Bioaugmentation for Groundwater Remediation

SERDP and ESTCP Remediation Technology Monograph Series
Series Editor: C. Herb Ward, Rice University

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SERDP and ESTCP have joined to facilitate the development of a series of monographs on remediation technology written by leading experts in each subject area. This volume provides a review of the state-of-the-art on bioaugmentation for groundwater remediation. Previously published volumes include:

- *In Situ* Bioremediation of Perchlorate in Groundwater
- *In Situ* Remediation of Chlorinated Solvent Plumes
- *In Situ* Chemical Oxidation for Groundwater Remediation
- Delivery and Mixing in the Subsurface: Processes and Design Principles for *In Situ* Remediation

Additional volumes planned for publication in the near future include:

- Chlorinated Solvent Source Zone Remediation
- Processes, Assessment and Remediation of Contaminated Sediment

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Preface

In the late 1970s and early 1980s, our nation began to grapple with the legacy of past disposal practices for toxic chemicals. With the passage in 1980 of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), commonly known as Superfund, it became the law of the land to remediate these sites. The U.S. Department of Defense (DoD), the nation's largest industrial organization, also recognized that it too had a legacy of contaminated sites. Historic operations at Army, Navy, Air Force, and Marine Corps facilities, ranges, manufacturing sites, shipyards, and depots had resulted in widespread contamination of soil, groundwater, and sediment. While Superfund began in 1980 to focus on remediation of heavily contaminated sites largely abandoned or neglected by the private sector, the DoD had already initiated its Installation Restoration Program in the mid-1970s. In 1984, the DoD began the Defense Environmental Restoration Program (DERP) for contaminated site assessment and remediation. Two years later, the U.S. Congress codified the DERP and directed the Secretary of Defense to carry out a concurrent program of research, development, and demonstration of innovative remediation technologies.

As chronicled in the 1994 National Research Council (NRC) report, "Ranking Hazardous-Waste Sites for Remedial Action," our early estimates on the cost and suitability of existing technologies for cleaning up contaminated sites were wildly optimistic. Original estimates, in 1980, projected an average Superfund cleanup cost of a mere $3.6 million per site and assumed only around 400 sites would require remediation. The DoD's early estimates of the cost to clean up its contaminated sites were also optimistic. In 1985, the DoD estimated the cleanup of its contaminated sites would cost from $5 billion to $10 billion, assuming 400–800 potential sites. A decade later, after an investment of over $12 billion on environmental restoration, the cost-to-complete estimates had grown to over $20 billion, and the number of sites had increased to over 20,000. By 2007, after spending over $20 billion in the previous decade, the estimated cost to complete the DoD's known liability for traditional cleanup (not including the Munitions Response Program for unexploded ordnance) was still over $13 billion. Why did we underestimate the costs of cleaning up contaminated sites? All of these estimates were made with the tacit assumption that existing, off-the-shelf remedial technology was adequate to accomplish the task, that we had the scientific and engineering knowledge and tools to remediate these sites, and that we knew the full scope of chemicals of concern.

However, it was soon and painfully realized that the technology needed to address the more recalcitrant environmental contamination problems, such as fuels and chlorinated solvents in groundwater and dense nonaqueous phase liquids (DNAPLs) in the subsurface, was seriously lacking. In 1994, in the "Alternatives for Ground Water Cleanup" document, the NRC clearly showed that as a nation we had been conducting a failed 15-year experiment to clean up our nation's groundwater and that the default technology, pump-and-treat, was often ineffective at remediating contaminated aquifers. The answer for the DoD was clear. The DoD needed better technologies to clean up its contaminated sites, and better technologies could only arise through a better scientific and engineering understanding of the subsurface and the associated chemical, physical, and biological processes. Two DoD organizations were given responsibility for initiating new research, development, and demonstrations to obtain the technologies needed for cost-effective remediation of facilities across the DoD: the Strategic Environmental Research and Development Program (SERDP) and the Environmental Security Technology Certification Program (ESTCP).

SERDP was established by the Defense Authorization Act of 1991, as a partnership of the DoD, the U.S. Department of Energy, and the U.S. Environmental Protection Agency; its mission is "to address environmental matters of concern to the Department of Defense and the Department of Energy through support of basic and applied research and development of technologies that can enhance the capabilities of the departments to meet their environmental obligations." SERDP was created with a vision of bringing the capabilities and assets of the nation to bear on the environmental challenges faced by the DoD. As such, SERDP is the DoD's environmental research and development program. To address the highest-priority issues confronting the Army, Navy, Air Force, and Marine Corps, SERDP focuses on cross-service requirements and pursues high-risk and high-payoff solutions to the DoD's most intractable environmental problems. SERDP's charter permits investment across the broad spectrum of research and development, from basic research through applied research and exploratory development. SERDP invests with a philosophy that all research, whether basic or applied, when focused on the critical technical issues, can impact environmental operations in the near term.

A DoD partner organization, ESTCP, was established in 1995 as the DoD's environmental technology demonstration and validation program. ESTCP's goal is to identify, demonstrate, and transfer technologies that address the DoD's highest priority environmental requirements. The program promotes innovative, cost-effective environmental technologies through demonstrations at DoD facilities and sites. These technologies provide a large return on investment through improved efficiency, reduced liability, and direct cost savings. The current cost and impact on DoD operations of environmental compliance is significant. Innovative technologies are reducing both the cost of environmental remediation and compliance and the impact of DoD operations on the environment, while enhancing military readiness. ESTCP's strategy is to select laboratory-proven technologies with potential broad DoD application and use DoD facilities as test beds. By supporting rigorous testing and evaluation of innovative environmental technologies, ESTCP provides validated cost and performance information. Through these tests, new technologies gain end-user and regulatory acceptance.

In the 17–21 years since SERDP and ESTCP were formed, much progress has been made in the development of innovative and more cost-effective environmental remediation technology. Since then, recalcitrant environmental contamination problems for which little or no effective technology had been available are now tractable. However, we understand that newly developed technologies will not be broadly used in government or industry unless the consulting engineering community has the knowledge and experience needed to design, cost, market, and apply them.

To help accomplish the needed technology transfer, SERDP and ESTCP have facilitated the development of a series of monographs on remediation technology written by leading experts in each subject area. Each volume will be designed to provide the background in process design and engineering needed by professionals who have advanced training and five or more years of experience. The first volume in this series, *In Situ Bioremediation of Perchlorate in Groundwater*, met a critical need for state-of-the-technology guidance on perchlorate remediation. The second volume, *In Situ Remediation of Chlorinated Solvent Plumes*, addressed the diverse physical, chemical, and biological technologies currently in use to treat what has become one of the most recalcitrant contamination problems in the developed world. The third volume, *In Situ Chemical Oxidation for Groundwater Remediation*, provided comprehensive, up-to-date descriptions of the principles and practices of *in situ* chemical oxidation for groundwater remediation based on a decade of intensive research, development, and demonstration. The fourth volume, *Delivery and Mixing in the Subsurface: Processes and Design Principles for In Situ Remediation,* described the principles of chemical delivery and mixing systems, and their design and implementation for effective *in situ* remediation. Other volumes will follow on such topics as the remediation of DNAPL-chlorinated solvent source zones and remediation of

contaminated sediments. Additional volumes will be written as new remediation technologies are developed and proven to be effective.

This volume, *Bioaugmentation for Groundwater Remediation*, provides a review of the past 10–15 years of intensive research, development, and demonstrations that have been at the forefront of developing bioaugmentation into a viable remedial technology. In addition, both a primer on the basic microbial processes involved in bioaugmentation as well as a thorough summary of the methodology for implementing the technology is provided within this volume. It is our intention that this will serve as a valuable resource for environmental remediation professionals who seek to understand, evaluate, and implement bioaugmentation. Topics addressed in this volume include:

- A brief history and overview of bioaugmentation (Chap. 1).
- A detailed review of the discovery of *Dehalococcoides* and the development of reductive dechlorination of chlorinated solvents as a remedial technology (Chap. 2).
- The state-of-the-science for the production and handling of *Dehalococcoides* bioaugmentation cultures (Chap. 3).
- An overview of a decision process for determining whether to implement bioaugmentation with *Dehalococcoides* (Chap. 4).
- Design considerations for implementing bioaugmentation (Chap. 5).
- A summary of microbial monitoring options during bioaugmentation with *Dehalococcoides* (Chap. 6).
- A thorough review of the use of bioaugmentation for treatment of chemicals other than the more common chlorinated solvents (TCE and PCE), including DCE (Chap. 7), aerobic cometabolism of chlorinated solvents (Chap. 8), carbon tetrachloride (Chap. 9), and MTBE (Chap. 10).
- An analysis of cost considerations needed to evaluate whether bioaugmentation should be considered for the treatment of chlorinated aliphatic compounds in groundwater (Chap. 11).
- An assessment of important unknowns and uncertainties that impact the state-of-the-science that underpins bioaugmentation development and implementation. This chapter, written in consultation with a broad range of experts in the remediation field, should help guide the research agenda on bioaugmentation (Chap. 12).

Each chapter in this volume has been thoroughly reviewed for technical content by two or more experts in each subject area covered. The editors and chapter authors have produced a well-written and up-to-date treatise that we hope will prove to be a useful reference for those making decisions on remediation of contaminated sites, for remediation practitioners, and for those involved in development of advanced technology for the *in situ* remediation of contaminated groundwater.

SERDP and ESTCP are committed to the development of new and innovative technologies to reduce the cost of remediation of soil, groundwater, and sediment contamination as a result of past operational and industrial practices. We are also firmly committed to the widest dissemination of these technologies to ensure that our investments continue to yield savings for not only the DoD, but also the nation. In sponsoring this monograph series, we hope to provide the broader remediation community with the most current knowledge and tools available in order to bring these technologies to bear on the remediation of contaminated sites.

Jeffrey A. Marqusee, PhD, Executive Director, SERDP and ESTCP
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About the Editors

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Dr. Stroo is a Principal Technical Advisor with HydroGeoLogic, Inc. He provides technical support on large remediation projects for private- and public-sector clients and has served as a technical advisor to the Strategic Environmental Research and Development Program (SERDP) and the Environmental Security Technology Certification Program (ESTCP) for over 10 years.

Dr. Stroo received his BS degrees in Biology and Soil Science from Oregon State University, his MS in Soil Science from West Virginia University, and his PhD in Soil Science (Soil Microbiology) from Cornell University.

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Dr. Stroo has served on several expert review panels for SERDP, other government agencies, and private companies. Recently, he served as co-chair of the SERDP workshops on Remediation of Chlorinated Solvents in Groundwater and Remediation of Dense Nonaqueous Phase Liquid (DNAPL) Source Zones. He is coeditor of the SERDP-facilitated monographs, *In Situ Bioremediation of Perchlorate in Groundwater* and *In Situ Remediation of Chlorinated Solvent Plumes*.

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Dr. Leeson served as chairperson of the Fourth, Fifth, and Sixth International *In Situ* and On-Site Bioremediation Symposium and was the founding Managing Editor for *Bioremediation Journal* from 1996 to 1999 and the Editor-in-Chief from 1999 to 2001. She has developed and implemented several expert panel workshops on topics ranging from contaminant bioavailability to chlorinated solvent-contaminated groundwaters.

C. Herb Ward

Dr. Ward holds the Foyt Family Chair of Engineering in the George R. Brown School of Engineering at Rice University. He is also Professor of Civil and Environmental Engineering and Ecology and Evolutionary Biology.

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Dr. Ward has been a member of the USEPA Science Advisory Board and served as Chair of the SERDP Scientific Advisory Board. He is the founding and current Editor-in-Chief of the international scientific journal *Environmental Toxicology and Chemistry*.

Dr. Ward received the Frederick George Pohland Medal for Outstanding Contributions to Bridging Environmental Research, Education, and Practice and the Brown and Caldwell Lifetime Achievement Award for Remediation in 2006, the Water Environment Federation Jack Edward McKee Medal for Achievement in Groundwater Restoration in 2007, and the Society for Industrial Microbiology and Biotechnology Charles Thom Award for bioremediation research in 2011.

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Dr. Aziz specializes in assessment and remediation of industrial and military sites impacted by a broad range of contaminants, including chlorinated solvents, polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, and xylenes (BTEX) and perchlorate. She has led the development, design, and implementation of several bioremediation/bioaugmentation technologies for public and private sector clients and has developed environmental software (BIOCHLOR) distributed through the U.S. Environmental Protection Agency (USEPA).

