building settlement reduction, dam safety, tunneling, erosion prevention, slope stabilization, impermeable and reactive barriers for groundwater protection, aquifer and energy storage, and carbon sequestration.

1.4 Biomining

Biomining is the use of acidophilic, chemolithotrophic microorganisms to facilitate the extraction of metals from sulfide or iron-containing ores or concentrate. The metal solubilization is a combination of chemical and biological processes. Since the metal is extracted into water, the process is also known as bioleaching or bio-oxidation. Metals for which this technique is mainly employed included copper, cobalt, nickel, zinc, and uranium. For gold and silver recovery, bioleaching bacteria are only applied to remove interfering metal sulfides from ores bearing the precious metals prior to cyanide treatment. Biomining has developed into a successful and expanding area of biotechnology (Rawlings and Johnson 2007; Dold 2008; Watling et al. 2010). The biomining organism that has been studied to the greatest extent by far is *Acidithiobacillus ferrooxidans*. The use of microbes in ore processing has some distinct advantages over the traditional physicochemical methods. Microbial extraction procedures do not require the high amounts of energy used during roasting or smelting and do not produce sulfur dioxide or other environmentally harmful gaseous emissions. They are also described as moderate capital investment and lower operating costs, making the technology potentially applicable to relatively low-grade ores in smaller deposits. Tailings from biomining operations are less chemically active, and the biological activity they can support is reduced by at least the extent to which they have already been bioleached.

There are two main types of processes for commercial-scale microbially assisted metal recovery (Anjum et al. 2012). Irrigation process involves the percolation of leaching solutions through crushed ore that have been stacked in columns, heaps, or dumps. Stirred tank-type processes employ continuously operating, highly aerated stirred tank reactors. Biohydrometallurgy is regarded as one of the most promising and revolutionary biotechnologies where the products are dissolved in aqueous solution, thereby rendering them more amenable to containment, treatment, and recovery under mild conditions. Consequently, the application of biohydrometallurgy in the recovery of metals from lean grade ores and wastes has made it an ecofriendly technology for enhanced metal production. On a commercial scale, challenges exist for the potential use of microorganisms for the engineered extraction of metals from the low grade ores. Environmental pollution problems regarding the acid mine drainage and industrial effluents would be minimized by more extensive exploitation of enhanced bioremediation techniques.

1.5 Bioremediation and Phytoremediation

Microorganisms and plants participate in the biodegradation and removal of hazardous contaminants to restore the polluted environment through bioremediation processes (Singh et al. 2009; Glick 2010). The majority of environmental contaminants are hydrophobic and sorb to soil particles. To access these contaminants, microbes may interact directly with the contaminant and/or soil particulates or secrete biosurfactants to mobilize the contaminant into the aqueous phase. A variety of in situ and ex situ bioremediation strategies have been employed aiming at promoting biodegradation of the chemical pollutants present in the contaminated medium. Some of the most widely distributed organic environmental contaminants are aliphatic and aromatic hydrocarbons, including alkanes, mono- and polycyclic aromatic, present in crude oils or in refined oil fuels or petrochemicals; halogenated aliphatic especially chlorinated species including chlorinated methanes, ethanes, and ethenes such as trichloroethene, dichloroethene, and perchloroethene and vinyl chloride; mono- and poly-halogenated phenols and benzoates, including pentachlorophenol; polychlorinated biphenyls; chloroaromatics including 2,4-dichlorophenoxyacetate, and related compounds; nitroaromatics including nitrobenzene and 2,4,6 trinitrotoluene; organophosphates; nitrate esters (including glycerol trinitrate); nitrogen heterocycles (including hexahydro-1,3,5-trinitrotriazine or RDX); polyesters, polyurethanes and nylon.

The nature of the microbial type or population which develops during bioremediation is influenced by a wide range of factors including the physical properties of the medium, salt concentration, solids content, surface properties, particle size, nature and concentration of contaminants, nature and concentration of nutrients present to support microbial growth, moisture, presence of oxygen or other electron acceptors, pH, and temperature (Singh et al. 2012). Contaminants in well-aerated environments are predominantly degraded by aerobic metabolic processes, whereas contaminants in a non-aerated subsurface environment may be anaerobically degraded. Aerobic pathways for biodegradation of organic contaminants including petroleum hydrocarbons, chlorinated hydrocarbons, nitroaromatics and nitrogen heterocycles, organophosphate derivatives, and plastics involve reaction mechanisms of dioxygenases, monooxygenases, hydroxylases, ligninases, and reductases among others. Anaerobic biodegradations typically involve initial activation mechanisms such as carboxylation, methylation, hydroxylation, dehydrogenation followed by anaerobic reductions, and hydrolytic reactions.

Microbes have potential to remediate metals in contaminated media by exploiting a combination of mechanisms including precipitation, sorption, biosurfactant complexation, biofilm entrapment, and metabolic uptake. Some microbes can also transform metals including radionuclides, for example, by reducing mercury into volatile form which can be removed. In metal bioremediation applications there is particular interest in exploiting microbes which have been isolated from mine tailings and heavy metal contaminated sites, as these strains

exhibit superior metal-scavenging capacities, greater resistance to metal toxicity (sometimes mediated by metal efflux mechanisms), and ionizing radiation.

A wide range of commercial bioremediation approaches for the contaminated environments have been implemented. They include soil bioremediation processes involving natural attenuation, in situ subsurface treatments, and use of biopiles, composting, landfarming, and bioreactor treatments. Crude oil spills at sea have been addressed through shorelines or deep sea bioremediation strategies. Monitored natural attenuation or intrinsic remediation process presumes that the environment contains sufficient nutrients to support the indigenous microbial population in the biodegradation of contaminants. Oxygen availability is often a rate-limiting factor although anaerobic biodegradation may also be important. In situ subsurface bioremediation approach involves designing a subsurface configuration to allow growth and contaminant biodegradation by the microbes present through efficient supply of nutrients, oxygen, or other electron acceptors through injector wells and air sparging systems into the unsaturated or saturated zone, generally below the contaminated plume. Processes may include a soil vapor extraction or other vapor containment system to prevent release of volatile contaminants from the site to the atmosphere. Engineered soil biopiles are typically fitted with a series of aeration pipes to distribute air and moisture to support growth and biodegradation processes in the contaminated soil. Landfarming bioremediation approach involves surface atmospheric aeration processes enhanced by frequent tilling and maintenance of moisture levels at 40–60 % saturation via sprinklers or other means. The process is applicable to in situ soils contaminated near the surface or to spreading and treating excavated soils at a designated landfarming location. 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. Advanced soil slurry bioreactors have advantages for bioremediation process similar to the general fermentation and other bioprocesses, namely those conditions in the mixture can approach homogeneity and processes can be optimally controlled to promote microbial growth and contaminant biodegradation. While capital and energy costs are high in these processes, high rates and extents of degradation may be achieved with high levels of process dependability and reliability.

Phytoremediation processes remove inorganic or organic contaminants from soil promoted by associations between plant roots and microbes in soil and aqueous media (Glick 2010). The mechanisms involved include phytodegradation, whereby root exudates from the plant enhance microbial growth and contaminant biodegradation in the root surroundings, rhizofiltration where soluble contaminants are sorbed onto or into the roots, phytoextraction involving uptake of metals by roots and their translocation in the plants may be harvested for disposal, and phytovolatilization, where contaminants taken up by the plant are possibly, but not always, modified and transpired into the atmosphere.

Although biological processes are typically implemented at a relatively low cost, implementation of bioremediation technologies requires knowledge of interdisciplinary sciences involving microbiology, chemistry, engineering, ecology, and

hydrogeology. 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. A number of potential hydrocarbon-degrading strains have been isolated and characterized using advanced molecular techniques in the last two decades and further increase in our understanding of the ecology of hydrocarbon-degrading microbial communities, nature of contaminants, soil chemistry, and engineering design of the appropriate treatment system will help in developing practical soil bioremediation strategies.

1.6 Extremophiles Biotechnology

Extremophiles, organisms living under extreme conditions, are divided into different categories according to the nature of their adaptation: thermo- (high temperature), psychro- (low temperature), halo- (high salt), acido- or alkali- (extreme low or high pH), and xero- (low water activity). The extremophiles are adapted to and limited by very narrow sets of environmental conditions, and they thrive in or require the extreme conditions. Extremotrophic or extremotolerant organisms can survive and proliferate under a wider set of environmental conditions. They tolerate extreme environments but normally grow better at moderate conditions including hot springs, shallow submarine hydrothermal systems, or hot-vent systems, where microorganisms can be found at temperatures above 100 °C. Extremophiles are also found in highly saline lakes, sometimes at salt conditions near that of saturation, and in environments with extreme pH values (acidic or alkaline). The sources of psychrophilic organisms include the cold polar seas and soils and Alpine glaciers, as well as deep-sea sediments, which are cold and are also at high pressures.

The use of extremophilic microorganisms in industrial processes has grown rapidly over the last two decades. Extremozymes from these microbes possess high stability and reduced risk of contamination of the organisms that produce them. Due to the superior properties of these enzymes, they are expected to form the bridge between biological and chemical processes. Typical examples are polymer-degrading enzymes like amylases, proteases, cellulases, pullulanases, and xylanases with their significant roles in chemical, food, pharmaceutical, paper, pulp, and waste-treatment industries (Fujinami and Fujisawa 2010). Other important products are cyclodextrins, compatible solutes, and polyunsaturated fatty acids. Extremozymes are also employed in the production of hydrogen gas.

Microorganisms can interact with heavy metals in a variety of ways that result in decreased metal mobility and solubility. Two biogeochemically important groups of microbes, the metal and sulfate-reducing bacteria, have suitable physiology for metal precipitation and immobilization. Several biopolymers such as biosurfactants, exopolysaccharides, and bioplastics have been developed from

halophilic microorganisms. Biosurfactants enhance the remediation of oil-contaminated soil and water. Polyhydroxyalkanoates (PHA) are intracellularly accumulated bacterial storage compounds. These biodegradable plastics have properties comparable to those of polyethylene and polypropylene and replace oil-derived thermoplastics in many applications. Biohydrometallurgy using autotroph extremophiles has made significant advances in development as a commercially viable technology for processing sulfide ores. Bioleaching has future potential for remediation of heavy metal contaminated materials. Bioprocesses have been determined as being one-third to one-half the cost of conventional chemical and physical remediation technologies. The application of bioremediation as an alternative remediation technology is becoming the technique of choice for environmental professionals.

Since thermophiles have a remarkable ability to tolerate fluctuations in pH, temperature, and environmental change, an attribute which offers a clear advantage in the development of a commercially viable biofuel process such as bioethanol, biodiesel, biobutanol, biohydrogen, and biogas from biomass fermentation (Tango and Islam 2002; Barnard et al. 2010). Methanogens play a crucial role in the production of biogas, whereas psychrophiles are being exploited for their cold-adapted lipases for use in biodiesel. Promising thermophilic bacteria are *Clostridium thermocellum*, *Thermoanaerobacterium saccharolyticum*, *T. thermohydrosulphuricum*, *T. ethanolicus*, and *Geobacillus stearothermophilus*. Psychrophiles have been exploited for their high value-added enzymes, biomolecules as food additives, antibiotics, and neutraceuticals, and bioremediation (Margesin and Feller 2010). Halo-alkaliphiles marine hydrocarbon-utilizing bacteria isolated from oil-contaminated sediments exhibit a remarkable salt tolerance and are represented by *Gammaproteobacteria*, such as *Marinobacter*, *Halomonas*, *Alcanivorax*, *Cycloclasticus*, and *Neptunomonas* (Sorokin et al. 2012). They produce a variety of enzymes (hydrolases and isomerases) biosurfactants, bioplastics, exopolysaccharides, lectins, liposomes, poly-(gamma-D-glutamic acid), food supplements, and colorants. Alkaliphilic microorganisms, capable of growing in alkaline environments produce industrially important extracellular proteases, cellulases, amylases, and lipases that are able to function under high alkaline pH values (pH >9.0) and play an important role in the detergent industry. Alkaline enzymes often show activities in a broad pH range, thermostability, and tolerance to oxidants compared to neutral enzymes. Extremophiles have also been utilized for the microbial degradation of petroleum pollutants.

1.7 Microbial Electrosynthesis and Bioelectrochemical Systems

Geobacter sulfurreducens and *G. metallireducens* can produce protein filaments with metallic-like conductivity, known as microbial nanowires that facilitate long-range electron transport (Lovley 2006). This knowledge was a paradigm shift in biological electron transfer in the emerging field of bioelectronics with important implications for geomicrobiology and biogeochemistry (Logan 2009). Microbial electrosynthesis processes are conducted in bioelectrochemical system (BES), consisting of an anode, a cathode and, a membrane separating the two. An oxidation process occurs at the anode (e.g., acetate oxidation or water oxidation), whereas a reduction process occurs at the cathode (e.g., O_2 reduction or H_2 evolution). The electrodes are surrounded by an electrolyte, the fluid around the electrode containing the reactants and/or products, which is generally an aqueous solution or wastewater. BES can also be operated in "microbial fuel cell" mode, in which they deliver power, in short-circuit mode.

Direct extracellular electron transfer (DEET), one of the most recently discovered forms of microbial electrosynthesis, offers the possibility of novel bioenergy such as waste to methane and cost-effective and sustainable bioremediation approaches (Malvankar and Lovley 2012). In DEET, microorganisms form direct electrical connections with insoluble materials that can either accept or donate electrons, e.g., Fe(III) oxides, electrodes, and even other microorganisms. Microorganisms capable of DEET have also major impact on the natural cycling of carbon, metals, and nutrients. Rapid advances in molecular biology and computational analysis have made it feasible to address bioremediation with a systems biology approach using DEET process (Rabaey and Rozendal 2010). Microorganisms such as *Geobacter* spp. with the capability of DEET play an important role in subsurface environments for effective remediation of groundwater contaminated with hydrocarbon fuels or uranium and similar contaminants associated with the mining and processing of nuclear fuel. It is postulated that a similar organic metallic-like conductivity may be an important mechanism for microorganisms to exchange electrons in syntrophic associations, such as those responsible for the conversion of organic wastes to methane in anaerobic digesters, which is a proven bioenergy technology.

More fundamental research is required to elucidate the features that confer metallic-like conductivity to the pili of *G. sulfurreducens* and to determine the diversity of microorganisms in which this strategy for long-range electron transport can be found. There has been an increase in recent years in the number of reports of microorganisms that can generate electrical current in microbial fuel cells. Although many new strains have been identified, few strains individually produce power densities as high as strains from mixed communities. Enriched anodic biofilms have generated power densities as high as 6.9 W per m^2 (projected anode area), approaching theoretical limits (Malvankar and Lovley 2012).

Microbial fuel cells (MFC) do not require the use of metal catalysts at the anode; instead they use microorganisms that biologically oxidize organic matter and transfer electrons to the anode. These electrons flow through a circuit to the cathode, where they combine with protons and a chemical catholyte, such as oxygen. The reduction of oxygen is usually catalyzed by a precious metal catalyst, such as platinum, and depending on the energy gain by the bacteria and energy losses at the cathode, a voltage of 0.3–0.5 V can usually be obtained for fuels such as glucose or acetic acid. Any biodegradable organic matter can be used as a source in an MFC for power generation, including simple molecules, such as carbohydrates and proteins, as well as complex mixtures of organic matter present in human, animal, and food-processing wastewaters.

Electrical current generation has been shown for four of the five classes of Proteobacteria, as well as the Firmicutes and Acidobacteria phyla. The yeast *Pichia anomala* has redox enzymes on its outer membrane and can produce current in an MFC, and the oxygenic phototrophic cyanobacterium *Synechocystis* sp. PCC 6803 was discovered to produce electrically conductive appendages or nanowires. We are not yet at the upper limits of maximum power densities for microorganisms in MFC (Logan 2009). A single *Escherichia coli* cell that weighs 2×10^{-13} g, doubles two times per hour, and has a volume of 0.491 μm^3 could theoretically produce 16,000 kW per m^3 (based on the volume of the cell). To put this power density in perspective, a person eating 8,400 J (2,000 cal) every day is consuming the equivalent of 100 W of continuous power or 1 kW per m^3 (assuming a body volume of 0.1 m^3). In the USA, around 1.5 % of the electricity produced is used for wastewater treatment, and around 4–5 % of the electrical energy is used for the whole water infrastructure (Malvankar and Lovley 2012). It seems likely that there are many microorganisms yet to be discovered that might be beneficial for electricity production.

1.8 Petroleum Microbiology and Biotechnology

Depending on oxygen input or the presence of other electron acceptors and appropriate nutrients, petroleum biodegradation in near or subsurface environments is carried out by either aerobic or anaerobic microorganisms. Biodegradation of hydrocarbons by aerobic bacteria is supported by oxygen, whereas in the absence of oxygen, anaerobic heterotrophic microorganisms require nitrate, sulfate, iron, manganese, or carbon dioxide as electron acceptors for biodegradation of hydrocarbons. The importance of microbial activities in petroleum oilfields and reservoirs has been recognized for a long time, but our knowledge of the diversity of bacteria growing in these ecosystems and their metabolic activities in situ is still limited (Ward et al. 2012).

Biological souring is commonly the consequence of secondary oil recovery, which impacts negatively the petroleum industry in several ways including higher production costs and lower value products, enhanced corrosion and reservoir

plugging by iron sulfide precipitation, and odor issues with low air quality (Williamson 2011). Both nonbiological (thermal decomposition of sulfur-containing hydrocarbons, thermochemical sulfate reduction, and pyrite dissolution) and biological mechanisms (enzymatic reduction of sulfate, thiosulfate, or elemental sulfur to sulfide to gain energy for growth by sulfate-reducing bacteria) are responsible for souring in oil reservoirs or oilfield systems (Agrawal et al. 2010). Microbial souring is generally controlled by applying mechanical and/or chemical treatments. However, biocides are generally expensive and require repeated application to be effective and their continued use may lead to the development of biocide-resistant microbial populations. Seawater containing nitrate has been effective in preventing souring in fields with temperatures above 60 °C (Voordouw 2011). Nitrate induces growth of heterotrophic nitrate-reducing bacteria (NRB) and sulfide-oxidizing NRB, thus inhibiting sulfidogenesis to shift the sulfate-reducing bacteria (SRB) community away from sulfide production. The NRBs compete for the same organics as the SRB and production of nitrite by NRB can strongly inhibit the growth of SRB.

Although capital costs for chemical injection are relatively low, injected water volumes and chemical requirements increase over time. More work and better understanding is needed to define long-term dosage requirements of nitrate/nitrite addition to make a cost-effective treatment process. Better nitrate injection strategies, possibly in combination with selective biocides, and reservoir simulation tools need to be developed to predict the success of various treatment options and better control of microbial souring.

The progressive depletion of high-quality light crudes has led to investigations on biochemical conversion of heavy crudes into lighter crudes utilizing extremophile bacterial species. From a practical perspective denitrogenation and desulfurization, demetallation processes need to be integrated (Singh et al. 2012). An effective biodenitrogenation and biodesulfurization process requires removal of sulfur and nitrogen through specific enzymatic attack of the C–N and C–S bonds, respectively, but without C–C bond attack, thereby preserving the fuel value of the residual products. Metal-containing fossil fuels that can be treated with enzymes (cytochrome c reductase and chloroperoxidase) include crude petroleum oil, distillate fractions, coal-derived liquid shale, bitumens, gilsonite, tars, and synthetic fuels derived from them.

Microbial enhanced oil recovery (MEOR) is applied to improve the mobility of oil through decreasing oil viscosity, dissolution of carbonates in the reservoir, physically displacing oil, and plugging of highly permeable areas in the reservoir to increase the sweep efficiency of water flooding. MEOR methods are considered more economical and environmentally friendly compared to conventional EOR. Mechanisms involved in MEOR include production of bioproducts (biosurfactants and biopolymers), gases (CO_2 and CH_4), biofilm growth, and microbial plugging. MEOR methods have been actively pursued in field conditions and more than 70 % of the low temperature oilfield wells treated by bacteria achieved increases in oil production rate (Gao and Zekri 2011). However, considerable uncertainty remains regarding process performance due to reservoir heterogeneity.

In recent years, there have been significant advances in biotechnology including biomolecular, metabolic, and protein engineering developments, which will undoubtedly result in creation of powerful biocatalysts for applications in enhanced oil recovery from petroleum reservoirs, biodemulsification of oilfield emulsions and slop oils, biorefining, and upgrading of crude oil and petroleum fractions. These advances would help us address the site heterogeneity issues, which require specifically tailored strategies and custom-designed technologies. In order to utilize the real potential of environmental microbes in petroleum industries, the use of a concerted approach combining conventional microbiology and biochemistry, genomics, and nano-biotechnology, along with geotechnology design is needed.

1.9 Bioprospecting Agents in Oil and Gas Exploration

Petroleum oil and gas prospecting involves a combination of seismic surveys and exploratory drilling into subsurface sedimentary formations once preliminary studies have ascertained the presence of a sedimentary basin. Samples from wells are then analyzed to identify and quantify a host of different indicators before decisions are made about further drilling. Hydrocarbons are migrated from subsurface petroleum reservoirs to surface environments and may lead to the use of hydrocarbon seep detection as an exploration tool in the petroleum industry. Environments where hydrocarbons seep to the surface are colonized by aerobic and anaerobic hydrocarbon-utilizing microorganisms and can be used as a bioprospecting tool to indicate the seep and hence subsurface reservoirs (Hubert and Judd 2010). Microorganisms, native to petroleum reservoir habitats, may be transported upwards with seeping hydrocarbon fluids to surface environments. The development of approaches depends on a thorough understanding of the microbiology of surface hydrocarbon seeps and deep petroleum reservoirs for hydrocarbon prospecting. Strategies also require development of quantitative detection for the indicator organisms. Thus integrating microbiological data with other prospecting approaches will further strengthen oil and gas exploration strategies.

1.10 Bionanotechnology

Bionanotechnology, an emerging area involving physics, engineering, biology, and chemistry disciplines, has revolutionized different industries with a great variety of products designed for applications in electronics, transportation, food, cosmetics, pharmaceutical, biomedical, energy, and environmental (Grieger et al 2010). Nanotechnology uses nanoparticles (1–100 nm) produced in the form of metals, metal oxides, semiconductors, polymers, carbon materials, organics or biological, and morphological forms such as spheres, cylinders, disks, platelets, hollow spheres, and tubes. The use of nano-scaled zero-valent iron particles (nZVI) to remediate

contaminated soil and groundwater has received increasing amounts of attention within the last decade, primarily due to its potential for broader application, higher reactivity, and cost effectiveness compared to conventional zero-valent iron applications and other in situ methods.

Microorganisms naturally produce colloidal nanoparticles, which play an important role in the transport, fate, transformation, and bioavailability of environmentally relevant substances (Alvarez and Cervantes 2011). Nanoparticles adsorbed on the bacterial surface have also been found to achieve 56 % higher DBT desulfurization compared with the control lacking nanoparticles. There is limited number of studies done for application of bionanotechnology in petroleum industry. Most experiments using nanoparticles at lab scale have been conducted in small reactors. More data are required on life cycle analysis and effectiveness of techniques in field. Assessment of potential exposure and toxicity of nanomaterial need to be further characterized as little is known about the transport and fate and ecological and human health impacts of nanoparticles used in environmental applications.

1.11 Conclusion

Systems (micro)biology is a new way to approach research in bio-ecosystems in every field of science and engineering either above-, near-, or subsurface. By this approach it may be possible to explore the new properties of microorganisms that arise from the interaction of genes, proteins, and other macromolecules and the environment. This is possible today due to the large numbers of genomic sequences which are becoming increasingly available. However, additional genomic sequences of the different microorganisms will be required to define the molecular adaptations to their environment and the interactions between the members of the community at large. The idea is to integrate fundamental microbial and biochemical knowledge with genomics to obtain an integrated picture of how a microbial cell or a community operates and more importantly how these interactions can be converted into more efficient biotechnology for the mankind.

References

Agrawal A, Vanbroekhoven K, Lal B (2010) Diversity of culturable sulfidogenic bacteria in two oil-water separation tanks in the north-eastern oil fields of India. Anaerobe 16:12–18

Alvarez LH, Cervantes FJ (2011) (Bio)nanotechnologies to enhance environmental quality and energy production. J Chem Technol Biotechnol 86:1354–1363

Anjum F, Shahid M, Akcil A (2012) Biohydrometallurgy techniques of low grade ores: a review on black shale. Hydrometallurgy 117–118:1–12

Banfield JF, Tyson GW, Allen EE, Whitaker RJ (2005) The search for a molecular-level understanding of the processes that underpin the earth's biogeochemical cycles. Rev Mineral Geochem 59:1–7

Barnard D, Casanueva A, Tuffin M, Cowan D (2010) Extremophiles in biofuel synthesis. Environ Technol 31:871–888

Croal LR, Gralnick JA, Malasarn D, Newman DK (2004) The genetics of geochemistry. Annu Rev Genet 38:175–202

DeJong JT, Brina M, Mortensen BM, Brian C, Martinez BC, Nelson C (2010) Bio-mediated soil improvement. Ecol Eng 36:197–210

Dold B (2008) Sustainability in metal mining: from exploration, over processing to mine waste management. Rev Environ Sci Biotechnol 7:275–285

Fujinami S, Fujisawa M (2010) Industrial applications of alkaliphiles and their enzymes – past, present and future. Environ Technol 31:845–856

Gao CH, Zekri A (2011) Applications of microbial-enhanced oil recovery technology in the past decade. Energ Sour A 33:972–989

Glick BR (2010) Using soil bacteria to facilitate phytoremediation. Biotechnol Adv 28:367–374

Grieger KD, Fjordbøge A, Hartmann NB, Eriksson E, Bjerg PL, Baun A (2010) Environmental benefits and risks of zero-valent iron nanoparticles (nZVI) for in situ remediation: risk mitigation or trade-off? J Contam Hydrol 118:165–183

Hubert C, Judd A (2010) Using microorganisms as prospecting agents in oil and gas exploration. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 2714–2725

Ivanov V, Chu J (2008) Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. Rev Environ Sci Biotechnol 7:139–153

Logan BE (2009) Exoelectrogenic bacteria that power microbial fuel cells. Nat Rev Microbiol 7:375–381

Lovley DR (2006) Bug juice: harvesting electricity with microorganisms. Nat Rev 4:497–508

Madsen EL (2011) Microorganisms and their roles in fundamental biogeochemical cycles. Curr Opin Biotechnol 22:456–464

Malvankar NS, Lovley DR (2012) Microbial nanowires: a new paradigm for biological electron transfer and bioelectronics. CheSusChem 5:1039–1046

Margesin M, Feller G (2010) Biotechnological applications of psychrophiles. Environ Technol 31:835–844

Rabaey K, Rozendal RA (2010) Microbial electrosynthesis – revisiting the electrical route for microbial production. Nat Rev Microbiol 8:706–716

Rawlings DE, Johnson DB (2007) The microbiology of biomining: development and optimization of mineral-oxidizing microbial consortia. Microbiology 153:315–324

Singh A, Kuhad RC, Ward OP (2009) Biological remediation of soils – an overview of global market and available technologies. In: Singh A, Kuhad RC, Ward OP (eds) Advances in applied bioremediation, vol 17, Soil biology series. Springer, Germany, pp 1–19

Singh A, Singh B, Ward OP (2012) Potential applications of bioprocess technology in petroleum industry. Biodegradation 23:865–880

Sorokin DY, Janssen AJH, Muyzer M (2012) Biodegradation potential of halo(alkali)philic prokaryotes. Crit Rev Environ Sci Technol 42:811–856

Tango MSA, Islam MR (2002) Potential of extremophiles for biotechnological and petroleum applications. Energ Sour 24:543–559

Van Nostrand JD, Wu L, He Z, Zhou J (2010) GeoChips for analysis of microbial functional communities. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, New York, pp 4040–4050

Voordouw G (2011) Production-related petroleum microbiology: progress and prospects. Curr Opin Biotechnol 22:401–405

Ward OP, Singh A, Van Hamme JD, Voordouw G (2012) Petroleum microbiology. In: Schmidt T, Schaechter M (eds) Topics in ecological and environmental microbiology. Academic, Waltham, MA, pp 625–643

Watling HR, Watkin ELJ, Ralph DE (2010) The resilience and versatility of acidophiles that contribute to the bio-assisted extraction of metals from mineral sulphides. Environ Technol 31:915–933

Williamson N (2011) Health and safety issues from the production of hydrogen sulphide. In: Whitby C, Skovhus TL (eds) Applied microbiology and molecular biology of oilfield systems. Springer, New York, pp 151–157

Chapter 2
Novel Molecular Tools to Assess Microbial Activity in Contaminated Environments

Nadine Loick and Christopher Weisener

2.1 Introduction

In order to understand the diversity and roles of microorganisms in global biogeochemical cycling, we must first recognize their distribution and several key factors controlling the environment. Bacterial cells arose ~3.5 billion years ago, based on fossils described by Schopf (1993) identifying "Cyanobacterium-like microorganisms." It has been estimated that there are approximately 4–6 $\times$ 10^{30} different prokaryotic cells placing prokaryotes as one of the dominant life forms on the planet (Santos Pontes et al. 2007). Given their long history it is not surprising that they have developed and adapted to a variety of—by human standards—hospitable and inhospitable environments. This explains their morphological, physiological, and in part their genetic diversity (Torsvik and Øvreås 2002). In a way bacteria are the "ultimate survivalists" because of their genetic variability allowing them to adapt relatively quickly. The ability of bacteria to adapt is a result of recombination events and induced mutations, which have allowed them to flourish during extreme environmental changes. Bacteria can often acquire or exchange genes via horizontal gene transfer (HGT) allowing them to adapt readily in any environment (Cohan 2001; Zeigler 2003; Santos Pontes et al. 2007). It is not surprising that bacteria have been recognized increasingly as a major catalyst in many biogeochemical reactions in both pristine and contaminated environments and play important roles in bioremediation, energy conversion, and biocatalysis in industrial and biomedical processes (Ferris 1993; Bull et al. 2000; Warren and Haak 2001; Crowe et al. 2007; Falkowski et al. 2008; Weisener et al. 2008; Schippers et al. 2010)

The biogeochemistry associated within environmental compartments is often complex. During the last 100 years pollution from varying sources such as mining, agriculture, burning of fossil fuels, and waste disposal activities into the hydrosphere

---

N. Loick • C. Weisener (✉)
Biomaterials Laboratory, GLIER/EES Department, University of Windsor, Windsor, ON, Canada N9B 3P4
e-mail: weisener@uwindsor.ca

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_2, © Springer-Verlag Berlin Heidelberg 2014

and the terrestrial environment has culminated to such extent that many pristine environments no longer exist (Mielke et al. 2004). This has led to elevated concentrations of metals and metalloids within these locations. In general, the behavior of contaminants, particularly metals, depends on interactions between physicochemical parameters such as temperature and pH, solubility and formation of minerals, e.g., the rates and types of metabolisms and the specific metal/metalloids involved (such as Au, Cu, Ni, Pb, Zn, Se, As), and more importantly the influence of microbial activity (Schippers et al. 2010). Physicochemical conditions within the sediment compartment can often control and influence bacteria since certain variables such as pore size density, pH gradients, oxygen depletion, and nutrient matter can restrict or enhance microbial colonization. Prior to the 1990s, prokaryotic activity in natural and contaminated environments was often investigated using culture-based methodologies (Baldwin et al. 2005). In part due to their importance for public health, vast progress was made identifying specific protagonists in clinical microbiology during this period, while less so in the environmental counterparts of that discipline. The difficulties of cultivating bacteria from natural environments, such as soils and freshwater, have made the study of microbial diversity a difficult and not straightforward task (Fry 2000; Nee 2003; Kemp and Aller 2004). This is partly due to the complex interdependence of bacterial species and the complex chemistries for media enrichments.

During the 1990s, however, something a kin to a revolution occurred with the advent of molecular-based approaches being directed toward environmental habitats. Giovannoni et al. (1990) were the first to phylogenetically analyze clone libraries created from eubacterial 16S rRNA extracted from picoplankton from the Sargasso Sea. Since then the use of molecular methods has resulted in a transformation in our understanding of microbial community structure in terms of function, phylogeny, and community composition within natural environments. This has led to a better understanding of the role microorganisms play in current and past biogeochemical systems. Continued advances and improvements to these approaches have provided data on their ecological roles and the evolution of bacterial species found in environmental samples. These new methods have led to the discovery of new bacterial species and function changing our concept of microbial diversity (Dunbar et al. 1999; Fry 2000; Kaeberlein et al. 2002; Jaspers and Overmann 2004; Santos Pontes et al. 2007). Although significant progress has been made, the complexities of environmental systems often require some degree of caution when evaluating the progression of events leading up to element and contaminant cycling. This holds especially true in contaminated environments since those are often unique and cannot necessarily be compared to naturally evolved geochemical systems. In part, the paucity in studies providing a greater understanding of such systems may be due to analytical challenges inherent in investigating such environmental systems in which many components are on a nano-scale and rely on a greater understanding of the bacterial community structure and function of the community as a whole. Some molecular methods used to investigate these consist of quantitative polymerase chain reaction (q-PCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction enzyme fragment length polymorphism (T-RLFP), and variations of fluorescent in situ hybridization

(FISH) and catalyzed reporter deposition-FISH (CARD-FISH). These molecular tools provide the unique opportunity to profile existing microbial ecophysiology and quantify individual groups of bacteria (Edwards et al. 2003; Huang et al. 2007; Fike et al. 2008; Norlund et al. 2009). Methods such as T-RLFP and DGGE have proven to be valuable preliminary assessment tools for fingerprinting more abundant microbial ribotypes within a community but often neglect the rarer types that may comprise the community. In this instance detailed metagenomic sequencing of relevant microbial species and communities may be required to shed light on the metabolic pathways and activities.

Recent advancements have led to a coupling of molecular-based approaches with physical characterization methods such as high-resolution microscopy, aiding in establishing the complexities of the interactions of bacteria with their environment and giving insights into mechanisms of contaminant cycling (Fike et al. 2008; Norlund et al. 2009). These investigations have contributed greatly to our understanding of complex bacteria/mineral relationships. FISH along with its variation CARD-FISH have proven to be very useful techniques for assessing bacteria mineral selectivity. Since they are based on the utilization of specific oligoprobes binding to their complementary DNA sequence and thereby labeling those organisms with that specific gene, certain parts of a community can be identified (Edwards et al. 2003; Peplies et al. 2006; Norlund et al. 2009). In fact, the combination of such molecular and spectroscopy-based techniques holds great promise for elucidating the complexities of bacterial communities and their influence on contaminants in sediment compartments (Neu et al. 2010). These methodologies will be discussed in a subsequent section of this chapter.

All of these methods can be used to assess microbial communities in terms of function, diversity, and population. With all molecular techniques there are inherent limitations, and caution should be used when interpreting results. For instance, fingerprinting techniques may only profile more abundant ribotypes within a community, while not detecting rarer ones. Metagenomic sequencing of microbial communities in impacted environments is a new perspective of metabolic activities and pathways for such systems (Hutchens 2009). The focus of this chapter will be on reviewing recent developments and advancements made to characterize microbial community composition and activity in contaminated environments with emphasis given to advantages and limitations. Future directions for elucidating microbial function in contaminated systems will be highlighted.

2.2 Using Culture-Based Techniques to Investigate Microbial Community Function and Development

Over the past 150 years microbiologists have relied on traditional approaches such as culture-based techniques, which rely on selective media, to identify microorganisms within a specific environment. This approach has resulted in a vast array of stored

pure culture collections from diverse habitats such as soils, marine estuaries, and freshwater ecosystems. Much of these investigations have derived from clinical studies as well as from studies investigating bacteria related to biogeochemical pathways in a quest to ascertain linkages to bacterial biodiversity. The classical approach to understanding diversity has relied on numerical taxonomy to compare isolates, identify them, and generally improve the taxonomy of the groups. The problem with this approach, however, is that it has led to confusion and uncertainties regarding the classification based on physiology and morphological comparisons. The development of molecular approaches described in this chapter has helped to shed light on these difficulties. These culture-independent approaches have illustrated that in most cases bacteria that grow as enrichment cultures are not the predominant species observed in natural systems (Fry 2004). The overall issue with using laboratory prepared media is that in some cases the numerically abundant species of bacteria will grow more slowly compared to less dominant species. An excellent review has been written by Fry (2004) who reviews some of the examples of the latest culture-based techniques used to successfully isolate dominant but uncultured bacteria using enrichment and micromanipulation techniques in a range of habitats.

2.3 Molecular Biological Analysis Techniques

While culture-based approaches, such as enrichments, are extremely useful for phylogenetic analyses of communities, they are limited to a few culturable organisms making up less than 1 % of the total microorganisms present in environmental samples (Ward et al. 1990; Amann et al. 1995). Additionally, culture-dependent methods are inherently biased through the choice of medium, selecting a specific portion of the present microbial community. The vast majority of microorganisms in the environment, though not having been cultured, have been characterized using culture-independent methods. Those methods are mainly based on the analysis of nucleic acids, lipids, and/or proteins from the sample matrix.

A wide variety of molecular techniques is available to analyze and characterize microbial community structure. Roughly these methods can be divided into those looking at certain fractions of the microbial community (Partial Community Analysis) and those looking at the whole microbial community (Whole Community Analysis).

2.3.1 Fingerprinting Techniques

One group of methods falling under the category of Partial Community Analysis is fingerprinting techniques. Those methods are techniques that characterize the structure of a microbial community based on the unique sequences of extracted nucleic acids (Muyzer 1999). In general fingerprinting techniques involve the

extraction of nucleic acids from the sample matrix, followed by amplification of specific molecular markers, such as the 16S rRNA gene, in a polymerase chain reaction (PCR). Results are restricted to differences within the sequences of these molecular markers. Pitfalls of PCR-based analyses from sample collection to analysis and cross checking of results have been reviewed by von Wintzingerode et al. (1997).

Fingerprinting methods are based on sequence variations between different organisms. Those differences result in different melting behaviors as well as differences in the locations of those parts of the sequences that represent restriction enzyme recognition sites. For different species, those sites are at different positions along the DNA strand. Two of the most commonly used techniques to analyze environmental samples of unknown microbial community composition are denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) and restriction fragment length polymorphism (RFLP), which both make use of sequence variations of PCR products amplified from environmental DNA.

Denaturing/temperature gradient gel electrophoresis (DGGE and TGGE) were introduced by Muyzer et al. (1993). Both techniques are based on electrophoresis of PCR amplified DNA fragments in a polyacrylamide gel, over either a gradient of increasing denaturants or temperature. Both methods rely on differences in the sequence-dependent melting behavior of double-stranded DNA. For this the extracted nucleic acids have to be amplified using primers that target specific molecular markers such as the 16S rRNA gene. For DGGE/TGGE the use of a 5′-GC clamped (30–50 nucleotides) forward primer is essential to avoid complete dissociation of the double strands. After loading the PCR product onto the gel, an electric current is applied, pulling the DNA fragments through the gel. Depending on the sequence of the amplicon, a higher or lower concentration of denaturant/temperature is needed for a complete dissociation of the double strands into single strands, causing the amplicons to stop moving through the gel at different positions. To determine the identities of bands separated on the gel, those bands can be excised from the gel and further analyzed via re-amplification, cloning and sequencing, or hybridization with molecular probes specific for particular taxonomic groups.

While these methods are valuable for the identification of changes in dominant species within a community, fingerprints resulting from DGGE/TGGE can be very complex, especially when using universal bacterial primers. This can lead to difficulties in separating bands of fragments with similar melting points. Additional care has to be taken, as rRNA operons of the same bacterium can show heterogeneity, leading to multiple bands and an overestimation of the microbial diversity. Also quantification of the extracted bands is not possible.

While DGGE and TGGE make use of the resulting different melting behaviors, various restriction fragment length polymorphism (RFLP) techniques make use of restriction enzyme recognition sites being present at different positions along the DNA strand. In amplified ribosomal DNA restriction analysis (ARDRA), the PCR product resulting from amplification of the extracted nucleic acids is digested with specific restriction enzymes. Depending on the location of the restriction enzyme

recognition sites, the DNA is cut into pieces of varying lengths. Subsequently the restricted fragments are separated via gel electrophoresis. This method has been used, e.g., by Smit et al. (1997) analyzing the effects of copper contamination on the soil microbial community. A challenge in the estimation of microbial diversity using this method is that the number of bands in the profile is larger than the number of different organisms in the sample, making the interpretation of the results difficult. This problem was resolved by Avaniss-Aghajani et al. (1994) who developed terminal restriction fragment length polymorphism (T-RFLP) analysis. In T-RFLP analysis, extracted nucleic acids are amplified using a 5′ fluorescently labeled primer. As with ARDRA, the resulting amplicons are digested with restriction enzymes. After digestion, the fragments are analyzed on an automatic DNA-sequencer, where only the terminal, fluorescently labeled fragments are detected.

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

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

highlighted the difficulties of implementing remediation strategies when dealing with mixed contaminations, as well as the importance of combining different analysis methods.

Other studies used those fingerprinting techniques when monitoring the effects of different remediation techniques such as amending mine tailings with a mixture of organic carbon sources to treat pore water and drainage (Lindsay et al. 2011), incorporating compost into a heavy metal-contaminated acidic soil (Farrell et al. 2010), or testing the effects of phytoremediation approaches (Martinez-Inigo et al. 2009; Tipayno et al. 2012) or landfarming on oil refinery sludge (Ros et al. 2010).

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

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

2.3.2 Quantitative-PCR

In studies where changes in the amounts of specific groups or species of organisms or the expression of certain genes are of interest, quantitative or real-time PCR (qPCR) is a method of choice. Wherever a gene sequence can be utilized that uniquely identifies the organisms of interest, qPCR is a commonly used method to quantify these specific DNA sequences.

Quantitative PCR is an advancement of PCR in which the amount of the amplified targeted DNA is measured during each cycle of the PCR reaction using an intercalating dye such as SYBR® Green or fluorescently labeled probes (TaqMan®). The increase in fluorescence is measured during the reaction and software calculates the concentration from the intensity during the early exponential phase of the reaction, when concentrations are proportional to the starting template concentration. Quantitative PCR is especially useful observing gene expression, if the sequence of the functional gene of interest is known. For instance, in a study by Mouser et al. (2009), the authors used qPCR to follow the expression of the nitrogen fixation gene, *nifD*, and the ammonium transporter gene, *amtB*, in *Geobacter* species while evaluating in situ bioremediation of uranium-contaminated groundwater. In another study, Yergeau

et al. (2012) used reverse transcription qPCR assays to confirm the active expression of hydrocarbon degradation genes by *Pseudomonas* and *Rhodococcus* species in Arctic biopile soils.

2.4 The Use of Gene Arrays for Community Structure Determinations

In environmental sciences, microarrays have mainly been used to provide a high-throughput and comprehensive view of the microbial community (Gentry et al. 2006). Similar to hybridization techniques, microarrays exploit complementary base pairing. Microarrays consist of multiple spots containing thousands to hundreds of thousands of probes of sequences of interest which will be used to bind complementary nucleic acids extracted from the sample. Based on the information they provide, microarrays can be divided into two major groups: those that investigate (1) phylogeny and those investigating (2) function (Rastogi and Sani 2011). Based on the target genes, Gentry et al. (2006) divided those arrays further into five major groups (a–e).

(1) Phylogenetic information can be gained from phylogenetic arrays (PGA) and community genome arrays (CGA). (a) PGAs are used to compare communities in different environments or follow changes in their composition over time and are based on conserved marker genes such as the 16S rRNA gene. (b) Community genome arrays on the other hand are made up of the whole genomic DNA extracted from cultured organisms and can be used to describe a community based on its relationship to the cultured organisms. (2) Functional information can be gained from functional gene arrays (FGA) and metagenomic arrays (MGA). (c) FGAs are used to gain information about the presence of known genes encoding proteins catalyzing biogeochemical processes of interest, such as sulfur, carbon, or nitrogen cycles, and can provide information about the microbial populations involved. (d) The probes used on metagenomic arrays on the other hand are made from environmental DNA and can be used without any prior knowledge about the sequences of the community. (e) A fifth type of array, the whole-genome open-reading frame array (WGA), can provide information on both phylogeny as well as function. Those arrays hold probes for all open-reading frames—the part of a gene that encodes a protein—and can be used to compare genomes as well as to analyze the interactions of different organisms at the transcriptional level. Specific applications of these five major types of arrays are discussed in a review by Gentry et al. (2006).

Microarrays have originally been developed for gene expression analysis of individual organisms or pure cultures and their use with environmental samples bears special challenges (Zhou and Thompson 2002; Gentry et al. 2006). Some difficulties are associated with the sample matrix, such as the presence of substances like humic acids, metals, and organic contaminants which can interfere with the hybridization step, or the fact that some environments contain only low levels of

biomass creating the need to amplify nucleic acids, which as mentioned before may introduce bias. The possibly biggest challenge is the vast number of unknown organisms in the sample and therefore unknown DNA sequences. Organisms without a corresponding probe on the microarray will be overlooked, even if they may be important and dominant in the system. Additionally, probes can cross-hybridize to similar unknown sequences resulting in a false signal due to binding of a different gene or to an underestimation of a signal due to a weaker binding of a slightly divergent sequence preventing a binding of the target sequence (Gentry et al. 2006). Nonetheless, microarrays have been successfully applied in microbial ecological studies.

Neufeld et al. (2006) designed a habitat-specific array to investigate the microbial community structures of hexachlorocyclohexane (HCH) contaminated and uncontaminated soils. The authors found strong correlations between HCH contamination and probe signals for organisms belonging to the genus *Sphingomonas* and to different organisms with acid-tolerant phenotypes. Using a functional gene array (GeoChip), Liang et al. (2009) examined the diversity of microbial communities along a contamination gradient along an oil field in China. With increasing contamination level, the authors found a decrease in the diversity and number of functional genes, as well as in archaea. A study by Xiong et al. (2010) investigated the effects of arsenic contamination on microbial communities. Comparing soil samples with different arsenic contamination levels from the rhizosphere of the arsenic accumulating plant *Pteris vittata* and non-rhizosphere areas the authors not only found that, in comparison to arsenic contaminated soil, the uncontaminated soil showed a higher heterogeneity and more unique genes, but they also detected distinct differences in arsenic resistance, sulfur reduction, phosphorus utilization, and denitrification genes between rhizosphere and non-rhizosphere samples. Their results suggest that bacteria associated with the rhizosphere of *P. vittata* play an important role in the plants soil arsenic uptake and accumulation.

2.5 Microbial Diversity Investigations Via Sequencing

Throughout the last five years the use of high-throughput sequencing (HTS) techniques has revolutionized the way microbial communities are analyzed (Logares et al. 2012). Instead of sequencing individual DNA clones, these methods sequence hundreds of thousands to tens of millions of DNA molecules in parallel. Since December 2008 three HTS platforms are commercially available, 454 (Roche/454 Life Sciences), Solexa (Illumina), and SOLiD (ABI), which can generate gigabases of sequences in a single experiment (Jones 2010). Compared to traditional sequencing, where obtained sequences are typically over 800 nucleotides in length, sequences obtained with these next-generation techniques are much shorter with 25–50 nucleotides (Illumina and ABI) and 200–400 nucleotides (454) in length (Graveley 2008). Those short sequences, however, make it difficult to match the sequences to the reference genome and gain sufficient information to relate DNA segments to species or functional groups. A comparison of the three different

platforms has been provided by Mardis (2008). Due to the larger fragment length obtained, the Roche/454 Life Science Sequencer is the most widely used HTS platform and its HTS process, including considerations and drawbacks such as the large amount of DNA needed as well as the required computing power for data analysis, has been reviewed by Jones (2010). New technologies are being developed at an incredible speed and existing techniques have constantly been improved in throughput as well as read length. Since 2010 further next-generation sequencing methods are available, such as the Ion Torrent (Life Technologies) and the SMRT (Pacific Biosciences). A more recent review of next-generation sequencing technologies with an emphasis on their application in environmental DNA analysis has been published by Shokralla et al. (2012). Figure 2.1, originally published in that review paper, displays the development and advances of next-generation sequencing methods.

In 2006, a study by Edwards et al. was the first one describing an application of HTS to environmental samples. The authors used 454-sequencing to compare microbial communities of two sites in an iron mine. Sequence analysis revealed distinct differences between the communities and their metabolic pathways. While the authors could explain much of the correlation between occurring microbial metabolism and the geochemical conditions, they highlighted the fact that many pathways in the environment are still unexplained. Bowman et al. (2012) advanced our knowledge of the effects and possible implications of climate change, using 454-sequencing to analyze microbial communities in the Arctic ice identifying microbial diversity to improve our understanding of the consequences of the potential disappearance of this environment.

In contaminated environments, metagenomic analyses are especially useful when studying the underlying pathways and investigating metabolic activities and bioremediation potential. Bioremediation is a potentially effective and inexpensive way to restore contaminated environments; however, its implementation is often limited by a lack of information on the microbial communities present and the factors influencing their growth and metabolism (Lovley 2003). Several studies have used cloning-based sequencing methods looking at genes, functions, and pathways to look at bioremediation of environments affected by different contaminants such as jet fuel (Brennerova et al. 2009), hydrocarbons and chlorinated solvents (Dojka et al. 1998), oil (Röling et al. 2002), acid mine drainage (Martins et al. 2009), or uranium (Seifert et al. 2008). Generally those studies have used sequencing in combination with other molecular techniques such as fingerprinting. While they give valuable information on specific genes and function, they were inherently biased and restricted to the genes used for analysis. HTS, however, is a method of particular interest as it can possibly lead to the discovery of novel organisms or genes that might be missed by traditional methods. Yergeau et al. (2012) were the first ones to use metagenomic sequencing analysis in a soil bioremediation experiment. The authors reported the presence of *Caulobacter* who "could be involved in alkane degradation in Arctic soils, a role that has not been previously reported." Additionally they found sequences related to uncultured or not classified microorganisms and genes, which potentially represent novel hydrocarbon degradation genes.

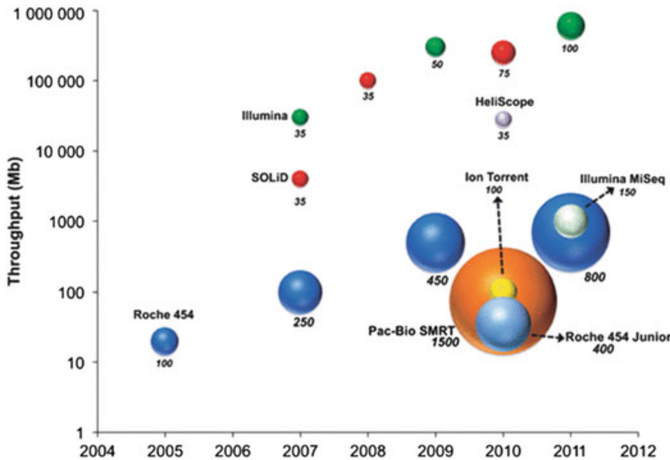


Fig. 2.1 Historical development of next-generation sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform [in base pairs (bp)]. *Colors* correspond to individual platforms (Shokralla et al. 2012)

2.6 Coupling Molecular Based with Microscopic and Isotopic Approaches

Advances made in the development of new molecular tools have contributed greatly to the advancement of our understanding of complex bacteria/mineral relationships in some environmental systems. As mentioned earlier, fluorescent in situ hybridization (FISH), similar to other hybridization methods, uses specific fluorescently labeled oligoprobes binding to their complementary DNA sequence. FISH along with its different variations, such as catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH) or MAR-FISH, the combination of FISH with microautoradiography (MAR), have proven invaluable for the detection and enumeration of specific bacterial taxa and the investigation of microbial activities in complex communities, as well as for imaging their spatial association with mineral phases (Edwards et al. 2003; Okabe et al. 2004; Peplies et al. 2006; Norlund et al. 2009; Tischer et al. 2012).

Like other molecular biological methods, the standard FISH technique has its limitations. One of those is that, when working with intact cells, not all bacterial and archaeal cells can undergo permeabilization by the oligonucleotide probes. Additionally, using rRNA probes hampers the identification of cells with low ribosome contents and thereby restricts the sensitivity of the method (Wagner et al. 2003). Many limitations have been overcome by advances made over the last 10 years improving the method itself as well as combining it with other methods. Some of those have been reviewed by Wagner et al. (2003) along with new trends in the use of FISH techniques to identify and analyze functional groups of microorganisms (Wagner and Haider 2012).

CARD-FISH has proven valuable for assessing complexities of microbial communities associated with hydrocarbon contaminated aquifer environments (Tischer et al. 2012). To investigate both bacteria and archaea simultaneously, the authors used a dual hybridization approach followed by two consecutive stainings. Additionally, the authors were able to improve the CARD-FISH detection for their materials by applying microwave irradiation during the permeabilization and hybridization steps. These improvements to the standard approach will allow more detailed investigations at higher spatial resolution to elucidate the ecology of possible hydrocarbon contaminant degraders (Tischer et al. 2012).

MAR-FISH can be used to obtain detailed information pertaining to the activity of defined groups of prokaryotic cells within natural environments. In this approach radioactively labeled substrates are used to determine the physiological activity of bacteria within a given environment system. The technique not only determines general physiological conditions but can also be used to investigate the ecophysiology of organisms defined by oligonucleotide probes (Ito et al. 2002; Wagner and Loy 2002). Nielsen et al. (2002) successfully quantified acetate consuming iron-reducing bacteria in activated sludge by incubating the biomass with radio-labeled acetate while inhibiting sulfate reducing and methanogenic prokaryotes. The authors were able to show that besides being useful for enumeration, MAR-FISH is also suitable to classify functional groups of bacteria phylogenetically.

Microscopy and imaging techniques are invaluable and irreplaceable methods in the analysis of complex environmental systems. Neu et al. (2010) reviewed some applications of laser scanning microscopy (LSM), magnetic resonance imaging (MRI), and scanning transmission X-ray microscopy (STXM). By combining different techniques, such as STXM using synchrotron radiation, laser scanning microscopy (LSM), and FISH-based oligonucleotide probes (e.g., immunogold), it is possible to examine phylogenetic identity and metabolic activity from individual cells thus differentiating processes at the species level similar to MAR-FISH. These microscopic techniques present a powerful way to visualize and examine mixed environmental microbial communities, especially as microbial communities often appear in films or as aggregates (Neu et al. 2010).

Other combinations of methods that have been used to differentiate and identify community structure and metabolic activity in natural sediments include the combination of CARD-FISH with Raman microspectroscopy (Huang et al. 2007; Wagner 2009) stable isotope labeling, and high resolution secondary ion mass spectrometry (SIMS) (Fike et al. 2008; Orphan and House 2009).

The combination of stable isotope characterization methods with genomic analysis provides direct insight into the expression of key functional genes and community structure. Stable isotope methods are extremely powerful since they allow the determination of chemical bonding environments associated with the microbial cells. For example, the incorporation of ^{13}C isotopic tracers into the cell membrane causes significant changes in the observed resonance spectra compared to its parent ^{12}C spectra (Huang et al. 2004). The observed changes result from modifications to the bond vibrational state brought on by the heavier isotope. The observed shift in vibrational spectra is termed a “red shift” due to the production of

longer wavelengths within the Raman Spectra associated with ^{13}C-labeled cells (Huang et al. 2004). While this approach maintains the resolution of the MAR-FISH technique, it does provide a link between highly resolved single cell studies and population-based sequence strategies, which rely on stable isotopes. Studies investigating naphthalene-degrading consortia in contaminated aquifers have proven the validity of the method (Huang et al. 2007). Here the authors incubated labeled ^{13}C contaminated ground water with a single-specific FISH probe to observe the in situ activity of pseudomonads as naphthalene degraders. By using this approach, the authors noted that the bacteria's direct metabolism could be quantified using the labeled compound. Interestingly, the authors noted differences in ^{13}C concentrations for the different ^{13}C-labeled pseudomonad cells they attributed those to differences in the activities of individuals, within different strains or species targeted by the oligonucleotide probe.

The application of nano-SIMS imaging, a higher resolution version of the SIMS technique, by itself or in combination with specific isotope labeling experiments has proven very successful for assessing ecophysiology of microbes in various environments (Fayek et al. 2005; Wagner 2009) and could be effective for elucidating bacterial effects on metals in mine waste spoils. For example, the analyses of stable isotopes of oxygen, iron, copper, and sulfur as well as low weight carboxylic acids using SIMS can yield valuable information on biogeochemical element cycling in mine dumps and has the potential of becoming a tool for the prediction and control of metals released in acid mine drainage (AMD) generation. What is still required is further development of other molecular-based approaches for assessing mine waste spoils.

2.7 Conclusions

Like culture-based methods, molecular techniques have their advantages and disadvantages and can be biased. Molecular analyses methods generally depend on the extraction of DNA or RNA from the sample matrix. Preferential or incomplete lysis of microbial cells can produce a distorted view of the community structure. Additionally, DNA can persist in the environment for prolonged periods, making the differentiation between dead, alive, and active cells difficult if not impossible (Romanowski et al. 1992; Nielsen et al. 2007). To overcome the problem of detecting DNA from dead as well as live cells, recently selective intercalating dyes ethidium monoazide (EMA) and propidium monoazide (PMA) have been used to exclude DNA from dead cells (Bae and Wuertz 2009; Nocker and Camper 2009; Taskin et al. 2011). DNA analyses are useful for studying the functional potential of the microbial community; it, however, does not provide any information on the activity of the microbial community or parts of it.

Information of bacterial activity can be gained from rRNA or mRNA extracted from environmental samples. The number of ribosomes in a cell (rRNA) is known to correlate with growth rate, with some exceptions having been found for slow growing bacteria (Quiros et al. 1989), while the detection of mRNA is a definitive

indicator of activity (Wellington et al. 2003). However, due to the possibility of the modification of proteins following mRNA transcription, its detection might not necessarily equate with the phenotypic expression of the targeted gene (Wellington et al. 2003). Those uncertainties, as well as possible biases during DNA/RNA amplification need to be taken into consideration, especially when analyzing environmental samples. T-RLFP and real-time PCR have proven to be valuable preliminary assessment tools for fingerprinting important functional members of the bacterial community. To shed light on the metabolic pathways and activities contributing to, e.g., metal mobility and biomineral-induced metal mobilization, further analyses are required. Sequencing methods have enabled researchers to detect slight changes in the composition of microbial communities in samples that may occur following alterations of the environment which can happen naturally or as a consequence of anthropogenic activities (Leininger et al. 2006; Fierer et al. 2007; Shokralla et al. 2012). However, even those methods are not foolproof and especially in natural systems it is not always clear if a change in community composition is directly related to the change of a specific environmental factor.

Overall, limitations of methods must be considered and a combination of methods is recommended to minimize misinterpretation as well as oversight of less abundant, but important members of the microbial community. Studies using a combination of methods, such as the study by Kao et al. (2010) who used DGGE and qPCR plus culturing (enrichments) in a bioremediation study at a petroleum–hydrocarbon-contaminated site, give a more comprehensive view of environmental occurrences such as success or failure of remediation strategies.

References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Mol Biol Rev 59:143–169

Avaniss-Aghajani E, Jones K, Chapman D, Brunk C (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. Biotechniques 17:144–146

Bae S, Wuertz S (2009) Discrimination of viable and dead fecal bacteroidales bacteria by quantitative PCR with propidium monoazide. Appl Environ Microbiol 75:2940–2944

Baldwin AJ, Moss JA, Pakulski JD, Catala P, Joux F, Jeffrey WH (2005) Microbial diversity in a Pacific Ocean transect from the Arctic to Antarctic circles. Aquat Microb Ecol 41:91–102

Bowman JS, Rasmussen S, Blom N, Deming JW, Rysgaard S, Sicheritz-Ponten T (2012) Microbial community structure of Arctic multiyear sea ice and surface seawater by 454 sequencing of the 16S RNA gene. ISME J 6:11–20

Brandt KK, Frandsen RJN, Holm PE, Nybroe O (2010) Development of pollution-induced community tolerance is linked to structural and functional resilience of a soil bacterial community following a five-year field exposure to copper. Soil Biol Biochem 42:748–757

Brennerova MV, Josefiova J, Brenner V, Pieper DH, Junca H (2009) Metagenomics reveals diversity and abundance of meta-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air-sparging bioremediation. Environ Microbiol 11:2216–2227

Bull AT, Ward AC, Goodfellow M (2000) Search and discovery strategies for biotechnology: paradigm shift. Microbiol Mol Biol Rev 64:573–606

Cohan FM (2001) Bacterial species and speciation. Syst Biol 50:513–524

Cordova-Kreylos AL, Cao Y, Green PG, Hwang H-M, Kuivila KM, LaMontagne MG, Van De Werfhorst LC, Holden PA, Scow KM (2006) Diversity, composition, and geographical distribution of microbial communities in California salt marsh sediments. Appl Environ Microbiol 72:3357–3366

Corsini A, Cavalca L, Zaccheo P, Crippa L, Andreoni V (2011) Influence of microorganisms on arsenic mobilization and speciation in a submerged contaminated soil: effects of citrate. Appl Soil Ecol 49:99–106

Crowe SA, O'Neill AH, Kulczycki E, Weisener CG, Roberts JA, Fowle DA (2007) Reductive dissolution of trace metals from sediments. Geomicrobiol J 24:157–165

Dojka MA, Hugenholtz P, Haackand SK, Pace NR (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl Environ Microbiol 64:3869–3877

Dunbar J, Takala S, Barns SM, Davis JA, Kuske CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 65:1662–1669

Edwards KJ, McCollom TM, Konishi H, Buseck PR (2003) Seafloor bioalteration of sulfide minerals: results from in situ incubation studies. Geochim Cosmochim Acta 67:2843–2856

Edwards RA, Rodriguez-Brito B, Wegley L, Haynes M, Breitbart M, Peterson DM, Saar MO, Alexander S, Alexander ECJ, Rohwer F (2006) Using pyrosequencing to shed light on deep mine microbial ecology. BMC Genomics 7:57

Falkowski PG, Fenchel T, Delong EF (2008) The microbial engines that drive earth's biogeochemical cycles. Science 320:1034–1039

Farrell M, Griffith GW, Hobbs PJ, Perkins WT, Jones DL (2010) Microbial diversity and activity are increased by compost amendment of metal-contaminated soil. FEMS Microbiol Ecol 71: 94–105

Fayek M, Utsunomiya S, Pfiffner SM, White DC, Riciputi LR, Ewing RC, Anovitz LM, Stadermann FJ (2005) The application of HR-TEM techniques and nano SIMS to chemically and isotopically characterize *Geobacter sulfurreducens* surfaces. Can Mineral 43:1631–1641

Ferris FG (1993) Microbial biomineralization in natural environments. Earth Sci 47:233–250

Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, Robeson M, Edwards RA, Felts B, Rayhawk S, Knight R, Rohwer F, Jackson RB (2007) Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Appl Environ Microbiol 73:7059–7066

Fike DA, Gammon CL, Ziebis W, Orphan VJ (2008) Micron-scale mapping of sulphur cycling across the oxycline of a cyanobacterial mat: a paired nanoSIMS and CARD-FISH approach. ISME J 2:749–759

Fry J (2000) Bacterial diversity and unculturables. Microbiol Today 27:186–188

Fry JC (2004) Culture-dependent microbiology. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM Press, Washington, DC

Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J (2006) Microarray applications in microbial ecology research. Microb Ecol 52:159–175

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63

Gough HL, Stahl DA (2011) Microbial community structures in anoxic freshwater lake sediment along a metal contamination gradient. ISME J 5:543–558

Graveley BR (2008) Power sequencing. Nature 453:1197–1198

Huang WE, Griffiths RI, Thompson IP, Bailey MJ, Whiteley AS (2004) Raman microscopic analysis of single microbial cells. Anal Chem 76:4452–4458

Huang WE, Stoecker K, Griffiths R, Newbold L, Daims H, Whiteley AS, Wagner M (2007) Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. Environ Microbiol 9:1878–1889

Huang L-N, Zhou W-H, Hallberg KB, Wan C-Y, Li J, Shu W-S (2011) Spatial and temporal analysis of the microbial community in the tailings of a Pb-Zn mine generating acidic drainage. Appl Environ Microbiol 77:5540–5544

Hutchens E (2009) Microbial selectivity on mineral surfaces: possible implications for weathering processes. Fungal Biol Rev 23:115–121

Ito T, Nielsen JL, Okabe S, Watanabe Y, Nielsen PH (2002) Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Appl Environ Microbiol 68:356–364

Jakobs-Schönwandt D, Mathies H, Abraham W-R, Pritzkow W, Stephan I, Noll M (2010) Biodegradation of a biocide (Cu-N-cyclohexyldiazenium dioxide) component of a wood preservative by a defined soil bacterial community. Appl Environ Microbiol 78:8076–8083

Jaspers E, Overmann J (2004) Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. Appl Environ Microbiol 70(8):4831–4839

Jones WJ (2010) High-throughput sequencing and metagenomics. Estuar Coast 33:944–952

Kaeberlein T, Lewis K, Epstein SS (2002) Isolating “uncultivatable” microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129

Kao C-M, Chen CS, Tsa F-Y, Yang K-H, Chien C-C, Liang S-H, Yang C-A, Chen SC (2010) Application of real-time PCR, DGGE fingerprinting, and culture-based method to evaluate the effectiveness of intrinsic bioremediation on the control of petroleum-hydrocarbon plume. J Hazard Mater 178:409–416

Kemp PF, Aller JY (2004) Bacterial Diversity in aquatic and other environments: what 16S rDNA libraries can tell us. FEMS Microbiol Ecol 47:161–177

Kent AD, Smith DJ, Benson BJ, Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl Environ Microbiol 69:6768–6776

Kim J, Koo S-Y, Kim J-Y, Lee E-H, Lee S-D, Ko K-S, Ko D-C, Cho K-S (2009) Influence of acid mine drainage on microbial communities in stream and groundwater samples at Guryong Mine, South Korea. Environ Geol 58:1567–1574

Kimura S, Bryan CG, Hallberg KB, Johnson DB (2011) Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy. Environ Microbiol 13:2092–2104

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442: 806–809

Liang Y, Li G, Van Nostrand JD, He Z, Wu L, Deng Y, Zhang X, Zhou J (2009) Microarray-basedanalysis of microbial functional diversityalong an oil contamination gradient in oil field. FEMS Microbiol Ecol 70:324–333

Lindsay MBJ, Wakeman KD, Rowe OF, Grail BM, Ptacek CJ, Blowes DW, Johnson DB (2011) Microbiology and geochemistry of mine tailings amended with organic carbon for passive treatment of pore water. Geomicrobiol J 28:229–241

Logares R, Haverkamp THA, Kumar S, Lanzén A, Nederbragt AJ, Quince C, Kauserud H (2012) Environmental microbiology through the lens of high-throughput DNA sequencing: synopsis of current platforms and bioinformatics approaches. J Microbiol Methods 91:106–113

Lovley DR (2003) Cleaning up with genomics: applying molecular biology to bioremediation. Nat Rev 1:35–44

Lu J, Chen T, Wu J, Chris Wilson P, Hao X, Qian J (2011) Acid tolerance of an acid mine drainage bioremediation system based on biological sulfate reduction. Bioresour Technol 102: 10401–10406

Mardis ER (2008) Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet 9: 387–402

Martinez-Inigo MJ, Perez-Sanz A, Ortiz I, Alonso J, Alarcon R, Garcia P, Lobo MC (2009) Bulk soil and rhizosphere bacterial community PCR-DGGE profiles and beta-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75:1376–1381

Martins M, Faleiro ML, Barros RJ, Veríssimo AR, Barreiros MA, Costa MC (2009) Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination. J Hazard Mater 166:706–713

Mielke HW, Gonzales CR, Powell ET, Le B, Quach VN (2004) PAHs and metals in soils of inner city and suburban New Orleans, Louisiana, USA. Environ Toxicol Pharmacol 18:243–247

Mouser PJ, N'Guessan AL, Elifantz H, Holmes DE, Williams KH, Wilkins MJ, Long PE, Lovley DR (2009) Influence of heterogeneous ammonium availability on bacterial community structure and the expression of nitrogen fixation and ammonium transporter genes during in situ bioremediation of uranium-contaminated groundwater. Environ Sci Technol 43:4386–4392

Muyzer G (1999) Genetic fingerprinting of microbial communities – present status and future perspectives. In: Proceedings, 8th international symposium on microbial ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Ňancucheo I, Johnson DB (2011) Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria. Microb Biotechnol 5:34–44

Nee S (2003) Unveiling prokaryotic diversity. Trends Ecol Evol 18:62–63

Neu TR, Manz B, Volke F, Dynes JJ, Hitchcock AP, Lawrence JR (2010) Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. FEMS Microbiol Ecol 72:1–21

Neufeld JD, Mohn WW, de Lorenzo V (2006) Composition of microbial communities in hexachlorocyclohexane (HCH) contaminated soils from Spain revealed with a habitat-specific microarray. Environ Microbiol 8:126–140

Nielsen JL, Juretschko S, Wagner M, Nielsen PH (2002) Abundance and phylogenetic affiliation of iron reducers in activated sludge as assessed by fluorescence in situ hybridization and microautoradiography. Appl Environ Microbiol 68:4629–4636

Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D (2007) Release and persistence of extracellular DNA in the environment. Environ Biosafety Res 6:37–53

Nocker A, Camper AK (2009) Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol Lett 291:137–142

Norlund KI, Southam G, Tyliszczak T, Hu Y, Karunakaran C, Obst M, Hitchcock AP, Warren LA (2009) Microbial architecture of environmental sulfur processes: a novel syntrophic sulfur-metabolizing consortia. Environ Sci Technol 43:8781–8786

Okabe S, Kindaichi T, Ito T (2004) MAR-FISH – an ecophysiological approach to link phylogenetic affiliation and *In situ* metabolic activity of microorganisms at a single-cell resolution. Microbes Environ 19:83–98

Orphan VJ, House CH (2009) Geobiological investigations using secondary ion mass spectrometry: microanalysis of extant and paleo microbial processes. Geobiology 7:360–372

Peplies J, Lachmund C, Glöckner FO, Manz W (2006) A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl Environ Microbiol 72:4829–4838

Quiros LM, Parra F, Hardisson C (1989) Structural functional analysis of ribosomal subunits from vegetative Mycelium and spores of *Streptomyces antibioticus*. J Gen Microbiol 135:1661–1670

Rastogi G, Sani RK (2011) Molecular techniques to assess microbial community structure, function, and dynamics in the environment. In: Ahmad I, Ahmad F, Pitchel J (eds) Microbes and microbial technology: agricultural and environmental applications. Springer Science + Business Media, New York, p 516

Röling WFM, Milner MG, Jones DM, Lee K, Daniel F, Swannell RJP, Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl Environ Microbiol 68:5537–5548

Romanowski G, Lorenz MG, Sayler GS, Wackernagel W (1992) Persistence of free plasmid DNA in soil monitored by various methods, including a transformation assay. Appl Environ Microbiol 58:3012–3019

Ros M, Rodriguez I, Garcia C, Hernandez T (2010) Microbial communities involved in the bioremediation of an aged recalcitrant hydrocarbon polluted soil by using organic amendments. Bioresour Technol 101:6916–6923

Santos Pontes D, Lima-Bittencourt CI, Chartone-Souza E, Amaral Nascimento AM (2007) Molecular approaches: advantages and artifacts in assessing bacterial diversity. J Ind Microbiol Biotechnol 34:463–473

Schippers A, Breuker A, Blazejak A, Bosecker K, Kock D, Wright TL (2010) The biogeochemistry and microbiology of sulfidic mine waste and bioleaching dumps and heaps, and novel Fe(II)-oxidizing bacteria. Hydrometallurgy 104:342–350

Schopf JM (1993) Microfossils of the early archaean apex chart: new evidence of the antiquity of life. Science 260:640–646

Seifert J, Erler B, Seibt K, Rohrbach N, Arnold J, Schlömann M, Kassahun A, Jenk U (2008) Characterization of the microbial diversity in the abandoned uranium mine Königstein. In: Merkel BJ, Hasche-Berger A (eds) Uranium, mining and hydrogeology. Springer, Berlin, pp 733–742

Shokralla S, Spall JL, Gibson JF, Hajibabaei M (2012) Next-generation sequencing technologies for environmental DNA research. Mol Ecol 21:1794–1805

Smit E, Leeflang P, Wernars K (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol Ecol 23:249–261

Tan G-L, Shu W-S, Zhou W-H, Li X-L, Lan C-Y, Huang L-N (2009) Seasonal and spatial variations in microbial community structure and diversity in the acid stream draining across an ongoing surface mining site. FEMS Microbiol Ecol 70:277–285

Taskin B, Gozen AG, Duran M (2011) Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification. Appl Environ Microbiol 77:4329–4335

Thavamani P, Malik S, Beer M, Megharaj M, Naidu R (2012) Microbial activity and diversity in long-term mixed contaminated soils with respect to polyaromatic hydrocarbons and heavy metals. J Environ Manag 99:10–17

Tipayno S, Kim C-G, Sa T (2012) T-RFLP analysis of structural changes in soil bacterial communities in response to metal and metalloid contamination and initial phytoremediation. Appl Soil Ecol 61:137–146

Tischer K, Zeder M, Klug R, Pernthaler J, Schattenhofer M, Harms H, Wendeberg A (2012) Fluorescence in situ hybridization (CARD-FISH) of microorganisms in hydrocarbon contaminated aquifer sediment samples. Syst Appl Microbiol 35(8):526–532. doi:10.1016/j.syapm.2012.01.004

Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245

von Wintzingerode F, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229

Wagner M (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. Annu Rev Microbiol 63:411–429

Wagner M, Haider S (2012) New trends in fluorescence in situ hybridization for identification and functional analyses of microbes. Curr Opin Biotechnol 23:96–102

Wagner M, Loy A (2002) Bacterial community composition and function in sewage treatment systems. Curr Opin Biotechnol 13:218–227

Wagner M, Horn M, Daims H (2003) Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. Curr Opin Microbiol 6:302–309

Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345:63–65

Warren LA, Haak EA (2001) Biogeochemicalcontrols on metalbehaviour in freshwater environments. Earth Sci Rev 54:261–320

Weisener CG, Babechuk MG, Fryer BJ, Maunder C (2008) Microbial dissolution of silver jarosite: examining its trace metal behaviour in reduced environments. Geomicrobiol J 25:415–424

Wellington EMH, Berry A, Krsek M (2003) Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. Curr Opin Microbiol 6:295–301

Xiong J, Wu L, Tu S, Van Nostrand JD, He Z, Zhou J, Wang G (2010) Microbial communities and functional genes associated with soil arsenic contamination and the rhizosphere of the arsenic-hyperaccumulating plant *pteris vittata* L. Appl Environ Microbiol 76:7277–7284

Yergeau E, Sanschagrin S, Beaumier D, Greer CW (2012) Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. PLoS One 7(1):e30058

Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53:1893–1900

Zhou J, Thompson DK (2002) Challenges in applying microarrays to environmental studies. Curr Opin Biotechnol 13:204–207

Chapter 3
Advanced Molecular and Microspectroscopy Toolbox for Deciphering Soil Diazotroph Diversity

Anu Kalia and Vir R. Parshad

3.1 Introduction

Microorganisms exhibit richest diversity on earth with their prominence and importance as scavengers/decomposers or pathogens having role in the maintenance of several environmental processes like cycling and maintenance of organic and inorganic nutrients by decomposition of plant and animal wastes, as bioremediation agencies, and as vital product modifiers/generators. Deciphering their role in specific niche from environmental perspective demands development of acumen in novel detection and identification techniques for easy screening and ascertaining of role/function.

Microbial diversity is defined as species richness (species evenness and species difference as genetic and phenetic diversity) in a given habitat, i.e., on land, in fresh water, or in sea, or as parasites or symbionts. Limited information is available regarding microbial diversity in soil (bulk or rhizosphere), aquifers (fresh water, brackish, or marine), and in association to higher organisms (plant/animal as parasites, commensals, or mutualists) as less than 5 % of total microbial diversity of world is known. Only 5,000 bacteria among estimated 300,000–1,000,000 estimated prokaryotic species in Bergey's manual of Systematic Bacteriology (Kirk et al. 2004), very low percent among estimated 1,500,000 fungal species, speck is known among approximately 7.5×10^{29} marine viruses, and no data are available for soil and subsurface viral species (Pace 1997). Today's awakening hosts tomorrows benefits and hence identification of microdiversity, isolation of novel prokaryotic forms, genetic and functional characterization of new forms enable us to better understand the usefulness of healthy microbial diversity and this awareness would inculcate generation plans to conserve and protect microbiodiversity (Kalia and Gupta 2005a).

A. Kalia (✉) • V.R. Parshad
Electron Microscopy and Nanoscience Laboratory, College of Agriculture, Punjab Agricultural University, Ludhiana 141004, Punjab, India
e-mail: kaliaanu@gmail.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_3, © Springer-Verlag Berlin Heidelberg 2014

The phenetic and genetic plasticity of microbes as well as their single cell structure do not have distinct traits that haunt our ability to estimate microbial diversity, to define species occurrence (distribution and localization owing to spatial heterogeneity), as well as to quantify the extent of its contribution. Thus molecular approaches offer great potential in microbial ecology studies to obtain microbial diversity, identification of specific/functional genes, and detection of microbial activity as well as their obscured physiological and ecological role in situ (Siering 1998).

Molecular techniques have been instrumental in isolation of new microbes (Persing et al. 2004). An entirely new domain in the archaebacteria kingdom has been added making the total domain number to be three and are termed as euryarchaeota (cultivated methanogens), crenarchaeota (thermophiles/thermoacidophiles), and korarchaeota (entirely unculturable archae identified as gene sequences pJP27 and pJP28 from obsidian pool in Hot springs of Yellowstone caldera). This has been possible by ribotyping the 16S rRNA sequence of the sample water from the edges of the obsidian pool (Pace 1997). New molecularly characterized isolates not only enrich our general and basic understanding of the cellular machinery and processes; it may be harnessed at various subcellular levels (transcriptome, proteome, metabolome) for more pronounced economical purposes as designing of clinical diagnostic or identification kits (Kalia and Gupta 2005b; Lynch et al. 2004). Riesenfeld et al. (2004) have advocated that these uncultured bacteria could harbor novel antibiotic resistance genes to function as reservoirs of novel antibiotic resistance markers.

The microbes occur as flocs or aggregates termed hot spots in almost all the known ecological niches with maximum diversity and abundance recorded in surface waters, ocean floors over continental shelves, and the top few inches of soil (may contain more than 2 tons of fungal and bacterial biomass and approximately 10,000 bacterial species per 100 g soil) (Torsvik et al. 1990). The term soil microbes embraces soil bacteria, fungi, viruses, and protists (protozoa) and plays a prominent role in global biogeochemical cycling of elements (nitrogen, phosphorus, sulfur). Among these, the nitrogen fixers or diazotrophs include exclusively prokaryote members of domains *Archaea* and bacteria that exhibit diverse range of extent of interaction with rhizosphere and plant roots are of particular significance in terrestrial system. These microbes are involved in biological nitrogen fixation (BNF) contributing 176 million tons N annually and involves nitrogenase enzyme complex based reduction of atmospheric di-nitrogen (N_2) to ammonium (NH_4^+) (Kennedy and Islam 2001). Nitrogen fixers could be categorized on the basis of the extent of interaction as free living, loose associative, and symbiotic (Kalia and Gupta 2002).

As soil forms a heterogeneous medium having components varying both in terms of physical structure as well as chemical properties (Ladd et al. 1996), the distribution of microbes like diazotrophs can be substantially different in soil volume from one cubic centimeter (cm3) to the next cm3 (Izquierdo and Nusslein 2006; Nunan et al. 2003). Practically, diazotroph distribution in soil is affected by a

variety of abiotic (physical like texture, pH, moisture, temperature, and chemical like soil organic carbon content and nitrogen content) factors with soil texture (Riffkin et al. 1999), soil nitrogen level (Limmer and Drake 1998), and above ground vegetation cover/type (Bardgett et al. 1999) being the most influential. However, a variety of biotic factors like rhizosphere microflora diversity and microbial interactions with meso/macrofauna and plant roots also alters the soil diazotrophic counts and types or predominance of certain genera in particular communities (Roesch et al. 2010). This chapter explores the advanced molecular and microspectroscopy tools for identification and estimation of types, abundance, and role of diazotrophs in various niches.

3.2 Paradox of Microbial Diversity Studies

Microbial diversity studies suffer the great paradox of methodological limitations that greatly rely on culturing microbes on defined media, though only 1 % of majority bacteria and very few fungi are culturable accounting for ~99 % to exist in physiological states elusive of traditional culturing (Torsvik et al. 1998). The temporal and spatial variability of microbes in particular niches be it soil, aquatic, or subterranean regions create a bias/overestimation while studying, analyzing, and interpreting diversity indices as dominant microflora/fauna may get highlighted sheathing less abundant, physiologically dormant cysts/spores or dead cells. The results of microbes cultured under lab conditions depend on selection of optimum growth medium/conditions and growth characters (spreading colonies or colony–colony inhibition) and cannot provide information on microstructure and architecture of microbial aggregates (flocs, biofilms, etc.), are often too slow and inefficient to provide real-time and meaningful information about bioreactor processes. The paradox enhances the need for describing the diversity of free-living soil diazotrophs using cultivation-based strategies (Burgmann et al. 2004). Though certain culture-independent biochemical techniques such as fatty acid or phosholipid fatty acid methyl ester (FAME/PLFA) analysis identify microbes on basis of presence of signature fatty acids analyzed by GC, though limitations due to fatty acid composition, occurrence of numerous fatty acids in a single species or presence of common fatty acid in several species are of prime concern. Therefore, molecular approaches have been developed and successfully applied to describe diazotroph communities in different niches in the soil systems like forest, pasture, agricultural, wetland, and rhizospheres (Burgmann et al. 2004).

Diverse markers (biochemical, serological, and molecular), which are specific macromolecular entities present in specific microbial types that help in identification of particular species in mixed community niches or samples, are being explored for microbial, ecological, and interaction studies.

3.2.1 Markers for Enumeration of Microbial Diversity

Microbes could be deciphered by employing morphological, biochemical, as well as molecular markers; however, molecular markers possess ability for rapid detection of unculturable/fastidious microbes under in vitro conditions. Molecular markers are genetic traits used to identify species or species subpopulations by its different alleles and include primarily molecular probes, i.e., labeled oligonucleotide designed on basis of specific short stretches of conserved sequences in genes coding for r-RNA (5s, 16s, or 23s rRNA for prokaryotes and 18s rRNA for eukaryotes), interspersed regions, repetitive sequences, or restriction fragment polymorphic DNA sequences in genophore or extrachromosomal plasmids of microbial cells used to specifically identify a certain taxon. However apart from gross G + C content and nucleic acid reassociation protocols, all other techniques require amplification of target DNA sequences by using primers or probes that further result in obtaining genomic profiles allowing identification from phylum level to down variety or strain specific hierarchy. The specificity of these primers and probes allows specific hybridization with respective sequences in particular forms/species observable both under gnotobiotic/in vitro as well as in situ conditions. Novel molecular beacons (small 10–20 mer fluorescent labeled nucleic acid probes) have quencher attached near fluorescent molecule to decrease autofluorescence of the molecule (Antony and Subramaniam 2001).

3.2.2 Markers for Enumeration of Introduced Microbes

Several markers have been discovered or designed for enumeration of or pinpointing presence/absence of introduced microbes in a given niche or environmental sample (Gamalero et al. 2003). These include serological markers like surface proteins or oligosaccharides that exhibit immunological response leading to formation of antibodies that can be utilized for identification of species, strains, or serovars.

Molecular techniques for detection of introduced microbes involve the use of specific genetic markers as antibiotic resistance, enzymatic/chromogenic, luminescent, and fluorescent markers that help in elucidating the relative presence or absence and abundance of microbes in a specific sample or in particular niches.

Antibiotic resistance markers (particularly rifampicin, kanamycin, and streptomycin resistance) have been utilized most in microbial, ecological, and survival kinetics studies of microbes particularly rhizospheric interaction of introduced strains. Several genes encoding metabolic enzymes such as *xyl*E, *gus*A, and *lac*Z genes encoding catechol 2,3-dioxygenase, β-glucuronidase, and β-galactosidase provide chromogenic reactions on reacting with respective substrates (catechol, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside and X-gal) and could be used as markers to detect, quantify, and to localize introduced microbes. Bioluminescent

marker genes (*lux* operon *lux* ABCDE of *Vibrio fischeri*, eukaryotic *luc* genes) are sensitive markers that provide quantifiable light signals useful in both detection and localization of introduced microbial types. Fluorescent markers like 27 kDa stable and protease-resistant green fluorescent protein (GFP) and its variants cyan FP and yellow FP are of great significance, but their instability under anaerobic conditions and generation of interference signal by soil particles limit their capability for wider use.

3.3 Molecular Toolbox for Deciphering Microbial Diversity

The molecular detection techniques make use of combination of one or more of the above molecular markers to yield reliable detection of microorganisms (Raes and Bork 2008). These techniques are highly reproducible, high throughput, amenable to automation, and reliable for identification of particularly uncultured segment of microbial diversity (Fig. 3.1). The DGGE/TGGE and RFLP (ARDRA and T-RFLP) techniques provide the temporal and spatial changes in the microbial community analysis and the characteristic DNA fingerprints (Aeinas et al. 1997) or banding patterns so obtained could be compared for marking presence or absence of certain known prokaryotes or ruling out the other forms (Liu et al. 1997). The hybridization technique, FISH is most versatile for detection of microbes in situ in array of samples, viz., soil, root, rhizosphere, food, water, sediment, etc. A novel magnetotactic rod (MHB-1) was detected in sediment from Northern Germany lake by FISH sharing 91 % 16S rRNA sequence similarity to *Magnetobacterium bavaricum* (Flies et al. 2005).

Microarrays and DNA chips are also rapid and sensitive techniques for determining the genetic diversity of microbes in the sample. These arrays include specific or universal probes dot blotted on glass or polypropylene matrix surfaces of few millimeter diameter with number around 60,000 probes/chip that can hybridize with fluorescently labeled PCR pool or cDNA pool (Wilson et al. 2002). This offers high reproducibility and semiquantitative analysis for mapping variability in microbial diversity and allows rapid comparison of diversity in terms of number and activity of microbes. Microbial diversity analysis using probe hybridization of PCR products or vice versa in microarray analysis of PCR products help in identification of microbes at the group or species level directly without further sequencing (Dahllof 2002). Larsen et al. (2001) have reported the abundance and community structure of viruses and other prokaryotic forms in marine water samples using Flow Cytometery, DGGE, and PFGE. The PFGE DNA separation technique was first introduced by Schwartz and Cantor (1984) and includes resolution of extremely large DNA fragment (ranging from 30–50 kb to 10 Mb) and their separation in agarose. This technique is useful for the characterization of the larger plasmid like *sym* megaplasmids of rhizobiaceae members or DNA fragments.

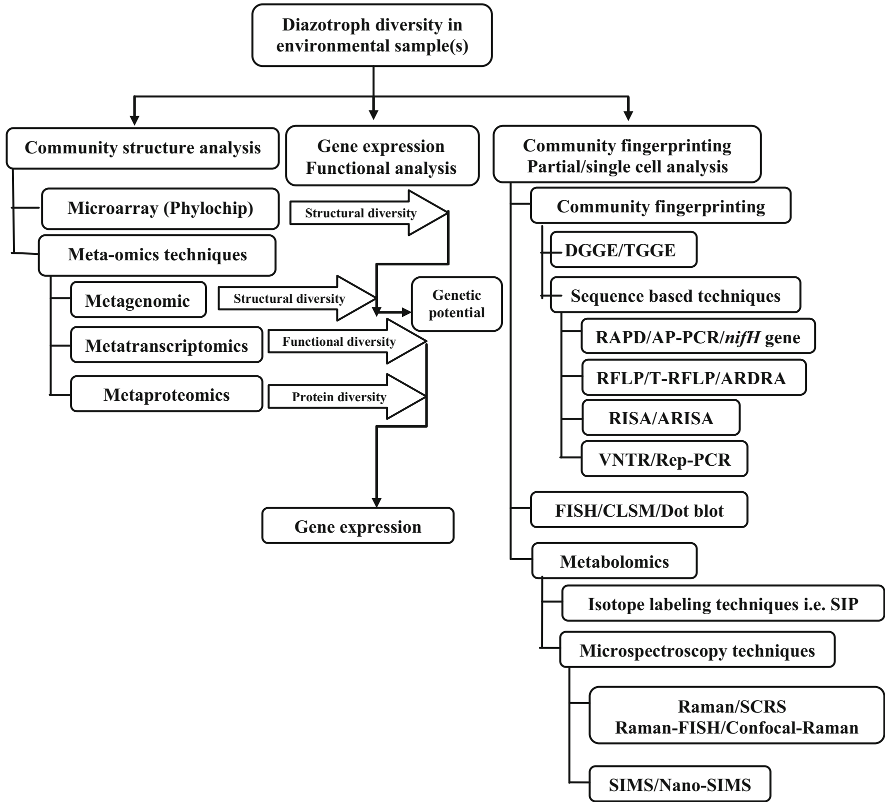


Fig. 3.1 Molecular and microspectroscopy toolbox for deciphering diazotroph diversity. *DGGE* Denaturing Gradient Gel Electrophoresis, *TGGE* Temperature Gradient Gel Electrophoresis, *RAPD* Random Amplified Polymorphic DNA, *AP-PCR* Arbitrary Primed-Polymerase Chain Reaction, *RFLP* Restriction Fragment Length Polymorphism, *T-RFLP* Terminal Restriction Fragment Length Polymorphism, *ARDRA* Amplified Ribosomal DNA Restriction Analysis, *RISA* Ribosomal Intergenic Spacer Analysis, *ARISA* Automated Ribosomal Intergenic Spacer Analysis, *VNTR* Variable Number Tandem Repeats, *Rep-PCR* Repetitive sequence based PCR, *FISH* Fluorescent In Situ Hybridization, *CLSM* Confocal Laser Scanning Microscopy, *SIP* Stable Isotope Probing, *SCRS* Single Cell Raman Spectroscopy, *SIMS* Secondary Ion Mass Spectrometery

3.4 Molecular Toolbox for Deciphering Diazotroph Diversity

The molecular toolbox for deciphering diazotroph diversity comprises of various tools among which following tools are most popularly utilized.

3.4.1 Microarrays

These provide high-throughput and comprehensive view of microbial communities in environmental samples and may include the global genome diversity and functional activity analyzing systems like Phylochip (16S rRNA gene microarray containing ~30,000 16S rRNA probes accuracy of which depends strongly on the choice of primers, DeSantis et al. 2007), Geochip (functional gene arrays containing conserved domains of genes (>24,000 probes) involved in specific metabolic pathways, He et al. 2007) and community genome arrays using high-throughput pyro-sequencing technique (contains highly specific signature gene sequences from known cultured microbial species, Pinto and Raskin 2012). The basic principle involves DNA extraction from sample, PCR amplification, fluorescent labeling of PCR amplicons followed by direct hybridization to molecular probes adsorbed/immobilized on microarray plate and scoring of signals by epifluorescence/confocal laser scanning microscopy. The hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. It is a versatile technique for rapid analysis of large number of samples in replicates; however, it cannot be used for identification and detection of novel microbes.

The functional gene array (FGA) includes all the functional genes involved in various biogeochemical, ecological, and environmental processes providing in situ community metabolic potential and hence are the microarrays of choice to decipher the diazotrophic microbial diversity. Biological nitrogen fixation is exclusively prokaryotic phenomena and involves expression of diverse (*ntr*, *nod*, *nif*, *fix*) genes in rhizospheric diazotrophs and certain competent plant hosts. The *nifH* and *nif D* gene products are more critical since the nifH gene sequence is highly valuable for phylogenetic and diversity analyses (Santos et al. 2012). Zhang et al. (2007) have reported that functional diazotroph diversity diagnostics could be performed by employing *nifH*-based oligonucleotide microarray containing 196 probes for both diversity and activity analysis of diazotrophs in roots of Namibian wild rice. Commercially, DIM-Array Nitrogen Cycle system microarrays (designed and manufactured by Dimole and Arrayit) are available which not only measure but also report the effect of agricultural management practices, fertilizer treatments, and environmental factors on the quantity and proportion of bacteria involved in key processes of the nitrogen cycle in the rhizosphere (http://arrayit.blogspot.in/2012/06/soil-microbe-test.html).

3.4.2 Gel Electrophoresis-Based PCR Techniques

Gel electrophoresis-based PCR techniques include agarose or polyacrylamide gel electrophoresis analysis of direct sample DNA or target DNA products after PCR amplification. The PCR-based gel electrophoresis methods however involve target

sequence amplification, which may include different markers (16s/23s rDNA sequences, RFLP, RAPD, ITS, or minisatellite VNTR regions) followed by analysis of separated DNA bands/banding patterns on gels.

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) techniques involve extraction of microbial DNA from sample and amplification using PCR universal primers targeting 16s or 18s rRNA sequences having 5′ end with 35–40 bp GC clamp that ensures a part of DNA to remain double stranded followed by denaturation by running through polyacrylamide gel with an increasing gradient concentration of formamide and urea acting as denaturants. Partly denatured or fully denatured molecules stop migrating in the gel and DNA fragments occupy different positions in the gel according to their base composition and sequence variation. These denatured DNA fragments after differential migration in the polyacrylamide gel provide bands that theoretically may represent single species (Muyzer et al. 1993; Miller et al. 1999). This technique also enables excision and subsequent sequencing of bands, allowing species identification using existing databases (Arias et al. 2005). Orr et al. (2011) have reported the diversity and activity of nitrogen fixers in organic and conventionally managed soils using *nifH*-based DGGE and quantitative PCR techniques. Vollu et al. (2012) have reported the utility of DGGE profiling for deciphering diazotroph diversity. They observed difference in the *nifH*-based PCR-DGGE profiles of rhizosphere soil and root of *Chrysopogon zizanioides* followed by generation of clone libraries. TGGE is similar to the above one with high temperature a type of denaturing agent (Miethling et al. 2000; Emmerling et al. 2002).

3.4.3 Sequence-Based PCR Techniques

These techniques include using one of several signature conserved universal or specific sequences to decipher the presence of a specific type species or genera.

Random amplified polymorphic DNA (RAPD)/arbitrary primed PCR (AP-PCR) and nifH gene analysis molecular markers can be used to classify microbes particularly bacteria upto the level of strain, serotypes, and subtypes among serotypes. AP-PCR utilizes RAPD molecular markers for characterization and identification of different types of microbe in a given sample. The molecular approach to study diazotroph diversity is primarily based on PCR amplification of nifH marker gene that contains phylogenetic information and codes for nitrogenase reductase enzyme involved in nitrogen fixation. Gosal et al. (2012) have reported the application of *nif* H marker to decipher the rhizospheric soil diazotroph diversity in wheat grown fields of Punjab. However, due to presence of multiple copies of *nif* gene within a genome as well as due to presence of alternative nitrogenase systems, the use and authenticity of this marker may jeopardize because of over/under estimation of diazotroph diversity (Burgmann et al. 2004). A report by Sevilla et al. (2001) suggests the problem with the use of *nif H* degenerate primer marker that exhibited similarity between *nif* HDK of *Gluconacetobacter diazotrophicus* and other

N-fixing bacteria. Moreover, certain diazotrophs exhibit low genetic diversity on isolation from various environments as in *G. diazotrophicus*.

Restriction fragment length polymorphism (RFLP), T-RFLP, and ARDRA based PCR techniques make use of DNA restriction fragment length polymorphisms in genome or in rDNA sequences for studying microbial diversity. The rDNA operon (*rrn*) is a particularly useful target for the development of nucleic acid hybridization- and PCR-based assays. In prokaryotes, the rDNA operon encodes the 16S (*rrs*), 23S (*rrl*), and 5S (*rrf*) rRNA genes. The target DNA molecule is amplified, given restriction enzyme (4, 5, or 6 base pair cutting) treatment followed by fractionating DNA fragments by agarose or non-denaturing PAGE to obtain different banding patterns that could be compared or analyzed to measure structural changes in microbial communities.

Amplified ribosomal DNA restriction analysis (ARDRA) is RFLP analysis of 16s or 23s rDNA patterns of prokaryotes that provides alteration in prokaryotic diversity patterns in given sample (Gich et al. 2000). The use of ARDRA for diazotroph diversity analysis from a variety of rhizosphere soils of various plants have been reported (Weber et al. 1999; Gosal et al. 2012) and usually ARDRA profiles exhibit high diversity for diazotrophic isolates.

The limitation of most of RFLP techniques could be overruled by terminal restriction fragment (TRF) length polymorphism (T-RFLP) technique. As it is rapid, high-throughput, offers high resolution, and allows semiquantitative analysis, it is a sensitive tool appropriate for analyzing endophytic microbial communities allowing identification of genera by using T-RFLP Analysis Program (TAP) software linked to Ribosomal Database Project database and involves differentiation according to the patterns derived from cleavage of their DNA. It involves use of fluorescent phosphoramidite-labeled PCR amplicons that simplifies the banding pattern analysis of complex communities and allows detection of only labeled terminal restriction fragments. The amplicons are restriction enzyme digested, separated either by gel or capillary gel electrophoresis. The separated, labeled fragments are then densitometrically detected and a profile based on fragment lengths is generated (Buckley and Schmidt 2001). Thus, in T-RFLP, the specific fingerprint of a community is revealed by analyzing the polymorphism of a certain gene (Iwata et al. 2012; Huang et al. 2011; Yeager et al. 2005). Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA) is a type of microbial typing protocol that involves fingerprinting of ribosomal intergenic spacer sequences for studying microbial diversity. Although the 16S rRNA gene has been most widely used (Klindworth et al. 2012), the 16S–23S rDNA IGS region has received increased attention as a target in molecular detection and identification schemes (Tan et al. 2001). In contrast to rRNA genes, which are remarkably well conserved throughout most bacterial species, the IGS regions exhibit a large degree of sequence diversity and length variation. Even within species, the IGS sequence variation may be very high, thus allowing intraspecies strain differentiation, as recently also shown for rhizobial strains. The technique basics are same as of the above-discussed techniques with only difference that lies in the sequence used for comparing/detecting isolates

(Gros et al. 2006). The small and large ribosomal subunit RNA (16S/23S) that contain intergenic spacer sequences having sequence as well as length variability could be amplified by PCR using specific primers and product, could be fractionated by gel electrophoresis, and provide strain-specific patterns allowing frequent identification. The gel banding patterns are either silver stained (RISA) or are quantified using fluorescent microscope (ARISA) since the primers are fluorescently labeled. This technique is versatile in terms of type of sample that could be analyzed as big range of sample could be quantified by same protocol or with minor alterations or pretreatment steps (Ranjard et al. 2001).

Microsatellite/VNTR sequences/REP-PCR is a genomic fingerprinting technique that utilizes the interspersed repetitive DNA sequences belonging to three different families (REP, ERIC, and BOX sequences) for comparison and characterization up to the level of subspecies or strain. Microbial genomes contain short repetitive DNA sequences (1–10 bp) that are diagnostic in providing limited knowledge on diversity as well as providing valuable information on taxonomic hierarchy of a particular microbe. Primers specific and complementary to these repetitive sequences are designed and PCR amplification is performed for the denatured sample DNA. The PCR reaction products are separated by gel electrophoresis and patterns could be analyzed for determining the taxonomic hierarchy of the microbes or in identifying novel microbes. The diverse communities' primers matching interspersed repetitive DNA sequences and the rep-PCR can generate a genomic fingerprint to distinguish bacteria at a fine level.

Several studies have demonstrated the usefulness of repetitive sequence-based polymerase chain reaction (rep-PCR) to fingerprint a large variety of bacteria and to study microbial diversity in natural ecosystems (Liu et al. 1997). A study of the diversity of endophytic bacteria present in seeds of a deepwater rice variety revealed the presence of seven types of BOX-PCR fingerprints. The 16S rDNA nucleotide sequence and BIOLOG system of bacterial identification were utilized to identify new bacterial genus *Pantoea agglomerans* from rice roots (Verma et al. 2001). Quantitative PCR techniques like mpn-PCR (Fredslund et al. 2001) and taxon-specific Q-PCR (Fierer et al. 2005) can be more useful for identifying microbial diversity.

3.5 Meta-Omics

The meta-omics techniques unravel the gross genome, transcriptome, proteome, and metabolome of whole community of microbes inhabiting a given niche. These approaches are based on identification and use of certain peculiar signature DNA, mRNA, proteins, characteristic metabolites, and other biomarkers that can help in verifying the ecological relevance and geographical prevalence of the process (Madsen 2011). However, to draw out better understanding of the relative benefits of various microbes and their extent of participation in particular process, integration of approaches like community proteogenomics combine metagenomics and

metaproteomics that involves extraction of total DNA and proteins from the same sample allowing linking of biological functions to phylogenetic identity with greater confidence (Rastogi and Sani 2011) and thus can be helpful in unraveling the complex environmental niches like rhizosphere and phyllosphere (Delmotte et al. 2009).