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Mr. Cox has helped to pioneer the development of MNA and EISB technologies, including bioaugmentation, for remediation of chlorinated solvents, propulsion energetics (perchlorate, n-nitrosodimethylamine [NDMA]), and explosives (RDX, TNT) in porous media and fractured bedrock. He works with private sector interests and government research programs to develop innovative *in situ* treatment technologies and to demonstrate and validate their use at field-scale for widespread commercial use. As part of his *in situ* remediation research, development, and implementation work, he has authored over 30 professional publications and articles regarding

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Dr. Criddle began his academic career in 1989 as a faculty member at Michigan State University (MSU). While at MSU, he served as Project Director for the Schoolcraft project, a field-scale test of bioaugmentation that involved faculty from diverse disciplines and institutions, staff scientists, students, consultants and outreach education to members of the public and officials in Michigan. The National Ground Water Association (NGWA) named it one of two Outstanding Remediation Projects for 2002. Since 1998, he has been a member of the Stanford faculty, serving as Associate Chair in 2003 and as Director of a bioremediation field project at the U.S. Department of Energy (DOE) Field Research Center in Oak Ridge, Tennessee (2000–2006). This project entailed a multi-year series of experiments focused on *in situ* sequestration of uranium in groundwater and involved faculty from several universities, DOE scientists, and many students. Dr. Criddle has over 100 peer-refereed publications and is coauthor with award-winning artist Larry Gonick of *The Cartoon Guide to Chemistry* (2006), a widely used supplementary text for high school and freshman chemistry.

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Dr. Edwards is internationally known for her work on anaerobic bioremediation, the application of molecular biology and metagenomics to uncover novel microbial processes, and the transition of laboratory research into commercial practice to develop bioremediation and bioaugmentation strategies for groundwater pollutants. Dr. Edwards and Geosyntec Consultants were recognized with the 2009 National Science and Engineering Research Council of Canada Synergy Award for their long-standing partnership that led to the development of KB-1®, a successful commercial microbial bioaugmentation culture. She has been inducted into the Canadian Academy of Engineering and is a Fellow of the American Association for the Advancement of Science.

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Dr. Koenigsberg was a Founder of Regenesis, where he coinvented or managed the development of the company's major products including ORC®, HRC®, MRC® and Regenox®. Also during his tenure, Regenesis produced one of the first commercial *Dehalococcoides* products (BDI®) and associated deoxyribonucleic acid (DNA) probes (Census℠) through the work of Dr. Frank Löffler at the Georgia Institute of Technology. Upon retiring from Regenesis, Dr. Koenigsberg was a Partner at WSP and a Principal at ENVIRON. In 2010, he received a Lifetime Achievement Award from the Association for Environmental Health & Sciences (AEHS) Foundation. He is a member of several editorial and advisory boards and is an Adjunct Professor at California State University at Fullerton where he also serves on the Dean's Advisory Council.

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CHAPTER 1

BIOAUGMENTATION FOR GROUNDWATER REMEDIATION: AN OVERVIEW

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1.1 INTRODUCTION

1.1.1 Background: The Pollution Problem

As industry has increased over the ages, so has human impact on the environment, especially with the advent of the Industrial Revolution. This period has been marked by the introduction of xenobiotic compounds. These were originally defined by Leisinger (1983) as "guest" chemicals that are not natural to the environment, or anthropogenic ("man-made") compounds whose structure is relatively new and foreign to microbes that are otherwise very capable of degrading organic waste (Leisinger, 1983; Timmis et al., 1994). However, recent work suggests many if not most of these xenobiotics also have natural origins as well (Gribble, 1998; Keppler et al., 2002). Nevertheless, many anthropogenic compounds can present a difficult challenge for the environment, as natural systems are not adapted for rapid degradation of these compounds, which often have unusual chemical bonds or halogen substitutions.

For the majority of the past 200 years, treatment and disposal of industrial waste was not a priority, as exemplified by the dumping of waste into the ground or rivers with the idea that "dilution is the solution to pollution." Only in the latter half of the twentieth century was concern over the fate of the environment brought to the forefront. Even so, it was not until the publishing of Rachel Carson's *Silent Spring* in 1962 and public outcry over incidents such as Love Canal in 1978 (Beck, 1979) that environmental pollution was concretely linked with human health, leading in part to the establishment of the Environmental Protection Agency in the United States (USEPA) in 1970 and the ensuing environmental protection acts, such as the Superfund Program established in 1980 (http://www.epa.gov/aboutepa/history/topics/index.html; accessed June 18, 2012). As of 2012, there are around 1,300 Superfund sites in the United States that contain various inorganic and organic contaminants (http://www.epa.gov/superfund/sites/npl/index.htm; accessed June 18, 2012), and there are still hundreds of thousands of contaminated sites requiring cleanup (USEPA, 2004).

The problem of environmental pollution spans the globe and insidiously affects human and environmental health. Many countries that have adopted modern industrial processes have discovered the legacy of polluted environments. Nations that are rapidly increasing in either population or chemical use, such as India and China, realize that their natural resources cannot support the burden of uncontrolled chemical disposal. While pollution prevention and sustainable development measures are preferred, in many cases, the damage has already been done. Remediation offers the chance to reduce pollutant levels. There are numerous proposed remediation technologies, incorporating chemical, physical and biological processes.

Despite the availability of so many options, a good remediation strategy that is effective, efficient and economical can be elusive. To this end, there are a number of tools available online

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to aid in the decision-making process such as the Decision Support Tools (http://www.frtr.gov/decisionsupport/; accessed June 18, 2012) and the Hazardous Waste Clean-Up Information site CLU-IN (http://clu-in.org/; accessed June 18, 2012). These software enable users to weigh the various remediation options against the characteristics of their specific site and pollutants. One of the technologies available for remediation is bioaugmentation (a specific type of strategy used in implementing bioremediation). The appropriate application and control of this technology is the subject of this volume.

1.1.2 Definitions: General Bioremediation Terminology

Due in part to the relatively low cost of biological processes, bioremediation is an increasingly popular approach to remediation. Bioremediation is the use of organisms (usually microorganisms) to clean up contaminated sites by degradation (breaking carbon bonds) or transformation (changing the bond structure or redox state) of pollutants to produce nontoxic compounds. As of 2009, bioremediation was the most common technology used to remediate polluted soils and groundwater (Figure 1.1).

For simplicity, the term "degradation" will be used in this text to refer to both processes, unless specifically stated. There are several classes of bioremediation technologies available, such as monitored natural attenuation (MNA, which often relies heavily on natural biodegradation), biostimulation, phytoremediation and bioaugmentation. The phrase "natural attenuation" refers to the intrinsic capacity of the environment to degrade or transform a contaminant within a reasonable timeframe. In the United States, all possible processes can participate. In Europe, most countries require demonstration of biological processes. As a remediation strategy, MNA involves no overt action on the part of the remediator, but it should be monitored to ensure that the degradation is proceeding in a timely fashion with no undesired metabolites. Biostimulation is the next step up from natural attenuation, wherein physical and/or chemical treatment enhances the natural biodegradation (e.g., oxygen added to maintain aerobic processes). This strategy requires careful calculation and extensive knowledge of the polluted site on the part of the practitioner to choose the right stimulation for the organisms that already exist at the site. In certain cases, the organisms that can degrade a target pollutant either do not exist at the site or are not present in sufficient numbers for a "timely" treatment, and that is where bioaugmentation might provide an advantage over the other bioremediation strategies.

Bioaugmentation is the addition of biocatalysts (generally bacteria, but it also could involve the addition of fungi, genes or enzymes) to degrade target pollutants, either *in situ* or *ex situ*. In most commercial applications, bioaugmentation involves the addition of mixed cultures of bacteria that have been derived from natural environments and demonstrated to be capable of rapid biodegradation of problematic contaminants. In rare cases, additions of genetically-engineered microorganisms (GEMs) also have been tested, but GEMs have yet to be

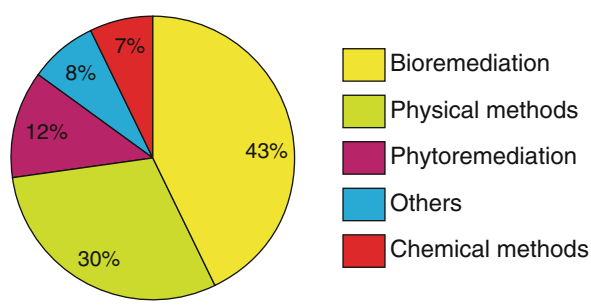

Figure 1.1. The use of common remediation methods by percentage as of 2009 (adapted from Pandey et al., 2009).

commercially-successful augmentation agents. Bioaugmentation also could be beneficial when a mixture of pollutants must be degraded by a mixture of specific bacteria. It does not refer to the addition of plant species (phytoremediation), although the two techniques can work well together as exemplified by rhizoremediation, which is discussed later.

1.1.3 Chapter Overview

This chapter aims to establish the fundamentals of bioaugmentation, from which the reader can then put into context the remainder of this volume. This volume focuses on the use of bioaugmentation for chlorinated solvent remediation in groundwater, but its uses are not limited to these compounds. We will discuss the history, status and prospects for bioaugmentation in environmental remediation in general, focusing on the key issues that influence the practice and potential for the technology to improve the effectiveness and/or reduce the costs for *in situ* bioremediation. It is intended to serve as an introduction to the remaining chapters and an overview of the technology for the general audience. The reader will be referred to different chapters for further elaboration on the ideas and concepts presented.

1.2 DEVELOPMENT OF BIOAUGMENTATION FOR GROUNDWATER BIOREMEDIATION

1.2.1 Historical Development of Bioaugmentation

The idea of adding microbes to perform reactions is an ancient technology, such as the use of microbial inocula to make fermented beverages like beer and wine, and dairy products such as cheese and yogurt (Singer et al., 2005). Bioaugmentation also has been used more recently in agriculture, with the addition of nitrogen-fixing bacteria to rhizospheres and the manipulation of bacteria to encourage plant growth, control pathogens and improve soil structure (van Veen et al., 1997; Gentry et al., 2004). Bioaugmentation for pollutant removal evolved from earlier bioremediation efforts, which focused on eliminating physical and chemical barriers to the degradation of the targeted pollutant(s) by indigenous microorganisms. The largest initial biostimulation successes were most often those associated with straightforward removal of environmental limitations (such as the lack of oxygen) and relied on the presence of large numbers of native microorganisms capable of degrading the targeted compound(s). For example, the treatment of petroleum hydrocarbons, such as those found in gasoline and diesel, often used pumping techniques to circulate oxygen and other nutrients through the subsurface where the indigenous bacteria were capable of degrading the contaminants (e.g., the Raymond Process) (Raymond, 1976).

The concept of adding bacteria to polluted media stems from the use of bacteria in compost piles and septic tanks, such as when bacteria were used in the early 1980s to target the degradation of pollutants in wastewater systems (Goulding et al., 1988). Bioaugmentation for treating contaminated soils and groundwater was initially considered in the 1980s and early 1990s, with the growing acceptance of bioremediation to treat petroleum hydrocarbons and wood preserving wastes. The increasing use and perceived deficiencies of *in situ* bioremediation led to a proliferation of vendors offering microbial inoculants to improve groundwater and soil bioremediation. By 1992, there were at least 75 bioaugmentation cultures available commercially for *in situ* bioremediation.

Most of these inoculants were composed of common soil microorganisms grown under aerobic conditions, and there was generally little characterization of these microbial cultures.

The majority of the inocula were for treating fuel hydrocarbons and/or polycyclic aromatic hydrocarbons (PAHs), but roughly 10% claimed the ability to treat halogenated aliphatic compounds (Major and Cox, 1992). Bioaugmentation cultures for hydrocarbon degradation were tested in several well-monitored studies, including controlled field trials following the Exxon Valdez oil spill of 1989. In most cases, bioaugmentation inocula had little effect on the rate or extent of removal of fuel hydrocarbons (Tagger et al., 1983; Lee and Levy, 1987; Venosa et al., 1996). Numerous studies demonstrated that populations of oil-degrading bacteria in soil and water increase in the presence of oil (Lee and Levy, 1987; Button et al., 1992; Atlas, 1993; Prince, 1993), and results from field trials of bioaugmentation were generally no better than biostimulation alone (Atlas and Bartha, 1972; Swannell and Head, 1994).

In many cases, the effectiveness of commercial bioaugmentation cultures has been difficult to assess. Complete biodegradation pathways often were not understood or documented, and few controlled field trials were performed. Many doubted the ability of the added microbes to thrive, or even survive, long enough to degrade the contaminants (Goldstein et al., 1985). In addition, drastic changes in the ecosystem (e.g., aerobic to anaerobic) also slowed the microbial community transition and adaptation to the targeted pollutant(s). The prevailing ecological theory was that the microbial strains present at a site were those that were best suited to their niche, so the natural communities would remain stable even when subjected to moderate levels of biotic or abiotic stress (Suflita et al., 1989). Furthermore, the general consensus in the early 1990s was that the genetic potential to degrade most if not all contaminants already existed in the environment and could be expressed by manipulation of environmental conditions.

1.2.2 Recent Developments: Bioaugmentation with *Dehalococcoides* for Reductive Dehalogenation of Chlorinated Ethenes

Due to these early disappointments, developments in the area of bioaugmentation were met with skepticism, and there was relatively little research interest until the chlorinated ethene pollution problem was recognized in the late 1990s. The bioremediation of chlorinated ethenes often had been unsuccessful using conventional bioremediation techniques. Few indigenous organisms were capable of complete degradation, with long lag times and incomplete treatment (e.g., the "*cis*-1,2-dichloroethene [*cis*-DCE] stall") being typical. Reductive dechlorination of perchloroethene (PCE; also termed perchloroethylene or tetrachloroethylene) and trichloroethene (TCE) was recognized as early as 1983 (Bouwer and McCarty, 1983). The observation that highly chlorinated compounds were degraded under anaerobic conditions (Vogel et al., 1987; Mohn and Tiedje, 1990) led to an increase in the stimulation of anaerobic conditions *in situ* for the degradation of these compounds, although the identity of the responsible organisms was not known. Research demonstrated that each subsequent reductive dechlorination step was slower than the preceding one, often resulting in the accumulation of vinyl chloride (VC), with VC being a carcinogenic gas more hazardous than the more chlorinated compounds.

As a result, researchers temporarily abandoned the idea of anaerobic biodegradation of PCE and TCE, and for several years, research focused on the use of aerobic cometabolic biodegradation of these compounds (Fogel et al., 1986; Little et al., 1988; Oldenhuis et al., 1989). However, cometabolic biodegradation proved difficult to implement successfully. In general, the ineffective treatment of chlorinated compounds was due, in some cases, to the time needed for growth of the competent microorganisms to sufficient numbers (Morse et al., 1998; Ellis et al., 2000). In other cases, competent microorganisms did not exist at the cleanup sites, and this is where bioaugmentation normally proves its advantage.

Vinyl chloride was finally shown to be completely reduced to ethene by a unique group of organisms (*Dehalococcoides* spp.) (Freedman and Gossett, 1989). In the case of chlorinated solvents, two bacterial groups (*Dehalococcoides* and *Dehalobacter*) have been relatively well studied in the laboratory, and in the case of *Dehalococcoides*, its presence has been correlated with the ability of the "natural" microbiota to completely degrade chlorinated solvents (Hendrickson et al., 2002). This breakthrough and subsequent research that further revealed the unique capabilities of this group of bacteria made effective bioaugmentation possible (Duhamel et al., 2002; Cupples et al., 2003; He et al., 2003). Recognition that complete dechlorination was achievable even at concentrations near the solubility limit for the most highly chlorinated compounds led to even greater interest in this process and the potential for using bioaugmentation to enhance *in situ* bioremediation of chlorinated solvents (DiStefano et al., 1991; Adamson et al., 2003; Stroo et al., 2010).

As this volume shows, bioaugmentation has become a more accepted and successful technique in recent years mainly due to the success with chlorinated solvents. Bioaugmentation with *Dehalococcoides* spp. to remediate chlorinated solvents has become a viable commercial practice and has been used at several hundred sites (Figure 1.2). In addition, bioaugmentation with aerobic bacteria capable of cometabolically degrading chloroethenes has been used at approximately 150 chlorinated solvent sites (personal communication, Michael Saul, CL-Solutions, Inc., March 24, 2010). Bioaugmentation with aerobic cometabolic bacteria is not discussed at length in this volume, largely because of the lack of peer-reviewed literature describing demonstrations of the process. However, there is growing commercial use and information on case studies (www.cl-solutions.com; accessed June 18, 2012).

In the case of *Dehalococcoides* bioaugmentation, it is well-documented that it can improve bioremediation performance by increasing the rate of biological treatment and decreasing the

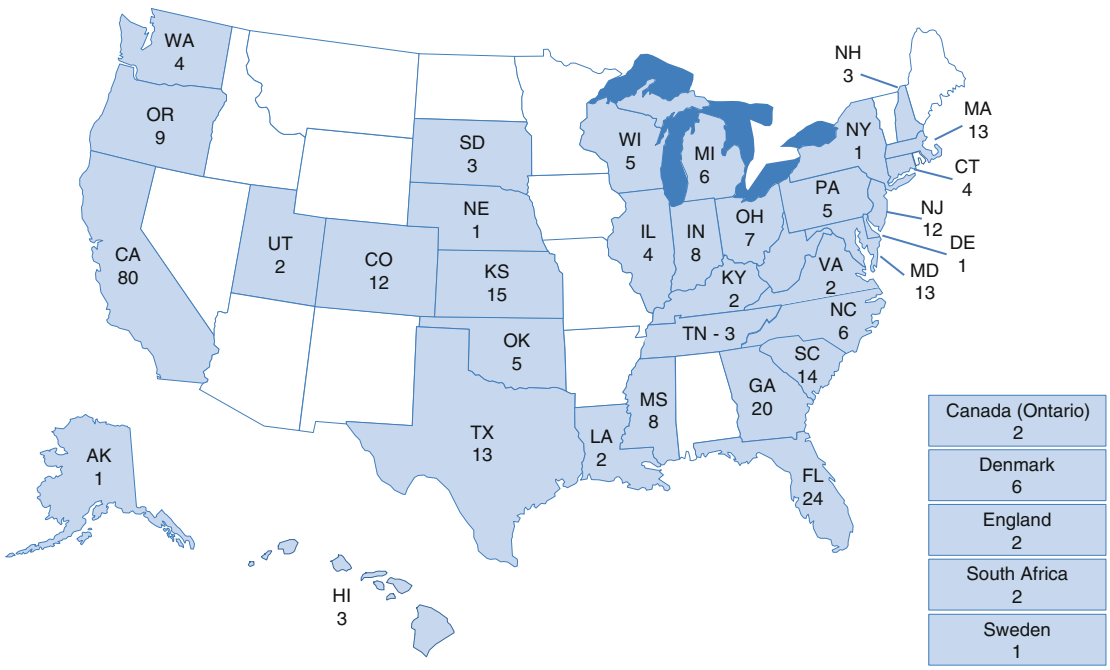

Figure 1.2. Recent census of bioaugmentation applications using *Dehalococcoides* spp. for site cleanup. Figure based on information provided in 2009 by R. J. Steffan (Shaw Environmental & Infrastructure, Inc., Lawrenceville, NJ), R. L. Raymond, Jr. (Terra Systems, Inc., Wilmington, DE) and P. C. Dennis (SiREM, Guelph, Ontario, Canada).

time before the onset of complete dechlorination (ESTCP, 2005; Lendvay et al., 2003; Major et al., 2002; Maes et al., 2006; Hood et al., 2008). The use of *Dehalococcoides* spp. for bioaugmentation is discussed later in this chapter and is a primary focus of this volume. This technique represents a remarkable success story for bioaugmentation, partly because it is based on a rare combination of circumstances: (1) the limited distribution, abundance or capacity of organisms mediating complete dehalorespiration; (2) the widespread problem of groundwater contamination with halogenated solvents, especially the chlorinated ethenes; and (3) the ability of dehalorespiring organisms to survive and colonize the subsurface after additions of fermentable substrates and establishment of anaerobic conditions.

1.3 TYPES OF BIOAUGMENTATION

Once the decision is made to use bioaugmentation, there are several variations available, as summarized in Figure 1.3. The choice of strategy depends on the site parameters and the pollutant of interest. Bioaugmentation also can be used in combination with other remediation strategies. The following sections discuss the benefits and drawbacks of the various types of bioaugmentation. The types of bioaugmentation are divided into two categories: those that are currently practiced and those that are still in the experimental stages. Current bioaugmentation practices are discussed briefly below and in detail in other chapters in this volume. Potential future bioaugmentation strategies also are discussed.

1.3.1 Currently Practiced Methods

Most of the commercial bioaugmentation that is currently practiced relies on the application of microorganisms, or those options in the cell bioaugmentation category. The variations discussed in this section represent only some of the potential bioaugmentation technologies.

1.3.1.1 Preadapted Bacterial Strains or Consortia

Among the more successful bioaugmentation techniques has been the use of preadapted bacterial strains or consortia. These strains can be isolated or enriched from other contaminated sites. However, if the site of interest already has the capacity to degrade the pollutant, though not at

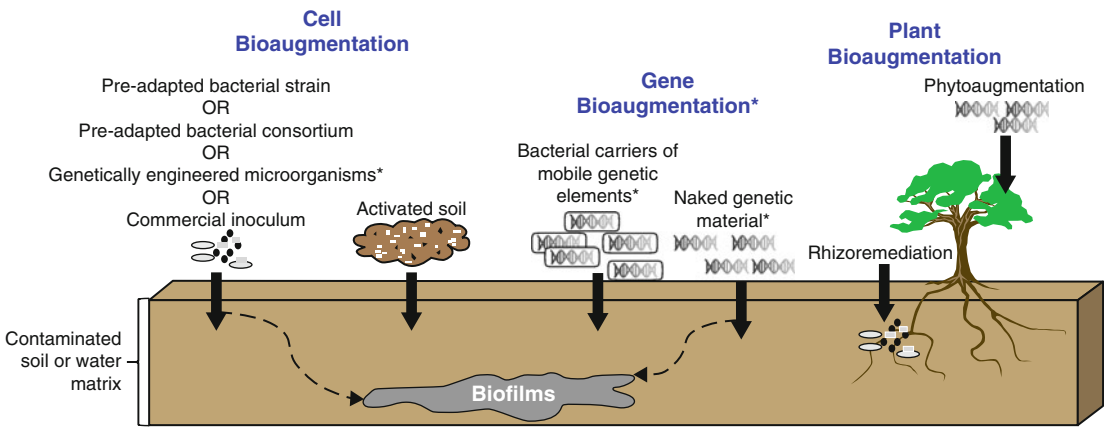

Figure 1.3. Summary of different bioaugmentation methods. * denotes methods that are yet to be commercially practiced.

a satisfactory rate, then the site organisms may be preadapted or enriched for use as an inoculum. Presumably, the microorganisms that exist at that site are already accustomed to the temperature, pH and nutrient availability, and are therefore better suited for use at that site (Bento et al., 2005). However, if there are no existing strains at the site that degrade the pollutant, or if the numbers of indigenous degraders are low, or if there are multiple pollutants that must be degraded sequentially, then it might be necessary to use a "foreign" inoculum, like an enrichment from a different site or a commercial inoculum. For example, bioaugmentation with *Dehalococcoides* is common at chlorinated ethene sites where indigenous degraders often are present but at very low numbers.

Bacteria in the environment often form relationships with other bacteria in the system – whether commensal or predatory. A consortium of bacteria often performs better as an inoculum since the bacteria are already with a community of other bacteria that synergistically support the activity of interest, namely pollutant degradation. For example, addition of a consortium capable of PAH degradation resulted in more extensive degradation than any of the strains individually (Jacques et al., 2008). Similar results have been reported for petroleum hydrocarbons (Richard and Vogel, 1999). The bacteria do not need to be extracted and enriched; the soil itself can be exposed to the contaminant and enriched for degradation to give an inoculum called "activated soil" (Otte et al., 1994; Barbeau et al., 1997). The benefit of activated soil is that it develops a consortium in the soil itself, thus negating the use of artificial media and the biases that introduces.

1.3.1.2 Commercial Inocula

There are a number of commercially available inocula that target different pollutants (Table 1.1). These inocula can be delivered by several methods including injection, mixing, relying on bacterial chemotaxis, from a reactor on the surface or as a spray. The success of these inocula depends partially on the application method and the strains therein, but it mainly depends on the chemical and biological characteristics of the polluted site. In groundwater applications, the focus of this volume, inocula are typically delivered via injection wells or direct injection equipment such as Geoprobe© systems.

1.3.1.3 Bioaugmentation in Combination with Plants and Phytoaugmentation

Plants are already used in bioremediation in a process called phytoremediation, in which plants either degrade pollutants (directly or indirectly through plant-associated bacteria), volatilize or accumulate pollutants (Suresh and Ravishankar, 2004; Kramer, 2005). This technique has been tested in a number of field studies (Vangronsveld et al., 2009; van Aken and Geiger, 2011). Plants have the advantages of roots that reach into the subsurface forming a system called the rhizosphere, and they have wide seed distribution capacities. Plants naturally take up heavy metal pollutants through their roots during growth (Padmavathiamma and Li, 2007). To expand on their intrinsic capabilities, genetic modification has been widely considered, although rarely applied (Cherian and Oliveira, 2005).