With the information flow from high-throughput sequencing technologies like Roche 454 GS-FLX (generates 750 Mbp of sequencing data), Illumina/Solexa GAIIx, MiSeq (generates1.5 Gb per day) or HiSeq2000 (produces >50 Gb per day), Life/APG, HeliScope/Helicos BioSciences sequencing platforms (Caporaso et al. 2012; Pinto and Raskin 2012), as well as the advanced bioinformatics tools with improved algorithms like QIME (operational taxonomic units, Foster et al. 2012; Gonzalez and Knight 2012), large scale sequence data could be obtained, compared, and analyzed for accurate, reliable, and rapid identification of microbial forms in a complex environmental sample. These techniques have been instrumental in deciphering the metagenomes annotated as Genomic Encyclopedia of Bacteria and Archaea (GEBA) project (http://www.jgi.doe.gov/programs/GEBA/) and the Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) (Roling et al. 2010)

3.5.1 Metagenomics

Metagenomics is analysis of global genomic DNA (virtually capturing and sequencing of about all the genes) directly extracted from whole community of organisms inhabiting a particular niche providing a view of the community structure (species phylogeny, richness, and distribution) and functional (metabolic) potential of a community thereby providing reference genes and genomes for metatranscriptomics (Carvalhais et al. 2012). Metagenomic protocols involve genomic DNA extraction from microbial cells in an environmental sample followed by random DNA shearing to obtain short fragments which are cloned, sequenced in either a random or targeted fashion, and reconstructed into a consensus sequence (Guazzaroni et al. 2009). Soni and Goel (2011) have deciphered the diversity of *nifH* in their metagenome and performed in silico study of sequenced shotgun clones to propose the evolution of *nifH* gene from nearest adjacent genes/regions. This approach can help in identification of new genes as reported by Riaz et al. (2008) who have showed occurrence of N-acyl homoserine lactone degrader, QlcA peptide (belonging to family of zinc-dependent metallohydrolases) to be distantly related to other NAHL-lactonases discovered in *Agrobacterium*, *Bacillus*, *Photorhabdus*, and *Rhizobium*.

3.5.2 Metatranscriptomics

Metatranscriptomics provides a snapshot of transcriptional profiles that correspond to discrete populations within a microbial community at the time of sampling which

indicates potential activities and regulatory mechanisms of complex microbial communities (Carvalhais et al. 2012; Vercruysse et al. 2012). The technique is versatile for deciphering immediate microbial gene regulatory responses to changed environment at the time of sampling and heralds unraveling functional soil bacterial diversity since majority of bacteria exhibit transcriptional gene control that enables rapid adaption to altered environmental conditions (Moran 2009). Hence it is useful in understanding the timing and regulation of complex microbial processes within communities and consortia, as well as microbial dexterity in response to changing conditions (Moran 2009). With the advent of advanced RNA extraction techniques from soil (Wang et al. 2012) and massive parallel cDNA or RNA sequencing (Ozsolak et al. 2009), improvements in transcription start site mapping, strand-specific measurements, gene fusion detection, small RNA characterization, and detection of alternative splicing events (Ozsolak and Milos 2011) have paved toward quantification of RNA from complex samples. The metatranscriptomic studies for assessing nitrogen fixation process in rhizosphere have become more accurate by functional categorization of transcripts using new comparison-analysis databases (Kyoto Encyclopedia of Genes and Genomes, Clusters of Orthologous Groups, and evolutionary genealogy of genes: Non-supervised Orthologous Groups) having groups of genes assigned for different functional pathways (e.g., denitrification or nitrogen fixation) based on the similarity of protein orthologs from sequenced isolate microbial genomes.

3.5.3 Metaproteomics

The community proteomics or metaproteomics involves global proteomic characterization of microbial proteins in environmental samples for exploration of microbial communities mostly uncultivated and uncharacterized populations using high performance mass spectrometry-based technology platforms (Wilmes and Bond 2009). It is an established useful complement of functional metagenomics and can be used for phylogenetic classification of bacterial species by using 2D maps (Dopson et al. 2004) or mass spectrometry-based peptide sequencing (Dworzanski and Snyder 2005). Since proteins provide more direct information about microbial metabolic reactions (can help identify active microbes via database analysis using level of homology to other species) and regulatory cascades (post-transcriptional regulation of metabolic processes) compared to functional genes or corresponding mRNAs, are not prone to bias for certain templates as in PCR-based nucleic acid techniques, and are ideal for quantitative analysis, they seem to be promising alternative markers for molecular ecological studies (Benndorf et al. 2007).

Metaproteomics demands applying novel sample processing and extraction techniques for protein isolation from complex environmental samples (Chourey et al. 2010; Benndorf et al. 2007). As the extraction of low abundance proteins from very small quantity of complex environmental samples is tedious, application of novel nanotechnology-based techniques like nanoelectrophoresis,

nanochromatography, and magnetic biobeads technologies (Ivanov et al. 2006) feature a good alternative. Data obtained by proteomics on functions of specific proteins extend the scope of the study from community composition to identification of niche-specific genes (Berlec 2012). Environmental proteomics allows to relate protein presence to biogeochemical processes and to identify the source organisms for specific enzymes (Schulze 2004). The proteomic studies of the rhizosphere nitrogen cycle involve plant–bacterial interaction proteome analysis particularly for diazotroph–legume partners to decipher impacts of plants on the bacterial proteomes, and vice versa (Cheng et al. 2010). Metaproteomic studies of the phyllosphere of certain crop and model plants have been reported; however, the rhizosphere metaproteome are still in their infancy and very recent first reports have been published by Wang et al. (2011) and Wu et al. (2011). Since extraction of microbial proteins from rhizosphere/phyllosphere samples is quite difficult due to interference of high abundance plant-specific proteins, combined metaproteogenomic studies are being performed which have been instrumental to segregate the functional gene potential of different dinitrogen fixing microbial genera in rice plant rhizosphere (Knief et al. 2012).

3.5.4 Meta-Metabolomics

Meta-metabolomics is the study of number of primary and secondary metabolites (hydrophilic carbohydrates, volatile alcohols, ketones, amino and non-amino organic acids, hydrophobic lipids, antibiotics, pigments, and non-ribosomal peptides, cofactors) with the peculiar signature ones the number of which are dependent upon the sample, extraction process, and method of analysis. No single extraction process and separation/detection system can identify and quantitate every metabolite present in the sample with the current protocols allowing detection of 4,200 metabolites in one profile (Fiehn and Weckwerth 2003) probably due to high variability in the chemical structures and properties as well as the dynamic nature of the products generated from metabolic reactions (Tang 2011). The technical jet-setters for deciphering complete metabolome from a complex environmental sample demands advancement in the already known arsenal of metabolome analytical tools, i.e., nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) like the features of simultaneous separation and detection of metabolites using ultrahigh-accuracy mass analyzers, availability of fully sequenced genomes and novel data integration, and modeling and standardization software (Tang 2011). This holds promise for isolation and identification of diverse metabolites which led to the mushrooming of novel terms and techniques like ecological metabolomics or ecometabolomics that not only identifies total number of metabolites but also can assess ambiguities resulting from environmental influences on cellular expressions (Sardans et al. 2011).

Metabolome studies could provide enormous information regarding the exact amount of nitrogen transformation reactions happening during the reduction of

atmospheric nitrogen to ammonia in soil by diazotrophs as well as could be useful to improve understanding of nitrogen metabolism in holobionts, i.e., legume host–diazotroph interactions (Allwood et al. 2012; Desbrosses et al. 2005). Hernandez et al. (2009) have reported the alterations in transcriptome and metabolome profiles of nodules of *Phaseolus vulgaris–Rhizobium tropici* under different phosphorus availability in terms of differential gene expression and decrease in amino acid content while increase in organic and polyhydroxy acids contents under P-deficient conditions. Above all the internal partitioning, assimilation, and use of nitrogen in crop plant as studied from the differences between sink and source metabolic profiles could be better understood by metabolomic studies (Jeong et al. 2004).

3.6 Isotope Labeling Techniques

3.6.1 Stable Isotope Probing

The stable isotopes (do not emit radiation) can be used to label phylogenetically informative cellular macromolecules like phospholipid fatty acids, DNA, or RNA for in situ phylogenetic characterization of soil microorganisms so as to establish a causal relationship to function within the community (Sims 2007). The technique is termed stable isotope probing (SIP) (Bhat et al. 2010) and provides valuable information rather more definitive detection of physiologically or biogeochemically active microbial populations associated with a specific process like C or N assimilation in soil since it allows differentiation of active microbes from inactive community members (Tiunov 2007). Though useful, the technique suffers a drawback that addition of intermediates of the labeled substrate could lead to use by other species not involved in the pathway resulting in false positive inferences (Manefield et al. 2006). The SIP studies thus redefine the relative role of particular microbe/microbial communities and links phylogenetic diversity to activity and the sensitivity of the roles/activities performed by microbes/microbial communities in response to altered abiotic–biotic conditions particularly soil management practices (Sims 2007). Moreover, the technique is most useful in deciphering the in situ functional redundancy concept of ecology, experimentally in microbial communities (Wagner 2009).

The in situ SIP technique can be used to observe substantial fractionation of the isotopes during nitrogen assimilation processes as nitrification and ammonification among the native soil microorganisms that involves ppm changes in $\delta^{15}N$ (Robinson 2001). The technique can also provide the vertical gradient of heavy N accumulation in soil, the value of which could be correlated with the potential nitrification and ammonification activities suggesting use of $\delta^{15}N$ profiles to be used for integral evaluation of nitrogen mineralization rate (Vervaet et al. 2002).

3.6.2 Fluorescent In Situ Hybridization–Microautoradiography

It is a microscopy-based technique involving simultaneous visualization of identity and specific activity (Wagner 2009), i.e., metabolic state of microorganisms at the single-cell level by coupling of FISH with MAR facilitating the phylogenetic identification of substrate-active cells that consume the radioactive substrate within complex microbial communities without a priori knowledge of the microbial community of interest (Rogers et al. 2007). The basic technique has several steps including (1) incubation of sampled soil with radioactive substrate like amino acids, glucose, and acetate; (2) fixation and extraction of cells; (3) FISH by using newer marker dyes like Cy dyes (Cy3), Alexa dyes, FAM, and TAM followed by (4) MAR detects decay of radioisotopic compounds (release beta decay particles) incorporated in microbial cells via exposure of a thin layer of highly sensitive photographic silver emulsion by using bright-field/phase contrast microscopy. This method is being very useful in identification of metabolic activities related to carbon, iron, phosphorus, hydrogen, and sulfur metabolism in rhizosphere, sediments, lakes, ocean planktons (Musat et al. 2012), as well as could be used to study plant–microbial interactions (Rogers et al. 2007).

3.7 Microspectroscopy-Based Techniques

These techniques combine visible/infrared/laser microscopy (like FISH/FTIR/CLSM) with already known arsenal of spectroscopy techniques [like Raman/Secondary Ion Mass Spectrometry (SIMS)] to decipher the metabolic potentials of diverse microbes in a given environmental sample.

3.7.1 Raman Microspectroscopy/Single Cell Raman Spectroscopy

Raman microspectroscopy involves illuminating aqueous or dry sample with laser-generated monochromatic photons thereby causing rotational and vibrational changes in sample molecules to obtain spectra (Raman intensity vs. wave number graph) that can be analyzed to identify materials (Musat et al. 2012). Invariably Raman spectroscopy provides the molecular signature of the sample of varied origin (physical, chemical, and biological) and high information content or quantitative data, excellent tolerance to water interferences, and no need for fixation or sectioning of the specimen for microecophysiology analysis (Li et al. 2012). It is a noninvasive and label-free technology, allowing in vivo and multiple parameter analysis of individual living cells.

Recent reports show the ability of Raman spectrometry to analyze the chemical composition of the extracellular polymer substances (EPS), microorganisms embedded in EPS, as well as other substances inside biofilm (inorganic compounds and humic substances) (Schwartz et al. 2009). A single cell Raman spectrum usually contains more than 1,000 Raman bands which provide rich and intrinsic information of the cell (e.g., nucleic acids, protein, carbohydrates, and lipids), reflecting cellular genotypes, phenotypes, and physiological states. A Raman spectrum serves as a molecular "fingerprint" of a single cell, making it possible to differentiate various cells including bacterial, protistan, and animal cells without prior knowledge of the cells.

3.7.2 Raman-FISH/Confocal Raman

This combo technique allows determination of chemical bonding patterns associated with biological molecules within individual microbial cells by Raman vibrational spectroscopy along with FISH that identifies specific microbe in complex environments (Huang et al. 2007). The basic method involves incubation of sample with a substrate labeled with a stable isotope (^{15}N, ^{13}C) followed by generation of spectral profiles of microbial cells at single-cell resolution by Raman microscopy (Wagner 2009). As the proportion of stable isotope incorporation in cells affects the amount of light scattered, measurable peak shifts toward the red region (Red shift) for labeled cellular components are observed (Xie and Li 2003). The confocal Raman provides much higher resolution and overcomes many of the limitations associated with conventional SIP/fluorescent in Situ Hybridization–Microautoradiography (FISH-MAR) techniques (Wagner 2009).

3.7.3 Secondary Ion Mass Spectrometry

This is a combinatorial microscopy-analytical mass spectrometry technique that could investigate the stable isotopic composition of materials, i.e., offers exciting opportunities for studying the relationship between bacterial activity and the spatial heterogeneity of the physical environment of the soil (O'Donnell et al. 2007). It is a new tool in the study of biophysical interfaces in soil for biogeochemistry and soil ecology studies as it allows precise, spatially explicit, elemental, and isotopic analysis at the nm scale (Rennert et al. 2012; Boxer et al. 2009).

A heavy ion beam (Cs^+ or O^-) is bombarded across the sample surface that results in sputtering or release of secondary ions (negatively charged such as $^{12}C^-$, $^{13}C^-$, $^{16}O^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, and $^{28}Si^-$ and positively charged such as $^{23}Na^+$, $^{28}Si^+$, $^{39}K^+$, and $^{40}Ca^+$) on a range of biological materials at the subcellular scale which could be sorted and identified on basis of their mass to charge values from a series of spatially referenced spectra to create an isotope map of the sample

surface enabling a spatial resolution at a nanoscale (~50–150 nm) to image isotope uptake by single microbial cells (Kilburn et al. 2010). This technique can be used to image about five different isotopes from the same area simultaneously resulting in extraction of data from quantitative isotope ratios from individual components offering elucidation of ion and molecule transport processes into cells and their distribution within cells (Guerquin-Kern et al. 2005; Lechene et al. 2007). Ploug et al. (2010) have reported the use of Nano-SIMS to understand the carbon and nitrogen fluxes in marine cyanobacteria *Aphanizomenon* sp. colonies with net C:N fixation ratio of individual cells to be 7.3 ± 3.4. This exemplifies the ability of this technique to directly observe and quantify the metabolic activity of microorganisms in their natural environment so as to understand the relative contributions of different groups of microbes to major microbially catalyzed processes (Wagner 2009).

Nano-SIMS is a variant of SIMS technique involving nanoscale spatial resolution of the nutrient isotope in the soil matrix/microbial cell/plant cell. It is very useful in deciphering biological processes such as nutrient (carbon, nitrogen, phosphorus) cycling by identifying the spatial location of microorganisms and their activity within the soil matrix as well as for prediction of gross process rates arising from community-level activity in soil (Herrmann et al. 2007a). Moreover, it is versatile in terms of spatial resolution and sensitivity for identifying the various stages of occurrence of nitrogen or nitrogenous compounds in microbial cells and rhizosphere soil and their uptake by plant roots, i.e., nutrient portioning in soil–microbe–plant continuum (Herrmann et al. 2007b; Clode et al. 2009; Rennert et al. 2012). The soil microbes are known to reduce atmospheric nitrogen as ammonia followed by its transformation to nitrate, nitrite, and finally denitrification. However, certain recent Nano-SIMS based reports confirm that higher plants have the ability to capture organic nitrogen compounds like amino acids directly from soil (Jones et al. 2005).

3.8 Conclusions

The molecular methods discussed in this chapter are unique regarding their use efficiency and versatility in deciphering microbes in different types of environmental samples. Though useful molecular techniques have certain constraints that have to be addressed such as inability to create or design really universal primers that could amplify all the known and unknown sequences without PCR biases (Babalola 2003). Moreover the information provided by various groups is fragmentary and there is scarcity of comparison databases (Curtis and Sloan 2004), which demands advent of new techniques or advancements before jumping on to conclusions that molecular methods alone or in combo of one or two could actually provide ensemble microbial diversity. New software is now made available for quick and easy analysis of large amount of sequence data by virtue of expanding rDNA sequence databases. These are in public domain and are accessible for comparing the results of sequence analysis and protocols across globe via World Wide Web.

Another major challenge of new yet uncharacterized microbes identified by molecular microbial techniques is for microbial ecologists regarding comparison of this diversity in different environments. Bohannan and Hughes (2003) have proposed and developed three statistical approaches, viz., parametric estimation, nonparametric estimation, and community phylogenetics to address the above challenge, as a combination of these tools with molecular biology techniques allows the rigorous estimation and comparison of microbial diversity in different environments.

The future of molecular studies for deciphering uncultured and novel microbes is far superior as new advancements are adding up in our present knowledge of molecular methods. New advancements as production of nucleic acid chips will help provide rapid, quantifiable results on presence or absence of specific signature nucleic acids when the sample ssDNA/RNA would bind with the probe molecule bound to chip matrix. This technique would be better than FISH in terms of reproducibility, rapidity, and reuse of the same chip several times for different hybridization reactions (Siering 1998). Moreover knowing and correlating microbial presence in situ with detailed physical, chemical, and genetic characterization is desirable to relate the functionality of a gene at the microscale in a particular niche (Turnbaugh and Gordon 2008).

References

Aeinas SG, Rodriguez-Valera F, Pedros-Alio C (1997) Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16S rDNA. FEMS Microbial Ecol 24:27–40

Allwood JW, Heald J, Lloyd AJ, Goodzcre R, Mur LAJ (2012) Separating the inseparable: the metabolomic analysis of plant-pathogen interactions. In: Hardy NW, Hall RD (eds) Plant metabolomics: methods and protocols, methods in molecular biology, vol 860, pp 31–49. doi:10.1007/978-1-61779-594-7_3

Antony T, Subramaniam V (2001) Molecular beacons: nucleic acid hybridization and emerging applications. J Biomol Struct Dyn 19:497–504

Arias ME, González-Pérez JA, González-Vila FJ, Ball AS (2005) Soil health – a new challenge for microbiologists and chemists. Int Microbiol 8:13–21

Babalola OO (2003) Molecular techniques: an overview of methods for the detection of bacteria. Afr J Biotechnol 2(12):710–713

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS, Davis WJ (1999) Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Funct Ecol 13:650–660

Benndorf D, Balcke GU, Harms H, von Bergen M (2007) Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. ISME J 1:224–234. doi:10.1038/ismej.2007.39

Berlec A (2012) Data obtained by proteomics on functions of specific proteins extends the scope of the study from community composition to identification of niche-specific genes. Plant Sci 193–194:96–102

Bhat MI, Rasool F, Bhat MA (2010) Applications of stable and radioactive isotopes in soil science. Curr Sci 98:1458–1471

Bohannan BJM, Hughes J (2003) New approaches to analyzing microbial biodiversity data. Curr Opin Microbiol 6:282–287

Boxer SG, Kraft ML, Weber PK (2009) Advances in imaging secondary ion mass spectrometry for biological samples. Annu Rev Biophys 38:53–74

Buckley DH, Schmidt TM (2001) The structure of microbial communities in soil and the lasting impact of cultivation. Microb Ecol 42:11–21

Burgmann H, Widmer F, Von Sigler W, Zeyer J (2004) New molecular screening tools for analysis of free-living diazotrophs in soil. Appl Environ Microbiol 70:240–247

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624

Carvalhais LC, Dennis PG, Tyson GW, Schenk PM (2012) Application of metatranscriptomics to soil environments. J Microbiol Methods 91:246–251. doi:10.1016/j.mimet.2012.08.011

Cheng Z, McConkey BJ, Glick BR (2010) Proteomic studies of plant-bacterial interactions. Soil Biol Biochem 42:1673–1684

Chourey K, Jansson J, VerBerkmoes N, Shah M, Chavarria KL, Tom LM, Brodie EL, Hettich RL (2010) Direct cellular lysis/protein extraction protocol for soil metaproteomics. J Proteome Res 9:6615–6622

Clode PL, Kilburn MR, Jones DL, Stockdale EA, Cliff JB, Herrmann AM, Murphy DV (2009) In situ mapping of nutrient uptake in the rhizosphere using nanoscale secondary ion mass spectrometry. Plant Physiol 151:1751–1757

Curtis TP, Sloan WT (2004) Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Curr Opin Microbiol 7:221–226

Dahllof I (2002) Molecular community analysis of microbial diversity. Curr Opin Biotechnol 13:213–217

DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 53(3):371–383

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C, Vorholt JA (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci USA 106:16428–16433. doi:10.1073/pnas.0905240106

Desbrosses GG, Kopka J, Udvardi MK (2005) Lotus japonicas metabolic profiling. Development of gas chromatography-mass spectrometry resources for the study of plant-microbe interactions. Plant Physiol 137:1302–1318

Dopson M, Baker-Austin C, Bond PL (2004) First use of two-dimensional polyacrylamide gel electrophoresis to determine phylogenetic relationships. J Microbiol Methods 58:297–302

Dworzanski JP, Snyder AP (2005) Classification and identification of bacteria using mass spectrometry-based proteomics. Expert Rev Proteomics 2(6):863–878

Emmerling C, Schloter M, Hartmann A, Kandeler E (2002) Functional diversity of soil organisms: a review of recent research activities in Germany. J Plant Nutr Soil Sci 165:408–420

Fiehn O, Weckwerth W (2003) Deciphering metabolic networks. Eur J Biochem 270:579–588

Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Micobiol 71:4117–4120

Flies CB, Peplies J, Schuler D (2005) Combined approach for characterization of uncultivated magnetotactic bacteria from various aquatic environments. Appl Environ Microbiol 71:2723–2731

Foster JA, Bunge J, Gilbert JA, Moore JH (2012) Measuring the microbiome: perspectives on advances in DNA-based techniques for exploring microbial life. Brief Bioinform 13 (4):420–429. doi:10.1093/bib/bbr080

Fredslund L, Ekelund F, Jacobsen CS, Johnsen K (2001) Development and application of a most-probable-number-pcr assay to quantify flagellate populations in soil samples. Appl Environ Microbiol 67:1613–1618

Gamalero E, Lingua G, Berta G, Lemanceau P (2003) Methods of studying root colonization by introduced beneficial bacteria. Agronomic 23:407–418

Gich FB, Amer E, Figueras JB, Abella CA, Balaguer MD, Poch M (2000) Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). Int Microbiol 3:103–106

Gonzalez A, Knight R (2012) Advancing analytical algorithms and pipelines for billions of microbial sequences. Curr Opin Biotechnol 23:64–71

Gosal SK, Saroa GS, Vikal Y, Cameotra SS, Pathania N, Bhanot A (2012) Isolation and molecular characterisation of diazotrophic growth-promoting bacteria from wheat rhizospheric soils of Punjab. Soil Res 49:725–732, http://dx.doi.org/10.1071/SR11136

Gros R, Monrozier JL, Faivre P (2006) Does disturbance and restoration of alpine grassland soils affect the genetic structure and diversity of bacterial and N_2-fixing populations? Environ Microbiol 8:1889–1901. doi:10.1111/j.1462-2920.2006.01106.x

Guazzaroni ME, Beloqui A, Golyshin PN, Ferrer M (2009) Metagenomics as a new technological tool to gain scientific knowledge. World J Microbiol Biotechnol 25:945–954. doi:10.1007/s11274-009-9971-z

Guerquin-Kern JL, Wu TD, Qunitana C, Croisy A (2005) Progress in analytical imaging of the cell by dynamic secondary ion mass spectrometry (SIMS microscopy). Biochim Biophys Acta 1724:228–238

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1:67–77. doi:10.1038/ismej.2007.2

Hernandez G, Valdes-Lepez O, Ramrez M, Goffard N, Weiller G, Aparicio-Fabre R, Fuentes SI, Erban A, Kopka J, Udvardi MK, Vance CP (2009) Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus stressed common bean plants. Plant Physiol 151:1221–1238

Herrmann AM, Clode PL, Fletcher IR, Nunan N, Stockdale EA, O'Donnell AG, Murphy DV (2007a) A novel method for the study of the biophysical interface in soils using nano-scale secondary ion mass spectrometry. Rapid Commun Mass Spectrom 21:29–34

Herrmann AM, Ritz K, Nunan N, Clode PL, Pett-Ridge J, Kilburn MR, Murphy DV, O'Donnell AG, Stockdale EA (2007b) Nano-scale secondary ion mass spectroscopy-a new analytical tool in biogeochemistry and soil ecology: a review article. Soil Biol Biochem 39:1835–1850

Huang WE, Stoecker K, Griffiths R, Newbold L, Daims H, Whiteley AS, Wagner M (2007) Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. Environ Microbiol 9:1878–1889. doi:10.1111/j.1462-2920.2007.01352.x

Huang LN, Tang FZ, Song YS, Wan CY, Wang SL, Liu WQ, Shu WS (2011) Biodiversity, abundance, and activity of nitrogen-fixing bacteria during primary succession on a copper mine tailings. FEMS Microbiol Ecol 78:439–450

Ivanov YD, Govorun VM, Bykov VA, Archakov AI (2006) Nanotechnologies in proteomics. Proteomics 6(5):1399–1414

Iwata K, Yu SS, Azlan NNA, Omori T (2012) Ammonia accumulation of novel nitrogen-fixing bacteria. In: Sammour R (ed) Biotechnology – molecular studies and novel applications for improved quality of human life. InTech, pp. 13–24. http://www.intechopen.com/books/biotechnology-molecular-studies-and-novel-applications-for-improvedquality-of-human-life/ammonia-accumulation-of-novel-nitrogen-fixing-bacteria

Izquierdo JA, Nusslein K (2006) Distribution of extensive *nifH* gene diversity across physical soil microenvironments. Microb Ecol 51:441–452

Jeong ML, Jiang H, Chen HS, Tsai CJ, Harding SA (2004) Metabolic profiling of the sink-to-source transition in developing leaves of quaking aspen. Plant Physiol 136:3364–3375

Jones DL, Shannon D, Junvee-Fortune T, Farrar JF (2005) Plant capture of free amino acids is maximized under high soil amino acid concentrations. Soil Biol Biochem 37:179–181

Kalia A, Gupta RP (2002) Nodule induction in non-legumes by Rhizobia. Indian J Microbiol 42:183–193

Kalia A, Gupta RP (2005a) Conservation and utilization of microbial diversity. NBA Scientific Bulletin Number 1, National Biodiversity Authority, Chennai, pp 1–40

Kalia A, Gupta RP (2005b) Proteomics: a paradigm shift. Crit Rev Biotechnol 25:173–198

Kennedy IR, Islam N (2001) The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. Aust J Exp Agric 41:447–457

Kilburn MR, Jones DL, Clode PL, Cliff JB, Stockdale EA, Herrmann AM, Murphy DV (2010) Application of nanoscale secondary ion mass spectrometry to plant cell research. Plant Signal Behav 5(6):760–762

Kirk LK, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 54:169–188

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glockner FO (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:1–11. doi:10.1093/nar/gks808

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, vonMering C, Vorholt JA (2012) Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 6(7):1378–1390

Ladd JN, Forster RC, Nannipieri P, Oades JM (1996) Soil structure and biological activity. Soil Biochem 9:23–78

Larsen A, Castberg T, Sandaa RA, Brussaard CPD, Egge J, Heldal M, Paulino A, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Population dynamics and diversity of phytoplankton, bacteria and viruses in a seawater enclosure. Mar Ecol Prog Ser 221:47–57

Lechene C, Luyten Y, McMahon G, Distel D (2007) Quantitative imaging of nitrogen fixation by individual bacteria in animal cells. Science 317:1563–1566

Li M, Xu J, Romero-Gonzalez M, Banwart SA, Huang WE (2012) Single cell Raman spectroscopy for cell sorting and imaging. Curr Opin Biotechnol 23:56–63

Limmer C, Drake HL (1998) Effect of carbon, nitrogen, and electron acceptor availability on anaerobic N_2 Fixation in beech forest soil. Soil Biol Biochem 30:153–158

Liu WT, Marsh TL, Cheng H, Forney IJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lynch JM, Benedetti A, Insam H, Nuti MP, Smalla K, Torsvik V, Nannipieri P (2004) Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biol Fertil Soils 40:363–385

Madsen EL (2011) Microorganisms and their roles in fundamental biogeochemical cycles. Curr Opin Biotechnol 22:456–464

Manefield M, McDougald D, Rice S, Kjelleberg S (2006) Bacterial communication: when does a metabolite become a signal. Microbiol Aust 27:115–117

Miethling R, Wieland G, Backhaus H, Tebbe CC (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. Microb Ecol 40:43–56

Miller KM, Ming TJ, Schulze AD, Withler RE (1999) Denaturing Gradient Gel Electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. Biotechniques 27:1016–1030

Moran MA (2009) Metatranscrpitomics: eavesdropping on complex microbial communities. Microbe 4(7):329–335

Musat N, Foster R, Vagner T, Adam B, Kuyper MMM (2012) Detecting metabolic activities in single cells, with emphasis on nanoSIMS. FEMS Microbiol Rev 36:486–511

Muyzer G, Waal ECD, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nunan N, Wu KJ, Young IM, Crawford JW, Ritz K (2003) Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil. FEMS Microbiol Ecol 44:203–215

O'Donnell AG, Young IM, Rushton SP, Shirley MD, Crawford JW (2007) Visualization, modelling and prediction in soil microbiology. Nat Rev Microbiol 5:689–699

Orr CH, James A, Leifert C, Cooper JM, Cummimgs SP (2011) Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. Appl Environ Microbiol 77(3):911–919. doi:10.1128/AEM.01250-10

Ozsolak F, Milos PM (2011) RNA sequencing: advances, challenges and opportunities. Nat Rev Genet 12:87–98

Ozsolak F, Platt AR, Jones DR, Reifenberger JG, Sass LE, McInerney P, Thompson JF, Bowers J, Jarosz M, Milos PM (2009) Direct RNA sequencing. Nature 461:814–818

Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740

Persing DH, Tenover FC, Tang YW, Versalovic J, Unger ER, Relman DA, White TJ (2004) Molecular microbiology diagnostic principles and practice. ASM Press, NW, Washington, DC, p 632

Pinto AJ, Raskin L (2012) PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. PLoS One 7(8):e43093. doi:10.1371/journal.pone.0043093

Ploug H, Musat N, Adam B, Moraru CL, Lavik G, Vagner T, Bergman B, Kuypers MMM (2010) Carbon and nitrogen fluxes associated with the cyanobacterium *Aphanizomenon* sp. in the Baltic Sea. ISME J 4(9):1215–1223. doi:10.1038/ismej.2010.53

Raes J, Bork P (2008) Molecular eco-systems biology: towards an understanding of community function. Nat RevsMicrobiol 6(9):693–699. doi:10.1038/nrmicro1935

Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl Environ Microbiol 67 (10):4479–4487

Rastogi G, Sani RK (2011) Molecular techniques to assess microbial community structure, function, and dynamics in the environment. In: Ahmad I, Ahmad F, Pichtel J (eds) Microbes and microbial technology: agricultural and environmental applications. Springer, New York, pp 29–57. doi:10.1007/978-1-4419-7931-5_2

Rennert T, Totsche KU, Heister K, Kersten M, Thieme J (2012) Advanced spectroscopic, microscopic, and tomographic characterization techniques to study biogeochemical interfaces in soil. J Soils Sediments 12:3–23

Riaz K, Elmerich C, Moreira D, Raffoux A, Dessaux Y, Faure D (2008) A metagenomic analysis of soil bacteria extends the diversity of quorum-quenching lactonases. Environ Microbiol 10 (3):560–570

Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6(9):981–989

Riffkin PA, Quigley PE, Kearney GA, Cameron FJ, Gault RR, Peoples MB, Thies JE (1999) Factors associated with biological nitrogen fixation in dairy pastures in south-western victoria. Aust J Agric Res 50:261–272

Robinson D (2001) d15N as an integrator of the nitrogen cycle. Trends Ecol Evol 16(3):153–162

Roesch LFW, Fulthorpe RR, Jaccques RJS, Bento FM, Camargo FAO (2010) Biogeography of diazotrophic bacteria in soils. World J Microbiol Biotechnol 26:1503–1508

Rogers SW, Moorman TB, Ong SK (2007) Fluorescent in situ hybridization and micro-autoradiography applied to ecophysiology in soil. Soil Sci Soc Am J 71(2):620–631

Roling WFM, Ferrer M, Golyshin PN (2010) Systems approaches to microbial communities and their functioning. Curr Opin Biotechnol 21:532–538

Santos PCD, Fang Z, Mason SW, Setubal JC, Dixon R (2012) Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 13:162, http://www.biomedcentral.com/1471-2164/13/162

Sardans J, Penuelas J, Rivas-Ubach A (2011) Ecological metabolomics: overview of current developments and future challenges. Chemoecology 21:191–225

Schulze W (2004) Environmental proteomics-what proteins from soil and surface water can tell us: a perspective. Biogeosci Discuss 1:195–218, http://www.biogeosciences.net/bgd/1/195/

Schwartz DC, Cantor CR (1984) Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 37:67–75

Schwartz T, Jungfer C, Heibler S, Friedrich F, Faubel W, Obst U (2009) Combined use of molecular biology taxonomy, Raman spectrometry, and ESEM imaging to study natural biofilms grown on filter materials at waterworks. Chemosphere 77:249–257

Sevilla M, Burris RH, Gunapala N, Kennedy C (2001) Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and *nif*⁻ mutant strains. Mol Plant Microbe Interact 14:359–366

Siering PL (1998) The double helix meets the crystal lattice: the power and pitfalls of nucleic acid approaches for biomineralogical investigations. Am Mineral 83:1593–1607

Sims GK (2007) Stable isotope probing to investigate microbial function in soil. Recent Res Dev Soil Sci 2:1–23

Soni R, Goel R (2011) *nifH* homologs from soil metagenome. Ekologija 57(3):87–95

Tan Z, Hurek T, Vinuesa P, Muller P, Ladha JK, Reinhold-Hurek B (2001) Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. Appl Environ Microbiol 67(8):3655–3664

Tang J (2011) Microbial metabolomics. Curr Genomics 12:391–403

Tiunov AV (2007) Stable isotopes of carbon and nitrogen in soil ecological studies. Biol Bull 34:395–407

Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787

Torsvik V, Daae FL, Sandaa RA, Ovreas L (1998) Review article: novel techniques for analyzing microbial diversity in natural and perturbed environments. J Biotechnol 64:53–62

Turnbaugh PJ, Gordon JI (2008) An invitation to the marriage of metagenomics and metabolomics. Cell 134:708–713

Vercruysse M, Fauvart M, Beullens S, Braeken K, Cloots L, Engelen K, Marchal K, Michiels J (2012) A comparative transcriptome analysis of *Rhizobium etli* bacteroids: specific gene-expression during symbiotic non-growth. Mol Plant Microbe Interact 24:1553–1561. doi:10.1094/MPMI-05-11-0140

Verma SC, Ladha JK, Tripathi AK (2001) Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep-water rice. J Biotechnol 91:127–141

Vervaet H, Boeckx P, Unamuno V, Van Cleemput O, Hofman G (2002) Can d15N profiles in forest soils predict NO^{3-} loss and net N mineralization rates. Biol Fertil Soils 36:143–150

Vollu RE, Blank AF, Seldin L, Coelho MRR (2012) Molecular diversity of nitrogen-fixing bacteria associated with *Chrysopogon zizanioides* (L.) Roberty (vetiver), an essential oil producer plant. Plant Soil 356:101–111

Wagner M (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. Annu Rev Microbiol 63:411–429

Wang HB, Zhang ZX, Li H, He HB, Fang CX, Zhang AJ, Li QS, Chen RS, Guo XK, Lin HF, Wu LK, Lin S, Chen T, Lin RY, Peng XX, Liu WX (2011) Characterization of metaproteomics in crop rhizospheric soil. J Proteome Res 10:932–940

Wang Y, Hayatsu M, Fuji T (2012) Extraction of bacterial RNA from soil: challenges and solutions. Microbes Environ 27(2):111–121

Weber OB, Baldani VLD, Teixeira KRS, Kirchhof G, Baldani JI, Dobereiner J (1999) Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. Plant Soil 210:103–113

Wilmes P, Bond P (2009) Microbial community proteomics: elucidating the catalysts and metabolic mechanisms that drive the Earth's biogeochemical cycles. Curr Opin Microbiol 12(3):310–317

Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS (2002) High density microarray of small subunit ribosomal DNA probes. Appl Environ Microbiol 68:2535–2541

Wu LK, Wang HB, Zhang ZX, Lin R, Zhang ZY, Lin WX (2011) Comparative metaproteomic analysis on consecutively *Rehmannia glutinosa* – monocultured rhizosphere soil. PLoS One 6: e20611

Xie C, Li Y (2003) Confocal micro-Raman spectroscopy of single biological cells using using optical trapping and shifted excitation difference techniques. J Appl Phys 93:2982–2986

Yeager CM, Northup DE, Grow CC, Barns SM, Kuske CR (2005) Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. Appl Environ Microbiol 71(5):2713–2722

Zhang L, Hurek T, Reinhold-Hurek B (2007) A *nifH*-based oligonucleotide microarray for functional diagnostics of nitrogen-fixing microorganisms. Microb Ecol 53:456–470

Chapter 4
Haloalkaliphilic Bacteria: Molecular Diversity and Biotechnological Applications

Megha K. Purohit, Vikram H. Raval, and Satya P. Singh

4.1 Introduction

Extremophiles live in the unusual habitats and can potentially serve in verity applications (Horikoshi 2008; Horikoshi et al. 2011). The groups of bacteria being able to grow in the presence of salt and alkaline pH are referred to as haloalkaliphiles. The dual extremities make them interesting models for fundamental research and exploration of biotechnological potential (Boominadhan et al. 2009; Purohit and Singh 2011; Pandey et al. 2012). They are distributed in the hypersaline and alkaline environments, many in natural hypersaline brines in arid, coastal, and even deep sea locations. Their novel characteristics and capacity for large-scale culturing make haloalkaliphiles potentially valuable in biotechnology (Capes et al. 2012). Most of the studies on haloalkaliphilic bacteria so far have focused on the diversity and phylogenetic analysis, while limited information is available on the enzymatic and other biotechnological potential. Maintenance of the stability and activity in high salt is a challenge for halophilic and haloalkaliphilic proteins (Dodia et al. 2008a, b; Wang et al. 2009). With the present state of the knowledge and molecular tools, the search for further horizons on the diversity and applications would be expanded (Purohit and Singh 2009; Siddhpura et al. 2010).

Towards capturing the unculturable, metagenomics has allowed the mining of the huge microbial potential and understanding of the dynamics, properties, and functions of these organisms (Schmeisser and Stelle 2001; Purohit and Singh 2009; Siddhpura et al. 2010; Singh et al. 2012; Raval et al. 2012). Since the diversity and phylogenetic complexity of the environments can range over orders of magnitude, the potential for applications of the haloalkaliphilic organisms in biotechnology appears quite vast. The extensive studies are required as only limited attention has been paid to haloalkaliphiles, particularly from the moderately saline habitats

M.K. Purohit • V.H. Raval • S.P. Singh (✉)
Department of Biosciences, Saurashtra University, Rajkot, India
e-mail: satyapsingh@yahoo.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_4, © Springer-Verlag Berlin Heidelberg 2014

(Dodia et al. 2008a, b; Joshi et al. 2008; Purohit and Singh 2011; Pandey et al. 2012).

4.2 Saline Habitats

While the saline habitats are relatively simple in ecological aspect, it's equally complex compared to other terrestrial habitats in terms of the diversified metabolism among the constituent groups. The population adapts itself to poly-extremities of the salt and alkalinity. The significant diversity of the saline and hypersaline habitats is reflected in the microbial communities (Oren 2002a, b). Saline habitats with the saturating salt concentrations provide suitable model of the organizational structure for studies in microbial ecology. The hypersaline aquatic habitats have salt concentrations greater than those of normal sea water (3–4 %, w/v). Several halophilic biomes are the saline lakes; evaporate lagoon sediments and coastal salterns. Saline soils and the salt-excreting surfaces of animals are among the least explored habitats (Grant and Mwatha 1998). Since past few decades, these habitats have emerged as the potential ecosystems harboring novel biocatalysts. In view of the scientific achievements during the past decade, in the form of research papers, reviews, and patents, Trincone proposed three major future focus areas: the marine enzymes, the marine natural products, and processes related to biocatalysis (Trincone 2010).

Alkaliphilic microorganisms are widely distributed in nature and can be found in almost all environments with the alkalinity. However, few naturally occurring alkaline environments, such as soda soils, lakes, and deserts, harbor a wide range of these microbes (Kumar et al. 1997). Their ecology has been studied in detail by Grant and Tindall (1986) and Grant et al. (1990). Other habitats include dilute alkaline springs, desert soils, and soils containing decaying proteins or forest soil (Langsworthy 1978; Horikoshi and Akiba 1982; Durham 1987). The pH values of these environments are commonly around 10 and above. The man-made alkaline environments were found to be the effluents from food, textiles, tanneries, potato processing units, paper manufacture units, calcium carbonate kilns, detergents, and other industrial processes (Gee et al. 1980; Joshi and Ball 1993). Highly saline and alkaline environments are relatively rare in the world compared with high saline but neutral environments. However, there is a possibility that such environments harbor a unique microbial population (Tindall et al. 1980, 1984; Grant 1988; Lodwick et al. 1991; Kostrikina et al. 1991; Litchfield and Gillevet 2002; Oren 2008). The best sources for halophilic alkaliphilic have been the extreme soda lakes of the Wadi Natrum in Egypt and Lake Magadi in Kenya (Grant and Tindall 1980). The study of this unique group of microorganisms has aroused interest because of the extreme tolerance of haloalkalophiles to the two environmental extremes, salinity, and high pH. Further, in this category there are moderate thermophiles, with growth temperatures of approximately 40 °C.

4.3 Extremophiles

Extremophiles are often described as the organisms that are adapted to their environments by optimizing their metabolic processes. The extreme conditions may be with respect to the physiological conditions, such as temperature, pressure, and radiation; nutritional, carbon and nitrogen sources along with other nutrients; and geochemical parameters, such as salinity and pH. The microbial life in these extremities is highly diverse and can be found under the extremes of temperature, pressure, radiation, desiccation, salinity, pH, oxygen species, redox potential, metals, and gases (van den Burg 2003). The nomenclature of these organisms is based on the basis of their adaptation strategies. The organisms thriving in harsh acidic conditions are called acidophiles; those that live in the hot water springs and hydrothermal vents are called thermophiles. The microbes that grow facultatively at low and suboptimal temperatures are named psycrophiles. In the similar manner, those adapted to high salt are referred as halophiles, while the communities able to grow in alkaline pH are called alkaliphiles. Further, the organisms classified as barophiles grow at high pressure in the deep oceans.

4.3.1 Halophiles and Alkaliphiles

Halophiles dominate the hypersaline environments where salt concentration is more than 2.5 M. Therefore, the halophiles and their enzymes are active and stable in high salt (Mevarech et al. 2000). The halophiles include prokaryotic and eukaryotic microorganisms able to balance the osmotic pressure of the environment and resist the denaturing effects of salts. They are loosely classified as slightly, moderately, or extremely halophilic, based on their requirement for NaCl. The extremely halophilic archaea are well adapted to saturating concentrations of NaCl and have developed number of novel strategies, such as the enzymes that function in saturated salts, purple membrane that allows phototrophic growth, sensory rhodopsins that mediate the phototactic response and gas vesicles that promote cell flotation. The non-halophilic microorganisms able to tolerate high salt concentrations are referred as halotolerant or extremely halotolerant organisms (Kushner and Kamekura 1988; de la Haba et al. 2011). The halophiles are distributed in hypersaline environments around the globe, such as natural hypersaline brines in arid, coastal, and even deep sea locations. The artificial salterns used to mine salts from the sea may also harbor extreme halophiles. Their novel characteristics and suitability for the large-scale cultivation make them highly valuable for biotechnology (Das Sarma and Arora 2001). The microorganisms display a normal distribution pattern based on their pH requirement for the optimal growth. The majority of these microorganisms proliferate well at near-neutral pH. The number of the microorganisms decreases as the pH deviates from the neutral. The alkalophilic bacteria in the soil account for 1/10 to 1/100 of the neutrophilic bacteria. However,

some neutrophilic organisms are capable of growth even at extreme pH conditions. This is primarily due to the special physiological and metabolic systems, adopted by the bioenergetic membrane and transport mechanisms, enabling their survival and multiplication under such adverse conditions (Krulwich and Guffanti 1983; Krulwich 1986; Krulwich et al. 1990). Such microorganisms can be described as pH-dependent extremophiles. The organisms with pH optima for growth in excess of pH 9 are defined as alkaliphiles, which include prokaryotes, eukaryotes, and archaea. The alkalophilic microorganisms constitute a diverse group that thrives in the alkaline environments. They are categorized into two broad groups: alkaliphiles and alkalitolerants. The term alkaliphiles is referred for those organisms that are capable to growth above pH 10, with the optimal growth around pH 9, but unable to grow at pH 7 or less, while the alkalitolerants are capable of growing at pH values in excess of 10, but have optimal growth at the neutrality (Krulwich 1986). The extreme alkaliphiles have been further subdivided into two groups: facultative and obligate alkaliphiles. The facultative alkaliphiles with the optimal growth at pH 10 or above can grow well at neutrality, while the obligate alkalophiles are unable to grow at neutrality (Krulwich and Guffanti 1989).

4.3.2 Haloalkaliphiles

The haloalkaliphiles are adapted to grow at high salinity and alkaline pH. These properties make them interesting systems for the fundamental research and the exploration of biotechnological potential. Studies on the haloalkaliphiles started in early 1980s and during the later decades the focus has been on the ecology, physiology, and taxonomy. The work on these microbes revealed significant diversity in highly saline and alkaline lakes. The occurrence of the haloalkaliphiles was probably first described by Tindall and co-workers in 1984. The haloalkaliphilic bacteria, beyond the boundaries of their conventional habitats; Soda Lakes and Dead Seas have been reported from India. The occurrence and heterogeneity of the haloalkaliphilic bacteria from the man-made and natural saline habitats along the Coastal Gujarat in India have been investigated over the last several years (Dodia et al. 2006, 2008a, b; Joshi et al. 2008; Nowlan et al. 2006; Patel et al. 2005, 2006a, b; Pandey and Singh 2012; Pandey et al. 2012; Purohit and Singh 2012). Besides the diversity and phylogeny of these bacteria, the secretion of extracellular enzymes and the stability of the extracellular enzymes are also investigated. The majority of the isolates can grow over a wide range of salt concentration 5–30 % (w/v) and pH 7–10. The salt and pH requirement varies among the isolates obtained from the same sites indicating extensive diversity. With the increasing extremity of pH and salt during the enrichment for the isolation, the diversity and number of the haloalkaliphilic bacteria decreased (Singh et al. 2012).

4.4 Adaptive Strategies

4.4.1 Salt Adaptation

The halophilic proteins are highly acidic in nature and thus would denature in low salt. The other strategies are the exclusion of salt from the cytoplasm and to synthesize and accumulate organic solutes which are noninterfering with the enzymes. The organisms having organic solutes in the cell can adapt to a broader salt concentration range (Cho 2005; Kunte 2006). Majority of the halophilic bacteria and the halophilic methanogenic archaea accumulate organic solutes as a strategy to maintain osmotic balance. There are a number of compatible solutes, which include glycine, betaine, ectoine, and other amino acid derivatives, sugars, and sugar alcohols. Many are either uncharged or zwitterionic (Galinski 1986; Roberts 2005, 2006).

The "high-salt-in strategy" is not limited to the *Halobacteriaceae*. The *Halanaerobiales (Firmicutes)* also accumulate salt rather than organic solutes. A third, phylogenetically unrelated group of the organisms accumulates KCl. The third group, in which the "high-salt-in-strategy" was recently identified, is the aerobic red extremely halophilic Salinibacter ruber (Bacteroidetes) isolated from the saltern crystallizer brines (Oren et al. 2004). Analysis of its genome revealed many points of resemblance with the *Halobacteriaceae*, probably resulting from the extensive horizontal gene transfer.

4.4.2 Compatible Solutes

The osmotic pressure of the surrounding as compared to the cytoplasm within the cell is very high due to the high ionic composition of the hypersaline habitats. The strategies for osmoregulation in a several haloalkaliphilic organisms have been studied and described. The halotolerant and moderate halophiles either synthesize or take up specific organic molecules as their compatible solutes to balance the high external osmotic pressure. *Halomonas elongata* and obligate halophilic archaeon *Methanohalophilus portucalensis* maintain high concentration of glycine betaine as compatible solutes from the environment. The glycine betaine transport in *Halobacillus halophillus* depends upon the intracellular Cl^- concentrations. Haloalkaliphilic, sulfur-oxidizing bacterium, *Thioalkalivibrio versutus* strain produced glycine betaine as a main organic compatible solute under high NaCl concentrations. Certain organisms also synthesize sugars such as sucrose or trehalose as their compatible solutes to balance the external osmotic pressure (Welsh 2000). A haloalkaliphilic methanotrophs accumulates sucrose and 5-oxo-1-proline, in addition to the synthesis of ectoine at high NaCl concentrations. With respect to archaea, unusual solutes such as β-amino acids, *N*-acetyl-β-lysine, mannosylglycerate, and di-myo-inositol phosphate are synthesized. In these cases, the uptake of the solutes is preferred against de novo synthesis. There are instances where

some solutes are not only produced in response to salt but also to temperature stress (Muller et al. 2005).

The organisms survive under high salt concentrations and at low temperatures mainly due to the accumulation of the compatible solute, glycine betaine, which is *N*-trimethyl derivative of glycine. It's accumulated at high concentration in the cell through synthesis, uptake, or combination of both. *Bacillus subtilis* possess three transport systems for glycine betaine: the secondary uptake system opuD and two binding-protein-dependent transport systems, opuA and opuC (proU). The secondary transport system betP is involved in glycine betaine accumulation in *Corynebacterium glutamicum* (Sleator et al. 1999). The characterization and disruption of betL, a gene involved in glycine betaine uptake in *Listeria monocytogenes* has been studied. Growth at 10 % NaCl and temperature as low as 20 °C has been reported for this organism. The ability of the organism to survive both high salt and low temperatures is attributed mainly to the accumulation of the compatible solute glycine betaine. The genetic basis of glycine betaine uptake in gram-positive bacteria and even in plants has been studied extensively (Kappes et al. 1996; Sakamoto and Murata 2001; Boscari et al. 2002; Sulpice et al. 2003).

4.4.3 Sodium Pump

The extremely halophilic archaea and few bacteria have developed an active Na^+ and K^+ antiport leading to the exclusion of Na^+ from the cell to compensate high external concentrations of Na^+. The H^+ gradient is also used for either the excretion of Na^+ or ATP formation. The findings on the salt adaptation indicate that the adaptation might have been among the earliest developments during the evolution among the Halophiles (Oren 1999, 2000).

4.4.4 Chloride Pump

Two groups of bacteria, anaerobic *Halanaerobiales* and the aerobic extremely halophilic *Salinibacter* rubber, demonstrate high requirement for chloride. These organisms accumulate inorganic salts rather than organic osmotic solutes. Thus, chloride has specific functions in halo-adaptation in some groups of halophilic microorganisms (Muller and Oren 2003).

4.4.5 Osmoregulation

As discussed in earlier sections, osmoregulation is a fundamental phenomenon developed by bacteria, fungi, plants, and animals to balance osmotic stress. The

accumulation of compatible solutes protects the cells and allows growth. The solutes can be either taken up from the environment or synthesized in the cell preventing water loss from the cell and plasmolysis. Since the water permeability of the cytoplasmic membrane is high, imposed imbalances between turgor pressure and the osmolality gradient across the bacterial cell wall are short in duration. Bacteria respond to osmotic upshifts in three overlapping phases: dehydration (loss of some cell water), adjustment of cytoplasmic solvent composition, and rehydration and cellular remodeling. Responses to osmotic downshifts also proceed in three phases: water uptake (phase I), extrusion of water and cosolvents (phase II), and cytoplasmic cosolvent re-accumulation and cellular remodeling (phase III) (Munns 2005).

4.4.6 pH

According to several studies, the alkaliphiles maintain a neutral cytoplasmic pH. In some cases, the cell wall plays a key role in protecting the cell from the alkaline environment. In addition to peptidoglycan, alkaliphilic *Bacillus* spp. contain certain acidic polymers such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid. The negative charges on the acidic non-peptidoglycan components may give the cell surface its ability to absorb sodium and hydronium ions and repulse hydroxide ions. This, as a consequence, may help cells to grow under alkaline environments. The sodium ions in the surrounding environment have apparently proved essential for effective solute transport through the membranes of alkaliphilic *Bacillus* spp. These characteristics may provide better adaptive mechanisms to haloalkaliphilic bacteria, because they require high salt concentration along with high pH.

4.4.7 Light

Many halophilic archaea live in shallow evaporation pond encountering high temperature and ultraviolet light. Such organisms harbor a special retinal pigments, carotenoids, which provide protective barrier against the ultraviolet light. *Salinibacter ruber*, a red extreme halophile, phylogenetically belonging to the Bacteroidetes branch of the Bacteria, coexists with Archaea of the family Halobacteriaceae in NaCl-saturated saltern crystallizer ponds and in other hypersaline environments at halite saturation. Carotenoid pigments such as C-40, substituted carotenoid (“salinixanthin”) and Retinal pigments Bacteriorhodopsin (“xanthorhodopsin”), halorhodopsin, and sensory rhodopsins are derived from Salinibacter ruber, while C-50 bacterioruberins, bacteriorhodopsin, halorhodopsin, and sensory rhodopsins are reported from other halobacteriaceae (Oren 2008).

4.5 Phylogeny of Haloalkaliphiles

The microbial diversity has focused renewed emphasis and in this context, haloalkaliphiles hold great significance among the microbial world. The efforts to understand the diversity of the halophilic microorganisms have indicated that what we know is just a small fraction of the huge microbial diversity existing in various saline habitats (Singh et al. 2012; Bagheri et al. 2012). Both molecular and classical studies have revealed the presence of moderately to extremely halophilic microorganisms in a wide range of the saline environments. The studies have revealed the extensive diversity of the aerobic/anaerobic halophilic bacteria and archaea in the moderate to saline or hypersaline environments (Jacob and Irshaid 2012). During the recent years, culture-dependent and culture-independent approaches have been employed to explore and understand the microbial diversity (Stewart 2012).

4.5.1 The Phylogeny

Although the genetic data and molecular techniques are extensively being used for the identification and phylogenetic relatedness of the prokaryotes and archaebacteria during the last many years, the traditional phylogenetic methods have their own importance. The parameters include ecological and growth characteristics of the organisms. The halophilic organisms display wide variations in biochemical, metabolic, and physiological properties. The phylogeny of the halophiles expands the physicochemical boundaries for life and will extend the maximum salinity that can support the microbial growth (Wackett 2012). During the past decade, studies on the ecology, physiology, and taxonomy of the haloalkaliphiles/haloarchaea revealed significant diversity in highly saline and alkaline environment (Hoover et al. 2003; Capes et al. 2012). The metabolic groups of the halophiles includes oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens (Hedi et al. 2009).

4.5.1.1 Morphological Features and Microbial Diversity

Moderate to hyper halophilic/haloalkaliphilic bacteria possess round, regular shape and opaque/smooth texture. The variability in the colony characters decreases with the increasing salt concentrations, indicating reduced diversity with the increasing extremity (Dodia et al. 2006, 2008a, b; Joshi et al. 2008; Purohit and Singh 2011, 2012; Pandey 2012; Purohit 2012; Rawal et al. 2012; Pandey et al. 2012; Rawal 2012; Raval 2012). Certain bacteria possess red to brownish pigmentation, an inherent feature of some haloalkaliphiles. The moderate halophiles/haloalkaliphiles have been widely reported with Gram negative characteristics, while information relating to Gram positive is rather scarce (Doronina et al. 2003; Loiko et al. 2003;

Hoover et al. 2003, Purohit and Singh 2011, 2012; Rawal et al. 2012; Pandey et al. 2012). The coccid cell shape are widely observed among the bacteria enriched at higher NaCl concentrations and pH, where as the rod shape was frequently distributed at lower range of salt (Banciu et al. 2004; Romano et al. 2002). Such observations are useful for primary characterization and could be used to assess the initial level diversity among the isolates (Joshi et al. 2008; Oren 2008; De la Haba et al. 2011).

4.5.1.2 Metabolic Diversity

In the era of the increasing emphasis on the molecular tools and chronometers, the metabolic and physiological features of the organisms are still important in diversifying the microorganisms. In fact, the metabolic diversity of the halophiles existing in nature is as significant as their phylogenetic diversity. The biochemical and metabolic blueprints of the organism are responsible for the bioenergetics, biosynthesis, and biodegradation.

4.5.1.3 Biochemical Profiling

The microorganisms have their own identifying biochemical characteristics. The variation in oxygen requirements reflects the differences in bio-oxidative enzyme systems of the organisms. The majority of the microbial processes that occur at low salt concentrations can also be found up to reasonably high salinities (Das Sarma and Arora 2001). Autotrophic nitrification and methanogenesis processes usually do not occur at salt concentrations above 100–150 g/l (Oren 2008). The bioenergetics strategies could be related to the adaptation process of the microbes (Guzman et al. 2010).

Photosynthetic processes are not energy deficient, and dissimilatory processes require large amount of energy (Hamana et al. 2012). The synthesis of organic osmotic compounds or the accumulation of KCl uses salt to balance the salinity of the medium (Saum et al. 2012). Halophiles generally cannot utilize urea and tryptophan suggesting the absence of the relevant enzymes, urease and tryptophanase (Romano et al. 2002; Reddy 2008). Similarly, the ability of the organisms to metabolize different sugars is also one of the approaches to diversify them. The utilization of the disaccharides along with simple carbon sources suggested the adaptation of different metabolic pathways for the energy generation (Ates et al. 2011).

4.5.1.4 Antibiotic Resistance and Antagonistic Effect

The halophiles have been recognized as valuable sources of novel biomolecules, which also include antimicrobials (Dodia et al. 2008a, b; Jacob and Irshaid 2012).

However, molecular and genetic basis of the antibiotic resistance in the halophiles is not yet explored at greater length. Resistance genes, in general, are plasmid born (Vargas and Nieto 2004). The differential responses are generated by the organisms, where certain genes for the protein and cell wall synthesis might be repressed at higher salt concentrations (Asha et al. 2005).

4.5.2 Molecular Diversity

The widespread applications of the molecular techniques in studying microbial communities has greatly enhanced our understanding of the microbial diversity and functions in the natural environment leading to avenues for the novel commercially viable products (Stewart 2012; Garapati and Suryanarayana 2012).

4.5.2.1 PCR-Based Approaches

PCR-based techniques allow the classification of microorganisms based on particular genetic markers and the profiling of the complex microbial communities based on the sequence diversity (Bach et al. 2001). DNA-based technology for the identification of bacteria usually implies the 16S rRNA genes (Purohit and Singh 2011; Amoozegar et al. 2012). Molecular approaches based on 16S rRNA gene sequence analysis provide the basis for the direct investigation of the community structure, diversity, and phylogeny of the microorganisms in almost any environment (Phillips et al. 2012). The combination of the Fatty Acid Methyl Ester (FAME) profile coupled with the analysis of the 16S rRNA gene sequences could be highly significant (Surve et al. 2012). Further, genetic fingerprinting techniques can also be used for the phylogenetic identification (Jacob 2012). In order to get insight into the structure and functional relationship at the genetic level, the whole genome sequencing of the halophiles/haloalkaliphiles is the need of the hour as reflected by some of the recent publications (Yeganeh et al. 2012; Rawal et al. 2012).

4.5.2.2 Diversity of the Unculturables

The molecular approaches developed during the past couple of decades have allowed to access and study the microbial diversity in its entirety, irrespective of their ability to grow under the laboratory conditions. This has opened the unexplored doors, allowing access to the vast range of the natural products (Gilbert 2010; Glockner et al. 2010). The advent of the culture-independent techniques has changed the way the microbial communities have been looked at and explored (Eamonn et al. 2012). The technological advances in sequencing and cloning along with the improvements in annotation and comparative sequence analysis have

generated valuable information on the microbial ecology and tapping the vast arena of the biological wealth (Raes et al. 2007; Glockner et al. 2010; Ferrer et al. 2011).

4.6 Biotechnological Applications of the Haloalkaliphiles

Studies on the haloalkaliphiles stem from their importance in the ecology of the saline habitats and relevance in varied industrial application (Rothschild and Manicinelli 2001; Karan et al. 2012). The economically viable products are metabolites, extracellular enzymes, osmotically active substances, exo-polysaccharides, and special lipids (Li et al. 2012; Dawson et al. 2012). The search and the development of novel products which can function at the extreme environmental conditions have focused the interest of the scientific communities during the recent years (Ruiz et al. 2012).

4.6.1 Compatible Solutes

As discussed in the above sections, the compatible solutes are excellent stabilizers. Ectoine has been shown to protect skin from UVA-induced cell damage (Desmarais et al. 1997). RonaCare™ Ectoin, produced by Merck KgaA, Darmstadt, is used as a moisturizer in the cosmetics and skin care products. Ectoine and derivatives have been patented as moisturizers in cosmetics (Montitsche et al. 2000).

4.6.2 Antimicrobial Substances

The biological diversity of the marine environment has enormous scope for the discovery of the novel natural products (Kokare et al. 2004). The extremophiles have been recognized as valuable sources of novel bioproducts and this may include antimicrobials (Fiedler Forsyth et al. 1971; Hassanshahian and Mohamadian 2011).

4.6.3 Bacteriorhodopsin

Certain extremely halophilic and haloalkaliphilic bacteria contain membrane bound retinal pigments called bacteriorhodopsin (BR) and halorhodopsin (HR). These pigments can be useful as the special light modulators, artificial retina, neural network optical computing devices, and volumetric and associative memories. Recently, the cloning and functional expression of archaerhodopsin gene from *Halorubrum xinjiangense* was successfully achieved in *Escherichia coli*, where

the purple membrane was fabricated into films depending on the light-on and light-off stimuli (Feng et al. 2006).

4.6.4 Biosurfactants and Exopolysaccharides

Biosurfactants enhance the remediation of oil-contaminated soil and water and have potential for pollution treatment in marine environments and coastal region (Banat et al. 2000). The biosurfactants from the extremophiles can be used for in situ microbially enhanced oil recovery (MEOR).

Exopolysaccharide (EPS) producers could be interesting source for MEOR, where polymers with appropriate properties act as emulsifiers and control the mobility (Hamana et al. 2012).

4.6.5 Food Biotechnology

Haloalkaliphilic microorganisms play important role in various fermentation processes that take place in the presence of salt and alkaline pH. They produce various compounds that give characteristic taste, flavor, and aroma to the products. Certain halophiles and haloalkaliphiles are used in the production of an Asian (Thai) fish sauce, where fish is fermented in concentrated brine (Thongthai and Suntinanalert 1991; Akolkar et al. 2010). Halophiles can also be used for the production of single cell proteins which is used as additive for nutritive supplements (Taran and Bakhtiyari 2012).

4.6.6 Degradation of Aromatic Compound and Pollutants

There are only few reports related to the degradation of the aromatic compounds under highly saline conditions by the halophilic and haloalkaliphilic bacteria. The ability to oxidize hydrocarbons in the presence of salt and alkaline pH would be useful for the biological treatment of the saline ecosystems contaminated with the petroleum products (Margesin and Schinner 2001; Gayathri and Vasudevan 2010; Feng et al. 2012).

4.6.7 Enzymes

Haloalkaliphiles possess range of biocatalysts which enable them to survive under the extreme conditions of the salinity and alkaline pH (Debashish et al. 2005). Among

the enzymes; proteases, carbohydrases, amylase, and peroxidases are most explored (Essghaier et al. 2012; Pandey et al. 2012; Purohit and Singh 2011). The unique features of the enzymes are reflected in their stability to function at the elevated physicochemical conditions, such as pH and salt (Antranikian et al. 2005; Ferrer et al. 2011; Purohit and Singh 2011).

4.6.8 Computational Approach

There is constant need for developing new ways of comparing multiple sets of data, in the wake of the generation of huge number of sequences and information. In silico analysis could be possible on account of the features of varied halophilic/ haloalkaliphilic organisms (Ukani et al. 2010). Further, the comparative genomics approaches and bioinformatics web-based tools are used for the identification and annotation of the genes. This approach could help in addressing the hypothetical proteins existing in the extremophiles (Sardeshmukh et al. 2012).

4.7 Conclusions

Although there is significant advancement in studies related to halophiles and haloalkaliphils, the genomics and proteomics with respect to the microbial diversity of saline habitats are in inceptions. Further studies would provide information and insights into the adaptation of these microbes and exploration of the potential application avenues. The future studies would, therefore, focus on the diversity, molecular phylogeny, population dynamics, and the structural basis of the protein stability under the multitude of extreme conditions.

References

Akolkar AV, Durai D, Desai AJ (2010) *Halobacterium sp.* SP1 (1) as a starter culture for accelerating fish sauce fermentation. J Appl Microbiol 109(1):44–53

Amoozegar ML, Bagheri M, Didari M, Shahzedeh Fazeli SA, Schumann P, Sanchez-Porro C, Ventosa A (2012) *Saliterribacillus persicus* gen. nov., sp. nov., a moderately halophilic bacterium isolated from the Iranian hypersaline lake Aran-Bidgol. Int J Syst Evol Microbiol. doi:ijs.0.041640-0v1-ijs.0.041640

Antranikian G, Vorgias CE, Bertoldo C (2005) Extreme environments as a resource for microorganisms and novel biocatalysts. Adv Biochem Eng Biotechnol 96:219–262

Asha KRT, Vinitha DA, Kiran SG, Manjusha W, Sukumaran N, Selvin J (2005) Isolation and cultivation of Halophilic archaea from solar salterns located in peninsular coast of India. Int J Microbiol. doi:10.5580/15cb

Ates O, Oner ET, Arga KA (2011) Genome-scale reconstruction of metabolic network for a halophilic extremophile, *Chromohalobacter salexigens* DSM 3043. BMC Syst Biol 5:12

Bach HJ, Hartmann A, Schloter M, Munch JC (2001) PCR primers and functional probes for amplification and detection of bacterial genes for extracellular peptidases in single strains and in soil. J Microbiol Methods 44:173–182

Bagheri M, Amoozegar MA, Schumann P, Didari M, Mehrshad M, Sproer C, Cristina Sanchez-Porro C, Ventosa A (2012) *Ornithinibacillus halophilus* sp. nov., a moderately halophilic Gram-positive endospore-forming bacterium from an Iranian hypersaline lake. IJSEM. doi:10.1099/ijs.0.041350-0

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508

Banciu H, Sorokin DY, Galinski EA, Muyzer G, Kleerebezem R, Kuenen JG (2004) *Thialkalivibrio halophilussp*. nov, a novel obligately lithoautotrophic, facultatively alkaliphilic, and extremely salt-tolerant, sulfur-oxidizing bacterium from a hypersaline alkaline lake. Extremophiles 8:325–334

Boominadhan U, Rajakumar R, Sivakumaar PKV, Melvin M (2009) Optimization of protease enzyme production using Bacillus sp. isolated from different wastes. Joe Bot Res Int 2(2):83–87

Boscari A, Mandon K, Dupont L (2002) *BetS* is a major glycine betaine/proline betaine transporter required for early osmotic adjustment in *Sinorhizobium meliloti*. J Bacteriol 184:2654–2663

Capes MD, DasSarma P, DasSarma S (2012) The core and unique proteins of haloarchaea. BMC Genomics 13:39

Cho BC (2005) Heterotrophic flagellates in hypersaline waters. In: Gunde-Cimerman N, Oren A, Plemenitas A (eds) Adaptation to life at high salt concentrations in archaea, bacteria, and Eukarya. Springer, Dordrecht, Netherlands

Das Sarma S, Arora P (2001) Halophiles. Encyclopedia of Life Science. Nature Publishing Group, London, pp 1–9

Dawson KS, Freeman KH, Macalady JL (2012) Molecular characterization of core lipids from halophilic archaea grown under differen*t salinity conditions*. Org Geochem 48:1–8

De la Haba RR, Sanchez-Porro C, Marquez MC, Ventosa A (2011) Taxonomy of halophiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo

Debashish G, Malay S, Barindra S, Joydeep M (2005) Marine enzymes. Adv Biochem Eng Biotechnol 96:189–218

Desmarais D, Jablonski PE, Fedarko NS, Roberts MF (1997) 2-Sulfotrehalose, a novel osmolyte in haloalkaliphilic archaea. J Bacteriol 179:3146–3153

Dodia MS, Joshi RH, Patel RK, Singh SP (2006) Characterization and stability of extracellular alkaline proteases from moderately halophilic and alkaliphilic bacteria isolated from saline habitat of coastal Gujarat, India. Braz J Microbiol 37:244–252

Dodia MS, Bhimani HG, Rawal CM, Joshi RH, Singh SP (2008a) Salt dependent resistance against chemical denaturation of alkaline protease from a newly isolated Haloalkaliphilic *Bacillus* sp. Bioresour Technol 99:6223–6227

Dodia MS, Rawal CM, Bhimani HG, Joshi RH, Khare SK, Singh SP (2008b) Purification and stability characteristics of an alkaline serine protease from a newly isolated *Haloalkaliphilic bacterium* sp. AH-6. J Ind Microbiol Biotechnol 35:121–131

Doronina NV, Darmaeva T, Trotsenko Y (2003) *Methylophaga natronica* sp. nov., a new alkaliphilic and moderately halophilic, restricted-facultative methylotrophic bacterium from soda lake of the Southern Transbaikal region. J Syst Appl Microbiol 26:382–389

Durham DR (1987) Utility of subtilisin GX as a detergent additive. J Appl Bacteriol 63:381–386

Eamonn PC, Roy DS, Julian RM, Colin H (2012) Functional metagenomics reveals novel salt tolerance loci from the human gut microbiome. ISME J. doi:10.1038/ismej.2012.38

Essghaier B, Hedi A, Bejji M, Jijakli H, Boudabous A, Sadfi-Zouaoui N (2012) Characterization of a novel chitinase from a moderately halophilic bacterium, *Virgibacillus marismortui* strain M3-23. Ann Microbiol 62:835–841

Feng J, Liu H, Chu J, Zhou P, Tang J, Liu S (2006) Genetic cloning and functional expression in *Escherichia coli* of an archaerhodopsin gene from *Halorubrum xinjiangense*. Extremophiles 10 (1):29–33

Feng TC, Cui CZ, Dong F, Feng YY, Liu YD, Yang XM (2012) Phenanthrene biodegradation by halophilic *Martelella* sp. AD-3. J Appl Microbiol. doi:10.1111/j.1365-2672.2012.05386.x

Ferrer M, Guazzaroni ME, Richter M et al (2011) Taxonomic and functional metagenomic profiling of the microbial community in the anoxic sediment of a sub-saline shallow lake (Laguna de Carrizo, Central Spain). Microb Ecol 62:824–837

Fiedler Forsyth MP, Shindler DB, Gochnauer MB, Kushner DJ (1971) Salt tolerance of intertidal marine bacteria. Can J Microbiol 17:825–828

Galinski EA (1986) Osmoadaptation in bacteria. Adv Microb Physiol 37:273–328

Garapati UK, Suryanarayana T (2012) Isolation of two strong poly (U) binding proteins from moderate halophile *Halomonas eurihalina* and their identification as cold shock proteins. PLoS One 7(4):e34409

Gayathri KV, Vasudevan N (2010) Enrichment of phenol degrading moderately halophilic bacterial consortium from saline environment. J Biorem Biodegrad. doi:10.4172/2155-6199.1000104

Gee JM, Lund BM, Metcalf G, Peel JL (1980) Properties of a new group of alkalophilic bacteria. J Gen Microbiol 117:9–17

Gilbert J (2010) Aquatic metagenome library generation and analysis. Nature. doi:10.1007/978-3-540-77587-4-340

Glockner J, Kube K, Shrestha P, Marc W, Glockner FO, Reinhardt R, Liesack W (2010) Identification of novel catalyst by caseetee construction from sponges through metagenomic approaches. Environ Microbiol 12(5):1218–1229

Grant WD (1988) Bacteria from alkaline, saline environments and their potential in biotechnology. J Chem Technol Biotechnol 42:291–294

Grant WD, Mwatha WE (1998) Bacteria from alkaline, saline environments. In: Ishida Y, Maruyama Y, Morita RY, Uchida A, Hattori T (eds) Recent advances in microbial ecology. Japan Scientific Societies Press, Japan

Grant WD, Tindall BJ (1980) The isolation of alkalophilic bacteria. In: Gould GW, Corry JCL (eds) Microbial growth and survival in extremes of environments. Academic, London

Grant WD, Tindall BJ (1986) The alkaline saline environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic, London

Grant WD, Mwatha WE, Jones BE (1990) Alkaliphiles: ecology, diversity and applications. FEMS Microbiol Rev 75:255–270

Guzman D, Quillaguaman J, Munoz M, Hatti-Kaul R (2010) *Halomonas andesensis sp*. nov., a moderate halophile isolated from the saline lake Laguna Colorada in Bolivia. Int J Syst Evol Microbiol 60:749–753

Hamana K, Kamekura M, Onishi A, Kazawa T, Matsuzaki S (2012) Polyamines in photosynthetic eubacteria and extreme-halophilic archaebacteria. J Biochem 97:1653–1658

Hassanshahian M, Mohamadian J (2011) Isolation and characterization of *Halobacterium* in Iran. Jundishapur J Microbiol 4:S59–S65

Hedi A, Sadfi N, Fardeau ML et al (2009) Studies on the biodiversity of halophilic microorganisms isolated from El-Djerid Salt Lake (Tunisia) under aerobic conditions. Int J Microbiol. doi:10.1155/2009/731786

Hoover RB, Pikuta EV, Bej AK, Marsic D, Whitman WB, Tang J, Krader P (2003) *Spirochaeta americana* sp. nov., a new haloalkaliphilic, obligately anaerobic spirochaete isolated from soda Mono Lake in California. Int J Syst Evol Microbiol 53:815–821

Horikoshi K (2008) Past, present and future of extremophiles. Extremophiles 12:1–2

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms: a new microbial world. Japan Scientific Societies Press, Tokyo, Japan

Horikoshi M, Nakajima S, Masahito U, Mukaiyama T (2011) Extremophiles handbook bioorganisms – K Japan Science and Technology Agency. Exploratory Research for Advanced Technology (ERATO). Macroscopic Quantum Control Project 2:113–8656

Jacob NP (2012) Investigating Arabia mountain: a molecular approach. Science 335:1588

Jacob JH, Irshaid FI (2012) Biochemical and molecular taxonomy of a mild halophilic strain of *Citrobacter* isolated from Hypersaline environment. Res J Microbiol 7:731–738

Joshi A, Ball B (1993) Extracellular alkaline enzymes of facultative bacteria of $CaCO_3$ kilns near Jabalpur. Indian J Microbiol 33:179–183

Joshi RH, Dodia MS, Singh SP (2008) Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium. Biotechnol Bioprocess Eng 13:552–559

Kappes RM, Kempf B, Bremer E (1996) Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J Bacteriol 178:5071–5079

Karan R, Capes MD, Das Sarma S (2012) Function and biotechnology of extremophilic enzymes in low water activity. Aquat Biosyst 8:4

Kokare CR, Mahadik KR, Kadam SS, Chopade BA (2004) Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* sp. AH1 from the west coast of India. Curr Sci 86:593–597

Kostrikina NA, Zvyagintseva IS, Duda VI (1991) Ultrastructural organization of the extremely haloalkalophilic archaebacteria *Natronobacterium pharaonis* and *Natronococcus ocultus*. Microbiology 59:710–713

Krulwich TA (1986) Bioenergetics of alkalophilic bacteria. J Membr Biol 89:113–125

Krulwich TA, Guffanti AA (1983) Physiology of acidophilic and alkalophilic bacteria. Adv Microb Physiol 24:173–214

Krulwich TA, Guffanti AA (1989) Alkalophilic bacteria. Annu Rev Microbiol 43:435–463

Krulwich TA, Guffanti AA, Seto-Young D (1990) pH homeostasis and bioenergetic work in alkalophiles. FEMS Microbiol Rev 75:271–278

Kumar CG, Tiwari MP, Jany KD (1997) Screening and isolation of alkaline protease producers from soda soils of Karnal, India. In: Proceedings of first national symposium on extremophiles, 20–21 Mar 1997, Hamburg, Germany

Kunte HJ (2006) Osmoregulation in bacteria: compatible solute accumulation and osmosensing. Environ Chem 3:94–99

Kushner DJ, Kamekura M (1988) Physiology of halophilicneubacteria. In: Rodriguez-Valera F (ed) Halophilic bacteria, vol 1. CRC Press, Boca Raton, FL

Langsworthy TA (1978) Microbial life in extreme pH values. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, New York

Li X, Wang HL, Li T, Yu HY (2012) Purification and characterization of an organic solvent-tolerant alkaline cellulase from a halophilic isolate of *Thalassobacillus*. Biotechnol Lett 34:531–1536

Litchfield CD, Gillevet PM (2002) Microbial diversity and complexity in hypersaline environments: a preliminary assessment. J Ind Microbiol Biotechnol 28:48–55

Lodwick D, Ross HNM, Walker JA, Almond JW, Grant WD (1991) Nucleotide sequence of the 16S ribosomal RNA gene from the haloalkalophilic archaeon (archaebacterium) *Natronobacterium magadii* and the phylogeny of halobacteria. Syst Appl Microbiol 14:352–357

Loiko NG, Soina VS, Sorokin DY, Mitiushina LL, El'-Registan GI (2003) Resting forms of gram negative chemolithoautotrophic bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum*. Microbiologiia 72:328–337

Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5:73–83

Mevarech M, Frolow F, Gloss LM (2000) Halophilic enzymes: proteins with a grain of salt. Biophys Chem 86:155–164

Montitsche L, Driller H, Galinski E (2000) Ectoine and ectoine derivatives as moisturizers in cosmetics. US Patent 060071

Muller V, Oren A (2003) Metabolism of chloride in halophilic prokaryotes. Extremophiles 7 (4):261–266

Muller V, Spanheimer R, Santos H (2005) Stress response by solute accumulation in archaea. Curr Opin Microbiol 8:729–736

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663
Nowlan B, Dodia MS, Singh SP, Patel BKC (2006) *Bacillus okhensis sp*. nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan. Int J Syst Evol Microbiol 56:1073–1077
Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348
Oren A (2000) Life at high salt concentrations. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 3rd edn. Springer, New York
Oren A (2002a) Diversity of halophilic microorganisms: environments, phylogeny, physiology, applications. J Ind Microbiol Biotechnol 28:56–63
Oren A (2002b) Halophilic microorganisms and their environments. Kluwer, Dordrecht
Oren A (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Systems 4:2. doi:10.1186/1746-1448-4-2
Oren A, Rodriguez-Valera F, Anton J, Benlloch S, Rossello-Mora R, Amann R, Coleman J, Russell NJ (2004) Red, extremely halophilic, but not archaeal: the physiology and ecology of *Salinibacter ruber*, a bacterium isolated from saltern crystallizer ponds. In: Ventosa A (ed) Halophilic microorganisms. Springer, Berlin, pp 63–76
Pandey S (2012) Stability and activity of enzymes from halophilic and haloalkaliphilic bacteria in the presence of organic solvents. PhD thesis, Saurashtra University
Pandey S, Singh SP (2012) Organic solvent tolerance of an α-amylase from haloalkaliphilic bacteria as a function of pH, temperature, and salt concentrations. Appl Biochem Biotechnol 166:1747–1757
Pandey S, Rakholiya K, Raval VH, Singh SP (2012) Catalysis and stability of an alkaline protease from a Haloalkaliphilic Bacterium under non-aqueous conditions as a function of pH, salt and temperature. J Biosci Bioeng 114:251–256
Patel RK, Dodia MS, Singh SP (2005) Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. Proc Biochem 40:3569–3575
Patel RK, Dodia MS, Joshi RH, Singh SP (2006a) Production of extracellular halo-alkaline protease from a newly isolated Haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India. World J Microbiol Biotechnol 22:375–382
Patel RK, Dodia MS, Joshi RH, Singh SP (2006b) Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic Bacillus sp. Process Biochem 41:2002–2009
Phillips K, Zaidan F, Elizondo OR, Lowe KL (2012) Phenotypic characterization and 16S rDNA identification of culturable non-obligate halophilic bacterial communities from a hypersaline lake, La Sal del Rey, in extreme South Texas (USA). Aquat Biosyst. doi:10.1186/2046-9063-8-5
Purohit MK, Singh SP (2009) Assessment of various methods for extraction of metagenomic DNA from saline habitats of coastal Gujarat (India) to explore molecular diversity. Lett Appl Microbiol 49:338–344. doi:10.1111/j.1472-765X.2009.02663.x
Purohit MK, Singh SP (2011) Comparative analysis of enzymatic stability and amino acid sequences of thermostable alkaline proteases from two haloalkaliphilic bacteria isolated from Coastal region of Gujarat, India. Int J Biol Macromol 49:103–112
Purohit MK, Singh SP (2012) Metagenomics of saline habitats with respect to bacterial phylogeny and biocatalytic potential. In: Satyanarayana T, Johri BN, Prakash A (eds) Microorganisms in sustainable agriculture and biotechnology. Springer, New York
Purohit MK (2012) Cloning, over-expression and characterization of alkaline proteases from cultivatable and non-cultivable halophilic and/or haloalkaliphilic bacteria isolated from saline habitats of coastal Gujarat. PhD thesis, Saurashtra University
Raes J, Husenholts P, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P (2007) Qualitative phylogeny assessment of microbial communities in diverse environment. Sci Exp. doi:1-2/10:1126
Raval VH (2012) Biochemical characterization and molecular studies on the alkaline proteases from Haloalkaliphilic bacteria isolated from Gujarat coasts. PhD thesis, Saurashtra University
Raval VH, Purohit MK, Singh SP (2012) Diversity, population dynamics and biocatalytic potential of cultivable and non-cultivable bacterial communities of the saline ecosystems. In: Trincone

A (ed) Marine enzymes for biocatalysis: sources, biocatalytic characteristics and bioprocesses of marine enzymes. Biohealthcare Publishing, Oxford

Rawal CM (2012) Cloning, over expression, and characterization of alkaline protease from halophilic/haloalkaliphilic bacteria. PhD thesis, Saurashtra University

Rawal CM, Raval VH, Bhimani HD, Bhensdadia DV, Kothari CR, Patel AB, Bhatt VD, Parmar NR, Sajnani MR, Koringa PG, Joshi CG, Kothari RK, Singh SP (2012) Whole-genome shotgun sequencing of the extremophile *Alkalibacillus haloalkaliphilus* C-5, of Indian origin. J Bacteriol 194:4775. doi:10.1128/JB.01031-12

Reddy LVA (2008) Purification and characterization of an organic solvent and detergent-tolerant novel protease produced by *Bacillus* sp.RKY3. J Chem Technol Biotechnol 8:1526–1533

Roberts MF (2005) Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems 1:5. doi:10.1186/1746-1448-1-5

Roberts MF (2006) Characterization of organic compatible solutes of halotolerant and halophilic microorganisms. In: Rainey FA, Oren A (eds) Methods in microbiology, extremophiles. Elsevier-Academic, Amsterdam

Romano I, Orlando P, Gambacorta A et al (2002) Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L., plipastatin, and surfactin. Extremophiles 6(6):499–506

Rothschild LJ, Manicinelli RL (2001) Life in extreme environments. Nature 409:1092–1101

Ruiz DM, Paggi RA, Gimenez MI, De Castro RE (2012) Autocatalytic maturation of the Tat-dependent halophilic subtilase *Nep* produced by the archaeon *Natrialba magadii*. J Bacteriol 194:3700–3707

Sakamoto A, Murata N (2001) The use of bacterial choline oxidase, a glycine betaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiol 125:180–188

Sardeshmukh NS, Sanmukh SG, Nakkeeran E (2012) Comparative functional genomics approach for the annotation of proteins in Unclassified Halophilic archaeon DL31. Nat Prec. hdl:10101/npre.2012.6784.1

Saum SH, Pfeiffer F, Palm P, Rampp M, Schuster SC, Muller V, Oesterhelt D (2012) Chloride and organic osmolytes: a hybrid strategy to cope with elevated salinities by the moderately halophilic, chloride-dependent bacterium *Halobacillus halophilus*. Environ Microbiol. doi:10.1111/j.1462-2920.2012.02770.x

Schmeisser C, Stelle S (2001) Metagenomics, biotechnology with non-culturable microbes. Appl Microbiol Biotechnol 75:356–364

Siddhpura PK, Vanparia S, Purohit MK, Singh SP (2010) Comparative studies on the extraction of metagenomic DNA from the saline habitats of Coastal Gujarat and Sambhar Lake, Rajasthan (India) in prospect of molecular diversity and search for novel biocatalysts. Int J Biol Macromol 47:375–379

Singh SP, Raval VH, Purohit MK, Pandey S, Thumar JT, Gohel SD, Akbari VG, Rawal CM (2012) Haloalkaliphilc bacteria and actinobacteria from the saline habitats: new opportunities for biocatalysis and bioremediation. In: Satyanarayana T, Johri BN, Prakash A (eds) Microorganisms in environmental management: microbes and environment. Springer, New York, pp 415–429

Sleator RD, Gahan CGM, Abee T (1999) Identification and disruption of *BetL*, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28 Appl. Environ Microbiol 65:2078–2083

Stewart EJ (2012) Growing uncultured bacteria. J Bacteriol 194:4151–4160

Sulpice R, Tsukaya H, Nonaka H, Mustardy L, Chen TH, Murata N (2003) Enhanced formation of flowers in salt-stressed Arabidopsis after genetic engineering of the synthesis of glycine betaine. Plant J 36:165–176

Surve VV, Patil MU, Dharmadhikari SM (2012) FAME and 16S rDNA sequence analysis of halophilic bacteria from solar salterns of Goa: A comparative study. Int J Sci Res Publ 2:1

Taran M, Bakhtiyari S (2012) Production of single cell protein by a halophilic micro-organism using glucose as carbon source: optimization of process variables in extreme conditions by Taguchi experimental design. Global Adv Res J Microbiol 1:041–046

Thongthai C, Suntinanalert P (1991) Halophiles in Thai fish sauce (nampla). In: Rodriguez-Valera F (ed) General and applied aspects of halophilic microorganisms. Plenum, New York

Tindall BJ, Mills AA, Grant WD (1980) An alkalophilic red halophilic bacterium with a low magnesium requirement from a Kenyan soil lake. J Gen Microbiol 116:257–260

Tindall BJ, Ross HNM, Grant WD (1984) *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkalophilic archaebacteria. Syst Appl Microbiol 5:41–57

Trincone A (2010) Potential biocatalysts originating from sea environments. J Mol Catal B Enzym 66:241–256

Ukani H, Purohit MK, Georrge JJ, Paul S, Singh SP (2010) HaloBase. Development of database system for halophilic bacteria and archaea with respect to proteomics, genomics and other molecular traits. J Sci Ind Res 70:976–981

van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6:213–218

Vargas C, Nieto J (2004) Genetic tools for the manipulation of moderately halophilic bacteria of the family *Halomonadaceae*. Methods Mol Biol 267:183–208

Wackett LP (2012) Halophilic microorganisms. Environ Microbiol Rep 4:467–468

Wang F, Hao J, Yang C, Sun M (2009) Cloning, expression, and identification of a novel extracellular cold-adapted alkaline protease gene of the marine bacterium strain YS-80-122. Appl Biochem Biotechnol 162:1497–1505

Welsh DT (2000) Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol Rev 24:263–290

Yeganeh LP, Azarbaijani R, Sarikhan S, Mousavi H, Ramezani M, Amoozegar MA, Shahzadeh Fazeli A, Salekdeh GH (2012) Complete genome sequence of *Oceanimonas* sp. GK1, a halotolerant bacterium from Gavkhouni Wetland in Iran. J Bacteriol 194:2123–2124

Chapter 5
Recent Trends in Bioremediation

Asha A. Juwarkar, Rashmi R. Misra, and Jitendra K. Sharma

5.1 Introduction

The global environment is under great stress due to urbanization and industrialization as well as population pressure on the limited natural resources. The problems are compounded by drastic changes that have been taking place in the lifestyle and habits of people. The environmental problems are diverse and sometimes specific with reference to time and space. The nature and the magnitude of the problems are ever changing, bringing new challenges, and creating a constant need for evolving newer and more appropriate technologies.

The major environmental threats include organic aqueous waste (pesticides), organic liquids (solvents from dry-cleaning), oils (lubricating, automotive, hydraulic, and fuel oils), and organic sludge/solids (painting operations, tars from dyestuffs intermediates). Mostly soil contaminations are the result of accidental spills and leaks. It originates from cleaning of equipment, residues left in used containers and outdated materials, use of excessive pesticides in agriculture, landfill leachate, which contains mixtures of organic compounds [e.g., benzene, toluene, ethylbenzene, and xylene (BTEX), chlorinated hydrocarbons, pesticides, medicals], and inorganic compounds (e.g., heavy metals, macrocomponents) (Gallego et al. 2001; Christensen and Bzdusek 2005). Other sources of chemical contaminants include improperly managed landfills, automobile service and maintenance establishments, photographic film processors, and household wastes, which include pesticides, paint products, household cleaners, and automotive products (Cameotra and Makkar 2010).

There are several conventional techniques (chemical treatment process) available to treat some of these chemicals, but due to their cost, end products which in turn are again toxic, these techniques fail to completely eradicate these chemicals.

A.A. Juwarkar (✉) • R.R. Misra • J.K. Sharma
Eco-System Division, CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur 440020, India
e-mail: juwarkar@gmail.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_5, © Springer-Verlag Berlin Heidelberg 2014

There is an urgent need to develop new technologies which are cost-effective and eco-friendly. In this context, biotechnology has tremendous potential to cater to the need and holds hope for environmental protection and management (Hatti-Kaul et al. 2007; Azadi and Ho 2010). One of the green technologies to treat these hazardous chemicals is bioremediation.

Bioremediation is an increasingly popular alternative to conventional chemical methods for treating waste compounds and media with the possibility to degrade contaminants, since it uses natural microbial activity mediated by different consortia of microbial strains. Many studies on bioremediation have been reported and the scientific literature has revealed the progressive emergence of various advances in bioremediation techniques. This chapter emphasizes on recent trends in bioremediation techniques/processes for decontamination of different environmental matrices.

5.2 Bioremediation

Bioremediation can be defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition. Bioremediation may be employed in order to attack specific contaminants, such as chlorinated pesticides that are degraded by bacteria, or a more general approach may be taken, such as oil spills that are broken down using multiple techniques including the addition of biosurfactant to facilitate decomposition of crude oil by bacteria (Juwarkar et al. 2008).

Bioremediation may be either aerobic (Wiegel and Wu 2000; Bedard and May 1996) or anaerobic (Komancova et al. 2003). Due to the problem associated with either of this method to treat highly complex compounds, sometime sequential anaerobic–aerobic bioremediation processes are also adopted to remediate contaminated sites (Master et al. 2002). Remediation using fungal strains in some cases proved as a highly effective remediation approach (Kubátová et al. 2001). Figure 5.1 shows schematic representation of bioremediation process. Based on removal of waste, bioremediation can be classified into in situ and ex situ technique.

5.2.1 In Situ Bioremediation Technologies

In situ techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance. Following are some of the in situ bioremediation techniques (Fig. 5.2).

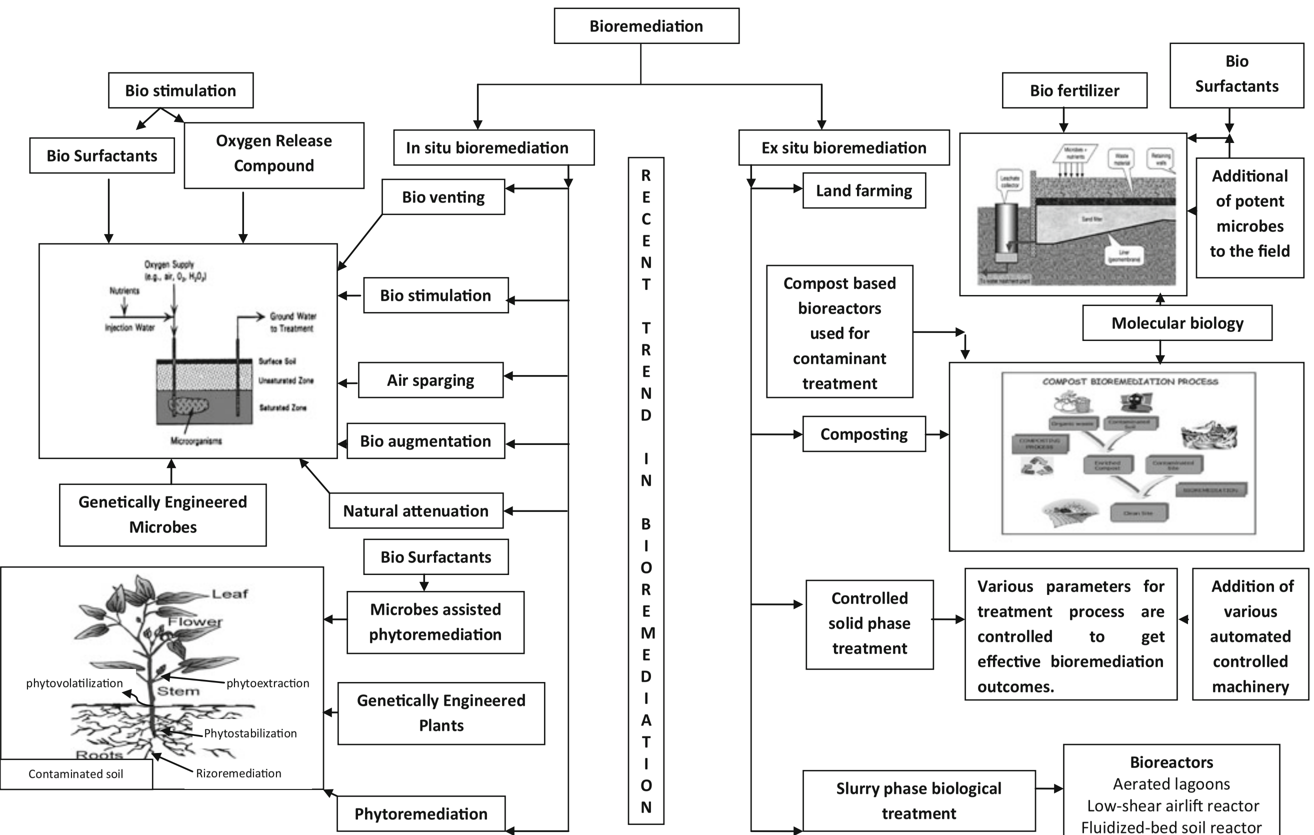


Fig. 5.1 Schematic representation of bioremediation process

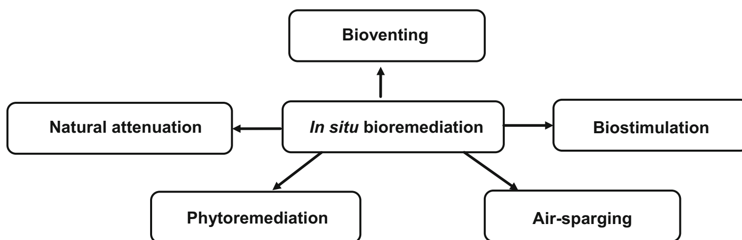


Fig. 5.2 In situ bioremediation technologies

5.2.1.1 Bioventing

Bioventing is the one of the most common in situ treatment technique. This technology is designed primarily to treat soil contamination by fuels, nonhalogenated volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs), pesticides, and herbicides. The technology requires presence of indigenous organisms capable of degrading the contaminants of interest, as well as nutrients necessary for growth. This is an aerobic process involving air supply for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. Table 5.1 shows several advances in the field of bioventing during last decade.

5.2.1.2 Biostimulation

Biostimulation is one of the in situ treatment techniques for treatment of subsurface region by addition of water-based solutions carrying nutrients, electron acceptor, or other amendments. These technologies are designed primarily to treat soil and groundwater contamination by fuels, nonhalogeneted VOCs, SVOCs, pesticides, and herbicides. The technology requires the presence of indigenous organisms capable of degrading the contaminants of interest. Coupling biostimulation process with advanced tools and techniques enhances the bioremediation process as shown in Table 5.1.

5.2.1.3 Air Sparging

Air sparging is an in situ treatment process which usually applies to contaminated ground water by injecting air below the water table. This technology is designed primarily to treat groundwater contamination by fuels, nonhalogenated VOCs, SVOCs, pesticides, organics, and herbicides. The technology requires presence of indigenous organisms capable of degrading the contaminants of interest, as well as nutrients necessary for growth and specific contaminant availability. Table 5.1 shows some of the findings in air-sparging field in the last decade.

Table 5.1 Application of in situ bioremediation processes

Contaminant	Bioremediation process	References
Bioventing		
Diesel oil in unsaturated soil in a mesocosm	Bioventing with nutrient addition and inoculation with an oil-degrading bacterium	Møller et al. (1996)
Gasoline-contaminated soil	Bioventing	Shewfelt et al. (2005)
TCE	Cometabolic bioventing for removal of TCE in the unsaturated zone in a soil column using methane as growth substrate	Sui et al. (2006)
Petroleum hydrocarbons and organic chemicals in ground water	Effects of nitrogen source on bioventing of gasoline-contaminated soil	Shewfelt and Zytner (2001)
Diesel fuel-contaminated soil	Effects of nitrogen source on biodegradation of diesel fuel-contaminated soil	Zytner et al. (2001)
Petroleum hydrocarbon	Bioventing using wind	Ryan et al. (2012)
Gasoline-contaminated soil	Bioventing	Mark et al. (2002)
Biostimulation		
Ammonia	Molecular-based technique (ammonia monooxygenase (amoA) gene)	Krishnani et al. (2009)
RB5 dye solutions	Decolorization of RB5 dye solutions using microbial consortium acclimatized from activated sludge from a textile effluent treatment plant	Dafale et al. (2008)
TCE	Stable carbon isotope analysis for dechlorination of TCE	Hirschorn et al. (2007)
Oil-contaminated coastal marsh	Nitrogen and phosphorus addition	Garcia-Blanco et al. (2007)
Mining soils contaminated with hydrocarbons	Biostimulation of the native microbial consortium	Salinas-Martinez et al. (2008)
Dechlorination of PCB	Biostimulation by adding ferrous sulfate	Zwiernik et al. (1998)
Uranium	Pilot-scale in situ bioremediation technique using microbes	Xu et al. (2010)
1,2,4-TCB-contaminated soils	Bioremediation technique using microbes	Wang et al. (2010)
Polyacyclic aromatic hydrocarbons in contaminated soil	Biostimulation and bioaugmentation in the presence of copper(II) ions	Atagana (2006)
Chlorinated hydrocarbons	Biostimulation	Hirschorn et al. (2007)
Soil artificially contaminated with Kerosene	Biostimulation	Agarry et al. (2012)
Sulfate	Biostimulation using functional gene approach	Luciana et al. (2012)
Air sparging		
Petroleum hydrocarbons	Air sparging	Heron et al. (2002), Gidarakos and Aivalioti (2008)

(continued)

Table 5.1 (continued)

Contaminant	Bioremediation process	References
Chlorinated aliphatic hydrocarbons (CAHs) in groundwater	Co-metabolic air sparging (propane as the cometabolic substrate)	Tovanabootr et al. (2001)
Crude oil from soil	Air sparging assisted stirred tank reactors	Urum et al. (2005)
Groundwater contaminated with trichloroethylene TCE	Pulsed air sparging system	Kim et al. (2007)
Contaminant	Two phase flow model for air sparging	Gao et al. (2012)
Soil highly contaminated with kerosene and BTEX	Air sparging	Kabelitz et al. (2009)
Bioaugmentation		
Polyacyclic aromatic hydrocarbons in contaminated soil	Biostimulation and bioaugmentation in the presence of copper(II) ions	Atagana (2006)
Chlorpyrifos in soil	Plasmid-mediated bioaugmentation	Zhang et al. (2012)
Chlorinated solvents-contaminated clay	Electrokinetic-enhanced bioaugmentation	Mao et al. (2012)
PCBs	De-chlorinating culture	Bedard and May (1996)
2,3,4,5,6-pentachlorobiphenyl	Granular anaerobic methanogenic microbial consortium for declorination	Natarajan et al. (1996)
PAHs-contaminated soil	Bioaugmentation in bio-slurry phase reactor	Nasseri et al. (2010)
Phytoremediation		
Arsenic	Spider brake (*Pteriscretica* L.) plants	Ebbs et al. (2010)
Lead and zinc	*Arundodonax*	Kos et al. (2003)
Obsolete pesticides	*Mey*. *Kochiascoparia* (L.) *Schrad*, and *Xanthium strumarium* L., *Artemisia annua* L., *Kochiasieversiana* (Pall.) C.A.	Nurzhanova et al. (2010)
Soil contaminated with cadmium and lead	*Zea mays*	Mojiri (2011)
Heavy metal	Chelating agent	Dipu et al. (2012)

5.2.1.4 Bioaugmentation

Bioaugmentation is the introduction of a group of natural microbial strains or a genetically engineered variant to treat contaminated soil or water. It involves group of microbes like bacteria, protozoa, nematodes, rotifers, and fungi capable of degrading organic compounds. Some of the findings introducing specific microbes or microbial consortium to the treatment process are shown in Table 5.1.

5.2.1.5 Natural Attenuation

Natural attenuation is a proactive approach that focuses on the verification and monitoring of natural remediation processes (Khan et al. 2004) also known as passive remediation, in situ bioremediation, intrinsic remediation, bioattenuation, and intrinsic bioremediation. Natural attenuation is an in situ treatment method that uses natural processes to contain the spread of contamination and to reduce the concentration and amount of pollutants at contaminated sites (Boparai et al. 2008; Khan et al. 2004). This means the environmental contaminants are undisturbed while natural attenuation works on them. Natural attenuation processes are often categorized as destructive or nondestructive (Gelman and Binstock 2008). Target contaminants include fuels, nonhalogenated VOCs, SVOCs, pesticides, and herbicides. The process may be applied to halogenated organics, but it requires longer treatment times. Also, the technology is applicable to especially hydrophobic contaminants such as high molecular weight PAHs that tend to sorb very tightly to soil particles and have very low rates of migration. Often, communities of adopted degraders will mineralize such contaminants quickly after they desorb from soil particles.

5.2.1.6 Phytoremediation

Phytoremediation is an emerging technology that uses plants for the treatment/mineralization of pollutants. Pollutants can be taken up inside plant tissues (phytoextraction), adsorbed to the roots (rhizofiltration), transformed by plant enzymes (phytotransformation), volatilize via plants into the atmosphere (phytovolatilization), degraded by microbes in the root zone (rhizoremediation) or incorporated to soil material (phytostabilization) (Salt et al. 1998; Pilon-Smits 2005).

Phytoremediation may be used for remediation of soil and groundwater contaminated with toxic heavy metals, radionuclides, organic contaminants such as chlorinated solvents, BTEX compounds, nonaromatic petroleum hydrocarbons, nitrotoluene ammunition wastes, and excess nutrients (Schnoor et al. 1995). Table 5.1 shows some of the recent trends in field of phytoremediation.

5.2.2 *Ex Situ Bioremediation Technologies*

Ex situ bioremediation techniques are those that are applied to soil and groundwater at the site which has been removed via excavation (soil) or pumping (water) (Fig. 5.3).

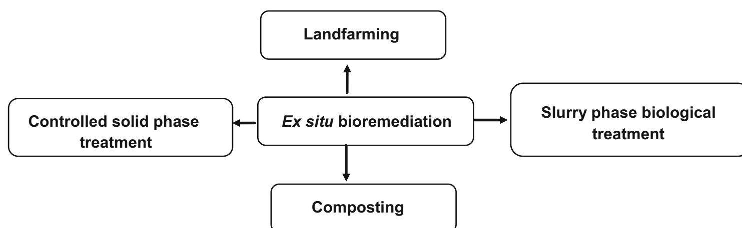


Fig. 5.3 Ex situ bioremediation technologies

5.2.2.1 Landfarming

Landfarming technology involves the application of contaminated material that has been excavated onto the soil surface and periodically tilled to mix and aerate the material (Harmsen et al. 2007; Maciel et al. 2009). Sometimes, in cases of very shallow contamination, the top layer of site may simply be tilled without requiring any excavation. Liners or other methods may be used to control leachate. This technology is designed primarily to treat soil contamination by fuels, nonhalogenated VOCs, SVOCs, pesticides, and herbicides. The process may be applied to halogenated organics but is less effective. Although the technology is very simple and inexpensive, it does require large amount of space, and reduction in contaminant concentrations may sometimes be due to volatilization rather than biodegradation (Souza et al. 2009; Sanscartier et al. 2010). Table 5.2 shows the recent trends in landfarming field.

5.2.2.2 Composting

Composting involves combining contaminated soil with nonhazardous organic matter such as manure or agricultural wastes which supports the growth of microbes. Table 5.2 shows some of the findings in composting field.

5.2.2.3 Controlled Solid Phase Treatment

This process includes preparation of treatment beds, biotreatment cells, and soil piles or composting. Moisture, heat, nutrients, oxygen, and pH can be controlled to enhance biodegradation. These technologies differ from landfarming since the treatment processes are often enclosed to control off-gases. Typically, excavated material is mixed with soil amendments and placed on a treatment area that includes leachate collection systems and some of aeration.

Like landfarming, these technologies require a lot of space and excavation of contaminated material. One advantage, however, of contained ex situ methods is that toxic byproducts or metabolites formed during the biodegradation process (e.g.,

Table 5.2 Application of ex situ bioremediation processes

Contaminant	Bioremediation process	References
Land farming		
Hydrocarbon-contaminated soils	Land farming	Paudyn et al. (2008)
Hydrocarbon	Land farming	Souza et al. (2009)
Hydrocarbon with refinery sludge	Land farming	Marin et al. (2005)
High-molecular weight petroleum hydrocarbons (HCs)	Land farming	Sanscartier et al. (2009)
Oil refinery sludge	Land farming in semiarid condition	Marin et al. (2005)
Crude oil-contaminated soil	Slurry-phase biological treatment and land farming techniques	Kuyukina et al. (2003)
Composting		
Olive mill wastewater sludge	Composting	Abid et al. (2007)
Bioremediation of hydrocarbon-contaminated soil	Composting using organic manure	Atagana (2008)
Triazines-contaminated soil	Composing and vermicomposting	Delgado Moreno and Pena (2009)
Phenolic compounds	Microbial and fungal composting	Ghaly et al. (2011)
Heavy metal	Compost	Vargas-GarcíaMdel et al. (2012)
Solid phase treatment		
Soil contaminated with polycyclic aromatic hydrocarbons	Solid phase treatment	Negri et al. (2004)
Soil contaminated with pentachlorophenol	Solid phase treatment using lignin-degrading fungi	Richard et al. (2006)
Sediment toxicity identification in an agricultural stream	Solid phase treatment	Phillips et al. (2006)
Slurry-phase biological treatment		
Nitro phenol	Slurry-phase biological treatment	Wang et al. (2005)
PAHs-contaminated soil	Bio-slurry-phase reactor	Nasseri et al. (2010)
Crude oil-contaminated soil	Slurry-phase biological treatment and land farming techniques	Kuyukina et al. (2003)
2,4-Dinitrotoluene and 2,6-dinitrotoluene	Slurry-phase biological treatment	Zhang et al. (2000)
Pyrene-contaminated soil	Bio-slurry-phase reactors	Venkata Mohan et al. (2008)

vinyl chloride from TCE) are contained. Table 5.2 shows some of the findings in controlled solid phase treatment field.

5.2.2.4 Slurry-Phase Biological Treatment

These technologies involve the treatment of excavated soil in the controlled environment of a bioreactor. Excavated soil is processed to separate stones and rubble and then mixed with water to a predetermined concentration depending upon the concentration of contaminants, rate of biodegradation, and physical nature of the soils. Usually slurries contain 10–40 % solids. Electron acceptors and nutrients are

added to the reactor, and parameters such as pH and temperature are controlled to optimize biological processes. Also, the reactor may be inoculated with specific organisms if a suitable population is not present. Targeted contaminants include petrochemicals, solvents, pesticides, wood preservatives, explosives, petroleum hydrocarbons, and other organic chemicals.

Bioreactors are favored over in situ biological techniques for heterogeneous soils, low permeability soils, areas where underlying groundwater would be difficult to capture, or when quicker treatments are required. Similar to solid phase ex situ treatments, they have the advantage of containing toxic metabolites such as vinyl chloride. Slurry-phase treatment tends to be quick, but more expensive, than controlled solid phase treatment. Table 5.2 shows some of the findings in slurry-phase biological treatment field.

5.2.3 Factors Affecting Bioremediation

- Energy sources
- Bioavailability
- Bioactivity and biochemistry
- pH
- Temperature
- Toxicity of compound
- Water content and geological character
- Nutrient availability
- External electron availability

5.2.4 Merits and Demerits of Bioremediation

Merits
- Effective
- Economical
- Eco-friendly
- Less toxic by-products generation
- On-site treatment possible
- Does not affect natural flora

Demerits
- More effective for readily biodegradable compounds
- Specific environmental condition required
- Specific microflora required
- Long time required to remove or transform contaminant

To overcome these demerits associated with traditional bioremediation process many advancement have been made. Some of the recent outbreaks in the field of bioremediation are discussed in the following section.

5.3 Recent Trends in Bioremediation

Advances in biotechnological tools and techniques and their application in biological system to enhance the bioremediation processes helped overcome various limitations associated with traditional bioremediation processes. Applications of several tools and techniques which enhance bioremediation are discussed below.

5.3.1 Application of Biosurfactants in Bioremediation Process for Bioavailability of Hydrophobic Pollutants

Bioavailability of targeted compounds was one of the major problems in bioremediation. This was mainly attributed to the hydrophobic nature of contaminants. Use of most chemical surfactants helps to overcome these problems associated with hydrophobic contaminants. Looking for green and cost-effective technology, it was found that many biological molecules are amphiphilic and partitions preferentially at interphases (Banat et al. 2000). Microbial compounds, which exhibit particularly high surface and emulsifying activity, are classified as biosurfactants (Banat et al. 2000).

Biosurfactants are surface-active substances synthesized by living cells. Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, eco-friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions, and potential applications in environmental protection (Banat et al. 2000; Rahman et al. 2002). Biosurfactants are widely available and highly diverse because of different genes of microbes producing them (Ron and Rosenberg 2002; Bodour et al. 2004). Hence, use of biosurfactants will reduce the hydrophobicity of compounds and make it readily available to the biological system for their remediation. Table 5.3 shows the application of biosurfactants in bioremediation.

5.3.2 Role of Oxygen Releasing Compounds in Enhancing Aerobic Bioremediation Process

Oxygen is typically the rate limiting factor in aerobic bioremediation at many sites. The degradation of petroleum hydrocarbons occurs much faster under aerobic

Table 5.3 Recent trends in bioremediation

Outline of findings	References
Biosurfactants	
Bioremediation of diesel	Tribelli et al. (2012)
Bacterial bio surfactant for hydrocarbon remediation	Eddouaouda et al. (2011)
Lipid biosurfactant	Franzetti et al. (2010)
Application of biosurfactant in phenanthrene biodegradation	Reddy et al. (2010)
Bio surfactant enhancing the biodegradation of hydrocarbon in soil	Kang et al. (2010)
Biosurfantant in heavy metal study	Asçi et al. (2010)
Multimetal contaminant bioremediation	Juwarkar et al. (2008)
Oxygen releasing compounds	
Soil contaminated with kerosene	Agarry et al. (2012)
Hydrocarbon bioremediation	Odencrantz et al. (1996)
Microbes assisted phytoremediation	
Microbial consortium for pulp mill effluent bioremediation	Kumar et al. (2012)
Soil bacteria to facilitate phytoremediation	Glick (2010)
Microbes assisted phytoremediation for copper remediation	Chen et al. (2006)
Microbes assisted phytoremediation for chromium remediation	Singh et al. (2010)
Microbes assisted phytoremediation for zinc remediation	Juwarkar and Singh (2010)
Molecular biology	
Genomics approach for bioremediation	Lovely (2003)
Microbial community analysis at heavy metal-contaminated groundwater using metagenomics approach	Hemme et al. (2010)
Use of gene probes in aerobic in situ bioremediation of TCE	Hazen et al. (2009)
DNA-based stable isotope probing and pyrosequencing for analysis of microbial community of petroleum-contaminated arctic soils	Bell et al. (2011)
Proteogenomic analysis of the citrate synthase protein during bioremediation of U(VI)	Wilkins et al. (2011)
Bioinformatics	
In-silico biodegradation pathways for 1-naphthyl methylcarbamate	Jaimini et al. (2012)
Perturbedmetabolic network analysis	Ideker et al. (2001)
Nanotechnology	
Nanowires in bioremediation	Malvankar and Lovely (2012)
Use of nanoparticles in soil–water bioremediation processes	Duran (2008)
Silver nanoparticle in textile effluent bioremediation	Duran et al. (2010)
Nanoparticle for Cr(VI) bioremediation processes	Telling et al. (2009)

conditions compared to anaerobic conditions. Therefore, the addition of oxygen can significantly increase the remediation rate. Application of oxygen releasing compound (ORC) is one way to enhance the rate of bioremediation process. ORC releases oxygen slowly when it comes in contact with water. One patented ORC product is similar to Milk of Magnesia. It is most frequently used to address dissolved phase contamination, such as total petroleum hydrocarbons (TPH) and

BTEX, as well as contamination in the capillary fringe zone. It can be applied using retrievable filter socks placed in monitoring wells or as a slurry mixture.

5.3.3 Microbes Assisted Phytoremediation

Phytoremediation in combination with microbes tackles the targeted compounds and improves remediation process. Many researchers in this area have carried out extensive works which have potential field applications. Some of the outcomes of application of these techniques are reported in Table 5.3.

Some of the advance techniques in the field of bioremediation which has drawn attention of researchers in recent past are genetic engineering; culture of recombinant microorganisms; cells of animals and plants; metabolic engineering; hybridoma technology; bioelectronics; nanobiotechnology; protein engineering; transgenic animals and plants; tissue and organ engineering; immunological assays; genomics and proteomics; and bioseparations and bioreactor technologies (Gavrilescu and Chisti 2005; Kulshreshtha 2012). These techniques reflect promising perspective towards the remediation, but at the same time need lots of research to implement these modern and fascinating technologies at the field level. Genetic engineering techniques are used in the bioremediation process such as genetically modified organisms (microbes), but their field applications are strictly regulated due to several ecological risks associated with these organisms. Lab scale studies carried out by different researchers around the globe are shown in Table 5.3. Some studies show application of transgenic plants and transgenic microbes in the field of phytoremediation (Pieper and Reineke 2000; Eapen et al. 2007).

5.3.4 Role of Molecular Biological Tools and Techniques in Bioremediation Process

Considering the microbial world which is very vast and their functioning in the specific environment varies and is considered to be the part of their coculturing effect. Most of the microbes are uncultivable in laboratory condition, but their role in the particular niche cannot be neglected particularly in the process like bioremediation. In order to trace the microbial population and their role in the environment, molecular techniques prove to be a boon in this field. The genes involved in the bioremediation of the targeted compounds and their respective enzymes can be traced and hence in turn the microbial floras involved in the bioremediation process are traceable. It also helps to record the metabolic pathway followed by the organism to degrade the particular compounds. Table 5.3 shows the application of molecular biology in bioremediation.

5.3.5 Application of Bioinformatics as a Tool to Acquire Pre-information of Cellular Processes Associated with Bioremediation

Bioinformatics tools have been developed to identify and analyze various components of cells such as gene and protein functions, interactions, and metabolic and regulatory pathways. Bioinformatics analysis will facilitate and quicken the analysis of cellular process, to understand the cellular mechanism to treat and control microbial cells as factories. The next decade will belong to understanding molecular mechanism and cellular manipulation using the integration of bioinformatics. Bioinformatics has wide application in bioremediation for the structure–function determination and pathways of biodegradation of xenobiotics (Fulekar and Sharma 2008). Some of the applications of bioinformatics in bioremediation processes are reported in Table 5.3.

5.3.6 Role of Nanotechnology in Bioremediation Process

Nanotechnology is an emerging branch of science which has attracted the interest of researchers involved in varied subject due to its size and effective results towards common problem. It involves varied applications including designing, characterization, production, and application of particles by controlling their size to nanoscale. Nanoworld seems to be fascinating and highly promising approach to clean the environment contaminated with pollutants. Some of the recent applications of nanotechnology in the field of remediation are shown in Table 5.3.

There are many more advances in field of bioremediation which helps to overcome various hurdles in bioremediation process while treating complex compounds. With the introduction of new tools and technologies every day in the field, new insight to research is added.

5.4 Conclusion

Bioremediation is one of the green approaches to clean the planet. It is promising, efficient, eco-friendly, and cost-effective. Advances in bioremediation seem to be highly attractive, but these technologies need field trials in order to gain market value. In most cases, single technology fails to work in field due to various environmental factors associated and the toxicity of targeted compound. Hence, there is a need to develop hybrid technologies which can fit to ever-changing environmental conditions and toxicity of the compound. There is huge demand of scientific and engineering inputs to be incorporated to these technologies for field applications.

Acknowledgment The authors would like to gratefully acknowledge Dr. S.R. Wate, Director, CSIR-NEERI for giving the permission to publish the manuscript.

References

Abid N, Chamkha M, Godon JJ, Sayadi S (2007) Involvement of microbial populations during the composting of olive mill wastewater sludge. Environ Technol 28:751–760

Agarry SE, Owabor CN, Yusuf RO (2012) Enhanced bioremediation of soil artificially contaminated with kerosene: optimization of biostimulation agents through statistical experimental design. J Pet Environ Biotechnol 3:3, http://dx.doi.org/10.4172/2157-7463.1000120

Asçi Y, Nurbas M, Açikel YS (2010) Investigation of sorption/desorption equilibria of heavy metal ions on/from quartz using rhamnolipid biosurfactant. J Environ Manage 91:724–731

Atagana H (2006) Biodegradation of polyacyclic aromatic hydrocarbons in contaminated soil by biostimulation and bioaugmentation in the presence of copper(II) ions. World J Microbiol Biotechnol 22:1145–1153

Atagana HI (2008) Compost bioremediation of hydrocarbon contaminated soil inoculated with organic manure. Afr J Biotechnol 7:1516–1525

Azadi H, Ho P (2010) Genetically modified and organic crops in developing countries: a review of options for food security. Biotechnol Adv 28:160–168

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508

Bedard DL, May RJ (1996) Characterization of the polychlorinated biphenyls in sediments of woods pond: evidence for microbial declorination of aroclors 1260 in-situ. Environ Sci Technol 30:237–245

Bell TH, Yergeau E, Martineau C, Juck D, Whyte LG, Greer CW (2011) Identification of nitrogen-incorporating bacteria in petroleum contaminated arctic soils by using [15N] DNA-based stable isotope probing and pyrosequencing. Appl Environ Microbiol 77:4163–4171

Bodour AA, Guerrero-Barajas C, Jiorle BV, Malcomson ME, Paull AK, Somogyi A, Trinh LN, Bates RB, Maier RM (2004) Structure and characterization of flavolipids, a novel group of biosurfactants produced by *Flavobacterium* sp. MTN11. Appl Environ Microbiol 70:114–120

Boparai HK, Shea PJ, Comfort SD, Machacek TA (2008) Sequencing zerovalent iron treatment with carbon amendments to remediate agrichemical-contaminated soil. Water Air Soil Pollut 193:189–196

Cameotra SS, Makkar RS (2010) Biosurfactant-enhanced bioremediation of hydrophobic pollutants. Pure Appl Chem 82:97–116

Chen Y, Wang Y, Wu W, Lin Q, Xue S (2006) Impacts of chelate-assisted phytoremediation on microbial community composition in the rhizosphere of a copper accumulator and non-accumulator. Sci Total Environ 356:247–255

Christensen ER, Bzdusek PA (2005) PAHs in sediments of the Black River and the Ashtabula River, Ohio: source apportionment by factor analysis. Water Res 39:511–524

Dafale N, Nageswara Rao N, Meshram SU, Wate SR (2008) Decolorization of azo dyes and simulated dye bath wastewater using acclimatized microbial consortium Biostimulation and halo tolerance. Bioresour Technol 99:2552–2558

Delgado Moreno L, Pena A (2009) Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. Sci Total Environ 407:1489–1495

Dipu S, Kumar AA, Thanga SG (2012) Effect of chelating agents in phytoremediation of heavy metals. Remed J 22:133–146

Duran N (2008) Use of nanoparticles in soil-water bioremediation processes. J Soil Sci Plant Nutr 8:33–38

Duran N, Marcato PD, Alves OL, Da Silva JPS, De Souza GIH, Rodrigues FA, Esposito E (2010) Ecosystem protection by effluent bioremediation: silver nanoparticles impregnation in a textile fabrics process. J Nanopart Res 12:285–292

Eapen S, Singh S, D'Souza SF (2007) Advances in development of transgenic plants for remediation of xenobiotic pollutants. Biotechnol Adv 25:442–451

Ebbs S, Hatfield S, Nagarajan V, Blaylock M (2010) A comparison of the dietary arsenic exposures from ingestion of contaminated soil and hyperaccumulating pteris ferns used in a residential phytoremediation project. Int J Phytoremediation 11:121–132

Eddouaouda K, Mnif S, Badis A, Younes SB, Cherif S, Samira Ferhat S, Mhiri N, Chamkha M, Sayadi S (2011) Characterization of a novel biosurfactant produced by *Staphylococcus* sp. strain 1E with potential application on hydrocarbon bioremediation. J Basic Microbiol 52:408–418

Franzetti A, Gandolfi I, Smyth TJP, Banat IM (2010) Production and applications of trehalose lipid biosurfactants. Eur J Lipid Sci Technol 112:617–627, Special Issue: Microbial biosurfactants

Fulekar MH, Sharma J (2008) Bioinformatics applied in bioremediation. Innov Rom Food Biotechnol 2:28–36

Gallego JL, Loredo J, Llamas JF, Vazquez F, Sanchez J (2001) Bioremediation of diesel-contaminated soils: evaluation of potential in situ techniques by study of bacterial degradation. Biodegradation 12:325–335

Gao S, Meegoda JN, Liming H (2012) A dynamic two-phase flow model for air sparging. Int J Numer Anal Meth Geomech. doi:10.1002/nag.2109

Garcia-Blanco S, Venosa AD, Suidan MT, Lee K, Cobanli S, Haines JR (2007) Biostimulation for the treatment of an oil-contaminated coastal salt marsh. Biodegradation 18:1–15

Gavrilescu M, Chisti Y (2005) Biotechnology – a sustainable alternative for chemical industry. Biotechnol Adv 23:471–499

Gelman F, Binstock R (2008) Natural attenuation of MTBE and BTEX compounds in a petroleum contaminated shallow coastal aquifer. Environ Chem Lett 6:259–262

Ghaly A, Zhang B, Dave D (2011) Biodegradation of phenolic compounds in creosote treated wood waste by a composting microbial culture augmented with the fungus *Thermoascus aurantiacus*. Am J Biochem Biotechnol 7:90–103

Gidarakos EL, Aivalioti MV (2008) In-well air sparging efficiency in remediating the aquifer of a petroleum refinery site. J Environ Eng Sci 7:71–82

Glick BR (2010) Using soil bacteria to facilitate phytoremediation. Biotechnol Adv 28:367–374

Harmsen J, Rulkens WH, Sims RC, Rijtema PE, Zweers AJ (2007) Theory and application of landfarming to remediate polycyclic aromatic hydrocarbons and mineral oil contaminated sediments; beneficial reuse. J Environ Qual 36:1112–1122

Hatti-Kaul R, Tornvall U, Gustafsson L, Borjesson P (2007) Industrial biotechnology for the production of bio-based chemicals – a cradle-to-grave perspective. Trends Biotechnol 25:119–124

Hazen T, Chakraborty R, Fleming J, Gregory I, Bowman J, Jimenez L, Zhang D, Pfiffner S, Brockman F, Sayler G (2009) Use of gene probes to assess the impact and effectiveness of aerobic in situ bioremediation of TCE. Arch Microbiol 191:221–232

Hemme CL, Deng Y, Gentry TJ, Fields MW, Wu L, Barua S, Barry K, Tringe SG, Watson DB, He Z (2010) Metagenomic insights into evolution of a heavy metal-contaminated groundwater microbial community. ISME J 4:660–672

Heron G, Gierke JS, Faulkner B, Mravik S, Wood L, Enfield CG (2002) Pulsed air sparging in aquifers contaminated with dense nonaqueous phase liquids. Ground Water Monit Remediat 22:73–82

Hirschorn SK, Grostern A, Lacrampe-Couloume G, Edwards EA, MacKinnon L, Repta C, Major DW, Sherwood Lollar B (2007) Quantification of biotransformation of chlorinated hydrocarbons in a biostimulation study: added value via stable carbon isotope analysis. J Contam Hydrol 94:249–260

Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlett DR, Aebersold R, Hood L (2001) Integrated genomic and proteomic analyses of a systematically perturbedmetabolic network. Science 292:929–934

Jaimini D, Shabnam AA, Sarkar C (2012) In-silico feasibility of novel biodegradation pathways for 1-naphthyl methylcarbamate. Am-Euras J Toxicol Sci 4:89–93

Juwarkar AA, Singh SK (2010) Microbe-assisted phytoremediation approach for ecological restoration of zinc mine spoil dump. Int J Environ Poll 43:236–250

Juwarkar AA, Dubey KV, Nair A, Singh SK (2008) Bioremediation of multi-metal contaminated soil using biosurfactant – a novel approach. Indian J Microbiol 48:142–146

Kabelitz N, Machackova J, Imfeld G, Brennerova M, Pieper DH, Heipieper HJ, Junca H (2009) Enhancement of the microbial community biomass and diversity during air sparging bioremediation of a soil highly contaminated with kerosene and BTEX. Appl Microbiol Biotechnol 82:565–577

Kang SW, Kim YB, Shin JD, Kim EK (2010) Enhanced biodegradation of hydrocarbons in soil by microbial biosurfactant, sophorolipid. Appl Biochem Biotechnol 160:780–790

Khan FI, Husain T, Hejazi R (2004) An overview and analysis of site remediation technologies. J Environ Manag 71:95–122

Kim HM, Hyun Y, Lee KK (2007) Remediation of TCE contaminated groundwater in a sandy aquifer using pulsed air sparging: laboratory and numerical studies. J Environ Eng 133:380–388

Komancova M, Jurcova I, Kochankova L, Burkhard J (2003) Metabolic pathways of polychlorinated biphenyls degradation by *Pseudomonas* sp. Chemosphere 50:537–543

Kos B, Grčman H, Leštan D (2003) Phytoextraction of lead, zinc and cadmium from soil by selected plants. Plant Soil Environ 49(12):548–553

Krishnani KK, Shekhar MS, Gopikrishna G, Gupta BP (2009) Molecular biological characterization and biostimulation of ammonia—oxidizing bacteria in brackishwater aquaculture. J Environ Sci Health A 44:1598–1608

Kubátová A, Erbanová P, Eichlerová I, Homolka L, Nerud F, Aek V (2001) PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatusin* contaminated soil. Chemosphere 43:207–215

Kulshreshtha S (2012) Current trends in bioremediation and biodegradation. J Bioremed Biodeg 3: e114, org/10.4172/2155-6199.1000e114

Kumar V, Dhall P, Kumar R, Singh YP, Kumar A (2012) Bioremediation of agro-based pulp mill effluent by microbial consortium comprising autochthonous bacteria. ScientificWorldJournal 2012:127014

Kuyukina MS, Ivshina IB, Ritchkova MI, Philp JC (2003) Bioremediation of crude oil-contaminated soil using slurry-phase biological treatment and land farming techniques. Soil Sediment Contam 12:85–99

Lovely D (2003) Cleaning up with genomics – applying molecular biology to bioremediation. Nature 1:35–43

Luciana PP, Sage RH, Elizabeth MP, Kenneth FR, Amy P (2012) Effect of bioaugmentation and biostimulation on sulfate-reducing column startup captured by functional gene profiling. FEMS Microbiol Ecol 82(1):135–47

Maciel BM, Santos ACF, Dias JCT, Vidal RO, Dias RJC, Gross E, Cascardo JCM, Rezende RP (2009) Simple DNA extraction protocol for a 16S rDNA study of bacterial diversity in tropical landfarm soil used for bioremediation of oil waste. Genet Mol Res 8:375–388

Malvankar NS, Lovley DR (2012) Microbial nanowires: a new paradigm for biological electron transfer and bioelectronics. ChemSusChem 5:1039–1046

Mao X, Wang J, Ciblak A, Cox EE, Riis C, Terkelsen M, Gent DB, Alshawabkeh AN (2012) Electrokinetic-enhanced bioaugmentation for remediation of chlorinated solvents contaminated clay. J Hazard Mater 213–214:311–317

Marin JA, Hernandez T, Garcia C (2005) Bioremediation of oil refinery sludge by landfarming in semiarid condition. Environ Res 98:185–195

Mark H, Kirsten S, Hung L, Richard GZ (2002) Bioventing of gasoline contaminated soil under varied laboratory conditions. SCE/EWRI of ASCE Environmental Engineering Conference, Niagara

Master ER, Lai VV, Kuipers B, Cullen WR, Mohn W (2002) Sequential anaerobic–aerobic treatment of soil contaminated with weathered Aroclor 1260. Environ Sci Technol 36:100–103

Mojiri A (2011) The potential of Corn (*Zea mays*) for phytoremediation of soil contaminated with cadmium and lead. J Biol Environ Sci 5:17–22

Møller J, Winther P, Lund L, Kirkebjerg K, Westermann P (1996) Bioventing of diesel oil-contaminated soil: comparison of degradation rates in soil based on actual oil concentration and on respirometric data. J Ind Microbiol Biotechnol 16:110–116

Nasseri S, Kalantary RR, Nourieh N, Naddafi K, Mahvi AH, Baradaran N (2010) Influence of bioaugmentation in biodegradation of PAHs-contaminated soil in bio-slurry phase reactor. J Environ Health Sci Eng 7:199–208

Natarajan MR, Wu WM, Nye J, Wang H (1996) Dechlorination of polychlorinated biphenyl congeners by an anaerobic microbial consortium. Appl Microbiol Biotechnol 46:673–677

Negri M, Manfredini A, Saponaro S, Sorlini C, Bonomo L, Valle A, Zanardini E (2004) Solid phase treatment of an aged soil contaminated by polycyclic aromatic hydrocarbons. J Environ Sci Health A Tox Hazard Subst Environ Eng 39:1–17

Nurzhanova A, Kulakow P, Rubin E, Rakhimbayev I, Sedlovskiy A, Zhambakin K, Kalugin S, Kolysheva E, Erickson L (2010) Obsolete pesticides pollution and phytoremediation of contaminated soil in Kazakhstan. Application of phytotechnologies for cleanup of industrial, agricultural, and wastewater contamination. NATO Science for Peace and Security Series C: Environmental Security, pp 87–111

Odencrantz JE, Johnson JG, Koenigsberg SS (1996) Enhanced intrinsic bioremediation of hydrocarbons using an oxygen-releasing compound. J Remed 6:99–114

Paudyn K, Rutter A, Kerry Rowe R, Poland JS (2008) Remediation of hydrocarbon contaminated soils in the Canadian Arctic by landfarming. Cold Regions Sci Technol 53:102–114

Phillips BM, Anderson BS, Hunt JW, Huntley SA, Tjeerdema RS, Kapellas N, Worcester K (2006) Solid-phase sediment toxicity identification evaluation in an agricultural stream. Environ Toxicol Chem 25:1671–1676

Pieper DH, Reineke W (2000) Engineering bacteria for bioremediation. Curr Opin Biotechnol 11:262–270

Pilon-Smits EAH (2005) Phytoremediation. Annu Rev Plant Biol 56:15–39

Rahman KSM, Thahira-Rahman J, McClean S, Marchant R, Banat IM (2002) Rhamnolipid biosurfactants production by strains of *Pseudomonas aeruginosa* using low cost raw materials. Biotechnol Prog 18:1277–1281

Reddy MS, Naresh B, Leela T, Prashanthi M, Madhusudhan N, Dhanasri G, Devi P (2010) Biodegradation of phenanthrene with biosurfactant production by a new strain of Brevibacillus sp. Biores Technol 101:7980–7983

Richard TL, Walker PL, Gossett JM (2006) Effects of oxygen on aerobic solid-state biodegradation kinetics. Biotechnol Prog 22:60–69

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252

Ryan CD, Jim L, Mehdi B (2012) Sustainable wind-driven bioventing at a petroleum hydrocarbon–impacted site. Remediation 22:65–78

Salinas-Martinez A, Delgado E, Pérez Andrade H, Hanad-Marroquin LA, Medrano-Roldán H (2008) Development of a bioremediation process biostimulation of native microbial consortium through the heap leaching technique. J Environ Manage 88:115–119

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Phys 49:643–668

Sanscartier D, Laing T, Reimer K, Zeeb B (2009) Bioremediation of weathered petroleum hydrocarbon soil contamination in the Canadian High Arctic: laboratory and field studies. Chemosphere 77:1121–1126

Sanscartier D, Reimer K, Zeeb B, George K (2010) Management of hydrocarbon contaminated soil through bioremediation and landfill disposal at a remote location in Northern Canada. Can J Civil Eng 37:147–155
Schnoor JL, Licht LA, McCutcheton SC, Wolfe NL, Carreira LH (1995) Phytoremediation of organic and nutrient contaminants. Environ Sci Technol 29:318A–323A
Shewfelt K, Zytner RG (2001) The effects of nitrogen source and supply on bioventing of gasoline contaminated soil. In: Proceedings of the petroleum hydrocarbons and organic chemicals in ground water: prevention, detection, and remediation, Houston, TX, pp 265–272
Shewfelt K, Lee H, Zytner RG (2005) Optimization of nitrogen for bioventing of gasoline contaminated soil. J Environ Eng Sci 4:29–42
Singh NK, Rai UN, Singh M, Tripathi RD (2010) Impact of rhizobacteria on growth and chromium accumulation in *Scirpus lacustris* L. grown under chromium supplementation. J Environ Biol 31:709–714
Souza TS, Hencklein FA, Angelis DF, Gonçalves RA, Fontanetti CS (2009) The Allium cepa bioassay to evaluate landfarming soil, before and after the addition of rice hulls to accelerate organic pollutants biodegradation. Ecotoxicol Environ Saf 72:1363–1368
Sui H, Li X, Huang G, Jiang B (2006) A study on cometabolic bioventing for the *in situ* remediation of trichloroethylene. Environ Geochem Health 28:147–152
Telling ND, Coker VS, Cutting RS, van der Laan G, Pearce CI, Pattrick RAD, Arenholz E, Lloyd JR (2009) Remediation of Cr(VI) by biogenic magnetic nanoparticles: an X-ray magnetic circular dichroism study. Appl Phys Lett 95:163–701
Tovanabootr A, Semprini L, Dolan ME, Azizian M, Magar VS, Debacker D, Leeson A, Kempisty CD (2001) Cometabolic air sparging field demonstration with propane to remediate trichloroethene and cis-dichloroethene. In: 6th International in situ and on site bioremediation symposium, San Diego, pp 145–153
Tribelli PM, Di Martino C, López NI, RaigerIustman LJ (2012) Biofilm lifestyle enhances diesel bioremediation and biosurfactant production in the Antarctic polyhydroxyalkanoate producer Pseudomonas extremaustralis. Biodegradation 23(5):645–651
Urum K, Pekdemir T, Ross D, Grigson S (2005) Crude oil contaminated soil washing in air sparging assisted stirred tank reactor using biosurfactants. Chemosphere 60:334–343
Vargas-GarcíaMdel C, López MJ, Suárez-Estrella F, Moreno J (2012) Compost as a source of microbial isolates for the bioremediation of heavy metals: in vitro selection. Sci Total Environ 431:62–67
Venkata Mohan S, Prasanna D, Purushotham Reddy B, Sarma PN (2008) Ex situ bioremediation of pyrene contaminated soil in bio-slurry phase reactor operated in periodic discontinuous batch mode: Influence of bioaugmentation. Int Biodeterior Biodegradation 62:162–169
Wang JL, Zhao G, Wu LB (2005) Slurry-phase biological treatment of Nitro phenol using bioaugmentation technique. Biomed Environ Sci 18:77–81
Wang F, Dörfler U, Schmid M, Fischer D, Kinzel L, Scherb H, Munch J, Jiang X, Schroll R (2010) Homogeneous inoculation vs. microbial hot spots of isolated strain and microbial community: what is the most promising approach in remediating 1,2,4-TCB contaminated soils? Soil Biol Biochem 42:331–336
Wiegel J, Wu Q (2000) Microbial reductive dehalogenation of polychlorinated biphenyls. FEMS Microbiol Ecol 32:1–15
Wilkins MJ, Callister SJ, Miletto M, Williams KH, Nicora CD, Lovley DR, Long PE, Lipton MS (2011) Development of a biomarker for *Geobacter* activity and strain composition; proteogenomic analysis of the citrate synthase protein during bioremediation of U(VI). Microb Biotechnol 4:55–63
Xu M, Wu W, Wu L, He Z, Van Nostrand J, Deng Y, Luo J, Carley J, Ginder-Vogel M, Gentry T (2010) Responses of microbial community functional structures to uranium pilot-scale in situ bioremediation. ISME J 4:1060–1070

Zhang C, Joseph B, Shirley FN, Jim CS (2000) Slurry-phase biological treatment of 2,4-dinitrotoluene and 2,6-dinitrotoluene: role of bioaugmentation and effects of high dinitrotoluene concentrations. Environ Sci Technol 34:2810–2816

Zhang Q, Wang B, Cao Z, Yu Y (2012) Plasmid-mediated bioaugmentation for the degradation of chlorpyrifos in soil. J Hazard Mater 221–222:178–184

Zwiernik MJ, John F, Quensen III, Boyd SA (1998) $FeSO_4$ amendments stimulate extensive anaerobic PCB dechlorination. Environ Sci Technol 32:3360–3365

Zytner RG, Salb A, Brook TR, Leunissen M, Stiver WH (2001) Bioremediation of diesel fuel contaminated soil. Can J Civil Eng 28:131–140

Chapter 6
Heavy Metal Bioremediation and Nanoparticle Synthesis by Metallophiles

Arvind Sinha, Rajeshwari Sinha, and Sunil K. Khare

6.1 Introduction

Microbes encounter different types of metals and metalloids in the environment. Therefore, they may interact with them, sometimes to their benefit, at other times to get rid of the detrimental effect (Ehrlich 1997). Metal rich biotopes are encountered both as natural and anthropogenic origin. Some of the microorganisms have adapted themselves for metal rich biotopes and are referred as metallophiles. They are adapted through different physiological processes, viz. redoxolysis, acidolysis, complexolysis, alkylation, biosorption, bioaccumulation and complex formation (Brandl and Faramarzi 2006). In combination or alone, these processes protect the cells from the adverse effect of the metals by changing the nature of the metals, toxicity or mobility (Mapelli et al. 2011). The metallophiles play vital roles in metal speciation, biogeochemical cycling and biomineralization. Such resistance mechanisms form the basis for their use in bioremediation, biomining, nanoparticle fabrication, biomineralization and many more important processes (Gadd 2007). Such microbial cells or biological processes at times craft molecules and macromolecules with an unparalleled level of structural control (Brown et al. 2000). The chapter encompasses various aspects of microbial metal interactions and their applications in heavy metal bioremediation and nanoparticle biosynthesis. The possible mechanisms were also elucidated.

A. Sinha • R. Sinha • S.K. Khare (✉)
Enzyme and Microbial Biochemistry Lab, Department of Chemistry, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
e-mail: skhare@rocketmail.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_6, © Springer-Verlag Berlin Heidelberg 2014

6.2 Microbial Heavy Metal Interaction

Metals play an integral role in the life processes of microorganisms. Metals like sodium, potassium, calcium, magnesium, manganese, nickel, chromium, cobalt, copper, iron and zinc are essential and required as micronutrients. These are used for proper functioning of different physiological processes like redox reactions, stabilization of molecules through electrostatic interactions, for regulation of osmotic pressure and as the cofactors in various enzymes (Bruins et al. 2000).

Many other metals like silver, cadmium, lead and mercury are non-essential and have no defined biological roles (Gadd 2010). These metals exert potential toxicity to the microorganisms. Toxicity of non-essential metals is either mediated by the displacement of essential metals from their native binding sites or through interactions with essential biomolecules. However, both essential and non-essential metals at high concentration damage the microbial cell membrane, disrupt cellular activities, alter enzyme functioning and damage the structure of DNA (Jomova and Valko 2011).

For their entry into the cells, microorganisms usually have two types of metal uptake systems: (1) Non-specific, chemiosmotic gradient driven transport across the cytoplasmic membrane. This is also termed as 'open gate' mechanism. (2) Substrate specific, slower and energy driven transport. The process often uses ATP as the energy source (Nies and Silver 1995).

Many divalent metal cations like Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} are structurally quite similar. Also, the structure of oxyanions such as chromate resembles that of sulphate. Same is true for arsenate and phosphate. In order to differentiate between structurally very similar metal ions, the microbial uptake system is tightly and specially regulated. Yet, at times, metal transport systems are unable to differentiate among metal ions having resemblance in charge and ionic radius. Hence, high concentrations of non-essential metals are also transported inside the cell by the 'open gate'. This is one of the reasons for metal mediated toxicity in the microorganisms (Nies 1999).

Since metal ions cannot be degraded or modified like toxic organic compounds, microorganisms adopt different mechanisms for the metal resistance (Bruins et al. 2000; Gadd 2010). Major mechanisms of metal resistance are: (1) Exclusion by permeability barrier (2) Intra and extracellular sequestration (3) Active efflux pumps (4) Enzymatic reduction and (5) Reduction in the sensitivity of cellular targets towards metal ions. One or more of these resistance mechanisms, alone or in combination, allow microorganisms to tolerate and function in the metal contaminated environments. Some of the common reactions/metal transformations are summarized in Figs. 6.1 and 6.2.

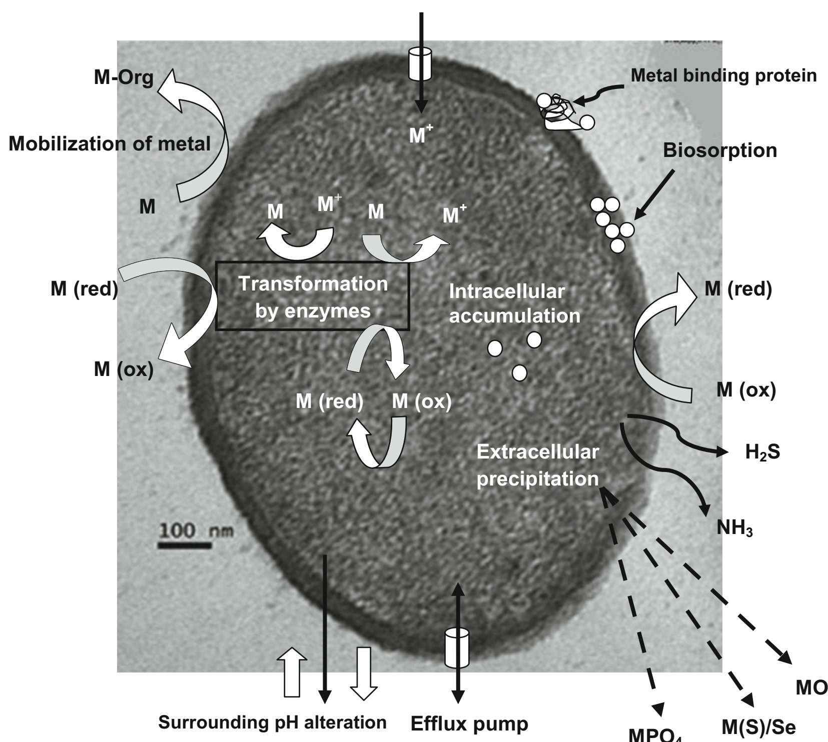


Fig. 6.1 Various physicochemical processes operational during interaction of microbes with metal(s). (*Circles*, M, M^{+}), metal or metal ions; $MHPO_4$, metal phosphate; M(S)/Se, metal sulphides/selenides; MO, metal oxide

6.3 Biotechnological Application of Metal Resistant Bacteria

Metals can impregnate through the microbial cell membrane. They interact with the cellular proteins, enzymes and the DNA. This interaction may lead to an alteration in cellular structure and functionality. Metal resistants, however, accumulate, transform or mobilize these metals with various cellular mechanisms. They possess an endogenous ability to exquisitely regulate their physiology to overcome the toxic effect of the external metal environment. During the process, different inorganic materials are synthesized or transformed (Klaus-Joerger et al. 2001). Thus, these microbes in combination with bioremediation can be harnessed to convert environmentally problematic metals into 'high-end' important and functional metal nanoparticles (Lloyd et al. 2011). Synthesis of nanoparticles having different chemical compositions and sizes/shapes with controlled monodispersity is one of the major challenges for their sustainable use. Currently, employed physical and chemical methods for the synthesis of nanoparticles have certain associated

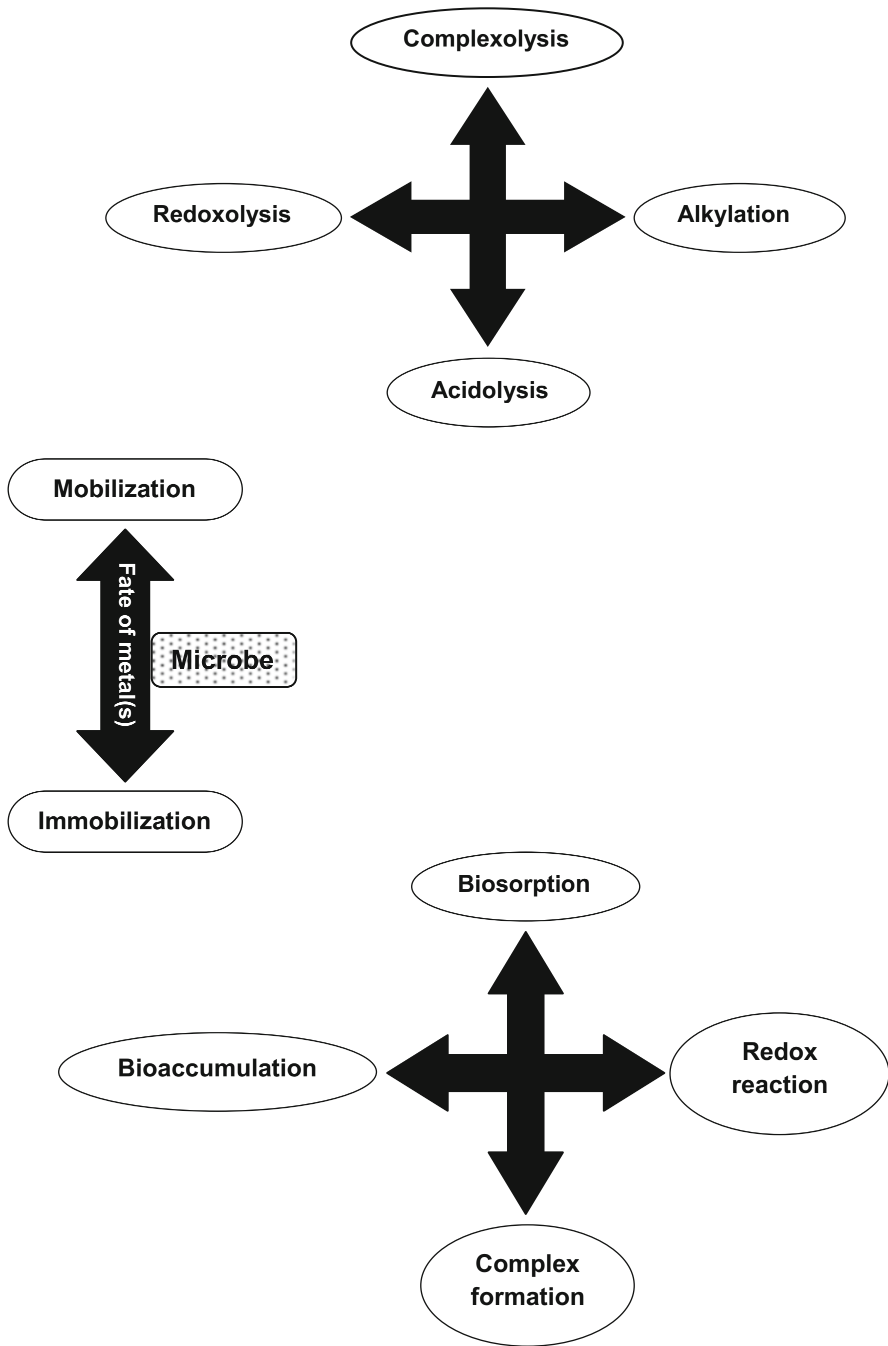


Fig. 6.2 Schematic diagram showing microbial reactions for mobilization and immobilization of metal(s)

problems such as stability, uncontrolled crystal growth and aggregation (Narayanan and Sakthivel 2010). Since the biological system has a strict control over their physiology; biosynthesis of nanoparticles can lead to desirable product, i.e. nanoparticles with good monodispersity having same chemical composition, shape and size (Klaus-Joerger et al. 2001; Mandal et al. 2006).

6.3.1 Bioremediation

Based on different physiological mechanism the metal bioremediation by microbes is broadly divided into two categories, namely (1) Metabolism dependent and (2) non-metabolism dependent.

6.3.1.1 Metabolism Dependent

Metabolism-dependent processes are the property of only viable cells. These are with the outcome of active defence mechanism of the living cell towards toxic metal in the surroundings. The toxic metals are firstly transported across the cell membrane. The transport is mediated by open gate or ATP driven processes, which are influenced by metabolic state of the cell, pH, availability of organic and inorganic nutrients, and other metabolites inside the cell (Gadd 2010).

6.3.1.2 Non-metabolism Dependent

Non-metabolism dependent processes are based on the physical adsorption, ion exchange, chemical sorption processes (Vijayaraghavan and Yun 2007). These are independent of metabolic state of the cells. The metal uptake is governed by physico-chemical interactions between the metal and functional groups present on the cell surface (Giotta et al. 2011).

In physical adsorption the metal ions are adsorbed due to Van der Waals' forces. Dead biomass of algae, fungi and yeasts adsorb the metal ion on their surface by this phenomenon. The adsorption process involves a solid phase called sorbent or biosorbent and a liquid phase normally water containing a dissolved metal ion. The metal ions bind to biosorbent. The process continues till equilibrium is established between the amount of solid-bound metals molecules and its part remaining in the solution. After some time, the biosorbent becomes enriched with metal ions (Fu and Wang 2011).

Since the biological cell walls contain polysaccharides, different bivalent metal ions are exchanged with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of Na^+, K^+, Ca^{2+} and Mg^{2+}. These ions are exchanged with metal ions such as Cu^{2+}, Cd^{2+}, Co^{2+} and Zn^{2+}, resulting in the biosorptive uptake of heavy metals (Kuyucak and Volesky 1988).

Table 6.1 Microbial heavy metal bioremediation

Microorganism	Metal/ metal ion	Removal	Process	Reference
Bacteria				
Pseudomonas sp. MBR	Ni^{2+}	–	Reduction of Ni^{2+} to Ni^0	Zhan et al. (2012)
Enterobacter sp.	Hg^{2+}	–	Bioaccumulation	Sinha and Khare (2012)
Bacillus megaterium	Se^{4+}	–	Reduction of Se^{4+} to Se^0	Mishra et al. (2011)
Bacillus sp.	Mn^{2+}	–	Bioaccumulation as MnO_2	Sinha et al. (2011)
Geobacter sulfurreducens	U^{6+}	–	Reduction of U^{6+} to U^{4+}	Cologgi et al. (2011)
Serratia marcescens	U^{6+}	90–92 %	Biosorption	Kumar et al. (2011)
Pseudomonas seleniiprae-cipitatus sp. nov	Se^{4+}	–	Reduction of Se^{4+} to Se^0	Hunter and Manter (2011)
Auricularia polytricha and *Tremella fuciformis*	Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}	85–75 %	Biosorption	Mo et al. (2010)
Arthrobacter ramosus strain G2	Hg^{2+}	90 %	Reduction of Hg^{2+} to volatile elemental Hg^0	Bafana et al. (2010)
Pseudomonas sp.	Mn^{2+}	109 mg/g	Biosorption	Gialamouidis et al. (2010)
Bacillus sp. L14	Cu^{2+}, Cd^{2+}, Pb^{2+}	21.3–80.5 %	Biosorption and bioaccumulation	Guo et al. (2010)
Staphylococcus capitis	Cr^{6+}	81–89 %	Reduction of Cr^{6+} to Cr^{3+}	Zahoor and Rehman (2009)
Rhodobacter sphaeroides	Cd^{2+}	97 %	Biosorption	Bai et al. (2008)
Leptothrix discophora SP-6	Mn^{2+}	90 %	Oxidation of Mn^{2+} as MnO_2	Burger et al. (2008)
Desulfotomaculum nigrificans	Zn^{2+}	70 %	Bioprecipitation and biosorption	Radhika et al. (2006)
Klebsiella pneumoniae M426	Hg^{2+}	99 %	Conversion of Hg^{2+} into volatile sulphur compound	Essa et al. (2005)
Fungus				
Aspergillus niger	As	–	Biosorption and bioaccumulation	Mukherjee et al. (2010)
Auricularia polytricha *Tremella fuciformis*	Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}	12.4–20.3 mg/g 16.4–19.9 mg/g	Biosorption	Mo et al. (2010)

(continued)

Table 6.1 (continued)

Microorganism	Metal/ metal ion	Removal	Process	Reference
Fusarium sp. A19 *Penicillium* sp. A1	Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}	–	Biosorption Bioaccumulation	Pan et al. (2009)
Aspergillus sp. 1 *Rhizopus* sp.	Cd^{2+}, Cr^{6+}	2.7–1.2 mg/g 2.7–4.3 mg/g	Biosorption	Zafar et al. (2007)
Acremonium sp. KR21-2	Mn^{2+}	–	Oxidation of Mn^{2+} to Mn^{4+}	Miyata et al. (2006)
Algae				
Ulva lactuca	Cu^{2+}, Zn^{2+}, Cd^{2}, Pb^{2+}	32–54 mg/g	Biosorption	Areco et al. (2012)
Spirogyra sp.	Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}	90–95 %	Biosorption	Rajfur et al. (2010)
Chlorella vulgaris	U^{6+}	45–90 %	Biosorption	Vogel et al. (2010)
Mastocarpus stellatus	Cd^{2+}	90 %	Biosorption	Herrero et al. (2008)
Ulothrix cylindricum	As^{3+}	67.2 mg/g	Biosorption	Tuzen et al. (2009)
Catenella repens	U^{6+}	>90 %	Biosorption	Bhat et al. (2008)
U. reticulata	Zn^{2+}	135.5 mg/g	Biosorption	Senthilkumar et al. (2006)

In complexation the metals are removed from the solution by complex formation with the functional group present on the cell surface. Common functional groups that participate in metal complexation are carboxyl, amino and thiols present in polysaccharides and other polymers (Wang and Chen 2009).

Depending on the nature of cells, various mechanisms mentioned above can take place simultaneously or individually. Some of the microbial strains used for the remediation of heavy metal are enlisted in Table 6.1.

6.3.2 Synthesis of Nanoparticles

In recent years, the synthesis of nanoparticles has become an important aspect of nanotechnology. Various physical and chemical methods have been developed for this purpose. All these methods are put under two categories: (1) top-down and (2) bottom-up approach.

6.3.2.1 Top-Down Approach

In this approach, a suitable starting material is reduced in size step by step. The process uses the physical and lithographic principles of micro and nanotechnology to achieve desirable structure and sizes (to nanometre range) from starting materials (Dhingra et al. 2010; Merkel et al. 2010). The approach includes various physical methods like evaporation/condensation, vacuum deposition, arc discharge, laser ablation, milling and attrition. (Lam et al. 2000; Lue 2001).

6.3.2.2 Bottom-Up Approach

Bottom-up approach employs chemical means for the fabrication of the nanoparticles. It is done by the assembly of atom-by-atom, molecule-by-molecule or cluster-by-cluster, i.e. ionic, atomic or molecular units are assembled to form nanosized structures (Samineni and Goswami 2008; Thakkar et al. 2010). Initially nanostructured building blocks are formed which are subsequently assembled into the final material. Approach includes the methods like co-precipitation, sol-gel process, micro-emulsion, hydrothermal/solvothermal and templated synthesis (Cushing et al. 2004; Eastoe et al. 2006; Trewyn et al. 2007; Zhao et al. 2011).

6.3.2.3 Limitations of the Major Physical and Chemical Methods

The complexity of chemical ingredients (such as metal precursors, reducing agents, stabilizers and solvents), contamination of metal nanoparticles with organic stabilizers, use of toxic reducing agents, requirement of inert conditions and production of hazardous by-products are some major drawbacks of the currently applied processes (Djerdj et al. 2007; Sharma et al. 2007; Zhao et al. 2010). Costly equipments, complicated vacuum operation and requirement of stringent temperature and pressure make these processes more energy and capital intensive (Eastoe et al. 2006; Kim et al. 2009). The chemical and physical processes have further limitations of agglomeration of the particles, lesser control over monodispersity, particle shape and size distribution (Kannan et al. 2011; Stoerzinger et al. 2011; Zhao et al. 2008). In this context, use of different biological systems has gained quite a prominence, in recent years (Mandal et al. 2006; Mohanpuria et al. 2008).

6.4 Microbial Nanoparticle Synthesis

Over the year metal microbes interactions have been extensively exploited for bioremediation of heavy metals (Malik 2004; Narayanan and Sakthivel 2010). However, their use in nanoparticle synthesis by microbial systems has emerged

Table 6.2 Microbial nanobiosynthesis

Microorganism	Nanoparticle	Size (nm)	Localization of nanoparticle	Reference
Bacteria				
Brevibacterium casei SRKP2	CdS	10–30	Intracellular	Pandian et al. (2011)
Enterobacter sp.	$Hg_3(PO_4)_2$	2–5	Intracellular	Sinha and Khare (2011)
Shewanella oneidensis	Pd	5–10	Periplasmic space	Ogi et al. (2011)
Pseudomonas aeruginosa	Ag	13	Extracellular	Kumar and Mamidyala (2011)
Bacillus subtilis	Au Ag	7.6 ± 1.8, 7.3 ± 2.3 6.1 ± 1.6	Intra and extracellular Extracellular	Satyanarayana et al. (2010)
Lactobacillus sp.	TiO_2	8–35	Extracellular	Jha et al. (2009a)
Bacillus cereus	Ag	4–5	Intracellular	Babu and Gunasekaran (2009)
Klebsiella pneumoniae	Ag	1–6	Extracellular	Mokhtari et al. (2009)
Brevibacterium casei	Co_3O_4	–	Extracellular	Kumar et al. (2009)
Shewanella oneidensis MR-1	UO_2	3.0 ± 0.1	Extracellular	Burgos et al. (2008)
Bacillus selenitireducens	Te	200–1,000	Intracellular and extracellular	Baesman et al. (2007)
Rhodopseudomonas capsulata	Au	10–20	Intracellular	He et al. (2007)
Shewanella algae	Pt	5	Periplasm	Konishi et al. (2007)
Sulfurospirillum barnesii	Te	<50	Intracellular and extracellular	Baesman et al. (2007)
M. gryphiswaldense	Fe_3O_4	35–120	Intracellular	Lang and Schuler (2006)
Actinobacter sp.	Fe_2O_3	50–150	Extracellular	Bharde et al. (2005)
Escherichia coli	CdS	2–5	Intracellular	Sweeney et al. (2004)
Rhodococcus sp.	Au	5–15	Intracellular	Ahmad et al. (2003b)
Fungi				
Sclerotium rolfsii	Au	25	Cell free extract	Narayanan and Sakthivel (2011a)
Cylindrocladium floridanum	Au	5–35	Cell surface	Narayanan and Sakthivel (2011b)
Rhizopus stolonifer	Ag	3–20	Fungal filtrate	Banu et al. (2011)
Aspergillus niger	Ag	3–30	Extracellular	Jaidev and Narasimha (2010)
Streptomyces hygroscopicus	Ag	20–30	Extracellular	Sadhasivam et al. (2010)
Alternaria alternata	Ag	20–60	Extracellular	Gajbhiye et al. (2009)
Fusarium oxysporum	Pt	100–180	Intracellular	Govender et al. (2010)
Rhizopus oryzae	Au	10	Cell surface	Das et al. (2009)

(continued)

Table 6.2 (continued)

Microorganism	Nanoparticle	Size (nm)	Localization of nanoparticle	Reference
Saccharomyces cerevisiae	TiO_2	8–35	Extracellular	Jha et al. (2009a)
Saccharomyces cerevisiae	Sb_2O_3	2–10	Extracellular	Jha et al. (2009b)
Trichoderma asperellum	Ag	13–18	Extracellular	Mukherjee et al. (2008)
Aspergillus flavus	Ag	8.92 ± 1.61	Cell surface	Vigneshwaran et al. (2007)
Fusarium oxysporum	$BaTiO_3$	4–5	Extracellular	Bansal et al. (2006)
Thermomonospora sp.	Au	5–15	Extracellular	Ahmad et al. (2003a)
Fusarium oxysporum	Ag	8–40	Extracellular	Mukherjee et al. (2002)
Torulopsis sp.	PbS	2–5	Intracellular	Kowshik et al. (2002b)
Algae				
Tetraselmis suecica	Au	79	Extracellular	Shakibaie et al. (2010)
Chlorella vulgaris	Ag	<30	Extracellular	Xie et al. (2007)
Sargassum wightii	Au	8–12	Extracellular	Singaravelu et al. (2007)
Yeast				
Schizosaccharomyces pombe	CdS	2–2.5	Intracellular	Kowshik et al. (2002a)

as a new concept only recently. Although, naturally synthesized nanoparticles like siliceous nanoparticles by diatoms (Milligan and Morel 2002), magnetite nanoparticles by magnetotactic bacteria (Bharde et al. 2005) and gypsum and calcium layers by S-layer bacteria (Pum and Sleytr 1999), occurred in nature, the first report of intracellular synthesis of iron rich magnetic nanoparticles by magnetotactic bacteria was received only in 1975 (Blakemore 1975). Many reports on controlled microbial nanoparticle synthesis have been received since then. Some of the interesting examples of microbial nanobiosynthesis are described in Table 6.2.

6.5 Mechanism of Microbial Nano-biosynthesis

The mechanism of nanoparticle synthesis by the microorganism is not very clear so far. It has been proposed that the nanoparticle synthesis is the result of self-defence mechanism in response to the toxic environment (Hallmann et al. 1997). The production or secretion of proteins molecules, lipopolysaccharides, phospholipids and polyphosphates have been implicated in protecting the cells from metal toxicity. Microbial cells release specific enzymes, which remove toxic metals by variety of

reactions. Nanoparticles are probably the result of some such reactions. However, the precise mechanism of nanoparticles synthesis is yet to be understood. Generally, two types of nanoparticles, namely (1) metallic nanoparticles and (2) compound nanoparticles are synthesized by microorganisms. The synthesis is either intracellular or extracellular. According to Zhang et al. (2011), the process of nanoparticle formation starts with the trapping of the metal ions by the cells, probably by (1) electrostatic interaction and/or (2) by secretion of substances like extracellular polymers that make the metal ions to adhere to the cell. Since most of the microbial cells have negatively charged outer cell wall, the electrostatic interactions between the positively charged ions and the negatively charged groups play an important role in holding the metal ions (Manti et al. 2008). The secretion of adhesive polymeric substances further stabilizes the metal ions on the cell wall. The metal ions which are transported inside the cells either by nutrient exchange or by substance diffusion become the source for the intracellular nanoparticles. Inside cytoplasm metal ions are reacted by the enzyme secreted to detoxify them. These are either precipitated out onto the cell surface or retained inside (Zhang et al. 2011). During the synthesis of metallic nanoparticles, metal ions are reduced with the help of certain enzymes like NADH-dependent reductase (Mukherjee et al. 2002). For the synthesis of compound nanoparticles, involvement of oxido-reductases is predicted. It is believed that cellular metal binding proteins provide amino acid moieties, which serve as nucleation sites for the synthesis of nanoparticles (Naik et al. 2002). Finally, the nuclei grow and accumulate as intracellular or extracellular nanoparticles. These are further stabilized by the protein molecules (Yoshimura 2006).

The microbially synthesized extracellular nanoparticles have advantage of easy recovery but lack in monodispersity whereas the intracellularly synthesized nanoparticles have better monodispersity (Narayanan and Sakthivel 2010). Some enzymes and biomolecules involved in microbial nanoparticle synthesis are enlisted in Table 6.3. A number of other biomolecules like fatty acids, amino acids and polyphates have been known to assist in synthesis of nanoparticles. However, along with these enzymes and biomolecules, some other electron mediators are also needed to facilitate the desired nanocrystals.

6.6 Conclusions

Microorganisms showing resistance to metals are also dubbed as metallophiles. These can be effectively be used in remediation of environmental problematic heavy metals along with the synthesis of valuable metal nanoparticles and other metal compounds. They are efficient in controlling the monodispersity of nanoparticles which is one of the prerequisite for the application of nanoparticles and thus can be harvested for obtaining valuable nanoparticles which are otherwise difficult to obtain. However, the biological nanoparticle synthesis is still in infancy. For their commercial exploitation, many improvements like process optimization

Table 6.3 Cellular enzymes and biomolecules involved in microbial nanoparticle synthesis

Enzyme/biomolecules involved	Microorganism	Nanoparticle	Localization	Reference
Bacteria				
90 kDa protein	*Tetrathiobacter kashmirensis*	Se	Intracellular	Hunter and Manter (2008)
Nitroreductase	*Escherichia coli* *Enterobacter cloacae* *Klebsiella pneumonia*	Ag	Extracellular	Shahverdi et al. (2007)
NADPH-dependent enzymes and carotenoids	*Rhodobacter capsulatus*	Au	Intracellular	Feng et al. (2007)
Fungi				
Dimeric hydrogenase (44.5 and 39.4 kDa)	*Fusarium oxysporum*	Pt	Extracellular	Govender et al. (2010)
Compactin	*Penicillium brevicompactum*	Ag	Extracellular	Shaligram et al. (2009)
Nitrate reductase	*Aspergillus niger*	Ag	Extracellular	Gade et al. (2008)
Sulfite reductase (35.6 kDa) and phytochelatin Nitrate reductase and phytochelatin	*Fusarium oxysporum*	Au Ag	Extracellular	Kumar et al. (2007a, b)
55 and 13 kDa proteins	*Verticillium* sp., Actinomycete	Magnetite	Extracellular	Bharde et al. (2006)
80–10 kDa proteins	*Thermomonospora* sp.	Au	Extracellular	Ahmad et al. (2003a)
Glutathiones	*Colletotrichum* sp.	Au	Extracellular	Shankar et al. (2003)
66 and 10 kDa proteins	*Fusarium oxysporum*	Au	Extracellular	Mukherjee et al. (2002)
Yeast				
Membrane-bound quinine or oxidoreductase	*Saccharomyces cerevisiae*	Sb_2O_3	Intracellular	Jha et al. (2009b)
Peptidoglycan reducing sugars	*Schizosaccharomyces cerevisiae*	Au	Intracellular	Lin et al. (2005)

for better control over particle size, monodispersity and higher yield are needed. Rules need to be defined for microbial growth conditions, medium composition, favouring efficient synthesis.

References

Ahmad A, Senapati S, Khan MI, Kumar R, Sastry M (2003a) Extracellular biosynthesis of monodisperse gold nanoparticles by a novel extremophilic actinomycete *Thermomonospora* sp. Langmuir 19:3550–3553

Ahmad A, Senapati S, Khan MI, Kumar R, Ramani R, Srinivas V, Sastry M (2003b) Intracellular synthesis of gold nanoparticles by a novel alkalotolerant actinomycete *Rhodococcus* species. Nanotechnology 14:824–828

Areco MM, Hanela S, Duran J, Afonso MDS (2012) Biosorption of Cu(II), Zn(II), Cd(II) and Pb (II) by dead biomasses of green alga *Ulva lactuca* and the development of a sustainable matrix for adsorption implementation. J Hazard Mater 213–214:123–132

Babu MMG, Gunasekaran P (2009) Production and structural characterization of crystalline silver nanoparticles from *Bacillus cereus* isolate. Colloids Surf B Biointerfaces 74:191–195

Baesman SM, Bullen TD, Dewald J, Zhang D, Curran S, Islam FS, Beveridge TJ, Oremland RS (2007) Formation of tellurium nanocrystals during anaerobic growth of bacteria that use Te oxyanions as respiratory electron acceptors. Appl Environ Microbiol 73:2135–2143

Bafana A, Krishnamurthi K, Patil M, Chakrabarti T (2010) Heavy metal resistance in *Arthrobacter ramosus* strain G2 isolated from mercuric salt-contaminated soil. J Hazard Mater 177:481–486

Bai H-J, Zhang Z-M, Yang G-E, Li B-Z (2008) Bioremediation of cadmium by growing *Rhodobacter sphaeroides*: Kinetic characteristic and mechanism studies. Bioresour Technol 99:7716–7722

Bansal V, Poddar P, Ahmad A, Sastry M (2006) Room temperature biosynthesis of ferroelectric barium titanate nanoparticles. J Am Chem Soc 128:11958–11963

Banu A, Rathod V, Ranganath E (2011) Silver nanoparticle production by *Rhizopus stolonifer* and its antibacterial activity against extended spectrum β-lactamase producing (ESBL) strains of Enterobacteriaceae. Mater Res Bull 46:1417–1423

Bharde A, Wani A, Shouche Y, Joy PA, Prasad BLV, Sastry M (2005) Bacterial aerobic synthesis of nanocrystalline magnetite. J Am Chem Soc 127:9326–9327

Bharde A, Rautaray D, Bansal V, Ahmad A, Sarkar I, Yusuf SM, Sanyal M, Sastry M (2006) Extracellular biosynthesis of magnetite using fungi. Small 1:135–141

Bhat SV, Meloa JS, Chaugule BB, D'Souza SF (2008) Biosorption characteristics of uranium (VI) from aqueous medium onto *Catenella repens*, a red alga. J Hazard Mater 158:628–635

Blakemore R (1975) Magnetotactic bacteria. Science 190:377–379

Brandl H, Faramarzi MA (2006) Microbe metal interactions for the biotechnological treatment of metal containing solid waste. Particuology 4:93–97

Brown S, Sarikaya M, Johnson E (2000) A genetic analysis of crystal growth. J Mol Biol 299:725–735

Bruins MR, Kapil S, Oehme FW (2000) Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45:198–207

Burger MS, Mercer SS, Shupe GD, Gagnon GA (2008) Manganese removal during bench scale biofiltration. Water Res 42:4733–4742

Burgos WD, McDonough JT, Senko JM, Zhang G, Dohnalkova AC, Kelly SD, Gorby Y, Kemner KM (2008) Characterization of uraninite nanoparticles produced by *Shewanella oneidensis* MR-1. Geochim Cosmochim Acta 72:4901–4915

Cologgi DL, Lampa-Pastirk S, Speers AM, Kelly SD, Reguera G (2011) Extracellular reduction of uranium via Geobacter conductive pili as a protective cellular mechanism. Proc Natl Acad Sci U S A 108:15248–15252

Cushing BL, Kolesnishenko VL, O'Connor CJ (2004) Recent advances in the liquid-phase syntheses of inorganic nanoparticles. Chem Rev 104:3893–3946

Das SK, Das AR, Guha AK (2009) Gold nanoparticles: microbial synthesis and application in water hygiene management. Langmuir 25:8192–8199

Dhingra R, Naidu S, Upreti G, Sawhney R (2010) Sustainable nanotechnology: through green methods and life cycle thinking. Sustainability 2:3323–3338

Djerdj I, Arčon D, Jagličić Z, Niederberger M (2007) Nonaqueous synthesis of manganese oxide nanoparticles, structural characterization, and magnetic properties. J Phys Chem C 111:3614–3623

Eastoe J, Hollamby MJ, Hudson L (2006) Recent advances in nanoparticle synthesis with reversed micelles. Adv Colloid Interface Sci 128–130:5–15

Ehrlich HL (1997) Microbes and metals. Appl Microbiol Biotechnol 48:687–692

Essa AMM, Macaskie LE, Brown NL (2005) A new method for mercury removal. Biotechnol Lett 27:1649–1655

Feng Y, Yu Y, Wang Y, Lin X (2007) Biosorption and bioreduction of trivalent aurum by photosynthetic bacteria *Rhodobacter capsulatus*. Curr Microbiol 55:402–408

Fu F, Wang Q (2011) Removal of heavy metal ions from wastewaters: a review. J Environ Manage 92:407–418

Gadd GM (2007) Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol Res 111:3–49

Gadd GM (2010) Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156:609–643

Gade AK, Bonde PP, Ingle AP, Marcato P, Duran N, Rai MK (2008) Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles. J Biobased Mater Bioenergy 2:1–5

Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M (2009) Fungus mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 5:382–386

Gialamouidis D, Mitrakas M, Liakopoulou-Kyriakides M (2010) Equilibrium, thermodynamic and kinetic studies on biosorption of Mn(II) from aqueous solution by *Pseudomonas* sp., *Staphylococcus xylosus* and *Blakeslea trispora* cells. J Hazard Mater 182:672–680

Giotta L, Mastrogiacomo D, Italiano F, Milano F, Agostiano A, Nagy K, Valli L, Trotta M (2011) Reversible binding of metal ions onto bacterial layers revealed by protonation induced ATR-FTIR difference spectroscopy. Langmuir 27:3762–3773

Govender Y, Riddin TL, Gericke M, Whiteley CG (2010) On the enzymatic formation of platinum nanoparticles. J Nanopart Res 12:261–271

Guo H, Luo S, Chen L, Xiao X, Xi Q, Wei W, Zeng G, Liu C, Wan Y, Chen J, He Y (2010) Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium *Bacillus* sp. L14. Bioresour Technol 101:8599–8605

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43:895–914

He S, Guo Z, Zhang Y, Zhang S, Wang J, Gu N (2007) Biosynthesis of gold nanoparticles using the bacteria *Rhodopseudomonas capsulate*. Mater Lett 61:3984–3987

Herrero R, Lodeiro P, Rojo R, Rodrı’guez ACP, de Vicente MES (2008) The efficiency of the red alga Mastocarpus stellatus for remediation of cadmium pollution. Bioresour Technol 99:4138–4146

Hunter WJ, Manter DK (2008) Bio-reduction of selenite to elemental red selenium by *Tetrathiobacter kashmirensis*. Curr Microbiol 57:83–88

Hunter WJ, Manter DK (2011) *Pseudomonas seleniipraecipitatus* sp. nov.: a selenite reducing γ-Proteobacteria isolated from soil. Curr Microbiol 62:565–569

Jaidev LR, Narasimha G (2010) Fungal mediated biosynthesis of silver nanoparticles, characterization and antimicrobial activity. Colloids Surf B Biointerfaces 81:430–433

Jha AK, Prasad K, Kulkarni AR (2009a) Synthesis of TiO_2 nanoparticles using microorganisms. Colloids Surf B Biointerfaces 71:226–229

Jha AK, Prasad K, Prasad K (2009b) A green low cost biosynthesis of Sb_2O_3 nanoparticles. Biochem Eng J 43:303–306

Jomova K, Valko M (2011) Advances in metal induced oxidative stress and human disease. Toxicology 283:65–87

Kannan N, Mukunthan KS, Balaji S (2011) A comparative study of morphology, reactivity and stability of synthesized silver nanoparticles using *Bacillus subtilis* and *Catharanthus roseus* (L.) G. Don. Colloids Surf B Biointerfaces 86:378–383

Kim J, Kim D, Veriansyah B, Kang JW, Kim J-D (2009) Metal nanoparticle synthesis using supercritical alcohol. Mater Lett 63:1880–1882

Klaus-Joerger T, Joerger R, Olsson E, Granqvist C-G (2001) Bacteria as workers in the living factory: metal accumulating bacteria and their potential for materials science. Trends Biotechnol 19:15–20

Konishi Y, Ohno K, Saitoh N, Nomura T, Nagamine S, Hishida H, Takahashi Y, Uruga T (2007) Bioreductive deposition of platinum nanoparticles on the bacterium *Shewanella algae*. J Biotechnol 128:648–653

Kowshik M, Desmukh N, Vogel W, Urban J, Kulkarni SK, Paknikar KM (2002a) Microbial synthesis of semiconductor CdS nanoparticles, their characterization and their use in the fabrication of an ideal diode. Biotechnol Bioeng 78:583–588

Kowshik M, Vogel W, Urban J, Kulkarni SK, Paknikar KM (2002b) Microbial synthesis of semiconductor PbS Nanocrystallites. Adv Mater 14:815–818

Kumar CG, Mamidyala SK (2011) Extracellular synthesis of silver nanoparticles using culture supernatant of *Pseudomonas aeruginosa*. Colloids Surf B Biointerfaces 84:462–466

Kumar SA, Abyaneh MK, Gosavi SW, Kulkarni SK, Pasricha R, Ahmad A, Khan MI (2007a) Nitrate reductase mediated synthesis of silver nanoparticles from $AgNO_3$. Biotechnol Lett 29:439–445

Kumar SA, Abyaneh MK, Gosavi SW, Kulkarni SK, Ahmad A, Khan MI (2007b) Sulfite reductase mediated synthesis of gold nanoparticles capped with phytochelatin. Biotechnol Appl Biochem 47:191–195

Kumar U, Vivekanand K, Poddar P (2009) Real time nanomechanical and topographical mapping on live bacterial cells *Brevibacterium casei* under stress due to their exposure to Co^{2+} ions during microbial synthesis of Co_3O_4 nanoparticles. J Phys Chem B 113:7927–7933

Kumar R, Acharya C, Joshi SR (2011) Isolation and analysis of uranium tolerant *Serratia marcescens* strains and their utilization for aerobic uranium U(VI) bioadsorption. J Microbiol 49:568–574

Kuyucak N, Volesky B (1988) Biosorbents for recovery of metals from industrial solutions. Biotechnol Lett 10:137–142

Lam C, Zhang YF, Tang YH, Lee CS, Bello I, Lee ST (2000) Large scale synthesis of ultrafine Si nanoparticles by ball milling. J Cryst Growth 220:466–470

Lang C, Schuler D (2006) Biogenic nanoparticles: production, characterization, and application of bacterial magnetosomes. J Phys Condens Matter 18:S2815–S2828

Lin Z, Wu J, Xue R, Yang Y (2005) Spectroscopic characterization of Au^{3+} biosorption by waste biomass of *Saccharomyces cerevisiae*. Spectrochim Acta A Mol Biomol Spectrosc 61:761–765

Lloyd JR, Byrne JM, Coker VS (2011) Biotechnological synthesis of functional nanomaterials. Curr Opin Biotechnol 22:509–515

Lue J-T (2001) A review of characterization and physical property studies of metallic nanoparticles. J Phys Chem Solids 62:1599–1612

Malik A (2004) Metal bioremediation through growing cells. Environ Int 30:261–278

Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P (2006) The use of microorganisms for the formation of metal nanoparticles and their application. Appl Microbiol Biotechnol 69:485–492

Manti A, Boi P, Falcioni T, Canonico B, Ventura A, Sisti D, Pianetti A, Balsamo M, Papa S (2008) Bacterial cell monitoring in wastewater treatment plants by flow cytometry. Water Environ Res 80:346–354

Mapelli F, Marasco R, Balloi A, Rolli E, Cappitelli F, Daffonchio D, Borin S (2011) Mineral microbe interactions: biotechnological potential of bioweathering. J Biotechnol 157 (4):473–481. doi:10.1016/j.jbiotec.2011.11.013

Merkel TJ, Herlihy KP, Nunes J, Orgel RM, Rolland JP, DeSimone JM (2010) Scalable, shape specific, top-down fabrication methods for the synthesis of engineered colloidal particles. Langmuir 26:13086–13096

Milligan AJ, Morel FMM (2002) A proton buffering role for silica in diatoms. Science 297:1848–1850

Mishra RR, Prajapati S, Das J, Dangar TK, Das N, Thatoi H (2011) Reduction of selenite to red elemental selenium by moderately halotolerant *Bacillus megaterium* strains isolated from Bhitarkanika mangrove soil and characterization of reduced product. Chemosphere 84:1231–1237

Miyata N, Tani Y, Maruo K, Tsuno H, Sakata M, Iwahori K (2006) Manganese(IV) oxide production by *Acremonium* sp. strain KR21-2 and extracellular Mn(II) oxidase activity. Appl Environ Microbiol 72:6467–6473

Mo Y, Pan R, Huang HW, Cao LX, Zhang RD (2010) Biosorption of Cd(II), Cu(II), Pb(II) and Zn (II) in aqueous solutions by fruiting bodies of macrofungi (*Auricularia polytricha* and *Tremella fuciformis*). Huan Jing Ke Xue 31:1566–1574

Mohanpuria P, Rana NK, Yadav SK (2008) Biosynthesis of nanoparticles: technological concepts and future applications. J Nanopart Res 10:507–517

Mokhtari N, Daneshpajouh S, Seyedbagheri S, Atashdehghan R, Abdi K, Sarkar S, Minaian S, Shahverdi HR, Shahverdi AR (2009) Biological synthesis of very small silver nanoparticles by culture supernatant of *Klebsiella pneumonia*: the effects of visible light irradiation and the liquid mixing process. Mater Res Bull 44:1415–1421

Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M (2002) Extracellular synthesis of gold nanoparticles by the fungus *Fusarium oxysporum*. ChemBioChem 5:461–463

Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK, Ghatak J, Tyagi AK, Kale SP (2008) Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. Nanotechnology 19:075103–075110

Mukherjee A, Das D, Mondal SK, Biswas R, Das TK, Boujedaini N, Khuda-Bukhsh AR (2010) Tolerance of arsenate-induced stress in *Aspergillus niger*, a possible candidate for bioremediation. Ecotoxicol Environ Saf 73:172–182

Naik RR, Stringer SJ, Agarwal G, Jones SE, Stone MO (2002) Biomimetic synthesis and patterning of silver nanoparticles. Nat Mater 1:169–172

Narayanan KB, Sakthivel N (2010) Biological synthesis of metal nanoparticles by microbes. Adv Colloid Interface Sci 156:1–13

Narayanan KB, Sakthivel N (2011a) Facile green synthesis of gold nanostructures by NADPH dependent enzyme from the extract of *Sclerotium rolfsii*. Colloids Surf A Physicochem Eng Asp 380:156–161

Narayanan KB, Sakthivel N (2011b) Synthesis and characterization of nano gold composite using *Cylindrocladium floridanum* and its heterogeneous catalysis in the degradation of 4-nitrophenol. J Hazard Mater 189:519–525

Nies DH (1999) Microbial heavy metal resistance. Appl Microbiol Biotechnol 51:730–750

Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14:186–199

Ogi T, Honda R, Tamaoki K, Saitoh N, Konishi Y (2011) Direct room temperature synthesis of a highly dispersed Pd nanoparticle catalyst and its electrical properties in a fuel cell. Powder Technol 205:143–148

Pan R, Cao L, Zhang R (2009) Combined effects of Cu, Cd, Pb, and Zn on the growth and uptake of consortium of Cu-resistant *Penicillium* sp. A1 and Cd-resistant *Fusarium* sp. A19. J Hazard Mater 171:761–766

Pandian SRK, Deepak V, Kalishwaralal K, Gurunathan S (2011) Biologically synthesized fluorescent CdS NPs encapsulated by PHB. Enzyme Microb Technol 48:319–325

Pum D, Sleytr UB (1999) The application of bacterial S-layers in molecular nanotechnology. Trends Biotechnol 17:8–12

Radhika V, Subramanian S, Natarajan KA (2006) Bioremediation of zinc using *Desulfotomaculum nigrificans*: bioprecipitation and characterization studies. Water Res 40:3628–3636

Rajfur M, Kłos A, Wacławek M (2010) Sorption properties of algae *Spirogyra* sp. and their use for determination of heavy metal ions concentrations in surface water. Bioelectrochemistry 80:81–86

Sadhasivam S, Shanmugam P, Yun YS (2010) Biosynthesis of silver nanoparticles by *Streptomyces hygroscopicus* and antimicrobial activity against medically important pathogenic microorganisms. Colloids Surf B Biointerfaces 81:358–362

Samineni P, Goswami D (2008) Coded nanoscale self-assembly, Pramana. J Phys 7:1345–1351

Satyanarayana RA, Chien-Yen C, Chien-Cheng C, Jiin-Shuh J, Hau-Ren C, Min-Jen T, Cheng-Wei F, Jung-Chen W (2010) Biological synthesis of gold and silver nanoparticles mediated by the bacteria *Bacillus subtilis*. J Nanosci Nanotechnol 10:6567–6574

Senthilkumar R, Vijayaraghavan K, Thilakavathi M, Iyer PVR, Velan M (2006) Seaweeds for the remediation of wastewaters contaminated with zinc(II) ions. J Hazard Mater B 136:791–799

Shahverdi AR, Minaeian S, Shahverdi HR, Jamalifar H, Nohi AA (2007) Rapid synthesis of silver nanoparticles using culture supernatants of Enterobacteria: a novel biological approach. Process Biochem 42:919–923

Shakibaie M, Forootanfar H, Mollazadeh-Moghaddam K, Bagherzadeh Z, Nafissi-Varcheh N, Shahverdi AR, Faramarzi MA (2010) Green synthesis of gold nanoparticles by the marine microalga *Tetraselmis suecica*. Biotechnol Appl Biochem 57:71–75

Shaligram NS, Bule M, Bhambure R, Singhal RS, Singh SK, Szakacs G, Panday A (2009) Biosynthesis of silver nanoparticles using aqueous extract from the compactin producing fungal strain. Process Biochem 44:939–943

Shankar SS, Ahmad A, Pasricha R, Sastry M (2003) Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. J Mater Chem 13:1822–1826

Sharma NC, Sahi SV, Nath S, Parsons JG, Gardea-Torresdey JL, Pal T (2007) Synthesis of plant mediated gold nanoparticles and catalytic role of biomatrix embedded nanomaterials. Environ Sci Technol 41:5137–5142

Singaravelu G, Arockiamary JS, Kumar VG, Govindaraju K (2007) Novel extracellular synthesis of monodisperse gold nanoparticles using marine alga, *Sargassum wightii greville*. Colloids Surf B Biointerfaces 57:97–101

Sinha A, Khare SK (2011) Mercury bioaccumulation and simultaneous nanoparticle synthesis by *Enterobacter* sp. cells. Bioresour Technol 102:4281–4284

Sinha A, Khare SK (2012) Mercury bioremediation by mercury accumulating *Enterobacter* sp. cells and its alginate immobilized application. Biodegradation 23:25–34

Sinha A, Singh VN, Mehta BR, Khare SK (2011) Synthesis and characterization of monodispersed orthorhombic manganese oxide nanoparticles produced by *Bacillus* sp. cells simultaneous to its bioremediation. J Hazard Mater 192:620–627

Stoerzinger KA, Lin JY, Odom TW (2011) Nanoparticle SERS substrates with 3D Raman active volumes. Chem Sci 2:1435–1439

Sweeney RY, Mao C, Gao X, Burt JL, Belcher AM, Georgiou G, Iverson BL (2004) Bacterial biosynthesis of cadmium sulfide nanocrystals. Chem Biol 11:1553–1559

Thakkar KN, Mhatre SS, Parikh RY (2010) Biological synthesis of metallic nanoparticles. Nanomedicine 6:257–262

Trewyn BG, Slowing II, Giri S, Chen HT, Lin VSY (2007) Synthesis and functionalization of a mesoporous silica nanoparticle based on the sol-gel process and applications in controlled release. Acc Chem Res 40:846–853

Tuzen M, Sarı A, Mendil D, Uluozlu OD, Soylak M, Dogan M (2009) Characterization of biosorption process of As(III) on green algae *Ulothrix cylindricum*. J Hazard Mater 165:566–572

Vigneshwaran N, Ashtaputre NM, Varadarajan PV, Nachane RP, Paralikar KM, Balasubramanya RH (2007) Biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus*. Mater Lett 61:1413–1418

Vijayaraghavan K, Yun YS (2007) Utilization of fermentation waste (*Corynebacterium glutamicum*) for biosorption of reactive black 5 from aqueous solution. J Hazard Mater 141:45–52

Vogel M, Günther A, Rossberg A, Li B, Bernhard G, Raff J (2010) Biosorption of U(VI) by the green algae *Chlorella vulgaris* in dependence of pH value and cell activity. Sci Total Environ 409:384–395

Wang J, Chen C (2009) Biosorbents for heavy metals removal and their future. Biotechnol Adv 27:195–226

Xie J, Lee JY, Wang DIC, Ting YP (2007) Silver nanoplates: from biological to biomimetic synthesis. ACS Nano 1:429–439

Yoshimura H (2006) Protein assisted nanoparticle synthesis. Colloids Surf A Physicochem Eng Asp 282–283:464–470

Zafar S, Aqil F, Ahmad I (2007) Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technol 98:2557–2561

Zahoor A, Rehman A (2009) Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. J Environ Sci 21:814–820

Zhan G, Li D, Zhang L (2012) Aerobic bioreduction of nickel(II) to elemental nickel with concomitant biomineralization. Appl Microbiol Biotechnol 96(1):273–281. doi:10.1007/s00253-011-3827-9

Zhang X, Yan S, Tyagi RD, Surampalli RY (2011) Synthesis of nanoparticles by microorganisms and their application in enhancing microbiological reaction rates. Chemosphere 82:489–494

Zhao N, Nie W, Liu X, Tian S, Zhang Y, Ji X (2008) Shape and size controlled synthesis and dependent magnetic properties of nearly monodisperse Mn_3O_4 nanocrystals. Small 4:77–81

Zhao T, Sun R, Yu S, Zhang Z, Zhou L, Huang H, Du R (2010) Size controlled preparation of silver nanoparticles by a modified polyol method. Colloids Surf A Physicochem Eng Asp 366:197–202

Zhao C-X, He L, Qiao SZ, Middelberg APJ (2011) Nanoparticle synthesis in microreactors. Chem Eng Sci 66:1463–1479

Chapter 7
Mineralogical and Geochemical Controls in Biomining and Bioremediation

Bernhard Dold

7.1 Introduction

Biomining is generally defined as a biotechnological process to enhance metal recovery by oxidative dissolution of sulfide ore minerals catalyzed by the activity of prokaryotic microorganisms like bacteria and archaea (Donati and Sand 2007; Rawlings and Johnson 2007). The bioleaching process is mainly used for metal liberation from copper sulfide ores (Domic 2007). New approaches also involve the reductive dissolution of oxide ores (Ni-laterites) by microbial interactions (Hallberg et al. 2011).

Many of the current large-scale biomining operations, especially in Cu-sulfide ores, are inefficient with recoveries mostly in the range of 5–10 %, with some exception of up to 40 %. This is in most cases due to inappropriate parameters for selection of leachable material. For example, in copper mining, like in porphyry copper systems, the cutoff ore grade (typical range between 0.3 and 0.6 wt.% Cu) defines if the material is to be sent to the flotation process or leached in low-ore grade (0.1–0.4 wt.% Cu) run-off-mine (ROM) dumps, heaps, or additionally crushed (Fig. 7.1). By using the cutoff as selection criteria, the properties and suitability for bioleaching of the mineral assemblage is ignored, as some key minerals like chalcopyrite are not acid soluble in sulfuric acid (Table 7.1).

Most of the currently exploited Cu-sulfide mines contain mainly chalcopyrite as target mineral, which is not acid leachable, thus it must be oxidized by microorganisms in order to solubilize the copper. This process at ambient conditions is relatively inefficient, as the typical bacterial communities (*Acidithiobacillus ferrooxidans* and *Leptospirillum* spp.) present at these conditions are not able to oxidize chalcopyrite efficiently. Thermophilic population dominated by archaea (e.g., *Sulfolobus* spp.) are able to oxidize chalcopyrite efficiently with acceptable

B. Dold (✉)
SUMIRCO.EIRL Bernhard Dold Sustainable Mining Research & Consult.EIRL Casilla 28, 4130000 San Pedro de la Paz, Chile
e-mail: Bernhard.Dold@gmail.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_7, © Springer-Verlag Berlin Heidelberg 2014

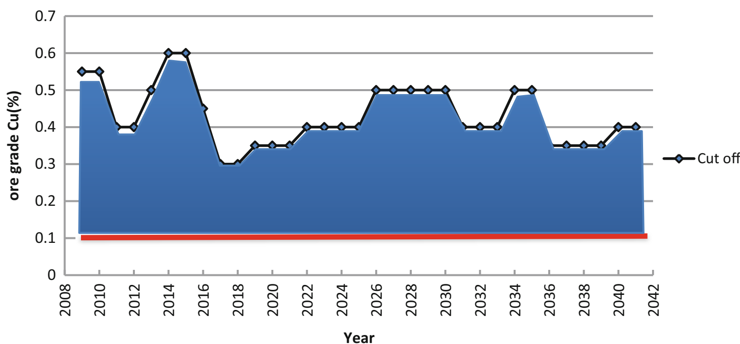


Fig. 7.1 Typical evolution of the cutoff grade during the evolution of a mine operation. Ore with ore grades above cutoff will be crushed, milled, and floated. Material below cutoff and above 0.1 wt.% Cu (*blue area*) will be sent to leach dumps (ROM or crushed to increase the reactive surface). Material below 0.1 wt.% Cu (*red line*) is normally classified as waste rock

Table 7.1 Solubility in water and in sulfuric acid (H_2SO_4) of some selected metals sulfide and oxide minerals in the different zone of a copper deposit

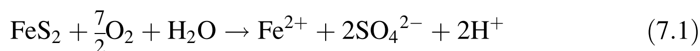
	Water soluble	Acid soluble	Acid insoluble	Zone
Pyrite			X	Primary
Chalcopyrite			X	Primary
Molybdenite			X	Primary
Wolframite (WS_2)			X	Primary
Arsenopyrite			X	Primary
Bornite		X		Primary
Galena		X		Primary
Sphalerite		X		Primary
Chalcocite–digenite		X		Enriched
Covellite		X		Enriched
Chalcanthite	X			Oxidation
Eriochalcite	X			Oxidation
Atacamite		X		Oxidation
Azurite–malachite		X		Oxidation
crysocolla		X		Oxidation

kinetics only above 50 °C as applied in some stirred tank operations for concentrates (du Plessis et al. 2007). In this chapter the influences of the mineralogy and geochemical conditions on the effectiveness of biomining operations and further potential for research and applications are discussed.

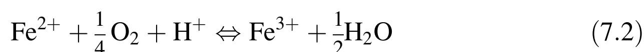
7.2 Sulfide Oxidation

Sulfide oxidation occurs when sulfide-rich rock units are exposed to the atmosphere and to water for example by erosion or is enhanced by anthropogenic activities like mining (Dold 2010). It is a natural process, which started to occur in geological times with the first presence of an atmosphere and has been going on ever since and might have an important role on the global iron cycle (Dold et al. 2013). This process is responsible for the formation of supergene ore deposit and the main environmental problem of mining industry called acid mine drainage (AMD). However, it can be used as a process for solubilization of metals from sulfide ores in biomining (Dold 2008).

The most common sulfide mineral in the earth crust is pyrite (FeS_2). Oxidation of pyrite takes place in several steps including the formation of the metastable secondary products ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), schwertmannite [between $Fe_8O_8(OH)_6SO_4$ and $Fe_{16}O_{16}(OH)_{10}(SO_4)_3$], goethite [FeO(OH)], and the more stable secondary jarosite [$KFe_3(SO_4)_2(OH)_6$] and hematite (Fe_2O_3) depending on the geochemical conditions (Bigham et al. 1996; Cornell and Schwertmann 2003). Oxidation of pyrite may be considered to take place in three major steps: (1) oxidation of sulfur [Eq. (7.1)]; (2) oxidation of ferrous iron [Eq. (7.2)]; and (3) hydrolysis and precipitation of ferric complexes and minerals [Eq. (7.3)]. The kinetics of each reaction is different and depends on the conditions prevailing in the system.

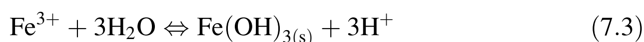


$$FeS_2 + \frac{7}{2}O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4{}^{2-} + 2H^+ \quad (7.1)$$

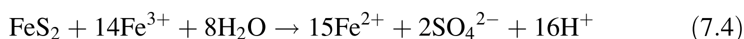


$$Fe^{2+} + \frac{1}{4}O_2 + H^+ \Leftrightarrow Fe^{3+} + \frac{1}{2}H_2O \quad (7.2)$$

Reaction rates strongly increased by microbial activity (e.g., *Acidithiobacillus* spp. or *Leptospirillum* spp.)



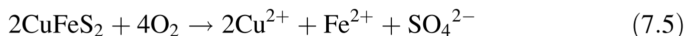
$$Fe^{3+} + 3H_2O \Leftrightarrow Fe(OH)_{3(s)} + 3H^+ \quad (7.3)$$



$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4{}^{2-} + 16H^+ \quad (7.4)$$

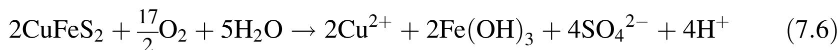
Equation (7.1) describes the initial step of pyrite oxidation in the presence of atmospheric oxygen. Once ferric iron is produced by oxidation of ferrous iron, especially at low pH conditions, it becomes the primary oxidant [Eq. (7.4)] of pyrite and the reaction would be strongly accelerated by microbiological activity [Eq. (7.2)] (Ehrlich 1996; Moses et al. 1987; Nordstrom et al. 1979; Rimstidt and Vaughan 2003). Under abiotic conditions the rate of oxidation of pyrite by ferric iron is controlled by the rate of oxidation of ferrous iron, which decreases rapidly with decreasing pH. Below about pH 3 the oxidation of pyrite by ferric iron is about ten to a hundred times faster than by oxygen (Ritchie 1994).

Chalcopyrite is one of the main target mineral in copper mining. In order to solubilize the copper, chalcopyrite should be oxidized and complete oxidation of chalcopyrite may be written as:



$$2CuFeS_2 + 4O_2 \rightarrow 2Cu^{2+} + Fe^{2+} + SO_4{}^{2-} \quad (7.5)$$

The above reaction is without the acid production. However, the combination of ferrous iron oxidation and ferrihydrate hydrolysis is the main acid producing process.



$$2CuFeS_2 + \frac{17}{2}O_2 + 5H_2O \rightarrow 2Cu^{2+} + 2Fe(OH)_3 + 4SO_4{}^{2-} + 4H^+ \quad (7.6)$$

Chalcopyrite, together with molybdenite, is known as one of the most resistant sulfides to oxidation (Plumlee 1999). Rimstidt et al. (1994) reported that the oxidation rate of chalcopyrite increases with increasing ferric iron concentration, but with an oxidation rate of 1–2 orders of magnitude less than pyrite.

It has been known for nearly 50 years that *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* obtain energy by oxidizing Fe^{2+} to Fe^{3+} from sulfides (Bryner et al. 1967) with increased rate of reaction (7.2) up to the factor of about 100 over abiotic oxidation (Singer and Stumm 1970). More recent results show that a complex microorganism community is responsible for sulfide oxidation (Baker and Banfield 2003; Johnson and Hallberg 2002; Johnson et al. 1993; Nordstrom 2000). The initial step of pyrite oxidation does require an elaborated sequence of different geochemical reactions that dominate at different pH ranges (Nordstrom and Southam 1997). Schippers and Sand (1999) have demonstrated that acid-insoluble metal sulfides are chemically reacted by iron (III) hexahydrate ions, generating thiosulfate, which is then oxidized to sulfuric acid (Table 7.1). The acid-soluble sulfides are attacked by iron (III) and protons, resulting in the formation of elemental sulfur via intermediary polysulfides.

Acidithiobacillus spp. form nano-environments to grow on sulfide mineral surfaces. These nano-environments can develop thin layers of acidic water that do not affect the bulk pH of the water chemistry. With progressive oxidation, the nano-environments may change to microenvironments. Evidence of acidic microenvironments in the presence of near neutral pH for the bulk water can be inferred from the presence of jarosite (forms around pH 2) in certain soil horizons and mine tailings where the current water pH is neutral (Carson et al. 1982; Dold et al. 2011a). Barker et al. (1998) observed the solution pH decreased from near neutral at the mineral surface to 3–4 around microbial colonies living within confined spaces at interior colonized cleavage planes of biotite.