The relationship between plants and bacteria can be manipulated to encourage pollutant degradation. Plant growth promoting rhizobacteria (PGPR), reviewed recently by Zhuang et al. (2007), colonize the rhizosphere in either a symbiotic or free-living manner. They increase plant growth by producing growth stimulating compounds, preventing disease and increasing nutrient uptake. PGPR in combination with the plants are able to sequester metals more efficiently than either plants or bacteria alone. Rhizoremediation uses plants to help support bacterial growth during remediation (Kuiper et al., 2004; Cases and de Lorenzo, 2005). In recent trials,

Table 1.1. Examples of Commercially Available Bioaugmentation Inocula[a]

Manufacturer (website)	Product Name
Chlorinated volatile organic compounds	
BCI Labs (www.bcilabs.com)	BCI-e, -a, -t
BioRenova (www.biorenova.us)	Chloroclean Inoculum
CL-Solutions (www.cl-solutions.com)	CL-Out®
EOS Remediation (www.eosremediation.com)	ENV-TCA20™, PJKS-1™, BAC-9™
Osprey Biotechnics (www.ospreybiotechnics.com)	Munox® XL Plus-6
Regenesis (www.regenesis.com)	Bio-Dechlor INOCULUM© Plus (BDI)
The Shaw Group, Inc. (www.shawgrp.com)	Shaw Dechlorinating Culture - SDC-9™
SiREM (www.siremlab.com)	KB-1®, KB-1® Plus
Fuel hydrocarbons	
BioWorld (www.adbio.com)	BioWorld Bioremediation
CL-Solutions (www.cl-solutions.com)	Petrox™
Environmental Restoration Services, LLC (www.environmentalrestorationservices.com)	System E.T.20
Fluid Tech Inc (www.fluid-tech-inc.com)	Pristine Sea II
Oppenheimer Biotechnology (www.obio.com)	Oppenheimer Formula
Osprey Biotechnics (www.ospreybiotechnics.com)	Munox® XL Plus-1, Plus-2, Plus-5,
QM Environmental Services, Ltd (www.qmes.nl)	Microcat®-HX, -PR, -XRC
Sarva Bio Remed, LLC (www.sarvabioremed.com)	SpillRemed (Marine)®, SpillRemed (Industrial)®, AgroRemed®, BilgeRemed®, HydroRemed®,
SpillAway (www.spillaway.co.uk)	BioW™, OWS-200™, NavalKleen II™, NavalKleen SCF™, HC-300™, Liquid/Dry Remediact™
PAHs	
FMC Corp. (previously Adventus) (http://environmental.fmc.com/)	DARAMEND®
Osprey Biotechnics (www.ospreybiotechnics.com)	Munox® XL Plus-5
Benzene, toluene, ethylbenzene and total xylenes	
Sarva Bio Remed, LLC (www.sarvabioremed.com)	HydroRemed®
SpillAway (www.spillaway.co.uk)	HC-200™
Methyl tertiary butyl ether	
BioWorld (www.bioworldusa.com)	BioWorld Bioremediation
EOS Remediation (www.eosremediation.com)	ENV735™, ENV736™
Heavy metals	
FMC Corp. (previously Adventus) (http://environmental.fmc.com/)	EHC®-M
Biomedy (www.biomedy.com)	BioLeach
Planteco Environmental Consultants, LLC (www.planteco.com)	MMATs®
Grease/fats	
QM Environmental Services, Ltd (www.qmes.nl)	Microcat®-AD, -DNT-RF
SpillAway (www.spillaway.co.uk)	GTO™, SEP-700™

[a]All web sites accessed 30 May 2012

plant root exudates encouraged microbial growth, leading to better bioremediation by those bacteria (Gentry et al., 2004; Kuiper et al., 2004). In return, microbial products such as surfactants and siderophores can enhance metal mobility and plant uptake (Zaidi et al., 2006; Lebeau et al., 2008).

Phytoaugmentation is the addition of bacterial genes into plants to confer degradation capacities (Gentry et al., 2004). These transgenic plants offer the benefits of phytoremediation, such as an extensive root system that can process large amounts of pollutant and the ability to sequester pollutants. They also can degrade compounds more thoroughly than non-modified plants. While this technology has not yet been marketed, there are a wide variety of pollutants that can be targeted (Abhilash et al., 2009; Sylvestre et al., 2009; Van Aken, 2009).

1.3.2 Potential Bioaugmentation Strategies

Bioaugmentation is a rapidly developing field of study, as evidenced by the growing number of publications over the last decade (Figure 1.4). Many of these publications focus on the future of bioaugmentation and what new techniques can be used to improve bioaugmentation success. Some astounding and promising discoveries have been made, especially with the rapid progress in molecular biology capabilities and the genetic manipulation of microorganisms. Some of the bioaugmentation methods that have been proposed but not yet widely implemented in the field are discussed below.

1.3.2.1 Genetically Engineered Microorganisms

In the event that an appropriate pollutant-degrading strain does not exist or results in toxic or dead-end metabolites, there is the option of adding GEMs, in which genes are either introduced into a host microbe or existing genes in a bacterium are altered (Garbisu and Alkorta, 1999; Sayler and Ripp, 2000; Gentry et al., 2004; Khomenkov et al., 2008).

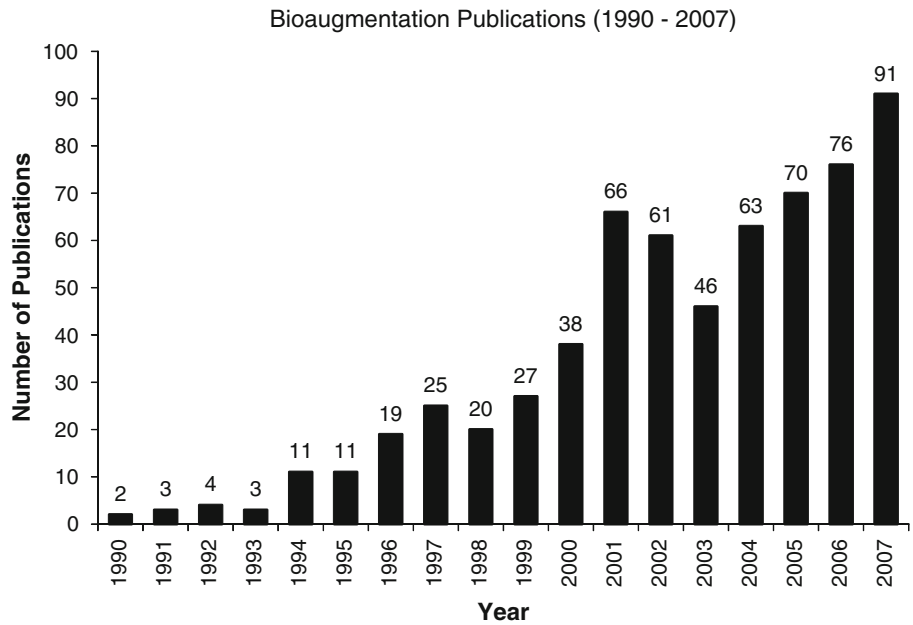

Figure 1.4. Number of international bioaugmentation publications (1991–2007) (adapted from the Science Citation Index).

The techniques used to create GEMs have been discussed by Sayler and Ripp (2000) and Cases and de Lorenzo (2005). GEMs should be particularly useful for xenobiotics that have only recently appeared in the environment and compounds for which no degradation pathways have been established – such as those with multiple double bonds, aromatic structures or with multiple halogen substitutions, like polychlorinated biphenyls (PCBs) – or for compounds that require multiple degradation steps (Khomenkov et al., 2008). GEMs can be optimized to have high degradation activity. For example, the genetic elements that control the level of gene expression, like the transcriptional promoter and terminator sequences, can be designed to over-express the degradation genes. A similar result may be obtained by changing the number of copies of the gene. Monitoring the location and spread of GEMs assists with both determining the success of bioaugmentation and controlling the release of GEMs. To this end, luminescent tags and other methods of tracking have been implemented (Valdman et al., 2004).

The proposed application of GEMs is subject to some of the same public concerns as other genetically modified organisms (GMOs), such as the unmitigated spread of the organisms, transfer of genetic material and disruption of the natural flora (Kappeli and Auberson, 1997; Davison, 2005). There are a number of ways to control the spread of GEMs and their genetic material, but the most common is the use of molecular methods (Davison, 2005). The horizontal transfer of antibiotic resistance genes can be eliminated by avoiding the use of antibacterial resistance as a selection marker during strain construction. Another partial solution to prevent the genes from transferring to other organisms would be to avoid the use of plasmids and maintain the genes on the chromosome, although this is not a fail-proof solution (Gentry et al., 2004). One control strategy, which has been implemented with GMOs, is the use of suicide elements to biologically contain the organisms to the site and the application, as illustrated in Figure 1.5 (Contreras et al., 1991; Davison, 2005). In this system, a control element, which could be modulated by the user, would target a killing element that would induce cell death.

While it is unlikely that any control measure to prevent GEMs from spreading will achieve complete control, the possible benefits of GEMs for bioremediation should be weighed against the risks. Other than contamination of industrial systems, it is unlikely that a true health risk would evolve from the application of GEMs for pollutant degradation (Urgun-Demirtas et al., 2006). A recent review examined regulation of the use of GMOs in the United States,

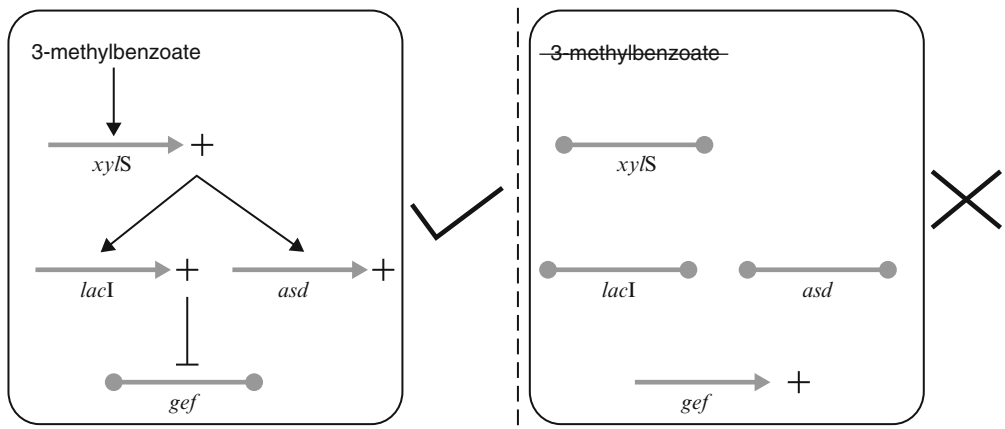

Figure 1.5. Example of a control strategy for GEMs (adapted from Davison, 2005). When the pollutant of interest, 3-methylbenzoate, is present, it activates *xylS*, which then positively activates the transcription of the *asd* gene (for the essential diaminopimellic acid) and *lacI* gene. *LacI* represses the transcription of a toxin, *gef*. If the substrate of interest is not present, *xylS* is not activated, and the cell dies from lack of diaminopimellic acid and *gef* toxin production.

illustrating the USEPA's use of regulation to arrive at a better understanding of the impacts of GMOs (Sayre and Seidler, 2005). Ideally, regulations would allow research to proceed under realistic field conditions and facilitate the use of "safe" technologies while still protecting the environment and the public. One way to sidestep this issue was suggested in a study that used killed genetically-modified *Escherichia coli* that had over-expressed atrazine chlorohydrolase to remediate a site contaminated with atrazine (Strong et al., 2000).

The success of GEMs in the field remains uncertain. Since their creation and optimization would have occurred in the laboratory under favorable and perhaps unrealistic conditions, there is always some doubt whether inoculated GEMs will be able to survive in natural environments. However, it appears that some GEMs may have specific advantages over indigenous organisms, such as tolerance for high levels of a pollutant, or simply not affected by the other microflora (Lenski, 1993; Ripp et al., 2000; Bott and Kaplan, 2002). In one field study, the bacterium *Pseudomonas fluorescens* HK44, containing a bioluminescent gene (lux) within the promoter for naphthalene catabolic genes, was used to both degrade and monitor the presence and degradation of naphthalene (Ripp et al., 2000). The hurdles encountered during this endeavor have been reviewed, and the use of GEMs in general has been discussed in recent reviews (Sayler and Ripp, 2000; Cases and de Lorenzo, 2005). In another field release, *Pseudomonas putida* W619-TCE, known to degrade TCE, was inoculated in the roots of poplar trees to reduce TCE transpiration during phytoremediation (Weyens et al., 2009). These technologies are still new and uncertain, and the regulations controlling them are expected to be revised periodically.

1.3.2.2 Gene Bioaugmentation

Bioremediation, in its most simplistic form, relies on enzymes that catalyze biodegradation. These enzymes are proteins that are coded by genes carried in the bioremediating organism. In gene bioaugmentation, the goal is to circumvent the problems inherent in sustaining inoculated organisms in the contaminated system and instead encourage the uptake of the genes themselves into the indigenous microbes.

Catabolic mobile genetic elements (MGEs) are ideal for gene bioaugmentation. MGEs are pieces of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) that can be easily transmitted between organisms and include plasmids, transposons, bacteriophage-related elements and genomic islands. Degradation genes often are found on MGEs. For example, *Dehalococcoides* strains involved in chlorinated ethene degradation can transfer reductive dehalogenation genes on MGEs, possibly phages (West et al., 2008). Two recent reviews on the topic have compiled lists of existing MGEs (Top et al., 2002; Nojiri et al., 2004).

The most likely method to accomplish gene bioaugmentation potentially is to inoculate the contaminated media with organisms carrying MGEs. These organisms could then transfer the MGE to the indigenous microbes, and the fate of the added organisms would be unrelated to the degradation of the pollutant. There are three general methods by which the inoculated strains could transfer DNA – transformation, conjugation and transduction. Bacteria in the contaminated medium that are naturally competent could incorporate extracellular DNA directly through natural transformation. Conjugation involves the direct transfer of genetic material from one cell to another, but conjugation is limited by the compatibility of the donor and receiving bacteria. Finally, transduction uses a bacteriophage (bacterial virus) to transfer genetic material between organisms.

There are several hurdles for successful gene bioaugmentation. First, the donating organisms must survive long enough to transfer the genetic material. Second, the DNA must be compatible with the accepting strains. Plasmids are one type of MGE that can be transferred by

conjugation, but they are limited by plasmid compatibility and the survival of the plasmid in the organism. MGEs that integrate into the chromosome, which also may include plasmids and transposons carried on plasmids, have a better chance of staying in the organism and being propagated. Finally, once the genes are in the host, there still remains the problem of gene expression and successful protein folding. A plasmid may have a large host range, but still have low expression of gene product (Kiesel et al., 2007).