When mine water, rich in ferrous and ferric iron, reaches the surface it may fully oxidize, hydrolyze, and may precipitate to ferrihydrite (fh), schwertmannite (sh), goethite (gt), or jarosite (jt) depending on pH–Eh conditions and availability of key elements such as potassium and sulfur (Fig. 7.2). Jarosite, schwertmannite, and ferrihydrite are metastable compared to goethite (Bigham et al. 1996). The hydrolysis and precipitation of iron hydroxides (and to a lesser degree, jarosite) would produce most of the acid in this process. If pH is less than about 2, ferric hydrolysis products like $Fe(OH)_3$ are not stable and Fe^{3+} remains in solution.

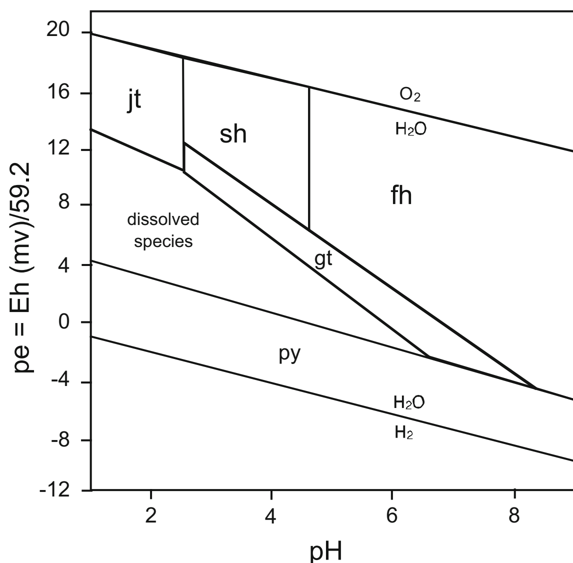
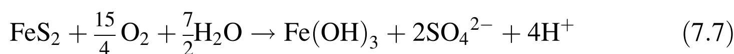


Fig. 7.2 pe–pH diagram for Fe-S-K-O-H system at 25 °C; total log activities of $Fe^{2+} = -3.47$; $Fe^{3+} = 3.36$ or -2.27; $SO_4^{2-} = -2.32$; $K^+ = -3.78$; log Kso values for solid phases for *gt* goethite, *jt* K-jarosite, *fh* ferrihydrite, *sh* schwertmannite are 1.40, −12.51, 4.5, and 18.0, respectively. *Py* pyrite. Mean composition of the schwertmannite used for the development of this pe–pH diagram was $Fe_8O_8\ (OH)_{4.8}\ (SO_4)_{1.6}$. After Bigham et al. (1996), note that these stability fields have to be interpreted as indicative, as the thermodynamic data published from schwertmannite and ferrihydrite show high variability (Majzlan et al. 2004)

Note that the net reaction of complete oxidation of pyrite, hydrolysis of Fe^{3+}, and precipitation of iron hydroxide (sum of reactions 1, 2, and 6) produces 4 mol of H^+ per mole of pyrite; thus, pyrite oxidation is the most efficient producer of acid among the common sulfide minerals.



$$FeS_2 + \frac{15}{4}O_2 + \frac{7}{2}H_2O \rightarrow Fe(OH)_3 + 2SO_4^{2-} + 4H^+ \quad (7.7)$$

The precipitation of these secondary Fe(III) hydroxides can form coatings (Huminicki and Rimstidt 2009) and cemented layers (Blowes et al. 1991; Dold et al. 2009; Graupner et al. 2007), which can decrease the oxidation rates and change the flow direction of the pore solution. Evangelou and Zhang (1995) reported increased oxidation rates of pyrite by addition of HCO_3^- due to the formation of pyrite surface Fe(II)-CO_3 complexes resulting in a limitation of the iron availability. Additionally, the formation of silica gel can encapsulate minerals and limit the interaction with the environment (Evangelou 2001). This can lead to the formation of cemented layers, which limit oxygen and water flow as well as form preferential flow regimes (Graupner et al. 2007; Rammlmair et al. 2008). The formation of these secondary mineral phases can not only limit

the access of bacteria to the sulfide mineral surface but can also inhibit the bacterial activity by co-precipitation of the bacterial cell into the mineral phase (Hedrich et al. 2011). The secondary mineralogy of the oxidation and cemented zones can scavenge environmentally critically elements under oxic condition. This effect can be reversed, when placed under reducing conditions like during bioremediation and has to be considered before a remediation approach is chosen.

7.3 How Is Biomining Done Today?

There are several criteria, which decide nowadays to which type of process the mined material is send. If a new mining operation is starting, there is often an oxidation zone on the surface present, which might contain some economic value in form of metal oxides (chalcanthite, azurite, malachite, chrysocolla, or Au and Ag concentrations among others). This oxide mineral assemblage is water soluble or acid leachable (Table 7.1), so that these metals can easily be recovered by an acid leach process, mainly using sulfuric acid. If the deposit shows important secondary enrichment due to supergene processes, as for example the porphyry copper deposits in northern Chile, the mineral assemblage is dominated by secondary sulfide minerals like covellite and chalcocite–digenite, which are also acid soluble. If the two latter cases are important in the system, usually a separated acid leach operation is installed in order to recover the soluble metals by solvent extraction electro-winning process (SX-EW).

If exploitation goes further on toward the primary zone or the oxide and enriched zone are less important, the criteria of decision making is the cutoff grade as explained above. In this case not the mineral assemblage, but only the element concentrations of the mayor target elements decide the process applied. This can then lead to a very low recovery of the target elements due to their mineral assemblage.

There are several options how the leaching or bioleaching is done in the mining operation (Fig. 7.3):

1. In small leach piles (or heaps) of some meters high (Fig. 7.3a), where the material is exposed to acid leach by sulfuric acid irrigation (pH ~ 1) some days or weeks up to months. Sometime the material is crushed before or sulfuric acid is mixed with water in a reaction tank to fracture the material. The pregnant leach solutions (PLS) are recovered by underlying geomembrane basement liners (Fig. 7.4 and Fig. 7.6). The PLS is send to an SX-EW plant for metal recovery and then recycled to the leach pile. In these operations usually oxide ores or material from the supergene enriched zones is exploited. Afterwards, the material is replaced by fresh ore, and the residual is deposited on waste dumps. As in these operations usually high-ore grade (e.g., 1–2 wt.% Cu) is processed, the waste material still can contain 0.4–0.8 wt.% of Cu and the resulting waste

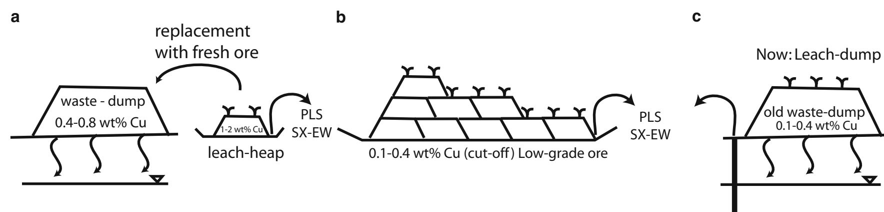


Fig. 7.3 Schematic designs of (bio) leaching and biomining operations. (**a**) Acid leach heaps with final deposition on waste dump (Fig. 7.4). (**b**) Permanent bioleach dump (Fig. 7.5). (**c**) Former waste dump redefined to leach dump. Potential groundwater contamination is highlighted

dumps normally do not have impermeabilization, so that they can be a important source of groundwater contamination.

2. Permanent leach deposits (leach dumps) for low-grade sulfidic ore material (i.e., Escondida, Chile; Figs. 7.3a and 7.5). In this case material with ore grade below cutoff and normally above 0.1–0.2 wt. Cu is placed above an impermeabilized surface (usually by geotextile). When the first level is completed, new material is placed above in order follow on the leach process. Additionally, artificial ventilation ensures the air supply for the microbiological activity. Characterization of the microbial community is undertaken (Demergasso et al. 2005; Zepeda et al. 2009), however, mineralogical control is often missing. In cases that supergene enrichment is important, recovery is relatively high (up to 41 %) (Zepeda et al. 2007). However, as these operations are mainly from porphyry copper deposit (e.g., Escondida, Chuquicamata), only temperatures in the range for a mesophile community are reached, so that chalcopyrite leaching is not very efficient.
3. Waste dumps, which, due to increased metal prices are redefined into leach dumps. They are usually only irrigated sulfuric acid and no baseliner to recover PLS is present. Instead, it is tried to recover the PLS by a series of piezometer or wells in order to recover the metals from the groundwater. This practice has to be evaluated very critically as often no detailed knowledge on the hydrogeology is available and therefore the flow directions are little know, resulting in a high risk for groundwater contamination by PLS.
4. The trend in metal mining goes clearly into lower-ore grade and greater volumes mined; thus also the mobilized non-economic or low-grade material is increasing, so that, for example, in Chileans copper mines waste dumps or leach dumps are in planification to completely new dimensions with the associated technical challenges. For example, at the Andina porphyry copper mine (Central Chile) a dump is planed with 4 km length and 1 km high containing 1,900 Mt of low-grade material (Weibel et al. 2011). The challenge in this type of deposits is to design a dump, which can produce its own impermeabilization in order to prevent infiltrations to the groundwater and to enable the PLS recovery on a long-term perspective and optimize the leach kinetics.

Fig. 7.4 Leach pile or heap operation (acid leach) for high-grade copper oxide ores

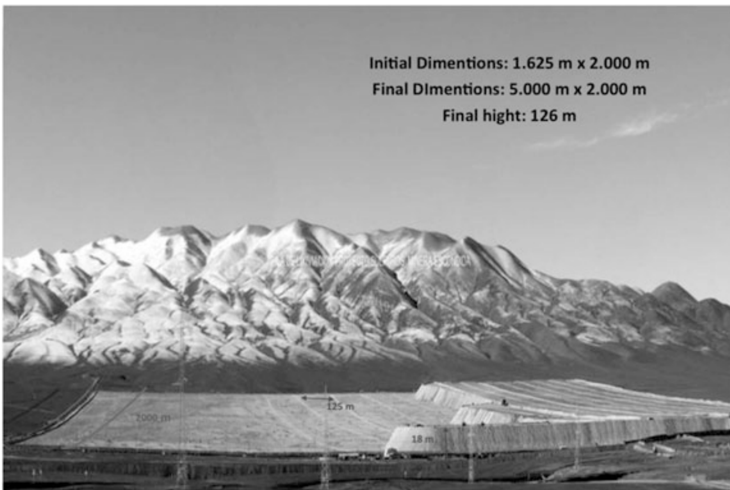


Fig. 7.5 Overview of the bioleaching operation for low-ore grade material at the Escondida, porphyry copper deposit, Northern Chile

5. Tank or Reactor leaching operations are used to leach mainly concentrates or high-ore grade material with thermophile archaea communities (du Plessis et al. 2007).

Fig. 7.6 Collection facility for PLS of a mayor (bio) leaching operation. After SX-EW, the solution is recycled to the leaching process

7.4 Main Drawbacks of Today's Bioleaching Operations

(A) The most important drawback today is the inadequate selection of the material for the leaching process. This has to be seen as the main reason for the low recovery of these systems. In order to improve this it must be clear that a mineral deposits is exploited, not a metal deposit. Characterization and decision making are nowadays done on behalf of the ore grade of a material (i.e., the concentration of the target element), but the mineralogical association of this element is ignored. The mineral association is key to evaluate extraction of a certain element by the chosen mining process. Thus, not the ore grade but the mineralogical association of the target element has to be the process selection criteria.

(B) In case of porphyry copper deposits, only around 2 % of pyrite is available for heat production during sulfide oxidation. This is usually not enough to reach the thermophile temperature range (>50 °C). Thus, the mesophilic bacterial community, dominated by *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, are not able to oxidize efficiently chalcopyrite. Therefore, the primary ore mineralogy (cp, bn) is not suitable for bioleaching operations in porphyry copper systems.

(C) Most of these leach dumps are built with ROM material. This ensures a relatively good aeration as the coarser material is in the lower part of each level of the dump, which promotes advection through the chimney effect. However, some operations crush additionally the material in order to increase

surface for the oxidation process. This limits the airflow through the system and can so decrease the oxidation kinetics. Some operations have additionally ventilation infrastructure, which increases the operation costs (e.g., Escondida).

(D) Many of the leach operations are managed at pH of 1 or even below. This pH is lower than the optimum range for the iron and sulfur oxidizers, hence might also play a role in the low efficiency of the operation. The low pH is generally used to prevent precipitation of secondary minerals like Fe(III)hydroxides and sulfates like, for example, jarosite and/or goethite.

(E) The chemistry of the PLS is often ignored. In many of these operations, the PLS are recycled after the metal recovery. This is done to increase oxidation kinetics as the PLS have high concentrations of ferric iron, the predominant oxidant in this system. Due to evaporation during recycling, the concentrations of the solutes in the PLS tend to increase. As the solubility of the secondary minerals like jarosite is a function of pH, Eh, and concentrations, this increase of solutes makes it possible that, for example, jarosite might precipitate at lower pH as the ferric iron concentration increases during recycling (Figs. 7.6 and 7.7). The precipitation of secondary minerals like jarosite might inhibit sulfide minerals from oxidation by coating and cemented layer formation and thus lowers the recovery.

(F) The presence of other minerals like ferrihydrite, goethite, and schwertmannite in the leach material has to be considered, as they might dissolve due to the low pH conditions and increase the concentrations of ferric iron as an additional source, and thus decrease the pH where jarosite can precipitate. These secondary mineral precipitation can inhibit the sulfides from oxidation and change the solution flow path and make sectors of the leach pile inaccessible for the leach solution (Rammlmair et al. 2008).

7.5 How Can We Improve the Biomining Process?

To increase sulfide oxidation kinetics for bioleaching purpose, following parameters should be considered:

(A) *pH*: The chemolithotrophic autotroph iron- and sulfur-oxidizer have their optimum in a pH range of 2–3. However, most of the bioleaching operations manage pH conditions around 1 or even lower due to constant irrigation with sulfuric acid and evaporation effects, which is below the optimum pH for the microbial activity and might be a reason for lower microbial activity (Hallberg 2010). This low pH prevent precipitation of secondary Fe(III)hydroxides, which could coat the sulfides and lower recovery. It must also be considered that the gangue mineralogy can be responsible of an important acid consumption, slowing down the recovery at the beginning due to increased pH conditions.

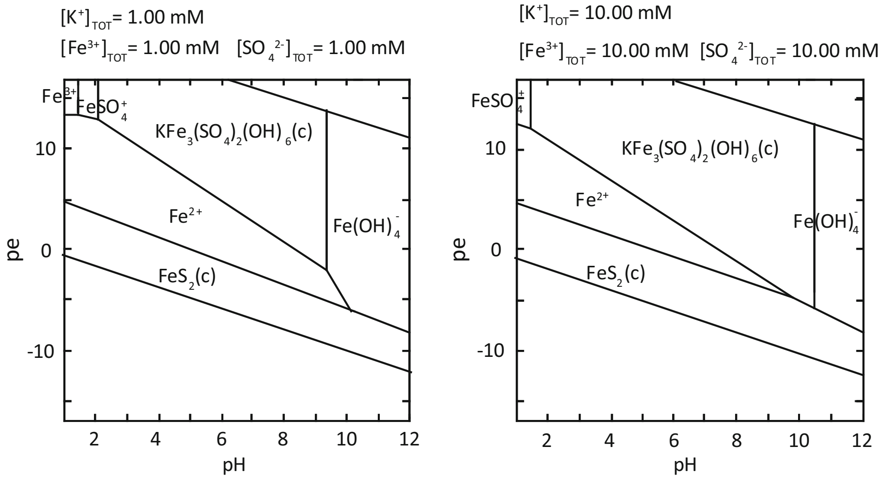


Fig. 7.7 pH–Eh diagrams of the system Fe-K-S at concentrations typical for a PLS (**a**) and with an increase (**b**) to show how the stability field of jarosite expands toward lower pH

(B) *Redox potential*: The redox potential (Eh) must be operated and maintained constantly in oxidizing conditions. Saturation with the leach solution has to be avoided.

(C) *Temperature*: *Acidithiobacillus* spp. or *Leptospirillum* spp. have their optimum temperature between 20 and 40 °C (Johnson and Hallberg 2003). As sulfide oxidation is an exothermic reaction, increased temperatures are often reached in bioleaching operations. However, to effectively operate bioleaching of chalcopyrite, 40–50 °C or higher are needed in order to stimulate conditions for thermophile archaea, which have shown increased recovery of chalcopyrite oxidation under stirred tank conditions (Rawlings and Johnson 2007). In laboratory cell tests it has been shown that Cu release is enhanced at constant 40 °C and 100 % humidity (Dold et al. 2011b), in relation to the standard wet–dry cycles of the Standard humidity cell tests ASTM D5744-96. An elegant way to increase the heat production during sulfide oxidation can be the addition of pyrite, which was separated by an additional flotation step from the tailings. This would have a dual positive effect of increasing temperature in the leach dump to increase recovery, and lowering the acid potential of the tailings, where it might lead to AMD and produce high environmental damages and costs.

(D) *Humidity and air flow*: Increased humidity can prevent precipitation of secondary soluble salts, which can inhibit the sulfide surface from reaction. As for sulfide oxidation atmospheric oxygen is needed at least at the beginning of the process (in a later stage Fe^{3+} will be the principal oxidant), and bacteria community is autotroph, a good airflow throughout the system has to be ensured during the operation. In Escondida, Chile, this is ensured by a giant

ventilation system. Specific designs of the leach dumps in order to promote the chimney effect could enhance aeration at a lower energy cost.

(E) *Control of the geochemistry of the PLS*: The water chemistry of the leach solution have to be controlled constantly in order to ensure optimum conditions for the microbial community and to limit the risk of mineral precipitations, which can coat the target minerals and change the flow path of the leach solutions.

7.6 Bioremediation

As explained before, sulfide oxidation is the biogeochemical process, which leads to the solubilization of sulfides and the subsequent formation of acid rock drainage (ARD). In biomining, this process is accelerated in a controlled environment in order to extract the metal content. If this process occurs uncontrolled in mine waste, it produces the most important environmental problem of the mining industry, called acid mine drainage, and needs remediation. There are different approaches in order to control and remediate the damage produced by AMD formation. The main principle of these approaches is to invert or close the biogeochemical cycle back from the oxidizing environment, which is responsible for acid production and metal liberation and mobilization, into a reducing environment, in order to remove the contaminant elements by precipitation or sorption processes from solution. Below the term bioremediation usually the precipitation of secondary sulfide minerals and the formation of alkalinity triggered by microbial activity is understood and used for remediation of mine waste systems like tailings (Dold et al. 2011a), waste dumps (Schippers et al. 2010), and pit lakes (Klapper et al. 1998; Fauville et al. 2004; Knoller et al. 2004). Sometimes also sorption processes onto organic matter falls below this definition, as well as phytoremediation (Sheoran et al. 2012).

The microbiology of bioremediation process is extensively reviewed (Johnson 2002, 2003; Johnson and Hallberg 2002; Hallberg and Johnson 2005). Two principal approaches in bioremediation of mine waste can be distinguished:

1. Treatment of AMD by enhanced biogeochemical processes (reduction, precipitation, sorption) in treatment facilities (Hallberg 2010) and constructed (Hallberg and Johnson 2005) or natural wetlands (Kalin 2001) or permeable reactive barriers (Benner et al. 2000; Blowes et al. 2000).
2. Change of the geochemical conditions of the contamination source (e.g., mine tailings and waste dumps) to reducing environment in order to stop sulfide oxidation and AMD formation (Diaby 2008; Dold et al. 2011a).

In case of AMD treatment, the AMD chemistry is the key in order to guarantee a proper function of the treatment process and also decides if the process can function in an inorganic way or microbial intervention is needed. For example, the formation of the exotic deposit at the Chuquicamata mine, Northern Chile, is a good example

of natural attenuation of ARD. The source of the ARD was the exposure of the porphyry copper ore body to natural oxidation through erosion and uplift of the Andes. The West-fault triggered the formation of a gravel filled paleo-channel, in which the acid, Cu, and Fe(III) rich-ARD were channeled in an oxic environment downstream. Close to the source, at the beginning of the paleo-channel, Fe(III) hydroxides precipitated and scavenged oxyanions like molybdate and arsenate, due to their known behavior of sorption onto Fe(III)hydroxides at low pH condition. Therefore Mo, one of the target elements in this system, is enriched close to the ARD source. Copper maintains mobile under the prevailing acid pH conditions and flew downstream, were it encountered two types of bedrock. On the east site of the channel prevailed bedrock altered by propylitic alteration, which adds a neutralization potential to the rock in the form of calcite. The other bedrock is mainly based on granodiorite and andesitic gravels, without any carbonates. This difference of bedrock controls the buffer capacity and subsequent the final pH conditions. The propylitic zone is able to buffer the pH to near neutral pH, necessary for the observed precipitation of chrysocolla, whereas the gravels only are able to buffer toward pH 5–6, where atacamite will form (Dold 2006). Thus, this system is mainly pH controlled, triggering the precipitation of the different mineral in a neutralization sequence and forming a secondary ore deposit, a modern way of pollution control.

In case of AMD treatment, there are two scenarios to consider. One is a neutral ferrous iron-rich plume and the other is an acid ferrous iron-rich plume out-groping from a mine waste system (tailings or waste dumps) or underground mines. In the first case, the contact with the atmosphere will initiate the autooxidation of the ferrous iron to ferric iron and initiate the hydrolysis of the Fe(III)hydroxides (ferrihydrite and/or goethite). If the ferrous plume is contaminated by oxyanions (e.g., As, Mo, Se, S) these will tend to be scavenged by these secondary precipitates. In this process still no bacterial interaction is needed.

If the ferrous plume is acid ($<$ pH 4), then iron oxidizers will oxidize ferrous to ferric iron and the hydrolysis of schwertmannite and/or jarosite and goethite is initiated. Again, if oxyanions are in solution, they will be scavenged by the Fe(III) hydroxides. Bivalent metal cations can maintain in solution and can be extracted selectively. The most important role of microbial activity in these systems is now to use the ability of some microorganisms to modulate the redox state of a target element selectively in order to extract the element as pure as possible from solution in order to be able to recover the element as an economic value, instead of an environmental cost.

Many of the so-called bioremediation or microbiological treatment methods for AMD control are based on the action of heterotrophic iron and sulfate-reducing bacteria, which initiate the precipitation of secondary sulfide minerals in a wetland or a permeable reactive barrier. The main drawback of these methods is that they are not able to treat the AMD for selective metal recovery; instead, a mixture of organic matter and metal sulfides is produced, which itself represent a hazardous waste material, which has to be managed in a proper way. Thus, new research goes into the selective recovery of the metals from industrial waste solutions like AMD,

in order to recover as much economic values as possible (Hallberg 2010; Kamradt et al. 2012).

In case of acid lake remediation, the heterotrophic approach has shown good results specially in pit lakes formed by coal mining where mainly iron and the acidity are the main problems to solve (Geller et al. 1998). In order to control and to limit an AMD source (tailings impoundment, waste dump, underground mine, pit lakes) by a bioremediation approach, usually the system is changed from an oxic environment toward a reducing environment. This can be reached by addition of organic matter or simply by flooding or is often done by implementation of geotechnical and soil covers, phytoremediation, or by implementation of wet covers with a wetland (Dold et al. 2011a).

As expressed above, natural processes can scavenge some of the pollutants in a very effective way. For example, oxyanions like arsenate are always associated to the Fe(III) hydroxides in an oxic AMD environment; this can be in a waste dump, in the oxidation zone from a tailings impoundment (Dold and Fontboté 2001; Smuda et al. 2007), or in the unsaturated zone of an underground mine or in the pit lakes walls. By the change toward reducing and increased pH conditions, the sorbent, in this case the Fe(III)hydroxides, will undergo reductive dissolution and liberate the sorbed arsenic into solution, thus, by solving a problem producing another, as happened in case of the flooding of the Wismut uranium mines (Jenk et al. 2009). Therefore, before a remediation approach is selected, a thorough knowledge of the mineralogy and element speciation is needed in order to predict geochemical stability and behavior during the remediation process.

7.7 Conclusions

Nowadays biomining operations are in most cases highly inefficient, mainly due to the lack of mineralogical, geochemical, and microbiological characterization and control before and during the operations. Most of them are not effectively bioleaching but mainly acid leaching operations and have therefore an enormous potential for process improvement. The goal must be to optimize these operations by a thorough characterization of the mineral assemblage and the microbiological community present or to be developed and to ensure the biogeochemical parameters for an optimized operation. Especially, the selection criterion (today cutoff grade) for the definition of the leachable units has to be changed toward the mineral assemblage.

Special attention has to be given to the impermeabilization of these operation, in order to be able to recover the PLS on a long-term perspective in the future and also with the objective to prevent groundwater contamination.

In bioremediation, special focus has to be given that the microbial community supposed to do the job has the suitable geochemical conditions in order to function in the optimum range. Mineralogical and geochemical studies of the element and mineral speciation have to be performed in order to prevent negative secondary

pollution effects. Thus, biomining or bioremediation approaches need a detailed knowledge of the mineralogy and the geochemistry to predict and manage a proper function of the biogeochemical systems.

References

Baker BJ, Banfield JF (2003) Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152

Barker WW, Welch SA, Chu S, Banfield JF (1998) Experimental observations of the effects of bacteria on aluminosilicate weathering. Am Mineral 83:1551–1563

Benner SG, Gould WD, Blowes DW (2000) Microbial populations associated with the generation and treatment of acid mine drainage. Chem Geol 169:435–448

Bigham JM, Schwertmann U, Traina SJ, Winland RL, Wolf M (1996) Schwertmannite and the chemical modeling of iron in acid sulfate waters. Geochim Cosmochim Acta 60:2111–2121

Blowes DW, Reardon EJ, Jambor JL, Cherry JA (1991) The formation and potential importance of cemented layers in inactive sulfide mine tailings. Geochim Cosmochim Acta 55:965–978

Blowes DW, Ptacek CJ, Benner SG, McRae CWT, Bennett TA, Puls RW (2000) Treatment of inorganic contaminants using permeable reactive barriers. J Contam Hydrol 45:123–137

Bryner LC, Walker RB, Palmer R (1967) Some factors influencing the biological and non-biological oxidation of sulfide minerals. Trans Soc Mining Eng AIME 238:56–65

Carson CD, Fanning DS, Dixon JB (1982) Alfisols and ultisols with acid sulfate weathering features in Texas. In: Kittrick JA, Fanning DS, Hossner LR (eds) Acid sulfide weathering, vol 10. Soil Science Society of America Publicaiton, Madison, WI, pp 127–146

Cornell RM, Schwertmann U (2003) The iron oxides. Wiley-VCH, Weinheim, 664 p

Demergasso CS, Galleguillos PA, Escudero G, Zepeda A, Castillo D, Casamayor EO (2005) Molecular characterization of microbial populations in a low-grade copper ore bioleaching test heap. Hydrometallurgy 80:241–253

Diaby N (2008) Biogeochemical evolution of a marine shore tailings deposit during bioremediation. University of Lausanne, Lausanne

Dold B (2006) Geochemical modeling of the exotic copper mineralization at the Exotica deposit, Chuquicamata, Chile. In: XI Congreso Geologico Chileno, vol 2, Antofagasta, Chile, pp 274–250

Dold B (2008) Sustainability in metal mining: from exploration, over processing to mine waste management. Rev Environ Sci Biotechnol 7:275–285

Dold B (2010) Basic concepts in environmental geochemistry of sulfide mine-waste management. In: Kumar S (ed) Waste management. Intech Open Access, Rijeka, pp 173–198 (http://www.intechopen.com/books/show/title/waste-management)

Dold B, Fontboté L (2001) Element cycling and secondary mineralogy in porphyry copper tailings as a function of climate, primary mineralogy, and mineral processing. J Geochem Explor 74:3–55

Dold B, Wade C, Fontbote L (2009) Water management for acid mine drainage control at the polymetallic Zn-Pb-(Ag-Bi-Cu) deposit of Cerro de Pasco, Peru. J Geochem Explor 100:133–141

Dold B, Diaby N, Spangenberg JE (2011a) Remediation of a marine shore tailings deposit and the importance of water-rock interaction on element cycling in the coastal aquifer. Environ Sci Technol 45:4876–4883

Dold B, Weibel L, Cruz J (2011b) New modified humidity cells test for acid rock drainage prediction in porphyry copper deposits. EnviroMine, Santiago de Chile

Dold B, Gonzalez-Toril E, Aguilera A, Lopez-Pamo E, Bucchi F, Cisternas M-E, Amils R (2013) Acid rock drainage and rock weathering in Antarctica – important sources for iron cycling in the Southern Ocean. Environ Sci Technol 47(12):6129–6136

Domic EM (2007) A review of the development and current status of copper bioleaching operations in Chile: 25 years of successful commercial implementation. In: Rawlings DE, Johnson DB (eds) Biomining. Springer, Heidelberg, pp 81–96

Donati ER, Sand W (2007) Microbial processing of metal sulfides. Springer, Dordrecht, 314 p

du Plessis CA, Batty JD, Dew DW (2007) Commercial applications of thermophile bioleaching. In: Rawlings DE, Johnson DB (eds) Biomining. Springer, Heidelberg, pp 57–80

Ehrlich HL (1996) Geomicrobiology. Dekker, New York, 719 p

Evangelou VP (2001) Pyrite microencapsulation technologies: principles and potential field application. Ecol Eng 17:165–178

Evangelou VP, Zhang YL (1995) A review; pyrite oxidation mechanisms and acid mine drainage prevention. Crit Rev Environ Sci Technol 25:141–199

Fauville A, Mayer B, Frommichen R, Friese K, Veizer J (2004) Chemical and isotopic evidence for accelerated bacterial sulphate reduction in acid mining lakes after addition of organic carbon: laboratory batch experiments. Chem Geol 204:325–344

Geller W, Klapper H, Salomons WE (1998) Acidic mining lakes: acid mine drainage, limnology and reclamation. Springer, Berlin

Graupner T, Kassahun A, Rammlmair D, Meima JA, Kock D, Furche M, Fiege A, Schippers A, Melcher F (2007) Formation of sequences of cemented layers and hardpans within sulfide-bearing mine tailings (mine district Freiberg, Germany). Appl Geochem 22:2486–2508

Hallberg KB (2010) New perspectives in acid mine drainage microbiology. Hydrometallurgy 104:448–453

Hallberg KB, Johnson DB (2005) Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine. Sci Total Environ 338:53–66

Hallberg KB, Grail BM, du Plessis CA, Johnson DB (2011) Reductive dissolution of ferric iron minerals: a new approach for bio-processing nickel laterites. Miner Eng 24:620–624

Hedrich S, Lunsdorf H, Kleeberg R, Heide G, Seifert J, Schlomann M (2011) Schwertmannite formation adjacent to bacterial cells in a mine water treatment plant and in pure cultures of ferrovum myxofaciens. Environ Sci Technol 45:7685–7692

Huminicki DMC, Rimstidt JD (2009) Iron oxyhydroxide coating of pyrite for acid mine drainage control. Appl Geochem 24:1626–1634

Jenk U, Meyer J, Paul M (2009) Flooding of Wismut's uranium mines after closure – key findings and unexpected effects. In: Securing the future and 8th ICARD, Skelleftea, Schweden

Johnson DB (2002) Acid mine drainage: bioremediation, the candidate process. In: Eccles HE (ed) Bioremediation. Taylor and Francis, London

Johnson DB (2003) The microbiology of acidic mine waters. Res Microbiol 154:466–473

Johnson DB, Hallberg KB (2002) Pitfalls of passive mine water treatment. Rev Environ Sci Biotechnol 1:335–343

Johnson DB, McGinness S, Ghauri MA (1993) Biogeochemical cycling of iron and sulfur in leaching environments. FEMS Microbiol Rev 11:63–70

Kalin M (2001) Biogeochemical and ecological considerations in designing wetland treatment systems in post-mining landscapes. Waste Manag 21:191–196

Kamradt A, Borg G, Schaefer J, Kruse S, Fiedler M, Romm P, Schippers A, Gorny R, Du Bois M, Bieligk C, Liebetrau N, Nell S, Friedrich B, Morgenroth H, Wotruba H, Merkel C (2012) An integrated process for innovative extraction of metals from Kupferschiefer mine dumps, Germany. Chem Ing Tech 84:1694–1703

Klapper H, Friese K, Scharf B, Schimmele M, Schultze M (1998) Ways of controlling acid by ecotechnology. In: Geller W, Klapper H, Salomons W (eds) Acidic mining lakes – acid mine drainage, limnology and reclamation. Springer, Berlin, pp 401–416

Knoller K, Fauville A, Mayer B, Strauch G, Friese K, Veizer J (2004) Sulfur cycling in an acid mining lake and its vicinity in Lusatia, Germany. Chem Geol 204:303–323

Majzlan J, Navrotsky A, Schwertmann U (2004) Thermodynamics of iron oxides: part III. Enthalpies of formation and stability of ferrihydrite (Fe(OH)3), schwertmannite (FeO(OH)3/4(SO4)1/8), and [epsiv]-Fe_2O_3 1. Geochim Cosmochim Acta 68:1049–1059

Moses CO, Nordstrom DK, Herman JS, Mills AL (1987) Aqueous pyrite oxidation by dissolved oxygen and by ferric iron. Geochim Cosmochim Acta 51:1561–1571

Nordstrom DK (2000) Advances in the hydrogeochemistry and microbiology of acid mine waters. Int Geol Rev 42:499–515

Nordstrom DK, Southam G (1997) Geomicrobiology of sulfide mineral oxidation. In: Banfield JF, Nealson KH (eds) Geomicrobiology, vol 35, Reviews in mineralogy. Mineralogical Society of America, Washington, DC, pp 361–390

Nordstrom DK, Jenne EA, Ball JW (1979) Redox equilibria of iron in acid mine waters. In: Jenne EA (ed) Chemical modeling in aqueous systems, vol 93, ACS symposium series. American Chemical Society, Washington, DC, pp 51–79

Plumlee GS (1999) The environmental geology of mineral deposits. In: Plumlee GS, Logsdon MJ (eds) The environmental geochemistry of ore deposits. Part A: Processes, techniques, and health issues, vol 6A, Reviews in economic geology. Society of Economic Geologists, Littleton, CO, pp 71–116

Rammlmair D, Grissemann C, Furche M, Noell U, Graupner T, Meima JA, Romero-Baena A (2008) Evidence of reduced water infiltration by microhardpans – electrical resistivity measurements at Pena de Hierro, Rio Tinto, Spain. In: Proceedings of the 9th international congress for applied mineralogy, ICAM 2009, the Australasian Institute of Mining and Metallurgy, Publication Series (8/2008), Brisbane, Australia, pp 349–356

Rawlings DE, Johnson DB (2007) Biomining. Springer, Berlin, 314 p

Rimstidt JD, Vaughan DJ (2003) Pyrite oxidation: a state-of-the-art assessment of the reaction mechanism. Geochim Cosmochim Acta 67:873–880

Rimstidt JD, Chermak JA, Gagen PM (1994) Rates of reaction of galena, spalerite, chalcopyrite, and arsenopyrite with Fe(III) in acidic solutions. In: Alpers CN, Blowes DW (eds) Environmental geochemistry of sulfide oxidation, vol 550, ACS symposium series. American Chemical Society, Washington, DC, pp 2–13

Ritchie AIM (1994) Sulfide oxidation mechanisms: controls and rates of oxygen transport. In: Jambor JL, Blowes DW (eds) Short course handbook on environmental geochemistry of sulfide mine-waste, vol 22. Mineralogical Association of Canada, Nepean, pp 201–244

Schippers A, Sand W (1999) Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. Appl Environ Microbiol 65:319–321

Schippers A, Breuker A, Blazejak A, Bosecker K, Kock D, Wright TL (2010) The biogeochemistry and microbiology of sulfidic mine waste and bioleaching dumps and heaps, and novel Fe (II)-oxidizing bacteria. Hydrometallurgy 104:342–350

Sheoran V, Sheoran AS, Poonia P (2012) Phytoremediation of metal contaminated mining sites. Int J Earth Sci Eng 5:428–436

Singer PC, Stumm W (1970) Acid mine drainage: the rate-determining step. Science 167:1121–1123

Smuda J, Dold B, Friese K, Morgenstern P, Glaesser W (2007) Mineralogical and geochemical study of element mobility at the sulfide-rich excelsior waste rock dump from the polymetallic Zn-Pb-(Ag-Bi-Cu) deposit, Cerro de Pasco, Peru. J Geochem Explor 92:97–110

Weibel L, Dold B, Cruz J (2011) Application and limitation of standard humidity cell tests at the Andina porphyry copper mine, CODELCO, Chile. In: SGA biennial meeting, Antofagasta, Chile

Zepeda V, Galleguillos F, Castillo D, Lastra M, Demergasso C (2007) Bacterial activity at low temperature in cultures derived from a low-grade copper sulphide bioleaching heap at the Escondida Mine, Chile. Adv Mater Res 20–21:543–546

Zepeda V, Galleguillos F, Urtuvia V, Molina J, Demergasso C (2009) Comparison between the bacterial populations from solutions and minerals in 1m test columns and the industrial low grade copper sulphide bioleaching process in the Escondida Mine, Chile. Adv Mater Res 71–73:63–66

Chapter 8
Microbial Recovery of Nickel from Lateritic (Oxidic) Nickel Ore: A Review

Lala Behari Sukla, Sunil Kumar Behera, and Nilotpala Pradhan

8.1 Introduction

Nickel is a strategically important metal. Nickel was used much earlier before it was scientifically discovered in 1751 by a Swedish scientist Cronstedt. Nickel is the 24th most abundant element in the Earth's crust, comprising about 3 % of the composition of the earth. It is the 5th most abundant element by weight after iron, oxygen, magnesium and silicon (Cempel and Nickel 2006). The wide applications of nickel in anti-corrosion materials manufacturing, stainless steel and alloy steel production, chemical industries, electrical and electronic equipment, etc. require vast sources of nickel. Sulphidic and lateritic (oxidic) are the two principal types of nickel deposits present in the world.

Laterites are oxidic ores extensively found in tropical regions of the world. They were formed by the laterisation process of ultramafic rocks. Laterites are the weathering products of the ultramafic rocks in the earth crust (Le et al. 2006). The warm climate and abundant rainfalls are the favourable environmental conditions for formation of laterites. Lateritic deposits are broadly classified into limonite and saprolite types. Limonites or the oxidic laterites are highly enriched with iron oxides; however, the saprolites are silicate-dominated lateritic minerals (Chang et al. 2010). Majority of the laterites found on the surface of earth crust are limonite $[(Fe, Ni)O(OH) \cdot nH_2O]$ types of lateritic ores. Iron oxide, goethite $[FeO(OH)]$, constitutes the major chemical constituent of limonitic minerals, with which nickel is associated (Simate et al. 2010; Golightly 1981).

Lateritic nickel deposits contribute about 80 % of the land-based nickel reserves of the world. However, only 40 % of global nickel is produced from laterites (Valix and Loon 2003). The underutilisation of the vast resources may be attributed to higher processing cost (Thomas 1995). Presently, most of the global nickel

L.B. Sukla (✉) • S.K. Behera • N. Pradhan
Bioresources Engineering Department, Institute of Minerals and Materials Technology (CSIR), Bhubaneswar 751013, Orissa, India
e-mail: suklalb@yahoo.co.in

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_8, © Springer-Verlag Berlin Heidelberg 2014

production comes from sulphide deposits. As a consequence the worldwide reserves of sulphide minerals are diminishing at the alarming rate (Jinhui et al. 2009). Further increase in mining cost of the underground sulphide deposits in addition to increasing environmental compliance persuading for extraction of nickel from laterites. In these circumstances exploitation of the unutilised abandoned laterite deposits for nickel extraction has become inevitable need of the day.

The conventional pyrometallurgical and hydrometallurgical methods are used for extraction of nickel from lateritic nickel. In pyrometallurgical methods ore is passed through certain processes operated at high temperatures like drying, calcination, roasting, reduction, smelting, etc., whereas hydrometallurgical methods involve treatment of acids and solvents with ores for the leaching of metals. There is also a process combining pyrometallurgical and hydrometallurgical methods which is called “Caron process”. These metallurgical routes are economically viable when the higher nickel containing minerals processed for nickel extraction. Since, majority of lateritic minerals are poor in nickel content as well as the nickel is non-stoichiometrically associated with the minerals in lateritic ores (Golightly 1981). Consequently the existing pyrometallurgical and hydrometallurgical routes become cumbersome for extraction of metal values from such minerals.

In this context, the scope of the microbial processing of low-grade oxidic nickel laterites has been gaining interest. Microbe-assisted bio-hydrometallurgical route offers many advantages over the conventional mineral processing (pyrometallurgical and hydrometallurgical) routes for processing of low-grade ores for its simple operating conditions, low energy and capital costs requirement, and environmentally friendly nature (Acevedo 2000; Castro et al. 2000). Biohydrometallurgy is an interdisciplinary process which involves application of microorganisms for extraction of metal values.

- *Bio*—Application of microorganisms (bacteria, fungi, etc.)
- *Hydro*—Medium of the process is aqueous environment
- *Metallurgy*—The process deals with extraction of metals and treatment of metal containing minerals and materials

Microbe-assisted solubilisation of metals from minerals and mineral complexes have been extensively studied for recovery of the metals values of copper, gold, cobalt and uranium (Rawlings 2002; Rawlings et al. 2003; Olson et al. 2003). The microorganisms which are the main tools in biohydrometallurgical operations for metal extraction may be categorised broadly into two groups as per their nutritional requirements, i.e. heterotrophic and autotrophic microorganisms. In these processes microbes are used as tools either for conversion of insoluble sulphides/oxides complexes of metal to aqueous soluble metal sulphates or as a pre-treatment agent to alter the mineral structure thereby making the mineral susceptible to lixiviants or leaching agents.

Heterotrophic microbes do not perform photosynthesis. Such microorganisms make use of organic carbon sources for their nutritional requirement. These microorganisms secrete hydroxycarboxylic (organic) acids as metabolic by products. Organic acids produced by these microbes are metal complexing and chelating

agents, hence these help in dissolution of nickel by lowering the pH of the medium (Tzeferis 1992). In contrast, the autotrophic microorganisms are photosynthetic in nature and they use carbon dioxide as the carbon source. However, the chemolithographic (autotrophic) microbes can rely upon reduced inorganic compounds (reduced iron and sulphur) for their survival. In this context for the processing of the laterites, the heterotrophic microbes especially fungi have been widely applied. The use of chemolithotrophic microorganisms in laterite processing has been discouraged as the laterites are devoid of reduced iron and sulphur compounds.

With this brief introduction, this chapter is reviewed upon the challenges and development in microbial processing of nickel laterites (oxidic ores).

8.2 Laterites

Nickel laterite deposits are found as weathering materials. Basically most of the lateritic nickel deposits are distributed in New Caledonia, Australia, Cuba, Brazil, Colombia, Greece, Philippines and Indonesia (Boldt and Queneau 1967). In Indian context, the lateritic deposited at ultra-basic belt of Sukinda, Odisha is the only nickel deposit (Swain et al. 2007). Laterite ores generally do not contain discrete nickel minerals; rather nickel is adsorbed within the secondary oxide and silicate minerals (Valix et al. 2001). Hence the nickel laterites are categorised as oxide deposit, clay silicate deposit and hydrous silicate deposit on the basis of their host minerals (Brand et al. 1998; Swamy et al. 2003). The oxide deposit of laterites consisting mainly of goethite [Fe(O)OH] as host mineral contains nickel of about 1.0–1.6 % (Simate et al. 2010; Sukla and Das 1987). Extraction of nickel from these lean grade lateritic ores has great importance because of the shortage of high-grade nickel sources in the Earth (Lee et al. 2005). The sulphidic ores with relatively higher nickel content are industrially exploited for extraction of nickel, whereas the lateritic (oxidic) ore is hardly utilised because of its mineralogical complexities and poor nickel content (Alibhai et al. 1993). The importance of oxidic nickel deposits further increased since it contains important value metal cobalt along with nickel.

8.3 Microbial Extraction of Nickel from Laterites

Looking to the challenges in processing of nickel laterites by the conventional metallurgical methods, the curiosity for the processes of microbial extraction of nickel from laterites was developed. Microbial routes of the mineral processing gained importance since it requires simple technological outlay for the treatment of low-grade nickel laterite ores which operate at low temperature and atmospheric pressure. Further the microbial processing involves low degree of process control

and ecofriendly process (Mulligan et al. 2004; Bosecker 2001). Therefore, curiosity gained for the microbial processing of nickel laterites for extraction of metal values.

8.3.1 Heterotrophic Microbes in Processing of Laterites

Fungi are the most widely studied microorganisms in the microbial processing of nickel laterites. Heterotrophic microorganisms such as fungal strains of *Aspergillus* and *Penicillium* and bacterial strains of *Bacillus* and *Pseudomonas* have been found to be effective in bioleaching process, especially of oxidic, siliceous and carbonaceous materials (Bosecker 1985; Sukla et al. 1993; Tzeferis 1994; Rezza et al. 2001; Mohapatra et al. 2007; Tang and Valix 2006; Behera et al. 2011). However, leaching of nickel laterites by using fungal strains of *Aspergillus* and *Penicillium* have been studied extensively. Since the organic acids produced by these microbes have metal-chelating properties, they play vital role in metal solubilisation (Gadd 1999). The microbial metabolites such as organic acids (citric, oxalic, gluconic, etc.), amino acids and exopolysaccharides produced by these microbes are also reported to be involved in bioleaching of the metals (Burgstaller and Schinner 1993; Tzeferis 1994; Castro et al. 2000; Le et al. 2006).

Following mechanisms have been reported to be involved in fungal bioleaching: (1) acidolysis, (2) complexolysis and (3) bioaccumulation (Bosecker 1985; Burgstaller and Schinner 1993; Tsekova et al. 2010).

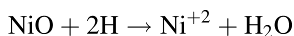
8.3.1.1 Acidolysis

Acidolysis is the major leaching mechanism involved in fungal bioleaching process. During the process, carboxylic acids produced by the fungi are involved in breakdown of the metal–oxygen bond in lateritic minerals. The proton ions contributed by the carboxylic acids first attack the oxygen atoms in the metal oxide compounds of laterites. Subsequently the protonated oxygen molecule is hydrolyzed; as a result the metal associated with the oxide minerals gets solubilised. The following equations represent generalised reaction during acidolysis (Burgstaller and Schinner 1993):

(i) Acid production:

$$\text{Glucose}/\text{Sucrose} \xrightarrow{\text{Fungus}} \text{Oxalic}/\text{Citric}/\text{Gluconic acid} \quad (8.1)$$

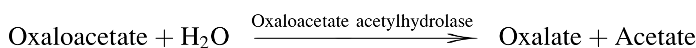
(ii) Proton attack



$$NiO + 2H \rightarrow Ni^{+2} + H_2O$$

8.3.1.2 Complex or Chelating Formation

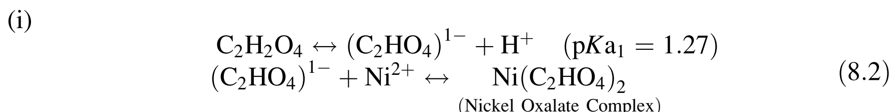
The growth of fungi in sugar (organic carbon) containing medium leads to the production of different organic acids, such as oxalic acid, citric acid, gluconic acid and fumaric acid. The biosynthesis of these acids by fungi involves glycolysis and tricarboxylic acid (TCA) cycles. In glycolysis glucose is converted to the pyruvate. Pyruvate is then oxidised to carbon dioxide and water in the TCA cycle, and at the same time, it accumulates the citric acid. The biosynthesis of oxalate in fungi can be formed from oxaloacetate in a C–C bond lysis reaction catalysed by oxaloacetate hydrolase (oxaloacetate acetylhydrolase (OAH), EC 3.7.1.1) and from the oxidation of glyoxylate and glycolaldehyde (Balmforth and Thomson 1984; Kubicek et al. 1988; Hammel et al. 1994). The widely proposed cellular pathway for oxalate production is through the breakdown of acetoacetate by OAH enzyme (Ruijter et al. 1999; Pedersen et al. 2000a, b). The key enzyme OAH which is located in the cytoplasm of *Aspergillus niger*, catalyses the conversion of oxaloacetate to oxalate and acetate (Kubicek and Rohr 1986; Kubicek et al. 1988). Many of the researchers have suggested that citric acid is more effective in dissolution of the lateritic minerals. However, Sukla and Panchanadikar (1993) reported that oxalic acid is more efficient among the organic acids for dissolution of nickel from lateritic chromite overburden. It might be due to effective dissolution of nickel bearing host minerals goethite in the chromite overburdens; hence the extraction of nickel is more with oxalic acid. Behera et al. (2012) observed that application of manganese supplement to the culture medium of *A. niger* improved oxalate secretion by the fungi. Further the oxalate secreted by the fungi was involved in better solubilisation of nickel from thermally pre-treated lateritic chromite overburden. The elevated oxalate secretion by the *A. niger* in response to addition of manganese attributed to enhancement of OAH activity. As earlier it was mentioned that out of several proposed pathways, that pyruvate generated during the process of glycolysis is transformed to oxaloacetate, which later on hydrolysed to oxalate and acetate by cytoplasmic enzyme OAH. The enzyme oxaloacetate acetylhydrolase OAH is located in the cytoplasm of *A. niger*, where it catalyses the conversion of oxaloacetate to oxalate and acetate (Kubicek et al. 1988). The OAH enzyme belongs to phosphoenolpyruvate mutase [PEPM/isocitrate lyase (ICL)] family. The members of this super family act upon α-oxycarboxylate substrate and cleave C–C or P–C bonds. All the PEPM/ICL superfamily members require divalent metal (manganese or magnesium) cofactors for their catalytic activities, where these cofactors play the role of mediators in the interaction between the enzyme and substrate (Chen et al. 2011).



$$\text{Oxaloacetate} + H_2O \xrightarrow{\text{Oxaloacetate acetylhydrolase}} \text{Oxalate} + \text{Acetate}$$

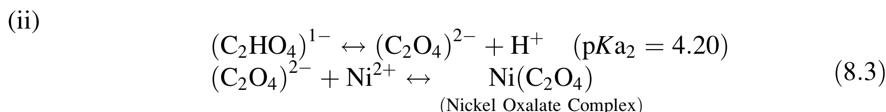
Oxalic acid has two carboxyl groups, so the possible complexes of nickel cation with oxalate anion are expressed as follows (Behera et al. 2011).

(i)



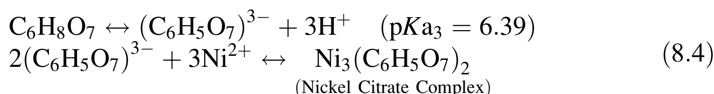
$$\begin{array}{l} C_2H_2O_4 \leftrightarrow (C_2HO_4)^{1-} + H^+ \quad (pKa_1 = 1.27) \\ (C_2HO_4)^{1-} + Ni^{2+} \leftrightarrow \underset{\text{(Nickel Oxalate Complex)}}{Ni(C_2HO_4)_2} \end{array} \tag{8.2}$$

(ii)



$$\begin{array}{l} (C_2HO_4)^{1-} \leftrightarrow (C_2O_4)^{2-} + H^+ \quad (pKa_2 = 4.20) \\ (C_2O_4)^{2-} + Ni^{2+} \leftrightarrow \underset{\text{(Nickel Oxalate Complex)}}{Ni(C_2O_4)} \end{array} \tag{8.3}$$

Similarly, nickel forms nickel citrate complexes with citric acid secreted by the fungi.



$$\begin{array}{l} C_6H_8O_7 \leftrightarrow (C_6H_5O_7)^{3-} + 3H^+ \quad (pKa_3 = 6.39) \\ 2(C_6H_5O_7)^{3-} + 3Ni^{2+} \leftrightarrow \underset{\text{(Nickel Citrate Complex)}}{Ni_3(C_6H_5O_7)_2} \end{array} \tag{8.4}$$

Likewise, nickel forms similar types of complexes with other carboxylic acid formed during the process.

Furthermore, (Behera and Sukla 2012) studied about the effect of synthetic surfactant polyoxyethylenesorbitan monolaurate (Tween-20) on bioleaching of nickel from lateritic chromite overburdens by *A. niger*. It was observed that addition of surfactant in low concentration favoured nickel recovery of pre-treated chromite overburdens during fungal bioleaching. The surfactant used in very low concentration favoured higher rate of sucrose consumption by *A. niger* for its culture medium. In addition to it, the average size of fungal micelle generated in the presence of surfactant was comparatively smaller than that of without surfactant. The smaller sized micelles of *A. niger* provided more surface area for microbe–mineral interaction. Hence, the microbial metabolites generated at mineral–microbe interface interacted more efficiently upon mineral matrix, as a result of which nickel recovery was improved the nickel extraction from pretreated chromite overburden.

8.3.1.3 Bioaccumulation of Metals

Fungus accumulates the metal ion from the solution during the leaching process to maintain the equilibrium between solid and dissolved metals, which in turn favours the continuous solubilisation of the metal (Burgstaller and Schinner 1993). Magyarosy et al. (2002) conducted experiments to study the nickel accumulation inside the *A. niger* cell. It was reported that nickel was actively uptaken by the fungal cell and accumulated inside the cell in the form of nickel–oxalate complex. However, the nickel accumulation in *A. niger* cell was inhibited by protonophores like carbonyl cyanide *p*-(trifluoromethoxy) and phenyl hydrazone (FCCP). These protonophores disrupt the proton gradient of the electron carriers in the electron

transport chain, thus the metal uptake and bioaccumulation are inhibited. It indicates that membrane transporters of the cell play prominent role in the metal uptake. Hence, the metal accumulation is a metabolically active phenomenon. Further, the heavy metal-resistant efficiency of these microorganisms is an added advantage for metal leaching (Burgstaller and Schinner 1993; Le et al. 2006).

Microbial recovery of nickel from low-grade nickel laterite ores have been studied by several authors in laboratory scale only. However, commercialisation of the process has been less successful due to its longer processing period coupled with poor metal recovery (Tang and Valix 2006). However, several authors reported that microbe-assisted leaching is comparatively efficient than chemical leaching since the microbiological activities are involved in metal leaching along with metal-chelating organic acid when compared with chemical leaching by organic acids (Burgstaller and Schinner 1993; Tzeferis 1994; Castro et al. 2000; Valix et al. 2001; Le et al. 2006). It was reported that physical attachment of the fungal cell onto mineral surfaces contributed high organic acid (formed at hyphal tips) concentration with adjacent to mineral surfaces without greatly affecting the pH of the whole medium (Alibhai et al. 1993).

The mineralogy of the ores has significant effect on the metal recovery during the leaching process. Nickel is present in an absorbed state within the goethite matrix of the lateritic ores. Due to low solubility and complex structure, extraction of nickel from the goethite matrix is very difficult. However, it has been reported that due to thermal pre-treatment of lateritic ores, nickel recovery is enhanced through bioleaching (Ruan et al. 2002; Valix and Cheung 2002; Mohapatra et al. 2008). Thermal pre-treatment alters the mineralogical constitution of the laterites by dehydroxylation of goethite matrix (Landers and Gilkes 2007; Jinhui et al. 2009; Mohapatra et al. 2009). Furthermore, Behera et al. (2011) found that as the consequent of thermal pretreatment, the laterites converted into a meso-porous like structure and the surface area of the laterite ore particle was also increased. As a result of which it improved the interaction of lixiviants with the ore particle and increased the recovery of nickel.

A summary of some of the studies conducted for extraction of metal values from different lateritic ores by using fungal strains is presented in Table 8.1. Citric and oxalic acids are the two prominent fungal metabolites involved in nickel leaching. However, citric acid has been suggested as the most effective leaching agent by some authors (Tzeferis 1994). The possible explanation for least effectiveness of oxalic acid might be attributed to precipitation of the leached nickel as nickel oxalate, which has very low solubility (Tzeferis 1994). Sukla and Panchanadikar (1993) found that nickel recovery from lateritic chromite overburdens was higher by oxalic because the oxalic acid effectively solubilises the iron oxides (goethite) in the ores. Tang and Valix (2006) suggested that the extent of metal dissolution is dependent up on the acid activity (hydronium ion concentration) rather than the type of metabolic acids involved.

Table 8.1 Microorganisms producing different organic acids during microbial processing of lateritic ores

Fungal strains	Organic acid produced by the fungal strains	Nickel recovery (%)	References
Aspergillus, *Penicillium*	Citric, oxalic	Up to 50–60	Alibhai et al. (1993)
Penicillium	Citric	Up to 72	Bosecker (1985)
Aspergillus, *Penicillium*	Citric, oxalic	55–60	Tzeferis (1994)
Aspergillus niger	Citric, oxalic	Up to 78	Castro et al. (2000)
Aspergillus	Citric acid, oxalic, gluconic	Up to 34	Mohapatra et al. (2007)
Aspergillus niger	Oxalic acid, citric acid	Up to 35	Behera et al. (2011)

8.3.2 Chemolithographic Microbes in Processing of Laterites

As it has been mentioned in introduction, the chemolithographic (autotrophic) microbes belong to iron and sulphur oxidising in nature and require reduced inorganic compounds of iron and sulphur for their metabolism and survival. The acidophilic autotrophic microbes are strictly chemolithotrophic, i.e. derive energy for metabolism from oxidation of sulphur or reduced iron and sulphur compounds. The use of chemolithographic acidophilic microbes in lateritic mineral processing has not been much reported, because nickel laterites contain no energy source (reduced inorganic compounds of iron and sulphur) to support their nutritional requirement. However, the sulphidic minerals have been successfully processed by these chemolithographic microorganisms for the extraction of metal values. The sulphidic minerals processing microorganisms belong to genera of *Acidithiobacillus, Leptospirillum, Acidimicrobium, Sulpholobus, Sulphobacillus, Metallosphaera*, etc. These are acidophilic in nature, show optimum growth at pH 2–4. Such microbes are autotrophic in nature as they use inorganic source of carbon (CO_2). Among these microbes, *Acidithiobacillus ferrooxidans* is more elaborately studied for bioleaching of sulphidic minerals. For sulphidic mineral processing, chemolithotropic bacteria belonging to the genus *Acidithiobacillus* involve oxidative bioleaching mechanism. The oxidative bioleaching mechanism involves the production of Fe^{3+} ion from the bio-oxidation of Fe^{2+} by *Acidithiobacillus* bacterial strain (Chen et al. 2011).

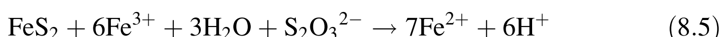
Two mechanisms namely thiosulphate mechanism and polysulphide mechanism have been proposed for bioleaching of acid-insoluble metal sulphides like pyrite (FeS_2) and molybdenite (MoS_2), and for acid-soluble metal sulphides such as sphalerite (ZnS), chalcopyrite ($CuFeS_2$), respectively (Schippers and Sand 1999; Rohwerder et al. 2003).

8.3.2.1 Thiosulphate Pathway

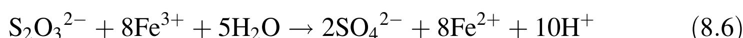
In thiosulphate mechanism metal is solubilised by ferric iron (generated by microbial process), which attack on the acid-insoluble metal sulphides such as pyrite (FeS_2), molybdenite (MoS_2) or tungstenite (WS_2) and thiosulphate is generated as main intermediate product. Further, the sulphate is generated as the main end product of the process (Rawlings et al. 2003).

The chemolithotrophic microbes belong to genera *Acidithiobacillus and Leptospirillum* and oxidise ferrous iron to ferric form under aerobic condition.

The active ferric iron acts as attacking reagent upon metal sulphide bond.

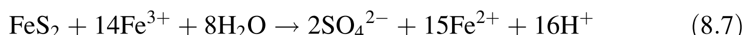


$$FeS_2 + 6Fe^{3+} + 3H_2O + S_2O_3{}^{2-} \rightarrow 7Fe^{2+} + 6H^+ \quad (8.5)$$



$$S_2O_3{}^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4{}^{2-} + 8Fe^{2+} + 10H^+ \quad (8.6)$$

The two above equations sum up to give the following overall equation:

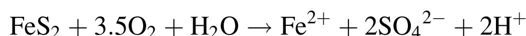


$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 2SO_4{}^{2-} + 15Fe^{2+} + 16H^+ \quad (8.7)$$

The main role of the microorganisms in this mechanism is to catalyse the regeneration of the consumed ferric ions under aerobic condition as mentioned below.

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O \quad (8.8)$$

The overall reaction based on the primary oxidant oxygen is given below:

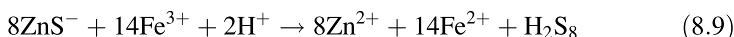


$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4{}^{2-} + 2H^+$$

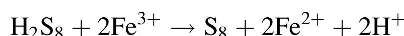
8.3.2.2 Polysulphate Pathway

In the polysulphide mechanism, metal was solubilised by the combined attack of ferric iron and proton on the acid-soluble metal sulphides such as sphalerite (ZnS), chalcopyrite (Cu_2S) or galena (PbS) and element sulphur is the main intermediate. The elemental sulphur is relatively stable, but can be oxidised to sulphate by sulphur-oxidising microbes (Rawlings et al. 2003).

The polysulphide pathway reaction mechanism of zinc sulphide bioleaching is stated below.



$$8ZnS^- + 14Fe^{3+} + 2H^+ \rightarrow 8Zn^{2+} + 14Fe^{2+} + H_2S_8 \quad (8.9)$$

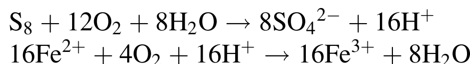


$$H_2S_8 + 2Fe^{3+} \rightarrow S_8 + 2Fe^{2+} + 2H^+$$

The role of microorganism in this mechanism is twofold:

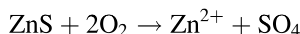
(i) To catalyse the regeneration of the ferric ions consumed for the chemical oxidation of the intermediary hydrogen sulphide into elemental sulphur via formation of polysulphides.
(ii) They catalyse the generation of sulphuric acid in order to maintain the supply of protons required in the first reaction step for the dissolution of the minerals.

Therefore further reaction steps are as follows.



$$S_8 + 12O_2 + 8H_2O \rightarrow 8SO_4^{2-} + 16H^+$$
$$16Fe^{2+} + 4O_2 + 16H^+ \rightarrow 16Fe^{3+} + 8H_2O$$

However the overall reaction is:

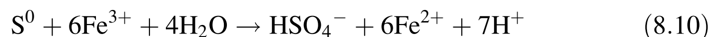


$$ZnS + 2O_2 \rightarrow Zn^{2+} + SO_4$$

It is evident from the above mechanism that a high microbial oxidation rate of ferrous to ferric iron is important for an efficient bioleaching process of sulphide minerals. Since the laterites minerals lack energy source for such chemolithotrophic microbes, hence the bio-oxidation process is hardly applied for the microbial processing of laterite ores.

8.3.2.3 Reduction Microbial Processing of Laterites

Recently developed anoxic reductive method is a new and novel approach for processing of lateritic minerals (ferric rich) by using *Acidithiobacillus ferrooxidans*. This bacterium reduces ferric iron to ferrous iron in anoxic condition with a suitable electron donor (elemental sulphur) and produces sulphuric acid. Hallberg et al. (2011) screened for the iron-reducing efficiency of *A. ferrooxidans* under anoxic condition and reported its abilities to solubilise nickel from a limonitic laterite ore. Behera et al. (2012) reported for the successfully extraction of nickel from lateritic chromite overburden by using *A. ferrooxidans* under anoxic environment. The anoxic microbial processing of the chromite overburden was carried out in $9K^-$ medium supplemented with elemental sulphur. It was reported that *A. ferrooxidans* reduced the ferric iron of goethite [FeO(OH)] phase of the laterite chromite overburden when incubated under anoxic conditions in presence of sulphur. Sulphur used in the medium for *A. ferrooxidans* acts as the source of electron for chemolithotropic mode of nutrition (Kucera et al. 2012). Fe^{2+} ions generated due to reduction of Fe^{3+} in the goethite during the microbial processing and the sulphur was oxidised to hydrogen sulphate (HSO_4^-) (which later on was converted to H_2SO_4) that generated acidity in the medium and was responsible for dissolution of nickel. This can be explained by Eq. (8.10) as reported by Brock and Gustafson (1976).



$$S^0 + 6Fe^{3+} + 4H_2O \rightarrow HSO_4^- + 6Fe^{2+} + 7H^+ \quad (8.10)$$

Under aerobic condition oxygen behaves as a terminal electron acceptor during chemolithotrophic mode of respiration because ferrous iron in acidic environment is spontaneously oxidised to ferric iron and generates free electron. Further, Fe^{2+}/Fe^{3+} redox couple has a very positive standard electrode potential (+770 mV at pH 2) which is close to the standard electrode potential of O_2/H_2O redox couple (O_2/H_2O: +820 mV at pH 7) as a result, only oxygen bears the potentiality to act as a natural electron acceptor (Rawlings 2005). Hence under anoxic condition elemental sulphur behaves as the electron donor and ferric iron in goethite of laterite overburden acts as electron acceptor and subsequently reduced to magnetite.

The recent developments suggest that microbial processing of oxidic nickel ores might be technically feasible. In this connection the reductive dissolution can be used for bio-processing of ferric oxide rich nickel minerals.

8.3.3 Challenges in Mineral Processing by Heterotrophic Microbes

Oxidic ores contain no energy source for chemolithoautotrophic bacteria and most of these microbes depend upon oxidation of sulphur or reduced iron and sulphur compounds. Therefore, the heterotrophic microbes have been selected by several researchers for microbial leaching of most of lateritic ores and minerals. Use of heterotrophic microorganisms in extraction of metal from lateritic ores has not been so much encouraged due to several drawbacks. The fungal strains used in mineral processing show optimum growth at neutral pH range, which become liable to microbial contamination. Maintaining sterile environment to carry out such processes at large scale is generally not economically feasible. An added concern to the process is the need for organic carbon source utilised by the heterotrophs during the process. However, organic wastes generated from agriculture, domestic activities, food and beverage industries can be scientifically exploited for the use of cheap growth substrates of fungi. Furthermore, the use of fungi presents setback in mineral processing because of undesirable production of excess fungal biomass. The fungal biomass and fungal mycelium often adsorb or accumulate the leached materials. Therefore, further recovery of metal from these fungal biomass and mycelium is an added cumbersome process.

8.3.4 Challenges in Mineral Processing by Chemolithotrophic Microbes

The acidophilic, iron- or sulphur-oxidising chemolithotrophic microorganisms have been studied more extensively in laboratory scale as well as in commercial scale (Rawlings 2005). The detail molecular mechanism of bioleaching of sulphidic minerals by the model bacterial strain *Acidithiobacillus* has been studied more elaborately. The strains of *Acidithiobacillus* bacteria have been used in industry scale operation for the recovery of copper and uranium (Acevedo 2000; Brierley and Brierley 2001; Kodali et al. 2004; Ndlovu et al. 2009; Panda et al. 2012). In recent developments the *Acidithiobacillus* has been applied for the extraction of nickel from laterites in anoxic microbial reduction leaching process (Hallberg et al. 2011; Behera et al. 2012). During such process elemental sulphur was used for anoxic microbial reduction leaching of nickel from laterites; however, the process can be made economically sound by using low grade sulphide minerals in place of elemental sulphur. This can also provide a platform for utilisation and treatment of waste low grade sulphide minerals and laterites for recovery of metal values. However, further studies are required on the molecular mechanism involved in the anoxic reduction process for the application of chemolithotrophic microorganisms in the microbial processing of nickel laterites.

8.4 Conclusion

Microbial mineral processing route is a simple and effective technology for extraction of metal values from lean grade ores and mineral. Processing of sulphide minerals with chemolithotrophic acidophilic microorganisms such as *Acidithiobacillus* and *Leptospirillum* converts insoluble metal sulphides into soluble metal sulphates. The oxidic or lateritic ores and minerals can be processed by heterotrophic microorganisms where metal extraction is due to the production of organic acids and chelating compounds by these organisms. The fungal-mediated processing of laterites and recovery of nickel have been reported but still there remains some challenges before this process can be applied in large scale. On the other hand, the use of the chemolithotrophic microorganisms for processing of nickel oxidic or laterite minerals have been discouraged, since the oxide minerals lack nutritional support (ferrous iron or reduced sulphur compounds) for such microbes. However, there is potential for anoxic reduction process using chemolithotrophic microorganisms for processing of oxide mineral by performing co-leaching of low-grade sulphide minerals with oxides mineral.

References

Acevedo F (2000) The use of reactors in biomining processes. Electronic J Biotechnol 3:184–194

Alibhai KAK, Dudeney AWL, Leak DJ, Agatzini S, Tzeferis P (1993) Bioleaching and bioprecipitation of nickel and iron from laterites. FEMS Microbiol Rev 11:87–96

Balmforth AJ, Thomson A (1984) Isolation and characterization of glyoxylate dehydrogenase from the fungus *Sclerotium rolfsii*. Biochem J 218:113–118

Behera SK, Sukla LB (2012) Microbial extraction of nickel from chromite overburdens in presence of surfactant. Trans Nonferrous Met Soc China 22:2840–2845

Behera SK, Panda PP, Singh S, Pradhan N, Sukla LB, Mishra BK (2011) Study on reaction mechanism of bioleaching of nickel and cobalt from lateritic chromite overburdens. Int Biodeterior Biodegradation 65:1035–1042

Behera SK, Panda PP, Saini SK, Pradhan N, Sukla LB, Mishra BK (2012) Recovery of nickel from chromite overburden, Sukinda using Aspergillus niger supplemented with manganese. Korean J Chem Eng 30(2):392–399

Boldt JR, Queneau P (eds) (1967) The winning of nickel: its geology, mining, and extractive metallurgy. Longmans, Toronto, pp 25–78

Bosecker K (1985) Leaching of lateritic nickel ores with heterotrophic microorganisms. In: Lawrence RW, Branion RMR, Ebner HG (eds) Fundamental and applied biohydrometallurgy. Proceedings of the sixth international symposium on biohydrometallurgy, Vancouver, BC, Canada, 21–24 Aug 1985, pp 367–382

Bosecker K (2001) Microbial leaching in environmental clean-up programmes. Hydrometallurgy 59:245–248

Brand NW, Butt CRM, Elias M (1998) Nickel laterites: classification and features. AGSO J Aust Geol Geophys 17:81–88

Brierley JA, Brierley CL (2001) Present and future commercial applications of biohydrometallurgy. Hydrometallurgy 59:233–239

Brock TD, Gustafson J (1976) Ferric iron reduction by sulphur and iron-oxidizing bacteria. Appl Environ Microbiol 32:567–571

Burgstaller W, Schinner F (1993) Leaching of metals with fungi. J Biotechnol 27:91–116

Castro IM, Fietto JLR, Vieira RX, Tropia MJM, Campos LMM, Paniago EB, Brandao RL (2000) Bioleaching of zinc and nickel from silicates using Aspergillus niger cultures. Hydrometallurgy 57:39–49

Cempel M, Nickel G (2006) Nickel: a review of its sources and environmental toxicology. Pol J Environ Stud 15:375–382

Chang Y, Zhai X, Li B, Fu Y (2010) Removal of iron from acidic leach liquor of lateritic nickel ore by goethite precipitate. Hydrometallurgy 101:84–87

Chen P, Yan L, Leng F, Nan W, Yue X, Zheng Y, Feng N, Li H (2011) Bioleaching of realgar by *Acidithiobacillus ferrooxidans* using ferrous iron and elemental sulfur as the sole and mixed energy sources. Bioresour Technol 102:3260–3267

Gadd GM (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. Adv Microb Physiol 41:47–92

Golightly JP (1981) Nickeliferous laterite deposits. Econ Geol 75:710–735

Hallberg KB, Grail BM, Plessis CA, Johnson DB (2011) Reductive dissolution of ferric iron minerals: a new approach for bio-processing nickel laterites. Miner Eng 24:620–624

Hammel KE, Mozuch MD, Jensen KA Jr, Kersten PJ (1994) H_2O_2 recycling during oxidation of the arylglycerol beta-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase. Biochemistry 33:13349–13354

Jinhui L, Xinhai L, Qiyang H, Zhixing W, Youyuan Z, Junchao Z, Wanrong L, Lingjun L (2009) Effect of pre-roasting on leaching of laterite. Hydrometallurgy 99:84–88

Kodali B, Rao MB, Narasu ML, Pogaku R (2004) Effect of biochemical reactions in enhancement of rate of leaching. Chem Eng Sci 59:5069–5503

Kubicek CP, Rohr M (1986) Citric acid fermentation. CRC Crit Rev Biotechnol 3:331–373

Kubicek CP, Kunar GS, Woehrer W, Roehr M (1988) Evidence for a cytoplasmatic pathway of oxalate biosynthesis in Aspergillus niger. Appl Environ Microbiol 54:633–637

Kucera J, Zeman J, Mandl M, Cerna H (2012) Stoichiometry of bacterial anaerobic oxidation of elemental sulfur by ferric iron. Antonie Van Leeuwenhoek 101(4):919–922, http://dx.doi.org/10.1007/s10482-012-9699-x

Landers M, Gilkes RJ (2007) Dehydroxylation and dissolution of nickeliferous goethite in New Caledonian lateritic Ni ore. Appl Clay Sci 35:162–172

Le L, Tang JA, Ryan D, Valix M (2006) Bioleaching nickel laterite ores using multi metal tolerant *Aspergillus foetidus* organism. Miner Eng 19:1259–1265

Lee HY, Kim SG, Oh JK (2005) Electrochemical leaching of nickel from low grade laterites. Hydrometallurgy 77:263–268

Magyarosy A, Laidlaw RD, Kilaas R, Echer C, Clark DS, Keasling JD (2002) Nickel accumulation and nickel oxalate precipitation by *Aspergillus niger*. Appl Microbiol Biotechnol 59:382–388

Mohapatra S, Bohidar S, Pradhan N, Kar RN, Sukla LB (2007) Microbial extraction of nickel from Sukinda chromite overburden by *Acidithiobacillus ferrooxidans* and *Aspergillus* strains. Hydrometallurgy 85:1–8

Mohapatra S, Sengupta C, Nayak BD, Sukla LB, Mishra BK (2008) Effect of thermal pretreatment on recovery of nickel and cobalt from Sukinda lateritic nickel ore using microorganisms. Korean J Chem Eng 25(5):1070–1075

Mohapatra S, Pradhan N, Mohanty S, Sukla LB (2009) Recovery of nickel from lateritic nickel ore using *Aspergillus niger* and optimization of parameters. Miner Eng 22:311–313

Mulligan CN, Kamali M, Gibbs BF (2004) Bioleaching of heavy metals from low grade mining ores using *Aspergillus niger*. J Hazard Mater 110:77–84

Ndlovu S, Simate GS, Gericke M (2009) The microbial assisted leaching of nickel laterites using a mixed culture of chemolithotrophic microorganisms. Adv Mater Res 71–73:493–496

Olson GJ, Brierley JA, Brierley CL (2003) Bioleaching review part B: progress in bioleaching: applications of the microbial processes by the mineral industries. Appl Microbiol Biotechnol 63:249–257

Panda S, Sanjay K, Sukla LB, Pradhan N, Subbaiah T, Mishra BK, Prasad MSR, Ray SK (2012) Insights into heap bioleaching of low grade chalcopyrite ores – a pilot scale study. Hydrometallurgy 125–126:157–165

Pedersen H, Christensen B, Hjort C, Nielsen J (2000a) Construction and characterization of an oxalic acid nonproducing strain of *Aspergillus niger*. Metab Eng 2:4–41

Pedersen H, Gem C, Nielsen J (2000b) Cloning and characterization of oah, the gene encoding oxaloacetate hydrolase in *Aspergillus niger*. J Mol Gen Genet 263:281–286

Rawlings DE (2002) Heavy metal mining using microbes. Annu Rev Microbiol 56:65–91

Rawlings DE (2005) Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. Microb Cell Fact 4:13

Rawlings DE, Dew D, du Plessis C (2003) Biomineralization of metal-containing ores and concentrates. Trends Biotechnol 21:38–44

Rezza I, Salinas E, Elorza M, de Sanz TM, Donati E (2001) Mechanism involved in bioleaching of an aluminosilicate by heterotrophic microorganisms. Process Biochem 36:495–500

Rohwerder T, Gehrke T, Kinzler K, Sand W (2003) Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl Microbiol Biotechnol 63:239–248

Ruan HD, Frost RL, Kloprogge JT, Duong L (2002) Infrared spectroscopy of goethite dehydroxylation: III. FT-IR microscopy of in situ study of the thermal transformation of goethite to hematite. Spectrochim Acta A Mol Biomol Spectrosc 58:967–981

Ruijter GJG, van de Vondervoort PJI, Visser J (1999) Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 145:2569–2576

Schippers A, Sand W (1999) Bacterial leaching of metal sulphides proceeds by two indirect mechanism via thiosulphate or via polysulphides and sulphur. Appl Environ Microbiol 65: 319–321

Simate GS, Ndlovu S, Walubita LF (2010) The fungal and chemolithotrophic leaching of nickel laterites – challenges and opportunities. Hydrometallurgy 103:150–157

Sukla LB, Das RP (1987) Kinetics of nickel dissolution from roasted laterites. Trans Indian Inst Met 40:351–353

Sukla LB, Panchanadikar VV (1993) Bioleaching of lateritic nickel ore using a heterotrophic micro-organism. Hydrometallurgy 32:373–379

Sukla LB, Panchanadikar VV, Kar RN (1993) Microbial leaching of lateritic nickel ore. World J Microbiol Biotechnol 9:255–257

Swain PK, Chaudhury GR, Sukla LB (2007) Dissolution kinetics of chromate overburden by using mineral acids. Korean J Chem Eng 24:932–935

Swamy YV, Kar BB, Mohanty JK (2003) Physico-chemical characterization and sulphatization roasting of low-grade nickeliferous laterites. Hydrometallurgy 69:89–98

Tang JA, Valix M (2006) Leaching of low grade limonite and nontronite ores by fungi metabolic acids. Miner Eng 19:1274–1279

Torries FT (1995) Comparative costs of nickel sulphides and laterites. Resour Pol 21:179–187

Tsekova K, Todorova D, Ganeva S (2010) Removal of heavy metals from industrial wastewater by free and immobilized cells of *Aspergillus niger*. Int Biodeterior Biodegradation 64:447–451

Tzeferis P (1992) Mechanisms important for bioleaching and metal accumulation by microorganisms. Metalleiologika Metallourgika Chron 2:85–107

Tzeferis PG (1994) Leaching of a low grade hematitic laterite ore using fungi and biologically produced acid metabolites. Int J Miner Process 42:267–283

Valix M, Cheung WH (2002) Study of phase transformation of laterite ores at high temperature. Miner Eng 15:607–612

Valix M, Loon LO (2003) Adaptive tolerance behaviour of fungi in heavy metals. Miner Eng 16: 193–198

Valix M, Tang JY, Cheung WH (2001) The effects of mineralogy on the biological leaching of nickel laterite ores. Miner Eng 14:1629–1635

Chapter 9
Subsurface Petroleum Microbiology

Ajay Singh, Jonathan D. Van Hamme, Ramesh C. Kuhad, Nagina Parmar, and Owen P. Ward

9.1 Introduction

Biogeochemical processes carried out by the microorganisms in near or deep subsurface sediments have been the subject of much interest during the last two decades (Magot et al. 2000; Van Hamme et al. 2003; Jones et al. 2008; Voordouw 2011). The possibility for living organisms to survive or thrive in extreme oilfield environments depends on the ecosystem physicochemical characteristics. Plate tectonics, basin, and petroleum system formation are believed to be the vital processes that affect deep subsurface biological systems and are integral to interactions between the biosphere and geosphere in petroleum reservoirs (Head et al. 2003). They alter the physical environment, control the formation and distribution of organic carbon, and influence delivery of nutrients and oxidants that can be utilized by resident subsurface microbes.

Petroleum reservoirs, generally found in porous and permeable sediments such as sandstones and limestones, exhibit a wide range of temperatures (30–180 °C), salinities (up to 300 g/L NaCl), and pressures (up to several hundred bars). Essentially anaerobic, these extreme subsurface ecosystems were long considered

A. Singh (✉)
Lystek International Inc., 1425 Bishop Street North, Unit 16, Cambridge, ON, Canada N1R 6J9
e-mail: asingh@lystek.com

J.D. Van Hamme
Department of Biological Sciences, Thompson Rivers University, Kamloops, BC, Canada V2C 5N3

R.C. Kuhad
Department of Microbiology, University of Delhi, South Campus, New Delhi 110021, India

N. Parmar
Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada M5B 2K3

O.P. Ward
Department of Biology, University of Waterloo, Waterloo, ON, Canada N2L 3G1

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39, DOI 10.1007/978-3-642-41837-2_9, © Springer-Verlag Berlin Heidelberg 2014

too hostile for microbial life. However, with the exception of those exhibiting very high temperature and salinity, there is now evidence that many petroleum reservoirs contain active and diverse populations of chemolithotrophic and heterotrophic microorganisms with a wide range of metabolic abilities (Magot et al. 2000; Head et al. 2003; Galperin and Kaplan 2011). Besides potentially being indigenous, some of these microbial communities may have been introduced as contaminants during reservoir flooding with seawater, during drilling processes, or after migration through aquifers from terrestrial and sub-terrestrial sites (Jones et al. 2008). Currently, the World's petroleum inventory is dominated by the biodegraded petroleum reservoirs, but determining the microbial processes that have led to the biodegradation of hydrocarbons remains a challenge due to the inherent difficulties in collecting representative samples from deep subsurface petroleum reservoirs. An overview of our current knowledge on subsurface petroleum microbiology including the microbial diversity and metabolic processes in petroleum oilfields and reservoirs are provided in this chapter together with a shorter section providing examples of microbial processes in deep aqueous environments.

9.2 Metabolic Processes

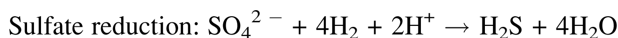
Although the role of microorganisms in subsurface petroleum transformation and the likelihood that they are almost exclusively anaerobic are now well accepted, important details such as the identities and specific metabolic activities of indigenous microbes are still obscure (Röling et al. 2003). It has been hypothesized that the majority of microbial activity occurs at the interface between oil-saturated and water-saturated regions of reservoirs, which is the zone of recharge with water-soluble nutrients and oxidants (Larter et al. 2003).

Geothermally heated oil reservoirs contain an excess of reduced electron donors including a range of hydrocarbons, H_2, and organic acids. The most commonly found fatty acids in many formation waters are acetate, butyrate, formate, propionate, and benzoate. These substrates may be oxidized by anaerobic microbial communities, which include sulfate-reducing bacteria (SRB) and methanogenic *Archaea*. More complex organic acids such as naphthenic acids are present in crude oil and amino acids may also accumulate during the kerogen maturation process (Foght 2010). Hydrogen, possibly resulting from abiotic reactions (mineral hydrolysis) at high temperature in the deep subsurface, may also act as a significant source of energy for the hydrogenotrophic microorganisms retrieved from oil reservoirs. The use of H_2 may favor the anaerobic oxidation of hydrocarbons in the presence of sulfate and CO_2 as terminal electron acceptors or may cause a shift in electron flow during fermentative processes, resulting in the formation of less-reduced end products of metabolism such as acetate, propionate, and isobutyrate. Hydrogenotrophic sulfate reducers and methanogenic *Archaea* have been frequently isolated from formation water and there are reports on homoacetogens having the ability to oxidize hydrogen and reduce CO_2 into acetate (Jones

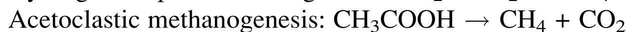
et al. 2008; Ollivier and Alazard 2010). In the deep biosphere, H_2 generated from abiotic (mineral hydrolysis) and biotic (hydrocarbon oxidation, fermentative processes) reactions seems to be a primary source of energy for methanogenic *Archaea* and SRB.

The type of metabolic processes occurring in oil reservoirs depends to a great extent on the availability of electron acceptors. Since many reservoirs have limited dissolved oxygen, it is unlikely to be available in significant amounts to enable efficient aerobic biodegradation (Skaare et al. 2011). Similarly, nitrate is relatively rare in oil reservoirs, except when specifically added to injection water to prevent souring. Dissimilatory iron-reducing microorganisms and methanogenic *Archaea* have been isolated from many production water samples and other extreme environments within the deep subsurface, linking subsurface hydrocarbon degradation to iron reduction and methanogenesis/CO_2 reduction (Head et al. 2003). The availability of fixed nitrogen likely does not limit microbial activity in reservoirs because ammonium ions buffered by reservoir minerals, N_2 gas, and N heterocycles could provide adequate N in situ. However, the availability of water-soluble nutrients like phosphorus and/or oxidants (terminal electron acceptors such as Fe^{3+}, $SO_4{}^{2-}$, or CO_2) is more likely to limit microbial activity.

Since stratal waters often contain sulfate and carbonate, sulfate reduction, methanogenesis, fermentation, and homoacetogenesis may be expected as the major metabolic processes within oil reservoirs (Ollivier and Alazard 2010):


Sulfate reduction: $SO_4{}^{2-} + 4H_2 + 2H^+ \rightarrow H_2S + 4H_2O$

Hydrogenotrophic methanogenesis: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$


Acetoclastic methanogenesis: $CH_3COOH \rightarrow CH_4 + CO_2$

Homoacetogenesis: $2HCO_3{}^- + H^+ + 4H_2 \rightarrow CH_3COO^- + 4H_2O$

9.3 Biotransformation of Petroleum Hydrocarbons

Microbial degradation of oil in petroleum reservoirs is a globally significant biogeochemical process. The physical and chemical changes that result from reservoir biodegradation of crude oil have important operational and economic consequences. Removal of the lighter saturated hydrocarbon fraction increases the polar fraction of resins and asphaltenes in the oil resulting in increased viscosity and production costs and reduced American Petroleum Institute (API) gravity and oil value. In addition, biodegradation results in increased acidity and sulfur content leading to corrosion problems during oil transport, refining, and processing. Out of Earth's estimated ten trillion barrel oil inventory, heavy oils and tars constitute around six trillion barrels (Head et al. 2003).

Hydrocarbon-degrading bacteria able to exploit petroleum hydrocarbons for producing energy and biomass were first isolated almost a century ago (Head et al. 2003). The two principal modes by which microorganisms can harvest energy for building the new cell material are respiration and fermentation. Biodegradation

of hydrocarbons by aerobic bacteria is supported by the presence of oxygen in petroleum reservoirs, whereas anaerobic heterotrophic microorganisms requiring nitrate, sulfate, iron, manganese, or carbon dioxide as electron acceptors are responsible for biodegradation of hydrocarbons in the absence of oxygen. Water controls both physical and physiological processes of microbial activity and hence plays an important role in subsurface microbial habitats. In order for a microbial cell to metabolize a compound, the enzymes of the cell must gain direct access to the compound and that must either be transported across the cell membrane into the bacterial cell or at least directly contact membrane-bound enzymes at the cell surface in the case of hydrophobic substrates such as saturated hydrocarbons (Wentzel et al. 2007). Substrate uptake takes place through either passive or facilitated diffusion at the point of contact (Van Hamme et al. 2003).

The effect of microbial activity on the total petroleum hydrocarbon composition in oils is well documented and reflects the removal of different compound classes of petroleum oils in anaerobic subsurface reservoirs (Magot et al. 2000; Head et al. 2003; Jones et al. 2008; Galperin and Kaplan 2011). The most degradable compounds are straight chain *n*-alkanes followed by the branched acyclic and monocyclic hydrocarbons, polycyclic steroidal and triterpenoidal hydrocarbons, and some aromatic hydrocarbons. Within the *n*-alkanes, multiple observations of anaerobic petroleum biodegradation in oil reservoirs, near-surface petroleum-contaminated soils, and controlled laboratory experiments have demonstrated a systematic relationship between decreasing relative degradation rates and increasing chain length for *n*-alkanes from *n*-C10 to *n*-C25 and higher (Aitken et al. 2004; Galperin and Kaplan 2011). In biodegraded reservoirs, alkylated naphthalenes and other two- and three-ringed aromatic hydrocarbons are only degraded after significant removal of *n*-alkanes and modification of acyclic isoprenoids such as pristane and phytane. This contrasts what occurs under aerobic conditions, where removal of aromatic compounds such as alkylated naphthalenes can occur before the alteration of *n*-alkanes can be observed.

A major fundamental difference between aerobic and anaerobic metabolism that affects subsurface biodegradation processes is the cellular energy yield resulting from the use of terminal electron acceptors with different free energies of formation. Anaerobic microbes gain considerably less energy from substrate biotransformation processes and, as a result, form much smaller amounts of biomass compared to aerobic microbes (Widdel and Rabus 2001). Aerobic degradation usually proceeds more rapidly and efficiently; consequently aerobic reactions require less free energy for initiation and yield more energy per reaction (Schink 2006; Wentzel et al. 2007). Laboratory experiments reveal that the cultivation of microorganisms with hydrocarbons as growth substrates under anaerobic conditions is more demanding with slower growth rates than cultivation of conventional anaerobes that grow along with facultative aerobic microorganisms (Widdel 2010). Doubling times of aerobic hydrocarbon degraders were in the range of several hours with high cell densities, whereas doubling times of anaerobic hydrocarbon degraders were in the order of days to even weeks (Widdel and Grundman 2010). These observations confirm earlier reports where comparison of hydrocarbon degradation under aerobic

and anaerobic conditions leads to a conclusion that rates of aerobic biodegradation of *n*-alkanes in the natural environment are high enough to be observable on a timescale measured in years to decades, whereas anaerobic degradation proceeds on a much longer timescale (Larter et al. 2003; Head et al. 2003).

Recently da Cruz et al. (2011) reported evidences from in vitro experiments that petroleum biodegradation could be jointly achieved by aerobic and anaerobic bacterial consortia in biofilms. In their study, an aerobic consortium depleted, in decreasing order, hydrocarbons > hopanes > steranes > tricyclic terpanes, while an anaerobic consortium depleted in the order of hydrocarbons > steranes > hopanes > tricyclic terpanes. Under subsurface conditions, anaerobic microorganisms could provide microquantities of oxygen to the aerobic microbiota by reducing salts like sulfate, nitrate, or perchlorate, for example, $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$. This oxygen might become trapped in biofilms and build microaerobic environments, stimulating aerobic bacterial growth and biodegradation. Gradual oxygen consumption would generate an anaerobic atmosphere and result in succession to anaerobic communities. Thus, the aerobic and anaerobic microorganisms might contribute to stepwise biodegradation in such a way that creates microenvironments that allow for the persistence of both microbial groups.

When in-reservoir oil biodegradation was first noticed, no anaerobic hydrocarbon-degrading microorganisms were isolated (Palmer 1993; Reuter et al. 1994). The prevalent occurrence of hydrocarbon biodegradation in shallow near-surface or deep petroleum reservoirs had been attributed to aerobic bacterial degradation stimulated by surface recharge of oxygen-bearing meteoric waters. The isolation of anaerobic bacteria and hydrocarbon degradation linked to nitrate reduction, iron reduction, sulfate reduction, and methanogenesis began to change this view in the 1990s, as all of these processes potentially play a role in oil biodegradation in anoxic petroleum reservoirs. Sulfate reduction and methanogenesis are the most likely processes responsible for in-reservoir hydrocarbon oxidation. Many SRB are known which can completely oxidize alkanes, saturated hydrocarbons that are quantitatively the most significant crude oil fraction. A multidisciplinary approach employing systematic laboratory anaerobic microcosm studies of oil degradation, microbial community analysis, petroleum geochemistry, isotopic analysis of reservoir gases, and modeling of oil biodegradation under reservoir conditions, was required to establish which anaerobic pathways are likely to be most significant for in-reservoir oil biodegradation (Jones et al. 2008; Carmona et al. 2009). Crude oil biodegradation, and the resulting patterns of compound removal observed in biodegraded petroleum reservoirs, was mimicked in oil-degrading laboratory microcosms under methanogenic and sulfate-reducing conditions. Distinct patterns of removal were observed under the two conditions that support the role of biological activity in petroleum maturation (Jones et al. 2008). A strong selection for CO_2-reducing methanogens and against acetoclastic methanogens was observed in oil-degrading microcosms inoculated with methanogenic communities. Although the reason for the predominance of syntrophic acetate oxidation over acetoclastic methanogenesis in this system is unclear, there is evidence of a detrimental effect of crude oil on acetoclastic

methanogens favoring alternative pathways of methanogenic alkane degradation via syntrophic acetate oxidation (Dolfing et al. 2008). However, there is a report on predominating acetoclastic methanogenesis in some methanogenic oil-degrading systems (Gieg et al. 2008).

Pathways for aerobic hydrocarbon metabolism are generally well known (Van Hamme et al. 2003), although new genes for alkane and aromatic hydrocarbon metabolism continue to be discovered. However, these new discoveries generally reveal that the approach to hydrocarbon metabolism is well conserved. Conversely, anaerobic hydrocarbon metabolism is not as well understood, with the majority of research being carried out only in the last 20 years. The approaches that anaerobic microorganisms use to activate hydrocarbons for oxidation and entry into central metabolism are hydroxylation with water, carboxylation, and fumarate addition. To date, anaerobic hydrocarbon metabolism has been described under a wide range of environmental conditions and for a range of alkanes and aromatics. For example, fumarate addition is now known to occur for alkyl- and trimethyl-benzenes, toluene, ethylbenzene, xylene, methylnaphthalenes, and cyclic and linear alkanes. Nitrate reducers, Fe^{3+} reducers, sulfate reducers, and anoxygenic photoheterotrophs have all been shown to use hydrocarbons as electron donors. Other terminal electron acceptors that can be used include humic acids, fumarate, and manganese. A number of recent reviews detail various aspects of anaerobic hydrocarbon metabolism (Fuchs et al. 2011; Meckenstock and Mouttaki 2011; Vogt et al. 2011).

As with aerobic metabolism, under anaerobic conditions the reduced hydrocarbon substrate is oxidized and reduced intermediates are not generated. A number of genome sequences have been reported for anaerobic hydrocarbon-degrading organisms, the most recent being *Desulfatibacillum alkenivorans* AK-01 (Callaghan et al. 2012), a sulfate-reducing bacterium able to oxidize C_{13} to C_{18} alkanes, and a range of alkenes, alcohols, organic, and fatty acids. Detailed analysis of this organism's genome has revealed some of the expected genes responsible for biochemical activities it has been shown to possess. As with other anaerobic hydrocarbon-utilizing microorganisms, the exact nature of all metabolic intermediates between parent hydrocarbon and acetyl-CoA in central metabolism is not known, although all intermediates are expected to be oxidized. As more genomes are sequenced and microbiologists learn how to grow more anaerobes in the laboratory, a clearer picture of anaerobic hydrocarbon metabolism, and the resulting impact on oil formations should emerge.

Shuqing et al. (2008) have reviewed and described in detail the geological background of the oil sands distribution in the western Canada and biodegradation in petroleum oil. The Western Canada Sedimentary Basin (WCSB) covers an area of 1,400,000 km^2 in the western part of North America. The oil sand deposits comprise of at least 85 % of the total immobile bitumen in place in the world and are an important source of economically recoverable oil. Oil sands are formed through the microbial biodegradation of light oils over millions of years, resulting in increased viscosity, sulfur, resin, asphaltenes, and metal content (Kryachko et al. 2012). There are three major oil sand deposits in the WCSB with exploitable bitumen reserves: Athabasca (~75,000 km^2; 0–500 m below ground surface), Cold

Lake (~22,000 km^2; 985–1,970 m below ground surface), and Peace River (8,000 km^2; 550–700 m below ground surface).

Oil compositions and physical properties as affected by biodegradation in the WCSB have been investigated by various groups (Brooks et al. 1988; Riediger et al. 1999; Obermajer et al. 2004; Bennett et al. 2006; Larter et al. 2006). Generally, biodegradation levels appear to intensify from west to east in the Cretaceous deposits. Current reservoir temperature at the Athabasca and Peace River are <10 °C and 16–22 °C, respectively. The Athabasca oil sands are severely affected by biodegradation (Harner et al. 2011). All *n*-alkanes, isoprenoid alkanes, and alkylated naphthalenes are completely removed. Most regular steranes are selectively removed with diasteranes being relatively concentrated or slightly attacked as well. Hopanes are in varying degrees converted to 25-norhopanes, which are often present in severely biodegraded oils and are generally considered to be the product of heavy biodegradation. The extent of biodegradation in the Peace River area is characterized by complete loss of the *n*-alkanes, acyclic isoprenoids, alkylbiphenyls, and methylated naphthalenes, but terpane and sterane biomarkers do not appear to have been affected, indicating that most of the oils suffered a moderate level of biodegradation.

9.4 Factors Affecting Occurrence of Biodegraded Reservoirs

A number of factors have been identified that may exert a major impact on the occurrence of biodegraded petroleum reservoirs. Reservoir temperature appears to be the most important factor based on the observation that biodegraded oil is rarely found in petroleum reservoirs at temperatures much in excess of 80 °C. According to the palaeopasteurization hypothesis, this is because the majority of microbial life does not persist above this temperature (Connan 1984), although many hyperthermophiles are known to exist in a variety of environments on the planet.

For example, hyperthermophilic microorganisms (*Thermococcus*, *Thermotoga*, and *Archaeoglobus* spp.) with optimal growth temperatures above 80 °C have been frequently isolated from reservoirs (Connan 1984; Grassia et al. 1996). Life at high temperatures presents some physiological challenges for the organisms, as in vitro studies at temperatures around 100 °C have demonstrated that some low molecular weight compounds such ATP and NAD have half lives of less than 30 min, and thermolabile amino acids like cysteine and glutamic acid are rapidly degraded (Orphan et al. 2000). Typically, deep subsurface petroleum reservoirs are characterized by high temperatures, with temperature increasing ~2–3 °C per 100 m depth, and biodegraded oils are found at depths of up to about ~4 km, with the most biodegraded reservoirs at up to 2.5 km below the sediment surface (Head et al. 2003). The paleopasteurization hypothesis notwithstanding, not all oil

reservoirs with a temperature less than 80 °C are biodegraded, which may be explained by other factors in their geological histories.

Salinity ranges of formation water can limit microbial activity significantly and appears to be another major factor that affects in-reservoir oil biodegradation. This is consistent with the observations that reservoirs with highly saline waters typically show limited oil biodegradation (Larter et al. 2006) and failure to culture microorganisms from reservoir waters with salinity greater than 100 g/L (Grassia et al. 1996). Similarly, the pH of formation waters can limit bacterial activity (Magot et al. 2000). Since pH is influenced by dissolution of gasses under high pressure, the pH of reservoir samples measured at atmospheric pressure does not necessarily reflect in situ pH, which is normally in the 3–7 range. Thus, for in vitro studies it is critical to consider pH when designing culture media or when making inferences about the lifestyles of indigenous microorganisms recovered from deep subsurface samples. Although pressure within oil reservoirs (up to 500 atm) can influence the physiological and metabolic properties of microbes, it is not considered to preclude their activity in situ.

As discussed above, microbial metabolic activities within an oil field or reservoir environments are governed by the availability of electron donors and acceptors. Due to deep subterranean environments being isolated from surface waters, their redox potentials tend to be very low, and some electron acceptors such as oxygen, nitrate, and ferric iron are generally absent. Stratal waters generally contain sulfate and carbonate, which have led to the assumption that the major metabolic processes occurring in such conditions are sulfate reduction, methanogenesis, acetogenesis, and fermentation (Head et al. 2010). The potential electron donors may include organic molecules and CO_2 and H_2 of geochemical or microbial origin. Availability of fixed nitrogen likely does not limit microbial activity in reservoirs because ammonium ions buffered by reservoir minerals, N_2 gas, and N-heterocycles could provide adequate N in situ, but the availability of water-soluble nutrients like phosphorus is more likely to limit microbial activity in situ (Foght 2010). Resins and asphaltenes are important fractions of crude oil that are composed of thousands of complex hydrocarbons which can also contain heteroatoms of nitrogen, sulfur, and oxygen. Although less studied, they are an abundant potential source of electron donors for anaerobic metabolism and their presence could explain the observation that diverse groups of strict anaerobes can grow with crude oil as a sole carbon and energy source without any significant modification of the alkanes or light aromatic compounds (Galperin and Kaplan 2011). Reservoir temperature, oil-charge histories with reservoir topology (relationships between oil and water volumes and interface areas), and nutrient supply from temperature-dependent mineral diagenesis appear to be the major controllers of the degree of oil biodegradation in the petroleum reservoirs (Head et al. 2003).

9.5 Microbial Diversity of Petroleum Oilfields

In anaerobic environments containing organic matter, the biogeochemical carbon, nitrogen, and sulfur cycles are intimately intertwined through diverse sulfate- and nitrate-reducing microbial communities that may use similar organic electron donors as carbon and energy sources, participate in syntrophic relationships, or share metabolic byproducts (Aitken et al. 2004; Hubert et al. 2009). For example, hydrogen sulfide produced by SRB can serve as an electron donor for nitrate reduction. The products of nitrate reduction include nitrite, which can inhibit sulfate reducers, but which also serves as an electron acceptor for the oxidation of organic or reduced sulfur-containing electron donors. Oil field microbial community composition, and their changes in response to the use of chemical addition, has been studied through culturing, by denaturing gradient gel electrophoresis, by sequencing of clone libraries, and, more recently, using metagenomic techniques (Bødtker et al. 2009; Gittel et al. 2009; van der Kraan et al. 2010; Kotlar et al. 2011; Ren et al. 2011; Stevenson et al. 2011; Hubert et al. 2012; Kryachko et al. 2012). Table 9.1 shows some of the microbial species isolated from petroleum oilfields (Magot et al. 2000; Tang et al. 2009; Ollivier and Alazard 2010).

9.5.1 Sulfate-Reducing Bacteria

SRB are widespread anaerobic microorganisms considered among the oldest microorganisms on the Earth (Wolicka and Borkowski 2007). SRB use sulfate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulfide which can be subsequently oxidized under oxic conditions by chemolithotrophic sulfur bacteria or under anoxic conditions by phototrophic sulfur bacteria (Fig. 9.1). In general SRBs are strict anaerobes but some of them have shown oxygen tolerance. SRB are ubiquitous and can be found in many natural and engineered environments where sulfate is present. SRB have been isolated from petroleum oilfields, hydrocarbon seeps, marine sediments, hydrothermal vents, mud volcanoes, and hypersaline microbial mats (Muyzer and Stams 2008; Tang et al. 2009). They have been detected in habitats with extreme pH values, such as acid-mine drainage sites, where the pH can be as low as 2 and in soda lakes, where the pH can be as high as 10. SRB are morphologically diverse with cell forms including cocci, curved types, rod, spiral, and disk shaped.

SRB are major contributors to biogeochemical cycling of carbon, sulfur, nitrogen, and phosphorus. In many petroleum hydrocarbon-contaminated environments, biological sulfate reduction is an important metabolic activity resulting in utilization of around 70 % of BTEX (benzene, ethylbenzene, toluene, xylene) compounds. SRB are also able to thrive in hydrocarbon seep areas and gas reservoirs where short chain hydrocarbons such as propane and butane are abundant (Kniemeyer

Table 9.1 Microbial diversity of petroleum reservoirs

Microbes isolated from oilfields	Temperature range (°C)	Salinity range (% NaCl)
Sulfate-reducing bacteria		
Archaeoglobus fulgidus	60–85	0.02–3
Desulfacinum infernum	40–65	0–5
Desulfacinum subterraneum	60	–
Desulfobacter vibrioformis	5–38	1–5
Desulfobacterium cetonicum	20–37	0–5
Desulfobulbus sp.	28–39	–
Desulfomicrobium apsheronum	4–40	0–8
Desulfotomaculum halophilum	30–40	1–14
Desulfotomaculum kuznetsovii	50–85	0–3
Desulfotomaculum nigrificans	40–70	0–4
Desulfotomaculum thermocisternum	41–75	0–5
Desulfovibrio gabonensis	15–40	1–17
Desulfovibrio longus	10–40	0–8
Desulfovibrio bastini	35–40	–
Desulfovibrio vietnamensis	12–45	0–10
Thermodesulfobacterium mobile	45–85	–
Thermodesulfobacterium thermophilum	65	–
Thermodesulforhabdus norvegicus	44–74	0–5.6
Methanogenic bacteria		
Methanobacterium bryantii	25–40	0–2
Methanobacterium ivanovii	10–55	0.09
Methanobacterium thermoaggregans	40–70	2–4
Methanobacterium thermoalcaliphilum	30–80	0–2
Methanobacterium thermoautrophicum	40–70	0–30
Methanocalculus halotolerans	25–45	5
Methanoculleus receptaculi	50–55	0–7.6
Methanothermococcus thermolithotrophicus	17–62	1.4–2.4
Methanohalophilus euhalobius	10–50	6
Methanohalobium evestigatum	30–60	15–30
Methanoplanus petrolearius	28–43	1–3
Methanosarcina mazei	10–50	0.1–2
Methanosarcina siciliae	20–50	2.4–3.6
Methermicoccus shengliensis	50–70	15–30
Fermentative bacteria		
Acetoanaerobium romashkovii	30–60	–
Anaerobaculum thermoterrenum	28–60	0–2
Dethiosulfovibrio peptidovorans	20–45	1–10
Geotoga petraea	30–55	0.5–10
Geotoga subterranea	30–60	0.5–10
Haloanaerobium acetoethylicum	15–45	6–20
Haloanaerobium congolense	20–45	4–24
Haloanaerobium salsugo	22–51	6–24
Petrotoga miotherma	35–65	0.5–10
Spirochaeta smaragdinae	20–40	1–10
Thermoanaerobacter brockii	37–75	0–4.5

(continued)

Table 9.1 (continued)

Microbes isolated from oilfields	Temperature range (°C)	Salinity range (% NaCl)
Thermotoga elfii	50–72	0–2.4
Thermotoga hypogea	56–90	0–1.5
Thermotoga subterranea	50–75	0–2.4

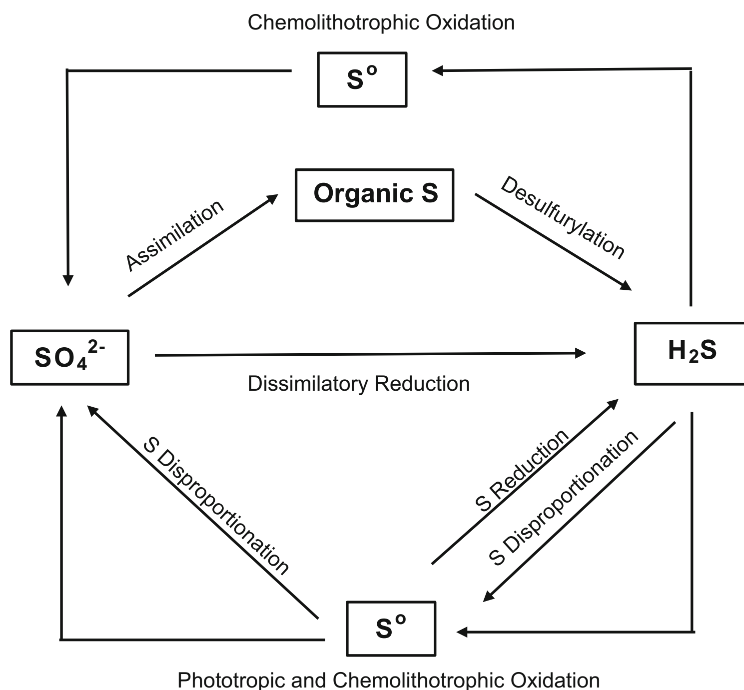


Fig. 9.1 Role of sulfate-reducing bacteria (SRB) in the biogeochemical transformation of sulfur

et al. 2007). The first extensive microbiological study describing the widespread presence of SRB in oil-producing wells was published by Bastin (1926).

Most of the information on the diversity of SRB in both natural and engineered ecosystems has been obtained by the use of marker genes such as 16S ribosomal RNA (rRNA) genes. Based on comparative analysis of 16S rRNA gene sequences, the known SRB can be grouped into seven phylogenetic lineages, five within the *Bacteria*, and two within the *Archaea* (Wolicka and Borkowski 2007; Muyzer and Stams 2008; Tang et al. 2009). Most of the sulfate reducers belong to various genera within the Deltaproteobacteria, followed by the Gram-positive SRB within the Clostridia (*Desulfotomaculum*, *Desulfosporosinus*, and *Desulfosporomusa*). *Desulfosporosinus* and *Desulfotomaculum* are able to produce endospores. Nitrospirae (*Thermodesulfovibrio*), Thermodesulfobacteria (*Thermodesulfo-bacterium*), and Thermodesulfobiaceae (*Thermodesulfobium*) lineages contain

only thermophilic sulfate reducers. Within the *Archaea*, SRB belong to the genus *Archaeoglobus* in the Euryarchaeota and to the genera *Thermocladium* and *Caldivirga* in the Crenarchaeota.

Metabolically, SRB are classified into two groups of complete oxidizers (acetate oxidizers), which have the ability to oxidize organic compounds to carbon dioxide, and incomplete oxidizers (non-acetate oxidizers), which carry out incomplete oxidation of organic compounds to acetate and CO_2 (Madigan and Martinko 2006). Although the growth kinetics for incomplete oxidizers is generally faster than the complete oxidizers, they are less versatile in terms of nutritional requirements. The group of complete oxidizers includes species belonging to the genera *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfonema*, *Desulfosarcina*, *Desulfoarculus*, *Desulfoacinum*, *Desulforhabdus*, *Desulfomonile*, *Desulfotomaculum acetoxidans*, *Desulfotomaculum sapomandens*, and *Desulfovibrio baarsii*. The incomplete oxidizers include *Desulfovibrio*, *Desulfomicrobium*, *Desulfobotulus*, *Desulfofustis*, *Desulfotomaculum*, *Desulfomonile*, *Desulfobacula*, *Archaeoglobus*, *Desulfobulbus*, *Desulforhopalus*, and *Thermodesulfobacterium*.

The ability of SRB in utilizing various hydrocarbons from crude oil has severe consequences for the petroleum industry both in the underground oil reservoirs and in surface facilities (Gieg et al. 2011; Singh et al. 2012). SRB activity increases H_2S concentrations (souring) in onshore and offshore oil reservoirs subjected to water flooding and creates associated problems such as contamination of oil and gas and produced water with sulfide, plugging of oil bearing rock formations, and accelerated corrosion in production, processing, and storage facilities (Uchiyama et al. 2010; Voordouw 2011). Indeed, SRB are the main organisms involved in the microbially induced corrosion processes (MICP) that occur commonly in oil and gas production and petrochemical processes equipment and pipelines (Duncan et al. 2009). Control of biogenic sulfide production, which improves the quality of produced oil and gas and decreases production costs, could be achieved through elimination of sulfate from water prior to injection, suppression of SRB with biocides or metabolic inhibitors such as nitrite and molybdate, and addition of nitrate to the injection water (Voordouw 2011).

9.5.2 Methanogenic Microorganisms

Methanogenic microbes can degrade hydrocarbons to methane under anaerobic conditions (Jones et al. 2008; Gray et al. 2010; Milkov 2011). Crude oil hydrocarbon degradation under methanogenic conditions in the laboratory mimics the characteristic sequential removal of compound classes seen in reservoir-degraded petroleum. The initial preferential removal of *n*-alkanes generates close to stoichiometric amounts of methane, principally by hydrogenotrophic methanogenesis. This degradation process is widespread in the geosphere. In comparison with other anaerobic processes, methanogenic hydrocarbon degradation is more sustainable

over geological time scales because replenishment of an exogenous electron acceptor is not required. As a consequence, this process has been responsible for the formation of the world's vast deposits of heavy oil, which far exceed conventional oil reserves such as those found in the Middle East. Studies of the organisms, syntrophic partnerships, mechanisms, and geochemical signatures associated with methanogenic hydrocarbon degradation have identified common themes and diagnostic markers for this process in the subsurface. These studies have also identified the potential to engineer methanogenic processes to enhance the recovery of energy assets as biogenic methane from residual oils stranded in petroleum systems (Gieg et al. 2008).

Methanogenic *Archaea* have been successfully isolated from saline oil well waters at a range of temperatures from different oil reservoirs (Ollivier et al. 1998; Dolfing et al. 2008). Halophilic methylotrophic methanogens, *Methanohalophilus euhalobius* and *Methanosarcina siciliae*, and the acetoclastic methanogen *Methanosarcina mazei*, capable of utilizing acetate, methanol, and methylamine but not $H_2 + CO_2$ for growth, have been isolated under both mesophilic and thermophilic conditions (Magot et al. 2000). Hydrogenotrophic mesophilic or thermophilic methanogens such as *Methanococcus thermolithotrophicus*, *Methanoplanus petrolearius*, *Methanocalculus halotolerans*, *Methanobacterium thermoautrophicum*, *M. bryantii*, *M. ivanovii*, and *M. thermoalcaliphilum* have been commonly isolated from oil reservoirs (Ollivier and Alazard 2010). Methanogenic *Archaea* may potentially act as iron-oxidizing microorganisms in the presence of CO_2 as terminal electron acceptor and therefore may also participate together with SRB in in situ biocorrosive processes (Dinh et al. 2004; Duncan et al. 2009).

Some syntrophs such as *Syntrophus* belong to the class delta-proteobacteria where many SRBs belong (Voordouw 2011). In the absence of sulfate, these syntrophs participate in consortia along with heterotrophic nitrate-reducing bacteria. Oil sand tailing ponds can emit up to 10^7 L of methane per day from conversion of naphtha hydrocarbon diluent used in bitumen extraction (Siddique et al. 2007). Similarly oil storage tanks that contain water can also evolve significant amounts of methane. Thus methanogenic hydrocarbon degradation offers the potential to convert oil that can no longer be economically produced to methane.

9.5.3 Fermentative Bacteria

The presence of fermentative bacteria has long been believed to be the consequence of introducing water and carbohydrates during drilling or oil recovery processes (Magot et al. 2000). However, isolation of thermophilic microbes such as *Geotoga* and *Petrotoga* species of the order Thermotogales from oil reservoirs suggests that these thermophilic microorganisms may be considered indigenous to these ecosystems. Other putative indigenous microorganisms isolated from hot oil reservoirs

include the moderately halophilic *Halanaerobium* sp.; anaerobic species of *Garciella* and *Petrobacter* using nitrate; *Deferribacter* spp. using iron and Mn as terminal electron acceptors with yeast extract, peptone, and casamino acid as energy sources; and finally the homoacetogenic bacterium *Acetogenium romashkowii*.

A wide range of mesophilic and thermophilic fermentative microorganisms have been isolated from oil reservoirs, including *Bacteria* (e.g., *Halanaerobium*, *Thermotoga*) and a few hyperthermophilic *Archaea* such as *Thermococcus* and *Pyrococcus* spp. (Ollivier and Cayol 2005). Besides their ability to ferment sugars to acids, gases, and solvents, several thermophiles have been shown to oxidize hydrogen in the presence of Fe^{3+} (*Thermococcus*, *Pyrococcus*, and *Thermotoga*) or thiosulfate (*Thermotoga*) as terminal electron acceptors, thus demonstrating that hydrogen oxidation in oil reservoirs is not restricted to SRB and methanogenic *Archaea* (Ollivier and Alazard 2010). Interestingly, besides *Thermotoga* spp., a number of mesophilic (*Dethiosulfovibrio*) and thermophilic bacteria (*Thermoanaerobacter*) can also reduce thiosulfate into sulfide and may contribute to biocorrosion and possibly also reservoir souring in oil field ecosystems. Some hyperthermophilic microorganisms such as *Thermococcus*, *Pyrococcus*, and *Thermotoga* have the ability to remove sulfur from crudes and produce sulfide, thus can also contribute to the oil souring during production. *Thermoanaerobacter brockii* is capable of using hydrogen as electron donor with improved consumption of amino acids and peptides in the presence of thiosulfate besides carbohydrates (Magot et al. 2000).

9.5.4 Metagenomics of Subsurface Oil Environments

Driven by increasing interest in microbial-enhanced oil recovery (MEOR), reservoir plugging and souring, biocorrosion, biodegradation of light hydrocarbons resulting in the production of heavy oils, and methane production from heavy oil formations, metagenomic studies are beginning to appear that shed additional light on the natural microbiological processes in the deep subsurface and the impacts that oil recovery techniques have on these processes.

Ren et al. (2011) used DGGE and relatively small 16S gene clone libraries to compare the *Bacterial* and *Archaeal* communities in both injection and production waters at a water-flooded petroleum reservoir in China. Interestingly, the microbial communities in produced waters were distinct from those in injection waters, indicating that injected microorganisms did not survive in the subsurface. In this case their goal was not to introduce organisms into the reservoir in an effort to stimulate activity, but the work does highlight the need to monitor produced waters in order to gain some understanding of what impacts microorganisms are having on the recovered oil. In contrast, a study in north Texas by Struchtemeyer et al. (2011) found that drilling muds prepared in open air environments at well sites could potentially introduce microorganisms into oil formations that can be recovered in

collected drilling waters. No information was specifically collected on microbial activity in these studies, and since complete metagenomes were not sequenced, no hypotheses can be made about hydrocarbon metabolic potential.

This lack of solid data relating to metabolic capacity is common at this stage, but will undoubtedly change as metatranscriptomic methods are applied. In general, microorganisms with the potential for low molecular weight hydrocarbon biodegradation, fermentative metabolism of carbohydrates and amino acids to ethanol, acetate, hydrogen, and carbon dioxide, and methane formation are observed in these studies. Indeed, as discussed above, these observations are supported by Jones et al. (2008) who showed that methanogenic petroleum biodegradation can explain the chemical composition of heavily degraded oils in the subsurface. As is always seen, straight chain alkanes are preferentially metabolized before aromatics, branched aromatics, and alkylated naphthalenes are depleted. Their main conclusion is that "identification of the pathways inherent in subsurface bio-degradation facilitates the engineering of processes to accelerate naturally slow methanogenic biodegradation to recover energy from heavy oilfields as methane, rather than oil." This area of research is quickly evolving as new models are proposed. Historically, it was believed that oxygen permeation into oil reservoirs resulted in oil degradation. However, with the relatively recent discovery of anaerobic hydrocarbon metabolism, thermodynamic studies have shown that anaerobic alkane and aromatic hydrocarbon biodegradation can result in the formation of methane (Dolfing et al. 2008).

The first deep subsurface metagenome encompassing sequencing of total DNA rather than just 16S genes was published in 2011 (Kotlar et al. 2011). Here, DNA was extracted from water recovered from an oil reservoir 2.5 km beneath the Norwegian Sea (85 °C, 250 bars) thought to be free of impact from previous human interventions. In the study, a modest amount of metagenomic data was used to characterize the overall microbial community in the reservoir. Interestingly, the genera of *Bacteria* and *Archaea* found in the reservoir are quite similar to those found in terrestrial environments, although the reason for this is unclear. Having said that, evidence for thermophilic or thermotolerant organisms was found as would be expected. The authors focused mainly on identifying the organisms present, and methanogens and sulfate-reducing bacteria appeared to dominate. They did not describe any attempts to reconstruct metabolic potential within the reservoir through annotation of known metabolic genes, something that would be interesting for future work. Even more interesting will be to carry out metatranscriptomic experiments if sampling procedures can be developed and to focus on putative genes that may currently have no known function.

9.6 Petroleum Microbiology in Deep Aqueous Environments

Another aspect of subsurface petroleum microbiology relates to how microbes deal with petroleum hydrocarbons in deep aqueous environments. Two examples, the Deepwater Horizon marine oil spill and hydrocarbon degradation in the large fine tailings lakes resulting from oil recovery from the Canadian Oil Sands, will be considered here.

The recent BP Deepwater Horizon oil spill in the Gulf of Mexico, the largest marine oil spill in history, casts some light on how crude oil is degraded in deep aqueous marine environments. A strategy which was adopted in addressing this oil spill was to apply oil dispersants as well as physical dispersion strategies as the oil exited the well head, close to the ocean floor to prevent large oil slicks from reaching the water surface, and moving toward the coast.

Physical, chemical, and microbial studies confirmed substantial aerobic hydrocarbon biodegradation associated with the dispersed oil plumes, which resulted in a reduction in dissolved oxygen concentration, relative to oxygen concentration in water outside the oil plume. Nevertheless, the oxygen concentration in the petroleum plume or cloud was never decreased to anoxic levels (Hazen et al. 2010; Atlas and Hazen 2011). The biodegradation activity in the plume was also reflected by the greater microbial counts in the plume (~10^5) compared to ~10^3 in the surrounding waters. At an early point in the spill, propane and ethane degradation was considered the primary drivers of respiration, and later on essentially all released methane was considered to be respired, mediated by a dominant presence of methanotrophs (Valentine et al. 2010; Kessler et al. 2011). Rapid degradation of alkanes and substantial degradation of PAHs were observed in the cloud. While more than 900 subfamilies of bacteria, from 62 phyla, only 16 subfamilies of the gamma-proteobacteria were found to be enriched in the cloud and three families of the class *Oceanospirillales* predominated (Hazen et al. 2010).

Nevertheless, functional gene microarray investigations confirmed that populations of microbes associated with both aerobic and anaerobic biodegradation of hydrocarbon components were present in the oil plume (Lu et al. 2012). For example, the *bbs* gene (beta-oxidation of benzylsuccinic acid), derived from *Azoarcus* and *Thauera* species, involved in anaerobic degradation of toluene, was enriched in the cloud. Anaerobic petroleum-degrading bacteria were also associated with marsh sediments, of Southeast Louisiana, which had been contaminated with crude oil from the BP spill (Boopathy et al. 2012).

The Athabasca Basin in Alberta, Canada, is estimated to contain trillions of barrels of heavy bitumen petroleum. The water used in the oil extraction process results in the generation of very large and deep tailing ponds containing fine suspended materials and residual bitumen suspended in water which densify to what are known as mature fine tailings (MFT). A small portion of naphtha, used as a diluting agent for bitumen processing, and which contains C_3–C_{14} aliphatic and

aromatic hydrocarbons, ends up in the MFTs, together bitumen-derived higher molecular weight aliphatic and ringed hydrocarbon structures.

These MFTs become anaerobic and exhibit bubbles of gas at the pond surface (Salloum et al. 2002). The microbial population in these deep ponds includes denitrifying bacteria, sulfate reducers, and methanogens. Methane biogenesis, both from naphtha and the higher molecular weight hydrocarbon substrates has been observed (Siddique et al. 2007, 2011). Given that methane is an undesirable greenhouse gas, understanding and controlling the microbial processes which generate methane in MFT ponds will be important to minimizing environmental impacts of these processes. In that regard, addition of sulfate inhibits methane generation from MFTs may represent a solution, although the associated generation of H_2S may represent a problem (Salloum et al. 2002).

9.7 Conclusion

As fossil energy resources dwindle, the need to better understand the distribution and occurrence of biodegraded petroleum deposits increases. While our knowledge of biodegraded petroleum reservoirs has advanced considerably in recent years, our knowledge of the processes, microorganisms involved, and quantitative understanding of the factors which control in-reservoir oil biodegradation remain far from complete. Petroleum geochemistry, geomicrobiology, and modeling have all contributed to our knowledge. Some basic questions have not yet been fully answered due to the difficulty in obtaining reliable or representative samples to ensure that indigenous microbial populations are really being examined! It is likely that nutrient supply exerts a control on in reservoir oil biodegradation, but what is the quantitative relationship between nutrient supply and degree of biodegradation? There are some indications that in-reservoir methanogenic crude oil degradation is driven through syntrophic acetate oxidation and dominated by methanogenic CO_2 reduction with acetoclastic methanogenesis assuming a secondary role. However, there is also evidence of more important roles for acetoclastic methanogenesis under some circumstances, so another important question is—what promotes subsurface methanogenic oil degradation through methanogenic CO_2?

References

Aitken CM, Jones DM, Larter SR (2004) Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature 431:291–294

Atlas RM, Hazen TC (2011) Oil biodegradation and bioremediation: a tale of the two worst spills in U.S. history. Environ Sci Technol 45:6709–6715

Bastin ES (1926) The problem of the natural reduction of sulphates. Bull Am Assoc Petrol Geol 10:1270–1299

Bennett B, Fustic M, Farrimond P (2006) 25-norhopanes: formation during biodegradation of petroleum in the subsurface. Org Geochem 37:787–797

Bødtker G, Lysnes K, Torsvik T, Bjørnestad E, Sunde E (2009) Microbial analysis of backflowed injection water from a nitrate-treated North Sea oil reservoir. J Ind Microbiol Biotechnol 36:439–450

Boopathy R, Shields S, Nunna S (2012) Biodegradation of crude oil from the BP oil spill in the marsh sediments of Southeast Louisiana, USA. Appl Biochem Biotechnol 167:1560–1568

Brooks PW, Fowler MG, MacQueen RW (1988) Biological marker conventional organic geochemistry of oil sands/heavy oils, Western Canada Basin. Org Geochem 12:519–538

Callaghan AV, Morris BE, Pereira IA, McInerney MJ, Austin RN, Groves JT, Kukor JJ, Suflita JM, Young LY, Zylstra GJ, Wawrik B (2012) The genome sequence of Desulfatibacillum alkenivorans AK-01: a blueprint for anaerobic alkane oxidation. Environ Microbiol 14:101–113

Carmona M, Zamarro MT, Blazquez B, Durante-Rodriguez G, Juarez JF, Valderrama JA, Barragan MJL, Garcia JL, Diaz E (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. Microbiol Mol Biol Rev 73:71–133

Connan J (1984) Biodegradation of crude oils in reservoirs. In: Brooks J, Welte DH (eds) Advances in petroleum geochemistry. Academic, London, pp 299–330

da Cruz GF, de Vasconcellos SP, Angolini CF, Dellagnezze BM, Garcia IN, de Oliveira VM, Dos Santos Neto EV, Marsaioli AJ (2011) Could petroleum biodegradation be a joint achievement of aerobic and anaerobic microorganisms in deep sea reservoirs? AMB Express 1:47. doi:10.1186/2191-0855-1-47

Dinh HT, Kuever J, Mubmann M, Hassel AW, Stratmann M, Widdel F (2004) Iron corrosion by novel anaerobic microorganisms. Nature 427:829–832

Dolfing J, Larter SR, Head IM (2008) Thermodynamic constraints on methanogenic crude oil biodegradation. ISME J 2:442–452

Duncan KE, Gieg LM, Parisi VA, Tanner RS, Tringe SG, Bristow J, Suflita JM (2009) Biocorrosive thermophilic microbial communities in Alaskan north slope oil facilities. Environ Sci Technol 43:7977–7984

Foght J (2010) Microbial communities in oil shales, biodegraded and heavy oil reservoirs, and bitumen deposits. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Heidelberg, pp 2161–2172

Fuchs G, Boll M, Heider J (2011) Microbial degradation of aromatic compounds – from one strategy to four. Nat Rev Microbiol 9:803–816

Galperin Y, Kaplan IR (2011) Review of microbial processes in the near-surface environment and their implications for the chemical fingerprinting of hydrocarbon fuels. Environ Forensics 12:236–252

Gieg LM, Duncan KE, Suflita JM (2008) Bioenergy production via microbial conversion of residual oil to natural gas. Appl Environ Microbiol 74:3022–3029

Gieg LM, Jack TR, Foght JM (2011) Biological souring and mitigation in oil reservoirs. Appl Microbiol Biotechnol 92:263–282

Gittel A, Sorensen KB, Skovhus TL, Ingvorsen K, Schramm A (2009) Prokaryotic community structure and sulfate reducer activity in water from high-temperature oil reservoirs with and without nitrate treatment. Appl Environ Microbiol 75:7086–7096

Grassia GS, McLean KM, Glenat P, Bauld J, Sheehy AJ (1996) A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. FEMS Microbiol Ecol 21:47–58

Gray ND, Sherry A, Hubert C, Dolfing J, Head IM (2010) Methanogenic degradation of petroleum hydrocarbons in subsurface environments remediation, heavy oil formation, and energy recovery. Adv Appl Microbiol 72:137–161

Harner NK, Richardson TL, Thompson KL, Best RJ, Best AS, Trevors JT (2011) Microbial processes in the Athabasca oil sands and their potential applications in microbial enhanced oil recovery. J Ind Microbiol Biotechnol 38:1761–1775

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Sharon E, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'haeseleer P, Holman HY, Osman S, Lu Z, Van Nostrand JD, Deng Y, Zhou J, Mason OU (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Head IM, Jones DM, Larter SR (2003) Biological activity in the deep subsurface and the origin of heavy oil. Nature 426:344–352

Head IM, Larter SR, Gray ND, Sherry A, Adams JJ, Aitken CM, Jones DM, Rowan AL, Huang H, Roling WFM (2010) Hydrocarbon degradation in petroleum reservoirs. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 3097–3109

Hubert C, Voordouw G, Mayer B (2009) Elucidating microbial processes in nitrate- and sulfate-reducing systems using sulfur and oxygen isotope ratios: the example of oil reservoir souring control. Geochim Cosmochim Acta 73:3864–3879

Hubert CRJ, Oldenburg TBP, Fustic M, Gray ND, Larter SR, Penn K, Rowan AK, Seshardri R, Sherry A, Swainsbury R, Voordouw G, Voordouw JK, Head IM (2012) Massive dominance of Epsilonproteobacteria in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. Environ Microbiol 14:387–404

Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Aitken CM, Bennett B, Huang H, Brown A, Bowler BF, Oldenburg T, Erdmann M, Larter SR (2008) Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 451:176–180

Kessler JD, Valentine DL, Redmond MC, Du MR, Chan EW, Mendes SD, Quiroz EW, Villanueva CJ, Shusta SS, Werra LM, Yvon-Lewis SA, Weber TC (2011) A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico. Science 331:312–315

Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M, Michaelis W, Classen A, Bolm C, Joye SB, Widdel F (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. Nature 449:898–901

Kotlar HK, Lewin A, Johansen J, Throne-Holst M, Haverkamp T, Markussen S, Winnberg A, Ringrose P, Aakvik T, Ryeng E, Jakobsen K, Drabløs F, Valla S (2011) High coverage sequencing of DNA from microorganisms living in an oil reservoir 2.5 kilometres subsurface. Environ Microbiol Rep 3:674–681

Kryachko Y, Dong X, Sensen CW, Voordouw G (2012) Compositions of microbial communities associated with oil and water in a mesothermic oil field. Antonie Van Leeuwenhoek 101:493–506

Larter S, Wilhelms A, Head I, Koopmans M, Aplin A, Di Primio R, Zwach C, Erdmann M, Telnaes N (2003) The controls on the composition of biodegraded oils in the deep subsurface – part 1: biodegradation rates in petroleum reservoirs. Org Geochem 34:601–613

Larter S, Adams J, Bennett B (2006) The controls on the composition of biodegraded oils in the deep subsurface. Part II. Geological controls on subsurface biodegradation fluxes and constraints on reservoir fluid property prediction. Bull Am Assoc Petrol Geol 90:921–938

Lu ZM, Deng Y, Van Nostrand JD, He ZL, Voordeckers J, Zhou AF, Lee YJ, Mason OU, Dubinsky EA, Chavarria KL, Tom LM, Fortney JL, Lamendella R, Jansson JK, D'haeseleer P, Hazen TC, Zhou JZ (2012) Microbial gene functions enriched in the deepwater horizon deep-sea oil plume. ISME J 6:451–460

Madigan MT, Martinko JM (2006) Brock biology of microorganisms, 11th edn. Prentice Hall, Upper Saddle River, NJ

Magot M, Ollivier B, Patel BKC (2000) Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 77:103–116

Meckenstock RU, Mouttaki H (2011) Anaerobic degradation of non-substituted aromatic hydrocarbons. Curr Opin Biotechnol 22:406–414

Milkov AV (2011) Worldwide distribution and significance of secondary microbial methane formed during petroleum biodegradation in conventional reservoirs. Org Geochem 42:184–207

Muyzer G, Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol 6:441–454

Obermajer M, Osadetz KG, Fowler MG (2004) Variable alteration in heavy crude oils of west-central Saskatchewan, Canada. Org Geochem 35:469–491

Ollivier B, Alazard D (2010) The oil reservoir ecosystem. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Heidelberg, pp 2261–2269

Ollivier B, Cayol J-L (2005) The fermentative, iron-reducing, and nitrate-reducing microorganisms. In: Ollivier B, Magot M (eds) Petroleum microbiology. American Society for Microbiology, Washington, DC, pp 71–88

Ollivier B, Fardeau ML, Cayol JL, Magot M, Patel BKC, Prensier G, Garcia JL (1998) Characterization of *Methanocalculus halotolerans* gen sp. nov., isolated from an oil-producing well. Int J Syst Bacteriol 48:821–828

Orphan VJ, Taylor LT, Hafenbradl D, Delong EF (2000) Culture dependent and culture-independent characterization of microbial assemblages associated with high temperature petroleum reservoirs. Appl Environ Microbiol 66:700–711

Palmer SE (1993) Effects of biodegradation and water washing on crude oil composition. In: Engel MH, Macko SA (eds) Organic geochemistry. Plenum, New York, pp 511–533

Ren H-Y, Zhang X-J, Rupert W, Gao G-J, S-x G, Zhao L-P (2011) Comparison of microbial community compositions of injection and production well samples in a long-term water-flooded petroleum reservoir. PLoS One 6:e23258

Riediger CL, Fowler MG, Snowdon LR (1999) Origin and alteration of lower cretaceous Mannville group oils from the provost oilfield, east central Alberta, Canada. Bull Can Petrol Geol 47:43–62

Röling WFM, Head IM, Larter SR (2003) The microbiology of hydrocarbon degradation in subsurface petroleum reservoirs: perspectives and prospects. Res Microbiol 154:321–328

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F (1994) Anaerobic oxidation of hydrocarbons in crude-oil by new types of sulfate-reducing bacteria. Nature 372:455–458

Salloum MJ, Dudas MJ, Fedorak PM (2002) Microbial reduction of amended sulfate in anaerobic mature fine tailings from oil sand. Waste Manag Res 20:162–171

Schink B (2006) Microbially driven redox reactions in anoxic environments: pathways, energetics, and biochemical consequences. Eng Life Sci 6:228–233

Shuqing Z, Haiping H, Luming L (2008) Biodegradation and origin of oil sands in the Western Canada Sedimentary Basin. Petrol Sci 5:87–94

Siddique T, Fedorak PM, MacKinnon MD, Foght JM (2007) Metabolism of BTEX and naphtha compounds to methane in oil sands tailings. Environ Sci Technol 41:2350–2356

Siddique T, Penner T, Semple K, Foght JM (2011) Anaerobic biodegradation of longer-chain n-alkanes coupled to methane production in oil sands tailings. Environ Sci Technol 45:5892–5899

Singh A, Singh B, Ward OP (2012) Potential applications of bioprocess technology in petroleum industry. Biodegradation 23:865–880

Skaare BB, Kihle J, Torsvik T (2011) Biodegradation of crude oil as potential source of organic acids in produced water. In: Lee K, Neff J (eds) Produced water: environmental risks and advances in mitigation technologies. Springer, New York, pp 115–126

Stevenson BS, Drilling HS, Lawson PA, Duncan KE, Parisi VA, Suflita JM (2011) Microbial communities in bulk fluids and biofilms of an oil facility have similar composition but different structure. Environ Microbiol 13:1078–1090

Struchtemeyer CG, Davis JP, Elshahed MS (2011) Influence of drilling mud formulation process on the bacterial communities in thermogenic natural gas wells of the Barnett Shale. Appl Environ Microbiol 77:4744–4753

Tang K, Baskaran V, Nemati M (2009) Bacteria of the sulphur cycle: an overview of microbiology, biokinetics and their role in petroleum and mining industries. Biochem Eng J 44:73–94

Uchiyama T, Ito K, Mori K, Tsurumaru H, Harayama S (2010) Iron corroding methanogen isolated from a crude-oil storage tank. Appl Environ Microbiol 76:1783–1788

Valentine DL, Kessler JD, Redmond MC, Mendes SD, Heintz MB, Farwell C, Hu L, Kinnaman FS, Yvon-Lewis S, Du M, Chan EW, Tigreros FG, Villanueva CJ (2010) Propane respiration jump-starts microbial response to a deep oil spill. Science 330:208–211

van der Kraan GM, Bruining J, Lomans BP, van Loosdrecht MC, Muyzer G (2010) Microbial diversity of an oil–water processing site and its associated oil field: the possible role of microorganisms as information carriers from oil-associated environments. FEMS Microbiol Ecol 71:428–443

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549

Vogt C, Kleinsteuber S, Richnow H-H (2011) Anaerobic benzene degradation by bacteria. Microb Biotechnol 4:710–724

Voordouw G (2011) Production-related petroleum microbiology: progress and prospects. Curr Opin Biotechnol 22:401–405

Wentzel A, Ellingsen TE, Kotlar H-K (2007) Bacterial metabolism of long-chain *n*-alkanes. Appl Microbiol Biotechnol 76:1209–1221

Widdel F (2010) Cultivation of anaerobic microorganisms with hydrocarbons as growth substrates. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 3787–3798

Widdel F, Grundman O (2010) Biochemistry of the anaerobic degradation of non-methane alkanes. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 909–924

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259–276

Wolicka D, Borkowski A (2007) The geomicrobiological role of sulphate-reducing bacteria in environments contaminated by petroleum products. Geomicrobiol J 24:599–607

Chapter 10
Nutrient Cycling: Potassium Solubilization by Microorganisms and Improvement of Crop Growth

Satyavir S. Sindhu, Priyanka Parmar, and Manisha Phour

10.1 Introduction

Potassium is a major and essential plant macronutrient and the most abundantly absorbed cation in higher plants. Potassium (K) plays an important role in the growth, metabolism, and development of plants. It activates plant enzymes, maintains cell turgor, enhances photosynthesis, reduces respiration, helps in transport of sugars and starches, helps in nitrogen uptake, and is also essential for protein synthesis. In addition to plant metabolism, potassium improves crop quality because it helps in grain filling and kernel weight, strengthens straw, increases disease resistance against pest and diseases, and also helps the plant to withstand stress. Without adequate potassium, the plants will have poorly developed roots, grow slowly, produce small seeds, and have lower yields. With the introduction of high-yielding crop varieties and hybrids during green revolution and with the progressive intensification of agriculture, the soils are getting depleted in potassium reserve at a faster rate and available soil K levels have also dropped due to leaching, runoff, and erosion (Sheng and Huang 2002a). As a consequence, potassium deficiency is becoming one of the major constraints in crop production, especially in coarse-textured soils and even in fine-textured soils. Therefore, crops do respond to K fertilization in soils.

Potassium deficiency is not as wide spread as that of nitrogen and phosphorus. However, many soils which were initially rich in K have become deficit due to heavy utilization by crops. Potassium deficiency symptoms usually occur first on the lower leaves of the plant and progress toward the top as the severity of the deficiency increases. One of the most common signs of potassium deficiency is the yellow searching or firing (chlorosis) along the leaf margin. In severe cases of potassium deficiency, the fired margin of the leaf may fall out. Potassium deficient

S.S. Sindhu (✉) • P. Parmar • M. Phour
Department of Microbiology, CCS Haryana Agricultural University, Hisar 125004, Haryana, India
e-mail: sindhuss@hau.ernet.in

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_10, © Springer-Verlag Berlin Heidelberg 2014

crops grow slowly, have poorly developed root systems, stalks are weak, and results in lodging of cereal crops. Long before the symptoms of K deficiency become visible, severe losses in terms of yield and quality could be caused to the crop (Khanwilkar and Ramteke 1993).

In recent years, there is a growing awareness regarding the importance of potassium in crop production in several parts of India. Potassium level has declined in different kind of soils over the years due to intensive cultivation and imbalanced fertilizer application. Therefore, application of potassium fertilizer to these soils gives positive response. India ranks 4th in consumption of potassium fertilizers after the USA, China, and Brazil as far as the total consumption of K fertilizers in the world is concerned (FAI 2007). Because, there is no reserve of K-bearing minerals in India for production of commercial K fertilizers, therefore, whole consumption of K fertilizers are imported in the form of muriate of potash (KCl) and sulfate of potash (K_2SO_4). On an average, 1.7 million tonnes of K is being imported annually in India (Anonymous 2003).

Other major essential macronutrients required for plant growth and development such as nitrogen (N) and phosphorus (P) are provided through application of nitrogenous and soluble phosphatic fertilizers. These chemical fertilizers are applied at high recommended doses, which cause environmental and economic problems (Brady 1990; Xie 1998). This necessitates the search to find an alternative indigenous source of P and K for plant uptake and to maintain K status in soils for sustaining crop production. Thus, direct application of rock phosphate and rock potassium materials may be agronomically more useful and environmentally safer than soluble P and K applied as chemical fertilizers (Rajan et al. 1996). However, P and K nutrients from rock materials are not readily available to the plant because these minerals are released slowly in the soil (Zapata and Roy 2004). Therefore, isolation and identification of microbial strains that are capable of solubilizing potassium minerals quickly in large quantity can conserve our existing resources and may avoid environmental pollution hazards caused by heavy application of chemical fertilizers.

10.2 Potassium Availability in the Soil

Among the major plant nutrients, potassium is most abundant in soils. It is also the seventh most common element in the earth crust and on an average, the surface layer (lithosphere) contains 2.5 % potassium. However, actual soil concentrations of this nutrient vary widely ranging from 0.04 to 3.0 % (Sparks and Huang 1985). Plants can take up potassium only from the soil solution and its availability is dependent upon the K dynamics as well as on total K content. There are three forms of potassium found in the soil, viz., soil minerals, nonexchangeable, and available. Soil minerals make up more than 90–98 % of soil potassium (Sparks and Huang 1985; Sparks 1987). It is tightly bound and most of it is unavailable for plant uptake. The second is nonexchangeable potassium which acts as a reserve to replenish

potassium taken up or lost from the soil solution. It makes up approximately 1–10 % of soil potassium and consists predominantly of interlayer K of non expanded clay minerals such as illite and lattice K in K minerals such as K-feldspars. Nonexchangeable K can also contribute significantly to the plant uptake (Memon et al. 1988; Sharpley 1989). Release of nonexchangeable K to the exchangeable form occurs when level of exchangeable and solution K is decreased by crop removal and/or by leaching and perhaps by large increase in microbial activity (Sparks 1987).

The third type of potassium found in the soil is the available potassium which constitutes 1–2 %. It is found either in the solution or as part of the exchangeable cation held by negative charge of clay minerals and organic matter in soils. The rate and direction of reactions between solution and exchangeable forms of K determine whether applied K will be leached into lower horizons, taken up by plants, converted into unavailable forms or released into available forms (Sparks 2000). Among three different forms of potassium in soils, the concentrations of soluble K in soils are usually very low, but the highest proportion of potassium in soils is in insoluble rocks and minerals (Goldstein 1994). A significant share of soil potassium occurs in unavailable form in soil minerals such as orthoclase and microcline (K-feldspars).

The potassium content of Indian soils varies from less than 0.5 to 3.0 %. Ghosh and Hasan (1980) have documented the state-wise available potassium status in India and categorized that 21 % districts are in low, 51 % are medium, and 28 % are having high available potassium. The average total potassium content of these soils is 1.52 % (Mengel and Kirkby 1987). However, total K is poorly correlated with available K and is rarely used to describe K fertility status of a soil. The immediate source of K for plants is the small amount which is in the soil solution and it ranges from 1 to 2 %. As K is removed, the equilibrium is disturbed; K in the nonexchangeable and soil mineral fraction will be drawn upon. The supply of K to the plants depends directly on the concentration of K in soil solution and indirectly on soil, which maintains this equilibrium (Sparks and Huang 1985). In mineral soils, K occurs in the form of silicate minerals, viz., muscovite, orthoclase, biotite, feldspar, illite, mica, vermiculite, smectite, etc. Total pool of soil K is extremely complex and this can be solubilized by microbes for uptake by plants and microorganisms through production of acids or exopolysaccharides (Ullaman et al. 1996; Rogers et al. 1998).

10.3 Solubilization/Mobilization of Potassium from Rocks or Mica

Significant areas of cultivated soils in India, Korea, and China are deficient in available potassium and have low crop productivity (Xie 1998). The use of plant growth promoting rhizobacteria including potassium-solubilizing bacteria as a

biofertilizer could work as a sustainable solution to improve plant nutrient uptake and production (Vessey 2003). Furthermore, direct application of potassium containing rock materials may be agronomically more useful and environmentally more feasible than soluble K (Rajan et al. 1996). Rock K materials are cheaper sources and most of them are readily available to a plant because the minerals are released slowly and their use as fertilizer causes significant yield increases of the various crops. The potassium present in the rock materials could be made available by the following mechanisms.

10.3.1 Bioweathering of Rocks

Bioweathering is a common geochemical process involved in erosion, decay, and decomposition of rocks and minerals mediated by living organisms (Barker et al. 1997; Burford et al. 2003). The bioweathering process plays a fundamental role in the release of nutrients from rocks and is associated with global environmental changes (Li et al. 2006). Microorganisms are the main bioweathering agents leading to rock degradation and soil formation. Microbes also provide nutrients, such as P, K, and silicon to support plant growth by changing environmental pH and oxidation reduction potential to solubilize rock minerals (Buss et al. 2007; Lian et al. 2008). Prokaryotic and eukaryotic algae also play an important role in colonizing barren or eroded land surfaces and contribute to soil development in different ecosystems. The algae cause corroding and weathering of rocks as they can grow on barren rocks on which other plants fail to grow. The weathering of rocks may be the result of carbonic acid formation from the respiratory CO_2 release of the algae and its subsequent reaction with water (Barker et al. 1998) or it may be associated with the metabolic products, i.e., production of organic acids. The organic acids produced by various microorganisms have been found to facilitate the weathering of minerals by directly dissolving K from rocks or through the formation of metalorganic complexes with silicon ions to bring the K into solution (Friedrich et al. 1991; Bennett et al. 1998).

Argelis et al. (1993) found that weathering of unaltered sand stone, granite, and lime stone was carried out by *Penicillium frequentans* and *Cladosporium cladosporoides*. They reported that both fungal species have the capacity to produce large amounts of oxalic, citric, and gluconic acids in broth culture that caused extensive deterioration of clay silicates, mica and feldspar from both sand stone and granite and also of calcite and dolomite from lime stone. Thus, filamentous fungi were also found to cause an extensive weathering of stone due to organic acid excretion.

10.3.2 Composting of Mica

Low-grade waste mica (8–10 % K_2O) is an alternative source of potassium, which is generated during the processing of mica sheets. Large amounts of mica waste are generated and dumped near the mica mines. This mica waste cannot be utilized by plants because most of the K is present as nonexchangeable form. However, this mica can be effectively used as a source of K fertilizer by chemical and/or biological modifications. One of the possible alternative viable technologies is the management of waste mica through composting technology (Nishanth and Biswas 2008). Rice straw, rock phosphate, and waste mica are inoculated with *Aspergillus awamori* and the acidic environment prevailing during composting has been reported to convert unavailable K into plant available form.

10.3.3 Solubilization of K-Bearing Minerals Using Microorganisms

Potassium-solubilizing microorganisms present in the soil could provide another alternative technology and these microbes play a key role in the natural K cycle. There are considerable population of K-solubilizing bacteria (KSB) in soil and rhizosphere (Sperberg 1958). A wide range of bacteria, such as members of the genus *Pseudomonas*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *Bacillus edaphicus*, and *B. circulans* were found to release potassium from potassium-bearing minerals but only a few bacteria such as *Bacillus mucilaginosus* and *Bacillus edaphicus* have high activity in mobilizing potassium in accessible form in soils (Lian et al. 2002; Sheng 2005; Li et al. 2006). Potassium- and phosphate-solubilizing bacteria (KSB and PSB) are extensively used as biofertilizers in Korea and China as significant areas of cultivated soils in these countries are deficient in soil-available K and P (Xie 1998). Their use in agriculture can reduce the use of agrochemicals and support ecofriendly crop production (Glick 1995; Requena et al. 1997; Sindhu et al. 2010). Therefore, the use of K-solubilizing bacteria as biofertilizer for agriculture improvement and environmental protection has been a focus of recent research (Sheng et al. 2003).

The first evidence of microbial involvement in solubilization of rock potassium was reported by Muntz (1890). Several microorganisms like *Aspergillus niger*, *Bacillus extroquens*, and *Clostridium pasteurianum* were found to grow on muscovite, biotite, orthoclase, microclase, and micas under *in vitro* conditions (Reitmeir 1951). Subsequently, a variety of soil microorganisms have been reported to solubilize silicate minerals (Bunt and Rovira 1955). The microorganisms like bacteria, fungi, and actinomycetes were found to colonize even on the surface of mountain rocks (Gromov 1957). Norkina and Pumpyanskaya (1956) reported that the silicate-solubilizing bacteria (SSB) *B. mucilaginous* subsp. *siliceous*, liberated potassium from feldspar and alumino-silicates. Duff and Webley (1959) reported

silicate-dissolving action of Gram-negative bacteria *Erwinia herbicola* or with *Pseudomonas* strains. Heinen (1960) reported the ability of *Bacillus caldolyticus* and *Proteus* sp. to grow and solubilize quartz. Webley et al. (1960) found that a *Pseudomonas* strain isolated from soil showed clearing zone in silicate medium. Webley et al. (1963) demonstrated that the silicaceous materials in rocks can be attacked through the metabolic products of microorganisms.

Alkasandrov et al. (1967) isolated different bacterial species which were found to dissolve potassium, silica, and aluminum from insoluble minerals. Purushothaman et al. (1974) reported the distribution of SSB in marine environments and suggested that these bacteria play a role in cycling of silicon in sea water. Avakyan et al. (1981) reported that *B. mucilaginosus* solubilized insoluble silicates. Belkanova et al. (1985) reported the cleavage of siloxane bond in quartz by *B. mucilaginosus*. Among the K-bearing silicate minerals, mica was found to weather readily (Tandon and Sekhon 1988). Li (1994) isolated K-solubilizing bacteria from soil, rock, and mineral samples. Bacterial isolate MCRCp1 was later identified as *B. mucilaginosus* based on morphological and physiological characters. Muralikannan (1996) isolated SSB from rice rhizosphere and tentatively identified as *Bacillus* sp. Kannan and Raj (1998) carried out enumeration of silicate- and phosphate-solubilizing bacteria from soil's tank sediments. Three out of 17 isolates were identified as *Bacillus* sp. based on biochemical characteristics. Lian (1998) also isolated silicate bacteria *B. mucilaginosus* from corn field.

Liu (2001) reported isolation of silicate bacteria *B. mucilaginosus* CS1 and CS2 from soil and these bacteria exhibited inhibitory activity on the growth of Gram-negative bacteria *E. coli*. They identified strain CS1 as *B. mucilaginosus*. It was reported that the ability of slime production by the *B. mucilaginosus* strain dissolved the silicates and also contributed in the colonization of rhizosphere as well as non-rhizosphere soil (Lin et al. 2002). Hutchen et al. (2003) isolated 27 strains of heterotrophic bacteria from feldspar-rich soil and studied dissolution of silicate mineral in liquid and solid minimal media. SSB were isolated from rice ecosystem in a medium containing 0.25 % insoluble magnesium trisilicate and reported that *Bacillus* sp. solubilized silicate minerals more efficiently under in vitro conditions (Raj 2004). Potassium-solubilizing rhizobacteria were isolated from the roots of cereal crops by the use of specific potassium-bearing minerals (Mikhailouskaya and Tcherhysh 2005).

Murali et al. (2005) isolated silicate solubilizers using modified Bunt and Rovira medium from soil samples collected from coconut palms. Majority of the silicate solubilizers were identified as *Bacillus* sp. and *Pseudomonas* sp. Hu et al. (2006) isolated two phosphate- and potassium-solubilizing strains KNP413 and KNP414 from the soil of Tianmu Mountain, Zhejiang Province (China). Both the isolates effectively dissolved mineral phosphate and potassium, while strain KNP414 showed higher dissolution capacity even than *Bacillus mucilaginosus* AS1.153, the inoculant of potassium fertilizer widely used in China. When grown on Aleksandrov medium, both strains were rod-shaped spore formers with a large capsule and they formed slimy translucent colonies. Based on G + C contents of DNA and 16S rRNA gene sequence similarity, strains KNP413 and KNP414 were

classified to the genus of *Paenibacillus*, i.e., *P. mucilaginosus*. Zhou et al. (2006) characterized *Bacillus mucilaginosus* which solubilized silicon from illite at 30 °C. The bacterium was identified as Gram-positive, rod-shaped endospore former with thick capsule. Sugumaran and Janarthanam (2007) isolated K-solubilizing bacteria from soil, rocks, and minerals samples, viz., microcline, orthoclase, muscovite mica. Among the isolates, *B. mucilaginosus* MCRCp1 solubilized more potassium by producing slime in muscovite mica. Phosphorus and potassium nutritional status in the soil were markedly improved through inoculation of this bacterium. Zhao et al. (2008) isolated mineral potassium-solubilizing bacterial strains with multiple activities relevant for beneficial plant–microbe interactions, i.e., potassium solubilization, production of indole acetic acid (IAA), and siderophore production.

Parmar (2010) isolated 70 bacterial isolates from the rhizosphere of wheat by using modified Aleksandrov medium plates (consisting of glucose, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCO_3$, 0.1 g; $FeCl_3$, 0.006 g; Ca_3PO_4, 2.0 g; mica powder, 2.0 g; and agar, 20.0 g). These bacterial isolates along with 67 standard reference strains were tested for potassium solubilization ability on Aleksandrov medium using mica as potassium source. Different rhizobacterial isolates were spotted on medium plates (Sindhu et al. 1999) and plates were incubated for 3 days at 28 ± 2 °C. Detection of potassium solubilization by rhizobacterial isolates was based upon the ability of solubilization zone formation. Twenty rhizobacterial isolates were found to solubilize potassium from mica powder.

Potassium-solubilizing fungi were isolated from ceramic industry soils and four fungal isolates gave the high ratio of clear zone on Aleksandrov agar supplemented with 0.5 % potassium aluminum silicate (Prajapati et al. 2012). Two fungal strains, i.e., KF1 and KF2 showed the highest available potassium in liquid medium containing potassium aluminum silicate. These isolates were characterized as *Aspergillus niger* and *Aspergillus terreus*. *A. terreus* showed more solubilization when grown in the presence of 1 % rock potassium (feldspar) than *A. niger*. Liu et al. (2012) isolated mineral-solubilizing *Paenibacillus* strain KT from a soil in Henan Province, China. After inoculation of this strain for 7 days, the concentrations of water-soluble Al, Ca and Fe released from the potassium-bearing rock in active bacterial culture were higher than from the control with autoclaved inoculum, but the concentration of water-soluble K in active bacterial culture was similar to that in the control. A potassium-solubilizing bacterium isolated from soil was characterized as *B. circulans* strain Z_{1-3}, which dissolved potassium from feldspar (Xiaoxi et al. 2012). Potassium-bearing rock material served as the sole source of potassium to support the growth of this strain.

10.4 Mechanisms Involved in Potassium Solubilization

Solubilization of illite and feldspar by microorganisms was reported due to the production of organic acids like citric, oxalic acid, and tartaric acids and also due to the production of capsular polysaccharides which helped in dissolution of minerals to release potassium (Sheng and He 2006; Liu et al. 2006).

10.4.1 Acid Production

Production of carboxylic acids like citric, tartaric, and oxalic acids has been reported as predominant mechanism contributing to K solubilization (Hazen et al. 1991; Styriakova et al. 2003; Sheng and He 2006). Silicate bacteria were found to dissolve potassium, silicon, and aluminum from insoluble K-bearing minerals such as micas, illite, and orthoclases by excreting organic acids which either directly dissolved rock K or chelated silicon ions to bring K into the solution (Aleksandrov et al. 1967; Friedrich et al. 1991; Ullman et al. 1996; Bennet et al. 1998). Organic acids can directly enhance dissolution by either a proton- or ligand-mediated mechanism. They can also indirectly enhance dissolution by the formation of complexes in solution with reaction products and as a consequence increase the chemical affinity for the overall dissolution (Ullman and Welch 2002). The potassium is made available to plants when the minerals are slowly weathered or solubilized (Bertsch and Thomas 1985).

Silicate bacteria were found to dissolve potassium, silica, and aluminum from insoluble minerals by liberation of phosphoric acids that solubilized apatite and released available form of nutrients from apatite (Heinen 1960). Moira et al. (1963) isolated several fungal isolates having the potential to release metal ions and silicate ions from minerals, rocks, and soils. The minerals used in this study were saponite and vermiculite. These fungal isolates were found to produce citric acid and oxalic acid that are mainly known to decompose or solubilize natural silicates and help in removal of metal ions from the rocks and soils. Vainberg et al. (1980) proposed that dissolution of minerals was caused by the formation of organic acids in the culture media. Berthelin (1983) demonstrated that potassium is solubilized from precipitated forms through production of inorganic and organic acids by *Thiobacillus*, *Clostridium*, and *Bacillus*. Production of carboxylic acids like citric, tartaric, and oxalic acids was associated with feldspar solubilization by *B. mucilaginosus* and *B. edaphicus* (Malinovskaya et al. 1990; Sheng and Huang 2002b). Organic compounds produced by microorganisms such as acetate, citrate, and oxalate were found to increase mineral dissolution rates in laboratory experiments and in the soil (Palmer et al. 1991). The production of gluconate promoted dissolution of silicates like albite, quartz, and kaolinite by subsurface bacteria (Duff et al. 1963; Vandevivere et al. 1994). Thus, production of organic acids such as acetate, citrate, and oxalate by microorganisms was found to increase mineral dissolution rate

(Hazen et al. 1991; Barker et al. 1998). Welch and Ullman (1993) found that the rate of plagioclase dissolution in solutions containing organic acids was more compared to inorganic acids and further showed that polysaccharides produced by the bacterium during the process of reproduction can combine with the minerals to form bacterial mineral complexes which leads to degradation of the minerals.

10.4.2 Production of Exopolysaccharides

Groudev (1987) reported that production of slime or acidic exopolysaccharides (EPS) contributed to the mechanism of releasing potassium from silicates. Liu et al. (2006) demonstrated that polysaccharides strongly adsorbed the organic acids and attached to the surface of the mineral, resulting in an area of high concentration of organic acids near the mineral. It was suggested that the extracellular polysaccharides adsorbed SiO_2 and this affected the equilibrium between the mineral and fluid phases and led to the reaction toward SiO_2 and K^+ solubilization. Welch and Vandevivere (2009) tested several naturally occurring polymers for their effect on mineral dissolution. Solutions of fresh microbial EPS extracted from subsurface microbes increased the dissolution rate of feldspars, probably by forming complexes with framework ions in solution. However, EPS was found to inhibit dissolution in experiments with both high- and low-molecular weight microbial metabolites by irreversibly binding to mineral surfaces.

10.4.3 Cumulative Effect of Different Mechanisms Leading to Potassium Solubilization

Jones and Handrecht (1967) reported that bacteria solubilized the insoluble silicates by production of CO_2, organic acids and exopolysaccharides. The solubilization of silicates was investigated using Kaolin quartz and sand as model substances. The chemical leaching of silicates was carried out using inorganic and organic acids as well as sodium hydroxide. The process was more effective in the alkaline than in the acid pH range. The transformation of crystalline biotite, mica, vermiculite, and certain rocks to amorphous state was found due to the action of some organic products of microbial metabolism (Weed et al. 1969). The slime-forming and K-solubilizing bacterium *Bacillus mucilaginosus* produced endoglucanase, cellobiase, protease, ribonuclease, deoxyribonuclease, and phosphomonoesterase (Tauson and Vinogrado 1988).

Bacteria have also been shown to accelerate the dissolution of silicates by the production of excess proton and organic ligands, and in some cases by the production of hydroxyl anion, extracellular polysaccharides, and enzymes (Berthelin and Belgy 1979; Hieberk and Bennett 1992; Vandevivere et al. 1994; Barker

et al. 1998). Hinsinger et al. (1992, 1993) reported that dissolution of trioctahedral mica structure-like phlogopite occurred in the rhizosphere of ryegrass (*Lolium multiflorum*) and rape (*Brassica napus*) probably due to proton excretion by roots. Other workers also found that production of protons, organic acids, siderophores, and organic ligands were involved in the weathering ability of the bacteria (Paul and Clark 1989; Grayston et al. 1996; Welch et al. 1999; Liermann et al. 2000). Sheng and He (2006) reported that solubilization of illite and feldspar by wild-type strain of *Bacillus edaphicus* and its four mutants was due to the production of organic acids like oxalic acid and tartaric acids and also due to production of capsular polysaccharides (CPS), which helped in dissolution of minerals to release potassium. In liquid cultures, five bacterial strains showed better growth on Suzhou illite than on Nanjing feldspar. Oxalic acid seemed to be more active agent for the solubilization of Nanjing feldspar. Oxalic and tartaric acids were likely involved in the solubilization of Suzhou illite. Similarly, decomposition of silicate minerals by *B. mucilaginosus* was found due to production of oxalate and citrate as well as to the polysaccharides which absorbed organic acids leading to decomposition of minerals (Liu et al. 2006).

Styriakova et al. (2003) reported that the activity of silicate-dissolving bacteria played a pronounced role in the release of Si, Fe, and K from feldspar and Fe-oxihydroxides. Increasing evidence also exists for a mechanism of direct silicate precipitation by bacteria via metal sorption at the cell membrane (Beveridge and Murray 1980; Beveridge and Fyfe 1985; Urruti and Beveridge 1994; Konhauser and Ferris 1996). In a study to assess the weathering of finely ground phlogopitite, trioctahedral mica with heterotrophic bacteria *B. cereus* and acidophilic *Acidithiobacillus ferrooxidans*, it was found that cultures enhanced the chemical dissolution of the mineral. The X-ray diffraction analysis of the phologopite samples before and after 24 weeks of contact with *B. cereus* cultures revealed a decrease in the characteristic peak intensities of phologopite, indicating destruction of individual structural planes of the mica. On the other hand, *Acidothiobacillus ferroxidans* cultures enhanced the chemical dissolution of the mineral and formed partially weathered interlayer from where K was expelled. This was coupled with the precipitation of k and jarosite (Styriakova et al. 2004).

Parmar (2010) studied the mechanism of K solubilization in 20 rhizobacterial isolates for potassium solubilization ability on Aleksandrov medium supplemented with mica as potassium source. Rhizobacterial isolate HWP47 caused solubilization of potassium in mica by acid production only and isolates HWP28 and HWP69 caused K solubilization by production of CPS and EPS. Another rhizobacterial isolate HWP38 solubilized potassium by production of acid and CPS, whereas six isolates caused solubilization by production of acid, CPS and EPS.

10.5 Environmental Factors Affecting Potassium Solubilization

Many indigenous soil microorganisms have the potential to absorb and mobilize the fixed form of nutrients from trace mineral sources. Different environmental factors such as pH, temperature, nutrients, oxygen, agitation, and nature of the rock material affected the rate of potassium solubilization. For example, the efficiency of potassium solubilization by different bacteria was found to vary with the nature of potassium-bearing minerals. Yakhontova et al. (1987) found that the intensity of degradation of silicate minerals by the bacterium was dependent on the structure and chemical composition of the mineral used. Potassium-dissolving ability of strain HM8841 was measured using Kietyote and Pegatolite in which 47 and 44.4 mg of soluble potassium was released after 38 h of incubation time. Sugumaran and Janarthanam (2007) studied the effect of K-solubilizing bacteria *B. mucilaginosus*, isolated from soil, rock, and mineral samples, on solubilization form microcline, orthoclase, and muscovite mica minerals. One of the slime-forming *B. mucilaginosus* strain MCRCp1 caused maximum potassium solubilization (4.29 mg/L) in media supplemented with muscovite mica, whereas the potassium released in microcline and orthoclase was only 1.26 mg/L and 0.85 mg/L, respectively. The K-solubilizing activity of the five slime-producing bacterial isolates varied from 1.90 to 2.26 mg/L from acid leached soil. MCRCp1 was found to have maximum activity (2.26 mg/L) to dissolve the silicate than other isolates. Zhou et al. (2007) reported that *Paenibacillus polymyxa* promoted dissolution of microperthite by direct and indirect mechanisms and enhanced the release of K, Al, and Si from the mineral. *P. polymyxa* and its metabolites were also found to promote dissolution of basalt (Zhou et al. 2008). Under the bacterial growth conditions, olivine was the most bioweathered mineral followed by augite but feldspar was found the most stable (Zhou et al. 2008).

Potassium release from minerals was affected by pH, dissolved oxygen, and bacterial strain used (Sheng and Huang 2002b). The content of potassium in solution was increased by 84.8–127.9 % by inoculation of bacteria as compared with the control. Welch et al. (1999) found that a variety of extracellular polysaccharides significantly enhanced the dissolution of plagioclase at pH 4 but had little effect at pH 7. Sheng et al. (2002) observed 35.2 mg/L potassium release from strains of potassium-solubilizing bacteria in 7 days at 28 °C at pH range from 6.5 to 8.0. Potassium-releasing characteristics of a bacterium from different minerals were studied by using soil column experiment (Badr 2006). Potassium and phosphorus solubilization capacity of SSB ranged from 490 to 758 mg/L at pH 6.5–8.0. More potassium was solubilized under aerobic condition than less aerobic conditions. The order of release of potassium was illite > feldspar > muscovite. The extent of potassium solubilization by *B. edaphicus* in the liquid media was more and better growth was observed on illite than feldspar (Sheng and He 2006).

Lian et al. (2007) studied a strain of thermophilic fungus *Aspergillus fumigatus* cultured with K-bearing minerals to determine if microbe–mineral interactions

enhance the release of mineral K. It was observed that the K solubilization rate showed a positive dependence upon pH when fungi and minerals were mixed directly and exhibited no correlations with solution acidity if cell–rock contact was restrained. Bacterial inoculation on mica material improved the water-soluble, exchangeable, and nonexchangeable K pools in soils. It influenced the K dynamics of soils into those pools which are relatively more available to plant (Basak and Biswas 2008).

Lopes-Assad et al. (2010) reported that *Aspergillus niger* strains CCT4355 and CCT911 solubilized 62–70 % potassium from the rock powder after 35 days in 125 mL Erlenmeyer flasks; however, the percent solubilization decreased at higher volumetric scales. The injection of filter-sterilized air into the medium enhanced the potassium solubilization. The authors suggested that production of organic acids such as oxalic, citric, or gluconic, depending on the pH of the medium, by the fungus may be a mechanism of rock solubilization. Parmar and Sindhu (2013) found that the amount of K released by the rhizobacterial strains ranged from 15 to 48 mg/L. Maximum K solubilization occurred with glucose as carbon source at 25 °C incubation temperature and 7.0 pH of the medium.

10.6 Effect of Inoculation of Potassium-Solubilizing Bacteria on Growth and Yield of Different Crops

Plant growth-promoting bacteria associated with plant roots may exert their beneficial effects on nutrient uptake and plant growth through a number of mechanisms such as N_2 fixation, production of phytohormones, siderophores, and transformation of nutrient elements such as phosphorus, potassium, and iron, when they are either applied to seeds or incorporated into the soil (Kloepper et al. 1989; Glick et al. 1999; Herridge et al. 2008; Sindhu et al. 2010). Moreover, rhizosphere bacteria have also been found to suppress various plant diseases (Weller 2007; Haas and Defago 2005; Sindhu et al. 2011).

10.6.1 Inoculation Effect of Potassium Solubilizers on Plant Growth and Yield

Beneficial effects of inoculation of K-solubilizing bacteria has been reported in sorghum (Zhang et al. 2004), cotton and rape (Sheng 2005), yam and tapioca (Clarson 2004), rice (Raj 2004), maize (Wu et al. 2005; Singh et al. 2010), egg plant (Ramarethinam and Chandra 2005), groundnut (Sugumaran and Janarthanam 2007), wheat (Sheng and He 2006; Singh et al. 2010; Parmar 2010), pepper (Supanjani et al. 2006), pepper and cucumber (Han et al. 2006), and forage crop sudan grass (Basak and Biswas 2008, 2010).

Increase in yield of maize and wheat by application of organo-minerals and inoculated with silicate bacteria was first reported by Aleksandrov (1958). Vintikova (1964) observed the beneficial effects of silicate bacteria on the yield of lucerne and maize. Khudsen et al. (1982) isolated potassium-solubilizing bacteria from rock and mineral samples which showed higher activity in potassium release from acid-leached soil and improved green gram's seedling growth. Zahro et al. (1986) studied the effect of inoculation of the silicate bacteria *Bacillus circulans* on the release of K and Si from different minerals and in different soils. Bacteria persisted for a long time and high population densities were detected after 14 months particularly in soils containing higher levels of organic matter. An increased yield in rice crop was observed due to inoculation of SSB (Muralikannan 1996; Kalaiselvi 1999).

The inoculation effect of potash mobilizer on egg plant recorded an increased potash uptake and plant biomass as compared to the control plants (Nayak 2001). Lin et al. (2002) observed 125 % increase in biomass, whereas K and P uptake were more than 150 % in tomato plant due to inoculation of silicate dissolving bacteria *B. mucilaginosus* strain RCBC13 in comparison to uninoculated plants. Sheng et al. (2003) studied the effect of inoculation of SSB *Bacillus edaphicus* on chilly and cotton which resulted in increased available P and K contents in plant biomass. Park et al. (2003) found that bacterial inoculation could improve phosphorus and potassium availability in the soils by producing organic acid and other chemicals and thereby stimulated growth and mineral uptake of plants. Zhang et al. (2004) reported the beneficial effect of potassic bacteria on sorghum, which resulted in increased biomass and increased contents of P and K in plants than the control. The beneficial effect of silicate-solubilizing *Bacillus* sp. was observed on grain yield and silica content of rice and available silica in soil (Raj 2004). Ramarethinam and Chandra (2005) recorded significantly increased egg plant yield, plant height and K uptake compared to control in a field experiment due to inoculation of potash-solubilizing bacteria *Frateuria aurantia*.

Mikhailouskaya and Tchernysh (2005) reported the effect of inoculation of K-mobilizing bacteria on severally eroded soils which were comparable with yields on moderately eroded soil without bacterial inoculation that resulted in increased wheat yield upto 1.04 t/ha. Sheng (2005) studied plant growth-promoting effects of potassium releasing bacterial strain *B. edaphicus* NBT on cotton and rape in K-deficient soil pot experiments. The inoculation of *B. edaphicus* resulted in increased root and shoot growth, and potassium content was increased by 30 and 26 %, respectively. The bacterial isolate was found to colonize and develop in the rhizosphere of both the crops.

Christophe et al. (2006) reported that *Burechulderia glathei* in association with pine roots significantly increased weathering of biotite. *B. glathei* PMB (7) and PML1 (12) was found to affect pine growth and root morphology, which was attributed to release of K from the mineral. Sheng and He (2006) recorded an increased root and shoot growth and also showed significantly higher N, P, and K contents of wheat plants components due to inoculation of *B. edaphicus* and its mutants in a yellow brown soil that had low available K. In the field experiment,

increased yield in tomato crop was recorded due to inoculation of silicate-dissolving bacteria *B. cereus* as a bioinoculant along with feldspar and rice straw (Badr 2006). Badr et al. (2006) studied the effect of bacterial inoculation combined with K and P bearing minerals on sorghum plants and reported increase in dry matter yield along with P and K uptake in three different soils, i.e., clay, sandy, and calcareous soils; 48, 65, and 58 % increase in dry matter, 71, 110, and 116 % uptake of P as well as 41, 93, and 79 % uptake of K, and improved fertility through inoculation of silicate dissolving bacteria. In a field experiment, increased rice grain yield was observed due to inoculation of SSB that recorded 5,218 kg/ha grain yield than the control yield of 4,419 kg/ha (Balasubramaniam and Subramanian 2006).

Sugumaran and Janarthanam (2007) reported that inoculation with slime-forming *B. mucilaginosus* strain MCRCp1 recorded increase in the plant dry matter by 125 % and oil content 35.4 % of groundnut plant. Available P and K increased from 6.24 to 9.28 mg/kg and 86.57 to 99.60 mg/kg, respectively, in soil due to inoculation of *B. mucilaginosus* MCRCp1 as compared to uninoculated control plants. Basak and Biswas (2008) found that inoculation of K-solubilizing *B. mucilaginosus* along with application of mica in sudan grass (*Sorghum vulgare* var. *sudanensis*) increased the biomass yield and uptake of K in both the soils. Significant correlation between biomass yield and K uptake by sudan grass and different pools of K in soils were observed. Parmar (2010) showed that inoculation of K-solubilizing isolate HWP47 in wheat (*Triticum aestivum* L.) variety WH711 caused 51.46 % increase in root dry weight (RDW) in soil at 60 days after sowing in pots. Similarly, 44.28 % increase in shoot dry weight (SDW) was found in HWP47-inoculated plants. Inoculation with isolate HWP47 showed 22.35 % increase in RDW and 73.68 % increase in SDW on addition of rock material. Isolates HWP15 and HWP47 also caused significant K uptake in the shoot tissues.

10.6.2 Coinoculation of Potassium Solubilizers with Other Beneficial Bacteria

Ciobanu (1961) showed that increase in the yield of cotton was by 50–94 % when *Azotobacter* (nitrogen-fixing bacteria) and silicate bacteria were applied simultaneously. Similarly, phosphorus-solubilizing bacteria and silicate bacteria were reported to play an important role in plant nutrition through the increase in P and K uptake by plant (Datta et al. 1982; Nianikova et al. 2002). The increased K uptake coupled with increased yield in yam and tapioca was observed by treating the plants with potassium mobilizer in conjunction with biofertilizers and chemical fertilizers (Clarson 2004). Similarly, Chandra et al. (2005) reported an increased yield by 15–20 % in yam and tapioca due to the potash solubilizer application and in combination with other biofertilizers like *Rhizobium*, *Azospirillum*, *Azotobacter*, *Acetobacter*, and PSM. Wu et al. (2005) found that inoculation of K solubilizer *B. mucilaginosus* along with P solubilizer *Bacillus megaterium* and N_2-fixer

Azotobacter chroococcum increased the growth and nutrient uptake significantly in maize crop. Bacterial inoculation also improved soil properties such as organic matter content and total N in soil. Han and Lee (2005) found that coinoculation of PSB (*B. megaterium*) and KSB (*B. mucilaginosus* strain KCTC3870) in combination with direct application of rock P and K materials into the soil resulted in increased N, P, and K uptake, photosynthesis and the yield of eggplant grown in nutrient-limited soil. The combined treatment resulted in increase of N, P, and K uptake in the shoot (14, 22, and 14 %, respectively) and in the root (11, 14, and 21 %). The treatment which combined both bacteria and mineral rocks further increased shoot dry weight by 27 % and root dry weight by 30 % over the control 30 days following planting.

Han et al. (2006) evaluated the combined potential of PSB (*B. megaterium* var. *phosphaticum*) and KSB (*B. mucilaginosus*) inoculation on pepper and cucumber in nutrient-limited soil. It was found that coinoculation of both PSB and KSB, and fertilization with rock P and K, increased the N, P, and K uptake in shoot (21, 31, and 33 %, respectively, for pepper and 29, 41, and 29 % for cucumber) and in root (16, 33, and 26 % for pepper; 29, 34, and 50 % for cucumber). The treatment including bacteria and mineral rocks, further increased plant growth, i.e., 26 % in shoot and 29 % in root dry weight for pepper, whereas 22 % in shoot and 27 % in root dry weight for cucumber plant in comparison to controls during 30 days following planting. The increase in plant growth by combining together, rock materials and both bacterial strains, suggested their potential use as biofertilizer.

Supanjani et al. (2006) reported that integration of P and K rocks with inoculation of phosphorus- and potassium-solubilizing bacteria increased P availability from 12 to 21 % and K availability from 13 to 15 %, in the soil as compared with control and subsequently improved nutrient N, P, and K uptake in *Capsicum annuum*. The integration approach of rocks and bacteria also increased plant photosynthesis by 16 % and leaf area by 35 % as compared to control. On the other hand, the biomass harvest and fruit yield of the treated plants were increased by 23–30 %, respectively. Similarly, the combined potential of phosphate-solubilizing bacteria *B. megaterium* var. *phosphaticum* and potassium-solubilizing bacteria, *B. mucilaginosus* was evaluated using pepper and cucumber as test crops (Vassilev et al. 2006). The outcome of the experiment showed that rock phosphorus and potassium applied either singly or in combination do not significantly enhanced availability of soil phosphorus and potassium indicating their unsuitability for direct application. However, coinoculation of PSB and KSB resulted in consistently higher P and K availability than the control.

Coinoculation of waste mica with potassium-solubilizing *B. mucilaginosus* and N_2-fixing *A. chroococcum* A-41 resulted in highest biomass production and nutrient acquisition by sudan grass (Basak and Biswas 2010). Coinoculation of bacterial strains maintained consistently higher amounts of available K and N in soils even at 150 days of crop growth. *Bacillus mucilaginosus* strain was found more effective K solubilizer than *Azotobacter chroococcum* strain A-41. Similarly, inoculation of maize and wheat plants with *Bacillus mucilaginosus*, *Azotobacter chroococcum*, and *Rhizobium* spp. significantly improved the biomass accumulation, potassium

content, and uptake by plants (Singh et al. 2010). *B. mucilaginosus* resulted in significantly higher mobilization of potassium than *A. chroococcum* and *Rhizobium* inoculation. Results revealed that PGPR could be used to mobilize potassium from waste mica, which in turn served as a source of potassium for plant growth. These results suggested that the treatment with P- and K-containing rock materials and inoculation with P- and K-solubilizing bacterial strains could be applied as a sustainable alternative to the use of chemical fertilizers. Thus, inoculation with PGPR including phosphate and potassium-solubilizing bacteria (PSB and KSB) as biofertilizers could be a sustainable solution to improve plant nutrition and crop production (Vessey 2003).

10.7 Possible Approaches to Increase Potassium Solubilization Efficiency and Its Application as Biofertilizer

Soil microorganisms play pivotal role in various biogeochemical cycles and are responsible for the cycling of nutrients in the plant utilizable form (Wall and Virginia 1999). Phosphate- and potassium-solubilizing microorganisms and other beneficial rhizobacteria cause the release of nutrients in plant utilizable form and exert beneficial effects on plant growth (Glick 1995; Sindhu et al. 2002; Marques et al. 2010). Thus, microbes influence aboveground ecosystems by contributing to plant nutrition, plant health, soil structure, and soil fertility. Therefore, microorganisms offer an environment friendly sustainable system and play a vital role in maintaining soil nutrient status.

Many rhizosphere bacteria are well known for their capacity to confer plant growth promotion and also increase resistance toward various diseases as well as abiotic stresses. These rhizobacteria often fail to confer these beneficial effects when applied in the field, which is often due to insufficient rhizosphere colonization (Lugtenberg et al. 2001). Lin et al. (2002) showed that silicate-dissolving bacteria increased 70 % in the rhizosphere soil and 20 % in non-rhizosphere soil, respectively. Sugumaran and Janarthanam (2007) also reported that the number of K-solubilizing bacterium *B. mucilaginosus* strain MCRCp1 increased to about 10^6–10^7 cfu/g in soil after 90 days of inoculation, whereas the count of K-solubilizing bacteria was only 10^3 g^{-1} in the control soil. New bacterial traits conferring strain survival in the rhizosphere have been found and opened a way to better understand specific signaling and the regulatory processes governing the plant-beneficial bacterial association (Matilla et al. 2007). Use of molecular techniques in genetic modification of microbial and plant biological activities allows their better functioning in the rhizosphere (Ryan et al. 2009) leading to substantial improvement in the sustainability of agricultural systems.

Plants could be selected by breeders with favorable traits or microorganisms can be engineered that increase nutrient accessibility, minimize biotic and abiotic

stresses, and suppress pathogenic microbes or that encourage the persistence of beneficial microorganisms (Weller 2007; Dey et al. 2009; Sindhu et al. 2009). The release of organic anions such as citrate and malate has been reported to improve availability of poorly soluble organic and inorganic phosphorus (Richardson et al. 2001; Ryan et al. 2001). Therefore, the release of organic anions from roots can have an important influence on plant growth and nutrition. Since many of the genes controlling these exudates have now been identified, it is possible to manipulate conditions in the rhizosphere by modifying their expression via genetic engineering. Moreover, different methods and techniques have been developed recently to characterize and conserve various agriculturally important microbial communities from different environments for their optimal utilization for agriculture (Kirk et al. 2004; Naik et al. 2008). The knowledge generated on biodiversity and genetic manipulation of phosphate- and potassium-solubilizing bacteria will be useful to design strategies for use of these strains as inoculants in organic agriculture.

Thus, complex interactions between the KSB, PSB, other PGPR, the plant, and the environment are responsible for the variability observed in solubilization of bound nutrients and their uptake leading to plant growth promotion. Future strategies are required to clone genes from KSB involved in solubilization of insoluble potassium and to transfer these genes into the bacterial strains having good colonization potential along with other beneficial characteristics such as nitrogen fixation. Further, the efficacy of potassium-solubilizing bacteria can be improved by developing the better cultural practices and delivery systems that favor their establishment in the rhizosphere. The applications of mixture of PGPRs with different beneficial activities including potassium and phosphate solubilization ability, may be a more ecologically sound approach because it may result in better colonization and better adaptation to the environmental changes occurring throughout the growing season. In near future, the biotechnological approaches used in manipulation of bacterial traits will lead to improved potassium solubilization and their inoculation as potassic biofertilizer will enhance plant growth and crop productivity for sustainable agriculture.

10.8 Conclusion

The use of low grade, locally available soil minerals such as mica, feldspar, and rock phosphate in combination with selected efficient strains of potassium-mobilizing bacteria as biofertilizers are urgently required to replace chemical fertilizers and for reducing the cost of crop cultivation. Although, many bacterial strains have been found to improve the growth of plants under pot house conditions, the extent of growth stimulation by bacterial strains under field conditions usually remains unexplored. Therefore, effective potassium-solubilizing and plant growth-promoting bacterium–plant systems must be tested under field conditions with specific crop experimental designs keeping in consideration of the soil type, plant

types grown, and the environmental factors. In addition, plant type/variety has also been found to influence the root colonization ability of the inoculated strains (Sheng 2005). Thus, competitive and effective bacterial strains must be selected from the pool of indigenous beneficial soil bacteria which could be adopted to the particular conditions of the inoculation site (Paau 1989; Sindhu and Dadarwal 2000). The application of efficient strains of potassium-solubilizing bacteria may find their use in the amelioration of potassium-deficient soils and further research could lead to an alternative mean of potassium nutrition for sustainable agriculture.

References

Aleksandrov VG (1958) Organo-mineral fertilizers and silicate bacteria. Dokl Akad Nauk 7:43–48

Aleksandrov VG, Blagodyr RN, Iiiev IP (1967) Liberation of phosphoric acid from apatite by silicate bacteria. Mikrobiyol Zh (Kiev) 29:111–114

Anonymous (2003) Agricultural statistics at a glance. Ministry of Agriculture Cooperation, New Delhi, pp 51–53

Argelis DT, Gonzala DA, Vizcaino C, Gartia MT (1993) Biochemical mechanism of stone alteration carried out by filamentous fungi living in monuments. Biogeochemistry 19:129–147

Avakyan ZA, Karavaiko GI, Mel'nikova EO, Krutsko VS, Ostroushhko YI (1981) Role of microscopic fungi in weathering of rocks from a pegmatite deposit. Mikrobiologiya 50:115–120

Badr MA (2006) Efficiency of K-feldspar combined with organic materials and silicate dissolving bacteria on tomato yield. J Appl Sci Res 2:1191–1198

Badr MA, Shafei AM, Sharaf El-Deen SH (2006) The dissolution of K and phosphorus bearing minerals by silicate dissolving bacteria and their effect on sorghum growth. Res J Agric Biol Sci 2:5–11

Balasubramaniam P, Subramanian S (2006) Assessment of soil test based potassium requirement for low land rice in udic haplustalf under the influence of silicon fertilization. Tamil Nadu Agricultural University, Kumulur, Truchirapalli, pp 621–712

Barker WW, Welch SA, Banfield JF (1997) Geomicrobiology of silicate minerals weathering. Rev Mineral 35:391–428

Barker WW, Welch SA, Welch SC, Banfield F (1998) Experimental observations of the effects of bacteria on aluminosilicate weathering. Am Mineral 83:1551–1563

Basak BB, Biswas DR (2008) Influence of potassium solubilizing microorganism (*Bacillus mucilaginous*) and waste mica on potassium uptake dynamics by sudan grass (*Sorghum vulgare* Pers) grown under two Alfisols. Plant Soil 317:235–255

Basak BB, Biswas DR (2010) Coinoculation of potassium solubilizing and nitrogen fixing bacteria on solubilization of waste mica and their effect on growth promotion and nutrient acquisition by a forage crop. Biol Fertil Soils 46:641–648

Belkanova NP, Karavajko GI, Avakyan ZA (1985) Cleavage of the siloxane bond in quartz by *Bacillus mucilaginosus*. Mikrobiologiya 54:27–30

Bennett PC, Choi WJ, Rogera JR (1998) Microbial destruction of feldspars. Miner Manage 8 (62A):149–150

Berthelin J (1983) Microbial weathering processes. In: Krumbein WE (ed) Microbial geochemistry. Blackwell Scientific Publications, Boston, MA, pp 223–262

Berthelin J, Belgy G (1979) Microbial degradation of phyllosilicates during simulate podzolization. Geoderma 21(4):297–310

Bertsch PM, Thomas GW (1985) Potassium status of temperature region soils. In: Munson RD (ed) Potassium in agriculture. ASA, CSSA and SSSP, Madison, WI, pp 131–162

Beveridge TJ, Fyfe WS (1985) Metal fixation by bacterial cell walls. Can J Earth Sci 22:1893–1898

Beveridge TJ, Murray RGE (1980) Sites of metal deposition in the cell walls of *Bacillus subtilis*. J Bacteriol 141:876–887

Brady NC (1990) The nature and properties of soils. Macmillan, New York, pp 351–380

Bunt JS, Rovira AD (1955) Microbiological studies of some sub Antarctic soils. J Soil Sci 6:119–128

Burford EP, Fomina M, Gadd GM (2003) Fungal involvement in bioweathering and biotransformation of rocks and minerals. Mineral Mag 67:1127–1155

Buss HL, Luttge A, Brantley SL (2007) Etch pit formation on iron silicate surfaces during siderophore-promoted dissolution. Chem Geol 240:326–342

Chandra K, Greep S, Ravindranath P, Sivathsa RSH (2005) Liquid biofertilizers. Regional Center for Organic Farming, Hebbal, Bangalore

Christophe C, Turpault MP, Freyklett P (2006) Root associated bacteria contribute to mineral weathering and to mineral nutrition in trees and budgeting analysis. Appl Environ Microbiol 72:258–1266

Ciobanu I (1961) Investigation on the efficiency of bacterial fertilizers applied to cotton. Cent Exp Ingras Bact Lucrari Stiint 3:203–214

Clarson D (2004) Potash biofertilizer for ecofriendly agriculture. Agro-clinic and Research Centre, Poovanthuruthu, Kottayam (Kerala), pp 98–110

Datta M, Banish S, Dupta RK (1982) Studies on the efficacy of a phytohormone producing phosphate solubilizing *Bacillus firmus* in augmenting paddy yield in acid soils of Nagaland. Plant Soil 69:365–373

Dey C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 32:1682–1694

Duff RB, Webley DM (1959) 2-ketoglutaric acid and natural chelator produced by soil bacteria. Chem Ind 44:1376–1377

Duff RB, Webley DM, Scott RO (1963) Solubilization of minerals and related materials by 2- ketogluconic acid producing bacteria. Soil Sci 5:105–114

FAI (2007) Fertiliser statistics 2006–2007. The Fertilizer Association of India, New Delhi

Friedrich S, Platonova NP, Karavaiko GI, Stichel E, Glombitza F (1991) Chemical and microbiological solubilization of silicates. Acta Biotechnol 11:187–196

Ghosh AB, Hasan R (1980) Soil fertility map of India. Indian Agricultural Research Institute, New Delhi

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanisms used by plant growth-promoting bacteria. Imperial College, London

Goldstein AH (1994) Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous mineral phosphates by Gram negative bacteria. In: Torriani-Gorni A, Yagil E, Silver E (eds) Phosphate in microorganisms: cellular and molecular biology. ASM, Washington, DC, pp 197–203

Grayston SJ, Vaugha D, Jones D (1996) Rhizosphere carbon flow in trees in comparison with annual plants. The importance of root exudation and its impact on microbial activity and nutrient availability. Appl Soil Ecol 5:29–56

Gromov BV (1957) The microflora of rocks and primitive soil in some northern regions of the USSR. Mikrobiologiya 26:52–54

Groudev SN (1987) Use of heterotrophic microorganisms in mineral biotechnology. Acta Biotechnol 7:299–306

Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3:307–319

Han HS, Lee KD (2005) Phosphate and potassium solubilizing bacteria effect on mineral uptake, soil availability and growth of eggplant. Res J Agric Biol Sci 1:176–180

Han HS, Supanjani E, Lee KD (2006) Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. Plant Soil Environ 52:130–136

Hazen TC, Jimenez L, Victoria GL (1991) Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microbiol Ecol 22:293–304

Heinen W (1960) Silicon metabolism in micorganisms. Arch Microbiol 37:199–210

Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Hieberk FK, Bennett PC (1992) Microbial control of silicate weathering in organic-rich groundwater. Science 258:278–281

Hinsinger P, Jaillard B, Dufey JE (1992) Rapid weathering of a trioctahedral mica by the roots of ryegrass. Soil Sci Soc Am J 65:977–982

Hinsinger P, Elsass F, Jaillard B, Robert M (1993) Root-induced irreversible transformation of trioctahedral mica in the rhizosphere of rape. J Soil Sci 44:535–545

Hu XF, Chen J, Guo JF (2006) Two phosphate and potassium solubilizing bacteria isolated from Tiannu mountain, Zhejiang, China. World J Microbiol Biotechnol 22:983–990

Hutchen SE, Valsami JE, Eldowney MS (2003) The role of heterotrophic bacteria in feldspar dissolution. Miner Manage 67:1151–1170

Jones LHP, Handrecht KA (1967) Silica in soils, plants and animals. Adv Agron 19:107–149

Kalaiselvi P (1999) Influence of silicate solubilizing bacteria on dissolution of silicate and potassium in rice soil added with graded levels of potassium and rice residues. M.Sc. (Agric) Thesis, Tamil Nadu Agricultural University, Coimbatore

Kannan NM, Raj SA (1998) Occurrence of silicate solubilizing bacteria in rice ecosystem. Madras Agric J 85:47–50

Khanwilkar SA, Ramteke JR (1993) Response of applied K in cereals in Maharashtra. In: Agriculture, pp 84–96

Khudsen D, Peterson GA, Prov PF (1982) Lithium, sodium and potassium. In: Page AL (ed) Methods of soil analysis, part (2) agronomy monograph 9, 2nd edn. ASA and SSSA, Madison, WI

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomus JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 58:169–188

Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol 7:39–44

Konhauser KO, Ferris FG (1996) Diversity of iron and silicate precipitation by microbial mats in hydrothermal waters. Iceland. Implications for Precambrian iron formations. Geology 24:323–326

Li YF (1994) The characteristics and function of silicate dissolving bacteria fertilizer. Soil Fertil 2:48–49

Li FC, Li S, Yang YZ, Cheng LJ (2006) Advances in the study of weathering products of primary silicate minerals, exemplified by mica and feldspar. Acta Petrol Mineral 25:440–448

Lian B (1998) Study on silicate bacteria dissolve potassium. Guizhou Science and Technology Press, Guiyang, pp 103–108

Lian B, Fu PQ, Mo DM, Liu CQ (2002) A comprehensive review of the mechanism of potassium release by silicate bacteria. Acta Mineral Sin 22:179–183

Lian B, Wang B, Pan M, Liu C, Tang HH (2007) Microbial release of potassium from K-bearing minerals by thermophilic fungus *Aspergillus fumigatus*. Geochem Cosmochim Acta 72:87–98

Lian B, Chen Y, Zhu LJ, Yang RD (2008) Progress in the study of weathering of carbonate rock by microbes. Earth Sci Front 15:90–99

Liermann LJ, Kalinowski BE, Brantley SL, Ferry JG (2000) Role of bacterial siderophores in dissolution of hornblende. Geochim Cosmochim Acta 64:587–602

Lin QM, Rao ZH, Sun YX, Yao J, Xing LJ (2002) Identification and practical application of silicate-dissolving bacteria. Agric Sci China 1:81–85

Liu GY (2001) Screening of silicate bacteria with potassium releasing and antagonistic activity. Chin J Appl Environ Biol 7:66–68

Liu W, Xu X, Wu S, Yang Q, Luo Y, Christie P (2006) Decomposition of silicate minerals by *Bacillus mucilaginosus* in liquid culture. Environ Geochem Health 28:133–140

Liu D, Lian B, Dong H (2012) Isolation of *Paenibacillus* sp. and assessment of its potential for enhancing mineral weathering. Geomicrobiol J 29:413–421

Lopes-Assad ML, Avansini SH, Rosa MM, de Carvalho JRP, Ceccato-Antonini SR (2010) The solubilization of potassium-bearing rock powder by *Aspergillus niger* in small-scale batch fermentations. Can J Microbiol 56:598–605

Lugtenberg BJJ, Dekkers L, Bloemberg CV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490

Malinovskaya IM, Kosenko LV, Votselko SK, Podgorskii VS (1990) Role of *Bacillus mucilaginosus* polysaccharide in degradation of silicate minerals. Mikrobiologiya 59:49–55

Marques APGC, Pires C, Moreira H, Rangel AOSS, Castro ML (2010) Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. Soil Biol Biochem 42:1229–1235

Matilla MA, Espinosa-Urgel M, Roderiguez-Herva JJ, Ramos JL, Ramos-Gonzalez MI (2007) Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. Genome Biol 8:R179

Memon YM, Fergus IF, Hughes JD, Page DW (1988) Utilization of non-exchangeable soil potassium in relation to soil types, plant species and stage of growth. Aust J Soil Res 26:489–496

Mengel K, Kirkby EA (1987) Principles of plant nutrition. Internl Potash Inst Bern, Switzerland, pp 200–210

Mikhailouskaya N, Tcherhysh A (2005) K-mobilizing bacteria and their effect on wheat yield. Latvian J Agron 8:154–157

Moira EK, Henderson MEK, Duff RB (1963) The release of metallic and silicate ions from minerals, rocks and soils by fungal activity. J Soil Sci 14:237–245

Muntz A (1890) Sur La decomposition des rockes *et al.* formation de la terrarable. Compt Rend Akad Sci Paris 110:1370–1372

Murali G, Gupta A, Nair RV (2005) Variations in hosting beneficial plant associated microorganisms by root (wilt) diseased and field tolerant coconut palms of west coast tall variety. Curr Sci 89:1922–1927

Muralikannan M (1996) Biodissolution of silicate, phosphate and potassium by silicate solubilizing bacteria in rice ecosystem. M.Sc. (Agric) Thesis, Tamil Nadu Agricultural University, Coimbatore

Naik PR, Raman G, Narayanan KB, Sakthivel N (2008) Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil. BMC Microbiol 8:230–243

Nayak B (2001) Uptake of potash by different plants with the use of potash mobilizing bacteria (*Frateuria aurantia*). M.Sc. (Agric) Thesis, QUAT, Bhubaneswar

Nianikova GG, Kuprina EE, Pestova OV, Vodolazhskaia SV (2002) Immobilizing of *Bacillus mucilaginosus,* a producer of exopolysaccharides, on chitin. Prikladnaia biokhimiia I. Mikrobiologiya 38:300–304

Nishanth D, Biswas DR (2008) Kinetics of phosphorus and potassium release from rock phosphate and waste mica enriched compost and their effect on yield and nutrient uptake by wheat (*Triticum aestivum*). Bioresour Technol 99:3342–3353

Norkina SP, Pumpyansakya LV (1956) Certain properties of silicate bacteria. Crop Sci Soc Jpn 28:35–40

Paau MA (1989) Improvement of *Rhizobium* inoculants. Appl Environ Microbiol 55:862–865

Palmer RJJ, Siebert J, Hirsch PR (1991) Biomass and organic acids in sandstone of a weathered building production by bacterial and fungal isolates. Microbiol Ecol 21:253–261

Park M, Singvilay O, Seok Y, Chung J, Ahn K, Sa T (2003) Effect of phosphate solubilizing fungi on P uptake and growth to tobacco in rock phosphate applied soil. Korean J Soil Sci Fertil 36:233–238

Parmar P (2010) Isolation of potassium solubilizing bacteria and their inoculation effect on growth of wheat (*Triticum aestivum* L. em. Thell.). M.Sc. Thesis submitted to CCS Haryana Agricultural University, Hisar

Parmar P, Sindhu SS (2013) Potassium solubilization by rhizosphere bacteria: influence of nutritional and environmental conditions. J Microbiol Res 3:25–31

Paul EA, Clark FE (1989) Soil microbiology and biochemistry. Academic, San Diego, CA

Prajapati K, Sharma MC, Modi HA (2012) Isolation of two potassium solubilizing fungi from ceramic industry soils. Life Sci Leaflets 5:71–75

Purushothaman A, Chandramohan D, Natarajan R (1974) Distribution of silicate dissolving bacteria in velar estuary. Curr Sci 43:282–283

Raj SA (2004) Solubilization on a silicate and concurrent release of phosphorus and potassium in rice ecosystem. In: Biofertilizer technology for rice based cropping system. Scientific Publishers, Jodhpur, India, pp 372–378

Rajan SSS, Watkinson JH, Sinclair AG (1996) Phosphate rock for direct application to soils. Adv Agron 57:77–159

Ramarethinam S, Chandra K (2005) Studies on the effect of potash solubilizing/mobilizing bacteria *Frateuria aurantia* on brinjal growth and yield. Pestology 11:35–39

Reitmeir RF (1951) Soil potassium. In: Norman AG (ed) Advances in agronomy, vol III, American Society of Agronomy. Academic, New York, pp 113–164

Requena BN, Jimenez I, Toro M, Barea JM (1997) Interactions between plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytiisoides*, a model legume for revegetation in Mediterranean semiarid ecosystems. New Phytol 136:667–677

Richardson AE, Hadobas PA, Hayes JE (2001) Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. Plant J 25:641–649

Rogers JR, Bennett PC, Choi WJ (1998) Feldspars as a source of nutrients for microorganisms. Am Mineral 83:1532–1540

Ryan PR, Delhaise E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560

Ryan PR, Dessaux Y, Thomashow LS, Weller DM (2009) Rhizosphere engineering and management for sustainable agriculture. Plant Soil 321:363–383

Sharpley AN (1989) Relationship between soil potassium forms and mineralogy. Soil Sci Soc Am J 52:1023–1028

Sheng XF (2005) Growth promotion and increased potassium uptake of cotton and rape by a potassium releasing strain of *Bacillus edaphicus*. Soil Biol Biochem 37:1918–1922

Sheng XF, He LY (2006) Solubilization of potassium bearing minerals by a wild type strain of *Bacillus edaphicus* and its mutants and increased potassium uptake by wheat. Can J Microbiol 52:66–72

Sheng XF, Huang WY (2002a) Study on the conditions of potassium release by strain NBT of silicate bacteria. Sci Agric Sin 35:673–677

Sheng XF, Huang WY (2002b) Mechanism of potassium release from feldspar affected by the strain NBT of silicate bacterium. Acta Pedol Sin 39:863–871

Sheng XF, He LY, Huang WY (2002) The conditions for releasing potassium by a silicate dissolving bacterial strain NBT. Agric Sci China 1:662–666

Sheng XF, Xia JJ, Chen J (2003) Mutagenesis of the *Bacillus edaphicus* strain NBT and its effect on growth of chilli and cotton. Agric Sci China 2:400–412

Sindhu SS, Dadarwal KR (2000) Competition for nodulation among rhizobia in *Rhizobium*-legume symbiosis. Indian J Microbiol 40:211–246

Sindhu SS, Gupta SK, Dadarwal KR (1999) Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of plant growth in green gram (*Vigna radiata*). Biol Fertil Soils 29:62–68

Sindhu SS, Sunita Suneja, Goel AK, Parmar N, Dadarwal KR (2002) Plant growth promoting effects of *Pseudomonas* sp. on coinoculation with *Mesorhizobium* sp. *Cicer* strain under sterile and wilt sick soil conditions. Appl Soil Ecol 19:57–64

Sindhu SS, Verma MK, Suman Mor (2009) Molecular genetics of phosphate solubilization in rhizosphere bacteria and its role in plant growth promotion. In: Khan MS, Zaidi A (eds) Phosphate solubilizing microbes and crop productivity. Nova, New York, pp 199–228

Sindhu SS, Dua S, Verma MK, Khandelwal A (2010) Growth promotion of legumes by inoculation of rhizosphere bacteria. In: Khan MS, Zaidi A, Musarrat J (eds) Microbes for legume improvement. Springer, New York, pp 195–235

Sindhu SS, Seema Dua, Sahu G (2011) Biological control of plant diseases. In: Rana MK (ed) Modern concepts of vegetable production. Biotechnology Books, Daryaganj, New Delhi, pp 470–517

Singh G, Biswas DR, Marwah TS (2010) Mobilization of potassium from waste mica by plant growth promoting rhizobacteria and its assimilation by maize (*Zea mays*) and wheat (*Triticum aestivum* L.). J Plant Nutr 33:1236–1251

Sparks DL (1987) Potassium dynamics in soils. Adv Soil Sci 6:1–63

Sparks DL (2000) Bioavailibility of soil potassium. In: Summer ME (ed) Handbook of soil science. CRC, Boca Raton, FL, pp D-38–D-52

Sparks DL, Huang PM (1985) Physical chemistry of soil potassium. In: Munson RD (ed) Potassium in agriculture. American Society of Agronomy, Madison, WI, pp 201–276

Sperberg JI (1958) The incidence of apatite solubilizing organisms in the rhizosphere and soil. Aust J Agric Res Econ 9:778–781

Styriakova I, Styriak I, Galko I, Hradil D, Bezdicka P (2003) The release of iron bearing minerals and dissolution of feldspar by heterotrophic bacteria of *Bacillus* species. Ceram Silicaty 47:20–26

Styriakova I, Bhatti TM, Bigham JM, Styriak I, Vourniess A, Tuoviness OH (2004) Weathering of phlogopite by *Bacillus cereus* and *Acidithiobacillus ferrooxidans*. Can J Microbiol 50:213–219

Sugumaran P, Janarthanam B (2007) Solubilization of potassium containing minerals by bacteria and their effect on plant growth. World J Agric Sci 3(3):350–355

Supanjani, Han HS, Jung SJ, Lee KD (2006) Rock phosphate potassium and rock solubilizing bacteria as alternative sustainable fertilizers. Agron Sustain Dev 26:233–240

Tandon HLS, Sekhon GS (1988) Potassium research and agricultural production in India. Fertilizer Development and Consultation Organization, New Delhi, pp 144

Tauson EL, Vinogrado S (1988) Extracellular enzymes of *Bacillus mucilaginosus*. Mikrobiologiya 57:236–240

Ullman WJ, Welch SA (2002) Organic ligands and feldspar dissolution. Geochem Soc 7:3–35

Ullman WJ, Kirchman DL, Welch SA (1996) Laboratory evidence by microbially mediated silicate mineral dissolution in nature. Chem Geol 132:11–17

Urruti MM, Beveridge TJ (1994) Formation of fine-grained metal and silicate precipitates on a bacterial surface (*Bacillus subtilis*). Chem Geol 116(3–4):261–280

Vainberg SN, Vlasov AS, Skripnik VP (1980) Enrichment of clay raw material using silicate bacteria. USSR Tr Mosk Khim Tekhnol Inst Im DI Mendeleeva 116:34–37

Vandevivere P, Welch SA, Ullman WJ, Kirchman DJ (1994) Enhanced dissolution of silicate minerals by bacteria at near neutral pH. Microbiol Ecol 27:241–251

Vassilev N, Medina A, Azcon R, Vassilev M (2006) Microbial solubilization of rock phosphate as media containing agro industrial wastes and effect of the resulting products on plant growth and phosphorus uptake. Plant Soil 287:77–84

Vessey KJ (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 25:557–586

Vintikova H (1964) A contribution to study on efficiency of silicate bacteria. Rostl Vyn 31:1219–1228

Wall DH, Virginia RA (1999) Control of soil biodiversity – in sight from extreme environments. Appl Soil Ecol 13:137–150
Webley DM, Duff RB, Mitchell WA (1960) A plate method for studying the breakdown of synthetic and natural silicates by soil bacteria. Nature 188:766–767
Webley DM, Henderson MEK, Taylor IF (1963) The microbiology of rocks and weathered stones. J Soil Sci 14:65–71
Weed SB, Davey CB, Cook MG (1969) Weathering of mica by fungi. Soil Sci Soc Am Proc 33:702–706
Welch SA, Ullman WJ (1993) The effect of organic acids on plagioclase dissolution rates and stoichiometry. Geochim Cosmochim Acta 57:2725–2736
Welch SA, Vandevivere P (2009) Effect of microbial and other naturally occurring polymers on mineral dissolution. Geomicrobiol J 12:227–238
Welch SA, Barker WW, Banfield JF (1999) Microbial extracellular polysaccharides and plagioclase dissolution. Geochim Cosmochim Acta 63:1405–1419
Weller DM (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250–256
Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM-fungi on maize growth: a greenhouse trial. Geoderma 125:155–166
Xiaoxi Z, Liu X, Tang J, Hu S, Jiang P, Li W, Xu L (2012) Characterization and potassium solubilizing ability of *Bacillus circulans* $Z_{1\text{-}3}$. Adv Sci Lett 10:173–176
Xie JC (1998) Present situation and prospects for the world's fertilizer use. Plant Nutr Fertil Sci 4:321–330
Yakhontova LK, Andreev PI, Ivanova MY, Nesterovich LG (1987) Bacterial decomposition of smectite minerals. Doklady Akademii Nauk USSR 296:203–206
Zahro MK, Monib M, Abdel-Al SHI, Heggol A (1986) Role of silicate bacteria in releasing K and silicone from biotite and orthoclase. Microbiol Res 139:349–357
Zapata F, Roy RN (2004) Use of phosphate rock for sustainable agriculture. FAO and IAEA, Rome
Zhang CJ, Tu GQ, Cheng CJ (2004) Study on potassium dissolving ability of silicate bacteria. Shaguan Coll J 26:1209–1216
Zhao F, Sheng XF, Huang Z (2008) Isolation of mineral potassium solubilizing bacterial strains from agricultural soils in Shandong province. Biodivers Sci 16:593–600
Zhou H, Zeng XX, Liu FF, Qiu GZ, Hu YH (2006) Screening, identification and desilication of a silicate bacterium. J Cent S Univ Tech 13:337–341
Zhou YF, Wang RC, Lu XC, Lu JJ (2007) Influence of microbe-mineral contact model on mineral dissolution: a primary study on microperthite dissolution by *Paenibacillus polymyxa*. Geol J China Univ 13:657–661
Zhou YF, Wang RC, Lu XC (2008) The effects of mineral surface properties on bacteria-mediated dissolution of basalt. Acta Petrol Mineral 27:59–66

Chapter 11
Phosphorus Cycling: Prospects of Using Rhizosphere Microorganisms for Improving Phosphorus Nutrition of Plants

Satyavir S. Sindhu, Manisha Phour, Sita Ram Choudhary, and Deepika Chaudhary

11.1 Introduction

Phosphorus (P) is a major essential macronutrient for biological growth and development. It is an essential element found in all living beings as part of proteins, nucleic acids, membranes, and energy molecules such as ATP, GTP, and NADPH. It is involved in many cellular essential processes including cell division, photosynthesis, breakdown of sugar, energy transfer, nutrient transport within the plant, expression and maintenance of genetic material, and regulation of metabolic pathways. In agriculture, P is the second major nutrient element in terms of quantitative requirement limiting plant growth preceded by nitrogen (Hinsinger 2001; Fernandez et al. 2007). It is found in soil, plants, and microorganisms in a number of organic and inorganic compounds. However, the total P content in an average soil is 0.05 % and only 0.1 % of the total P present in the soil is available to the plants. Even though some soils may have high levels of total P, they can still be P deficient due to low levels of soluble phosphate available to plants (Gyaneshwar et al. 2002). Thus, the pool of immediately available P must be replenished regularly to meet plant requirements (Bieleski 1973; Richardson and Simpson 2011).