The benefit of incorporating genes directly into indigenous microorganisms is that they are already adapted for survival in that environment and there is no need for the inoculated host bacteria to survive any longer than is necessary for gene transfer. There are a number of examples of successful plasmid transfers for degradation of pollutants in the laboratory (Top et al., 1998; Desaint et al., 2003; Bathe et al., 2005; Nancharaiah et al., 2008). However, this procedure conceivably could lead to the unmitigated spread of the gene if no control is engineered into the system. On the other hand, the genes might naturally be eliminated after the pollutant is degraded and the selective pressure for the genes is removed.

Evidence of transposons and other MGEs abound in bacterial genomes (Springael and Top, 2004; Shintani et al., 2005). The addition of specific MGEs simply accelerates the natural process of evolution (directing the content of the MGE such that there is pollutant degradation). Still, under current regulations and definitions, the use of gene bioaugmentation comes under the same rulings as GEMs. In the United States, under the USEPA's Toxic Substances Control Act (TSCA), the use of "new" microorganisms must be reported to the USEPA (USEPA, 1997). According to the Microbial Products of Biotechnology, Final Rule under TSCA Section 5 (USEPA, 1997), new microorganisms are those "created to contain genetic material from organisms in more than one taxonomic genera." Thus, different hosts of the same plasmid, even if the transfer occurred in the soil, are considered new microorganisms and would have to be reported. The European Community has similar laws, outlining the use of GMOs (EU, 2001). The USEPA's concern is the risk involved with these organisms due to "the significant likelihood of creating new combinations of traits, and the greater uncertainty regarding the effects of such microorganisms on human health and the environment." These are concerns mirrored by the public and by researchers in the field (Kappeli and Auberson, 1997; Urgun-Demirtas et al., 2006).

Clearly, the benefits of bioaugmentation can be increased by manipulating the degrading microorganisms. The key is to increase their efficacy while making them environmentally safe to use, whether by engineering programmed cell death or utilizing indigenous organisms. For both current and future bioaugmentation methods, the site characteristics and economic considerations play a major role in deciding what method will be appropriate. The following section discusses the key steps involved in making such a decision.

1.4 MAKING THE DECISION TO BIOAUGMENT

When presented with a contaminated site, a series of decisions must be made as to whether the site should be remediated and which remediation technique to use. If bioremediation is selected, practitioners then must decide whether to bioaugment. This decision is discussed more thoroughly in Chapter 4, but the general steps are summarized here. Bioremediation is one of several proven remediation technologies that include physical, chemical and biological approaches. It is important to understand that *in situ* bioremediation is not one technology, but rather a suite of related techniques for exploiting or enhancing desired biological activities. Therefore, even if bioremediation is selected, this does not imply bioaugmentation. An overview of the decision process taken before bioaugmentation is summarized in Figure 1.6.

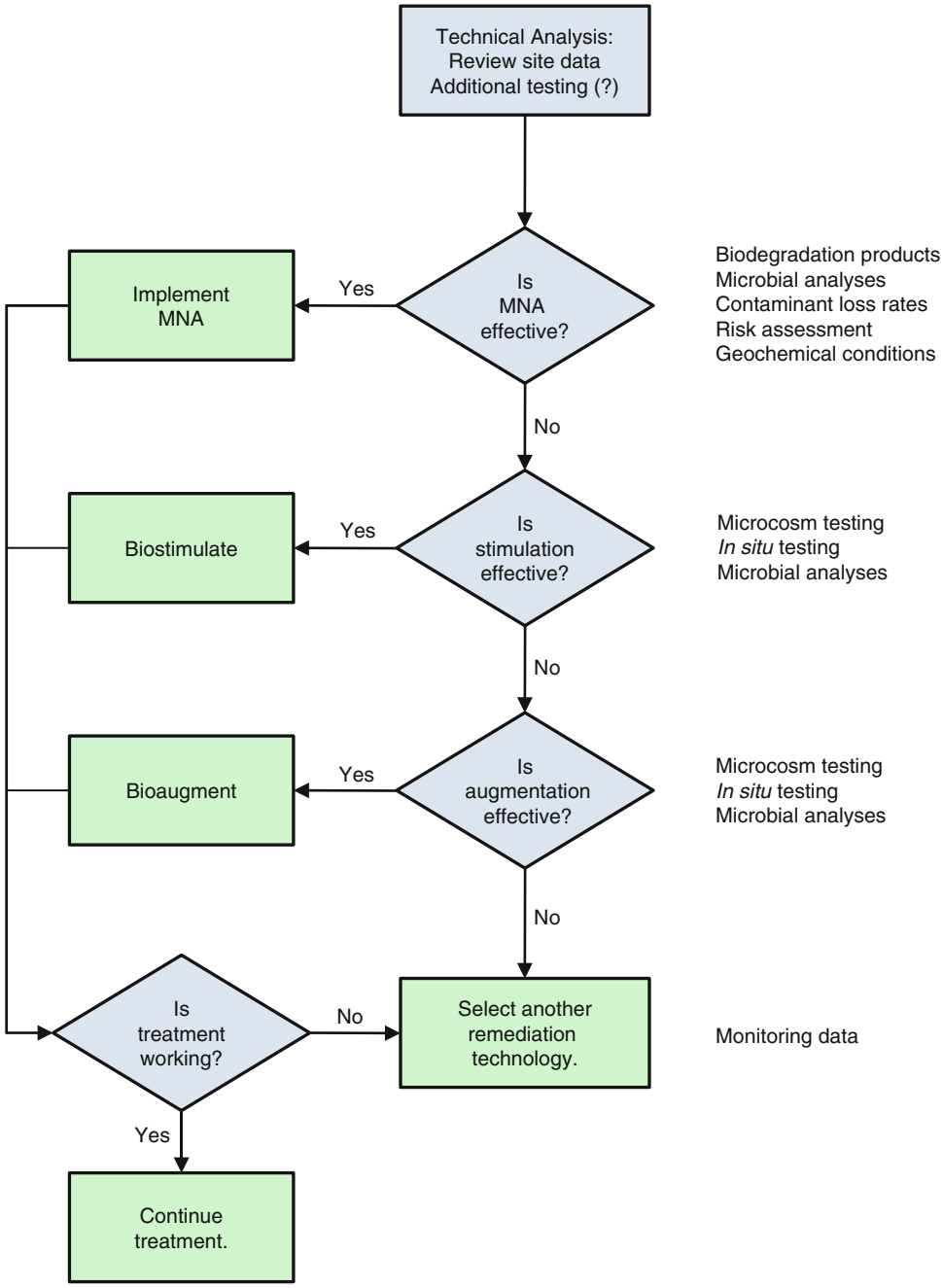

Figure 1.6. Overview of the bioaugmentation selection process. Information needs are listed along the right side of the decision flowpath.

The first bioremediation technique to be considered is MNA. The main cost for MNA comes from monitoring the pollutants, microbiota and biogeochemical conditions to ensure that degradation is proceeding in a timely and efficient manner. Due to its relative ease, MNA is a frequently selected remedy as reflected in the U.S. database of Records of Decision (RODs) (Figure 1.7).

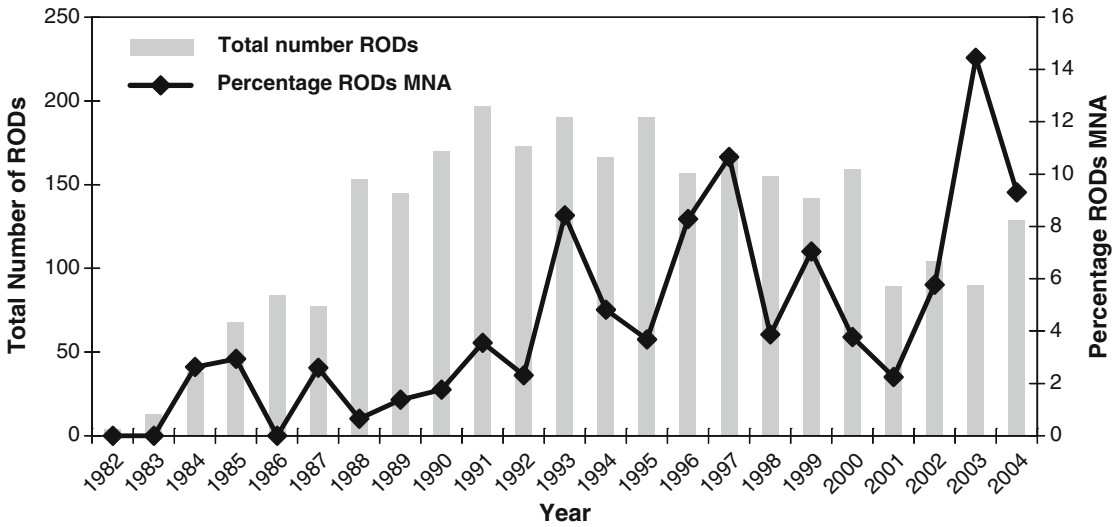

Figure 1.7. RODs for MNA at National Priorities List (NPL) sites (adapted from USEPA, 2007). The bars represent the number of RODs per year, and the line shows the percentage of those RODs that were for MNA for that year.

However, when natural attenuation processes are either nonexistent or not sufficiently protective or rapid, other more aggressive bioremediation techniques may be useful or necessary. In general, the simplest alternative is biostimulation through addition of nutrients and/or other reagents to promote the growth and activity of the desired organisms. However, if the necessary organisms are not present or are at low population levels, then bioaugmentation could provide an advantage. Often, due to cost and time issues, bioaugmentation is performed regardless of the actual degradation conditions at a site, to provide greater certainty and faster treatment. Chapter 5 of this book provides a more detailed discussion of bioaugmentation implementation in the context of chlorinated solvent degradation.

1.4.1 Technical Analysis/Site Evaluation

Proper site evaluation provides valuable information for any remediation strategy. The first step is a thorough analysis of the site to be remediated, with an eye for whether bioaugmentation is necessary and for any factors that would hinder degradation. Table 1.2 describes some of the factors that should be monitored, and the review by van Veen et al. (1997) details factors that inhibit inoculum survival. A good site evaluation can determine whether or not bioaugmentation will be successful, and thus save the responsible party both time and money.

Physical and chemical factors, such as pH, temperature, soil type, humidity, pollutant location and nutrient availability, play crucial roles in the success of bioaugmentation. While these factors can hinder any remediation strategy if out of the acceptable range, bioaugmentation is particularly susceptible to environmental conditions since living organisms are being injected *in situ*. The failure of bioaugmentation often has been tied to field scenarios that were not accurately mimicked by preliminary soil microcosms. Pollutant location also can limit the success of bioaugmentation. For example, if the pollutant is located deep in bedrock – like some dense nonaqueous phase liquids (DNAPLs) – then bioaugmentation is difficult because injections of organisms and amendments can be problematic. Some soil types might make it difficult for the bacteria to adhere (McGechan and Lewis, 2002). If there is more than one target pollutant, the use of different remediation strategies as well as multiple or sequential

Table 1.2. Environmental Factors Influencing Bioaugmentation Success

Type	Factor	Repercussion
Physical	Temperature	Affects inoculum growth rate
	Type of medium	Controls difficulty of inoculum injection
	Humidity	Affects inoculum growth rate, survival
Chemical	pH	Affects inoculum growth rate, survival
	Substrate availability	Controls degradation rate
	Nutrient availability	Affects inoculum growth rate, survival
	Competing e-acceptors	Affects inoculum growth rate, survival
	Other pollutants/toxins	May require more than one inoculum, remediation strategy
Biological	Competition	Affects inoculum growth rate
	Predation	Affects inoculum survival

bioaugmentations may be necessary. In some cases, the removal of one pollutant could enhance the natural attenuation of the remaining pollutants.

A different remediation strategy might be needed in the face of strongly unfavorable site conditions, such as multiple contaminants or extreme climate and pH. Such conditions are often the cause of MNA failure and the reason that practitioners turn to biostimulation and bioaugmentation. Unfortunately, bioaugmentation may not necessarily be an improvement over MNA – the site parameters might simply be unfavorable for bioremediation. In any case, each site needs to be carefully examined, as what works at one site is not guaranteed to work at another. Mixed pollutants can be treated with multiple inocula or multiple remediation strategies. High pollutant levels might require a more robust inoculum that is able to tolerate conditions that might kill other microorganisms. It has been noted that dechlorinating microbes are able to tolerate the high chlorinated solvent levels near a nonaqueous phase liquid (NAPL) source zone, and the microbes could even aid dissolution of the NAPL (Amos et al., 2008). There is also the possibility of using bacteria adapted to low pH or low temperatures, like psychrophiles (Margesin, 2007). However, commercial use of these bacteria would require considerable further research and possible genetic modification.

1.4.2 Select and Test Bioaugmentation Strategy

Once the site has been vetted and bioaugmentation is still deemed a feasible remediation strategy, then the type of bioaugmentation must be chosen. The previous section discussed a number of bioaugmentation possibilities. The type of pollutant and the site parameters will help determine the bioaugmentation strategy. For example, if the site is cocontaminated with metals but near a residential area, rhizoremediation might be appealing both for the efficacy of plants to accumulate metals and for the aesthetic appeal. Currently, preadapted microbial strains or commercial organisms have been used in field studies or at actual remediation sites. These commercial bioaugmentation inocula are being more thoroughly tested, due to past commercial products failing to meet their promised performance (Simon et al., 2004; Mathew et al., 2006; Brooksbank et al., 2007).

Even when using a commercial organism, it is generally preferable to test all methods in microcosms prior to use in the field, although this is infrequently practiced. Although there is

no guarantee that the method will work in the field, prior testing often can save time and money, and increase the likelihood of success. Care should be taken to ensure that the testing conditions are representative of field conditions. Unfortunately, this is often a time-consuming process, as microcosms must be given enough time to demonstrate detectable degradation and inoculum survival as compared to controls. In some cases, there are methods to accelerate this testing, such as the use of isotope labeling, where accelerated growth of the bioaugmentation strain can be detected with greater sensitivity by using isotopically labeled carbon dioxide (CO_2) (Hesselsoe et al., 2008). Once the microcosm test is successful and/or the treatment has been approved by the regulatory agencies, full-scale treatment can be implemented.

1.4.3 Implement the Treatment

Site-specific applications of bioaugmentation will naturally depend on the type of bioaugmentation strategy chosen and the problems that are foreseen by the site evaluation. The practitioner must decide on the inoculum type, the inoculum size and the mode of delivery, all three of which are interdependent. In all cases, the inoculum must first either be acquired or engineered and grown. Commercially available inocula are appealing because they are easy to use and readily available in large quantities. For preadapted bacterial inocula or activated soil, the bacteria/soil must first be obtained from a polluted site and acclimated to the pollutant. Next, the application rate must be determined, and several interrelated questions must be addressed:

- How many bacteria are needed per cubic meter?
- Does the inoculum addition need to be done aerobically or anaerobically?
- Are there any nutrients that need to be added?
- How will adequate distribution of the inoculum be ensured?

The effect of inoculum size on degradation rate or success depends on the site, the pollutant and the bioaugmenting organism (Vogel and Walter, 2002). More is not necessarily better when it comes to bioaugmentation, as a larger inoculum does not necessarily lead to faster degradation. Additionally, too much inoculum might overwhelm a system and lead to a loss of available nutrients. The application of commercially-available inocula depends on the manufacturer, but the form of the inoculum is tailored for the intended application. For example, for treating surface oil spills, QM Environmental Services, Ltd. provides Microcat®-XRC in a powder form for direct application to the spill. A lake or other body of water might benefit from either a spray (if the contaminant is on the surface) or addition of a liquid inoculum.

Most groundwater bioaugmentation strategies involve injecting the inoculum, although it is also possible to convert subsurface irrigation systems (Mehmannavaz et al., 2002). Many commercial inocula come in a liquid form that is ready for direct injection into the ground. In order to achieve more coverage, it is possible to inject into a strategically placed row of wells to create a biocurtain or biobarrier through which the groundwater will flow (Dybas et al., 2002; Hunter and Shaner, 2010). When injecting inocula into soil or contaminated groundwater, it is often difficult to ensure that the inoculum will be delivered effectively (so that it will not be carried away too quickly from the point of injection, for example), or that the inoculum will not be predated or outcompeted too quickly. One solution may be to use a carrier agent or encapsulating agent to deliver the inoculum, provide protection and/or nutrition and place the inoculum where the pollutant is located. Carrier agents tend to be clay or plant-derived compounds like peat, while encapsulating agents are gels, like alginate or polyacrylamide, that coat the cell but are flexible enough for injection and can be degraded (van Veen et al., 1997). These agents protect the inoculum against the environment (pH, predation, etc.) but target compounds can diffuse through (Gentry et al., 2004).

1.4.4 Monitoring Effectiveness

Once the bioaugmentation treatment is in place, it is necessary to monitor the presence of the inoculum and/or the degradation of the pollutant. Pollutant levels are primarily monitored to ensure the objective of the treatment – namely pollutant removal. It also would be ideal to monitor for the accumulation of toxic metabolites. Inoculum levels are monitored to ensure that the bacteria are alive and active and to be able to correlate pollutant reductions with microbial activity. Loss of inoculum would signal a need for reinoculation or use of a different inoculum. Ideally, once the treatment is complete, the inoculated strains should cease to be an active part of the system, and tracking the inoculum would verify this. There are several methods available for tracking the inoculum and pollutant degradation, including using microbiology, molecular biology or physicochemical techniques (Table 1.3).

Conventional microbiological techniques like plating and most probable number (MPN) counts take samples from the site of interest and then grow the organisms in the sample on defined media. In the case of plating, dilutions of the sample are spread onto agar plates with some kind of selective agent (usually the target compound) to isolate the degrading species and confirm their degradation activity. With MPN, the samples are diluted until the activity of interest can no longer be detected in liquid media.

Recent innovations include fluorescence *in situ* hybridization (FISH), which uses fluorescent probes that bind to a gene of interest (either phylogenetic or catabolic) so that organisms containing the target gene can be observed directly (Yang and Zeyer, 2003). Successful identification of the gene is observed using a fluorescent microscope or flow cytometry. If genetically-modified bacteria were to be used in the field, monitoring their presence and activity could be facilitated by incorporating a reporter gene – like the luc gene encoding firefly luciferase or the *gfp* gene encoding green fluorescent protein – downstream of the catabolic genes (Jansson et al., 2000).

Modern molecular methods avoid the pitfalls of culturing bacteria and can be especially useful with consortia or uncultured organisms because they use genetic material extracted directly from the medium. Molecular methods often revolve around the polymerase chain reaction (PCR) technique to monitor nucleic acid sequences – particularly the l6S ribosomal ribonucleic acid (rRNA) sequences – from the microbes of interest (Gentry et al., 2004). The benefit of PCR is that it amplifies a quantitatively small amount of target sample to a level where it can be detected either on gels or with fluorescent markers. PCR can be used to detect the presence of the gene, while real-time quantitative PCR (qPCR) can be used to quantify gene levels in a system (Van Raemdonck et al., 2006). Reverse-transcriptase PCR (RT-PCR) reflects what genes are being expressed, and involves extraction of messenger RNA (mRNA), reverse transcription of that RNA to DNA and amplification of the gene of interest. RT-qPCR combines the reverse transcription step with a quantitative PCR. Analysis of mRNA is currently considered a semi-quantitative method because it often is unstable. However, the presence of detectable mRNA demonstrates that the gene of interest is being expressed, and the results can indicate activity levels, particularly in comparison to other samples (ESTCP, 2005). If there are numerous genes or strains to be monitored, a microarray of the target genes can detect thousands of sequences (associated with those genes/strains) simultaneously (Johnson et al., 2008). Microarray analysis is performed by first labeling the sample genetic material, usually with fluorescent tags or radioactivity, and then hybridizing the sample with the microarray chip onto which the target genes have been affixed. The chip is then washed to remove the non-hybridized sample and read using the appropriate technology, like a fluorescence scanner. These and other molecular methods of monitoring bioaugmentation have been reviewed more thoroughly elsewhere (Saleh-Lakha et al., 2005), and are reviewed in Chapter 6 of this volume.

Table 1.3. Methods to Monitor Inoculum Survival and Pollutant Degradation

Target	Method Type	Name	Description	Quantitative?
Inoculum	Microbiology	Plating	Growth of the inoculum on plates demonstrates presence of the organism	Yes
		MPN	Dilution of the inoculated site medium to verify inoculum presence	Yes
		Microscopy	Hybridization of site media with a fluorescent probe specific for the inoculum	Semi
		Bioluminescent strains	Genetically-modified strains that carry a bioluminescent gene	Semi
	Molecular biology	PCR, RT-PCR of 16 S rRNA	Detection of the 16 S rRNA of the inoculated strains	Semi
		qPCR, RT-qPCR	Detection of the genes and transcripts of interest	Yes
		Microarray	DNA probes on a chip are used to detect multiple genes simultaneously	Semi
Pollutant	Microbiology	Metabolic biomarkers	Detecting biologically-specific pollutant degradation intermediates	No
		Push-pull test	Isotope-tagged pollutants are injected into the site and retrieved to evaluate degradation	No
	Physicochemistry	Microelectrodes	Use of electrodes to detect the presence of target pollutants	Yes
		Compound specific isotope analysis (CSIA)	Examination of pollutant isotope ratios to detect isotope fractionation	No
		Analytical chromatography	Extraction of the pollutant from the medium and direct detection based on chemical characteristics	Yes

The pollutant concentration itself can be monitored in a number of ways. Compounds that result from the biodegradation of certain pollutants can be used as markers, or more specifically metabolic biomarkers (Smets and Pritchard, 2003). Each metabolic biomarker should be an intermediate specific to the degradation of the pollutant of interest and be degraded easily to indicate ongoing degradation. In a push-pull test, isotope-tagged pollutants are injected into aquifers, briefly exposed to the bacteria, then quickly retrieved and analyzed for degradation (Scow and Hicks, 2005; Lee et al., 2010). Compound-specific isotope analysis (CSIA) exploits the preference of biological systems for certain stable isotopes, resulting in isotopic fractionation. CSIA is a powerful and sensitive technique that can be used to determine conclusively

whether a specific compound is being biodegraded *in situ*. For example, with carbon-based stable isotope analysis, the chlorinated ethenes remaining after biodegradation have a higher ^{13}C:^{12}C ratio than the original pollutants due to the biological preference for ^{12}C bonds, which are slightly weaker than ^{13}C bonds (Morrill et al., 2005). These tests require laboratory analyses and cannot be performed easily in the field. Microelectrodes, on the other hand, also can be used to detect byproducts of bacterial metabolism or the actual products of interest in the field (Satoh et al., 2003).

1.4.5 Other Considerations: Economics and Degradation Kinetics

In cleanup scenarios, the two main concerns are time (time required to meet remediation goals and/or the duration of site occupation) and cost (covered more thoroughly in Chapter 11). The time required for cleanup is controlled by the overall degradation kinetics, which in turn are controlled by the rate of catalysis and pollutant availability. If the rate-limiting step is the catalysis, then bioaugmentation with either a faster-degrading organism or more organisms will speed up the degradation, reduce time of cleanup and thus possibly reduce cost. If the site cannot support a large number of microbes, the bioaugmented population will diminish soon after inoculation. However, even if the site has to be bioaugmented multiple times, this might be a cost-efficient solution if it proves to speed site remediation. If, however, the rate-limiting step is pollutant availability, then no amount of bioaugmentation is going to help – it will, if anything, only incur cost and frustration and may in some cases increase cleanup time and cost by plugging wells or aquifers (Vogel, 1996). In this case, either the pollutant availability needs to be increased, such as by surfactants, and then bioaugmentation can be considered, or a different remediation method needs to be chosen.

The cost of site remediation is related to the level to which the pollutant must be reduced, which is determined by regulatory standards that vary from place to place. For bioremediation methods, contaminant removal to very low concentrations can prove problematic. Most bacteria must be exposed to a certain level of a substrate before the degradation pathways are induced. If the regulatory levels are lower than the induction levels, the bacteria are not going to degrade the pollutant unless some momentum exists in the system or other compounds are inducing the needed enzymes (He and Sanford, 2002). One solution is to preinduce the bioaugmented culture so that the degradation pathways are already activated, or to use bacteria that constitutively express the degradation pathway, meaning that they express the genes regardless of the pollutant level.

1.5 BIOAUGMENTATION ISSUES

Despite the apparent simplicity and efficacy of bioaugmentation, this technology remains controversial due to the inherent complexity of natural systems that do not behave like laboratory microcosms and the inability to control organisms released into the environment. While many bioaugmentation experiments in the laboratory show promising results, this success often does not translate at full scale in the field (Cases and de Lorenzo, 2005; Park et al., 2008). Before the late 1990s, bioaugmentation was overlooked due to its unreliable record (Pritchard, 1992; Thompson et al., 2005). Bioaugmentation can result in no visible increase in degradation and increased cost if the full-scale delivery of microorganisms to the site of interest fails or if there are mixing, localization and bioavailability issues. While bioaugmentation has become a common treatment for sites contaminated with chlorinated solvents, it has not fared as well with other pollutants. There are several criteria that must be addressed prior to bioaugmentation becoming a reliable remediation alternative for a particular pollutant. These

criteria, discussed in detail below, include development of bioaugmentation cultures, inoculum introduction and survival, increasing pollutant and nutrient bioavailability and reducing unwanted side-effects.