Phosphorus deficiency in soil is traditionally overcome by adding either phosphatic fertilizers (Khan et al. 2006) or it may be incorporated as leaf litter, plant residues, or animal remains. The phosphatic fertilizers are the world's second largest bulk chemical used in agriculture on earth (Goldstein et al. 1993; Goldstein 2007). After the addition of chemical phosphatic fertilizers, the extremely reactive soluble phosphate anions ($H_2PO_4^-$, HPO_4^{2-}) may form metal complexes with Ca in calcareous soils (Lindsay et al. 1989) and Fe^{3+} and Al^{3+} in acidic soils (Norrish and Rosser 1983). Thus, a large portion, i.e., 75–90 % of added P fertilizer in

S.S. Sindhu (✉) • M. Phour • S.R. Choudhary • D. Chaudhary
Department of Microbiology, CCS Haryana Agricultural University, Hisar 125004, Haryana, India
e-mail: sindhuss58@gmail.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39, DOI 10.1007/978-3-642-41837-2_11, © Springer-Verlag Berlin Heidelberg 2014

agricultural soils is precipitated/immobilized rapidly by iron, aluminum, manganese, and calcium complexes depending on soil type, soil pH, and existing minerals (Richardson et al. 2001b; Bünemann et al. 2006; Vu et al. 2008). Generally, a few days after fertilization, available phosphate levels can reach similar values to those before application (Sharpley 1985). Thus, due to the low P fertilizer efficiency, farmers often apply P fertilizers in excess of plant requirement to sustain crop production (Rodriguez and Fraga 1999) and this practice has resulted in a buildup of residual P and nonlabile inorganic P in the soil (Vu et al. 2008) leading to environmental problems such as water eutrophication (Correll 1998) and soil pollution. In order to meet current demands of food production by improving crop productivity, enhanced fertilization has provoked an intense scavenging of phosphorus mines worldwide and it is estimated that by 2060 these mines could be depleted (Gilvert 2009; Cordell et al. 2009). Therefore, there is an urgent need to explore alternative sources for better management of plant–soil–microbial P cycle to reduce our reliance on mineral fertilizers.

Utilization of microorganisms is an attractive approach to increase the availability of P in soil leading to enhanced crop production and to develop a more sustainable agricultural system under recent intensive, nutrient-extracting agricultural practices (Sanchez et al. 1997; Deubel and Merbach 2005; Richardson and Simpson 2011). The use of phosphate-solubilizing microorganisms (PSMs) is economical, ecofriendly, and has greater agronomic utility to compensate the expensive inorganic sources of P fertilizers. Thus, association between plant roots and phosphate-solubilizing microorganisms could play an important role in P nutrition in many natural agroecosystems (Rodriguez and Fraga 1999; Bagyaraj et al. 2000; Richardson et al. 2001b). Many phosphate-solubilizing bacteria including *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bradyrhizobium*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Salmonella*, *Serratia*, *Streptomyces*, and *Thiobacillus* have been isolated (Zhao and Lin 2001; Sindhu et al. 2009; Castagno et al. 2011; Azziz et al. 2012). Efficient phosphate-solubilizing fungi include the genus *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Sclerotium* (Zhao and Lin 2001). Inoculation of many phosphate-solubilizing microorganisms has been found to support growth of plants under nutrient imbalance conditions (Glick 1995; Igual et al. 2001; Wu et al. 2005).

Microbial-mediated solubilization of insoluble phosphates in the cultivated soils is generally attributed to production of organic acids by microorganisms (Kim et al. 1998a; Carrillo et al. 2002; Rodriguez et al. 2004). Organic acids including acetate, lactate, malate, oxalate, succinate, citrate, gluconate, ketogluconate, etc., can form complexes with the iron or aluminum in ferric and aluminum phosphates, thus releasing plant available phosphate into the soil (Jones 1998; Gyaneshwar et al. 2002). Moreover, the release of organic acid anions such as malate and citrate can mobilize soil P pools by reducing the number of binding sites for P fixation via chelation of Fe and Al (Gerke 1992) and by replacing P from adsorption sites (Nziguheba et al. 2000). Besides, the release of enzymes such as acid phosphatase

(Tarafder and Claassen 2003) and phytase (Richardson 2001) plays an important role in mobilization of organic P.

Recently, modest application of rock phosphate (RP) along with inoculation of phosphate-solubilizing microorganisms has been found to enhance P availability in soils with very low P status (Jones and Oburger 2011; Arcand and Schneider 2006). Thus, solubilization of RP by soil microorganisms serves a source of phosphorus for crops at lower cost with less technological sophistication. Therefore, an enormous amount of research has been conducted recently on isolation and characterization of PSMs from different soils with the objective of developing phosphatic biofertilizers. Considering the fact that world's high-quality sources of rock phosphate are finite and are distributed over only to few countries such as Morocco, the USA, China, Russia, etc., the world's supply of fossil resources is shrinking with the increasing demand of phosphatic fertilizers. This justifies the need to develop plants and/or agricultural systems that are more P efficient. For example, plant species particularly legumes, are capable of mobilizing P from less labile P pools than cereals (Kamh et al. 1999; Nuruzzaman et al. 2005a, b). Similarly, efficient phosphate solubilizing microorganisms could be used as inoculant for improving plant growth of agriculture and horticulture crops (Bagyaraj et al. 2000; Gyaneshwar et al. 2002; Khan et al. 2007; Naik et al. 2008). This chapter aims to identify the phosphate-solubilizing microorganisms from soil or rhizosphere and to understand the mechanism used for P solubilization. The inoculation responses of the phosphate-solubilizing microorganisms and their genetic manipulations to improve P solubilization capacity are presented in this chapter to improve the quality of agricultural inoculants for achieving enhanced crop productivity.

11.2 Phosphorus Cycling and Availability in Soils

The important reservoir of immobilized P in the soil is organic matter (Richardson 1994). The organic compounds making up the humus fraction are derived from surface vegetation, microbial protoplasm, or metabolic products of the microflora. The various inositol phosphates are often classified together as phytin or related substances and such organic matter components frequently accounts for 20–80 % of the entire organic P fraction. The phospholipid content of humus is invariably small and often 0.1–5 % or sometimes slightly more of the organic phosphorus is tied up in such compounds. A significant part of phospholipids may be phosphatidyl ethanolamine and phosphatidyl choline and these compounds are found in both plants and microorganisms. Phosphorus held within soil microorganisms constitutes a significant component of the total soil P and is estimated to account for around 2–10 % of total soil P. However, at different stages of soil development and within litter layers (soil surface), this may be as much as 50 % (Oberson and Joner 2005; Achat et al. 2010). Usually, soils rich in organic matter contain abundant organic P. Moreover, a good correlation exists between the concentrations of organic P, organic C, and total N. Ratios of organic C to organic P of 100–300:1

are common for mineral soils. Similarly, the nitrogen: organic phosphorus ratio may range from 5 to 20 parts of nitrogen for each part of P. The organic P level, therefore, is directly related to the concentration of other humus constituents, the P content being 0.3–1.0 % and 5–20 % of the C and N concentration, respectively.

Besides organic P, large quantities of the inorganic forms of P occur in minerals where the phosphate is part of the mineral structure, as insoluble calcium, iron or aluminum phosphates (Richardson et al. 2001b; Turan et al. 2006; Vu et al. 2008). Under acidic conditions, P ions are present as H_2PO_4 but are subjected to fixation with hydroxides of Al and Fe at pH below 5. Near neutral pH, HPO_4^{2-} ions are usually present. But above pH 8, the PO_4^{3-} ions form $[Ca_3(PO_4)_2]$ and its availability is reduced drastically. The P nutrient is estimated to be in insufficient amounts in most of the Indian soils as available P. According to the compilation of about 9.6 million soil tests for available P in Indian soils, it was reported that 49.3 % of areas covering different states and union territories are in the low category, 48.8 % in the medium category, and 1.9 % have high phosphorus status (Hasan 1994). Therefore, application of phosphatic fertilizers is unavoidable in intensive farming system. The source of P is only from phosphatic and sulfur rocks, which are nonrenewable sources and use of phosphatic fertilizers leads to the depletion of these resources. Thus, problem of P management in soil is also very tricky and more than 70–90 % of the applied phosphatic fertilizers get fixed in the soil rendering them unavailable for plant uptake under the ideal conditions (Larsen 1967; Holford 1997).

The role of the microbial biomass in the cycling of P in soil has recently received increased attention (Oberson and Joner 2005). Soil microorganisms effectively compete with plants for available orthophosphate from soil solution and also represent a significant pool of immobilized P that is temporarily unavailable to plants. However, significant amounts of P can be released from the microbial biomass in response to seasonal conditions when either carbon becomes limiting or soils undergo cycles of wetting and drying (Turner and Haygarth 2001; Bonkowski 2004). To be available to plants, orthophosphate must diffuse through the rhizosphere (Jakobsen et al. 2005) and as such will be in direct competition for uptake and immobilization by microorganisms. Subsequently, the rate of release of P from microorganisms or the turnover time for the microbial biomass within the rhizosphere will have major implication for P availability to plants. Radioactive-tracer studies indicated that orthophosphate released through microbial turnover contributes significantly to basal rates of mineralization in soil and estimations suggest a turnover time of the total microbial biomass in bulk soil of between 42 and 160 days depending on the farming system, whereas faster rates of turnover were observed in C-amended soils (Oehl et al. 2004; Bünemann et al. 2007). Achat et al. (2010) reported a faster cycling of a major component of the soil microbial P pool (accounting for 80 % of the total microbial P), with a turnover time of less than 10 days in an organic P-dominated forest soil. Recently, Bünemann et al. (2012) measured gross phosphorus fluxes in isotopic dilution studies with ^{33}P-labeled soils that included the biological processes of microbial P immobilization, remineralization of immobilized P, and mineralization of nonmicrobial soil organic P. The

results showed that inorganic P availability primarily affected microbial P immobilization which was the main component of gross P fluxes in both treatments.

Legumes have the capacity to mobilize more P from less residual inorganic P than cereals (Nuruzzaman et al. 2005b; Vu et al. 2008) and different legumes also differed in their capacity to utilize residual inorganic P from the rhizosphere. Hassan et al. (2012) compared the growth, P uptake, and the changes in rhizosphere soil P pools in five grain legumes in a soil with added P. Nodulated chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.), white lupin (*Lupinus albus* L.), yellow lupin (*Lupinus luteus* L.), and narrow-leafed lupin (*Lupinus angustifolius* L.) were grown in a loamy sand soil low in available P to which 80 mg P kg^{-1} was added and harvested at flowering and maturity. At maturity, growth and P uptake decreased in the following order: faba bean > chickpea > narrow-leafed lupin > yellow lupin > white lupin. Compared to the unplanted soil, the depletion of labile P pools (resin P and $NaHCO_3$-P inorganic) was greatest in the rhizosphere of faba bean (54 % and 39 %). Of the less labile P pools, NaOH-P inorganic was depleted in the rhizosphere of faba bean, while NaOH-P organic and residual P was most strongly depleted in the rhizosphere of white lupin. The results suggested that even in the presence of labile P, less labile P pools may be depleted in the rhizosphere of some legumes.

11.3 Microorganisms Involved in Solubilization of Inorganic Phosphorus

The insoluble phosphates predominant in saline and saline alkaline soils include tricalcium phosphate [$Ca_3(PO_4)_2$], carbonate apatites [$Ca_3(PO_4)_2 \cdot CaCO_3$], hydroxy apatites [$Ca_3(PO_4)_2 \cdot Ca(OH)_2$], oxi apatites [$Ca(PO_4)_2 \cdot CaO$], and fluor apatites [$Ca_3(PO_4)_2 \cdot CaF_2$], whereas hydroxyl phosphates of Fe and Al namely dufrenite, strengite [$Fe(OH)_2H_2PO_4$], varisite [$Al(OH)_2H_2PO_4$], etc., are usually present in acidic soils. These unavailable forms are converted to primary orthophosphate ($H_2PO_4^{-}$) and secondary orthophosphates ($H_2PO_4^{-2}$), which are available for plant growth. The ability of soil or rhizosphere bacteria to solubilize mineral phosphates is generally screened on a solid medium containing insoluble phosphate source such as tricalcium phosphate (TCP), apatite, rock phosphate (RP) and, in some cases, Fe and Al phosphates in agar media. The appearance of clearing zones around colonical growth of microorganisms indicates the ability to release Pi from the precipitate of insoluble phosphate and these bacterial strains are considered positive for P solubilization activity. Indicator medium containing dyes such as bromothymol blue (Krishanaraj 1996) or bromocresol green could also be used for better observation (Mehta and Nautiyal 2001; Gadagi and Tongmin 2002). The solubilization of different types of insoluble phosphates varies with the type of microorganisms, the type of phosphates available, media conditions, and available carbon source.

Stalstorm in 1903, first time demonstrated solubilization of TCP by soil bacteria in liquid and on solid media. Since then, a large number of heterotrophic and autotrophic soil microbes representing bacterial, actinomycetes, and fungal species have been identified as active P solubilizers. About 10–50 % of the bacterial isolates tested are capable of solubilizing calcium phosphates and counts of bacteria solubilizing insoluble P may range from 10^5 to 10^7 per gram of soil. Kucey et al. (1989) reported that PSM were present in almost all the soils although their number varied depending upon the soil and climatic conditions. PSM have been isolated from different sources such as, soil (Roychaudhary and Kaushik 1989), rhizosphere (Thakkar et al. 1993), compost (Thakkar et al. 1993; Gupta et al. 1993), rock phosphate (Bardiya and Gaur 1972; Gaur et al. 1973), and root nodules (Halder et al. 1991; Surange and Kumar 1993). The bacteria characterized as active phosphate solubilizers represented diverse groups ranging from autotrophs to heterotrophs, diazotrophs to phototrophs; fungi including mycorrhizal fungi both ectotrophic as well as endotrophic, and actinomycetes. Higher populations of bacteria and fungi capable of dissolving insoluble P were observed in the rhizosphere and rhizoplane of different crops as compared to non-rhizosphere soil (Katznelson and Bose 1959; Puente et al. 2004; Fankem et al. 2006). Tomar (2005) reported that the counts of phosphate-solubilizing bacteria (PSB) were more in chickpea rhizosphere followed by wheat and mustard. These PSB isolates showed large variation in P solubilization on Pikovskaya's medium.

The most important phosphate-solubilizing bacteria belong to genera *Bacillus* and *Pseudomonas*, though species of *Achromobacter*, *Alkaligenes*, *Brevibacterium*, *Corynebacterium*, *Serratia*, and *Xanthomonas* have also been found active in solubilizing insoluble P (Venkateswarlu et al. 1984). Phosphate-solubilizing *Pseudomonas* species isolated from rhizosphere of leguminous and cereal crops include *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, *P. liquifaciens*, *P. pickettii*, *P. putida*, *P. rathonis*, *P. savastanoi*, *P. striata*, and *P. stutzeri* (Rajarathinam et al. 1995; Cattelan et al. 1999). Naik et al. (2008) screened 443 fluorescent pseudomonad strains for the solubilization of tricalcium phosphate and reported that 80 strains (18 %) formed visible dissolution halos on Pikovskaya agar medium plates. Based on phenotypic characterization and 16S rRNA gene phylogenetic analyses, strains were identified as *Pseudomonas aeruginosa*, *P. mosselii*, *P. monteilii*, *P. plecoglosscida*, *P. putida*, *P. fulva* and *P. fluorescens*. The phosphate-solubilizing *Bacillus* species isolated from the rhizosphere of legumes and cereals like rice, maize, and oat, jute, and chilli include *Bacillus subtilis*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, and *B. polymyxa* (Barea et al. 1976; Gaind and Gaur 1991; Rajarathinam et al. 1995). Other P-solubilizing bacteria include species of bacteria like *Acinetobacter*, *Azotobacter chroococcum*, *Burkholderia cepacia*, *Erwinia herbicola*, *Enterobacter agglomerans*, *E. aerogenes*, *Kushneria* sp., *Nitrosomonas*, *Nitrobacter*, *Serratia marcescens*, *Synechococcus* sp., *Rahnella aquatilis*, *Micrococcus*, *Thiobacillus ferroxidans*, and *T. thiooxidans* (Banik and Dey 1983c; Kim et al. 1998a; Sheshardri et al. 2000; Zhu et al. 2011; Azziz et al. 2012). *Rhizobium* and *Bradyrhizobium* strains have also been found to solubilize RP or organic P

compounds effectively through the production of organic acids and/or phosphatases (Halder et al. 1991; Abd-Alla 1994).

Castagno et al. (2011) obtained 50 isolates from Salado river basin and 17 nonredundant strains were identified through BOX-PCR analysis. They were found to be related to *Pantoea*, *Erwinia*, *Pseudomonas*, *Rhizobium*, and *Enterobacter* genera via 16S rRNA gene sequence analysis. Viruel et al. (2011) characterized phosphobacteria from Puna, northwestern Argentina, and P-solubilizing activity was found to coincide with a decrease in pH values of the tricalcium phosphate medium for all strains after 72 h of incubation. Identification by 16S rDNA sequencing and phylogenetic analysis revealed that these strains belong to the genera *Pantoea*, *Serratia*, *Enterobacter*, and *Pseudomonas*. A moderately halophilic phosphate-solubilizing bacterium *Kushneria* sp. YCWA18 was isolated from the sediment of Daqiao saltern on the eastern coast of China (Zhu et al. 2011). The fastest growth of PSB was observed when the culturing temperature was 28 °C and the concentration of NaCl was 6 % (w/v). It was found that the bacterium can survive at a concentration of NaCl up to 20 %. The bacterium solubilized 283.16 μg ml^{-1} phosphorus in 11 days after being inoculated in 200 ml $Ca_3(PO_4)_2$ containing liquid medium and 47.52 μg ml^{-1} phosphorus in 8 days after being inoculated in 200 ml lecithin-containing liquid medium. The growth of the bacterium was concomitant with a significant decrease of acidity of the medium. Prasanna et al. (2011) selected thirty efficient PSB isolates among 226 colonies showing clear zone formation on Pikovskaya's agar medium, which were isolated from rice rhizosphere soils of Southern peninsular region of India. The isolated PSB strains released high amount of phosphorus from tricalcium phosphate and it ranged from 22.4 to 825.8 μg P ml^{-1} and the amount of phosphatase secreted into the medium ranged from 11.6 to 64 U. The efficient strains isolated from various rhizosphere soils were identified as *Enterobacter*, *Micrococcus*, *Pseudomonas*, *Bacillus*, *Klebsiella*, and *Serratia*. Among all the strains, A4 strain (*Enterobacter aerogenes*) released high amount of phosphorus.

Chookietwattana and Maneewan (2012) screened 84 halotolerant bacterial strains for solubilization of insoluble phosphate in the modified Pikovskaya broth and *Bacillus megaterium* strain A12ag showed highest phosphate solubilization under saline conditions. Panhar et al. (2012) showed that PSB populations were higher in rhizosphere of aerobic rice than non-rhizospheric soil and the highest population was found in Pikovskaya and *Pseudomonas* spp. (PS) medium, while the lowest was found in *Pseudomonas aeruginosa* (PA) medium plates. The highest P-solubilizing activity (69.58 %) was found in PSB9 strain grown in national botanical research institute's phosphate growth medium (NBRIP) plate. Singh et al. (2012) screened 35 bacterial isolates for their phosphate-solubilizing ability and 2 of them were identified through 16S rDNA sequencing as *Chryseobacterium* sp. PSR10 and *Escherichia coli* RGR13, respectively. Azziz et al. (2012) examined the abundance and diversity of phosphate-solubilizing bacteria (PSB) in a crop/pasture rotation experiment in Uruguay. The percentage of PSB relative to total heterotrophic bacteria ranged between 0.18 and 13.13 % and 12 isolates showed greatest solubilization activity and were characterized by 16S rDNA sequencing,

10 isolates belonged to the genus *Pseudomonas*, and 2 isolates showed high similarity with members of the genera *Burkholderia* and *Acinetobacter*. Shahid et al. (2012) isolated an *Enterobacter* sp. Fs-11 from sunflower (GeneBank accession no. GQ179978), which converted insoluble tricalcium phosphate to soluble phosphorus up to 43.5 μg ml^{-1} with decrease in pH of the medium up to 4.5 after 10 days incubation at 28 ± 2 °C in the Pikovskaya's broth.

The important P-solubilizing fungi belonged to genus *Aspergillus* and *Penicillium* (Asea et al. 1988; Reyes et al. 1999; Rashid et al. 2004). A few species of *Fusarium oxysporum*, *Trichoderma viride*, *Curvularia lunata*, *Sclerotium rolfsii*, *Alternaria teneuis*, *Humicola*, *Pythium*, *Phoma*, *Acrothecium*, *Morteirella*, *Paecilomyces*, *Rhizoctonia*, *Rhodotorula*, *Candida* sp., *Cunninghamella*, *Oideodendron*, *Pseudogymnoascus*, and *Trichoderma viride* were also found as good solubilizers of insoluble P. *Torula* sp. which are usually not present in soil, have been isolated from compost, and have been characterized for solubilization of TCP and RP by Singh et al. (1980). Among actinomycetes, *Actinomyces*, *Micromonospora*, *Nocardia*, and *Streptomyces* have been reported to solubilize mineral phosphate (Banik and Dey 1983a).

Recently, Tallapragada and Seshachala (2012) studied the native populations of phosphate-solubilizing bacteria and fungi in different rhizospheric soil samples obtained from betel vine plants (*Piper betel* L.). The phosphate-solubilizing capacity of bacteria and fungi revealed the dominance of *Aspergillus* species (26.1 mm) as major phosphate solubilizers, along with *Bacillus subtilis* (46.6 mm) among the bacteria that utilize tricalcium phosphate, potassium dihydrogen phosphate, and rock phosphate as phosphate sources. The other phosphorus-solubilizing microorganisms were *Bacillus* species, *Streptomyces*, *Aspergillus fumigatus*, *Nocardia*, actinomycetes, and certain yeasts. The population of phosphate-solubilizing bacterium *Bacillus subtilis* was 3×10^6 cfu g^{-1} and the population of fungus *Aspergillus niger* was 3×10^5 cfu g^{-1} in the rhizospheric zones of *Piper betel* plants.

The comparative solubilization pattern observed by the use of different PSM showed that TCP is most easily solubilized followed by ferric, aluminum, and RP (Banik and Dey 1981; Gaind and Gaur 1990; Kole and Hazra 1998). Strains of *Pseudomonas* spp. are capable of releasing 160.5–162.5 μg ml^{-1} in the medium containing TCP (Santhi 1998). Strains of *Acetobacter diazotrophicus* isolated from sugarcane were found to release 142–431 μg ml^{-1} Pi from TCP (Maheshkumar et al. 1999). The solubilization of TCP in liquid medium by different fluorescent *Pseudomonas* strains varied from 29 to 105 μg ml^{-1} on 10 days of inoculation and a significant drop in pH of Pikovskaya liquid medium was observed on 10 days of inoculation (Naik et al. 2008). Estimations of phosphate solubilization of different bacterial strains by other methods have been reported to range between 200 and 805 μg ml^{-1} (Nautiyal 1990). *P. fluorescens* strain NJ-101 isolated from agricultural soil was reported to release 74.6 μg ml^{-1} soluble phosphate from inorganic phosphate (Bano and Musarrat 2004). *Enterobacter agglomerans* strains were found to release Pi ranging from 82.6 to 551.3 μg ml^{-1} in medium containing hydroxyapatite (Kim et al. 1997). *Pseudomonas striata* has been reported to be more efficient than *Bacillus* spp. and *Aspergillus awamorii* in solubilizing TCP.

P. putida solubilized TCP to the extent of 50 % (Ostwal and Bhide 1972). Varsha et al. (1994) found that *Aspergillus awamorii* was best in solubilizing TCP (94 %) followed by dicalcium phosphate (54.5 %) and aluminum phosphate (31.8 %). However, ferric phosphate was best solubilized by *Aspergillus niger*.

Many bacteria capable of dissolving tricalcium phosphate fail to solubilize RP (Bardiya and Gaur 1972) and the organic phosphate-mineralizing bacteria or fungi do not prove to be efficient solubilizer of RP (Gaur et al. 1973). Among the different types of RP tested, Gufsa rock phosphate was solubilized maximum followed by Morocco, Jordan, Udaipur, Singhbhum, and Mussoorie rock phosphate (Singh et al. 1984). The growth and population of phosphate solubilizers was correlated with the extent of phosphate solubilized. Similarly, among China, Senegal, Hirapur, Udaipur, and Sonrai rock phosphate, Senegal rock phosphate was most efficiently solubilized by *Rhodotorula minuta* and *Saccharomyces cerevisiae* (Varsha and Patel 1995). Therefore, for effective solubilization of different phosphate types found in soil, it will be worthwhile to isolate rock phosphate dissolving microorganisms by enrichment culture techniques from such soils.

11.4 Mechanisms of Phosphorus Solubilization by Soil Microorganisms

Organic acids and protons are particularly effective in solubilizing precipitated or complexed forms of soil P or by facilitating the release of adsorbed orthophosphate or organic P through ligand exchange reactions (Ryan et al. 2001). Such mechanisms are widely demonstrable under laboratory and, in some cases, under controlled glasshouse conditions. However, their operation and quantification in field soils to directly supply P to plants is more difficult to assess. Moreover, plants themselves display a wide array of root morphological and physiological changes in response to P deficiency (Vance et al. 2003; Richardson et al. 2009b) and thus assessment of microbial verses plant-mediated processes for P mobilization is difficult. Nonetheless, microorganisms are integral to the cycling of soil P and enhancement of microbial activity in the rhizosphere has significant implication for the P nutrition of plants.

11.4.1 Solubilization of Inorganic Phosphorus

The amount of P solubilized under cultural conditions is dependent on the composition of the media and form of inorganic P precipitate used (including Ca-, Fe-, and Al-phosphates and various sources of rock phosphate) along with cultural and sampling procedures. Different mechanisms are employed by various phosphate solubilizing bacterial strains to solubilize bound form of phosphorus.

11.4.1.1 Production of Organic Acids

In most bacteria, mineral phosphate-solubilizing capacity has been shown to be related to the production of organic acids (Rodriguez and Fraga 1999; Shahid et al. 2012). Analyses of supernatants of growth of many phosphate-solubilizing bacteria showed the production of mono-, di-, and tricarboxylic acids (Table 11.1). The amount of acids liberated by these bacteria is more than 5 % of the carbohydrate consumed (Banik and Dey 1983a). A direct correlation between drop in pH and increase in available P of the culture media has been observed in certain cases (Agnihotri 1970; Liu et al. 1992). The most commonly produced acids include citric, fumaric, lactic malic, glyoxalic, succinic, tartaric, and α-ketobutyric acid secreted by *Bacillus megaterium*, *B. circulans*, *E. freundii*, *and Pseudomonas striata*. High performance liquid chromatography of cell-free supernatant of phosphate-solubilizing bacterium *Enterobacter* sp. Fs-11 showed that it produced malic acid and gluconic acid (2.43 and 16.64 μg ml^{-1}, respectively) in Pikovskaya's broth (Shahid et al. 2012). However, the fungi *A. awamorii* and *P. digitatum* were found to synthesize citric, succinic, and tartaric acid (Banik and Dey 1983a).

Glucose-derived gluconic acid (GA) produced in the periplasmic space of Gram-negative bacteria resulted in decrease of pH and seems to directly correlate with the phosphate-solubilizing activity (Goldstein and Liu 1987; Liu et al. 1992). It was shown that 60 mM gluconic acid resulted in the release of approximately 0.1 mM inorganic phosphate (Pi) and it was suggested that gluconic acid produced may cause the release of protons that finally solubilized the insoluble P (Goldstein 1995). The gluconic acid so produced may further oxidized to 2-keto gluconic acid, a very strong naturally occurring organic acid (pK_a—2.6). Thus, mineral phosphate solubilization phenotype is the result of gluconic and 2-keto gluconic acid production via the direct oxidation pathway involving enzymes located on the outer face of the cytoplasmic membrane. The enzymes include glucose dehydrogenases (GDH) that oxidize glucose to gluconic acid (Goldstein 1996) and the cofactor, pyrrolquinoline quinone (PQQ). It was proposed that direct glucose oxidation to gluconic acid is a major mechanism for mineral phosphate solubilization in Gram-negative bacteria.

Production of carboxylic anions is another important mechanism for phosphate mobilization by rhizosphere bacteria. Ryan et al. (2001) reported that among the carboxylic acids identified, dicarboxylic (oxalic, tartaric, malic, fumaric, malonic acids) and tricarboxylic (citric) acids are more effective for P mobilization. Thus, phosphate solubilization/mobilizing effect of microorganisms is due to a combined effect of pH and carboxylates (Puente et al. 2004; Rodriguez et al. 2006). Otani et al. (1996) reported that carboxylic anions are able to replace phosphate from sorption complexes by ligand exchange. Under acidic soil pH conditions, the phosphate ions are precipitated by Fe^{3+} and Al^{3+} and organic acids prevent such precipitation by chelation, forming metalo-organic molecules, e.g., ferric citrate by citric acid (Mortensen 1963). The chelation by dibasic acids may also lead to ion

Table 11.1 Organic acids produced by some Gram-negative bacteria

Bacteria	Organic acids produced	References
Acetobacter sp.	Gluconic acid	Galar and Bolardi (1995)
Azospirillum sp.	Gluconic acid	Rodriguez et al. (2004)
Enterobacter sp.	Malic, gluconic acid	Shahid et al. (2012)
Escherichia freundii	Lactic acid	Sperber (1958)
Pantoea eucalypti	Gluconic acid	Castagno et al. (2011)
Pseudomonas sp.	Gluconic acid	Illmer and Schinner (1992)
Pseudomonas sp.	Citric, gluconic acid	Taha et al. (1969)
Pseudomonas aeruginosa	Gluconic acid	van Schie et al. (1985)
Pseudomonas fluorescens	Gluconic acid, malic, succinic, lactic, fumaric, tartaric, and transaconitic acid	Henri et al. (2008)
Pseudomonas striata	Tartaric, citric acid	Gaur (1990)
Rhizobium leguminosarum	2-ketogluconic acid	Halder et al. (1991)
Sinorhizobium meliloti	Malic, succinic, fumaric acid	Bianco and Defez (2010)

exchanges with hydroxyl phosphates, forming hydroxyl salts of Fe and Al releasing the phosphate ions. Citrate has also been reported to release P from goethite (Geelhoed et al. 1999) or amorphous ferric hydroxides (Dye 1995). Oxalate was also found very effective but was not produced in sufficient amounts by the PSB strains tested. In general, the ability of different carboxylic anions to desorb P decreases with a decrease in the stability constants of Fe or Al-organic acid complex in the order: citrate > oxalate > malonate/malate > tartrate > lactate > gluconate > acetate > formate (Ryan et al. 2001). This result serves to confirm the ability of the strains tested in mobilizing P from insoluble sources, in particular those producing altogether citrate, malate, and tartarate.

Henri et al. (2008) isolated three *P. fluorescens* strains (CB501, CD511, and CE509) from acidic soils of Cameroon, having the ability to solubilize three phosphate types ($Ca_3(PO_4)_2$, $AlPO_4{\cdot}H_2O$, or $FePO_4{\cdot}2H_2O$). It was found that calcium phosphate (Ca-P) solubilization resulted from the combined effects of pH decrease and carboxylic acids synthesis. At pH 4, it was solubilized by most of the organic acids. However, the synthesis of carboxylic acids was the main mechanism involved in the process of aluminum phosphate (Al-P) and Fe-P solubilization. Both were mobilized at pH 4 by citrate, malate, tartarate, and on a much lower level by gluconate and transaconitate. Bianco and Defez (2010) reported that RD64 strain, a *Sinorhizobium meliloti* 1021 strain engineered to overproduce indole-3-acetic acid (IAA) and improved nitrogen fixation ability, was also found highly

effective in mobilizing P from insoluble sources such as phosphate rock (PR). Under P-limiting conditions, the higher level of P-mobilizing activity of RD64 than of the 1021 wild-type strain is connected with the upregulation of genes coding for the high-affinity P transport system, the induction of acid phosphatase activity, and the increased secretion into the growth medium of malic, succinic, and fumaric acids. *Medicago truncatula* plants nodulated by RD64 (*Mt*-RD64), when grown under P-deficient conditions, released larger amounts of another P-solubilizing organic acid, 2-hydroxyglutaric acid, than plants nodulated by the wild-type strain (*Mt*-1021).

In few other cases, the degree of solubilization was not necessarily correlated with acidity or with the decline in pH (Krishanaraj 1987; Asea et al. 1988). Solubilization of Ca-P has even been reported to occur even in the absence of organic acid (Illmer and Schinner 1992). An HPLC analysis of the culture suspension of *Pseudomonas* did not detect any organic acid even though the bacterium solubilized unavailable forms of P (Illmer and Schinner 1995). In each of these cases, acidification of the medium resulted and was postulated that H^+ excretion originating from NH_4 assimilation contributed to acidification (Parks et al. 1990). Krishanaraj (1996) derived MPS^- mutants from *Pseudomonas* and compared with their wild-type with respect to the Pi release in the TCP broth, drop in pH, and identification of organic acid released in the medium. It was found that a highly coordinated reaction caused the dissolution of insoluble P. In the event of P stress, glucose is utilized and gets converted to organic acids that provide H^+ and get cotransported into the external mileu with $H_2PO_4^-$ or HPO_4^{-2}. These reactions are hypothesized to involve the membrane enzymes and organic acid transporters.

11.4.1.2 Production of Inorganic Acids

The solubilization of inorganic P in some cases is attributed to the production and release of inorganic acids (Richardson 2001; Reyes et al. 2001). In the special case of ammonium- and sulfur-oxidizing chemoautotrophs, nitric acid and sulfuric acids are produced (Dugan and Lundgren 1965). The inorganic acids convert $Ca_3(PO_4)_2$ to di- and monobasic phosphates with the net result of an enhanced availability of the phosphorus to plants. Nitric or sulfuric acids produced during the oxidation of nitrogenous materials or inorganic compounds of sulfur react with RP and thereby increase the soluble P. Thus, oxidation of elemental sulfur is a simple and effective means of providing utilizable phosphates. For example, a mixture may be prepared with soil or manure, elemental sulfur, and RP. As the sulfur is oxidized to sulfuric acid by *Thiobacillus*, there is a parallel increase in acidity and net release of soluble P. Nitrification of ammonium salts also leads to a slight but significant liberation of soluble P from RP composts. However, biological sulfur or ammonium oxidation has never been adopted on a commercial scale because of the availability of cheaper and more efficient means of preparing fertilizers. Gaur (1990) observed solubilization of Mussourie rock phosphate (MRP) in soil amended with ammonium sulfate. The available P increased greatly in soil inoculated with PSM and the increase in

solubilization was more with fungal inoculation followed by bacteria and yeast. Application of 1 % farmyard manure further improved P solubilization. The structural complexicity and particle size of P and the quantity of organic acid secreted by microbes were also reported to affect P solubilization.

11.4.1.3 Other Mechanisms of Phosphate Solubilization

Although phosphate solubilization commonly requires acid production, other mechanisms may account for ferric phosphate mobilization. In flooded soil, the iron available as insoluble ferric phosphates may be reduced leading to the formation of soluble iron with concomitant release of P into solution. Such increases in the availability of P on flooding may explain why rice cultivated under water has a lower requirement for fertilizer P than the same crop grown in dry land agriculture. Phosphorus may also be made available for plant uptake by certain bacteria that librates H_2S. Fermentative microorganisms produce H_2S from sulfur-containing aminoacids, or anaerobic sulfate-reducing bacteria like *Desulphovibrio* and *Desulfatomaculum* causes reduction of sulfate to H_2S when the redox potential is low. Hydrogen sulfide reacts with ferric phosphate to yield ferrous sulfide and librates the phosphate.

Humic and fulvic acids are the other chelating substances produced during the decomposition of organic materials. Mishra et al. (1982) reported that 5 % solution of humic acid in alkali could solubilize 362 μg P per gram of RP. The action of humic and fulvic acid is due to the presence of hydroxyl, phenolic, and carboxyl groups (Banger et al. 1985). Respiratory H_2CO_3 production by plants and soil organisms has been found as an alternate mechanism of mineral phosphate solubilization (Juriank et al. 1986). The CO_2 produced in the rhizosphere due to decomposition of organic matter by microbes has also been reported to be involved in increased P availability to plants. The reaction may be with CO_2 directly or due to formation of carbonic acid which reacts with $Ca_3(PO_4)_2$ forming $CaHPO_4$ or $Ca(H_2PO_4)_2$ and $CaCO_3$. Rhizosphere acidification resulting from proton release during N_2 fixation (Tang et al. 1998; Hinsinger et al. 2003) is another process which enhances P availability in alkaline soils because the solubility of Ca phosphates increases with decreasing pH.

11.4.1.4 Isolation of Mineral Phosphate-Solubilizing (mps) Genes

The conversion of insoluble phosphates (both organic and inorganic) to a form accessible to the plants, like organophosphate, is an important trait for a plant growth promoting rhizobacteria (PGPR) for increasing plant yields. Molecular biology techniques are an advantageous approach for obtaining and characterizing improved PGPR strains (Rodriguez and Fraga 1999; Igual et al. 2001). Introduction or overexpression of genes involved in soil P solubilization in natural rhizospheric bacteria is a very attractive approach for improving the capacity of microorganisms

to apply as inoculants. Cloning and transfer of phosphate-solubilizing genes into microorganisms that do not have this capability may avoid the current need of mixing two populations of nitrogen-fixing and phosphate-solubilizing bacteria when used as inoculants (Bashan et al. 2000).

The repression of mineral phosphate-solubilizing activity was observed in the presence of increasing levels of inorganic P in the medium. Goldstein (1986) reported the complete inhibition of MPS activity by *Erwinia herbicola* by addition of 20 mM Pi in the medium. Similarly, it was found that externally added K_2HPO_4 inhibited the MPS activity of *Pseudomonas* Psd 201 (Krishanaraj 1996). The phosphate stress induction of MPS activity and repression of MPS activity by externally added Pi indicated the physiologically regulated gene expression of MPS activity in bacteria. Based on these observations, Goldstein (1986) proposed the existence of *mps* genes in *Erwinia herbicola*. Several genes were induced under P starvation in *E. coli* and constituted the Pho regulon. Recently, the transcriptional control of Pho regulon has been extensively studied in *E. coli* (Makino et al. 2007), *Bacillus subtilis* (Huelett et al. 2007), and *Saccharomyces cerevisiae* (Ogawa et al. 2007). Gene(s) involved in mineral phosphate solubilization from Gram-negative bacteria *Erwinia herbicola* were cloned using shotgun-cloning experiments (Goldstein and Liu 1987) and GDH-mediated dissimilatory bypass system, involving direct oxidation of glucose to gluconic acid in the periplasmic space was found responsible for the mineral phosphate solubilization in *Erwinia herbicola*. Expression of the *mps* gene allowed production of GA in *E. coli* HB101 and conferred the ability to solubilize hydroxyapatite (MPS^+ phenotype). MPS^- mutants of *E. coli* can synthesize GDH, but not PQQ; thus it did not produce GA. On screening a cosmid pHC76 library from *Erwinia herbicola*, they found that a 55 kb insert DNA was able to transform *E. coli*. Transposon mutagenesis of the cosmid construct pMCG 898 carrying a 4.5 kb insert showed that the essential gene was localized in a 1.8 kb region. Based on sequence comparison and minicell analysis, Liu et al. (1992) deciphered that the gene codes for an enzyme pyrrolquinoline quinone (PQQ), a cofactor for the enzyme glucose dehydrogenase (GDH). The cloned 1.8 kb locus encoded protein was found similar to the gene III product of a *pqq* synthesis gene complex from *Acinetobacter calcoaceticus* and to *pqq*E of *Klebsiella pneumoniae* (Liu et al. 1992). Coincidentally, nucleotide sequence analysis of a 7 kb fragment from *Rhanella aquatilis* genomic DNA that induced hydroxyapatite solubilization in *E. coli*, showed two complete open reading frames (ORFs), and a partial ORF. One of the cloned proteins showed similarity to *pqq*E of *E. herbicola*, *K. pneumoniae*, and *A. calcoaceticus* (Kim et al. 1998b), while the partial ORF is similar to the *pqq*C of *Klebsiella pneumoniae*. These genes complemented the cryptic *pqq* genes in *E. coli*, thus allowing GA production.

Another type of gene (*gab*Y) involved in GA production and MPS was cloned from *Pseudomonas cepacia* (Babu-khan et al. 1995). The deduced amino acid sequence was found similar to histidine permease membrane-bound components. In the presence of *gab*Y, GA is produced only if *E. coli* strain expresses a functional glucose dehydrogenase (*gcd*) gene. It was speculated that this ORF could be related to the synthesis of PQQ by an alternative pathway or the synthesis of a *gcd* cofactor

different from PQQ (Babu-khan et al. 1995). In addition, a DNA fragment from *Serratia marcescens* induced quinoprotein glucose-mediated gluconic acid production in *E. coli*, but showed no homology to *pqq* or *gcd* genes (Krishanaraj and Goldstein 2001). They suggested that this gene acted by regulating GA production under cell-signal effects. Other isolated gene JM109 (pKKY) involved in the MPS phenotype was obtained from genomic DNA fragment of *Enterobacter agglomerans* using cosmid (pHC79) genomic library (Kim et al. 1997). The complementation of this gene in *E. coli* JM109 showed the MPS activity, although the pH of the medium was not altered. These results indicate that acid production is an important way, but not the only mechanism, of P solubilization by bacteria (Illmer and Schinner 1995). All these findings demonstrate the complexity of MPS in different bacterial strains, but at the same time, offer a basis for better understanding of phosphate solubilization process.

11.4.1.5 Manipulation of MPS Genes for PGPR Improvement

Expression of the *mps* genes from *Ranella aquatilis* in *E. coli* supported a much higher GA production and hydroxyapatite dissolution in comparison with the donor strain (Kim et al. 1998b), suggesting that different genetic regulation of the *mps* genes might occur in both species. MPS mutants of *Pseudomonas* spp. showed pleiotropic effects, with apparent involvement of regulatory *mps* loci in some of them (Krishanaraj et al. 1999). Two distinct classes of mutants namely, non-solubilizers (MPS^{-}) and delayed expression types (MPS^{d}) were obtained through nitrosoguanidine and Tn5 mutagenesis of *Pseudomonas* strain Psd 201. These mutants also showed different phenotypic classes with respect to metabolic and cell surface properties. The nature of pleiotropies shown by these mutants indicated that these mutational lesions might have occurred in some of the regulatory *mps* loci since the level of expression of zone and time of solubilization got affected in some mutants (Krishanaraj et al. 1999). Gene bank of the MPS^{+} wild-type *Pseudomonas* sp. Psd 201 was mobilized from *E. coli* into MPS^{-} derivative strain *Pseudomonas* Psd 207. Two clones were isolated which could restore MPS^{+} phenotype to Psd 207 and had an insert of the size of 11.8 kb that might contain one or more *mps* loci.

Expression of the mineral phosphate-solubilizing genes (*mps* genes) in a different host could be influenced by the genetic background of the recipient strain, the copy number of the plasmids present, and metabolic interactions. An attempt to improve MPS in PGPR strains, using a PQQ synthase gene from *E. herbicola* was carried out (Rodriguez et al. 2000b). This gene was subcloned in a broad-host range vector pKT230. The recombinant plasmid was expressed in *E. coli* and transferred to PGPR strains of *Burkholderia cepacia* and *Pseudomonas aeruginosa*, using tri-parental conjugation. Several of the exconjugants that were recovered in the selection medium showed a larger clearing halo zone in medium with tricalcium phosphate as the sole P source. This indicated that heterologous expression of this gene in the recombinant strains, gave rise to improved MPS ability in these PGPRs.

A bacterial citrate synthase gene was reported to increase exudation of organic acids and P availability to the plant when expressed in tobacco roots (Lopez-Bucio et al. 2000). Citrate overproducing plants yielded more leaf and fruit biomass when grown under P-limiting conditions and required less P fertilizer to achieve optimal growth. This shows the putative role of organic acid synthesis genes in P uptake in plants.

11.4.2 Mineralization of Organic Phosphorus

The chief source of organic phosphorus compounds entering the soil is the vast quantity of vegetation that undergoes decay. Agricultural crops commonly contain 0.05–0.5 % P in their tissues and this element is found in several compounds or groups of substances in plants, i.e., phytin, phospholipids, nucleic acids, phosphorylated sugars, coenzymes, and related compounds. Phosphorus may also be present as inorganic orthophosphate, especially in vacuoles and internal buffers. The phosphorus in phytin, phospholipids, and nucleic acids is found as phosphate. The nucleic acids, RNA and DNA, consist of a number of purine and pyrimidine bases, pentose sugar, and phosphate. In bacterial cell, the bulk of P is in RNA, usually accounting for one-third to somewhat more than one-half of all the P. DNA contributes from 2 to 10 % of the total P content. The acid-soluble fraction of bacterial protoplasm contains ortho- and metaphosphate, sugar phosphates, many of the coenzymes, and adenosine phosphates.

In this process of organic phosphate solubilization, microorganisms convert the organic P to inorganic forms (Deubel et al. 2000). Thus, the bound element in the plant residue material and in soil organic matter is made available to succeeding populations of plants by the action of bacteria, fungi, and actinomycetes. The mineralization and immobilization of this element are related to the analogous reactions of nitrogen. As a rule, phosphate release is most rapid under conditions favoring ammonification (nitrogen mineralization). Thus, a highly significant correlation is observed between the rates of N and P conversion to inorganic forms and the nitrogen mineralized being from 8 to 15 times, the amount of phosphate made available. There is also a correlation between C (CO_2 release) and P mineralization (a ratio of 100 to 300:1). The results showed that the ratio of C:N:P mineralized microbiologically at the equilibrium condition is similar to the ratios of three elements in humus. Gross organic P mineralization under steady-state conditions can be quantified using isotopic dilution techniques (Achat et al. 2009a, b; Bünemann et al. 2007; Oehl et al. 2001). However, the biological processes of microbial immobilization, remineralization of immobilized P, and mineralization of nonmicrobial organic P likewise replenish phosphate ions in the soil solution (Frossard et al. 2000).

Phosphorus can be released from organic compounds in soil by three groups of enzymes: (1) nonspecific phosphatases, which perform dephosphorylation of phosphor ester or phosphor anhydride bonds in organic matter; (2) phytases, which

specifically cause P release from phytic acid; and (3) phosphonatases and C-P lyases enzymes that perform C-P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil. Availability of organic phosphate compounds for plant nutrition could be a limitation in some soils resulting from precipitation with soil particle ions. Therefore, the capability of enzymes to perform the desired function in the rhizosphere is a crucial aspect for their effectiveness in plant nutrition.

11.4.2.1 Nonspecific Acid Phosphatases

Utilization of organic P by plants and microorganisms requires mineralization (hydrolysis) of phosphorus-containing substrates by phosphatase enzymes which may be of either plant or microbial origin. In plants, this process includes the release from roots of extracellular phosphatases that are considered to be important for capture and recycling of organic P lost from roots or to allow greater access to soil organic P (Richardson et al. 2005). Enhanced phosphatase activity in the rhizosphere in response to P deficiency has been observed across a wide range of plant species and is commonly reported to be higher in P-deficient soils. Chen et al. (2002) showed that depletion of soil organic P was associated with a significant increase in the activity of both mono- and diester phosphatases.

Soil microorganisms produce a range of phosphatases when cultured in laboratory media and have the capacity to utilize P from various forms of organic P that occur in soil. This includes inositol phosphates (phytate and *myo*-inositol hexakisphosphate along with other isomers) and a predominant form of organic P identified in many soils (Lim et al. 2007; Turner 2007). When added to soils, organic P substrates (both mono- and diester) are rapidly hydrolyzed (Macklon et al. 1997). Conversely, when soil suspensions or soil extracts are treated with an excess of phosphatase activity, appreciable amounts of orthophosphate can be released (George et al. 2007). Bünemann (2008) reported that upto 60 % of the total organic P may typically be hydrolyzed by phosphatases with highest amounts being released by phytases (monoester phosphatases active against phytate). Both plant and microbial phosphatases are effective in releasing orthophosphate from soil organic P, with some evidence that microbial enzymes show higher efficiency for P release (Tarafdar et al. 2001). Increased mineralization of soil organic matter associated with higher microbial activity also occurs in the rhizosphere as a result of a microbial "priming effect" due to utilization of exudate C with subsequent mineralization of nutrients from soil organic matter (Cheng 2009).

A single phosphatase enzyme may catalyze the cleavage of ethyl phosphate, glycerophosphate, and phenyl phosphate. On the other hand, diesters may require different enzymes for their breakdown. Phosphatases acting on phospholipids and nucleic acids have diesters as their substrates. The phosphatase enzyme catalyzing hydrolysis of the monoesters often has distinct optima in pH for maximum activity, i.e., active at low pH ranges are acid phosphatases, whereas the enzymes active at

high pH ranges are termed as alkaline phosphatases. Bacterial nonspecific acid phosphatases (phosphohydrolases) (NSAPs) are formed by three molecular families, which have been designated as molecular class A, B, and C (Thallar et al. 1995a). From their cellular location, these enzymes seem to function as organic phosphoester scavengers, releasing inorganic phosphates from nucleotides and sugar phosphates, and thus providing the cell with essential nutrients (Beacham 1980; Wanner 1996).

Several genes involved in biosynthesis of acid phosphatase in Gram-negative bacteria have been characterized (Rossolini et al. 1998). These cloned genes encoding acid phosphatase represent an important source of material for genetic transfer to PGPR strains. For example, the *acp*A gene isolated from *Francisella tularensis* expresses an acid phosphatase with optimum action at pH 6 and with a wide range of substrate specificity (Reilly et al. 1996). Also, genes encoding nonspecific acid phosphatases class A (PhoC) and class B (NapA) isolated from *Morganella morganii* are very promising, since the biophysical and functional properties of the encoded enzymes were extensively studied (Thallar et al. 1994, 1995b). Besides, they are P-irrepressible enzymes showing broad substrate action and high activity around pH 6 and at 30 °C. Macaskie et al. (1997) reported on the successful use of class A NSAPs as tools for environmental bioremediation of uranium-bearing waste water and on heavy metal biomineralization, particularly nickel (Bonthrone et al. 1996; Baskanova and Macaskie 1997). Moreover, the transfer and expression of these genes encoding for NSAPs into plant growth-promoting rhizobacteria could result in bacterial strains with improved phosphate-solubilizing activity. Rodriguez et al. (2000a) isolated a gene from *Burkholderia cepacia* that facilitates phosphatase activity. This gene codes for an outer membrane protein that enhanced the synthesis of soluble phosphates in the medium and could be involved in P transport to the cell. Rodriguez et al. (2006) constructed a plasmid for the stable chromosomal insertion of the *pho*C phosphatase gene from *Morganella morganii* using the delivery system developed by Lorenzo et al. (1990). This plasmid was transferred to *Azospirillum* spp. and the strains with increased phosphatase activity were obtained. Two nonspecific periplasmic acid phosphatase genes (*nap*D and *nap*E) were cloned from *Rhizobium meliloti* (Deng et al. 1998, 2001). The *nap*A phosphatase gene from the soil bacterium *Morganella morganii* was transferred to *Burkholderia cepacia* IS-16, a strain used as biofertilizer, using the broad host range vector PRK293 (Fraga et al. 2001). An increase in extracellular phosphatase activity of the recombinant strain was achieved.

11.4.2.2 Phytases

Phytate is the major component of organic forms of P in soil (Richardson 1994). Phytate is the primary source of inositol in its basic form and the major stored form of phosphate in plant seeds and pollen. Monogastric animals are incapable of using the P bound in the phytate because their gastrointestinal tracts have low levels of

phytase activity. Thus, nearly all the dietary phytate phosphorus ingested by these species is excreted, resulting in P pollution in areas of intensive animal production. Supplemental microbial phytase in corn–soybean meal diets for swine and poultry effectively improved phytate phosphorus utilization by these animals and reduced their fecal P excretion by up to 50 % (Lei et al. 1993). Therefore, phytases have emerged as very attractive enzymes for industrial and environmental applications. Most phytases belong to high molecular weight acid phosphatases. The phytase enzyme librates phosphate from phytic acid or its calcium–magnesium salt (phytin) resulting in accumulation of inositol. Some species make intracellular phytase, while others excrete extracellular phytase enzymes. Moreover, some phytases are reasonably specific and act chiefly on inositol phosphates, whereas nonspecific phosphatases remove phosphorus from dissimilar organic compounds. Phytase activity is widespread and about 30–50 % of the bacterial isolates from soil synthesized this enzyme. Its activity in nature is enhanced by addition of carbonaceous materials that increase the size of community. Species of *Aspergillus*, *Rhizopus*, *Cunninghamella*, *Arthrobacter*, *Streptomyces*, *Pseudomonas,* and *Bacillus* have been found to synthesize the phytase enzyme.

The ability of plants to obtain P directly from phytate is very limited. However, the growth and P nutrition of *Arabidopsis* plants supplied with phytate was improved significantly when they were genetically transformed with the phytase gene (*phy*A) from *Aspergillus niger* (Richardson et al. 2001a). This resulted in improved P nutrition such that the growth and P content of the plant was equivalent to control plants supplied with inorganic P. The enhanced utilization of inositol phosphate by plants in the presence of soil microbes has also been reported (Richardson et al. 2001b). Therefore, developing agriculture inoculants with high phytase production would be of great interest for improving plant nutrition and reducing P pollution in soil.

Thermally stable phytase gene (*phy*) from *Bacillus* sp. DS11 (Kim et al. 1998d) and from *B. subtilis* VTT E-68013 (Kerovuo et al. 1998) have been cloned. Han et al. (1999) reported that 1.4 kb DNA fragment containing the coding region of the *phy*A gene from *Aspergillus niger* was expressed in *Saccharomyces cerevisiae*. The recombinant extracellular phytase from *S. cerevisiae* effectively hydrolyzed phytate phosphorus from corn or soybean meal in vitro. Acid phosphatase phytase genes from *E. coli* (*app*A and *app*A2 genes) have also been isolated and characterized (Rodriguez et al. 1999; Golovan et al. 2000). The bifunctionality of these enzymes makes them attractive for solubilization of organic P in soil. Richardson et al. (2001a) showed that when grown in defined media, utilization of phytate-P by grass and legume pasture species was improved by inoculation of bacterial isolate with high phytase activity. Also, neutral phytases have great potential for genetic improvement of plant growth-promoting rhizobacteria. Neutral phytase genes have been cloned from *B. subtilis* and *B. licheniformis* (Tye et al. 2002). For example, a *phy*A gene was cloned from the FZB45 strain of *B. amyloliquefaciens*, having plant growth promoting activity (Idriss et al. 2002). It showed the highest extracellular phytase activity and the diluted culture filtrates of these strains stimulated growth of maize seedlings under limited P in the presence of phytate. Culture filtrates

obtained from a phytase negative mutant strain, whose *phy*A gene was disrupted, did not stimulate plant growth. In addition, growth of maize seedlings was enhanced in the presence of purified phytase.

Plants genetically modified to release an extracellular fungal phytase (from *Aspergillus niger*) from roots showed similar novel ability to acquire P directly from phytate (Richardson et al. 2005). Assessment of rhizosphere soils after plant growth indicated a depletion of phytase-labile P that, although soil-type dependent, did not differ substantially between control and transgenic lines or to control soils without plants (Richardson et al. 2009b). This suggests that microorganisms are in fact a key driver in regulating the mineralization of phytate in soil and their presence within the rhizosphere may compensate for a plant's inability to otherwise acquire P directly from phytate. Thus, these experiments provided strong evidence that phytase activity can be important for stimulating plant growth under limited P in soil and support the potential of using phytase genes to improve or transfer the P-solubilizing trait to PGPR strains used as agricultural inoculants.

11.5 Plant Growth Stimulation by Inoculation of Phosphate-Solubilizing Bacteria

Inoculation of crop plants with P-mineralizing microorganisms resulted in enhanced crop productivity and thus provided evidence for microbially mediated P availability to plants. Various mechanisms are employed by microorganisms to enhance the capacity of plants to acquire P from soil including (1) increased root growth through hormonal stimulation of root growth by production of indole-3-acetic acid, gibberellins, or ACC deaminase enzyme (Richardson et al. 2009a; Malik and Sindhu 2011; Khandelwal and Sindhu 2012); (2) alteration of sorption equilibria that may result in increased net transfer of orthophosphate ions into soil solution or facilitate the mobility of organic P either directly or indirectly through microbial turnover (Seeling and Zasoski 1993); and (3) through induction of metabolic processes that are effective in directly solubilizing and mineralizing P from sparingly available forms of soil inorganic and organic P (Richardson et al. 2009a).

Inoculation of cereal or legume plants with different P-solubilizing microorganisms generally resulted in improved growth and P nutrition, especially under glasshouse conditions and in fewer cases under the field conditions (e.g., see reviews by Kucey et al. 1989; Rodriguez and Fraga 1999; Gyaneshwar et al. 2002; Sindhu et al. 2009; Zaidi et al. 2009; Khan et al. 2010). In some cases, inconsistent performance was observed under field conditions and it was commonly attributed to various factors that include lack of persistence and competiveness of introduced microorganisms in soil and poor understanding of actual mechanisms involved in growth promotion, where P-mobilization may not

necessarily be the primary mechanism (Sindhu and Dadarwal 2000; Richardson 2001; Zaidi et al. 2009).

11.5.1 Inoculation Effect of P-Solubilizing Bacteria on Crop Growth

The first evidence to show that inoculation of seedling with P-solubilizing bacteria increased the P uptake and yield of oat was performed by Gerretson (1948). Subsequently, improved plant growth responses and increased Pi uptake on addition of RP were reported (Banik and Dey 1983b; Bagyaraj et al. 2000; Sindhu et al. 2010). Phosphatic biofertilizers were first prepared in USSR using *Bacillus megaterium* var. *phosphaticum* as P-solubilizing bacteria and the product was named as "phosphobacterin." It was extensively used in collective farming for seed and soil inoculation to cover an area of 14 million hectares annually and reported to give 5–10 % increase in crop yields. Inoculation experiments conducted with phosphobacterin and other PSM for various crops like oat, wheat, potatoes, groundnut, peas, soybean, tomatoes, and tobacco showed an average 10–15 % increase in yields in about 30 % of the experiments conducted (Kundu and Gaur 1980a; Agasimani et al. 1994; Dubey 1997). The variations under field conditions are expected due to the effect of various environmental conditions and survival of the inoculant strains in the soil.

The agronomic influence of some commonly used phosphorus-solubilizing bacterial species is listed in Table 11.2. Inoculation of phosphorus-solubilizing bacteria along with RP resulted in increased availability of Pi for plant utilization (Hebbara and Suseeladevi 1990; Jisha and Alagawadi 1996). It was observed that inoculation of mineral phosphate-solubilizing bacteria (MPSB) along with application of 17.5 kg P ha^{-1} as Mussoorie rock phosphate (MRP) resulted in increased dry matter in chickpea and was as effective as single super phosphate application (Prabhakar and Saraf 1990). Kundu and Gaur (1984) observed positive effect on inoculation with a mixture of *Pseudomonas striata* and *Aspergillus awamorii* in rice crop. Increase in dry matter production and P uptake from 10 to 27 % and 15 to 34 %, respectively, was observed by inoculation of *Penicillium bilaji* in chernozemic soil with low P availability in wheat crop (Kucey 1987, 1988). The addition of RP (low P solubility) had little effect, while monoammonium phosphate (commercial fertilizer with high soluble P content) resulted in the highest yields and P uptake. The addition of *P. bilaji* to these P sources did not increase P availability but increased release of P from soil (Kucey 1987). Rachewad et al. (1992) reported that addition of PSB along with RP resulted in increased P uptake by sunflower under field conditions.de Freitas et al. (1997) observed that inoculation with PSB significantly increased the number and weight of pods and seed yield of canola (*Brassica napus*) but did not affect the P uptake. Saraf et al. (1997) showed that PSB inoculation increased seed yield (10.3 q ha^{-1}) of chickpea as compared to control

Table 11.2 Inoculation effect of phosphate-solubilizing bacteria on P uptake and crop yield

Bacteria	Crop	Conditions	Response	References
Pseudomonas putida	Canola	Greenhouse	Increased P uptake and yield	Lifschitz et al. (1987)
Pseudomonas sp.	Chickpea	Greenhouse	Increased P uptake and dry matter	Krishanaraj (1996)
Pseudomonas striata	Groundnut	Field	High pod yield and P uptake	Agasimani et al. (1994)
Bacillus subtilis, *B. circulans*, and *Aspergillus niger*	Mungbean	Field	Enhanced nodulation and grain yield	Gaind and Gaur (1991)
Enterobacter cloacae, *Burkholderia cepacia*, and *Serratia marcescens*	Bamboo	Greenhouse	Increased dry matter	Maheshkumar (1997)
Pseudomonas fluorescens	Maize	Greenhouse	Increased grain yield and P content	Henri et al. (2008)
Pseudomonas striata	Rice	Greenhouse	Increased P uptake and yield	Monod et al. (1989)
Pseudomonas striata	Soybean	Field	Increased yield and P content	Dubey (1997)
Pseudomonas striata and *Bacillus polymyxa*	Wheat	Greenhouse	Increased P uptake and yield	Kundu and Gaur (1980b)
Azospirillum lipoferum and *Bacillus megaterium*	Wheat	Greenhouse	Increased shoot P and shoot weight	El Komy (2005)
Bacillus spp. PSB9 and PSB16	Rice	Glass house	Increased P uptake in plants and higher plant biomass	Panhwar et al. (2011)
Bacillus sp.	Cotton	Field	Increased soil P and higher seed cotton yield	Qureshi et al. (2012)

(8.8 q ha^{-1}). Increased grain yield (13–69 %) and uptake of N and P was reported in chickpea by inoculation of PSB along with phosphatic fertilizers. Similarly, the grain and straw yield of chickpea was enhanced with increasing level of P (0–60 kg P_2O_5 ha^{-1}), which was further improved by inoculation of PSB (Sarawgi et al. 1999, 2000). Significantly higher yield (19.5 q ha^{-1}) was observed in soybean on PSB inoculation and on addition of 26.4 kg P ha^{-1} single super phosphate (SSP) as compared to control (16.3 q ha^{-1}) (Dubey 2001). Sharma (2003) observed that addition of RP with PSB increased grain yield (0.9–1.8 t ha^{-1}), N uptake (18–38 kg ha^{-1}), P uptake (2.7–6.6 kg ha^{-1}), and K- uptake (16–41 kg ha^{-1}) in rice–wheat cropping system.

Dey et al. (2004) found that inoculation of peanut with plant growth-promoting fluorescent pseudomonad isolate PGPR1, which solubilized TCP under in vitro conditions, significantly enhanced the pod yield (23–26 %, respectively), haulm yield, and nodule dry weight over the control during 3 years in field trials. Henri et al. (2008) conducted a greenhouse trial in *Zea mays* by inoculation of three *Pseudomonas fluorescens* strains (CB501, CD511, and CE509), having the ability

to solubilize the three phosphorus types. Inoculation of *P. fluorescens* strains showed positive effects on the growth, grain yield, and P uptake. The results revealed that strain CB501 was the best plant growth promoter with a global effect of +37 %, followed by strain CE509 (+21.2 %) and strain CD511 (+16.7 %). Thus, inoculation with phosphate-solubilizing *P. fluorescens* strains made more soluble P available to the growing maize plants. Bianco and Defez (2010) found that *Medicago truncatula* plants inoculated with P-mobilizing *Sinorhizobium meliloti* strain *Mt*-RD64 exhibited higher levels of dry-weight production than *Sinorhizobium meliloti*-1021 plants. P-starved *Mt*-RD64 inoculated plants showed significant increases in both shoot and root fresh weights when compared to P-starved *Sinorhizobium meliloti*-1021 plants. Ekin (2010) evaluated the effect of application of PSB *Bacillus* M-13, with and without varying amounts of phosphorus (P) fertilizer, on growth and yield of sunflower under field conditions. The PSB application was able to mobilize P efficiently in the sunflower and improved seed quality and oil yield. It also enhanced the head diameter, 1,000 seed weight, kernel ratio, and oil content and led to seed and oil yield increases of 15.0 and 24.7 % over no application, respectively. A much greater effect was observed when PSB was used in conjunction with P fertilizers. It was found that the highest seed yield of sunflower was achieved with about 50 kg P_2O_5 ha^{-1} when used in conjunction with PSB.

Inoculation of phosphate-solubilizing *Pantoea eucalypti* strains onto *Lotus tenuis* plants showed a significant plant growth-promoting activity (Castagno et al. 2011). Panhwar et al. (2011) evaluated the ability of two PSB strains, *Bacillus* spp. PSB9 and PSB16 on growth of aerobic rice (*Oryza sativa* L.) along with different doses of RP (0, 30 and 60 kg ha^{-1}) in glasshouse experiments. The PSB strains PSB9 and PSB16 solubilized significantly high amounts of P (20.05–24.08 mg kg^{-1}) compared to non-inoculated (19–23.10 mg kg^{-1}) treatments planted in plastic pots containing 3 kg soil. Significantly higher P solubilization (24.08 mg kg^{-1}) and plant P uptake (5.31 mg $plant^{-1}$) was observed with the PSB16 strain at the highest P level of 60 kg ha^{-1}. The higher amounts of soluble P in the soil solution increased P uptake in plants and resulted in higher plant biomass (21.48 g $plant^{-1}$) at 60 days of growth. PSB strains also increased plant height (80 cm) and improved root morphology in aerobic rice. Yousefi et al. (2011) performed the field experiment that included four soil types (clay, clay loam, loam, and sandy loam), three phosphorus fertilizer levels (0, 20, and 40 mg kg^{-1}), and four levels of phosphate-solubilizing microorganisms (PSM). Resulted indicated that the highest shoot dry matter was found in clay loam soil (21.5 g pot^{-1}) at the time of physiological maturity. Combined application of PSB and arbuscular mycorrhizal fungi (AMF) increased shoot dry matter yield, seed grain spike number, and grain yield by 52, 19, and 26 %, respectively, compared to the controls.

Chookietwattana and Maneewan (2012) observed that inoculation with halotolerant PSB *Bacillus megaterium* strain on tomato (*Lycopersicon esculentum* Mill cv. Seeda) significantly increased the germination percentage and germination index, especially at NaCl concentration between 30 and 90 mM and increased the seedling dry weight at NaCl concentration upto 120 mM. Singh et al. (2012) found

that seed inoculation of *Macrotyloma uniflorum* (horsegram) by phosphate-solubilizing *Chryseobacterium* sp. PSR10 strain showed better plant growth promotion in sterilized and unsterilized soil under greenhouse conditions. Seed inoculation in a field experiment with 50 % of the recommended dose of nitrogen and phosphorus fertilizers increased the plant growth, chlorophyll content, nitrate reductase activity, phosphorus content, and crop yield. Shahid et al. (2012) showed that inoculation of sunflower with *Enterobacter* sp. Fs-11 and its rifampicin-resistant derivative in sterile sand and natural soil resulted in increased plant height, fresh weight, dry weight, and total phosphorus contents as compared to uninoculated plants. Qureshi et al. (2012) reported that inoculation of cotton with P solubilizer *Bacillus* sp. produced significantly higher seed cotton yield 1,630 as compared to 1,511 kg ha^{-1} under field conditions in clay loam soil with pH 8.3. The highest seed cotton yield was observed at highest fertilizer level, i.e., 1,733 kg ha^{-1} with inoculum. The physical parameters like plant height, number of bolls per plant, boll weight, and soil available P were also found higher in the inoculated treatments.

11.5.2 Coinoculation of P-Solubilizing Bacteria with Other Beneficial Microbes

Several experiments conducted in legume and nonlegume crops by coinoculation of PSM with diazotrophs have shown synergistic effects with regard to increase in population of both bacteria and significant increase in crop yields in comparison to single inoculation (Kucey et al. 1989). The synergistic effect was observed after coinoculation of nitrogen-fixing bacteria with PSB. For example, the inoculation of phosphate-solubilizing bacteria either alone or in combination with *A. chroococcum* enhanced the yield and nutrient uptake of cotton and wheat in field trials (Kundu and Gaur 1980c, 1982). Increased phosphorus availability by *P. putida* to common bean plants on coinoculation with *Rhizobium phaseoli* has been found to increase nodulation of common bean (Grimes and Mount 1984). Seed inoculation with thermo-tolerant PSM (viz. *Bacillus subtilis*, *B. circulans*, and *Aspergillus niger*) improved nodulation, available P_2O_5 content of soil, root and shoot biomass, straw and grain yield, and P and N uptake by mungbean (Gaind and Gaur 1991). Soybean seeds inoculated with *Bradyrhizobium japonicum* along with inoculation of PSB showed significantly higher nodulation and yield (Chandra et al. 1995).

Increased nodulation, yield attributes, seed index, and seed yield have also been reported due to combined inoculation of *P. striata* and *B. japonicum* (Dubey 1997; Kumrawat et al. 1997). Similarly, significant increase in nitrogenase activity, growth, and grain yield of pea was found due to dual inoculation of *Rhizobium leguminosarum* and PSB (Srivastava et al. 1998). El Sayed (1999) observed that coinoculation of *Rhizobium leguminosarum* and P-solubilizing *Pseudomonas*

striata significantly increased the dry matter content, grain yield, and N and P uptake of lentil over the uninoculated control. Sonboir and Sarawgi (2000) reported increased nutrients uptake (N, P, and K), grain yield, and pods plant^{-1} with increasing level of P in chickpea that was further enhanced by inoculation of PSB. Jain and Singh (2003) found that *Rhizobium*, PSB, and potassium (50 kg ha^{-1}) increased P and N uptake by chickpea. Inoculation of PSB along with *Azospirillum* increased the grain and straw yield of barley by 6.1 and 9.2 % as compared to control (Yadav et al. 2004).

Mycorrhizal associations are best known to improve plant growth in nutritionally deficient soils by the stimulation of P uptake by fungal hyphae (Gianinazzi-Pearson 1996; Harrison 2005). Synergistic interaction between PSM and vesicular arbuscular mycorrhizal (VAM) fungi has been found and the positive responses were associated with low concentration of active calcium in soils. Ghosh and Poi (1990) reported improved nodulation, plant growth, P uptake, and PSM population due to combined inoculation with *Bacillus polymyxa* and *Glomus fasciculatum* in soybean, groundnut, mungbean, and lentil. Tilak et al. (1995) reported that dual inoculation with *Pseudomonas striata* and VAM fungi (*G. fasciculatum* and *G. mosseae*) significantly increased the bean yield, root biomass, and total P uptake by soybean plants over uninoculated control in alluvial sandy soils. The P-solubilizing bacteria behaved as mycorrhiza helper bacteria (MHB) because they promoted root colonization when associated with mycorrhizal fungi (Garbaye 1994). Toro et al. (1997) reported that combined inoculation of *G. intraradices* and *Bacillus subtilis* significantly increased plant biomass and N and P accumulation in onion plant tissues. The inoculated rhizobacteria released Pi from the added RP and at least 75 % of the P in dually inoculated plants was derived from the added RP. Kim et al. (1998c) observed a significantly higher soluble P concentration in tomato plants with the inoculation of PSB and AM fungi. Thus, these myco–rhizosphere interactions between bacterial and fungal plant association contributed to biogeochemical P cycling and promoted a sustainable nutrient supply to plants.

11.6 Conclusions and Future Prospects

Soil microorganisms play a pivotal role in various biogeochemical cycles and are responsible for the cycling of nutrients in the plant utilizable form (Wall and Virginia 1999; Sindhu et al. 2010; Richardson and Simpson 2011). These beneficial microbes influence the aboveground ecosystems by contributing to plant nutrition, plant health, soil structure, and soil fertility (Glick 1995; Sindhu et al. 2009). Various commercial products primarily based on microbial isolates capable of solubilizing P are widely promoted as plant growth promoting and developed as biofertilizers for extensive use in cropping systems for northern America, Australia, China, and India. For example, isolates of *Penicillium* spp., having the capacity to solubilize P under various laboratory conditions and the ability to colonize the

rhizosphere of a range of potential host plants, appeared to have high potential for development as inoculants (Kucey 1987; Wakelin et al. 2004; Harvey et al. 2009). On the other hand, in a recent evaluation of the performance of *Penicillium bilaii* inoculant on wheat crops across a range of 47 field experiments, Karamanos et al. (2010) reported no consistent benefit in terms of plant P nutrition and found no relationship between growth responses and any soil or environmental parameters, despite the majority of trials being responsive to P addition. In such cases, poor competitive ability and lack of persistence of inoculants in soils are commonly considered to be an important factor that may restrict their effectiveness (Sindhu and Dadarwal 2000; Richardson 2001). A key requirement for successful application of inoculants is the development of appropriate formulation and delivery systems to ensure survival and effective establishment of target microorganisms within the rhizosphere.

Opportunities for enhancing microbially mediated P availability in soils might be achieved by either management of existing populations of microorganisms to optimize their capacity to mobilize P or through the use of specific microbial inoculants. In addition, there is a need to better understand how soil properties and/or environmental factors may influence the efficacy or potential for P mobilization. Esberg et al. (2010) showed correlation between microbial respiration and changes in NaOH extractable P which suggested that microbial access to this fraction was greater. Moreover, stimulation of root growth or greater elongation of root hairs (Vessey and Heisinger 2001) by specific microorganisms may enhance plant P nutrition indirectly by allowing greater exploration of soil, rather than by direct increase in the availability of soil P. Moreover, microbial activity and community composition in the rhizosphere are influenced not only by availability of carbon but also by interaction with various plant- and microbially derived signal molecules (Badri et al. 2009; Bais et al. 2006). These secondary metabolites include flavonoids, phytoalexins, other antimicrobial compounds, and various phytostimulants (Xie and Yoneyama 2010) that may mimic or interfere with microbial signaling mechanisms through quorum sensing (e.g., *N*-acyl homoserine lactones; AHLs). Thus, quorum sensing has been found to play an important role in regulation of growth and function of various soil bacteria, including symbionts and some pathogens that are known to inhabit the rhizosphere (Barriuso et al. 2008; Teplitski et al. 2011).

Recently, different methods and techniques have been developed to characterize and conserve various agriculturally important microbial communities from different environments for their optimal utilization in agriculture (Kirk et al. 2004; Naik et al. 2008). Microbial communities in soil are highly diverse; bacteria alone may be represented by as many as 10^4 species per gram of soil with indications of more than one million distinct soil bacterial genomes (Torsvik et al. 2002; Gans et al. 2005). The knowledge generated on biodiversity and genetic manipulation of P-solubilizing bacteria will be useful to design strategies for use of these bacterial strains as inoculants in sustainable and organic agriculture. This includes ecological consideration of single microorganism (as inoculant) or different groups of soil microorganisms (as communities), how they interact in the rhizosphere or

within roots (endophytes), their ability to mobilize P from different soil fractions, and how soil and farm management practices influence these processes. Azziz et al. (2012) examined the abundance and diversity of phosphate-solubilizing bacteria (PSB) in a crop/pasture rotation experiment in Uruguay. In the first year of sampling, abundance of PSB was significantly higher in natural prairie (NP) and permanent pasture (PP) than in continuous cropping (CC). The percentage of PSB relative to total heterotrophic bacteria ranged between 0.18 and 13.13 %. PSB diversity also showed statistical differences among treatments, with PP populations more diverse than those present in CC. In the second year samples, no differences were found in PSB abundance or diversity. Similarly, George et al. (2009) found no differences in bacterial community structure in the rhizosphere or on the root surface (rhizoplane) of tobacco (*Nicotiana tabacum*) plants modified to release an extracellular fungal phytase as compared to control lines. By contrast, large differences in community structure occurred in response to soil treatments that were specifically implemented to modify P availability.

Thus, complex interactions in the rhizosphere between the PSB, other microorganisms, plant, and the environment are responsible for the variability observed in solubilization of bound phosphates, Pi uptake, and plant growth promotion. The inconsistency in performance of these inoculant strains is a major constraint to the wide spread use of PSB in commercial agriculture. Genetic manipulation of plants and microorganisms for key traits that are known to be associated with P-mobilization or growth promotion (George et al. 2005; Rodriguez et al. 2006), along with generation of specific mutants in key target genes for particular traits such as organic anion release in *Pseudomonas* spp. (Miller et al. 2010), could be useful for both elucidation of mechanisms and for quantifying their contribution to increased P availability in soil. Further, the efficacy of phosphate-solubilizing bacteria can be improved by developing the better cultural practices and delivery systems that favor their establishment in the rhizosphere. In near future, the biotechnological approaches used in manipulation of bacterial traits with improved efficiency of P solubilization in bacteria and their inoculation as phosphatic biofertilizer may enhance plant growth leading to improved crop productivity.

References

Abd-Alla MH (1994) Use of organic phosphorus by *Rhizobium leguminosarum* biovar *viceae* phosphatases. Biol Fertil Soils 18:216–218

Achat DL, Bakker MR, Augusto L, Saur E, Dousseron L, Morel C (2009a) Evaluation of the phosphorus status of P-deficient podzols in temperate pine stands: combining isotopic dilution and extraction methods. Biogeochemistry 92:183–200

Achat DL, Bakker MR, Morel C (2009b) Process-based assessment of phosphorus availability in a low phosphorus sorbing forest soil using isotopic dilution methods. Soil Sci Soc Am J 73:2131–2142

Achat DL, Morel C, Bakker MR, Augusto L, Pellerin S, Gallet-Budynek A, Gonzalez M (2010) Assessing turnover of microbial biomass phosphorus: combination of an isotopic dilution method with a mass balance model. Soil Biol Biochem 42:2231–2240

Agasimani CA, Mudalagiriyappa MV, Sreenivasa MH (1994) Response of groundnut to phosphate solubilizing microorganisms. Groundnut News 6:5–7

Agnihotri VP (1970) Solubilization of insoluble phosphates by some soil fungi isolated from nursery seed beds. Can J Microbiol 16:877–880

Arcand MM, Schneider KD (2006) Plant-and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock: a review. An Acad Bras Cienc 78:791–807

Asea PEA, Kucey RWN, Stewart JWB (1988) Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol Biochem 20:459–464

Azziz G, Bajsa N, Haghjou T, Taule C, Valverde A, Igual JM, Arias A (2012) Abundance, diversity and prospecting of culturable phosphate solubilizing bacteria on soils under crop–pasture rotations in a no-tillage regime in Uruguay. Appl Soil Ecol 61:320–326

Babu-khan S, Yeo C, Martin WL, Duron MR, Rogers R, Goldstein A (1995) Cloning of a mineral phosphate solubilizing gene from *Pseudomonas cepacia*. Appl Environ Microbiol 61:972–978

Badri DV, Weir TL, van der Lelie D, Vivanco JM (2009) Rhizosphere chemical dialogues: plant-microbe interactions. Curr Opin Biotechnol 20:642–650

Bagyaraj DJ, Krishanaraj PU, Khanuja SPS (2000) Mineral phosphate solubilization: agronomic implications, mechanism and molecular genetics. Proc Indian Natl Sci Acad B 66:69–82

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Banger KC, Yadav KS, Mishra MM (1985) Transformation of rock phosphate during composting and the effect of humic acid. Plant Soil 85:259–266

Banik S, Dey BK (1981) Solubilization of inorganic phosphate and production of organic acids by microorganisms isolated in sucrose-tricalcium phosphate agar plates. Zentralbl Microbiol 136:478–486

Banik S, Dey BK (1983a) Alluvial soil microorganisms capable of utilizing insoluble aluminium phosphates as a sole source of phosphorus. Zentralbl Microbiol 138:437–442

Banik S, Dey BK (1983b) Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate solubilizing microorganisms. Plant Soil 69:353–364

Banik S, Dey BK (1983c) Phosphate solubilizing potentiality of microorganisms capable of utilizing aluminium phosphate as a sole phosphate source. Zentralbl Microbiol 138:17–23

Bano N, Musarrat J (2004) Characterization of a novel carbofuran degrading *Pseudomonas* sp. with collateral biocontrol and plant growth promoting potential. FEMS Microbiol Lett 231:13–17

Bardiya MC, Gaur AC (1972) Rock phosphate dissolution by bacteria. Indian J Microbiol 12:269–271

Barea JM, Navarro E, Montoya E (1976) Production of plant growth regulators by rhizosphere phosphate solubilizing bacteria. J Appl Bacteriol 40:129–134

Barriuso J, Ramos Solano B, Fray RG, Camara M, Hartmann A, Gutiérrez Manero FJ (2008) Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. Plant Biotechnol J 6:442–452

Bashan Y, Moreno M, Troyo E (2000) Growth promotion of the sea-water irrigated oil seed halophyte *Salicornia bigelovii* inoculated with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp. Biol Fertil Soils 32:265–272

Baskanova G, Macaskie LE (1997) Microbially-enhanced chemisorption of nickel into biologically-synthesized hydrogen uranyl phosphate: a novel system for the removal and recovery of metals from aqueous solutions. Biotechnol Bioeng 54:319–329

Beacham IR (1980) Periplasmic enzymes in Gram negative bacteria. Int J Biochem 10:877–883

Bianco C, Defez R (2010) Improvement of phosphate solubilization and *Medicago* plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. Appl Environ Microbiol 76:4626–4632

Bieleski RL (1973) Phosphate pools, phosphate transport and phosphate availability. Annu Rev Plant Physiol 24:225–252
Bonkowski M (2004) Protozoa and plant growth: the microbial loop in soil revisited. New Phytol 162:617–631
Bonthrone KM, Baskanova G, Lin F, Macaskie LE (1996) Bioaccumulation of nickel by intercalation into polycrystalline hydrogen uranyl phosphate deposited via an enzymatic mechanism. Nat Biotechnol 14:635–638
Bünemann EK (2008) Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. Soil Biol Biochem 40:2116–2129
Bünemann EK, Marschner P, McNeill AM, McLaughlin MJ (2007) Measuring rates of gross and net mineralisation of organic phosphorus in soils. Soil Biol Biochem 39:900–913
Bünemann EK, Oberson A, Liebisch F, Keller F, Annaheim KE, Huguenin-Elie O, Frossard E (2012) Rapid microbial phosphorus immobilization dominates gross phosphorus fluxes in a grassland soil with low inorganic phosphorus availability. Soil Biol Biochem 51:84–95
Bünemann EK, Heenan DP, Marschner P, McNeill AM (2006) Long term effects of crop rotation, stubble management and tillage on soil phosphorus dynamics. Aust J Soil Res 44:611–618
Carrillo AE, Li CY, Bashan Y (2002) Increased acidification in the rhizosphere of cactus seedlings induced by *Azospirillum brasilense*. Naturewissenschaften 89:428–432
Castagno LN, Estrella MJ, Sannazzaro AL, Grassano AE, Ruiz OA (2011) Phosphate solubilization mechanism and *in vitro* plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado river basin (Argentina). J Appl Microbiol 110:1151–1165
Cattelan AJ, Hartel PG, Furhmann FF (1999) Screening of plant growth promoting rhizobacteria to promote early soybean growth. Soil Sci Soc Am J 63:1670–1680
Chandra K, Mukherjee PK, Karmakar JB, Sharma BK (1995) Effect of phosphate solubilizing bacteria on rhizobial symbiosis in soybean at rainfed conditions of Manipur. Environ Ecol 13:436–438
Chen CR, Condron LM, Davis MR, Sherlock RR (2002) Phosphorus dynamics in the rhizosphere of perennial ryegrass (*Lolium perenne* L.) and radiata pine (*Pinus radiata* D. Don.). Soil Biol Biochem 34:487–499
Cheng WX (2009) Rhizosphere priming effect: its functional relationships with microbial turnover, evapotranspiration, and C-N budgets. Soil Biol Biochem 41:1795–1801
Chookietwattana K, Maneewan K (2012) Screening of efficient halotolerant phosphate solubilizing bacterium and its effect on promoting plant growth under saline conditions. World Appl Sci J 16(8):1110–1117
Cordell D, Drangert JO, White S (2009) The story of phosphorus: global food security and food for thought. Global Environ Change 19:292–305
Correll DL (1998) The role of phosphorus in the eutrophication of receiving waters: a review. J Environ Qual 27:261–266
de Freitas JR, Banerjee MR, Germida JJ (1997) Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol Fertil Soils 27:358–364
Deng S, Summers ML, Kahn ML, Mc Dermontt TR (1998) Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase. Arch Microbiol 170:18–26
Deng S, Elkins JG, Da LH, Botero LM, McDermontt TR (2001) Cloning and characterization of second acid phosphatase from *Sinorhizobium meliloti* strain 104A14. Arch Microbiol 176:255–263
Deubel A, Merbach W (2005) Influence of microorganisms on phosphorus bioavailability in soils. In: Buscot F, Varma A (eds) Microorganisms in soils: roles in genesis and functions. Springer, Berlin, pp 177–191
Deubel A, Gransee A, Merbach W (2000) Transformation of organic rhizodepositions by rhizosphere bacteria and its influence on the availability of tertiary calcium phosphate. J Plant Nutr Soil Sci 163:387–392

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Micobiol Res 159:371–394

Dubey SK (1997) Coinoculation of phosphate solubilizing bacteria with *Bradyrhizobium japonicum* to increase phosphate availability to rainfed soybean on vertisol. J Indian Soc Soil Sci 45:506–509

Dubey SK (2001) Associative effect of nitrogen fixing and phosphate solubilizing bacteria in rainfed soybean (*Glycine max*) grown in vertisols. Indian J Agric Sci 71:476–479

Dugan P, Lundgren DG (1965) Energy supply for the chemoautotroph *Ferrobacillus ferrooxidans*. J Bacteriol 89:825–834

Dye C (1995) Effect of citrate and tartrate on phosphate absorption by amorphous ferric hydroxide. Fert Res 40:129–134

Ekin Z (2010) Performance of phosphate solubilizing bacteria for improving growth and yield of sunflower (*Helianthus annuus* L.) in the presence of phosphorus fertilizer. Afr J Biotechnol 9 (25):3794–3800

El Komy HMA (2005) Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. Food Technol Biotechnol 43:19–27

El Sayed SAM (1999) Influence of *Rhizobium* and phosphate solubilizing bacteria on nutrient and yield of lentil in New Valley (Egypt). Egypt J Soil Sci 39:175–186

Esberg C, du Toit B, Olsson R, Ilstedt U, Giesler R (2010) Microbial responses to P addition in six South African forest soils. Plant Soil 329:209–225

Fankem H, Nwaga D, Deubel A, Dieng L, Merbach W, Etoa FX (2006) Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. Afr J Biotechnol 5:2450–2460

Fernandez LA, Zalba P, Gomez MA, Sagardoy MA (2007) Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions. Biol Fertil Soils 43:805–809

Fraga R, Rodriguez H, Gonzalez T (2001) Transfer of the gene encoding the NapA acid phosphatase from *Morganella marganii* to a *Burkholderia cepacia* strain. Acta Biotechnol 21:359–369

Frossard E, Condron LM, Oberson A, Sinaj S, Fardeau JC (2000) Processes governing phosphorus availability in temperate soils. J Environ Qual 29:15–23

Gadagi RS, Tongmin SA (2002) New isolation method for microorganisms solubilizing iron and aluminium phosphates using dyes. Soil Sci Plant Nutr 48:615–618

Gaind S, Gaur AC (1990) Influence of temperature on the efficacy of phosphorus solubilizing microorganisms. Indian J Microbiol 30:305–310

Gaind S, Gaur AC (1991) Thermotolerant phosphate solubilizing microorganisms and their interaction with mungbean. Plant Soil 133:141–149

Galar ML, Bolardi JL (1995) Evidence for a membrane bound pyrrolquinoline quinine-linked glucose dehydrogenase in *Acetobacter diazotrophicus*. Appl Environ Microbiol 43:713–716

Gans J, Wolinsky M, Dunbar J (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309:1387–1390

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Gaur AC (1990) Phosphate solubilizing microorganisms as biofertilizer. Omega Scientific Publications, New Delhi, p 176

Gaur AC, Madan M, Ostwal KP (1973) Solubilization of phosphatic compounds by native microflora of rock phosphates. Indian J Exp Biol 11:427–429

Geelhoed JS, van Riemsdijk WH, Findenegg GR (1999) Simulation of the effect of citrate exudation from roots on the plant availability of phosphate adsorbed on goethite. Eur J Soil Sci 50:379–390

George TS, Richardson AE, Smith JB, Hadobas PA, Simpson RJ (2005) Limitations to the potential of transgenic *Trifolium subterraneum* L. plants that exude phytase when grown in soils with a range of organic P content. Plant Soil 278:263–274

George TS, Simpson RJ, Hadobas PA, Marshall DJ, Richardson AE (2007) Accumulation and phosphatase-lability of organic phosphorus in fertilised pasture soils. Aust J Agric Res 58:47–55

George TS, Richardson AE, Li SM, Gregory PJ, Daniell TJ (2009) Extracellular release of a heterologous phytase from roots of transgenic plants: does manipulation of rhizosphere biochemistry impact microbial community structure? FEMS Microbiol Ecol 70:433–445

Gerke J (1992) Phosphate, aluminium and iron in the soil solution of three different soils in relation to varying concentrations of citric acid. Zeitsch Pflanz Bodenk 155:339–343

Gerretson FC (1948) The influence of microorganisms on the phosphate intake by the plants. Plant Soil 1:51–81

Ghosh G, Poi SC (1990) Response of *Rhizobium*, phosphate solubilizing bacteria and mycorrhizal organisms in legume crops. Environ Ecol 16:607–610

Gianinazzi-Pearson V (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8:1871–1883

Gilvert N (2009) The disappearing nutrient. Science 461:716–718

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 32:145–148

Goldstein AH (1986) Bacterial solubilization of microbial phosphates: historical perspective and future prospects. Am J Altern Agric 1:51–57

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram-negative bacteria. Biol Agric Hortic 12:185–193

Goldstein AH (1996) Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by Gram-negative bacteria. In: Toriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 197–203

Goldstein AH (2007) Future trends in research on microbial phosphate solubilization: one hundred years of insolubility. In: Velazquez E, Roderguez-Barrueco C (eds) Proceedings of the first international meeting on microbial phosphate solubilization. Springer, Berlin, pp 91–96

Goldstein AH, Liu ST (1987) Molecular cloning and regulation of a mineral phosphate solubilizing gene from *Erwinia herbicola*. Biotechnology 5:72–74

Goldstein AH, Rogers RD, Mead G (1993) Separating phosphate from ores via bioprocessing. Biotechnology 11:1250–1254

Golovan S, Wang G, Zhang J, Forsberg CW (2000) Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can J Microbiol 46:59–71

Grimes HD, Mount MS (1984) Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol Biochem 16:27–30

Gupta R, Singal R, Shankar A, Kuhad RC, Saxena RK (1993) Modified plate assay for screening of phosphate solubilizing microorganisms. J Gen Appl Microbiol 40:255–260

Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245:83–93

Halder AK, Mishra AK, Chakrabarty PK (1991) Solubilizing of inorganic phosphates by *Bradyrhizobium*. Indian J Exp Biol 29:28–31

Han Y, Wilson DB, Lei XG (1999) Expression of an *Aspergillus niger* phytase gene (*phy*A) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 65:1915–1918

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Harvey PR, Warren RA, Wakelin S (2009) Potential to improve root access to phosphorus: the role of non-symbiotic microbial inoculants in the rhizosphere. Crop Pasture Sci 60:144–151

Hasan R (1994) Phosphorus fertility status of soils in India. Phosphorus research in India. In: Dev G (ed) Proceedings of group discussion, Indian Agricultural Research Institute, New Delhi. Principal Publications, Gurgaon, pp 8–12

Hassan HM, Marschner P, McNeill A, Tang C (2012) Growth, P uptake in grain legumes and changes in rhizosphere soil P pools. Biol Fertil Soils 48:151–159

Hebbara M, Suseeladevi L (1990) Effect of phosphorus solubilizing bacteria (PSB) on phosphorus availability to groundnut from rock phosphate. Curr Res 19:56–57

Henri F, Laurette NN, Annette D, John Q, Wolfgang M, François-Xavier E, Dieudonné N (2008) Solubilization of inorganic phosphates and plant growth promotion by strains of *Pseudomonas fluorescens* isolated from acidic soils of Cameroon. Afr J Microbiol Res 2:171–178

Hinsinger P (2001) Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil 237:173–195

Hinsinger P, Plassard C, Tang CX, Jaillard B (2003) Origins of root mediated pH changes in the rhizosphere and their response to environmental constraints: a review. Plant Soil 248:43–59

Holford ICR (1997) Soil phosphorus: its measurement and its uptake by plants. Aust J Soil Res 35:227–239

Huelett FM, Sun G, Liu W (2007) The Pho regulon of *Bacillus subtilis* is regulated by sequential action of two genetic switches. In: Toriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 50–54

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borris R (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant growth promoting effects. Microbiology 148:2097–2109

Igual JM, Valverde A, Ceravantes E, Velazquez E (2001) Phosphate solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. Agronomie 21:561–568

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 24:389–395

Illmer P, Schinner F (1995) Solubilization of inorganic calcium phosphates: solubilization mechanisms. Soil Biol Biochem 27:257–263

Jain LK, Singh P (2003) Growth and nutrient uptake of chickpea (*Cicer arietinum* L.) as influenced by biofertilizers and phosphorus nutrition. Crop Res 25:410–413

Jakobsen I, Leggett ME, Richardson AE (2005) Rhizosphere microorganisms and plant phosphorus uptake. In: Sims JT, Sharpley AN (eds) Phosphorus: agriculture and the environment. American Society for Agronomy, Madison, WI, pp 437–494