1.5.1 Development of Effective Bioaugmentation Cultures

Perhaps the biggest hurdle for bioaugmentation is to create an inoculum that will survive, grow and degrade the target pollutant(s) *in situ*. This chapter deals primarily with the practical aspects of bioaugmentation implementation and does not discuss the measures necessary to develop bioaugmentation strains/inocula. However, it is important for practitioners to understand the three basic criteria for a good bioaugmentation culture (Cases and de Lorenzo, 2005). First, the culture has to be able to survive long enough to impact the pollutant concentration in its new environment, unless it is only being used to transfer genetic material to other organisms. There are various methods by which its survival can be enhanced, such as the use of a delivery agent as previously discussed, but bioaugmentation cultures should be selected and cultured to enhance their *in situ* survival. Second, the organism needs to have a high degradation activity, although not necessarily a fast growth rate (Kuiper et al., 2004). Finally, control over the culture's longevity in the system is desirable to ensure the return of the ecosystem to its original state after treatment is complete. In many cases, it is preferable that the bioaugmented organism not outlive its usefulness in the system.

1.5.2 Successful Inoculum Delivery and Dispersion

Depending on the polluted site, delivery of the inoculum can vary in difficulty. In groundwater remediation, the inoculum often has to be injected into a well, where it must diffuse enough to obtain good coverage of the area but not leave the polluted site. The hydrogeology of the site can determine whether the inoculum can spread from the injection point or if injection is even possible. There are a few possibilities for increasing the dispersivity of cells, like the use of ultramicrobacteria that are more mobile due to their smaller size, the development of adhesion-deficient bacteria or the addition of surfactants (Gentry et al., 2004).

1.5.3 Inoculum Survival

Once the inoculum is delivered, it needs to survive long enough to perform its function. The type of inoculum – its robustness and rate of growth – can determine its survival. Some of the factors that inhibit inoculum survival/growth, such as pH, temperature and nutrient availability, have already been discussed under "Site Evaluation" in Section 1.4.1. Lack of nutrient availability can limit survival and the degradation process. Clay content or organic matter can limit growth by limiting nutrient availability by diffusion (Vogel, 1996). Nutrient or substrate availability can be enhanced with biostimulation or the use of surfactants. The nutrient issue also can be ameliorated by using a carrier agent that contains supplements (Gentry et al., 2004). Effective distribution of the inoculum throughout the subsurface will limit the concentration of organisms in any one area, thus increasing the amount of nutrients available per cell.

The other major factor inhibiting inoculum survival is not abiotic, but rather biotic, in the form of predation by other organisms (e.g., protozoa) and competition for nutrients. Some encapsulating agents provide protection against predation, and nutrients also can be included in the inoculum carrier agent or in a biostimulation process. But these are not necessarily long-term survival techniques and reinoculation may be necessary (Newcombe and Crowley, 1999;

Boon et al., 2000). Each ecosystem is unique, and there are no well-established methods for predicting inoculum survival.

The type of bioaugmentation agent used also can play a major role in the survival of the inoculum. A recurrent theme in strain selection is that bacteria from the site itself often make the best inoculant (Singer et al., 2005; Thompson et al., 2005). Enrichments from the site itself may have a higher chance of survival than commercial inocula, since they are already acclimated to the site parameters (El Fantroussi and Agathos, 2005). Gene bioaugmentation may be an even better choice in the future, as the survival of the inoculum itself is not necessary. The introduced organisms only have to survive long enough to transfer their MGEs. Direct gene bioaugmentation without the use of bacterial hosts would improve on this technology. However, the technique to deliver naked DNA that would encourage uptake by indigenous bacteria rather than its destruction has yet to be perfected. Predicting gene transfer frequencies also is difficult, and therefore performance cannot be evaluated easily.

1.5.4 Pollutant Bioavailability

Once the inoculum is in place, the introduced bacteria must obtain sufficient nutrients to survive and also must have access to the pollutant. Pollutant bioavailability can be a major factor in the time-scale of the treatment and thus the cost of remediation. Bioavailability is a serious concern for bioremediation of contaminants – such as chloroethenes – that form NAPLs because they are slowly released into the aqueous solution. If the pollutant is only slightly soluble, its concentration might not be high enough to induce the degradation pathways in microbes (Cases and de Lorenzo, 2005). One way to improve the bioavailability of the pollutant is to use surfactants to mobilize the pollutant (El Fantroussi and Agathos, 2005). Pollutants trapped in DNAPLs that would ordinarily take years for natural dissolution may be more quickly dislodged using surfactants that act either by forming micelles that encapsulate the pollutant or by reducing the interfacial tension between the pollutant and water. The combination of a surfactant foam with a bioaugmentation inoculum potentially can combine enhanced bioavailability and degradation capacities to speed up bioremediation (Rothmel et al., 1998).

1.5.5 Potential Undesirable Side-Effects

All possible impacts of bioaugmentation cannot be predicted. Certainly, bioaugmentation involves some potential risks, though to date experience has indicated the risks are minimal, and any such risks must be weighed against the benefits of pollutant removal (Gentry et al., 2004). Table 1.4 lists some examples of unanticipated side effects of bioaugmentation. The

Table 1.4. Examples of Unexpected Side-Effects of Bioaugmentation (adapted from Sayre and Seidler, 2005)

Microorganism	Use	Effect	Reference
Pseudomonas SR3	Biodegrades pentachlorophenol	Inhibits nodule number and size in *Lotus corniculatus* Inhibits substrate induced respiration	Pfaender et al., 1997
Pseudomonas putida PPO301 (pRO103)	Degrader of herbicide 2,4-D	Metabolic byproduct causes significant decreases in soil fungi	Short et al., 1991
Pseudomonas cepacia AC1100	Degrader of 2,4,5–T	Causes change in taxonomic diversity of soil microbiota	Bej et al., 1991

Note: 2,4-D – 2,4-dichlorophenoxyacetic acid; 2,4,5-T – 2,4,5-trichlorophenoxyacetic acid.

introduction of foreign material also introduces unknowns into the system, possibly resulting in undesired effects like toxic intermediates and clogging. Degradation of the pollutant might itself lead to secondary water quality impacts, such as taste or odor issues. The selection process used to develop the bioaugmentation strain might select bacteria with undesirable properties, such as enhanced antibiotic resistance (Davison, 2005). Introduction of a strain that can grow to large population numbers would almost certainly alter the microbial community structure (Coppotelli et al., 2008). Foreign genes could enter the gene pool and be horizontally transferred to the indigenous strains. Existing models are simply not sophisticated enough to predict these effects.

However, there are certain undesirable side-effects that are foreseeable and preventable. For example, in certain cases, injection of bacteria leads to clogging of the subsurface due to uncontrolled growth (Vogel, l996). In such cases, the choice of a slow-growing degrader may be favored over a fast-growing degrader that would quickly use up nutrients in a system and lead to clogging (Cases and de Lorenzo, 2005). The use of potentially pathogenic strains also should be avoided (Singer et al., 2005).

1.6 BIOAUGMENTATION TO REMEDIATE CHLORINATED COMPOUNDS

The primary focus of this volume is on bioaugmentation to remediate chlorinated solvent pollution. Chlorinated compounds are particularly difficult to degrade due to the presence of the halide, which often makes these compounds more recalcitrant to biodegradation than unsubstituted hydrocarbons. Halides can be bulky and often obstruct enzymes from reaching their target bonds, and they are electrophilic (like oxygen) and thus render oxidizing enzymes less useful. The most widely used chlorinated compounds were often chlorinated solvents, including PCE and trichloroethane (TCA), carbon tetrachloride (CT) and chlorinated aromatic compounds like chlorobenzene. Chlorinated solvents were heavily used as cleaning agents and to synthesize other chemicals. Due to use, spillage from tanks or pipes and improper disposal of these agents, chlorinated solvent contamination is widespread.

Besides being recalcitrant, these low solubility chlorinated solvents often sink through soil and aquifers to form DNAPL pools at the bottom of aquifers (Figure 1.8). These DNAPLs present hard-to-remediate source zones of contamination due to the pure product nature of DNAPLs and the difficulty of reaching them. As groundwater flows through these DNAPLs, it spreads soluble phase contamination to an even larger area.

The magnitude of this problem is reflected in part by the quantity of literature on the subject and the number of government and industry-sponsored research publications. The Interstate Technology & Regulatory Council (ITRC) has published a guide to evaluating and implementing *in situ* bioremediation strategies, including bioaugmentation, at sites contaminated with chlorinated ethenes (ITRC, 2008).

1.6.1 Chlorinated Aliphatic Hydrocarbons (CAHs): *Dehalococcoides* and the Chloroethenes

Chlorinated ethenes are the most prevalent groundwater contaminants and pose difficult remediation challenges, so this contamination is a major environmental concern and a sizeable commercial opportunity. As discussed earlier, chlorinated ethenes can be degraded to different degrees both aerobically and anaerobically. Under anaerobic conditions, PCE can be transformed by reduction past the toxic VC intermediate to the non-toxic gas ethene (Freedman

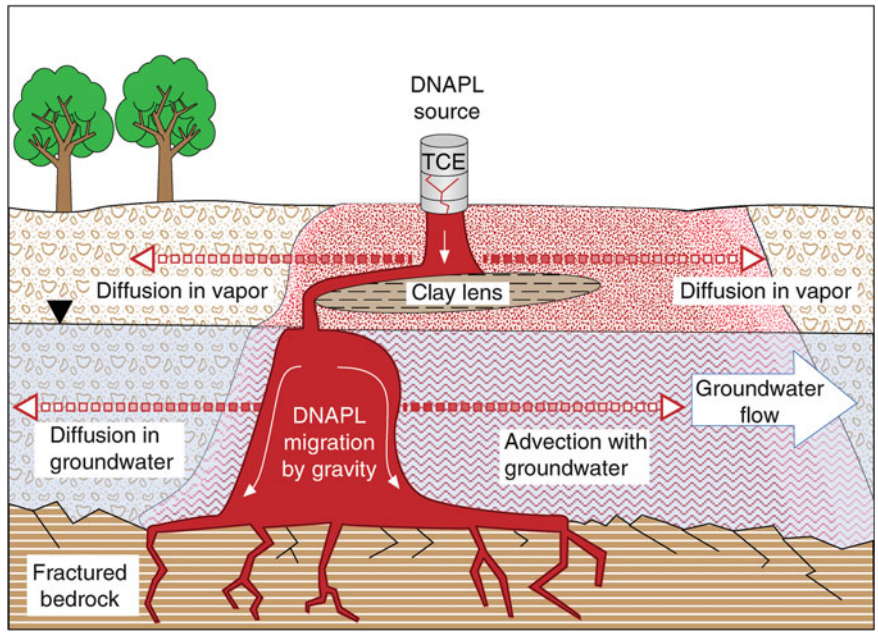

Figure 1.8. Conceptual diagram of a DNAPL-contaminated site (USEPA, 2007).

and Gossett, 1989). It has been shown that the organisms performing the dechlorination are using the CAH as an electron acceptor that is able to sustain growth of these organisms (Holliger et al., 1999). Presently, there are only a few known organisms that can degrade CAHs, including strains of *Dehalococcoides, Sulfospirillum* (formerly *Dehalospirillum*), *Desulfitobacterium* and *Dehalobacter* spp. (Damborsky, 1999). Thus far, the only microbes that have been found to degrade chlorinated ethenes all the way to ethene are members of the group *Dehalococcoides*. If these organisms are not present at the site to be bioremediated, bioaugmentation might be a benefit. Thus, in areas polluted with CAHs, it is clearly beneficial to either verify the presence of *Dehalococcoides* or to consider bioaugmentation with these organisms (ESTCP, 2005; Rahm et al., 2006; Lee et al., 2008). *Dehalococcoides* can be found in many polluted areas, but their absence has been correlated with CAH degradation stalling before conversion to ethene (Hendrickson et al., 2002). Further details regarding *Dehalococcoides* and chlorinated solvent biodegradation are provided in Chapter 2.

Naturally, there are several limitations to the use of such cultures, besides the limitations already detailed above for bioaugmentation in general. First, these organisms perform best under anoxic conditions and have a low tolerance for oxygen (ESTCP, 2005). They degrade the CAHs by reductive dechlorination, and thus should be kept under favorable redox conditions and with appropriate electron donors, such as lactic acid or another organic substrate. Judging from the number of inocula available and the number of sites in which they were applied, the use of these cultures has been a tremendous commercial success. Part of the intelligent application includes manipulation of the environment in order to induce hydrogen production under anaerobic conditions. This manipulation also aids the naturally occurring *Dehalococcoides*, which were found in 21 out of 24 sites examined (Hendrickson et al., 2002).

There have been a considerable number of field-scale studies of chlorinated solvent degradation, with varying levels of success. For example, bioaugmentation using an enriched culture from a different contaminated site was clearly demonstrated to increase the rate and extent of biodegradation at a CAH-contaminated site (Semprini et al., 2007). Similarly,

bioaugmentation was used to successfully remediate TCE in a well-monitored demonstration project at the Cape Canaveral Air Force Station, Florida (Hood et al., 2008). Similar results using a commercially available KB-1© inoculum were achieved at the Caldwell Trucking Facility in New Jersey (Kane et al., 2005) and Kelly Air Force Base (AFB), Texas (Major et al., 2002). A different culture was used with similarly successful results at Dover AFB (Ellis et al., 2000). A recent field demonstration successfully used gene biomarkers to track the dechlorination (Scheutz et al., 2008).