Jisha MS, Alagawadi AR (1996) Nutrient uptake and yield of sorghum (*Sorghum bicolor* L. Moench) inoculated with phosphate solubilizing bacteria and cellulolytic fungus in cotton stalk amended vertisol. Microbiol Res 151:1–5

Jones DL (1998) Organic acids in the rhizosphere – a critical review. Plant Soil 205:25–44

Jones DL, Oburger E (2011) Phosphorus in action. Soil Biol 100:169–198

Juriank JJ, Dudley LM, Allen MF, Knight WG (1986) The role of calcium oxalate in the availability of phosphorus in soils of semiarid regions: thermodynamic study. Soil Sci 142:255–261

Kamh M, Horst W, Amer F, Mostafa H, Maier P (1999) Mobilization of soil and fertilizer phosphate by cover crops. Plant Soil 211:19–27

Karamanos RE, Flore NA, Harapiak JT (2010) Re-visiting use of *Penicillium bilaii* with phosphorus fertilization of hard red spring wheat. Can J Plant Sci 90:265–277

Katznelson H, Bose B (1959) Metabolic activity and phosphate dissolving activity of bacterial isolates from wheat roots, rhizosphere and non-rhizosphere soil. Can J Microbiol 5:79–85

Kerovuo J, Lauracus M, Nurminen P, Kalkinen N, Apajalahti J (1998) Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64:2079–2085

Khan MS, Zaidi A, Wani PA (2006) Role of phosphate solubilizing microorganisms in sustainable agricultural – a review. Agron Sustain Dev 26:1–15

Khan MS, Zaidi A, Wani PA (2007) Role of phosphate solubilizing microorganisms in sustainable agriculture – a review. Agron Sustain Dev 27:29–43

Khan MS, Zaidi A, Ahemad M, Oves M, Wani PA (2010) Plant growth promotion by phosphate solubilizing fungi – current perspective. Arch Agron Soil Sci 56:73–98

Khandelwal A, Sindhu SS (2012) Expression of 1-aminocyclopropane-1-carboxylate deaminase in rhizobia promotes nodulation and plant growth of clusterbean (*Cyamopsis tetragonoloba* L.). Res J Microbiol 7:158–170

Kim KY, McDonald GA, Jordan D (1997) Solubilization of hydroxyapatite by *Enterobacter agglomerans* and cloned *Escherichia coli* in culture medium. Biol Fertil Soils 24:347–352

Kim KY, Jordan D, Krishnan HB (1998a) Expression of genes from *Rahnella aquatilis* that are necessary for mineral phosphate solubilization in *Escherichia coli*. FEMS Microbiol Lett 159:121–127

Kim KY, Jordan D, McDonald GA (1998b) Effect of phosphate solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol Fertil Soils 26:79–87

Kim KY, Jordan D, McDonald GA (1998c) *Enterobacter agglomerans*, phosphate solubilizing bacteria and microbial diversity in soil: effect of carbon sources. Soil Biol Biochem 30:995–1003

Kim YO, Lee JK, Kim HK, Yu JH, Oh TK (1998d) Cloning of thermostable phytase gene (*phy*) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. FEMS Microbiol Lett 162:185–191

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomus JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 58:169–188

Kole SC, Hazra JN (1998) Occurrence and acidity of tricalcium phosphate and rock phosphate solubilizing microorganisms in mechanical compost plants of Calcutta and an alluvial soil of West Bengal. Environ Ecol 16:344–349

Krishanaraj PU (1987) Studies on beneficial microorganisms in crop plants. M.Sc. thesis, UAS, Bangalore

Krishanaraj PU (1996) Genetic characterization of mineral phosphate solubilization in *Pseudomonas* sp. Ph.D. thesis, IARI, New Delhi

Krishanaraj PU, Goldstein AH (2001) Cloning of a *Serratia marcescens* DNA fragment that induces quino-protein glucose dehydrogenase-mediated gluconic acid production in *Escherichia coli* in the presence of *Serratia marcescens*. FEMS Microbiol Lett 205:215–220

Krishanaraj PU, Sadasivam KV, Khanuja PS (1999) Mineral phosphate solubilization defective mutants of *Pseudomonas* sp. express pleiotropic phenotypes. Curr Sci 76:1032–1034

Kucey RMN (1987) Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus solubilizing *Penicillium bilaji* strain and vesicular arbuscular mycorrhizal fungi. Appl Environ Microbiol 53:2699–2703

Kucey RMN (1988) Effect of *Penicillium bilaji* on the solubility and uptake of P and micronutrients from soil by wheat. Can J Soil Sci 68:261–270

Kucey RMN, Janzen HH, Legett ME (1989) Microbially mediated increases in plant-available phosphorus. Adv Agron 42:198–228

Kumrawat B, Dighe JM, Sharma RA, Katti GV (1997) Response of soybean to biofertilizer in black clay soils. Crop Res 14:209–214

Kundu BS, Gaur AC (1980a) Effects of phosphobacteria on yield and phosphate uptake by potato crop. Curr Sci 48:159

Kundu BS, Gaur AC (1980b) Effect of nitrogen fixing and phosphate solubilising microorganisms as single and composite inoculants on cotton. Indian J Microbiol 20:225–229

Kundu BS, Gaur AC (1980c) Establishment of nitrogen and phosphate solubilizing bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. Plant Soil 57:223–230

Kundu BS, Gaur AC (1982) Yield increase of wheat after inoculation with *A. chroococcum* and phosphobacteria. Curr Sci 51:291–293

Kundu BS, Gaur AC (1984) Rice response to inoculation with nitrogen fixing bacteria and PSM. Plant Soil 78:227–234

Larsen S (1967) Soil phosphorus. Adv Agron 19:151–210

Lei XG, Ku PK, Miller ER, Yokoyama MT, Ullrey DE (1993) Supplementing corn-soybean meal diets with microbial phytase maximizes phytate P utilization by winling pigs. J Anim Sci 71:3368–3375

Lifschitz R, Kloepper JW, Kozlowski M, Simonson C, Carlson J, Tipling EM, Zaleska T (1987) Growth promotion of canola (rape seed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Can J Microbiol 33:390–395

Lim BL, Yeung P, Cheng C, Hill JE (2007) Distribution and diversity of phytate-mineralizing bacteria. ISME J 1:321–330

Lindsay WLP, Vlek LG, Chien SH (1989) Phosphate minerals. In: Dixon JB, Weed SB (eds) Minerals in soil environment, 2nd edn. Soil Science Society of America, Madison, WI, pp 1089–1130

Liu ST, Lee LY, Jai CY, Hung CH, Chang YS, Wolfram JH, Rogers B, Goldstein AH (1992) Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in *E. coli* HB101: nucleotide sequence and probable involvement in biosynthesis of the co-enzyme pyrrolquinoline quinine. J Bacteriol 174:5814–5819

Lopez-Bucio J, de la Vega OM, Guevara-Garcia A, Herreera-Estrella I (2000) Enhanced phosphate uptake in transgenic tobacco plants that overproduce citrate. Nat Biotechnol 18:450–453

Lorenzo V, Herrero M, Jakubjik U, Timmis KN (1990) Mini-Tn5 transposon derivatives for transposon mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative eubacteria. J Bacteriol 172:6568–6572

Macaskie LE, Young P, Doyle TC, Roig MG, Diaz M, Monzano T (1997) Bioremediation of uranium-bearing waste water: biochemical and chemical factors affecting bioprocess application. Biotechnol Bioeng 53:100–109

Macklon AES, Grayston SJ, Shand CA, Sim A, Sellars S, Ord BG (1997) Uptake and transport of phosphorus by *Agrostis capillaris* seedlings from rapidly hydrolysed organic sources extracted from P^{32}-labelled bacterial cultures. Plant Soil 190:163–167

Maheshkumar KS (1997) Studies on microbial diversity and their activity in soil under bamboo plantations. M.Sc. thesis, UAS, Dharwad

Maheshkumar KS, Krishanraj PU, Alagawadi AR (1999) Mineral phosphate solubilizing activity of *Acetobacter diazotrophicus*: a bacterium associated with sugarcane. Curr Sci 76:874–875

Makino K, Amemura M, Kim SK, Nakata A, Shinagawa H (2007) Mechanism of transcriptional activation of the phosphate regulon in *Eschericia coli*. In: Toriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 5–12

Malik DK, Sindhu SS (2011) Production of indole acetic acid by *Pseudomonas* sp.: Effect of coinoculation with *Mesorhizobium* sp. *Cicer* on nodulation and plant growth of chickpea (*Cicer arietinum*). Physiol Mol Biol Plants 17:25–32

Mehta S, Nautiyal SC (2001) An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr Microbiol 43:51–56

Miller SH, Browne P, Prigent-Combaret C, Combes-Meynet E, Morrissey JP, O'Gara F (2010) Biochemical and genomic comparison of inorganic phosphate solubilization in *Pseudomonas* species. Environ Microbiol Rep 2:403–411

Mishra MM, Kapoor KK, Yadav KS (1982) Effect of compost enriched with Mussorie rock phosphate on crop yield. Indian J Agric Sci 52:674–678

Monod SPI, Gupta DN, Chavan AS (1989) Enhancement of phosphate availability and phosphorus uptake in rice by phosphate solubilizing culture. J Maharastra Agric Univ 14:178–181

Mortensen JL (1963) Complexing of metals by soil organic matter. Soil Sci Soc Am Proc 27:179–186

Naik PR, Raman G, Narayanan KB, Sakthivel N (2008) Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil. BMC Microbiol 8:230–243

Nautiyal CS (1990) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Norrish K, Rosser H (1983) Mineral phosphate. In: Lenaghan JJ, Katsantoni G (eds) Soils, An Australian view point. CSIRO, Melbourne, Academic Press, London, pp 335–361

Nuruzzaman M, Lambers H, Bolland MDA, Veneklaas EJ (2005a) Phosphorus uptake by grain legumes and subsequently grown wheat at different levels of residual phosphorus fertilizer. Aust J Agric Res 56:1041–1047

Nuruzzaman M, Lambers H, Bolland MDA, Veneklaas EJ (2005b) Phosphorus benefits of different legume crops to subsequent wheat grown in different soils of Western Australia. Plant Soil 271:175–187

Nziguheba G, Merckx R, Palm CA, Rao MR (2000) Organic residues affect phosphorus availability and maize yields in a Nitisol of western Kenya. Biol Fertil Soils 32:328–339

Oberson A, Joner EJ (2005) Microbial turnover of phosphorus in soil. In: Turner BL, Frossard E, Baldwin DS (eds) Organic phosphorus in the environment. CABI, Wallingford, pp 133–164

Oehl F, Oberson A, Probst M, Fliessback A, Roth HR, Frossard E (2001) Kinetics of microbial phosphorus uptake in cultivated soils. Biol Fertil Soils 34:31–41

Oehl F, Frossard E, Fliessbach A, Dubois D, Oberson A (2004) Basal organic phosphorus mineralization in soils under different farming systems. Soil Biol Biochem 36:667–675

Ogawa N, Hayashi N, Salto H, Noguchi K, Yamashita Y, Oshima Y (2007) Regulatory circuit for phosphatase genes in *Saccharomyces cerevisiae*: specific *cis*-acting sites in *Pho* promoters for binding the Pho4p. In: Toriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 56–62

Ostwal KP, Bhide VP (1972) Solubilization of tricalcium phosphate by soil *Pseudomonas*. Indian J Exp Biol 10:153–154

Otani T, Ae N, Tanaka H (1996) Phosphorus (P) uptake mechanisms of crops grown in soils with low P status: II. Significance of organic acids in root exudates of pigeonpea. J Soil Sci Plant Nutr 42:553–560

Panhar QA, Othman R, Rahman ZA, Meon S, Ismail MR (2012) Isolation and characterization of phosphate-solubilizing bacteria from aerobic rice. Afr J Biotechnol 11:2711–2719

Panhwar QA, Radziah O, Zaharah AR, Sariah M, Razi IM (2011) Role of phosphate solubilizing bacteria on rock phosphate solubility and growth of aerobic rice. J Environ Biol 32:607–612

Parks EJ, Olson GJ, Brickman PE, Baldi F (1990) Characterization of high performance liquid chromatography (HPLC) of the solubilization of phosphorus in iron ore by a fungus. Indian J Microbiol 5:83–190

Prabhakar M, Saraf CS (1990) Dry matter accumulation and distribution in chickpea as influenced by genotype, P source and irrigation level. Indian J Agric Sci 60:204–206

Prasanna A, Deepa V, Balkrishna Murthy P, Deecaraman M, Sridhar R, Dhandapani P (2011) Insoluble phosphate solubilization by bacterial strains isolated from rice rhizosphere soils from Southern India. Intern J Soil Sci 6:34–141

Puente ME, Bashan Y, Li CY, Lebsky VK (2004) Microbial populations and activities in the rhizoplane of rock-weathering desert plants. Root colonization and weathering of igneous rocks. Plant Biol 6:629–642

Qureshi MA, Ahmad ZA, Akhtar N, Iqbal A, Mujeeb F, Shakir MA (2012) Role of phosphate solubilizing bacteria (PSB) in enhancing P availability and promoting cotton growth. J Anim Plant Sci 22:204–210

Rachewad SN, Raut RS, Malewar GU, Ganure CK (1992) Effects of phosphate solubilizing biofertilizer on biomass production and uptake of phosphorus by sunflower. J Maharastra Agric Univ 17:480–481

Rajarathinam K, Balamurugan T, Kulasekarapandian R, Veersami S, Jayabalan M (1995) Isolation and screening of phosphate solubilizers from soil of Kamarajar district (Tamil Nadu). J Exotoxicol Environ Monit 5:155–157

Rashid M, Khalil S, Ayub N, Alam S, Latif M (2004) Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under *in vitro* conditions. Pakistan J Biol Sci 7:187–196

Reilly TJ, Baron GS, Nano F, Kuhlenschmidt MS (1996) Characterization and sequence of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. J Biol Chem 271:10973–10983

Reyes I, Bernier L, Simard RR, Tanguay P, Antoun H (1999) Characteristics of phosphate solubilization by an isolate of a tropical *Penicillium rugulosum* and two UV-induced mutants. FEMS Microbiol Ecol 28:291–295

Reyes I, Baziramakenga R, Bernier L, Antoun H (2001) Solubilization of phosphate rocks and minerals by a wild-type strain and two UV-induced mutants of *Penicillium rugulosum*. Soil Biol Biochem 33:1741–1747

Richardson AE (1994) Soil microorganisms and phosphorus availability. In: Pankhurst CE, Doube BM, Gupta VVSR (eds) Soil biota: management in sustainable farming systems. CSIRO, Highett, VIC, pp 50–62

Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Aust J Plant Physiol 28:897–906

Richardson AE, Simpson RJ (2011) Soil microorganisms mediating phosphorus availability. Plant Physiol 156:989–996

Richardson AE, Hadobas PA, Hayes JE (2001a) Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. Plant J 25:641–649

Richardson AE, Hadobas PA, Hayes JE, O'Hara CP, Simpson RJ (2001b) Utilization of phosphorus by pasteur plants supplied with meso-inositol hexaphosphate is enhanced by the presence of soil microorganisms. Plant Soil 229:47–56

Richardson AE, George TS, Hens M, Simpson RJ (2005) Utilization of soil organic phosphorus by higher plants. In: Turner BL, Frossard E, Baldwin DS (eds) Organic phosphorus in the environment. CABI, Wallingford, pp 165–184

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009a) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305–339

Richardson AE, Hocking PJ, Simpson RJ, George TS (2009b) Plant mechanisms to optimize access to soil phosphorus. Crop Pasture Sci 60:124–143

Rodriguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339

Rodriguez E, Han Y, Lei XG (1999) Cloning, sequencing and expression of an *Escherichia coli* acid phosphatase/phytase gene (*app*A2) isolated from pig colon. Biochem Biophys Res Commun 257:117–123

Rodriguez H, Gonzalez T, Selman G (2000a) Expression of a mineral phosphate solubilizing gene from *Erwinia herbicola* in two rhizobacterial strains. J Biotechnol 84:155–161

Rodriguez H, Rossolini GM, Gonzalez T, Jiping L, Glick BR (2000b) Isolation of a gene from *Burkholderia cepacia* IS-16 encoding a protein that facilitates phosphatase activity. Curr Microbiol 40:362–366

Rodriguez H, Gonzalez T, Goire I, Bashan Y (2004) Gluconic acid production and phosphate solubilization by the plant growth promoting bacterium *Azospirillum* spp. Naturwissenschaften 91:552–555

Rodriguez H, Fraga R, Gonzalez T, Bashan Y (2006) Genetics of phosphate solubilization and its potential applications for improving plant growth promoting bacteria. Plant Soil 287:15–21

Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thallar MC (1998) Bacterial non-specific acid phosphatases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci 54:833–850

Roychaudhary P, Kaushik BD (1989) Solubilization of Mussoorie rock phosphate by cyanobacteria. Curr Sci 58:569–570

Ryan PR, Delhaise E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560

Sanchez PA, Shepherd KD, Soule MJ, Place FM, Buresh RJ, Izac AN (1997) Soil fertility replenishment in Africa: an investment in natural resources capital. In: Replenishing soil fertility in Africa, Indianapolis. Proceedings, SSSA Special Publication No. 51, American Society of Agronomy, Madison, WI, pp 1–46

Santhi V (1998) Mechanism of mineral phosphate solubilization and growth promotion by diverse bacteria. M. Sc. thesis, University of Agricultural Sciences, Dharwad

Saraf CS, Shivkumar BG, Patil RR (1997) Effect of phosphorus, sulphur and seed inoculation on performance of chickpea (*Cicer arietinum*). Indian J Agron 42:323–328

Sarawgi SK, Tiwari PK, Tripathi RS (1999) Uptake and balance sheet of nitrogen and phosphorus in gram (*Cicer arietinum*) as influenced by phosphorus biofertilizers and micronutrients under rain-fed conditions. Indian J Agron 44:768–772

Sarawgi SK, Tiwari PK, Tripathi RS (2000) Growth, nodulation and yield of chickpea as influenced by phosphorus, bacterial culture and micronutrients under rain-fed conditions. Madras Agric J 86:181–185

Seeling B, Zasoski RJ (1993) Microbial effects in maintaining organic and inorganic solution phosphorus concentrations in a grassland topsoil. Plant Soil 148:277–284

Shahid M, Hameed S, Imran A, Ali S, van Elsas JD (2012) Root colonization and growth promotion of sunflower (*Helianthus annuus* L.) by phosphate solubilizing Enterobacter sp. Fs-11. World J Microbiol Biotechnol 28(8):2749–2758

Sharma SN (2003) Effect of phosphate solubilizing bacteria on the efficacy of Mussoorie rock phosphate in rice (*Oryza sativa*) – wheat (*Triticum aestivum*) cropping system. Indian J Agric Sci 73:478–481

Sharpley AN (1985) Phosphorus cycling in unfertilized and fertilized agricultural soils. Soil Sci Soc Am J 49:905–911

Sheshardri S, Kumaraswamy R, Lakshminarasimhan C, Ignacimuthu S (2000) Solubilization of inorganic phosphate by *Azospirillum halopraeferans*. Curr Sci 79:565–567

Sindhu SS, Dadarwal KR (2000) Competition for nodulation among rhizobia in *Rhizobium*-legume symbiosis. Indian J Microbiol 40:211–246

Sindhu SS, Verma MK, Mor S (2009) Molecular genetics of phosphate solubilization in rhizosphere bacteria and its role in plant growth promotion. In: Khan MS, Zaidi A (eds) Phosphate solubilizing microbes and crop productivity. Nova Science, Hauppauge, NY, pp 199–228

Sindhu SS, Dua S, Verma MK, Khandelwal A (2010) Growth promotion of legumes by inoculation of rhizosphere bacteria. In: Khan MS, Zaidi A, Musarrat J (eds) Microbes for legume improvement. Springer-Wien, NewYork, pp 195–235

Singh CP, Mishra MM, Yadav KS (1980) Solubilization of insoluble phosphates by thermophilic fungi. Ann Microbiol (Inst Pasteur) 131:289–296

Singh HP, Pareek RP, Singh PA (1984) Solubilization of rock phosphate by phosphate solubilizers in broth. Curr Sci 53:1212–1213

Singh AV, Chandra R, Goel R (2012) Phosphate solubilization by *Chryseobacterium* sp. and their combined effect with N and P fertilizers on plant growth promotion. Arch Agron Soil Sci 58:1–11

Sonboir HL, Sarawgi SK (2000) Nutrients uptake, growth and yield of chickpea as influenced by phosphorus, *Rhizobium* and phosphate solubilizing bacteria. Madras Agric J 87:149–151

Sperber JI (1958) Solution of apatite by soil microorganisms producing organic acids. Aust J Agric Res 9:778–781

Srivastava TK, Ahalwat LPS, Panwar JDS (1998) Effect of phosphorus, molybdenum and biofertilizers on productivity of pea. Indian J Plant Physiol 3:237–239

Stalstorm VA (1903) Beitrag, Zur Kenntrusder einwinsking sterilizer and in garung befindlieher strife any dil Loslishkerd der phosphorus are destrical cum phosphorus. Zentralb Bakteriol Abt 11:724–732

Surange S, Kumar N (1993) Phosphate solubilization under varying pH by *Rhizobium* from tree legumes. Indian J Exp Biol 31:427–429

Taha SM, Mahamood SAZ, Halim EI, Damaty A, Hafez AM (1969) Activity of phosphate dissolving bacteria in Egyptian soils. Plant Soil 31:149–160

Tallapragada P, Seshachala U (2012) Phosphate-solubilizing microbes and their occurrence in the rhizospheres of *Piper betel* in Karnataka, India. Turk J Biol 36:25–35

Tang C, Fang RY, Raphael C (1998) Factors affecting soil acidification under legumes II. Effect of phosphorus supply. Aust J Agric Res 49:657–664

Tarafdar JC, Yadav RS, Meena SC (2001) Comparative efficiency of acid phosphatase originated from plant and fungal sources. J Plant Nutr Soil Sci 164:279–282

Tarafder JC, Claassen N (2003) Organic phosphorus utilization by wheat plants under sterile conditions. Biol Fertil Soils 39:25–29

Teplitski M, Mathesius U, Rumbaugh KP (2011) Perception and degradation of N-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. Chem Rev 111:100–116

Thakkar J, Narsian V, Patel HH (1993) Inorganic phosphate solubilization by certain soil bacteria: solubilization of natural rock phosphates and pure insoluble inorganic phosphate by *Aspergillus awamorii*. Indian J Exp Biol 31:743–747

Thallar MC, Berlutti F, Schippa S, Lombardi G, Rossolini GM (1994) Characterization and sequence of PhoC, the principal phosphatase-irrepressible acid phosphatase of *Morganella morganii*. Microbiology 140:1341–1350

Thallar MC, Berlutti F, Schippa S, Lori P, Passariello C, Rossolini GM (1995a) Heterogenous patterns of acid phosphatases containing low molecular mass polypeptides in members of the family *Enterobacteriaceae*. Int J System Bacteriol 4:255–261

Thallar MC, Lombardi G, Berlutti F, Schippa S, Rossolini GM (1995b) Cloning and characterization of the NapA acid phosphatase/phosphotransferase of *Morganella morganii*. Identification of a new family of bacterial acid phosphatase encoding genes. Microbiology 140:147–151

Tilak KVBR, Saxena AK, Sadasivan KV (1995) Synergistic effects of phosphate solubilizing bacterium *Pseudomonas striata* and arbuscular mycorrhizae on soybean. In: Sujan Singh, Adholya A (eds) Mycorrhizae: biofertilizers for the future. Tata Energy Research Institute, New Delhi, pp 224–226

Tomar M (2005) Biodiversity of mineral phosphate solubilizing bacteria from chickpea, mustard and wheat rhizosphere. Ph.D. thesis submitted to CCS HAU, Hisar

Toro M, Azcon R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl Environ Microbiol 63:4408–4412

Torsvik V, Ovreas L, Thingstad TF (2002) Prokaryotic diversity – magnitude, dynamics, and controlling factors. Science 296:1064–1066

Turan M, Ataoglu N, Sahin F (2006) Evaluation of the capacity of phosphate solubilizing bacteria and fungi on different forms of phosphorus in liquid culture. J Sustain Agric 28:99–108

Turner BL (2007) Inositol phosphates in soil: amounts, forms and significance of the phosphorylated inositol stereoisomers. In: Tuner BL, Richardson AE, Mullaney EJ (eds) Inositol phosphates; linking agriculture and the environment. CABI, Wallingford, pp 186–206

Turner BL, Haygarth PM (2001) Biogeochemistry – phosphorus solubilization in rewetted soils. Nature 411:258

Tye AJ, Siu FK, Leung TY, Lim BL (2002) Molecular cloning and the biochemical characterization of two novel phytases from *Bacillus subtilis* 168 and *Bacillus licheniformis*. Appl Environ Microbiol 59:190–197

van Schie BJ, Hellingwerf KE, Vandijkan JP, Elferink MGL, Van Diji JM, Kuenen JG, Konigns N (1985) Energy transduction by electron transfer via a pyrroquinoline quinine dependent glucose dehydrogenase in *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticum* (var. Lowoffi). J Bacteriol 163:493–499

Vance CP, Uhde-Stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157:423–447
Varsha N, Patel HH (1995) Inorganic phosphate solubilization by some yeast. Indian J Microbiol 35:127–132
Varsha N, Thakkar J, Patel HH (1994) Isolation and screening of phosphate solubilizing fungi. Indian J Microbiol 34:113–118
Venkateswarlu B, Rao AV, Raina P (1984) Evaluation of phosphorus solubilization by microorganisms isolated from arid soils. J Indian Soc Soil Sci 32:273–277
Vessey JK, Heisinger KG (2001) Effect of *Penicillium bilaii* inoculation and phosphorus fertilisation on root and shoot parameters of field-grown pea. Can J Plant Sci 81:361–366
Viruel E, Lucca ME, Sineriz F (2011) Plant growth promotion traits of phosphobacteria isolated from Puna, Argentina. Arch Microbiol 193:489–496
Vu DT, Tang C, Armstrong RD (2008) Changes and availability of P fraction following 65 years of P application to a calcareous soil in a Mediterranean climate. Plant Soil 304:21–33
Wakelin SA, Warren RA, Harvey PR, Ryder MH (2004) Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol Fertil Soils 40:36–43
Wall DH, Virginia RA (1999) Control of soil biodiversity – in sight from extreme environments. Appl Soil Ecol 13:137–150
Wanner BL (1996) Phosphorus assimilation and control of the phosphate regulon. In: Niedhardt FC, Curtis R III, Ingraham JL, Lin EC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella:* cellular and molecular biology, 2nd edn. IASM Press, Washington, DC, pp 1357–1381
Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155–166
Xie XN, Yoneyama K (2010) The strigolactone story. Annu Rev Phytopathol 48:93–117
Yadav BN, Singh D, Singh SM (2004) Performance of barley (*Hordeum vulgare* L.) varieties under varying fertilizer levels and microbial inoculation. Agric Sci Digest 24:148–150
Yousefi AA, Khavazi K, Moezi AA, Rejali F, Nadian HA (2011) Phosphate solubilizing bacteria and arbuscular mycorrhizal fungi impacts on inorganic phosphorus fractions and wheat growth. World Appl Sci J 15:1310–1318
Zaidi A, Khan MS, Ahemad M, Oves M (2009) Plant growth promotion by phosphate solubilizing bacteria. Acta Microbiol Immunol Hungarica 56:263–284
Zhao XR, Lin QM (2001) A review of phosphate-dissolving microorganisms. Soil Fertil 3:7–11
Zhu F, Qu L, Hong X, Sun X (2011) Isolation and characterization of a phosphate-solubilizing halophilic bacterium *Kushneria* sp. YCWA18 from Daqiao saltern on the coast of Yellow sea of China. Evid Based Complem Altern Med, Article ID 615032, p 6

Chapter 12
Biotechnology in Enhanced Petroleum Oil Recovery

Ajay Singh, Nagina Parmar, and Owen Ward

12.1 Introduction

Enhanced oil recovery (EOR) methods are conventionally applied in the petroleum industry to recover residual oil from wells and oilfield emulsions. In petroleum oil well, residual high viscous oil is often located in areas inaccessible to fluids used for flooding, or the oil is adhered to sand or carbonate particles in the reservoir making it difficult to recover further and usually more than two-third of the oil in the reservoir is left unrecovered after primary and secondary extraction (Sen 2008; Brown 2010). Conventional EOR methods make use of chemicals (solvents, polymers, surfactants), injected gases (CO_2, N_2, flue gas), and thermal methods (steam flood, hot water) to extract remaining oil.

Oilfield emulsions, both oil-in-water and water-in-oil, are formed at various stages of exploration, production, oil recovery, and processing and represent a major problem for the petroleum industry. North American producers estimate that as much as 2 % of their oil production ends up as an emulsion during production and pipeline transport, which translates into millions of dollars in lost revenue and potential environmental damage (Becker 1997). Traditional physical and chemical de-emulsification methods to recover oil include centrifugation, heat treatment, electrical treatment, and chemicals containing soap, fatty acids, and long-chain alcohols. However, physical and chemical de-emulsification processes

A. Singh (✉)
Lystek International Inc., 1425 Bishop Street North, Unit 16, Cambridge, ON, Canada N1R 6J9
e-mail: asingh@lystek.com

N. Parmar
Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada M5B 2K3

O. Ward
Department of Biology, University of Waterloo, Waterloo, ON, Canada N2L 3G1

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_12, © Springer-Verlag Berlin Heidelberg 2014

are capital intensive, and emulsions often generated at the wellhead have to be transported to central processing facilities.

During last two decades, interest in using microorganisms and their product has continuously increased due to their biodegradability, no reliance on the cost of crude oil, and representation of a cost-effective and environmentally friendly alternative (Gao and Zekri 2011). Both microbial-enhanced oil recovery (MEOR) and biodemulsification methods apply microorganisms or their metabolic products (solvents, polymers, organic acids, and surfactants) to mobilize residual oil left over in the wells or de-emulsify petroleum oil emulsions (Zhou et al. 2008; Khire 2010). A brief overview of oil recovery methods is presented in this chapter.

12.2 Microbial-Enhanced Oil Recovery

MEOR methods have been actively pursued both in laboratory and field conditions with many successful attempts. Although more than 70 % of the low temperature oilfield wells treated by bacteria achieved increases in oil production rate, considerable uncertainty remains regarding process performance due to reservoir heterogeneity.

12.2.1 Mechanisms of MEOR

The residual oil is held in porous rocks by capillary pressure that is equal oil–water interfacial tension (IFT). MEOR methods improve the mobility of oil through decreasing oil viscosity, dissolution of carbonates in the reservoir, physically displacing oil, and plugging of highly permeable areas in the reservoir to increase the sweep efficiency of water flooding (Lazar et al. 2007; Elshahed 2010). MEOR mechanisms include microbial plugging, biofilm growth production of bioproducts (biosurfactants, biopolymers and solvents) and, gases (CO_2 and CH_4) (Youssef et al. 2009; Kaster et al. 2012). While biosurfactants reduce IFT to make residual oil flow, certain bacteria produces biopolymers that can plug the high-permeability zones with large pores, thus forcing injected water to sweep the oil in low permeability zones. Biofilms growing on the surface of the porous rock may lead to a change of surface properties and a decrease in permeability. Bacterial gases and solvents can dissolve in crude oil and reduce its viscosity while increasing reservoir pressure also leads to improved mobility and oil recovery (Voordouw 2011). Certain bacterial species can degrade the paraffin deposits near the wellbore region to improve permeability and oil production. Only bacteria are considered promising candidates for MEOR due to their higher tolerance of extreme reservoir properties in terms of high salinity, pH, temperature, pressure, and nutrient availability (Ward et al. 2009). Potentially useful MEOR isolates including extremely thermophilic anaerobes have been isolated and cultured in the laboratory (Brown 2010).

12.2.2 Field Applications of MEOR

MEOR has been tested in various oilfields around the world particularly in the USA, China, Malaysia, and Argentina with some success (Gao and Zekri 2011; Ward et al. 2012). Most of the successful MEOR treatments were conducted for formations with a low temperature (below 55 °C), low water salinity (less than 100,000 ppm), high water cut (above 75 %), and low production rate. Additional oil recovery with the MEOR in these field studies were reported in the range of 15,000–70,000 bbl. Although the reservoir heterogeneity significantly affects oil recovery efficiency, single-well stimulation treatment with MEOR may increase the rate of production from 0.2 to 0.4 ton of oil per day for 2–6 months without additional treatments. Microbial flooding processes are mostly used, where bacteria and nutrients were injected and carried deep into the reservoir with the normal water flooding operation. Selective plugging and biosurfactant production were believed to be the main contributor to the better oil recovery in the successful field studies. Reductions in IFT, crude oil viscosity, and paraffin content were also observed in some studies.

Although MEOR has potential for use in oil recovery from oil sands, there has been only limited number of studies on oil extraction from oil sands using MEOR (Harner et al. 2011). The oil sands deposits in Western Canada Sedimentary Basin (WCSB) cover an area of 1,400,000 km^2 in the western part of North America with the depth varying from 0 to 500 m and containing 6–18 % bitumen. The oil sands deposits are a mixture of a sand, clay, water, and bitumen and comprise of at least 85 % of the total immobile bitumen in place in the world (Huang et al. 2008). Common oil sand extraction methods decrease the viscosity of the bitumen through steam, chemical solvents, or hot air injection followed by hot water extraction and agitation process to facilitate separation of oil from the sand and water. Microbial communities, including sulfate-, nitrate-, and iron-reducing fermentative bacteria and methanogens, interact in the oil sands environment, metabolize crude oil compounds, and produce biosurfactants, solvents, gases, and acids to displace oil from mineral surfaces and reduce oil viscosity (Youssef et al. 2009; Harner et al. 2011).

12.3 Microbial De-emulsification of Petroleum Oil Emulsions

Oilfield emulsions are formed at various stages of petroleum production, processing, and transportation. While traditional physical de-emulsification methods are capital intensive, disposal of the chemical de-emulsifier in the aqueous phase and removal of the de-emulsifier from the oil phase create further complications in the chemical processes. Further, chemical treatment methods require a series of tests and tedious exercise to find a suitable emulsion-breaking chemical for

every emulsion type. Often, emulsions are transported to centralized facilities for oil recovery operations. On the other hand, microbial processes can be carried out at non-extreme conditions and an effective biodemulsification process may be used directly to treat emulsions on site at the wellhead, thus saving on transport and high capital equipment costs (Van Hamme et al. 2003).

A number of microbial species are known to possess de-emulsification properties (Table 12.1). Microbes exploit hydrophobic cell surfaces and the dual hydrophobic/hydrophilic nature of biosurfactants to displace the emulsifiers that are present at the oil–water interface in the biodemulsification process (Kosaric 1996; Singh et al. 2006; Huang et al. 2010). Some microbes produce certain compounds with de-emulsification properties, e.g., acetoin, polysaccharides, glycolipids, glycoproteins, phospholipids, and rhamnolipids (Das 2001; Singh et al. 2007). In a study to understand process mechanisms of demulsification using demulsifier from *Alcaligenes* sp. S-XJ-1, it was observed that the process appeared to begin with the adsorption of the biodemulsifiers onto the water–oil interface due to their amphiphilic nature (Wen et al. 2010; Liu et al. 2011). The reaction of the biodemulsifiers with the emulsifiers then results in the removal of thin liquid film from the surface of dispersed droplets to cause coalescence of droplets and phase separation.

Cell surface hydrophobicity (CSH) of bacteria plays a significant role in nonspecific adsorption to all kinds of biological or nonbiological surfaces and interfaces and bacterial migration and adsorption at the oil–water interface. The CSH of *Rhodococcus* sp. and *Alcaligenes* sp. is generally enhanced via accumulation of fatty acids on the cell surface to adhere to the oil phase (Chang et al. 2009; Huang et al. 2012). Demulsifying bacteria possessing a relatively high CSH and total unsaturated degree for the cell wall-bound fatty acids perform better demulsification activity. Generally Oleic acid (C18:1) and linoleic acid (C18:2) had a positive effect on the formation of CSH, while stearic acid (C18:0) and linolenic acid (C18:3) had the opposite effect.

Although the demulsification capability of most of the cultures is not affected by lyophilization or freezing and thawing, it can be completely destroyed by autoclaving or alkaline methanolysis or significantly reduced by washing of cells with any lipid solubilizing solvent such as *n*-pentane, *n*-hexane, kerosene or chloroform–methanol. Nutrient (carbon and nitrogen) sources in the growth media and cultural conditions significantly impact the biodemulsifying properties of the bacteria (Kosaric 1996; Das 2001; Huang et al. 2009; Singh et al. 2012). The cultures grown on petroleum fractions produce better biodemulsifiers compared to the cultures grown on carbohydrate sources (Nadarajah et al. 2002a, b). The biodemulsification activity also depends upon growth media, cell density, and age of the culture (Mohebali et al. 2012).

Mixed bacterial cultures with proven demulsifying ability have been tested on a range of oil emulsions obtained from different oil companies (Nadarajah et al. 2002a, b). The mixed culture grown on petroleum oil products caused separation of oil from water and solids in various oilfield emulsions within 24–96 h. The initial demulsification rate varied significantly among the various emulsions tested possibly due to the variation in the composition and viscosity of

Table 12.1 Applications of biotechnology in petroleum oil recovery

Biotechnology	Biocatalyst	Application
Microbial enhanced oil recovery (MEOR)	Biomass/bacteria (*Bacillus subtilis*, *Arthrobacter protoformiae*, *Serratia marcescens*); biosurfactants like rhamnolipid, trehaloselipids, surfactin, emulsan, viscosin (*Acinetobacter calcoaceticus*, *Arthrobacter paraffineus*, *Bacillus licheniformis*, *Corynebacterium fascians*, *Pseudomonas rubescens*); biopolymers like curdlan, dextran, pullulan, levan, xanthan (*Bacillus polymyxa*); solvents like acetone, butanol, propanediol (*Clostridium acetobutylicum*, *C. pasteurianum*, *Brevibacterium viscogenes*, *Xanthomonas campestris*); acids like propionic and butyric (*Clostridium* spp., *Enterobacter aerogenes*); Gases like CO_2, CH_4, H_2 (*Clostridium acetobutylicum*, *Clostridium tetanomorphum*, *Enterobacter aerogenes*, *Methanobacterium* sp.)	Tertiary oil recovery employing microbes or their products to mobilize residual oil to enhance crude oil recovery; biosurfactants and chemicals produced by microbes help in oil dissolution, viscosity reduction; selective biomass plugging, increased permeability; increased pressure and oil swelling
Biodemulsification	*Acinetobacter calcoaceticus*; *Bacillus subtilis*; *Corynebacterium petrophilum*; *Nocardia amarae*; *Ochrobactrum anthropi*; *Pseudomonas aeruginosa*; *Rhodococcus globerulus*	Oil recovery through de-emulsification of oil emulsions, oil solubilization, viscosity reduction, and wetting

the emulsions and the nature of emulsifier. Generally, emulsions with higher water content were found easier to break compared to the ones with lower water content. Due to variability in the properties of oilfield crude oil emulsions, inconsistencies have been experienced in performance of the different biodemulsification processes. Further research on biodemulsification processes with field emulsions needs to be aimed at development of more reliable and universally effective systems.

12.4 Conclusion

With current crude oil prices around \$85–100/barrel, there is strong economic incentive to recover residual oil from petroleum oil reservoirs. MEOR methods have been actively pursued both in laboratory and field conditions with many

successful attempts. However, considerable uncertainty remains regarding process performance due to reservoir heterogeneity. Oily wastes such as oil emulsions, slop oils, and oily sludges, which are produced at many stages of exploration, production, refining, and recovery contain significant amounts of oil associated with them. Biological methods for de-emulsification of oil emulsions and slop oils have been developed and evaluated at the laboratory scale and biodemulsifiers have shown potential in providing alternative to physicochemical oil recovery while operating at significantly lower cost than conventional processes such as centrifugation. Microbial de-emulsifiers represent potential alternatives to the chemicals, but most of the studies have been done on mechanism of biodemulsification in the laboratory conditions only. Field scale studies will further explore the potential of commercial biodemulsification application in the petroleum industry. Genetic manipulations on biodemulsifying organisms have not been attempted so far and would further help developing efficient biodemulsifiers.

References

Becker JR (1997) Crude oil waxes, emulsions, and asphaltenes. PennWell, Tulsa, OK

Brown LR (2010) Microbial enhanced oil recovery (MEOR). Curr Opin Microbiol 13:316–320

Chang W-N, Liu C-W, Liu H-S (2009) Hydrophobic cell surface and bioflocculation behavior of *Rhodococcus erythropolis*. Process Biochem 44:955–962

Das M (2001) Characterization of de-emulsification capabilities of a *Micrococcus* sp. Bioresour Technol 79:15–22

Elshahed MS (2010) Microbiological aspects of biofuel production: current status and future directions. J Adv Res 1:103–111

Gao CH, Zekri A (2011) Applications of microbial-enhanced oil recovery technology in the past decade. Energ Sourc A 33:972–989

Harner NK, Richardson TL, Thompson KL, Best RJ, Best AS, Trevors JT (2011) Microbial processes in the Athabasca oil sands and their potential applications in microbial enhanced oil recovery. J Ind Microbiol Biotechnol 38:1761–1775

Huang HP, Bennett B, Oldenburg T, Adams J, Larter SR (2008) Geological controls on the origin of heavy oil and oil sands and their impacts on in situ recovery. J Can Petrol Technol 47:37–45

Huang X-F, Liu J, Lu L-J, Wen Y, Xu J-C, Yang D-H, Zhou Q (2009) Evaluation of screening methods for demulsifying bacteria and characterization of lipopeptide bio-demulsifier produced by *Alcaligenes* sp. Bioresour Technol 100:1358–1365

Huang X-F, Guan W, Liu J, Lu L-J, Xu J-C, Zhou Q (2010) Characterization and phylogenetic analysis of biodemulsifier-producing bacteria. Bioresour Technol 101:317–323

Huang X-F, Li M-X, Lu L-J, Yang S, Liu J (2012) Relationship of cell-wall bound fatty acids and the demulsification efficiency of demulsifying bacteria *Alcaligenes* sp. S-XJ-1 cultured with vegetable oils. Bioresour Technol 103:530–536

Kaster KM, Hiorth A, Kjeilen-Eilertsen G, Boccadoro K, Lohne A, Berland H, Stavland A, Brakstad OG (2012) Mechanisms involved in microbially enhanced oil recovery. Transp Porous Med 91:59–79

Khire JM (2010) Bacterial biosurfactants and their role in microbial enhanced oil recovery (MEOR). Adv Exp Med Biol 672:146–157

Kosaric N (1996) Biosurfactants. In: Rehm H-J, Reed G, Puhler A, Stadler P (eds) Biotechnology. VCH, Weinheim, pp 697–717

Lazar I, Petrisor IG, Yen TF (2007) Microbial enhanced oil recovery (MEOR). Petrol Sci Technol 25:1353–1366

Liu J, Huang X-F, Lu L-J, Li M-X, Xu J-C, Deng H-P (2011) Turbiscan Lab® Expert analysis of the biological demulsification of a water-in-oil emulsion by two biodemulsifiers. J Hazard Mater 190:214–221

Mohebali G, Kaytash A, Etemadi N (2012) Efficient breaking of water/oil emulsions by a newly isolated de-emulsifying bacterium, *Ochrobactrum anthropi* strain RIPI5-1. Colloids Surf B Biointerfaces 98:120–128

Nadarajah N, Singh A, Ward OP (2002a) Evaluation of a mixed bacterial culture for de-emulsification of water-in-petroleum oil emulsions. World J Microbiol Biotechnol 18:435–440

Nadarajah N, Singh A, Ward OP (2002b) De-emulsification of petroleum oil emulsion by a mixed bacterial culture. Process Biochem 37:1135–1141

Sen R (2008) Biotechnology in petroleum recovery: the microbial EOR. Prog Energ Combust 34:714–724

Singh A, Van Hamme JD, Ward OP (2006) Petroleum microbiology. Part 2. Recovery, biorefining and biodegradation processes. Chimica Oggi (Chem Today) 24:65–67

Singh A, Van Hamme JD, Ward OP (2007) Surfactants in microbiology and biotechnology: part 2. Application aspects. Biotechnol Adv 25:99–121

Singh A, Singh B, Ward OP (2012) Potential applications of bioprocess technology in petroleum industry. Biodegradation 23:865–880

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549

Voordouw G (2011) Production-related petroleum microbiology: progress and prospects. Curr Opin Biotechnol 22:401–405

Ward OP, Singh A, Van Hamme JD, Voordouw G (2009) Petroleum microbiology. In: Schaechter M (ed) Encyclopedia of microbiology, 3rd edn. Elsevier, Amsterdam, pp 443–456

Ward OP, Singh A, Van Hamme JD, Voordouw G (2012) Petroleum microbiology. In: Schmidt T, Schaechter M (eds) Topics in ecological and environmental microbiology. Academic, San Diego, CA, pp 625–643

Wen Y, Cheng H, Lu L-J, Liu J, Feng Y, Guan W, Zhou Q, Huang X-F (2010) Analysis of biological demulsification process of water-in-oil emulsion by *Alcaligenes* sp S-XJ-1. Bioresour Technol 101:8315–8322

Youssef N, Elshahed MS, McInerney MJ (2009) Microbial processes in oil fields: culprits, problems, and opportunities. Adv Appl Microbiol 66:141–251

Zhou SQ, Huang HP, Liu YM (2008) Biodegradation and origin of oil sands in the Western Canada sedimentary basin. Petrol Sci 5:87–94

Chapter 13
The Chemistry of Removal of Inorganic Compounds from the Soil

Innocent Oseribho Oboh

13.1 Introduction

The solid state of soil comprises an average of 45 % of the total soil bulk and consists of mineral particles, organic matter and organic mineral particles. These play a very important role in giving the soil the ability to adsorb, exchange, oxidise, reduce, catalyse and precipitate chemicals specifically metal ions (Weber 1991). Soil also becomes a major reservoir for contaminants as it has the ability to bind to various organic or inorganic compounds. These compounds exist in the soil in many forms and different forces are needed to keep them bound to soil particles.

Unlike organic compounds, most inorganic compounds do not undergo microbial or chemical degradation (Kirpichtchikova et al 2006). Instead, the total concentration of these inorganic compounds in soil persists long after their introduction into the environment (Adriano 2003).

Metals, on the other hand, are natural components in soil. At higher concentrations, cationic metals will form insoluble hydroxide, carbonate or sulphide precipitates (Stumm and Morgan 1981; Evans 1989; Sposito 1989).

Contamination of soils can be brought about by industrial activities, such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertiliser and pesticide application and generation of municipal waste (Kabata-Pendias and Pendias 1989). This can in return negatively impact the environment alongside living organisms. However, soil contamination does not only affect living organisms in the subsurface, but also affects the plants that accumulate contaminants as they grow. Thus, contaminants enter the food chain with the potential to adversely impact public health (Ramakrishnan et al 2011).

On the other hand, contaminants in the soil can be washed out by ground water or rain, resulting in the dissemination of the contamination. However, this process

I.O. Oboh (✉)
Department of Chemical and Petroleum Engineering, University of Uyo, Uyo, Nigeria
e-mail: obohio2009@yahoo.com

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_13, © Springer-Verlag Berlin Heidelberg 2014

is not the preferred process for the removal of contaminants owing to the fact that the area being affected by the contaminants is larger. Hence, the process of remediation is more difficult and costly since the affected area grows (Vaudelet et al 2011).

The inorganic colloidal fraction of soil is responsible for the sorption process via mineral particles. It is comprised of clay minerals, oxides, sesquioxides and hydrous oxides. Specifically looking at the clay minerals, these are silicates that have originated from other forms of the compound. They include hydrous aluminium, magnesium or iron silicates (Dobrzanski and Zawadzki 1993). A type of clay mineral found in soils is typical for clays montmorillonite and illite. The unit cell is built from two silica tetrahedral layers, (Si_2O_5), surrounding an aluminium octahedral layer, $Al_2O_4(OH)_2$. Only weak Van der Waals forces exist between the two units allowing water, nutrients and chemicals to readily enter the interlayer regions and react with the inner surface and become immobilised. This also causes the ability to expand montmorillonite or illite when in contact with water, where the concentration can vary in the montmorillonite. Thus, the chemical formula can be written as $Al_2(OH)_2(Si_2O_5)_2{\cdot}nH_2O$ (Dobrzanski and Zawadzki 1993).

Soil surfaces can carry a net negative or positive charge depending on the nature of the surface and the soil pH. The permanent net negative charge on surfaces is due to charge imbalance resulting from the isomorphous substitution of Al^{3+} for Si^{4+} in the tetrahedral layers and/or substitution of Mg^{2+}, Fe^{2+}, etc., for Al^{3+} in the octahedral layers of aluminosilicate clays. The charge on the surface is not affected by changes in soil pH, and hence, it is termed as a permanent charged surface. Clay particles are usually negatively charged which is a very important factor as it influences sorption properties of the soil (Dube et al. 2001). pH-dependent charged surfaces are associated with the edges of clay minerals, with the surfaces of oxides, hydroxides and carbonates, as well as with organic matter (acid functional groups). There are at least two major possibilities as to how these charges are formed (Loughnan 1969). Firstly, the hydroxyl groups which exist on the edges and on the outer layers of minerals can dispose of hydrogen which is bonded covalently with oxygen. This process is pH dependent and the ability to split the hydrogen atom decreases when pH decreases. When the pH is above 6, hydrogen may easily by replaced by other ions like Ca^{2+}, Al^{3+}, Pb^{2+} and Cd^{2+} (Dube et al. 2001). The second process of creating negative charges is connected to the isomorphous ion replacement in the minerals. In the silica tetrahedral, Al^{3+} can replace the silicon ion Si^{4+} since these two have a similar ionic radius. However, Mg^{2+} and Fe^{2+} can exist only in the octahedral layers as supposed to Al^{3+}. The negative charge, which appears as a result of isomorphous ion replacement, is pH independent and therefore quite persistent (Dube et al. 2001).

In this chapter, after a brief introduction of input sources for inorganic compounds and their dynamics in soils, the chemistry or mechanism of various methods for their removal, is reviewed.

13.2 Naturally Present in Soils

The cationic metals occur naturally in soils as do oxides and hydroxides (Fe, Mn, Al). To a lesser extent they can occur as carbonates, phosphates, and sulphates and reduce in soils as sulphides due to their high insolubility. They could also be present in forms of salts with different solubilities (carbonate, sulphide and sulphate), oxides as well as in the mineral structure of soil minerals. A very specific group of inorganic pollutants consists of radio-nuclides, which are elements with radioactive isotopes. A very diverse group of chemical pollutants consists of substances causing soil salinity, acidification or alkalisation. They are primarily non-metallic anions such as chlorides and sulphates. As, Cr, Se and V can complex with O and typically exist as anionic species under relevant environmental conditions. The most common forms of As are arsenate (As(V)) and arsenite (arsenic(III)), which are present in soil solutions in the form of AsO_4^{3-} and AsO_3^{3-}, respectively. Cr can exist as chromate (Cr(VI) or CrO_4^{2-}). Similarly, Se can be present as selenates (SeO_4^{2-}) and selenites (SeO_3^{2-}) and V can be present as vanadate (VO_4^{3-}).

13.2.1 Contamination

Soil contamination can be characterised on the basis of different criteria for reclamation purposes. The most suitable criterion for pollutant characterisation is the chemical character of the compound or group of compounds that are the main soil pollutants. According to the chemical composition, this characteristic enables us to classify the pollutants into groups of chemical compounds with specific physical and chemical properties, such as water solubility and solubility in other solvents, volatility and susceptibility to degradation and biodegradation. In the case of contaminated soil, resulting from mining and post-mining activities, the most important pollutants are inorganic compounds like heavy metals, radio-nuclides, substances increasing the soil salinity, soil acidification or alkalisation and toxic anions. These characteristics of inorganic pollutants, except for oxyanions, other metallic pollutants occur in soil solutions in cationic forms, whereas non-metallic pollutions occur mostly as anions.

In the soil environment, metals can exist as cations, anions or neutral species and depending on which form they are present in, this can significantly affect the soils' sorption, solubility and mobility.

Chemical interactions that contribute to metal(loid) retention by colloid particles include the following.

13.2.2 Precipitation

Precipitation appears to be the dominant process in high pH soils or in the presence of anions such as SO_4^{2-}, CO_3^{2-}, OH^- and HPO_4^{2-} and when the concentration of the heavy metal(loid) ions is high (Naidu et al. 1996). Precipitation occurs when the ionic product of the dissolved metal(loid) exceeds the solubility product of that phase.

13.2.3 Sorption Process

Both soil properties and soil solution composition determine the dynamic equilibrium between metal(loid)s in solution and in the soil solid phase. The removal from solution implies that ions or compounds have been sorbed into the interfacial region between the soil solids and the solution phase.

Inorganic compounds are adsorbed mostly by chemical interaction with soil surfaces. These interactions range from purely electrostatic to strong covalent bonding. Charged solute species (ions) are attracted to the charged surface of soil particles by electrostatic attraction and/or through the formation of specific bonds (Mott 1981). Two reasons have been given for the effects of inorganic anions on the sorption of heavy metal(loid) cations (Naidu et al. 1994). Firstly, inorganic anions form ion-pair complexes with heavy metal(loid)s and thereby reduces the sorption of heavy metal(loid)s. Secondly, the specific sorption of ligand anions is likely to increase the negative charge on soil particles and therefore will increase the sorption of heavy metal(loid)s.

13.2.4 Complex Formation

Organic components of soil constituents have high affinity for heavy metal(loid) cations because of the presence of ligands or groups that can form chelates with metal(loid)s (Harter and Naidu 1995). With increasing pH, carboxyl, phenol, alcohol and carbonyl functional groups in soil organic matter dissociate, thereby increasing their affinity for metal(loid) cations. This is dependent on various factors including temperature and concentration.

13.3 Removal of Inorganic Compounds from Soil

13.3.1 Chemical Washing

Soil particles are cleaned by selectively transferring the contaminants into the soil solution. Since heavy metals are sparingly soluble and occur predominantly in the adsorbed state, washing the soils with water alone would not be sufficient to remove a susbstantial amount of cations in the leachates. Hence, chemical agents will have to be added to the washing water (Davis and Singh 1995). This is achieved by mixing the soil with aqueous solutions of acids, alkalis, complexants, surfactants and other solvents. The cleaned particles are then separated from the aqueous solution which is then treated to remove the contaminants (Dermont et al. 2008; CLAIRE 2007).

13.3.2 Phytoremediation

The chemistry of metal interaction with soil matrix is central to the phytoremediation concept. Lead, a major contaminant, is notorious for the lack of soil mobility primarily due to metal precipitation as insoluble phosphates, carbonates, hydroxides and oxides (Blaylock and Huang 1999). Thus, increasing metal solubility in the soil is an important prerequisite to enhance the potential for Pb phytoextraction. This can be achieved either by two mechanisms (convection or mass flow and diffusion), which may be responsible for metal transport from the bulk soil to the plant roots (Corey et al. 1981; Barber 1984). Due to the convection of soluble metals, ions move from soil solids to root surface. From the rhizosphere, water is absorbed by roots to replace water transpired by leaves. Water uptake from the rhizosphere creates a hydraulic gradient directed from the bulk soil to the root surface. Some ions are absorbed by the roots faster than the rate of supply via mass flow. Thus, a depleted zone is created in soil immediately and is adjacent to the root. This generates a concentration gradient directed from the bulk soil solution and soil particles holding the absorbed elements, to the solution in contact with the root surface. This concentration gradient drives the diffusion of ions towards the depleted layer surrounding the roots.

Plants have evolved specialised mechanisms to increase the concentration of metal ions in the soil solution. For, example, at low ion supply, plants may alter the chemical environment of the rhizosphere to stimulate the desorption of ions from soil solids into the solution. Such mechanisms include rhizosphere acidification due to H^+ extrusion from roots (Crowley et al. 1991). Protons compete and replace metal ions from binding sites, stimulating their desorption from soil solids into solution. In addition, some plants can regulate metal solubility in the rhizosphere by exuding a variety of organic compounds from the roots. Roots exude complex metal

ions keeping them in solution and available for uptake into roots (Romheld and Marschner 1986).

In general, sorption to soil particles reduces the activity of metals in the system. Thus, the higher the cation exchange capacity (CEC) of the soil, the greater the sorption and immobilisation of the metals. In acidic soils, metal desorption from soil-binding sites into solution is stimulated due to H^+ competition for binding sites. Soil pH affects not only metal bioavailability but also the very process of metal uptake into roots. This effect appears to be metal specific.

The pH controls metal speciation and binding by affecting the species distribution of dissolved ligands (e.g., phosphate, sulphate, carbonate and humic substances) and the surface charge of binding sites on DOM (organic matter which is present to act as an electron donor) and solid phases such as iron oxyhydroxides. Generally, at low pH, when surface sites are protonated, the sorption of cationic metals decreases and hence increases metal mobility. The converse occurs at high pH, which results in low metal solubility and greater sorption. The patterns of dissolution and sorption are reversed for metalloids, such as As, that exist as anionic species.

13.3.3 Introduction of Different Substances

This method is used for the immobilisation of inorganic contaminants such as heavy metals and is rarely applied to organic contaminants. The method is based on the introduction of the soil into different substances (cement, polyepoxide resin and zeolites), which strongly bonds with the contaminants or create sparingly soluble chemical associations (carbonates or phosphates).

The effects of this remediation technique include modification of the soil chemical properties, causing the immobilisation of heavy metals or chemical transformation into less mobile forms by pH changes. This method is often used in emergency cases to prevent the contaminated area from spreading, often in connection with phytostabilisation as a supporting technique. The process is mainly applied to low contaminated areas.

13.3.4 Electrokinetics Remediation

Electrokinetic remediation is an environmental technique specifically developed for the removal of contaminants in soil, sediments and sludge, even though it can be applied to any solid porous material (Reddy and Cameselle 2009). This method is based on the phenomenon of pollutant migration in an electric field (Acar 1993). Migrating particles have to have a permanent electric charge or have to be polarised, thus, the technique is used to remove heavy metals or polar compounds. Electrodes are inserted into the ground on opposite sites of the contaminated area.

The effect of the electric field induces the mobilisation and transportation of contaminants through the porous matrix towards the electrodes, where they are then collected, pumped out and treated. In other words, the contaminants are under the influence of an electromagnetic field that migrates through the soil within the cathode or anode area and are removed via chemical precipitation, adhesion to the electrodes' surfaces and or removing and processing the contamination beyond the remediated site.

Main electrodes, anode and cathode, are inserted into the soil matrix, normally inside a chamber which is filled with water or the appropriate solution to enhance the removal of contaminants. Typically, a voltage drop of 1 VDC/cm is applied to the main electrodes and due to several transportation mechanisms induced by the electric field, contaminants are transported out of the soil (Acar et al. 1990; 1995). These transportation mechanisms are discussed as follows:

Electromigration: This is the transportation of ions in solution in the interstitial fluid in the soil matrix towards the electrode of the opposite charge. Cations, which are positively charged particles, move towards the cathode (negative electrode), and anions, which are negatively charged particles, move towards the anode (positive electrode). The ionic migration or electromigration depends on the size and charge of the ion and the strength of the electric field.

Electro-osmosis: This mechanism is the net flux of water or interstitial fluid induced by the electric field. Electro-osmosis is a complex transport mechanism that depends on the electric characteristics of the solid surface, the properties of the interstitial fluid and the interaction between the solid surface and the components in solution. The electro-osmotic flow transports out of the porous matrix any chemical species in solution. Soils and sediments are usually electronegative (solid particles are negatively charged), so the electro-osmotic flow moves towards the cathode, whereas in electropositive solid matrices, the electro-osmotic flow moves towards the anode (Probstein and Hicks 1993).

Electrophoresis: It is the movement of charged particles based on colloidal size and bound contaminants through a stationary pore fluid that is relative to a low direct current or voltage gradient. When compared to ionic migration and electro-osmosis, mass transport by electrophoresis is negligible in low permeability soil systems. However, mass transport by electrophoresis may become significant in soil suspension systems and it is the mechanism for the transportation of colloids (including bacteria) and micelles.

Diffusion: This mechanism refers to the mass transport with respects to the concentration gradient, not the voltage gradient, as the previous mentioned transport mechanisms. During the electrokinetic treatment of contaminated soils, diffusion will appear as a result of the concentration gradients generated by the electromigration and electro-osmosis of contaminants. Diffusive transport is often neglected considering its lower velocity compared to electromigration and electro-osmosis.

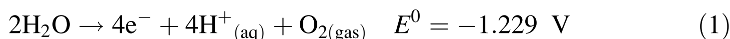
The two main transport mechanisms in electrokinetic remediation are electromigration and electro-osmosis, while diffusion is as a result of the

concentration gradient generated by the two main transport mechanisms in electrokinetic remediation (Pamukcu 2009). The extent of electromigration of a given ion depends on the conductivity of the soil, soil porosity, pH gradient, applied electric potential, initial concentration of the specific ion and the presence of competitive ions.

Electromigration is the major transport processes for ionic metals, polar organic molecules, ionic micelles and colloidal electrolytes. The electro-osmotic flow depends on the dielectric constant and viscosity of pore fluid as well as the surface charge of the solid matrix represented by the zeta potential. The zeta potential is a function of many parameters including the types of clay minerals and ionic species that are present including the pH, ionic strength and temperature. Electro-osmosis is considered the dominant transport process for both organic and inorganic contaminants that are in dissolved, suspended, emulsified or other such similar forms. Nonetheless, electro-osmotic flow though low permeability regions are significantly greater than the flow achieved by an ordinary hydraulic gradient, so the electro-osmotic flow is much more efficient in low permeability soils (Saichek and Reddy 2005). The application of an electric field to a moisten porous matrix also induces chemical reactions to take place in the soil and on the electrodes that decisively influences the chemical transportation and speciation of the contaminants alongside other constituents of the soil. Chemical reactions include acid–alkaline reactions, redox reaction, adsorption–desorption and dissolution–precipitation reactions.

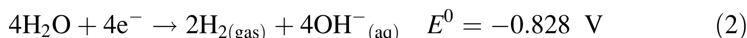
Such reactions can affect the speciation of the contaminants and therefore affect the transportation and contaminant removal efficiency (Yeung 2009). The main reaction in the electrochemical/electrokinetic systems is the decomposition of water that occurs at the electrodes. The electrolysis of water at the electrodes generate oxygen gas and hydrogen ions (H^+) due to the oxidation at the anode, whereas hydrogen gas and hydroxyl (OH^-) ions are generated due to the reduction at the cathode as represented in equations 1 and 2:

At Anode (Oxidation):



$$2H_2O \rightarrow 4e^- + 4H^+{}_{(aq)} + O_{2(gas)} \quad E^0 = -1.229\ \text{V} \tag{1}$$

At Cathode (Reduction):



$$4H_2O + 4e^- \rightarrow 2H_{2(gas)} + 4OH^-{}_{(aq)} \quad E^0 = -0.828\ \text{V} \tag{2}$$

Essentially, acid is produced at the anode because of the presence of aqueous H^+ ions and alkaline solution is produced at the cathode due to the presence of aqueous OH^- ions., Therefore, the pH in the cathode increases, while pH at the anode decreases. The migration of H^+ ions from the anode and OH^- from the cathode into the soil leads to dynamic changes in soil pH. H^+ is about twice as mobile as OH^-, so the protons dominate the system and an acid front moves across the soil until it meets the hydroxyl front in a zone near the cathode where the ions may recombine

to form water. Thus, the soil is divided in two zones with a sharp pH jump in between: a high pH zone close to the cathode and a low pH zone on the anode side.

The actual soil pH values will depend on the extent of transport of H^+ and OH^- ions and the geochemical characteristics of the soil. The implications of these electrolysis reactions are enormous in the electrokinetic treatment since they impact the absorption/desorption of the contaminants, the dissolution/precipitation reactions, chemical speciation and the degradation of the contaminants. Moreover, pH changes in the soil affecting the contaminant migration and the evolution of the electro-osmotic flow which is decisive in the removal of non-charged organic contaminants (Reddy and Cameselle 2009).

In electrokinetic remediation, it is also common to use of chemicals to enhance the dissolution and the transportation of the contaminants. The enhancing chemicals are going to interact with the soil and the contaminants. Therefore it is necessary to evaluate the geochemistry of the soil and the possible reactions with the enhancing chemicals. It is also important to consider the effect of pH at the same time in order to design a satisfactory system that can remove or eliminate the contaminants while still keeping the natural properties of the soil for its use after the remediation process.

13.4 Biochemical Transformation Processes

13.4.1 Reduction–Oxidation Reactions

Ar, Cr, Hg and Se are some of the metal(loid)s that are most commonly subjected to microbial redox reactions. These redox reactions are grouped into two categories: assimilatory and dissimilatory reactions. In assimilatory reactions, the metal(loid) substrate plays a role by acting as a terminal electron acceptor and thereby facilitating the organisms' growth. In the dissimilatory reactions, the metal(loid) substrate takes part in fortuitous reduction reactions coupled to oxidation of other substrates by the microorganism.

Arsenic in soils and sediments can be oxidised to arsenate [As(V)] by bacteria (Wakao et al. 1988; He and Hering 2009). Since As(V) is more strongly retained than arsenite [As(III)] by inorganic soil components, microbial oxidation results in the immobilisation of As. Under reducing conditions, As(III) is the dominant form of As in soils. Arsenite is much more toxic and mobile than As(V).

While Cr(III) is strongly adsorbed onto soil particles, Cr(VI) is only weakly adsorbed and is readily available for plant uptake and leaching into groundwater (James and Bartlett 1983). Thus, reduction of Cr(VI) to Cr(III) can enhance the immobilisation of Cr, thereby rendering it to be less bioavailable. Suitable conditions for Cr(VI) reduction occur when organic matter is present to act as an electron donor, and Cr(VI) reduction is enhanced in acidic rather than alkaline soils (Bolan et al. 2003).

13.4.2 Methylation/Demethylation

With the actions of microbes, volatilisation can occur as there is microbial conversion of metal(loid)s to their respective metallic, hydride or methylated form. As a result, these forms have low boiling points, high vapour pressures and are therefore susceptible for volatilisation. Methylation of As, Hg and Se is considered to be the major process of volatilisation in soils and sediments, releasing the methylated forms of these elements as toxic gas (Cernansky et al. 2009).

Two mechanisms of methylation which are transmethylation and fission have been found to be involved in the reduction of a compound to a methyl group. The first is transmethylation, which refers to the transfer of an intact methyl group from one compound (the methyl donor) to another compound (the methyl acceptor). The second mechanism involves fission of a compound (the methyl source), which does not necessarily contain a methyl group; this produces a 'one carbon fragment' that is subsequently captured by another compound (e.g. metalloid) and reduced to a methyl group (Bolan et al 2010).

13.5 Chemistry of Arsenic Compounds Removal from the Soil

As mentioned earlier, the most common forms of As are arsenate (As(V)) and arsenite (arsenic(III)), which are present in soil solution in the form of AsO_4^{3-} and AsO_3^{3-}, respectively. The introduction of the phosphate ions can induce the mobilisation of As in soil, thereby increasing its bioavailability (Smith et al. 2002). Both P and As belong to group 15 and have comparable dissociation constants for their acids and solubility products resulting $H_2AsO_4^-$ and $H_2PO_4^-$ ions to compete for the same sorption sites. Thus, phosphate addition is likely to enhance phytoremediation of As-contaminated soils.

A study by Woolson et al. (1973) showed that the addition of phosphate to soil contaminated by As displaced more than 65 % of the total As in the soil. Although the addition of phosphate increases As solubility, Peryea (1991) reported that desorption of As was dependent on soil type since there was no increase in As concentration in soil solution from volcanic soil (with high anion-fixing and pH-buffering capacity). This suggests that only large additions of P (>400 mg kg^{-1}) would affect the As solubility in these soils (Smith et al. 1998; Chen et al. 2002).

It has been noted that leaching of As from a column containing mineral soil combined with As-rich poultry manure increased with the addition of phosphate compounds. This was seen since the As concentration in the leachate was about ten times higher when $Ca(H_2PO_4)_2$ was used to leach the soil column as compared to $CaSO_4$ solution. In the presence of $Ca(H_2PO_4)_2$ solution, a maximum As

concentration of 800 μg L^{-1} was found in the leachate, much higher than the WHO maximum permissible limit of 10 μg L^{-1} for drinking water (Qafoku et al. 1999).

Arsenic uptake by plants is associated with the $H_2PO_4^-$ uptake mechanism, where presumably As(V) is taken up as a $H_2PO_4^-$ analogue (Pickering et al. 2000). The role of $H_2PO_4^-$ ions in enhancing the mobility of As, especially AsO_4^{2-} ions, comes to play (Bolan et al. 2010).

13.6 Large-Scale Application

Since the addition of fertilisers, such as the P fertiliser to As-contaminated soil, the increase of As solubility and mobility and the increase of plant uptake of soil As was observed (Creger and Peryea 1994). The use of P fertiliser is readily used to enhance As uptake by plants and is attracting growing interest. Tu and Ma (2003) suggested that for the efficient use of Chinese brake (*Pteris vitata* L.) for phytoremediation of As-contaminated soils, phosphate application may be of an important strategy. Davenport and Peryea (1991) observed that heavy use of monoammonium phosphate (MAP) or monocalcium phosphate (MCP) fertilisers significantly increased the amount of As leached from the soil. The results indicate that use of P fertilisers on such soils has the potential to greatly enhance downward movement of As (Peryea and Kammereck 1997). Thus, the increased mobilisation of As resulting from phosphate input can result in increased leaching to groundwater, especially in the absence of active plant growth. Attempts to use plants to remove As from soils need to take the various effects of phosphate into consideration (Bolan et al. 2010).

13.7 Conclusions

The chemistry for the removal of inorganic compounds as contamination found in urban environments has been discussed alongside some of the mechanisms behind the removal. It was noted that some factors are responsible for the mobilisation and bioavailability of these chemicals. Mobilisation processes such as phytoremediation, chemical washing and a lot of others have been seen to remove these contaminants from the soil. The pH value of the soil can be a factor that can affect the uptake of these contaminants from soil particles. Removal of metal(loid)s through phytoremediation techniques and the subsequent recovery of the metal (loid)s or their safe disposal have attracted increasing research and commercial interest.

However, a major drawback to mobilisation techniques may be that if plant growth is absent due to the phytotoxicity of metal(loid)s, the mobility of metal(loid) s is likely to be enhanced, thereby resulting in the contamination of groundwater sources.

References

Acar YB (1993) Principles of electrokinetic remediation. Environ Sci Technol 27:2638–2647

Acar YB, Gale RJ, Putnam GA, Hamed J, Wong RL (1990) Electrochemical processing of soils: theory of pH gradient development by diffusion, migration, and linear convection. J Environ Sci Health A Environ Sci Eng 25:687–714

Acar YB, Gale RJ, Alshawabkeh AN, Marks RE, Puppala S, Bricka M (1995) Electrokinetic remediation: basics and technology status. J Hazard Mater 40:117–137

Adriano DC (2003) Trace elements in terrestrial environments: biogeochemistry, bioavailability and risks of metals, 2nd edn. Springer, New York

Barber SA (1984) Soil nutrient bioavailability. Wiley, New York

Blaylock MJ, Huang JW (1999) Phytoextraction of metals. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals using Plants to clean up the environment. Wiley, New York, pp 53–70

Bolan NS, Adriano DC, Natesan R, Koo BJ (2003) Effects of organic amendments on the phytoavailability of chromate in mineral soil. J Environ Qual 32:120–128

Bolan N, Naidu R, Choppala G, Park J, Mora ML, Budianta D, Panneerselvam P (2010) Solute interactions in soils in relation to the bioavailability and environmental remediation of heavy metals and metalloids. Pedologist 53:1–18

Cernansky S, Kolencik M, Sevc J, Urik M, Hiller E (2009) Fungal volatilization of trivalent and pentavalent arsenic under laboratory conditions. Bioresour Technol 100:1037–1040

Chen TB, Fan ZL, Lei M, Huang ZC, Wei CY (2002) Effect of phosphorus on arsenic accumulation in As-hyperaccumulator *Pteris vittata* L. and its implication. Chin Sci Bull 47:1876–1879

CLAIRE (2007) Understanding soil washing, contaminated land: applications in real environments. Tech Rep TB13

Corey RB, Fujii R, Hendickson LL (1981) Bioavalability of heavy metal in soil–sludge systems. In: Proceedings of the 4th annual madison conference of applied research and practice on municipal and industrial waste, University of Wiscousin, Madison, WI, pp 449–465

Creger TL, Peryea F (1994) Phosphate fertilizer enhances arsenic uptake by apricot liners grown in lead-arsenate-enriched soil. HortScience 29:88–92

Crowley DE, Wang YC, Reid CPP, Szansiszlo PJ (1991) Mechanism of iron acquisition from siderophores by microorganism and plants. Plant Soil 130:179–198

Davenport JR, Peryea FJ (1991) Phosphate fertilizers influence leaching of lead and arsenic in soil contaminated with lead arsenate. Water Air Soil Pollut 57–58:101–110

Davis AP, Singh I (1995) Washing of zinc(II) from contaminated soil column. J Environ Eng 121:174–185

Dermont G, Bergeron M, Mercier G, Richer Lafleche M (2008) Soil washing for metal removal: a review of physical/chemical technologies and field applications. J Hazar Mat 152:1–31

Dobrzanski B, Zawadzki S (1993) Gleboznawstwo-Praca zbiorowa. PWRiL, Warszawa

Dube A, Zbytniewski R, Kowalkowski T, Cukrowska E, Buszewski B (2001) Adsorption and migration of heavy metals in soil. Pol J Environ Stud 10:1–10

Evans LJ (1989) Chemistry of metal retention by soils. Environ Sci Technol 23:1046–1056

Harter RD, Naidu R (1995) Role of metal-organic complexation in metal sorption by soils. Adv Agron 5:219–263

He YT, Hering JG (2009) Enhancement of arsenic(III) sequestration by manganese oxides in the presence of iron(II). Water Air Soil Pollut 203:359–368

James BR, Bartlett RJ (1983) Behavior of chromium in soils: VII. Adsorption and reduction of hexavalent forms. J Environ Qual 12:177–181

Kabata-Pendias A, Pendias H (1989) Trace elements in the soil and plants. CRC Press, Boca Raton, FL

Kirpichtchikova TA, Manceau A, Spadini L, Panfili F, Marcus MA, Jacquet T (2006) Speciation and solubility of heavy metals in contaminated soil using X-ray microfluorescence, EXAFS

spectroscopy, chemical extraction, and thermodynamic modeling. Geochim Cosmochim Acta 70:2163–2190

Loughnan FC (1969) Chemical weathering of the silicate minerals. Elsevier, New York

Mott CJB (1981) Anion and ligand exchange. In: Greenland DJ, Haynes MHB (eds) The chemistry of soil processes. Wiley, New York, pp 179–219

Naidu R, Bolan NS, Kookana RS, Tiller KG (1994) Ionic-strength and pH effects on the adsorption of cadmium and the surface charge of soils. Eur J Soil Sci 45:419–429

Naidu R, Kookana RS, Sumner ME, Harter RD, Tiller KG (1996) Cadmium adsorption and transport in variable charge soils: a review. J Environ Qual 26:602–617

Pamukcu S (2009) Electrochemical transport and transportation. In: Reddy KR, Cameselle C (eds) Electrochemical remediation technologies for polluted soils, sediments and groundwater. Wiley, New Jersey, pp 29–64

Peryea FJ (1991) Phosphate-induced release of arsenic from soils contaminated with lead arsenate. Soil Sci Soc Am J 55:1301–1306

Peryea FJ, Kammereck R (1997) Phosphate enhanced movement of arsenic out of lead arsenate contaminated topsoil and through uncontaminated subsoil. Water Air Soil Pollut 93:243–254

Pickering IJ, Prince RC, George MJ, Smith RD, George GN, Salt DE (2000) Reduction and coordination of arsenic in Indian mustard. Plant Physiol 122:1171–1177

Probstein RF, Hicks RE (1993) Removal of contaminants from soils by electric fields. Science 260:498–503

Qafoku NP, Kulier U, Summer ME, Radcliffe DE (1999) Arsenate displacement from fly ash in amended soils. Water Air Soil Pollut 114:185–198

Ramakrishnan B, Megharaj M, Venkateswarlu K, Sethunathan N, Naidu R (2011) Effects on microorganisms and their activities in soils. Rev Environ Contam Toxicol 211:63–120

Reddy KR, Cameselle C (2009) Overview of electrochemical remediation technologies. In: Reddy KR, Cameselle C (eds) Electrochemical remediation technologies for polluted soils, sediments and groundwater. Wiley, New Jersey, pp 3–28

Romheld V, Marschner H (1986) Mobilization of iron in the rhizosphere of different plant species. Adv Plant Nutr 2:155–204

Saichek RE, Reddy KR (2005) Electrokinetically enhanced remediation of hydrophobic organic compounds in soils: a review. Crit Rev Environ Sci Technol 35:115–192

Smith E, Naidu R, Alston AM (1998) Arsenic in the soil environment – a review. Adv Agron 64:149–195

Smith E, Naidu R, Alston A (2002) Chemistry of arsenic in soils: II. Effect of phosphorus, sodium and calcium on sorption. J Environ Qual 31:557–563

Sposito G (1989) The chemistry of soils. Oxford University Press, New York

Stumm W, Morgan JJ (1981) Aquatic chemistry, vol 2. Wiley, New York

Tu C, Ma LQ (2003) Effects of arsenate and phosphate on their accumulation by an arsenic-hyperaccumulator *Pteris vittata* L. Plant Soil 249:373–382

Vaudelet P, Schmutz M, Pessel M, Franceschi M, Guérin R, Atteia O (2011) Mapping of contaminant plumes with geoelectrical methods. A case study in urban context. J Appl Geophys 75:738–751

Wakao N, Koyatsu H, Komai Y, Shimokawara H, Sakurai Y, Shiota H (1988) Microbial oxidation of arsenite and occurrence of arsenite-oxidizing bacteria in acid mine water from a sulfur-pyrite mine. Geomicrobiol J 6:11–24

Weber JB (1991) Fate and behaviour of herbicides in soils. Appl Plant Sci 5:28–41

Woolson EA, Axely JH, Kearney PC (1973) The chemistry and phytotoxicity of arsenic in soil: II. Effect of time and phosphorus. Soil Sci Soci Am J 37:254–259

Yeung AT (2009) Geochemical processes affecting electrochemical remediation. In: Reddy KR, Cameselle C (eds) Electrochemical remediation technologies for polluted soils, sediments and groundwater. Wiley, New Jersey, pp 65–94

Chapter 14
Bioremediation of Contaminated Sites and Aquifers

Nagina Parmar, Ajay Singh, and Hammad Khan

14.1 Introduction

Industrial philosophies, employed on providing the globe with a rich, abundant, and profitable resource has revolutionized modern technology, enriched our entertainment and travelling abilities, and generated an energy sector prompt on using renewable sources. In a market so dependent on one resource, saturating consumer need with market availability can become difficult without sacrificing. Now imagine if that was water. In the midst of such ventures, understanding the potential ramifications of contamination, interference, or blockages occurring to such bodies of water, must be addressed. Water is an essential element making up all human life. It is what sustains us, what keeps our environment in equilibrium, and what regulates our economy. It is globally one of the most recognizable resources needed for survival, be it microscopic bacteria or full flesh beings, water supports all life and cannot be replaced (Christopherson and Byrne 2006). Water and water quality directly influence the health and prominence of a community. When a toxic chemical, an oil spill or a foreign invasive species, is introduced to such environments, the native equilibrium established in the ecosystems becomes disturbed (Zaporozec 2002).

Ecological catastrophes seen with the Exxon Valdez oil spill along the coast of Alaska, Kuwait's oil well fires across the Arabian peninsula and into the waters of the Persian Gulf, Chernobyl and Bhopal's chemical explosions in Ukraine and India, and of recent, the BP oil spill in the Gulf of Mexico have changed the way we think of a sustainable ecosystem (Hernan 2010). The state and survival of these ecosystems cannot be remediated naturally, as chemical contaminant turnover, in

N. Parmar (✉) • H. Khan
Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada M5B 2K3
e-mail: naginap@ryerson.ca

A. Singh
Lystek International Inc., Cambridge, ON, Canada N1R 6J9

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2_14, © Springer-Verlag Berlin Heidelberg 2014

means of detoxification is very slow. Ecosystems are fragile in nature and exposure to chemical contaminants such as polycyclic aromatic hydrocarbons (PAH), LNAPL monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (BTEX), DNAPLs, MTBEs, and chlorinated solvents expedite serious environmental concerns (Aitken et al. 1997; Dojka et al. 1998; Castro-Gutierrez et al. 2012). BTEX chemicals are components of petroleum products which are toxic and relatively soluble in water, thus having the ability to migrate from source point to other areas such as into aquifer and groundwater catchments (Lovely 1997). PAH chemicals are derived from gas manufacturing plants. These chemicals are characterized with high molecular weights, very low solubility in water with tendencies to bind tightly to soil constituents such as natural soil organic matter and are regarded as highly carcinogenic (Aitken et al. 1997). Trichloroethylene (TCE) is considered a halogenated volatile organic contaminant (VOC) which has been regarded as a carcinogenic and mutagenic compound (USEPA 1997). TCE is relatively insoluble in water and difficult to remove, which compounded with its toxicological properties, poses a great threat to environmental preservation (Pant and Pant 2009). Methyl tertiary butyl ether (MTBE) is a component of reformulated gasoline used as an octane enhancer, or oxygenate with the characteristic of being highly mobile and highly soluble in water (Davis and Erickson 2004). Natural crude oil seepage globally is estimated around 600,000 metric tons per year with a 200,000 metric tons range of uncertainty (Holliger et al. 1997). The exposure to such chemicals can form nonaqueous aggregates floating on the surface, which can prevent photorespiration in water dwelling plants by blocking sunlight and exchange of oxygen and carbon dioxide gases (Miller et al. 1978). These contaminants can also be ingested and transmitted to neighboring colonies by the marine fauna, causing further toxification internally, exposing skin and organs, and to potential breeding lines with toxic carcinogens that may lead to mutation and possibilities of reproductive sterilization to the upcoming progeny (Alvarez and Vogel 1991; Payne et al. 2003). Therefore, ecologists and other scientists concerned with remediating efforts are pushing for new means of cleanup, using mechanisms such as bioremediation, biostimulation, and bioaugmentation. These methods introduce noninvasive biological entities that facilitate biodegradation of PAH and BTEX contaminants, maximizing target areas of aerobic and anaerobic zones, thereby reducing chronic exposure and accelerating cleanup efforts from surface to subsurface areas (OTA 1991; Spies et al. 1996).

Bioremediation technology markets its success through its ability to establish and maintain conditions that promote accelerated biodegradation. Selecting specific bacteria for hydrocarbon degradation and introducing the desired genes into the indigenous population is one method used to speed up the recovery process. Remediation is often paralleled with biological requirements through the introduction of oxygen and electron derivatives that act as reservoirs promoting rapid ion exchange, metabolism, and reduction of LNAPL and DNAPL contaminants (Dojka et al. 1998). Through in situ and ex situ technologies, physical mechanisms such as pump and treat systems and fluidized bed reactor scan also are used in expediting bioremediation efforts (Davey and O'Tool 2000; Massol-Deya et al. 1995). These

methods generate robust biofilm colonies that can be directly pumped or applied into contaminated areas, degrade the hydrocarbon contaminant, and aid the indigenous population by reducing immediate toxicity and spread of the contaminant (Rodriguez-Martinez et al. 2006).

Applications must be carefully assessed before administering any foreign bacterium to native populations. Ecosystems are fragile. By screening and selecting an appropriate bioremediation, biostimulation, and bioaugmentation technologies, scientists can ensure that prolonged contamination and damage to the ecosystem is minimal and that the technologies introduced will exhibit minimal invasive attributes.

14.2 Contaminants Encountered in Aquifers and Water Bodies

Contaminants in water can exist as chemical forms of organic and inorganic waste, biological forms of bacteriological and microbial waste, and physical forms that interfere/block the natural growth of system (Nonner 2002). A report conducted by Cosgrove and Rijsbergman (2000) found rapidly growing cities, burgeoning industries, and the rise in the use of chemicals in agriculture have undermined the quality of many rivers, lakes, and aquifers. The impact of chemical waste generated by industries and gasoline manufacturing plants is highly dangerous and volatile over a relatively short period of time. However, agriculture waste generated through means of fertilizers, herbicides, and pesticides can be just as invasive or toxic if left uncontrolled, which can begin to slowly accumulate and transgress into toxic metabolites passed from one generation of the ecosystem and its recipients to the next. As mentioned earlier, some contaminants can exist as highly soluble compounds, moving with water currents, contaminating other systems, while others are nonsoluble but highly volatile when bound to organic soil matter (Aitken et al. 1997). Romijn (2002) further elaborates on this notion by correlating intrinsic groundwater vulnerability with contaminant load. The contaminant load could be described in terms of contaminant characteristics such as persistency, mobility, the mode of disposition or depth of introduction and hydraulic load, the source strength, concentration, recharge area, and duration of load. With these factors in mind, modeling the appropriate mechanism for remediation can be quite complex, requiring time, patience, and equivalent funds to exercise efforts. In this section, we will examine a few known contaminants that have historically been polluting water bodies for centuries and an insight into the environmental impact that has resulted will be discussed.

14.2.1 NAPL and BTEX Chemicals Contaminants

Nonaqueous phase liquids (NAPL's) and mono-aromatic hydrocarbons, principally benzene, toluene, ethylbenzenes, and xylenes (BTEX) are chemical contaminants often encountered in manufacturing gas plants and underground petroleum storage tanks (Aitken et al. 1997). Polycyclic aromatic hydrocarbons (PAHs) are relatively high molecular weight contaminants, which expedite major environmental concerns because both BTEX and PAH's poses carcinogenic capabilities when bound with organic sediment matter, and once bound these contaminants are difficult to be removed or degraded such as pyrene and phenanthrene. These larger molecular weight contaminants sink to the sediments and are categorized as DNAPLs (Aitken et al. 1997; Barathi and Vasudevan 2001; Castro-Gutierrez et al. 2012). BTEX contaminants are considered toxic and are relatively soluble in water, thus having the ability of migrating into groundwater catchments (Lovely 1997). BTEX are a class of chemicals known as volatile organic compounds (VOCs) which when exposed to living entities causes skin and sensory irritation, central nervous system (CNS) problems (tiredness, dizziness, headache, loss of coordination), and effects on the respiratory system. Prolonged exposure to BTEX contaminants may also affect the kidney, liver, and blood systems, thus leading to debilitating or inadequate body response, inevitably causing death (Mitra and Roy 2011). PAHs contaminants on the other hand, unlike BTEX, display poor water solubility properties, forming nonaqueous phases or films at the water surface or in sediment. These films pose several problems primarily due to their binding capacities. PAHs block sunlight from penetrating into the marine environment and act as a barrier between the water and air phase preventing adequate gas exchange (Spies et al. 1996). This may create a highly toxic and anoxic environment, where oxygen becomes a limiting agent and CO_2 and other gases released in the environment begin to accumulate without sufficient removal (Spies 2007). This consequently causes a shift in the equilibrium or natural balance of the system, leading to mass migration of contaminated entities out of the system and into another, mass death and toxification, and eventual mutation and fitness of the habitant species (Spies and Rice 2007). Spies and Rice (2007) examined the Exxon Valdez oil spill in the Gulf of Alaska. This was one of the largest documented incidents of petroleum sources released into a rich ecological environment. This incident revealed the true potential of PAHs, BTEX, chlorinated hydrocarbons, and other organic contaminants on marine plants and animals. When metabolized, PAH transformation products interfere with ion transport channels, the circulatory system loses its ionic balance and edema develops in the heart, leading to impaired circulation and morphological deformities and death (Springman et al. 2005). Furthermore, when reacted with sediment and other organic constituents of soil, PAH binds tightly to the matter and forms teratogens that travel along the water surface, with 7 PAH compounds being identified as carcinogenic (Aitken et al. 1997). These may extend beyond the impacted area and spread across the aquatic and mammalian food chain, exposing humans and animals all across the globe.

14.2.2 Contaminant Persistency in Aquifer Systems

In an aquatic medium, contaminants persist predominantly as colloidal, dissolved or particulate physical matter in which, agglomeration and sludge-like properties may develop (Singh et al. 2010). The source of media, water, moves towards a valley or catchment area where it collects and through engineering initiatives, it is distributed for diverse uses. The water travels through the alluvial sequence, which is described as the geological formation of layers differing in granulation, permeability, and stage of diagenesis. This determines the foundation of and/or surrounding material and porosity around the catchment or aquifer area (Polic and Pfendt 1994). As water percolates through groundwater and into the watershed, filtration between the sediment and alluvial system begins to accumulate large masses of pollutants such as organic contaminants into rifts/cracks and between sediment and rock minerals, which pose a greater problem. Polluted waters travel towards the catchment area and accumulate within the sediment through absorption, ion exchange, and co-precipitation which cause changes in pH, redox potential, and ionic strength within the sediment.

The effects of persistent organic contaminants are felt throughout the ecosystem, harboring many residual adverse effects post-containment and affecting growth and viability of the system. This was witnessed through the explosion at the Union Carbide India LTD (UCIL) pesticide facility in Bhopal, India, as documented by Johnson et al. (2009). Toxic gas (methyl isocyanate) and a concoction of organic pollutants spewed into the atmosphere, killing thousands and affecting over 500,000 inhabitants. Organic pesticides, carbaryl, aldicarb, and sevidol, described as gamma-hexachlorocyclohexane (HCH) compounds, polluted the area as primary contaminants, with secondary contamination coming from the production facility containment towers as they were incased with heavy metals such as mercury and chromium used as sealants and coolants. These chemicals are described as highly toxic, highly soluble in water and highly mobile in soils, which pose long-term threats to the ecology and quality of the groundwater system (Johnson et al. 2009). Studies investigating the repercussions of the 1984 accident have shown surrounding soil samples littered with heavy metals, chlorinated hydrocarbons, and organic pesticides, as well as persistence contamination throughout groundwater, vegetables, and breast milk samples around residential areas adjoining the UCIL facility. Furthermore, the total HCH pesticide concentration was also very prominent in all four samples suggesting the level of contamination may exist as a concoction of toxicants dispersed throughout subsurface soils and across the Madhya Pradesh region encompassing the city of Bhopal (Agarwal and Nair 2002).

14.3 Aerobic Bacteria in Surface Remediation

Bacteria and microorganisms alike serve many purposes within the environment, possessing the capabilities of dictating the success and viability of various ecosystems, often being used as tools to study changes or indications that reflect existing activities (Martani and Seto 1991). Such activities include environmental norms such as sustained fluctuations within temperature, humidity, nutrient availability, biotic activities, and slight pH variations (N'Guessan et al. 2010). However, environmental pollutants such as PAH's, BTEX contaminants, VOC's, MTBE, and DNAPL's, without adequate control, disturb ecological equilibriums and pose the capacity to cause immediate threats, which may prolong and persist if remediation efforts are not implemented (Pant and Pant 2009; Moran et al. 2007). Changes in the chemistry, nutrient availability, and mineral content may disturb biological entities managing the production and breakdown of organic compounds, as well, disturb the interactions between native biota and plants. Existing studies would suggest such a relationship is fragile; however, by understanding the physical and chemical properties of a contaminant with the interactive behavior of microbial entities, treatment options may be easier than initially thought. By simply selecting and stimulating appropriate microorganisms to the environment and introducing key factors that encourage continuous growth and catabolism, bioremediation efforts can be sustained when utilized effectively (Das and Chandran 2011).

Aerobic bacteria equipped with the capacity to metabolize contaminants are the primary organisms that instigate biological reduction. Selecting and stimulating aerobic bacteria to initiate surface contaminant remediation rely on key elements that determine the quality and viability of an aquifer system such as adequate nutrient availability, a pH between 6 and 9, and most importantly, the presence of oxygen and available terminal electron acceptors (TEAs) (Leahy and Colwell 1990; Atlas and Bartha 1992; Das and Chandran 2011).

Aerobic remediation treatment has shown to be most effective in reducing contaminant levels of aliphatic and aromatic petroleum hydrocarbons such as those characterized typically from gasoline and diesel fuels (EPA 2011). Furthermore, the aerobic bacteria residing between the subsurface and air–water interface aggregate together towards a contaminant, attracted by rich carbon compounds, forming a biofilm (Froehner et al. 2012). Such a formation can be used as an indicator for localizing the area of contaminant in the environment, targeting remediation efforts towards the source of contaminant and more importantly, allow scientists to strategically monitor the behavioral activities of the consortium and its level of biodegradation (Fuchs et al. 1996, 1997).

The degree of success is dependent on the level of available oxygen within the interface which has been described as one of the major contributing factors that limit microbial biodegradation of hydrocarbons due to the unavailability of oxygen as the final electron acceptor (Da Silva et al. 2004; Shi et al. 1999; Rodríguez-Martínez et al. 2006). A study conducted by Balkwill and Ghiorse (1985) studied

the behavior and activity of subsurface bacteria in shallow aquifers. The pair found communities within the aquifer to be metabolically active by the subsurface microflora, suggesting that a relative portion of the indigenous population holds the capacity to degrade aromatic and halogenated hydrocarbon pollutants.

Furthermore, Wilson et al. (1983a, b) proposed natural attenuation by such indigenous populations is possible in shallow aquifers as the aquifer sediments contain stable bacterial colonies, which when exposed to oligotrophic conditions, survive by metabolizing low levels of residual organic substances leached from the soil above. These substances serve as terminal electron acceptors (TEA) required for the metabolic breakdown mechanisms for aerobic, facultative, and heterotrophic bacteria through intrinsic bioremediation as the reaction ultimately converts hydrocarbons to carbon dioxide, water, and methane (EPA 2011; Dojka et al. 1998). Movement through the geochemical composition of the aquifer and the hydraulic conductivity of the sediment control the distribution of electron acceptors and nutrients in the subsurface, which without adequate remediation technologies can be limiting factors to aerobic biodegradation (EPA 2011). Direct sparging of air or oxygen, through an injection well, saturation of water with air or water prior to injection, or the addition of hydrogen peroxide directly into an injection well or reinjected water are few of the methods used to ensure oxygen is distributed throughout the area of contamination (EPA 2011). We will look at the different types of technologies offered for aerobic remediation later in this section; however, it is crucial to understand, in the aerobic treatment method, selection and utilization of indigenous and supplemented bacteria play a vital role in the biodegradation and remediation pathways; thus, we will look at the aerobic bacterial–contaminant interactions more in depth.

14.3.1 The Role of Aerobic Bacteria in Remediation in Surface Aerated Zones

Microbial biodegradation has been described as the ultimate mechanism naturally established by nature to combat a variety of stresses contaminating the environment. This process is complex and depends on the indigenous population of oil degrading bacteria and the quantity of contaminant present (Atlas 1992). The two main approaches identified to tackle contaminant remediation with respect to oil and hydrocarbon compounds through means of bioremediation are bioaugmentation and biostimulation. By naturally or artificially fixing a contaminated environment with continuous nutrients and substrates, coexisting oil degrading bacteria may be manipulated towards increasing the rate of remediation. Through metabolic activation and colonial proliferation, oil degraders can essentially control the rate of recovery, limit acute effects caused by contaminant poisoning, and increase localized containment (Das and Chandran 2011).

Reports of aerobic biodegradation in contaminated aquifers have brought forth a rash of new research, identifying commonly existing indigenous bacteria, fungi, and yeast that possess the ability to metabolize hydrocarbon chains, saturates, aromatics, asphaltenes, such as phenols and fatty acids, and resins (Colwell et al. 1977; Atlas 1985). Jones et al. (1983) studied biodegradation of contaminated marine sediments with alkyl aromatics derived from petroleum spills. They identified detectable traces of *Arthrobacter*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*, and *Rhodococus* species involved in degrading alkyl aromatics compounds. Bacteria, however, have been said to be most active petroleum degrading entities as they feed exclusively on hydrocarbons (Brooijmans et al. 2009; Yakimov et al. 2007)

With this in mind, correlation of Biology's Microlog galleries and the work of Castro-Gutiérrez et al. (2012), analyzing hydrocarbon degrading microflora in fuel contaminated aquifers found, various poly-aromatic hydrocarbon strains are characterized as being gram negative bacteria of strictly aerobic nature. The majority corresponded to the *Pseudomonas* species. *Pseudomonas* bacteria have the ability to degrade toxic chemicals of anthropogenic and natural origins, degrading contaminants of hydrocarbon, aromatic and polycyclic profiles (Palleroni 2005). The most frequent fuel degrading activity corresponded with the *Sphingomonas* species, which are recognized for their diverse metabolic pathway enabling the degradation of persistent PAH and other aromatic compounds (Stapleton et al. 2000; Shuttleworth et al. 2000). Similarly, *Geobacteraceae* spp. has been identified as important agents in the bioremediation of inorganic contaminants such as uranium and vanadium (Lovley et al. 1989). *Geobacter sulfurreducens* is capable of surviving in strenuous conditions low in nutrition and phosphate levels, which when exposed to a highly contaminated region, requires marginal nutrition and nitrogen fixation to accomplish subsequent degradation (Lovley et al. 1989; O'Neil et al. 2008; Bazylinski et al. 2000).

Trichloroethylene (TCE) is a halogenated volatile compound (VOC), its indications widely distributed through various processes such as textile, refrigeration, lubrication, adhesives pharmaceuticals, insecticides, and vinyl chloride production (Schettler et al. 1999). TCE contamination are believed to cause mutations and considered a carcinogen (USEPA 1997). Subsurface remediation through physical processes of excavation, extraction, and the pump and treat method, complexes with aquifer characteristics of geochemical sorption, and entrapment between sediment/rock layers have been disruptive in nature, costly and inefficient (Bankston et al. 2002; Beeman and Bleckmann 2002). The biodegradation potential of TCE and its intermediate products has been thoroughly investigated and has revealed much to do with intrinsic (biological) degradation that relies on three different metabolic pathways (Pant and Pant 2009). Two of the processes are considered anaerobic, the other is aerobic of which we will focus on in this section. Wilson and Wilson (1985) studied the concept of aerobic co-metabolism of TCE using methanotrophic bacteria pre-exposed to methane. The pair postulated that it was the methanotrophic bacteria that were metabolizing the TCE. This was verified by Little et al. (1988) and Alvarez-Cohen and McCarty (1991)

as they also found the methanotrophic bacteria metabolizing TCE; however, it was through the use of the methane monoxygenase (MMO) enzyme that aerobic degradation of TCE was possible. Henry and Grbic-Galic (1994) examined the mechanism itself, finding the MMO converts TCE into a TCE epoxide, which is then broken down into four intermediate compounds; carbon monoxide, glyoxylate, formate, and chlorinated acids in an aqueous environment such as an aquifer. These intermediates are subsequently metabolized into carbon dioxide and cell mass by the methanotrophs and heterotrophs. Phenol oxidizing and toluene oxidizing bacteria have also made headways in biological remediation, utilizing the aerobic approach for microbial degradation of various petroleum- and oil-based contaminants. Similar to the methanotrophs capable of utilizing MMO, toluene, or phenol oxidizing bacteria use an enzyme, oxygenase, to facilitate phenol or toluene degradation and mineralization (Pant and Pant 2009). *Pseudomonas putida* F1, a toluene-oxidizing bacteria can mediate co-metabolism by using toluene dioxygenase and toluene as the sole source of carbon (Lau et al. 1994). *Pseudomonas aeruginosa* JI104 is a bacterium that has been identified to utilize chlorinated ethenes and yield a complete mineralization of TCE. By using TCE as its sole source of carbon, *P. aeruginosa* can degrade the toluene contaminant aerobically through production of monooxygenase enzymes (Kitayama 1997). Intermediate compounds formed from the aerobic partial reductive dechlorination process during TCE degradation may yield *cis* and *trans*-dichloroethene (DCE) which may directly be used as carbon sources for oxidative degradation. Seven of such bacteria belonged to families of *Acinetobacter* species, *Bacillus* species, *Achromobacter*, *Klebsiella*, and *Pseudomonas* species (Olaniran et al. 2008). Furthermore, through bioaugmentation practices, the consortia of indigenous bacterium + selection of specific TCE degrading microorganisms can provide an environment rich in activity, prompted by degradation and utilization of the contaminant as the sole source of energy/carbon (Major et al. 2002; Ellis et al. 2000).

14.3.2 BTEX, VOC's, and MTBE Contaminants Remediation

Aquifer remediation efforts upon BTEX, NAPL's, and VOC's contamination have been well documented. The technologies rely on stimulating appropriate oil or hydrocarbon degrading bacteria, indicated with the production or inheritance of an oxygenase enzyme capable of utilizing the contaminant as a sole carbon source for degradation. This intrinsic or biological approach has diversified the network of available aerobes capable of reducing and mineralizing the contaminant to lesser forms, without interfering or permanently altering the geochemical equilibrium of the system; however, challenges do remain. Studying intrinsic bioremediation of hydrocarbons, van Bemmel (2010) delved into understanding the changes in the ecosystem before and after a BTEX-rich mineral oil contamination. One indication

of BTEX is a radical shift in the redox potential of the impacted groundwater. In slightly anoxic or anaerobic environments, the redox potential may require terminal electron acceptors to be artificially pumped into the system or may shift towards methanogenic forms. This methanogenic shift was explained earlier with co-metabolism of TCE by methane oxidizing bacteria utilizing the methane monooxygenase enzyme to systemically mineralize or oxidize the TCE completely or into its intermediates forms (Pant and Pant 2009). Furthermore, measuring the concentrations of dissolved oxygen (DO), nitrate, Iron (II), Carbon dioxide, methane, sulfate, and phosphorus can indicate appropriate remediation techniques, whether or not biostimulation is required as opposed to bioaugmentation. This must be assessed carefully as the geochemistry and geohydrology may indicate false positives for the measured shift in the redox potential through a natural course of weathering and other environmental factors (van Bemmel 2010; van Ras et al. 2007). In response, most terrestrial subsurface indigenous organisms have adapted or even have naturally selected to situate themselves in this contaminated environment due to the functionality of using the contaminant source as energy in reduced forms (Fredrickson et al. 1988).Thomas and Ward (1992) go on by further saying, as the contaminant (BTEX) concentration begins to increase in an area, oxygen concentration will begin to diminish as continuous metabolism by the oil/hydrocarbon degrading bacteria will drive a natural shift towards reduced DO levels and consequently an anaerobic state. Such claims can be rectified by thorough testing and simulations, mimicking the type of contaminant, the native bacteria present, and environmental constituents of the aquifer that influence the geochemistry of the system. Byl et al. (2001) analyzed the contaminated Karst aquifer in Southern Kentucky for evidence of geochemical and microbial fuel biodegrading activities. This region was selected due to the known presence of jet fuel containing BTEX constituents that have been contaminating the region over an extended period of time. These aquifers are characterized by bedrock, epikars, and regolith geology which are derived from their depth and hydrogeological setting composed of sand, gravel, clay, and carbonate rock (Wolfe et al. 1997). Due to complexities of the geochemical and hydrological nature in the karst aquifers, the USEPA had stated, natural attenuation and bioremediation guidelines are not applicable to these systems (USEPA 1997). As such, Byl et al. (2001) tested this paradigm against the revelations of subsurface bacteria associated with BTEX degradation from samples taken across all levels of rock moving down the karst aquifer. An inverse pattern, comparing DO levels and BTEX concentration, was found, suggesting DO concentrations were depleting as high concentrations of BTEX facilitated biological activity for the microorganisms present in the aquifer. Such microorganisms were identified as *Pseudomonas* sp., *Pseudomonas aureginosa*, and two groups of bacteria known as ammonia-oxidizing and iron oxidizing bacteria. The number of *P. aureginosa* generally increased as BTEX concentrations increased, suggesting that the bacteria have genetically inherited traits that mediate adaptation in BTEX contaminated environments. Furthermore, Chapelle (1993) indicated the *Pseudomonas* bacteria are capable of BTEX degradation due to their inheritance of a variety of enzymes that prompt systemic

metabolism, such as toluene di-oxygenase and catechol di-oxygenase. Ammonia oxidizing bacteria were identified to contain the mono-oxygenase enzyme, an anaerobic mode capable of BTEX degradation in the presence of DO (Bedard and Knowles 1989). Direct evidence of bacteria capable of degrading fuel in the karst aquifer came from tests analyzing indigenous bacteria from the karst grown directly on gasoline soaked media pads. A control test, placing indigenous bacteria on sterile media soaked pads containing dilution buffer only, were run parallel to the experiment. The results indicated successive growth and replication of the indigenous bacteria from the gasoline soaked media pads and no growth on the dilution buffer soaked pads, indicating the bacteria were using the gasoline solely as a food source. Based on this conclusion, the geochemistry in the karst aquifer, a system of unconsolidated regolith and bedrock, was of no interference for biological remediation of BTEX compounds (Byl et al. 2001). The concentration of bacteria present in the karst aquifers were comparable to the biological activity reported in contaminated sand derived aquifers, a system with sufficient percolation, aeration, and diverse microbial communities (Ghiorse and Wilson 1988). Thus, the technologies derived from sand aquifers can be applied to groundwater in regolith, epikarst, and karst aquifers (Newell et al. 2006). Natural attenuation then is very much possible as the aquifer lay abundant with BTEX degrading bacteria throughout aerobic and anaerobic regions, diversifying their role against the unique geochemistry when a carbon source such as BTEX is present (Byl et al. 2001).

Methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA), along with BTEX, are contaminants persistent in nature that have been a target of continued bioremediative studies. MTBE is categorized as a possible human carcinogen with a higher degree of mobility and found to be less biodegradable than MTBE. TBA, a metabolite of MTBE, is as an animal carcinogen with similar properties of MTBE found in ecosystems (Cirvello et al. 1995). It has been found approximately 385,000 confirmed LUST releases in the USA, with 25,000 LUST's containing MTBE (Johnson et al. 2000). Kane et al. (2001) analyzed aerobic biodegradation of MTBE by aquifer bacteria from leaking underground storage tanks (LUST). The presence of oxygen and water soluble gasoline components on the metabolism of MTBE and TBA by aquifer bacteria were investigated. The degree of MTBE mineralization and effect of MTBE consumption on the indigenous microbial community were also examined. Oxygenation in combination with bioaugmentation of an MTBE degrading consortium resulted in higher levels of MTBE degradation as compared to the indigenous population on its own (Salanitro et al. 2000). In context however, many reports have been compiled about the physical and organoleptic properties of MTBE that enable the contaminant to be highly soluble in water and thus difficult to remove (Davis and Erickson 2004). Aquifer sediment and groundwater were taken from different LUST sites in California. Palo Alto, Sacramento, Travis Air Force Base (TBA), and Sunnyvale. We will focus on Palo Alto, Sacramento, and TBA as each site has been characterized with containing anoxic sediments. Rapid MTBE degradation in microcosms containing sediment from LUST site in Palo Alto was observed, where 4.5 mg of MTBE/L was degraded to <0.1mg/L in 15 days. Maximum TBA concentrations

produced were recorded at 0.5mg/L. MTBE was reintroduced into the microcosm and rapid degradation, as previously, was also reported; however, TBA concentrations accumulated to 2 mg/L and persisted for 27 days. At the Travis AFB, rapid MTBE degradation was also seen; however, no TBA accumulation was reported. No significant MTBE degradation was shown in the microcosm containing samples from the LUST site in Sacramento. Aerobic conditions, where DO concentrations were measured, maintained constant levels throughout the experiment. The effects of water soluble gasoline components on MTBE degradation for Travis AFB and Palo Alto were also studied. In Palo Alto, dissolved gasoline components retarded MTBE degradation and caused TBA concentrations to accumulate higher and persisted >21 days longer when compared against void dissolved gasoline supplementation. In Travis AFB, no effect of the dissolved gasoline supplement was reported on MTBE degradation or TBA accumulation. In context, BTEX degradation from both sites was rapid, as such no inhibition of BTEX degradation was observed in the presence of MTBE (Kane et al. 2001). Deeb et al. (2001) suggested this finding of BTEX degradation situated during MTBE degradation experiments resulted from the inhibition of MTBE degrading strain PM1. PM1 uses MTBE as a sole carbon source and electron donor, which when in pure bacterial culture reported MTBE mineralization at 46 % which is what was to be expected (Hanson et al. 1999). Furthermore, when exposed to a consortium of biodegradable bacteria, BTEX degradation would take precedence due to its aromatic simplicity of hydrocarbon bonds. In contrast, MTBE is characterized by alkyl–ether bonds, which are relatively stable, unreactive due to difficulties in the cleavage of the ether bonds and generally resistant to microbial attack of the tertiary and quaternary structures (Mo et al. 1997; White et al. 1996; Squillace et al. 1997). This was also indicated by Landmeyer et al. (2001) as they too reported when gasoline (BTEX) is released with MTBE, degradation of hydrocarbons rapidly consume the available oxygen, resulting in MTBE not being degraded. As a remedy to MTBE degradation difficulties, sufficient oxygen supply, co-metabolism with the use of mono-oxygenase enzymes, and the elimination of easily biodegradable organic compounds in the contaminated subsurface are few indications reported by many to aid in MTBE degradation (Stocking et al. 2000).

14.3.3 In Situ and Ex Situ Bioremediation of Contaminated Sites and Aquifers

Bioremediation technology is a mode of systemic reduction of contaminants through the use of biological entities and the surrounding environment, to facilitate an active engagement of hydrologic and geologic conditions coupled against biological activity, a discipline known as biogeochemistry (Plaza et al. 2001). Many of the bioremediation technologies stem from two basic principles of remediation, in situ and ex situ remediation.

In situ remediation is a strategy geared towards stimulating the environment with the addition of organic and inorganic compounds to facilitate sustainable biodegradation of contaminants by the indigenous populations (Mitra and Roy 2011). Another way to encourage remediation efforts is to supplement or inject versatile microorganism within the contaminated aquifer site that possesses the ability to degrade various or specific contaminant forms. These methods of in situ remediation are referred to as biostimulation and bioaugmentation, respectively (Hazen 2010). These technologies are implemented primarily without physically removing the contaminant from the site, nor through the aid of external equipment or technologies that process and reintroduce remediated water and/or sediment into the system (Terrapex Environmental Ltd 2009). Other in situ technologies include chemical oxidation through the use of permanganate and persulfate, ozonation, in-well air stripping, vapor extraction through volatilization of volatile contaminants such as VOC's and pumping/extraction or pump, and treat systems that remove immiscible and DNAPL contaminants (Davis and Erickson 2004; Terrapex Environmental Ltd 2009). Many of these technologies have been implemented, with numerous articles, scholarly papers, and research initiatives dedicated to finding a sustainable function of eliminating persistent contaminants through active bioremediation. One of such was compiled by the USEPA, reporting on in situ groundwater remediation efforts. Through continuous analysis and review, the USEPA generalized in situ bioremediation as a technology most effective against short-chain, low molecular weight water soluble constituents in the forms of various petroleum, BTEX, and hydrocarbon sources. MTBE, long-chain, high molecular weight, and less soluble constituents require more abrasive or concentrated modes of bioremediation unsuitable for in situ form (USEPA 1995). Thorough understanding of the aquifer medium, its hydraulic conductivity, aquifer geology, and contaminant type determine the methodological approach towards selecting the in situ remediation technology thought to be most efficient. Permeability throughout the aquifer must be sufficient to allow perfusion of nutrients and microorganisms to areas of contaminant flow (Hazen 2010). In addition, any lateral flow through the stratified layers is dependent on the medium porosity, generally stating an aquifer of lower permeability and less stratification can lead to reduced effectiveness and extended remedial times (USEPA 1995). Recognition and compatibility of the indigenous community of organisms must also be communicated when introducing any form of supplement to an environment. The remediative technology does not want to impede on the natural balance once implemented nor hinder natural attenuation or intrinsic remediation after the technology has been removed/halted (Hazen 2010).

An in situ bioremediation system may operate through an engineered well system that treats the extracted groundwater with the addition of electron acceptors, nutrients, and other constituents for co-metabolism with the use of contaminant degrading microbes (Cusack et al. 1992). Infiltration galleries or injection wells can inject the treated groundwater back into the system, following a closed loop system that would continue to recirculate the water until remediation targets are achieved (USEPA 1995). Furthermore, aerobic treatment is often described as the most

effective mode of in situ remediation for petroleum and other hydrocarbon contaminants. Oxygen treatment methods include direct sparging of air or oxygen through an injection well, saturation of water with air or oxygen prior to reinjection or the addition of hydrogen peroxide directly into an injection well or reinjected water (USEPA 1995). Bioenhancement is an in situ process that applies these strategies mentioned above. Bioenhancement is described as a common approach involving pumping groundwater to the surface, dissolving necessary nutrients, electron acceptors, and/or co-substrates, and pumping the treated groundwater back into the contaminated aquifer. Another method enables enhancements directly sparged or injected into the subsurface with air or oxygen as release compounds (Stocking et al. 2000). Salanitro et al. (2001) used a potential MTBE degrading organism, *Rhodococcus*, with intermittent sparging using air or pure oxygen as a form of oxygenation. They described this remediation area as a development of a bio-barrier. Results after 9 months showed the levels of MTBE declined from 1 mg/L to only a few μg/L within the bio-barrier. Strategies for stimulating MTBE degradation was indicated by supplying oxygen, nutrients, and PM1 strain to the contaminated aquifer site. Levels of MTBE degradation reduced as much as 85 % of the contaminant within a year without prior exposure of PM1 strain to the contaminant site (Stavnes et al. 2002). Bioenhancement may also include the addition of a co-metabolite to aid in MTBE degradation. Successful co-metabolites found to degrade MTBE include iso-propanol, propane, *n*-butane, and diethyl ether (Steffan et al. 2000). Baker et al. (2002) used cyclohexane as a co-metabolite with the addition of oxygen and found an 80 % reduction of MTBE resulted over the time of treatment. Enhanced bioremediation of MTBE using mixed culture enriched on MTBE, designated as BC-4 and readily available oxygen (O_2), was tested. The results were directed accordingly: MTBE degradation with O_2 supplement only and MTBE degradation supplemented with O_2 + BC-4. Both methods proved to degrade MTBE well; however, O_2 + BC-4 was the better bioenhancement method of the two as it reduced the MTBE concentration in the aquifer from an estimated range of 2,000–8,000 μg/L to a remediated level of <5 μg/L, without TBA accumulation (Salanitro et al. 1999).

Ex situ bioremediation studies follow the underlining principle of the removal of subsurface layers, sediment, and groundwater believed to be heavily contaminated in a concentrated region, where in situ or dispersion characteristics are limited (Environment Canada 2002). The use of suspended growth bioreactors, development of aerated treatment beds for solid and slurry phase biological treatment, and fixed film bioreactors are few technologies indicated for ex situ bioremediation (Stocking et al. 2000). Migratory control of the contaminant plume can be accomplished using hydraulic controls such as pump and treat via the ex situ process of air stripping and granular activated carbon or by stimulating a contaminant degradation rate equal to or greater than the contaminant migration rate via bioreactors (NRC 1994; Stocking et al. 2000). Suspended growth bioreactors utilize concentrated suspensions of active cells to interact with the microorganisms in an enclosed vessel containing aqueous phase organic contaminants, dissolved oxygen, and nutrient supplements. High cell concentrations in the bioreactor are maintained via

decoupling of kinetic parameters of hydraulic retention time (HRT) and the mean cell residence time (MCRT), which allows for cell recycling (Shuler and Kargi 1992). Pitre and Steffan (1999) describes the importance of cell recycling of MTBE via ex situ remediation as the technology conserves the biomass of the slow growing MTBE population and ensures high cell concentrations of MTBE degraders in the remediation zone. Similarly, O'Connell and Moyer (2006) utilize a system of film fixed bioreactors known as fluidized bed bioreactors to conduct ex situ cell recycling for degradation of MTBE/TBA and VOC's. Recirculating liquid streams through a two phase system of solid sand and liquid water uses the biomass distributed within the bioreactor to remove VOC's. The HRT will manifest an appropriate association facilitated by the reaction in the presence of available DO and nutrients. Once sufficient oxygen concentrations are established, the fuel oxygenate consortia will actively degrade the MTBA and TBA contaminants. Furthermore, if BTEX contaminants are subjected to the technology as a mixture of BTEX + MTBE + VOC's + TBA in the bioreactor, tendencies for BTEX degrading bacteria to consume oxygen will be satisfied first, outcompeting fuel oxygenate degraders, due to the complexity of degradation. Tang and Sun (1997) indicated similar findings as they reported the slow growing MTBE degrading organisms require adequate time periods, growth nutrients, and abundant DO levels, without interference or competition from associated biodegrading organisms to be effective. In such a case, it is recommended to pretreat the slurry with granular activated carbon or air stripping of the BTEX compounds so the available oxygen supplements can be used for the more persistent MTBE contaminants. The end result of the bioreactor technology is mineralization of fuel oxygenates, including TBA, and petroleum hydrocarbons to carbon dioxide and water (O'Connell and Moyer 2006). These findings were similar to the findings of Fortin and Deshusses (1999) that have used biotrickling, a form of fixed film bioreactors, to convert 97 % of the influent MTBE to CO_2 using MTBE grown cultures. Fava et al. (2000) and Robles-Gonzalez et al. (2008) did similar studies using PAHs. Bioslurry reactors were used to determine the plausibility and potential of a bioremediation strategy in the final restoration process of a contaminated site and remediation of calcitrant compounds such as PAHs. It was determined that bioreactors with inoculums using strains of lower molecular weight and lower octanol–water coefficient and available DO can perform at optimal degradation rates under a bioreactor simulation where persistent PAHs are the source of interest (Cerniglia 1992). Rodriguez-Martinez et al. (2006) used the pump and treat method with a fixed film community of diesel degrading microorganisms to remediate the diesel plume. Their results dictated efficient removal rates, degrading 97–99 % of the total petroleum hydrocarbon load and a sustained HRT of 14 min after the start-up phase 10 days prior.

14.4 Anaerobic Technologies for Aquifer Bioremediation

Anaerobic bioremediation technologies have been at the helm for conducting, analyzing, and understanding remediation strategies to remove or degrade persistent contaminants that have penetrated deep within aquifers to anoxic regions. Typical aerobic treatment utilizes several methods of introducing oxygen as a terminal electron or supplement via air sparging, direct oxidation, air or oxygen injections, and an available and abundant consortium of aerobic degrading microorganisms (Major et al. 2002; USEPA 1995). Degradation of most hydrocarbon-based contaminants in the anaerobic mode struggle to use such remediative technologies, as the introduction of oxygen or any irreconcilable supplement to the environment, spells incompatibility, toxicity, and the possibility of recalcitrance to the organisms and fuel components dwelling in such regions (Rooney-Varga et al. 1999). Furthermore, these contaminants are characterized as either being highly soluble and mobile or highly insoluble and mobile as dense nonaqueous phase liquids (DNAPL's) at which adsorption to aquifer sediments limits bacterial degradation (Sheremata et al. 2000; Bourg et al. 1992). A report on the potential bioremediation of BTEX in petroleum contaminated aquifers using anaerobic technologies found significant portions of contaminated aquifers becoming anaerobic (Lovely 1997). This phenomenon stems from increased microbial respiration rates, due to increased metabolism by the indigenous biota, outweighing the atmospheric reoxygenation and diffusion rates into the aquifer (Lovely 1997). As a result, a shift towards anaerobic conditions market increased efforts towards finding alternatives to remediate contaminants such as BTEX, PAHs, MTBEs, and TCEs, which have been characterized by coinciding chemical and biological reduction zones tailored towards remediating the particular contaminant (Salanitro et al. 1997). We will venture into the anaerobic treatment method, looking into details about the remediation techniques and microorganisms indicated for the above-mentioned contaminants.

14.4.1 The Role of Anaerobic Bacteria in Aquifer Bioremediation

Bacterial diversity and activity throughout the aquifer hydrogeology insinuate interactions and variability mediated by nature, be that influenced by man or compounded naturally. In the presence of contaminant plumes, anaerobic biodegradation of BTEX, TCE, PAH, and MTBE is accomplished by specific biodegrading organisms complimented with alternate electron acceptors needed for the biotransformation into target CO_2, ethane, and complete mineralization (Salanitro et al. 1997; Pant and Pant 2009; Davis and Erickson 2004; Coates et al. 1997). For such processes, the selection and characterization of corresponding organism dictate anaerobic success, however, due to the persistency and alternative

chemical interactions in the various anaerobic zones; many articles have reported on the concoction of cultured bacteria as a whole in the contaminant site rather than individual organisms. Many reports have indicated sulfur reducing bacteria to be actively involved in oxidizing benzene (Weiner and Lovley 1998). Benzene is recognized for its toxic and persistent nature under in *situ* anaerobic conditions making it difficult for organisms to metabolize the compound. Furthermore, under sulfur reducing conditions, benzene degradation is minimal, if at all due to sulfur reduction becomes inhibited in the presence of Fe(III), as the Fe(III) reducing bacteria outcompete the sulfur reducing bacteria for available TEA's (Chapelle et al. 1996; Coates et al. 1996a, b; Lovley and Phillips 1987). This revelation led to experiments using supplemented benzene degrading sulfate reducing bacteria in Fe (III) containing sediment. From their results, it was suggested that the supplemented benzene degrading sulfate reducing bacteria were effective in stimulating benzene oxidation coupled with continuous sulfate reduction in Fe(III) containing sediments (Weiner and Lovley 1998). This suggests bioaugmentation with benzene oxidizing sulfate reducing bacteria can accelerate anaerobic benzene biodegradation, possibly reducing quantities quicker and more efficiently. Similarly, Coates et al. (1997) found persistent PAH compounds, naphthalene and phenanthrene to become oxidized to CO_2 under sulfur reducing conditions by sulfur reducing bacteria after a period of pre-exposure to high concentrations of PAHs. Furthermore, trace amounts of methane were also found, indicating PAH oxidation may have resulted from a co-metabolic process by bacteria other than sulfate reducers. Jahn et al. (2005) examined anaerobic degradation of BTEX compounds using enriched iron reducing cultures. Situated in an environment with high concentrations of BTEX hydrocarbons coupled with iron reduction with favorable TEAs and substrates, reports indicated BTEX oxidizing iron reducing organisms were fully capable of complete degradation of ethylbenzene and *o*-xylene by dissimilatory iron reduction. Studies have also shown toluene oxidation is done via iron, Fe(III), reducing microorganisms known as *Geobacter metallireducens* (Lovley et al. 1989; Lovley and Lonergan 1990). *G. metallireducens* is documented as the first organism capable of degrading aromatic hydrocarbons under strict anaerobic conditions, oxidizing toluene directly to CO_2 with Fe(III) serving as the sole TEA (Lovely 1997).

A bioremediation study to identify the microbial diversity involved in the Vega Baja, Puerto Rico contaminated aquifer using gene analysis of a biofilm GAC microbial community actively engaged in metabolism of the diesel hydrocarbon source revealed several families of bacteria present (Rodriguez-Martinez et al. 2006). The anaerobic sources in the biofilm community were derived from *Bacillus halodurans*, *Thauera aromatica*, and *Desulfotomaculum thermocisternum*. In addition, the anaerobic benzoate gene isolated from *Bacillus halodurans* and *Desulfotomaculum thermocisternum* initially reported low signals during biological remediation by the GAC biofilm community; however, the signal began to increase over time. Similarly, nirS and nirK genes involved in dissimilatory nitrate and nitrite reduction increased hybridization towards the end of the process, suggesting

both gene types are indicative of shifts towards anaerobic bacterial dominance during later stages.

TCE anaerobic biodegradation is another contaminant that has been identified with characteristics of being completely mineralized to a less abrasive constituent such as ethanes or ethenes. TCE undertakes complete sequential reductive dechlorination under anaerobic conditions (DeBruin et al. 1992; Freedman and Goseett 1989). The biodegradation of TCE using a stable carbon isotope and biomarkers were studied to identify the occurrence of microbial consortia during chloroethene biodegradation. Analysis indicated the presence of compatible bacteria and degradation attributes associated with *Dehalococcoides* spp., which translated into TCE degradation into ethene as intended (Imfeld et al. 2008).

14.4.2 Terminal Electron Acceptors in Anaerobic Bioremediation

Bioremediation in an anaerobic environment portrays an image of a desperate geochemical and geomicrobiological struggle to remediate contaminants persisting in a dark, deep, anoxic environments void of any life or sustainable interactions that initiate growth or progress. However much of the work around anaerobic bioremediation has revealed many organic contaminants are said to be naturally occurring or have some analogs in the environment that have enabled microorganisms to adapt, proliferate, and utilize various nutrients and essential electron sources to naturally attenuate the contaminant site (Fredrickson et al. 1988).

The availability of dissolved oxygen (DO) in subsurface contaminated aquifer warrants conditions of aerobic bioremediation, as the DO present in the aquifer serves as a carbon and energy/electron source. High organic contaminant plumes coupled with a vast microbial system of degradative interactions often mediate anaerobic conditions as DO replenishment and reaeration rates fall below microbial consumption rates around the organic plume (Barker et al. 1987; Chiang et al. 1989; McAllister and Chiang 1994). Thus, a shift towards anaerobic bioremediation is encountered, where oxygen as a carbon or electron source is no longer applicable and one or more new electron donor or electron acceptor must be utilized (Bouwer 1994). Nitrate (NO_3), sulfate (SO_4), iron as Fe(III), manganese as Mn(IV), and carbon dioxide (CO_2) have all been documented as attractive alternative electron acceptors for anaerobic bioremediation. These electron acceptors coincide with bacteria coupled interactions displaced throughout different regions of the aquifer, corresponding to the contaminant source when precursor electrons for metabolic activity become available (Salanitro et al. 1997; Nelson et al. 1994; Bouwer 1995; Wilson et al. 2005). Two forms of the electron utilization are established: one as an electron donor, which is derived from the hydrocarbon, chlorinated, or aromatic contaminant source, and the other is in the form of an electron acceptors such as NO_3, SO_4, Fe(III), Mn(IV), and CO_2 as mentioned above. The electron donor

transfers its electron to the electron acceptor, whereby establishing a redox potential in the contaminant region and subsequently synthesizing the cellular material required for metabolism (Bouwer 1995). The redox potential generated by the anaerobic electron sources yield less energy during substrate oxidation and electron transfer as indicated by Bouwer (1995); however, this variant may be supported by significant quantities of the substrate and electron source that enable continuous or prolonged activity. Furthermore, it is important to note in situ bioaugmentation of the indigenous population or of a supplemented anaerobe population and the biostimulation with nutrient and electron acceptors must complement each other. Inability to select appropriate constituents may result in the inhibition of the electron source and prevent reduction from taking place (Coates et al. 1996a, b). Many articles and research initiatives have described the role of electron acceptors, also referred to as terminal electron acceptors (TEAs) for BTEX, MTBE, PAHs, and TCE's remediation, respectively. We will examine each contaminant under anaerobic conditions and elaborate on the role of electron acceptors in anaerobic bioremediation.

14.4.2.1 BTEX and PAH Biodegradation in Anaerobic Conditions

BTEX biodegradation is one of the most documented remediation technologies captured in contaminated sites all across the world. BTEX constituents were degraded at significant rates by the anaerobic bacteria population using alternate electron acceptors, which translated into coupled nitrate reduction, iron reduction, sulfate reduction, and methanogenic reductions (Salanitro et al. 1997). Similarly, Baedecker et al. (1993) reported on the anaerobic biodegradation capabilities at crude oil spill sites where BTEX transformed into less nuisance constituents. Similar work by Lovely (1997) compiled data on BTEX oxidation via potential electron acceptors for anaerobic biodegradation. Fe(III) proved to be the most suitable electron accepting source for microorganisms, providing greatest electron accepting pools available in shallow aquifers (Christensen et al. 1994). Furthermore, the reduction of nitrate, Mn(IV), Fe(III), or sulfate and even methanogenesis are referred to as terminal electron acceptor processes. However, if Fe(III) or sulfate are available as TEAP's, sulfur reducing bacteria and iron reducing bacteria outcompete other reducing bacteria for the electron donor or carbon source (Lovley et al. 1994). This competitive behavior was also observed in the presence of Fe(III) reducers and sulfate reducers when abundant sulfate were present. Fe reducers utilized the electron donors first in the presence of Fe(III) electron acceptors, despite the high concentration of sulfate electron acceptors available in the aquifer for sulfate reducing biodegradation. Sulfate reducers in turn may reduce Fe(III), inhibiting the iron degrading bacteria, which can become problematic, as an intended complete mineralization to CO_2 may end up partially mineralized into more toxic constituents (Coleman et al. 1993).

The use of nitrate has also been documented in BTEX reduction studies. Much of the work has focused on the success of oxidizing toluene, ethylbenzene, and

xylene due to their compatibility with denitrifying microorganisms (Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Soerensen 1996). Moreover, optimal toluene degradation was also reported by Wilson et al. (1997) during batch microcosm studies with zero oxygen and nitrate as the electron acceptor. Prompt degradation activities of toluene resulted, except in the column where nitrate was absent, suggesting that under anaerobic conditions, removal of toluene is dictated by the presence of nitrate electron acceptors and corresponding nitrate reducers. Of the BTEX contaminants, benzene removal/remediation has been documented as being one of the highly persistent contaminants. Benzene degradation was observed to be slow under sulfate reducing conditions in freshwater aquifers (Chapelle et al. 1996; Lyngkilde and Christensen 1992; Patterson et al. 1993). However, benzene degradation was seen under similar conditions with the addition of enriched culture present in the aquifer sediments collected from the sulfate reducing zones in a jet fuel contaminated aquifer. The sulfur containing sediments were saturated with sulfur reducing organisms, thereby oxidizing the benzene and degrading it to CO_2 (Phelps et al. 1996).

Moreover, under Fe(III) reducing conditions and an equivalent TEAP supply, stimulation of Fe(III) reducing bacteria in sediments known to be less predominant, as compared to Fe(III) reducing bacteria, such as *Geobacter* spp., can manifest biodegradation of BTEX contaminants at higher levels. Thus, the potential of natural attenuation in an anaerobic Fe(III) reducing environment is a highly sought and realistic approach recognized for remediating BTEX contaminants (Lovley et al. 1989; Rooney-Varga et al. 1999).

PAH biodegradation in an anaerobic environment involves the biodegradation of poly-aromatic hydrocarbons, whereas BTEX are considered as mono-aromatic contaminants (Coates et al. 1996a, b; Rabus and Widdel 1996). These contaminants have been documented to degrade anaerobically; however, many reports have demonstrated the inability of PAHs to undergo degradation when subjected to strict anaerobic conditions (Bouwer and McCarty 1983). Studies marketing PAH degradation under anaerobic conditions are less abundant, as most studies have documented PAHs to persist in aerobic conditions and as a result, degrade well under various aerobic technologies (Coates et al. 1997). In such, anaerobic remediation has revealed Mn(IV), Fe(III), sulfate, and nitrate reduction to serve as the electron acceptors, with sulfate being documented as the apparent electron acceptor of choice for PAH oxidation to carbon dioxide (Aeckersberg et al. 1991; Al-Bashir et al. 1990; Durant et al. 1995). In conjunction, microbial metabolism and biotransformation of PAH requires previous or long-term exposure, as reports have indicated, PAHs were not degraded in soil and sediment samples containing PAHs, predicted to be during acute exposure. Harbor sediments in contrast that were chronically exposed to PAHs however were able to degrade PAHs to carbon dioxide as the microbial population had established metabolic competence for the contaminant (Coates et al. 1996a, b, 1997). Coates et al. (1997) demonstrated considerable degradation abilities of PAH under anaerobic conditions. Exposure to PAH contaminants, such as naphthalene, phenanthrene, and others, at a naval station in San Diego Bay revealed naphthalene, phenanthrene, and other PAHs

tested were readily oxidized to CO_2 within 37 days of remediation. When examined, sulfate was the required electron acceptor prompting PAH oxidation. When sediments were washed with sulfate free buffer, production of CO_2 stopped as the oxidation of naphthalene underwent inhibition in the absence of sulfate. Similarly, PAH exposure to sediments from Shelter Island in San Diego Bay revealed an absence of CO_2 production in the presence of sulfate. This finding correlated to exposure time studies of PAH, as Shelter Island sediments were only acutely exposed with a lag period for microbial adaptation, whereby at day 80, oxidation of naphthalene and phenanthrene were indicated through the production of CO_2 at the contaminant site.

14.4.2.2 TCE Biodegradation in Anaerobic Conditions

Chlorinated solvents in anaerobic environments have been described as being highly persistent and widely distributed among the deep layers of aquatic environment such as aquifers. The problems arise as chlorinated solvents tend to adsorb onto sediments and because of their solubility characteristic, tend to perfuse down the aquifer gradient towards lower elevations and not readily transformed by aerobic pump or air sparging technologies (Bouwer 1995). Thus, strategies for promoting anaerobic degradation of chlorinated solvents, such as TCE, have been proposed. The reductive dechlorination of TCE has the potential to be remediated using electron acceptors and electron donors (Major et al. 2002; Christ et al. 2005). Furthermore, TCE has been documented as being quite stable and requires the presence of an electron donating cocontaminant for anaerobic transformation (Battelle Memorial Institute et al. 2001). However, it has also been noted that in coupling of redox conditions and chlorinated solvent biotransformations, methods needs to be fully understood as the reductive chlorination process has been identified as a process that may require extended periods of time for complete mineralization (van Bemmel 2010; Bouwer 1995). Early studies on the anaerobic transformation of chlorinated solvents in field studies, fixed film reactors, sediment, and aquifer microcosms had revealed reductive dechlorination to lesser constituent metabolites under conditions of denitrification, sulfate reduction, or methanogenesis (Bouwer and McCarty 1983; Vogel and McCarty 1985, 1987; Wilson et al. 1986). Transformation of TCE under anaerobic conditions has been identified to occur via sequential reductive dechlorination to intermediate byproduct, dichloroethene (DCE), or can be dechlorinated into ethane or ethene through a complete TCE dechlorination process (Battelle Memorial Institute et al. 2001). Anaerobic microorganisms such as methanogens and sulfate reducers have readily been identified to interact with low molecular weight organic compounds or H_2 as electron donors during reductive dechlorination (Bouwer 1994). The biodegradability of TCE and its intermediate products have been identified via three metabolic processes, two of which readily reduce chlorinated solvents anaerobically (Wilson and Wilson 1985; Harker and Kim 1990). McCarty (1994) reported, during reductive dechlorination, chlorinated ethenes are used as electron

acceptors during the reductive dechlorination of chlorinated solvents. The metabolic mechanism behind sequential reductive dechlorination illustrates the removal of a chlorine atom is replaced by a hydrogen atom at each reaction step, producing hydrochloric acid (HCl) as a by-product (Mohn and Tiedje 1992). The reaction intermediates yield various configurations of DCE (cis, trans, and 1,1-DCE) and a vinyl chloride intermediate (VC); however, *cis*-DCE is identified as the more prevalent intermediate found after dechlorination has occurred (Bouwer 1994). Similar studies were conducted by the Battelle Memorial institute et al. (2001) in situ on tetrachloroethene (PCE) and TCE under anaerobic reductive technology, termed RABITT. In RABITT's attempt, reductive systems highly oxidized contaminants PCE and TCE are utilized as electron acceptors. Excess substrate is supplied to the native microbial consortium to serve as the electron donors to stimulate and prolong activities in the reductive pathway, which is coordinated with an ample supply of molecular hydrogen (H_2) to be transmitted into the system as an electron donor (Maymo-Gatell et al. 1995) The competition for H_2 by microbial methanogen and sulfate reducing bacteria have limited or have been the common cause of dechlorination failure (Fennell et al. 1997; Gossett et al. 1994). Microcosm experiments at Cape Canaveral air station revealed chlorinated solvents TCE and intermediates DCE and VC present in the groundwater at the site. As a remediative tool, reductive chlorination techniques were applied to the contaminant site, with organic electron donors (lactate, butyrate, propionate, benzoate, and yeast extract) serving to supply microbial activities. Field data revealed TCE was naturally being dechlorinated into DCE and subsequently VC prior to the introduction of electron donors. However, data recording dechlorination activity with the addition of organic electron prompted enhanced dechlorination values of TCE, DCE, and VC by 88.7 %, 90.6 %, and 66.3 %, respectively, as indicated by the significant increases in ethene concentrations (Battelle memorial Institute et al. 2001). A second case study conducted at a naval air station in Alameda, California, revealed elevated levels of TCE, DCE, and VC in the site aquifer, characterized as a stagnate highly contaminated water body. Analysis using butyrate as the electron donor, chosen for field injections due to its shorter lag times, dechlorinated TCE rapidly to ethene under sulfate reducing to methanogenic conditions. A 94 % reduction of TCE was concluded. Results for DCE and VC were unavailable as specifics; however, an average of 87 % total reduction of the chlorinated solvents identified in the aquifer were accounted for and reflected by subsequent ethene concentrations (Battelle memorial Institute et al. 2001). A third case study implementing RABITT techniques at a liquid storage and disposal site in Fort Lewis, Washington, revealed BTEX, TCE, DCE, and VC contaminants embalmed into the groundwater infiltrating through the highly contaminated trenches and pits. Field analysis revealed TCE and DCE were highly prevalent in the system; however, DCE transformation was being inhibited/ stalled at this dechlorination stage, reflecting in the formation of VC to ethene in only a few samples. It was reported that a long lag time was apparent during the initiation of DCE dechlorination due to supportive bacteria taking the role of DCE dechlorination; however, once initiated, VC to ethene transformed rapidly, suggesting

different bacteria capable of dechlorination of the intermediates were actively partaking in mineralization of DCE and VC. With the addition of an electron donor through injection wells, TCE concentrations reduced 99.94 % and DCE concentrations accumulated, however, at rates faster than they were transformed to VC as a result of the diverse consortium present at the site (Battelle memorial Institute et al. 2001). These three case studies have been provided by Battelle memorial Institute et al. (2001) as mediums for the RABITT project initiative.

14.4.2.3 MTBE Biodegradation in Anaerobic Environments

MTBE is an alkyl ether considered relatively stable, unreactive, capable of resisting microbial attack, and has been reported as a recalcitrant under aerobic and anaerobic conditions (Mo et al. 1997; Squillace et al. 1997; Jansen and Arvin 1990). Under anaerobic conditions, Yeh and Novak (1994) evaluated the MTBE biodegradation and found degradation paralleled with methanogenic conditions under low organic carbon content. However, in the presence of easily degradable organic compounds it has shown to inhibit MTBE degradation (Yeh and Novak 1994). Later work by Hurt et al. (1999a, b) and Wilson (1999) illustrated MTBE biodegradation is mediated via reduced methanogenic conditions. The use of humic substances as mediators in facilitating MTBE degradation was proposed by Finneran and Lovley (2001), using the reduction of Fe(III) as the oxidant, in contaminated freshwater aquifer sediments. The presence of TBA indicated with a decline in MTBE concentrations that MTBE was being degraded to the intermediate form under iron reducing conditions (Hurt et al. 1999a, b). Bradley et al. (1999) examined MTBE degradation supplemented with electron acceptors. Mineralization activities of MTBE across contaminated streambed sediments composed of sand, clay, and silt accounted for 15–66 % mineralization under natural conditions. This analysis was constructed under aerobic conditions; however, the diffusion of contaminants coupled with microbial references across the site may render natural MTBE degradation more difficult. MTBE degradation was shown to be possible with streambed organisms under denitrifying conditions. Using MTBE contaminated sediments obtained from the contaminant site and running comparative studies parallel with sediment obtained from an uncontaminated site, mineralization from both sediments was witnessed governed by the addition of nitrate as the electron acceptor under denitrifying conditions. Under methanogenic conditions and no nitrate supplementation, TBA was produced, which is regarded as an undesirable intermediate of partial mineralization of MTBE (Bradley et al. 2001). Electron acceptors are the preferred method of completely mineralizing MTBE to CO_2, with NO_3 being classified as being most effective amongst SO_4, Fe(III), and Mn(IV) electron acceptors (Davis and Erickson 2004). Landmeyer et al. (1998) showed MTBE was capable of being mineralized under Fe(III) reducing conditions in contaminated bed sediments of a fresh water stream. Under sulfate reducing conditions, MTBE was also reported to be completely mineralized from MTBE contaminated stream and lake sediments (Bradley et al. 2001). Finneran and Lovley

(2001) reported MTBE and TBA mineralization under iron reducing conditions supplemented with iron reducing culture. These findings support studies examined at a gasoline spill site in Parsippany, New Jersey, where iron reduction yielded partial MTBE degradation resulting in high TBA concentrations (Wilson et al. 2000). After examination the biologically available iron in the sediment used for the microcosmic analysis varied. Schmidt et al. (2004) did a stoichiometric analysis where it was revealed 6 mol of biologically available Fe(III) are needed to metabolize 1 mol of MTBE to TBA, whereas 30 mol are needed to completely mineralize MTBE to CO_2 and water. As a result, Fe(III) reducing bacteria were unable to fully mineralize MTBE, suggesting sufficient concentrations of electron acceptor Fe(III) were not available in the microcosm, required for complete MTBE degradation (Wilson et al. 2000).

14.4.3 Limitations and Capabilities of Geohydrological Bioremediation

Spills and leaks from underground storage tanks containing petroleum hydrocarbons, poly-aromatic hydrocarbons, MTBEs, and chlorinated solvents have resulted in worldwide contamination of both soil and the geohydrological system of aquifers and groundwater (EPA 1995; Rodriguez-Martinez et al. 2006; Das and Chandran 2011). Bioremediation technology has offered many mechanisms of coping, strategizing, and implementing technologies; however, limitations to the understanding, research, and practice make bioremediation a slow and cautious process as identified by all the authors cited in this chapter. Amongst the difficulties of bioremediation, the USEPA (1995), Brusseau (1998) and Kane et al. (2001) identified certain factors that hinder groundwater bioremediation such as the geology of the aquifer and surrounding waterbed, environmental conditions tailored to the region, and consequently, the microorganism inhabiting the contaminated aquifer site and the physicochemical properties of the contaminant.

In understanding the complexities concerning the aquifer, as was said earlier, the aquifer medium will determine the hydraulic conductivity. An aquifer, described with medium or lower permeability, will tend to exhibit minimal flow characteristic and require longer remediation times, with the potential of limited effectiveness (EPA 1995). Aquifers rich in calcium, magnesium, or iron may leach in with the groundwater and react with phosphate or carbon dioxide, which is typically available in the environment as a nutrient supplement for metabolic activities (Kinsella and Nelson 1993). The reaction reduces nutrient availability and may facilitate the production of crystalline precipitates, which can constrict flow channels and engineered remediation equipment such as injection wells and sparging points (Norris 1994). Similarly, oil spills in freshwater and marine environments as documented by Atlas (1985) have shown to become nitrogen and phosphorus limited as high levels of carbon dioxide indicate continuous metabolism.

Implications of unavailable nitrogen, as were mentioned earlier in Sects. 14.4.1 and 14.4.2, indicate nitrogen, in the form of nitrates as a TEA, may boast insufficient levels of the electron acceptor needed for the denitrification of certain contaminants and cause the contaminant to persist in the environment (Bouwer 1995; Davis and Erickson 2004; Baedecker et al. 1993). This can simply be corrected by the addition of bioenhancements such as nitrogen and phosphorus supplements; however, the limitations of any enhancement mechanism is the complex and at times hard to tabulate. Chaillan et al. (2006) describe the rate of nutrient consumption versus the rate of replenishment and have cautioned, without adequate understanding of the nutrient balance in the system, supplementing excessive nutrient concentrations can be a factor that also inhibits biodegradation activities. In addition, the introduction of oxygen has also been noted to react with dissolved iron, Fe(II), forming an insoluble precipitate of ferric oxide that can retard injection well operations (EPA 1995). Fe(II) is a byproduct of Fe(III) which is a TEA utilized by Fe(III) reducers under anaerobic conditions. If sequential aerobic–anaerobic technology was to be implemented, the production of ferric oxide could be an inevitable fail marker around the injection wells, which would limit the effectiveness of the aerobic metabolite bioremediation (Pant and Pant 2009; EPA 1995; Jahn et al. 2005). The addition of oxygen to anoxic environments did not result in MTBE degradation (Kane et al. 2001).

Limitations arise further when contaminant profiles deter microbial degradation. The susceptibility of PAHs to microbial degradation decreases as larger molecular weight and octanol–water partition coefficients of PAHs increase in the system (Cerniglia 1992). Pyrene is characterized as a large molecular weight molecule with a high octanol–water partition coefficient. If the initial inoculum is not large enough and cannot support microbial attack/degradation, there may be limited PAH degradation and the contaminant may persist (Daane et al. 2001). This was shown with results of PAH removal comparing naphthalene to pyrene and phenanthrene which are both larger molecular weight and octanol–water partition coefficients. Naphthalene was degraded up to 99.9 % whereas pyrene and phenanthrene were degraded only 18.5 % and 19.9 %, respectively, (Castro-Gutierrez et al. 2012). Furthermore, MTBE degradation was also found to be limited as a result of organisms or conditions that were not available for PM1 strain to efficiently degrade MTBE and may even be recalcitrant to biological degradation (Kane et al. 2001; EPA 1995).

Limitations also arise due to microbial competition within the aquifer for available TEAs and nutrients. Hydrocarbon contaminants such as BTEX have been documented to be degraded first by microbial reducers, limiting available TEA and electron donors, thus inhibiting larger targeted MTBE contaminants (Deeb et al. 2001). These abundant water soluble gasoline components, BTEX, have been reported by many others as well to take precedence under mixed slurries of organic plumes, inhibiting MTBE, TBA, and other large molecular highly soluble contaminants (Kane et al. 2001; Yeh and Novak 1994; Landmeyer et al. 2001). This high solubility has been speculated to also enhance MTBE diffusion into lower permeability regions, ultimately hindering remediation

capacity (Mackay et al. 1999). Sulfate reducers have also shown to be inhibited in the presence of Fe(III) as mentioned earlier as Fe(III) reducers are capable of outcompeting sulfate reducers for electron acceptors (Lovley and Phillips 1987). Increasing concentrations of TCE under stagnant conditions have been reported by Ely et al. (1997). Competition for available substrate (TCE vs. DCE) for microbial utilization have led to substrate toxicity, causing inhibition and inactivation of enzymes responsible for degrading TCE and other chlorinated VOCs. Competition for oxygen resources may also play an important role in remediation technologies as dictated by the use of chemical dispersants. Dispersants have been documented to exhibit high demands for oxygen, which when subjected for treatment in smaller, less mobile coastal or inland waters, creates an environment with limited oxygen reserves causing oxygen deprived organisms to perish, damaging the entire system (Dewling and McCarthy 1980).

Limitation due to environmental factors also plays a large role in bioremediation of contaminated aquifers. Temperature, pH, nutrient availability, water content, subsurface aeration, electron availability have been documented to affect the rate of degradation (Stocking et al. 2000). Nitrogen and phosphorus again have been documented to be limiting nutrients most often encountered in contaminated aquifers, with required ratios of carbon/nitrogen/phosphorus, depending on the nature of the contaminant to range between (100–300): 10: (0.05–1.0) (Alexander 1994). With regard to temperature, co-metabolic activities under TCE degradation resulted in increased lag times as temperature decreased (Fan and Scow 1993). Furthermore, increased temperatures resulted in increased enzymatic activity for every 10 °C increase up to 40–50 °C where inhibitory consequences are possible (Gerhardt et al. 1994).

14.5 Conclusions

Implications to public and marine health, sustainability of ecosystems and continuous monitoring, bridged with tighter penalties and legislation mandates progressive action towards assessing and controlling contaminants from polluting our waters. Bioremediation by means of bioenhancements, bioaugmentation, and natural attenuation have reported tremendous gains. Remediation technologies have been aimed at targeting the contaminants affecting the geohydrological conditions of the aquifer, instigating research efforts both aerobically and anaerobically to determine the most effective mode of remediating spills. Chemical contaminants such as BTEX and various DNAPL's such as PAHs, MTBE, and TCE have shown tremendous gains in the bioremediation field. All contaminants were shown to be degraded both aerobically and anaerobically, through the use of appropriate microorganisms, indigenous or augmented to facilitate desired degradation of the targeted contaminant. Reports have shown TEAs to be heavily involved in adequate biological function and consequently the rate of degradation. It has been seen that oxygen under aerobic conditions serves well as a TEA, showing great potentials in

degrading BTEX contaminants and other LNAPL's that are found in subsurface environments. Furthermore, situated under aerobic conditions, bioremediative technologies in situ and ex situ have engineered injection wells, air or oxygen sparging systems, and if needed, bioreactor and film fixed biological communities that are noninvasive and engineered to support continuous growth and degradation. Similarly, bioremediation under anaerobic conditions have also seen tremendous gains. DNAPL contaminants such as MTBE, TCE, PAHs, and benzene from BTEX are more persistent in nature and tend to diffuse down lower gradients of the aquifer and adsorb onto sediments making them harder to remediate and removed from the environment. However, through natural selection, reports have shown aquifer zones to be saturated with contaminant reducing bacteria appropriately appointed as NO_3, SO_4, Fe(III), CO_2, and H_2 reducers to be affixed with TEAs chemotactically attracted to corresponding regions. Contaminant degradation has shown to be oxidized well under anaerobic conditions where the production of CO_2 is a direct indication of MTBE, benzene, and PAHs degradation, while ethane corresponds with TCE degradation. Furthermore, specific bacteria isolated from the sediment and the contaminant slurries have indicated the presence of; *Dehalococcoides* spp., *Geobacter* spp., *Bacillus halodurans*, *Thauera aromatica*, *Desulfotomaculum thermocisternum*, *Arthrobacter* spp., *Mycobacterium*, *Pseudomonas* spp., *Sphingomonas*, and *Rhodococus* spp. These organisms facilitate continuous research efforts that pertain to genetic isolation of specific genes that market biological degradation towards targeted contaminants. However, naturally occurring environmental conditions do cause certain limitations to arise from either biological remediation technologies. Availability of TEAs, nutrients, and various electron donors, coupled with the competitive or suppressive indigenous or augmented microorganisms, can severely limit degradation attributes. Aquifer permeability, hydraulic conductivity, and environmental factors, such as pH and temperature, have also shown to be limiting factors that hinder bioremediation studies. However, continuous monitoring, analysis, and a coordinated strategic plan must be required before any technology can be implemented. These techniques have been shown to be an effective alternative to chemical remediation methods; however, with all great ventures in science and technology, patience and understanding is what dictates success. Research efforts and improvements in current and prospective technologies are in progress, leading the way towards broadening the horizons and potential of biological remediation of contaminated aquifers for the future.

References

Aeckersberg F, Bak F, Widdel F (1991) Anaerobic oxidation of saturated hydrocarbons to CO2 by a new type of sulfate reducing bacterium. Arch Microbiol 156:5–14

Agarwal R, Nair A (2002) Surviving Bhopal 2002: toxic present – toxic future. A report on human and environmental chemical contamination around the Bhopal disaster site. For the fact finding mission on Bhopal, Srishti, New Delhi

Aitken MD, Borden RC, Barlaz MA (1997) Limiting factors in the bioremediation of soil and groundwater contaminated with aromatic hydrocarbons. USGS water resource research grant proposal. U.S. Department of the Interior, U.S. Geological Survey. http://water.usgs.gov/wrri/96grants/seir13nc.htm

Al-Bashir BT, Cseh R, Leduc R, Samson R (1990) Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. Appl Microbiol Biotechnol 34:414–419

Alexander M (1994) Biodegradation and bioremediation. Academic, San Diego, CA

Altenschmidt U, Fuchs G (1991) Anaerobic degradation of toluene in denitrifying Pseudomonas sp. indication for toluene methyl-hydroxylation and benzoyl-CoA as central intermediate. Arch Microbiol 156:152–158

Alvarez PJJ, Vogel TM (1991) Substrate interactions of benzene, toulene and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. Appl Environ Microbiol 57:2981–2985

Alvarez-Cohen L, McCarty PL (1991) A co-metabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. Environ Sci Technol 25:1381–1387

Atlas RM (1985) Effects of hydrocarbons on microorganisms and bioremediation in arctic ecosystems. In: Engelhardt FR (ed) Petroleum effects in the arctic environment. Elsevier, London, pp 63–99

Atlas RM (1992) Petroleum microbiology. Encyclopedia of microbiology. Academic, Baltimore, MD, pp 363–369

Atlas RM, Bartha R (1992) Hydrocarbon biodegradation and oil spill remediation. Adv Microb Ecol 12:287–338

Baedecker MJ, Cozzarelli IM, Eganhouse RP, Siegel DI, Bennett PC (1993) Crude oil in a shallow sand and gravel aquifer-III. Biogeochemical reactions and mass balance modeling in anoxic groundwater. Appl Geochem 8:569–586

Baker KM, Butler BJ, Barker JF (2002) Field test of cometabolic remediation system for MTBE. Chemical abstracts. CAPLUS AN 2003:336664

Balkwill DL, Ghiorse WC (1985) Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl Environ Microbiol 50:580–588

Bankston JL, Sola DL, Komor AT, Dwyer DF (2002) Degradation of trichloroethylene in wetland microcosms containing broad leveled cattail and eastern cottonwood. Water Res 36:1539–1546

Barathi S, Vasudevan N (2001) Utilization of petroleum hydrocarbons by *Pseudomonas flourescens* isolated from a petroleum contaminated soil. Environ Int 26:413–416

Barker JF, Patrick GC, Major D (1987) Natural attenuation of aromatic hydrocarbons in a shallow sand aquifer. Ground Water Monit Rev 7:64–71

Battelle Memorial Institute, Air Force Research Laboratory, School of Civil and Environment Protection Engineering, United States Environmental Protection Agency and Naval Facilities Engineering Service Center (2001) Reductive anaerobic biological in-situ treatment technology (RABITT) treatment test interim report

Bazylinski DA, Dean AJ, Schuler D, Philips EJP, Lovley DR (2000) N2-dependent growth and nitrogenase activity in the metal metabolizing bacteria, *Geobacter* and *Magnetospirillum* species. Environ Microbiol 2:266–273

Bedard C, Knowles R (1989) Physiology, biochemistry, and specific inhibitors of CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiol Rev 53:68–84

Beeman RE, Bleckmann CA (2002) Sequential anaerobic-aerobic treatment of an aquifer contaminated by halogenated organics: field results. J Contam Hydrol 57:147–159

Bourg A, Mouvet C, Lerner DN (1992) A review of the attenuation of trichloroethylene in soils and aquifers. Q J Eng Geol Hydroge 25:359–370

Bouwer EJ (1994) Bioremediation of chlorinated solvents using alternative electron acceptors. In: Norris RD, Hinchee RE, Brown R et al (eds) Handbook of bioremediation. Lewis publishers, Boca Raton, FL, pp 149–169

Bouwer EJ (1995) Bioremediation of chlorinated solvents using alternate electron acceptors. In: Norris RD (ed) In-situ bioremediation of ground water and geological material: a review of technologies. DIANE publishing, Darby, PA. Section 8, pp 1–27

Bouwer EJ, McCarty PL (1983) Transformation of halogenated organic compounds under denitrification conditions. Appl Environ Microbiol 45:1295–1299

Bradley PM, Landmeyer JE, Chapelle FH (1999) Aerobic mineralization of MTBE and *tert*-butyl alcohol by stream-bed sediment microorganisms. Environ Sci Technol 33:1877–1879

Bradley PM, Chapelle FH, Landmeyer JE (2001) Methyl t-butyl ether mineralization in surface water sediment microcosms under denitrifying conditions. Appl Environ Microbiol 67:1975–1978

Brooijmans RJW, Pastink MI, Siezen RJ (2009) Hydrocarbon degrading bacteria: the oil spill cleanup crew. Microbial Biotechnol 2:587–594

Brusseau ML (1998) The impact of physical, chemical and biological factors on biodegradation. In: Serra R (ed) Proceedings of the international conference on biotechnology for soil remediation: scientific bases and practical applications. C.I.P.A. S.R.L, Milan, Italy, pp 81–98

Byl TC, Hileman GE, Williams SH, Farmer JJ (2001) Geochemical and microbial evidence of fuel biodegradation in a contaminated karst aquifer in southern Kentucky. In: Kuniansky EL (ed) U. S. geological survey karst interest group proceedings: USGS water resource investigations report 01–4011, St. Petersburg, FL

Castro-Gutiérrez VM, Rodríguez-Rodríguez CE, Vargas-Azofeifa I (2012) Hydrocarbon degrading microflora in a tropical fuel-contaminated aquifer: assessing the feasibility of PAH bioremediation. Int J Environ Res 6:345–352

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

Chaillan F, Chaineau CH, Point V, Sailot A, Oudot J (2006) Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings. Environ Pollut 144:255–265

Chapelle FH (1993) Ground-water microbiology and geochemistry. Wiley, New York, p 424

Chapelle FH, Bradley PM, Vroblesky DA, Lovley DR (1996) Measuring rates of biodegradation in a petroleum hydrocarbon contaminated aquifer. Ground Water 34:691–698

Chiang CY, Salanitro JP, Chai EY, Colthart JD, Klein CL (1989) Aerobic biodegradation of benzene, toluene and xylene in a sandy aquifer: data analysis and computer modeling. Ground Water 27:823–834

Christ JA, Ramsburg CA, Abriola LM, Pennell KD, Loffler FE (2005) Coupling aggressive mass removal with microbial reductive dechlorination for remediation of DNAPL source zones: a review and assessment. Environ Health Perspect 113:465–477

Christensen TH, Kjeldsen P, Albrechtsen H-J, Heron G, Nielsen PH, Bjerg PL, Holm PE (1994) Attenuation of landfill leachate pollutants in aquifers. Crit Rev Environ Sci Technol 24:109–202

Christopherson RW, Byrne ML (2006) Geosystems: an introduction to physical geography, Canadian edn. Pearson Education Canada Inc. Toronto, ON, pp 251–281

Cirvello JD, Radovsky A, Heath JA, Farnell DR, Landamond C III (1995) Toxicity and carcinogenicity of t-butyl alcohol in rats and mice following chronic exposure in drinking water. Toxicol Ind Health 11:151–166

Coates JD, Anderson RT, Lovley DR (1996a) Anaerobic hydrocarbon degradation in petroleum contaminated harbor sediments under sulfate reducing and artificially imposed iron reducing conditions. Environ Sci Technol 30:2784–2789

Coates JD, Anderson RT, Lovley DR (1996b) Anaerobic oxidation of polycyclic aromatic hydrocarbons under sulfate reducing conditions. Appl Environ Microbiol 62:1099–1101

Coates JD, Woodward J, Allen J, Philip P, Lovley DR (1997) Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum contaminated marine harbor sediments. Appl Environ Microbiol 64:3589–3593

Coleman ML, Hedrick DB, Lovley DR, White DC, Pye K (1993) Reduction of Fe (III) in sediments by sulfate reducing bacteria. Nature 361:436–438

Colwell RR, Walker JD, Cooney JJ (1977) Ecological aspects of microbial degradation of petroleum in the marine environment. Crit Rev Microbiol 5:423–445

Cosgrove WJ, Rijsbergman FR (2000) World water vision: Making water everybody's business. Earthscan Publications, London

Cusack F, Singh S, McCarthy C, Grieco J, Derocco M, Nguyen D, Lappinscott H, Costerton JW (1992) Enhanced oil recovery – 3 dimensional sandpack simulation of ultramicrobacteria resuscitation in reservoir formation. J Gen Microbiol 138:647–655

Da Silva M, Alvarez LB, Alvarez PJ (2004) Enhanced anaerobic biodegradation of benzene, toluene, ethylbenzene, xylene ethanol mixtures in bioaugmented aquifer columns. Appl Environ Microbiol 70:4720–4726

Daane LL, Harjono I, Zylstra GJ, Haggblom MM (2001) Isolation and characterization of polycyclic aromatic hydrocarbon degrading bacteria associated with the rhizosphere of salt march plants. Appl Environ Microbiol 67:2683–2691

Das N, Chandran P (2011) Microbial degredation of petroleum hydrocarbon contaminants: an overview. SAGE-Hindawi Biotechnology Research International

Davey ME, O'Tool GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867

Davis LC, Erickson LE (2004) A review of bioremediation and natural attenuation of MTBE. American Institute of Chemical Engineers. Environ Prog 23:243–252

DeBruin WP, Kotterman MJJ, Posthumus MA, Schraa G, Zehnder AJB (1992) Complete biological reductive transformation of tetrachloroethene to ethene. Appl Environ Microbiol 58:1996–2000

Deeb RA, Hu HY, Hanson JR, Scow KM, Alverex-Cohen L (2001) Substrate interactions in BTEX and MTBE mixtures by an MTBE degrading isolate. Environ Sci Technol 35:312–317

Dewling RT, McCarthy LT (1980) Chemical treatment of oil spills. Environ Int 3:155–162

Dojka MA, Hugenholtz P, Haack SK, Pace NR (1998) Microbial diversity in a hydrocarbon and chlorinated solvent contaminated aquifer undergoing intrinsic bioremediation. Appl Environ Microbiol 64:3869–3877

Durant ND, Wilson IP, Bouwer EJ (1995) Microcosm studies of subsurface PAH degrading bacteria from a former manufactured gas plant. J Contam Hydrol 17:213–237

Ellis DE, Lutz E, Odom JM, Buchanan RJ, Bartlett CL, Lee MD (2000) Bioaugmentation for accelerated in-situ anaerobic bioremediation. Environ Sci Technol 34:2254–2260

Ely RL, Williamson KJ, Hyman MR, Arp DJ (1997) Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects and bacterial response. Biotechnol Bioeng 54:520–534

Environment Canada (2002) Ex-situ remediation technologies for contaminated sites. Copyright 2002 Environment Canada. http://www.on.ec.gc.ca/pollution/ecnpd/tabs/tab23-e.html

Evans PJ, Mang DT, Kim KS, Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol 57:1139–1145

Fan S, Scow KM (1993) Biodegradation of trichloroethylene and toluene by indigenous microbial populations in soil. Appl Environ Microbiol 59:1911–1981

Fava F, Di Gioia D, Marchetti L (2000) Role of the reactor configuration in the biological detoxification of a dump site polychlorobiphenyl contaminated soil in lab scale slurry phase conditions. Appl Microbiol Biotechnol 53:243–248

Fennell DE, Gossett JM, Zinder SH (1997) Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. Environ Sci Technol 31:918–926

Finneran KT, Lovley DR (2001) Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol. Environ Sci Technol 35:1785–1790

Fortin NY, Deshusses MA (1999) Treatment of methyl *tert-b*utyl ether vapors in biotrickling filters 2. Analysis of the rate limiting step and behavior under transient conditions. Environ Sci Technol 33:2987–2991

Fredrickson JN, Hicks RJ, Li SW, Brockman FJ (1988) Plasmid incidence in bacteria from deep surface sediments. Appl Environ Microbiol 54:2916–2923
Freedman DL, Goseett JM (1989) Biological reductive dechlorination of tetrachloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 55:2144–2151
Froehner S, Machado KS, Dombroski LF, Nunes AC, Kishi RT, Bleninger T, Sanez J (2012) Natural biofilms in freshwater ecosystems: Indicators of the presence of polycyclic aromatic hydrocarbons. Water Air Soil Pollut 223:3965–3973
Fuchs S, Haritopoulou T, Wilhelmi M (1996) Biofilms in freshwater ecosystems and their use as a pollutant monitor. Water Sci Technol 34:137–140
Fuchs S, Haritopoulou T, Shafer M, Wilhelmi M (1997) Heavy metals in freshwater ecosystems introduced by urban rainwater runoff – monitoring of suspended solids, river sediments and biofilms. Water Sci Technol 36:277–282
Gerhardt P, Murray RGE, Wood WA, Krieg NR (1994) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC
Ghiorse WC, Wilson JT (1988) Microbial ecology of the terrestrial subsurface. Adv Appl Microbiol 33:107–172
Gossett JM, DiStefano TD, Stover MA (1994) Biological degradation of tetrachloroethylene in methanogenic conditions. U.S. air force technical report no. AL/EQ-TR-1983-0026, USAF Armstrong Laboratory, Environics Directorate, Tyndall AFB, FL
Hanson JR, Ackerman CE, Scow KM (1999) Biodegradation of *tert-b*utyl ether by a bacterial pure culture. Appl Environ Microbiol 65:4788–4792
Harker AR, Kim Y (1990) Trichloroethylene degradation by two independent aromatic degrading pathways in *Alcaligenes eutrophus* JMP 134. Appl Environ Microbiol 56:1179–1181
Hazen TC (2010) In situ: groundwater bioremediation. In: Timmons KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Chapter 13
Henry SM, Grbic-Galic D (1994) Bioremediation of trichloroethylene in methanotrophic systems and implications for process applications. In: Chaudhry GR (ed) Biological degradation and bioremediation of toxic chemicals. Dioscorides, Portland, pp 314–344
Hernan RE (2010) This borrowed earth: lessons from the fifteen worst environmental disasters around the world. Palgrave Macmillan, New York, pp 1–11
Holliger C, Gaspard S, Glod G, Heijman C, Schumacher W, Schwarzenbach RP, Vazquez F (1997) Contaminated environments in the subsurface and bioremediation: organic contaminants. FEMS Microbiol Rev 20:517–523
Hurt KL, Wilson JT, Beck FP, Cho JS (1999a) Anaerobic biodegradation of MTBE in a contaminated aquifer. In: Alleman BC, Leeson A (eds) Proceeding of the fifth international in situ and on site bioremediation symposium, vol 5. Battelle, Columbus, pp 103–108
Hurt K, Wilson J, Beck F, Cho J (1999b) Anaerobic biodegradation of MTBE in a contaminated aquifer. In: Alleman B, Leeson A (eds) Natural attenuation of chlorinated solvents, petroleum hydrocarbons and other organic compounds, vol 1. Battelle, Columbus, OH, pp 103–108
Imfeld G, Nijenhuis I, Nikolausz M, Zeiger S, Paschre H, Drangmeister J (2008) Assessment of in situ degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system. Water Res 42:871–882
Jahn MK, Haderlein SB, Meckenstock RU (2005) Anaerobic degradation of benzene, toluene, ethylbenzene and *o*-xylene in sediment free iron reducing enrichment cultures. Appl Environ Microbiol 71:3355–3358
Jansen HM, Arvin F (1990) Solubility and degradability of the gasoline additive MTBE, methyl *tert*-butyl ether, and gasoline compounds in water. In: Arendt F, Hinsenveld M, van den Brink WJ (eds) Contaminated soil '90. Kluwer, Dordrecht, The Netherlands, pp 445–448
Johnson RF, Pankow D, Bender D, Price C, Zogorski J (2000) MTBE: to what extent will past releases contaminate community water supply wells? Environ Sci Technol 34:210A–217A
Johnson S, Sahu R, Jadon N, Duca C (2009) Contamination of soil and water inside and outside the union carbide India limited, Bhopal. Center for Science and Environment 41, Tughlakabad Institutional Area

Jones DM, Douglas AG, Parkes RJ, Taylor J, Giger W, Schaffner C (1983) The recognition of biodegraded petroleum derived aromatic hydrocarbons in recent marine sediments. Mar Pollut Bull 14:103–108

Kane SR, Beller HR, Legler TC, Koester CJ, Pinkart HC, Halden RU, Happel AM (2001) Aerobic biodegradation of methyl *tert-b*utyl ether by aquifer bacteria from leaking storage tank sites. Appl Environ Microbiol 67:5824–5829

Kinsella JV, Nelson MJK (1993) In situ bioremediation: site characterization, site design and full scale field remediation of petroleum hydrocarbon and trichloroethylene contaminated groundwater. In: Flathman PE, Jerger DE (eds) Bioremediation field experience. CRC Press, Boca Raton, FL

Kitayama A (1997) A study on biodegradation of aromatic hydrocarbons. Doctoral thesis, The University of Tokyo, Tokyo, Japan

Landmeyer JE, Chapelle FH, Bradley PM, Pankow JF, Church CD, Tratnyek PG (1998) Fate of bioremediation in a gasoline contaminated aquifer (1993-98). Ground Water Monit Remediat 18:93–102

Landmeyer JE, Chapelle FH, Herlong HH, Bradley PM (2001) Methyl *tert*-butyl ether biodegradation by indigenous aquifer microorganisms under natural and artificial oxic conditions. Environ Sci Technol 35:1118–1126

Lau PCK, Bergeron H, Labbe D, Wang Y, Brousseau R, Gibson DT (1994) Sequence and expression of the tod GIH genes involved in the last three steps of toluene degradation by *Pseudomonas putida* F1. Gene 146:7–13

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315

Little CD, Palumbo AV, Herbes SE, Lidstrom ME, Tyndall RL, Gilmer PJ (1988) Trichloroehtylene biodegradation by methane oxidizing bacterium. Appl Environ Microbiol 54:591–596

Lovely DR (1997) Potential for anaerobic bioremediation of BTEX in petroleum contaminated aquifers. J Ind Microbiol Biotechnol 18:75–81

Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol and *p*-cresol by the dissimilatory iron reducing organisms, GS-15. Appl Environ Microbiol 56:1858–1864

Lovley DR, Phillips EJP (1987) Competitive mechanisms for inhibition of sulfate reduction and methane production in the zone of ferric reduction in sediments. Appl Environ Microbiol 53:2636–2641

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Philips EJP, Seigel DI (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339:297–300

Lovley DR, Chapelle FH, Woodward JC (1994) Use of dissolved H2 concentrations to determine the distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ Sci Technol 28:1205–1210

Lyngkilde J, Christensen TH (1992) Fate of organic contaminants in the redox zones of a landfill leachate pollution plume (Vejen, Denmark). J Contam Hydrol 10:291–307

Mackay DM, Finarson MD, Wilson RD, Fowler B, Scow K, Hyman M, Naas C, Schirmer M, Durrant GD (1999) Field studied of in situ remediation of an MTBE plume at site 60, Vandenberg air force base, California. In: Proceeding of the 2000 petroleum hydrocarbons and organic chemicals in ground water: prevention, detection, and remediation. National Ground Water Association, Westerville, OH, pp 145–157

Major DW, McMaster MM, Cox EE, Edwards E, Dworatzek S, Hendrickson E et al (2002) Filed demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethane. Environ Sci Technol 3:5106–5116

Martani E, Seto M (1991) Effects of mineral nutrients on the degradation of 2,4-dichlorophenol at low concentrations by microbial community and bacterial isolates. J Pestic Sci 16:1–7

Massol-Deya AA, Whallon J, Hickey RF, Tiedje JM (1995) Channel structures in aerobic biofilms of fixed film reactors treating contaminated groundwater. Appl Environ Microbiol 61:769–777

Maymo-gatell X, Tando V, Gossett JM, Zinder SH (1995) Characterization of an H2-utiilization enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis. Appl Environ Microbiol 61:3928–3933

McAllister PM, Chiang CY (1994) A practical approach to evaluating natural attenuation of contaminants in ground water. Ground Water Monit Remediat 14:161–173

McCarty PL (1994) An overview of anaerobic transformation of chlorinated solvents. In: Symposium on intrinsic bioremediation of ground water. Research report no. EPA 540/R-94/515

Miller MC, Alexander VR, Barsadate J (1978) The effects of oil spill on phytoplankton in arctic lake and ponds. Arctic 31:192–218

Mitra S, Roy P (2011) BTEX: a serious ground-water contaminant. J Environ Sci 5:394–398

Mo K, Lora CO, Wanken AF, Javanmardian M, Yang X, Kulpa CF (1997) Biodegradation of methyl *tert*-butyl ether by pure bacterial cultures. Appl Microb Biotechnol 47:69–72

Mohn WW, Tiedje JM (1992) Microbial reductive dehalogenation. Microbiol Rev 56:482–507

Moran MJ, Zogorski JS, Squillace PJ (2007) Chlorinated solvents in groundwater of the United States. Environ Sci Technol 41(1):74–81

N'Guessan AL, Elifantz H, Nevin KP, Mouser PJ, Methe B, Woodward TL, Manley K, Williams KH, Wilkens MJ, Larsen JT, Long PE, Lovley DR (2010) Molecular analysis of phosphate limitation in *Geobacteraceae* during the bioremediation of an uranium contaminated aquifer. ISME J 4:253–266

National Research Council (NRC) (1994) Alternatives for groundwater cleanup. National Academy Press, Washington, DC

Nelson MJK, Compeau G, Maziarz T, Mahaffey WR (1994) Laboratory treatability for assessment of field applicability. In: Jerger DE, Exner JH, Flathman PE (eds) Bioremediation field experience. Lewis publisher, Boca Raton, FL, pp 59–80

Newell CJ, Cowie I, McGuire TM, McNab W (2006) Multi-year temporal changes in chlorinated solvent concentrations at 23 MNA sites. J Environ Eng 132:653–663

Nonner JC (2002) Sources of groundwater contamination. In: Zaporozec A (ed) Groundwater contamination inventory: a methodological guide. IHP-VI series on groundwater no. 2. UNESCO 2002

Norris RD (1994) In situ bioremediation of soils and groundwater contaminated with petroleum hydrocarbons. In: Norris RD, Hinchee RE, Brown RA, McCarty PL, Semprini L, Wilson JT et al (eds) Handbook of bioremediation. CRC Press, Boca Raton, FL

O'Connell J, Moyer E (2006) Ex-situ remediation of fuel oxygenates and petroleum hydrocarbons using bioreactors. In: Petroleum hydrocarbons and organic chemicals in groundwater: prevention, assessment and remediation conference. National Groundwater Association, pp 149–163

O'Neil RA, Holmes DE, Coppi MV, Adams LA, Larrahondo MJ, Ward JE et al (2008) Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation. Environ Microbiol 10:1218–1230

Office of technology assessment (OTA) (1991) Bioremediation of marine oil spills: an analysis of oil spill response technologies, OTA-BP-O-70, Washington, DC

Olaniran AO, Pillay D, Pillay B (2008) Aerobic biodegradation of dichloroethenes by indigenous bacteria isolated from contaminated sites in Africa. Chemosphere 73:24–29

Palleroni N (2005) Genus I. *Pseudomonas*. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergley's manual of systematic bacteriology. Springer, New York, pp 323–379

Pant P, Pant S (2009) A review: advances in microbial remediation of trichloroethylene (TCE). J Environ Sci 22:116–126

Patterson BM, Pribac F, Barber C, Davis GB, Gibbs R (1993) Biodegradation and retardation of PCE and BTEX compounds in aquifer material from western Australia using large-scale columns. J Contam Hydrol 14:261–278

Payne JF, Mathieu A, Collier TK (2003) Ecotoxicological studies focusing on marine and freshwater fish. In: Douben PT (ed) PAH: an ecotoxicological perspective. Wiley, Toronto, ON, pp 191–224

Phelps CD, Kazumi J, Young LY (1996) Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction. FEMS Microbiol Lett 145:433–437

Pitre MP, Steffan R (1999) Biotreatment of MTBE contaminated groundwater in membrane bioreactor. Envirogen Report.

Plaza G, Ulfig K, Hazen TC, Brigmon RL (2001) Use of molecular techniques in bioremediation. Acta Microbiol Pol 50:205–218

Polic P, Pfendt P (1994) Alluvial aquifer contamination: exchangeable heavy metals and factors affecting their spatial distribution. Water Air Soil Pollut 74:155–167

Rabus R, Widdel F (1996) Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl Environ Microbiol 62:1238–1241

Robles-Gonzalez IV, Fava F, Poggi-Varaldo HM (2008) A review on slurry bioreactors for bioremediation of soil and sediments. Microb Cell Fact 7:5

Rodríguez-Martínez EM, Pérez EX, Schadt CW, Zhou J, Massol-Deyá AA (2006) Microbial diversity and bioremediation of a hydrocarbon-contaminated aquifer (Vega Baja, Puerto Rico). Int J Environ Res Public Health 3:292–300

Romijn E (2002) Groundwater quality and contamination. In: Zaporozec A (ed) Groundwater contamination inventory: a methodological guide. IHP-VI Series on groundwater no. 2. UNESCO 2002

Rooney-Varga JN, Anderson RT, Fraga JI, Ringelberg D, Lovley DR (1999) Microbial communities associated with anaerobic benzene degradation in a petroleum contaminated aquifer. Appl Environ Microbiol 65:3056–3063

Salanitro JP, Wisniewski HL, Byers DL, Neaville CC, Schroder RA (1997) Use of aerobic and anaerobic microcosms to assess BTEX biodegradation in aquifers. Ground Water Monit Remediat 17:210–221

Salanitro JP, Johnson PC, Spinnler GE, Bruce CL (2000) Field-scale demonstration of enhanced MTBE through aquifer bioaugmentation and oxygenation. Environ Sci Technol 34:4152–4162

Salanitro JP, Spinnler GE Maner P, Wisneiwski H, Johnson PC (1999) Potential for MTBE bioremediation - in situ inoculation of specialized culture. In: Proceedings of conference on petroleum hydrocarbon and organic chemicals in groundwater, Houston, TX, 17–20 Nov 1999

Salanitro JP, Spinnler GE, Maner PM, Tharpe DL, Pickle DW, Wisniewski HL, Johnson PC, Bruce C (2001) In situ bioremediation of MTBE using biobarriers of single or mixed cultures. In: Leeson A, Alleman BC, Alvarex PJ, Magar VS (eds) Bioaugmentation, biobarriers and biogeochemistry, vol 8. Battelle, Columbus, OH, pp 61–71

Schettler T, Solomon G, Valenti M, Huddle A (1999) Generations at risk: reproductive health and the environment. MIT Press, Cambridge, pp 1–397

Schmidt TC, Schirmer M, Weiss H, Haderlein SB (2004) Microbial degradation of methyl *tert*-butyl alcohol in the subsurface. J Contam Hydrol 70:173–203

Schocher RJ, Seyfried B, Vazquez F, Zeyer J (1991) Anaerobic degradation of toluene by pure culture of denitrifying bacteria. Arch Microbiol 157:7–12

Sheremata TW, Yong RN, Ghoshal S (2000) Anaerobic biodegradation of trichloroethylene sorbed by a surrogate soil organic matter. J Environ Qual 29:1033–1040

Shi Y, Zwolinski MD, Schreiber ME, Bahr JM, Sewell GW, Hickey WJ (1999) Molecular analysis of microbial community structures in prestine and contaminated aquifers: field and laboratory microcosm experiments. Appl Environ Microbiol 65:2143–2150

Shuler ML, Kargi F (1992) Bioprocess engineering: basic concepts. PTR Prentice Hall Inc., Englewood Cliffs, NJ

Shuttleworth KL, Sung J, Kim E, Cerniglia CE (2000) Physiological and genetic comparison of two aromatic hydrocarbon degrading *Sphingomnas* strains. Mol Cell 10:199–205

Singh MJ, Someshekar RK, Prakash KL, Shivanna K (2010) Investigation of heavy metals in crystalline aquifer groundwater from different valleys of Bangalore, Karnataka. J Geogr Region Plan 3:262–270

Soerensen PL (1996) Soil should be cleaned at the criminal scene. Ingenioeren, No. 39

Spies RB (2007) Long-term ecological change in the Northern Gulf of Alaska. Elsevier B.V, Amsterdam

Spies RB, Rice S (2007) Contaminants. In: Spies RB (ed) Long-term ecological change in the Northern Gulf of Alaska. Elsevier, Amsterdam, pp 241–247

Spies RB, Rice SD, Wolfe DA, Wright BA (1996) The effect of the exxon valdez oil spill on Alaskan coastal environment. In: Proceedings of the 1993 exxon valdez oil spill symposium. American Fisheries Society. Bethesda, MD, pp 1–16

Springman KR, James GK, Anderson J, Emlen JM (2005) Contaminants as viral cofactors: assessing indirect population effects. Aquat Toxicol 71:13–23

Squillace PJ, Pankow FJ, Korte NF, Zogorski JS (1997) Review of the environmental behavior and fate of methyl *tert*-butyl ether. Environ Toxicol Chem 16:1836–1844

Stapleton RD, Bright NG, Sayler GS (2000) Catabolic and genetic diversity of degradative bacteria from fuel hydrocarbon contaminated aquifers. Microb Ecol 39:211–221

Stavnes SA, Fleischmann JJ, Hunt S, Goetz J, Kemper M, Roulier M, Hristova K, Scow K, Knutson K, Mahaffee WR, Slomczynski DJ, Davis-Hoover WJ (2002) MTBE bioremediation with BioNets containing isolite, PM1, solid oxygen source (SOS) or air. In: Gavaskar AR, Chen ASC (eds) Remediation of chlorinated and recalcitrant compounds. Battelle, Columbus, OH, pp 990–997

Steffan RJ, Vainberg S, Condee CW, McClay K, Hatzinger P (2000) Biotreatment of MTBE with a new bacterial isolate. In: Wickramanay GB, Gavaskar AR, Alleman BC, Magar VS (eds) Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Battelle, Columbus, OH, pp 165–173

Stocking AJ, Deeb RA, Flores AE, Stringfellow W, Talley J, Brownell R, Kavanaugh MC (2000) Bioremediation of MTBE: a review from a practical perspective. Biodegradation 11:187–201

Tang WT, Sun PT (1997) Field evaluation of biological and non-biological treatment technologies to remove MTBE/oxygenates from petroleum product terminal waste waters. Shell Development Company, Houston TX. American Petroleum Institute, Washington, DC. Publication Number 4655

Terrapex Environmental Ltd (2009) In-situ/ex-situ remediation. Copyright 2009. All rights reserved. http://www.terrapex.com/in-situ-ex-situ-remediation.asp

Thomas JM, Ward CH (1992) Subsurface microbial ecology and bioremediation. J Hazard Mater 32:179–194

United States Environmental Protection Agency (USEPA) (1995) In situ groundwater bioremediation. In: Directive No. EPA 510-B-95-007 How to evaluate alternative cleanup technologies for underground storage tank sites: a guide for corrective action plan reviewers

United States Environmental Protection Agency (USEPA) (1997) Use of monitored natural attenuation at superfund, RCRA correction action and underground storage tank sites. In: Directive No. 92004.4-17P Office of solid waste and emergency response US EPA, Washington, DC

van Bemmel JBM (2010) Intrinsic bioremediation of hydrocarbons. In: Timmins KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 4510–4515

van Ras NJP, Winters RO, Lieten SH, Dijkhuis JE, Henssen MJC, van Hattem WA, Lethbridge G (2007) Sustainability of natural attenuation of aromatics (BTEX). NICOLE Network

Vogel TM, McCarty PL (1985) Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride and carbon dioxide under methanogenic conditions. Appl Environ Microbiol 49:1080–1083

Vogel TM, McCarty PL (1987) Abiotic and biotic transformations of 1,1,1-trichloroehtane under methanogenic conditions. Environ Sci Technol 21:1208–1213

Weiner JM, Lovley DR (1998) Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene oxidizing enrichment. Appl Environ Microbiol 64:775–778

White GF, Russel NJ, Tidswell EC (1996) Bacterial scission of ether bonds. Microbiol Rev 60:216–232

Wilson J (1999) Natural attenuation of MTBE in ground water: what do we need to know? In: Proceedings of the 2000 petroleum hydrocarbons and organic chemicals in ground water: prevention, detection, and remediation
Wilson JT, Wilson BH (1985) Biotransformation of trichloroethylene in soil. Appl Environ Microbiol 49:242–243
Wilson JT, McNabb JF, Balkwill DL, Ghiorse WC (1983a) Enumeration and characterization of bacteria indigenous to a shallow water table aquifer. Ground Water 21:134–142
Wilson JT, McNabb JF, Wilson BH, Noonan MJ (1983b) Biotransformation of selected organic pollutants in ground water. Dev Ind Microbiol 24:225–233
Wilson JT, Leach LE, Henson M, Jones JN (1986) In situ biorestoration as a groundwater remediation technique. Ground Water Monit Rev 6:56–64
Wilson LP, D'Adamo PC, Bouwer EJ (1997) Bioremediation of BTEX, naphthalene and phenanthrene in aquifer material using mixed oxygen/nitrate electron acceptor conditions. EPA/600/SR-97/103
Wilson JT, Cho JS, Wilson BH, Vardy J (2000) Natural attenuation of MTBE in the subsurface under methanogenic conditions. EPA/600/R-00/006
Wilson JT, Adair C, Kaiser PM, Kolhatkar R (2005) Anaerobic biodegradation of MTBE at a gasoline spill site. Ground Water Monit Remediat 25:103–115
Wolfe WJ, HAugh CJ, Webbers A, Diehl TH (1997) Preliminary conceptual models of occurrence, fate and transport of chlorinated solvents in karts aquifers of Tennessee: U.S. Geological Survey water-resources investigations report 97-4097, p 80
Yakimov MM, Timmis KN, Golyshin PN (2007) Obligate oil degrading marine bacteria. Curr Opin Biotechnol 18(3):257–266
Yeh CK, Novak JT (1994) Anaerobic biodegradation of gasoline oxygenates in soils. Water Environ Res 66:744–752
Zaporozec A (2002) Groundwater contamination inventory: a methodological guide – Introduction. IHP-VI series on groundwater no. 2. UNESCO 2002

Index

A
Acetoclastic methanogenesis, 155
Acidithiobacillus ferrooxidans, 4, 119, 144
Acid lake remediation, 132
Acid mine drainage (AMD), 26, 29, 129
Acidolysis, 140
Acidophiles, 63
Aerobic bacteria, 155–156
Aerobic hydrocarbon metabolism, 158
Agricultural inoculants, 218
Air-sparging, 84
Alcohol, 250
Alkaliphilic microorganisms, 62
Alkalitolerants, 64
AMD. *See* Acid mine drainage (AMD)
American Petroleum Institute (API), 155
Ammonia oxidizing bacteria, 271
Amplified ribosomal DNA restriction analysis (ARDRA), 21, 45
Anaerobic bioremediation, 279
Anaerobic hydrocarbon metabolism, 158
Anoxygenic photoheterotrophs, 158
Antibiotic resistance markers, 40
API. *See* American Petroleum Institute (API)
Aquifers, 284
Archaebacteria, 38
ARDRA. *See* Amplified ribosomal DNA restriction analysis (ARDRA)
Arsenate, 249
Aspergillus niger, 141
Autotrophic microorganisms, 138

B
Bacteriorhodopsin, 67
Beneficial microbes, 223
Benzene, toluene, ethylbenzene and xylene (BTEX), 262, 264
BES. *See* Bioelectrochemical system (BES)
Bioaccumulation, 142–143
Bioattenuation, 87
Bioaugmentation, 86, 262
Bioavailability, 256
Bioavailability of contaminants, 22
Biocementation, 3
Bioclogging, 3
Biocorrosion, 166
Biodegradation, 6, 266
  of hydrocarbons, 156
  of *n*-alkanes, 156–157
Biodegraded petroleum reservoirs, 154, 159
Biodemulsification, 242
Biodemulsifiers, 242
Biodenitrogenation, 11
Biodesulfurization, 11
Biodiversity, 1
Bioelectrochemical system (BES), 9–10
Bioenhancement, 274
Biofertilizer, 179
Biofilms, 240
Biofuel, 8
Biogeochemical
  cycle, 17, 130
  processes, 1–2, 24, 130, 153
  reactions, 17
Biohydrometallurgy, 4, 138
Bioinformatics, 94
Bioleaching, 119, 140
Biological nitrogen fixation (BNF), 38
Biological souring, 10–11
BIOLOG system, 46
Bioluminescent marker genes, 40–41

N. Parmar and A. Singh (eds.), *Geomicrobiology and Biogeochemistry*, Soil Biology 39,
DOI 10.1007/978-3-642-41837-2, © Springer-Verlag Berlin Heidelberg 2014

Bio-mediated improvement of soil properties, 3–4
Biomineralization, 101, 216
Biomining, 4, 119
Biomolecules, 111
Bionanotechnology, 12–13
Biopolymers, 240
Bioprospecting tool, 12
Bioreactors, 6, 90, 274
Bioremediation, 5–7, 17, 26, 130–132
Bioslurry reactors, 275
Biostimulation, 84, 262
Biosurfactants, 91, 240, 242
Biotrickling, 275
Bioventing, 84
Bioweathering, 178
Bivalent metal ions, 105
BNF. *See* Biological nitrogen fixation (BNF)
BTEX. *See* Benzene, toluene, ethylbenzene and xylene (BTEX)
BTEX degradation, 272

C
Calcite, 3
Capsular polysaccharides (CPS), 184
Carboxylic acids, 140
Carotenoid, 67
Carotenoid pigments, 67
Catalyzed reporter deposition, 27
Catechol di-oxygenase, 271
Cell surface hydrophobicity (CSH), 242
CGA. *See* Community genome arrays (CGA)
Chalcopyrite, 119, 121
Chemical
  contaminants, 81
  oxidation, 273
  treatment process, 81
Chemolithographic acidophilic microbes, 144
Chemolithotrophic, 128
  microorganisms, 4, 139
  sulphur bacteria, 161
Chlorinated VOCs, 286
Chlorosis, 2, 175
Chromate, 249
Citrate synthase, 214
Citric acid, 141
Coal mining, 132
Coinoculation, 189, 222
Combinatorial microscopy-analytical mass spectrometry, 52
Co-metabolism of TCE, 270
Community genome arrays (CGA), 24
Complete oxidizers, 164
Composting, 88
Contaminant characteristics, 263
Contamination, 247
Copper sulfide ores, 119
CO_2-reducing methanogens, 157
CPS. *See* Capsular polysaccharides (CPS)
Crystalline, 284
CSH. *See* Cell surface hydrophobicity (CSH)
Culture based techniques, 19–20
Cycling of nutrients, 190
Cycling of P in soil, 202

D
De-emulsification methods, 239
Deep subsurface, 1–2, 153
Deepwater Horizon marine oil spill, 168
Dehalococcoides spp, 278
Deltaproteobacteria, 163
Denaturing gradient gel electrophoresis (DGGE), 44
Denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), 21
Denitrification, 285
Desulfatibacillum alkenivorans, 158
Desulfotomaculum thermocisternum, 277
Diazotrophs, 38
Dissolved organic matter (DOM), 252
Dissolved oxygen (DO), 270, 278
DNA chips, 41
DNA fingerprints, 41

E
Ecosystems, 261
Electrokinetic, 252
Electromigration, 253, 254
Electron donor, 283
Electrophoresis, 253
Electroplating, 247
Engineered soil biopiles, 6
Enhanced oil recovery (EOR), 239
Environmental contaminants, 5
Environmental factors, 9, 185
Equilibrium, 250
Exopolysaccharides (EPS), 183
Ex situ bioremediation, 87–90, 274
Extracellular nanoparticles, 111
Extremophiles, 7, 61, 63–64
Extremozymes, 7

F
Farmyard manure, 211
Fe^{2+}, 248
Fe (III) containing sediment, 277
Feldspar, 182
Fermentation, 155
Fermentative bacteria, 165–166
Ferric phosphate mobilization, 211
Ferrihydrite, 121
FGA. *See* Functional gene arrays (FGA)
Fingerprinting techniques, 21
Flow cytometery, 41
Fluorescent in-situ hybridization (FISH), 27
Fluorescent markers, 40
Fluorescent pseudomonad strains, 204
Formation waters, 160
Functional gene arrays (FGA), 23, 43
Functional metagenomics, 48
Fungal phytase, 218

G
GA. *See* Gluconic acid (GA)
Gel-electrophoresis, 22
Gel-electrophoresis based PCR, 43–44
Gene microarray, 168
Genetic engineering techniques, 93
Genomic fingerprinting technique, 46
Geobacter metallireducens, 277
Geobacter sulfurreducens, 9
GeoChip, 25, 43
Geomicrobiology, 2
Gibberellins, 218
Gluconacetobacter diazotrophicus, 45
Gluconic acid (GA), 208
Glucose oxidation, 208
Glycine betaine, 65
Goethite, 137
Groundwater contamination, 124–125

H
Haloalkaliphiles, 61
Halobacteriaceae, 67
Halophilic archaea, 63
Halophilic methanogenic archaea, 65
Halophilic methylotrophic methanogens, 165
Halotolerant, 65
Heavy bitumen petroleum, 168
Heavy metal bioremediation, 101
Heavy metal contaminated acidic soil, 23
High-throughput sequencing (HTS), 25
Homoacetogenesis, 155
Homoacetogens, 154–155
H_2PO_4, 257
Humic acid, 211
Hybridization technique, 41
Hydrocarbon-degrading bacteria, 155
Hydrogenotrophic methanogenesis, 155
Hydrophobic cell surfaces, 242
Hydrophobic contaminants, 87
Hydrophobic substrates, 156
Hypersaline, 61

I
Immobilization, 252
Indole-3-acetic acid, 218
Inorganic acids, 210
Inorganic anions, 250
In situ bioremediation, 82, 273
Insoluble phosphates, 203
Intracellular nanoparticles, 111
In-well air stripping, 273
Iron-reducing bacteria, 28
Iron reducing microorganisms, 155
Iron silicates, 248
Isoprenoids, 156

K
K dynamics, 176
K fertilization in soils, 175

L
Landfarming, 6, 88
Laser scanning microscopy (LSM), 28
Laterites, 137
Lateritic nickel deposits, 139
Leptospirillum ferrooxidans, 127
Limonites, 137
LSM. *See* Laser scanning microscopy (LSM)

M
Macronutrient, 175
Macronutrient for biological growth, 199
Magnetic resonance imaging (MRI), 28
Magnetotactic bacteria, 110
MAR. *See* Microautoradiography (MAR)
Mature fine tailings (MFT), 168
MCP. *See* Monocalcium phosphate (MCP)
Mechanism for mineral phosphate-
solubilization, 208
Membrane-bound enzymes, 156

MEOR. *See* Microbial enhanced oil recovery (MEOR)
MEOR mechanisms, 240
Metabolic breakdown mechanisms, 267
Metabolic interactions, 213
Metabolome, 49
Metagenome, 47, 167
Metagenomic arrays, 24
Metagenomics, 47
Metagenomic studies, 166
Metal bioremediation, 101
Metalloids, 101
Metallophiles, 101–112
Metal microbe interaction, 108
Metal recovery, 119
Metals, 102
Metal transport systems, 102
Meta-metabolomics, 49–50
Meta-omics techniques, 46–50
Metaproteomics, 48–49
Metatranscriptomics, 47–48
Meteoric waters, 157
Methanogenesis, 279
Methanogenic *Archaea*, 154, 165
Methanogenic microbes, 164
Methanogenic petroleum biodegradation, 167
Methanogens, 281
Methanotrophic bacteria, 269
Methylation/demethylation, 256
MFC. *See* Microbial fuel cells (MFC)
MFT. *See* Mature fine tailings (MFT)
Mg^{2+}, 248
Mica, 179
MICP. *See* Microbially induced corrosion processes (MICP)
Microarrays, 24, 41
Microautoradiography (MAR), 27
Microbe-mineral interaction, 142
Microbes assisted solubilisation of metals, 138
Microbial diversity, 37, 68
Microbial electrosynthesis, 9–10
Microbial enhanced oil recovery (MEOR), 11, 166, 240
Microbial extraction of nickel, 139–148
Microbial flooding, 241
Microbial fuel cells (MFC), 10
Microbial interactions, 119
Microbially-assisted metal recovery, 4
Microbially induced corrosion processes (MICP), 164
Microbial nanoparticle synthesis, 108–110
Microbial nanowires, 9
Microbial P immobilization, 214
Microbial recovery of nickel, 143
Microbiodiversity, 37
Microecophysiology analysis, 52
Microspectroscopy, 39
Mineralization, 276
Mineral phosphate solubilizing genes, 213
Minerals, 119
Mixed bacterial cultures, 242
Mobilization, 257
Molecular detection techniques, 41
Molecular probes, 40
Molecular techniques, 38, 93
Molecular toolbox, 42
Monoammonium phosphate, 219
Monocalcium phosphate (MCP), 257
Mono-oxygenase, 272
Montmorillonite, 248
MRI. *See* Magnetic resonance imaging (MRI)
Muriate of potash, 176
Muscovite mica minerals, 185
Mycorrhizal associations, 223

N

Nanobiotechnology, 93
Nanoparticles, 12, 101
Nano-SIMS, 53
Nanotechnology, 48–49, 94
Naphthenic acids, 154
NAPL. *See* Non-aqueous phase liquids (NAPL)
Natural attenuation, 87, 267
Neutrophilic bacteria, 63
Nickel, 137
Nitrate reducers, 158
Nitrate-reducing bacteria (NRB), 11
Nitrate reductase, 222
Nitrogenase, 38, 222
Nitrogen-fixing bacteria, 188, 222
Non-aqueous phase liquids (NAPL), 264
NRB. *See* Nitrate-reducing bacteria (NRB)
Nutrient uptake, 186

O

Octanol-water coefficient, 275
Oil degrading bacteria, 267
Oilfield emulsions, 239
Oil sands, 158, 241
Oil-water interfacial tension (OIT), 240
Oligonucleotide probes, 27
ORCs. *See* Oxygen releasing compounds (ORCs)
Organic acids, 138–139, 182, 208

Organic phosphate solubilization, 214
Osmoregulation, 66–67
Oxidation, 255
Oxidic ores, 137
Oxygenation, 271
Oxygen releasing compounds (ORCs), 91–93

P
PAH. *See* Polycyclic aromatic hydrocarbons (PAH)
PAH degradation, 280
Palaeopasteurization hypothesis, 159
Partial Community Analysis, 20
PCR. *See* Polymerase chain reaction (PCR)
PCR amplified DNA fragments, 21
PCR-based analyses, 21
Persistent organic contaminants, 265
Petroleum industry, 239
Petroleum oil well, 239
Petroleum reservoirs, 153
PGA. *See* Phylogenetic arrays (PGA)
PHA. *See* Polyhydroxyalkanoates (PHA)
Phenanthrene, 277, 280, 285
Phosholipid fatty acid methyl ester (PLFA), 39
Phosphatase, 214
Phosphate solubilizing bacteria, 180, 200
Phosphate solubilizing fungi, 200
Phosphatic biofertilizers, 219
Phosphatic fertilizers, 199
Phosphonatases, 215
Phosphorus, 199
Phosphorus deficiency in soil, 199
Photosynthesis, 189
Phototrophic sulphur bacteria, 161
Phylogenetic analyses of communities, 20
Phylogenetic arrays (PGA), 24
Phylogeny of the halophiles, 68
Physical adsorption, 105
Phytase gene, 217
Phytases, 214–215
Phytate phosphorus utilization, 217
Phytoextraction, 251
Phytoremediation, 6, 87
Picoplancton, 18
Plant growth-promoting bacteria, 186
Plant growth-promoting rhizobacteria, 211
Plant metabolism, 175
PLFA. *See* Phosholipid fatty acid methyl ester (PLFA)
Polycyclic aromatic hydrocarbons (PAH), 262
Polyhydroxyalkanoates (PHA), 8
Polymerase chain reaction (PCR), 20–21
Polysulfide mechanism, 144
Porphyry copper deposits, 127
Potassium, 175
Potassium-solubilizing microorganisms, 179
Precipitation, 250
Pseudomonas aureginosa, 270
Pseudomonas fluorescens, 220
P solubilization activity, 203
Pyrite, 121

Q
Quantitative-PCR, 23–24

R
Raman microspectroscopy, 51–52
Random amplified polymorphic DNA (RAPD), 44
16S rDNA nucleotide sequence, 46
RDNA operon, 45
Redox potential, 129
Reductive dechlorination, 281
Reductive dissolution, 119
Remediation, 262
  approach, 82
  technique, 252
Repetitive sequence-based polymerase chain reaction (rep-PCR), 46
Reservoir properties, 240
Reservoir souring, 166
Restriction fragment length polymorphism (RFLP), 21, 45
Rhizobacterial isolates, 181, 184
Rhizosphere, 251
  bacteria, 190
  microflora diversity, 39
Rock minerals, 178
Rock phosphate, 179, 203
RRNA operons, 21

S
Saprolites, 137
Selective plugging, 241
Siderophores, 184
Silicate minerals, 177
Silicate solubilizing bacteria (SSB), 179
Single super phosphate, 220
Sinorhizobium meliloti, 221
Slurry phase treatment, 90
Stable isotope probing (SIP), 50
Stable isotopes, 50

Stimulation of root growth, 224
Stratal waters, 160
Subsurface petroleum microbiology, 154
Sulfate-reducing bacteria (SRB), 11, 131, 154, 161–164
Sulfate reduction, 155
Sulfide ore, 119
Sulfide oxidation, 121–124
Sulfidic ore material, 125
Sulfulobus spp., 119, 120
Sulfuric acid, 128
Sulphides, 247
Sulphides/oxides complexes, 138
Sulphidic ores, 139
Surfactants, 251
Sustainable agriculture, 191
Syntrophs, 165

T
TCE. *See* Trichloroethylene (TCE)
Temperature gradient gel electrophoresis (TGGE), 44
Terminal restriction fragment length polymorphism (T-RFLP), 22
Thermophilic fermentative microorganisms, 166
Thioalkalivibrio versutus, 65
Thiosulphate mechanism, 144
Toluene di-oxygenase, 271
Toluene oxidizing bacteria, 269
Toxicant-induced stress, 22
Transcriptional gene control, 48
Transgenic plants, 93
Transportation, 255
Transposon mutagenesis, 212
T-RFLP. *See* Terminal restriction fragment length polymorphism (T-RFLP)
Tricalcium phosphate (TCP), 203
Trichloroethylene (TCE), 268

V
Vapor extraction, 273

W
Whole-genome open reading frame array (WGA), 24

X
Xenobiotics, 94