The use of bioaugmentation to remediate chlorinated ethene pollution has enjoyed greater success than any other bioaugmentation approach for several reasons. First, the organisms that can degrade these compounds are not ubiquitous and are generally not common in contaminated environments, unlike the case for petroleum degraders. Also, CAH degradation has profited from greater interest and research than other pollutants, with the result that there are now proposed protocols for CAH remediation, like the reductive anaerobic biological *in situ* treatment technology (RABITT) (Morse et al., 1998). The use of bioaugmentation to degrade chlorinated ethenes has been succinctly detailed in a white paper (ESTCP, 2005).

1.6.2 Applications for Other Chlorinated Compounds

There are numerous chlorinated compounds other than CAHs, and these also present difficult cleanup challenges. These pollutants include PCBs used in a wide variety of applications including dielectric fluids and flame retardants, and carbon tetrachloride used in fire extinguishers, refrigerants and cleaning agents. PCB contamination is widespread and persistent. *Dehalococcoides* strains are able to dechlorinate highly chlorinated PCBs (Fennell et al., 2004). There have been few studies on the use of bioaugmentation for enhanced degradation of PCBs at the field scale, although it has been tested in microcosms (Winchell and Novak, 2008). One co-culture has been found to be able to couple PCB degradation with growth and could make for a good bioaugmentation inoculum (May et al., 2008).

Carbon tetrachloride is a widespread groundwater contaminant whose use has been discontinued. Chapter 9 discusses in depth the use of bioaugmentation to remediate CT, which may represent another promising target for bioaugmentation. A bioaugmentation pilot experiment showed positive results with the degradation of carbon tetrachloride by *Pseudomonas stutzeri* KC without an accumulation of formaldehyde (Dybas et al., 1998, 2002).

1.7 BIOAUGMENTATION TO REMEDIATE OTHER CONTAMINANTS

Several reviews have summarized the key literature regarding bioaugmentation (Gentry et al., 2004; Scow and Hicks, 2005). There is a gradient of success that seems to correlate with the chemical nature of the pollutant. For example, bioaugmentation has been more successful for compounds that are absent or rare in natural systems than for those more commonly found at high concentrations. Thus, chlorinated solvents, which are naturally present at low concentrations, respond better to bioaugmentation than petroleum products, which have existed at high concentrations in natural systems for millennia. The genes to degrade newly introduced xenobiotics may not have yet evolved or be widespread, and thus only a few bacteria are capable of their degradation. The energy yield available to an organism from metabolizing the chemical also may be important, as competition may be more intense for higher-energy substrates. For example, the yield from chlorinated ethene respiration decreases as the number of chlorines decrease, and the number of indigenous bacteria that can gain energy from

halorespiration also decreases with decreasing chlorine number. It seems reasonable that bioaugmentation will be most successful for contaminants with similar characteristics, and there likely will be relatively little competition from any indigenous bacteria.

The following sections review successful field-scale bioaugmentation strategies based on pollutant type. The sections address the use of bioaugmentation to remediate organic contaminants, metals and mixed pollutants. The discussion focuses on field studies of bioaugmentation to the extent possible, since promising microcosm approaches have not always proven successful under field conditions.

1.7.1 Petroleum and BTEX

Petroleum products consist primarily of aliphatic hydrocarbons, although they also contain toxic and carcinogenic aromatic hydrocarbons (notably benzene, toluene, ethylbenzene and total xylenes, known as BTEX compounds). The major petroleum compounds are not necessarily difficult to biodegrade in most natural environments, and degradation pathways for most petroleum constituents are well-established. Petroleum contamination is widespread but usually can be treated biologically through natural attenuation or biostimulation alone, as oxygen and inorganic nutrients are typically the limiting factors (Swannell and Head, 1994). However, this depends on the site parameters, as bioaugmentation also has been shown to accelerate the bioremediation of diesel pollution (Bento et al., 2005). It is often desirable to remediate spills and leaks quickly and efficiently, so there long has been a perceived need for bioaugmentation cultures, and there are a variety of commercially-available products to bioremediate oil spills (Table 1.1). Like chlorinated solvents, petroleum hydrocarbons and BTEX are among the better studied pollutants in terms of remediation strategies. The Exxon Valdez spill increased public awareness of the idea of bioremediation and bioaugmentation, though bioaugmentation at the spill was not the critical step (Glaser, 1993). Most sites, even in pristine areas, contain bacteria ready to degrade petroleum hydrocarbons.

As mentioned earlier (Section 1.2.1), careful field studies generally have not shown a need or significant benefit from bioaugmentation for petroleum product removal (Van Hamme et al., 2003). Plant-assisted bioaugmentation might prove more successful, as plants are both aesthetically more pleasing and are often already present at the interfaces typically present at petroleum spills (Cohen, 2002; Juhanson et al., 2007). Bioaugmentation and phytoaugmentation also could be implemented as precautionary measures around areas prone to leaks and spills (Lendvay et al., 2003). Bioaugmentation could be more useful in removing petroleum product cocontaminants, like BTEX, rather than the petroleum itself (Park et al., 2008). Petroleum-related contamination also can coincide with other petroleum product wastes, like cyanide and heavy metals. In these cases, metal resistant bacteria might need to be added if the indigenous community is inhibited by the metals.

1.7.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are found in wood preservatives, mothballs and some petroleum products. They are composed of multiple aromatic rings in various conformations. Among the most common PAHs are anthracene, chrysene, naphthalene, pyrene and benzo[a]pyrene. These compounds are often toxic, mutagenic and lipophilic, making them difficult contaminants to treat, as they accumulate in soil organic matter and therefore are not readily bioavailable for microbial degradation (Cerniglia, 1992; Wilson and Jones, 1993; Bamforth and Singleton, 2005). There are mixed reviews on the efficacy of bioaugmentation for PAH degradation, and it has yet to

become an accepted approach (Atagana et al., 2003; Coppotelli et al., 2008; Tam and Wong, 2008). White rot fungi (notably isolates of *Phanerochaete chrysosporium*) have been studied as bioaugmentation agents for PAHs (and other recalcitrant compounds), but this approach has had little field-scale success (e.g., Bumpus, 1989; Field et al., 1992; Pointing, 2001). PAHs are often found in complex chemical mixtures, and a consortium of bacteria may be better equipped to bioaugment such a mixture than a single culture inoculum (Jacques et al., 2008). These larger PAH compounds, such as benzo[a]pyrene originally thought to be recalcitrant, might be more effectively degraded by GEMs (Samanta et al., 2002).

1.7.3 Methyl Tert-Butyl Ether (MTBE)

MTBE is a gasoline additive that replaced tetraethyl lead as an antiknock agent in the 1980s and that also serves as a fuel oxygenate. Consequently, many gasoline spills also are accompanied by MTBE contamination. Small amounts of MTBE in drinking water (low microgram per liter [μg/L] concentrations) impart an unpleasant taste, and larger amounts pose a possible health risk. The USEPA Federal Drinking Water Guideline for MTBE is 20–40 μg/L, although some states have lower standards. The MTBE problem is exacerbated by its relatively high solubility in water and the fact that it is biodegraded more slowly than other gasoline components, such as BTEX compounds. As a result, MTBE plumes often can be larger and more persistent than BTEX plumes. In this sense, MTBE can be a useful indicator of gasoline spills, preceding the supposedly more harmful BTEX components. However, it also can result in a need to treat significantly larger areas and greater volumes than the BTEX contamination alone.

MTBE is a relatively stable compound that is difficult to degrade due to its ether bond. Various reviews detail remediation efforts on MTBE, stressing that aerobic conditions are ideal for MTBE degradation (Deeb et al., 2000; Stocking et al., 2000; Zanardini et al., 2002; Häggblom et al., 2007), although anaerobic MTBE biodegradation also occurs (Finneran and Lovley, 2001; Lopes Ferreira et al., 2006). One complication with MTBE contamination is that it is usually accompanied by BTEX contamination and other gasoline products. Thus, any remediation strategy should not interfere with the ability to degrade the other pollutants, which are often more toxic than MTBE. Biodegradation of MTBE – and its breakdown product tert-butyl alcohol (TBA) – is clearly possible, but it has proven difficult to treat these compounds in groundwater (Deeb et al., 2000). Further details on bioaugmentation of MTBE are provided in Chapter 10 of this volume.

Field studies of bioaugmentation to degrade MTBE have demonstrated the need for aerobic conditions. At Port Hueneme, California, an enriched mixed culture and oxygen injection were combined to successfully remediate MTBE, although MTBE biodegradation also occurred in the oxygen-only control plot after a lag period (Salanitro et al., 2000). In a second study at the same site, only oxygen was needed to enhance remediation, and bioaugmentation did not increase effectiveness (Smith et al., 2005). This study employed a qPCR method that had been developed to monitor the presence of a bioaugmentation strain (PM-1) proven capable of rapid and complete MTBE degradation (Hristova et al., 2001).

1.7.4 Pesticides

Pesticides, particularly those of the organochlorine family, represent a generally more xenobiotic class of compounds, having been manufactured and released into the environment only recently. These compounds are often aromatic and chlorinated, thus being difficult to degrade. Pesticides are able to seep through the soil to contaminate groundwater. The success

of bioaugmentation with these compounds is varied (Singh et al., 2006). For example atrazine (2-chloro-4-[ethylamine]-6-[isopropylamine]-s-triazine) was introduced as an herbicide in the late 1960s. Repeated inoculation of the soil with atrazine-degrading organisms removed 72% of the atrazine under field conditions after 11 weeks (Newcombe and Crowley, 1999).

Another herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), was the subject of a successful field-scale gene bioaugmentation study in which bacteria carrying a 2,4-D degrading plasmid pJP4 were able to transfer the plasmid to indigenous organisms that successfully expressed the proteins, with transconjugants representing about 10% of the culturable population (Newby et al., 2000). Similar plasmid transfer in some gene-bioaugmented soils has resulted in successful 2,4-D degradation (Pepper et al., 2002).

Hexachlorocyclohexane (HCH, whose gamma isomer is commonly known as lindane), a now-banned, highly-chlorinated insecticide (a gamma-aminobutyric acid [GABA] inhibitor), is still found in high residual concentrations in areas where it was produced or used. HCH and related chlorinated pesticides are resistant to biodegradation, and often have very low risk-based cleanup levels because they are biomagnified. In one field-scale pilot test in India, a single-species bioaugmentation inoculum was used to successfully remediate a site contaminated with HCH (Raina et al., 2008). The investigators used local products to grow and store the inoculum, thus reducing cost and increasing the feasibility of bioaugmentation in economically stressed regions.

1.7.5 Metals

Metals, particularly heavy metals, sometimes accumulate in areas due to industrial activity. These metals, such as cadmium, mercury, lead, zinc, chromium and nickel, can either be transformed to a less toxic version of the metal or accumulated and sequestered to reduce bioavailability or facilitate removal. Microorganisms can reduce and precipitate metals such as hexavalent chromium (Cr[VI]) and radionuclides such as uranium that are less soluble in reduced forms. The technology has been successfully demonstrated in field-scale testing, and several bacterial cultures have been isolated and cultured during field testing (Vrionis et al., 2005).

In situ bioremediation is likely to be an important technology for treating several metals and radionuclides in soils and groundwater, but so far bioaugmentation has not proven necessary or beneficial (Hazen and Tabak, 2005; Wu et al., 2006). The sequestration process also can be aided by plants or in biofilms (Singh et al., 2006). Bioaugmentation can be performed to increase plant growth and thus plant uptake and sequestration (Zaidi et al., 2006; Lebeau et al., 2008). Rhizoremediation also can be a successful, plant-dependent bioaugmentation strategy (Kuiper et al., 2004). Depending on the metal and on the soil, microorganisms can increase metal bioavailability (although sometimes they also do the opposite) by changing soil pH or by secreting compounds like biosurfactants and siderophores that increase metal solubility and potential mobility.

1.7.6 Mixed Pollutants

Contaminated sites often contain more than one pollutant, and such mixtures can complicate the remediation strategy considerably. The orchestration of such a site cleanup can involve more than one remediation strategy and, if the strategy is bioaugmentation, more than one round or type of inoculation with different strains. One notable example is the inhibition of reductive dechlorination of TCE in the presence of TCA, a common cocontaminant (Duhamel et al., 2002). Inhibitory pollutants should be removed prior to bioaugmentation for other target compounds. For example, at sites contaminated with mixtures that include heavy metals, the metals often can

inhibit degradation of other contaminants. Thus, in a soil cocontaminated with 2,4-D and cadmium (II) (Cd[II]), different cadmium-resistant inocula were used to reduce the Cd(II) concentrations to a level where 2,4-D degradation could be accomplished by a second inoculum or by gene bioaugmentation (Roane et al., 2001; Pepper et al., 2002). In another example, soil from a decommissioned industrial area in Italy was remediated in microcosms using a two-step bioaugmentation process (Baldi et al., 2007). The first step involved heavy metal removal by a *Klebsiella* culture known to create a metal-sequestering gel. In the second step, the remaining organic pollutants were removed by fungi that were inhibited at the original free heavy metal concentration.

1.8 SUMMARY

Bioaugmentation – the addition of biocatalysts to promote the degradation of pollutants – has undergone a remarkable evolution over the last 30 years. It was viewed initially with enthusiasm by researchers and practitioners, leading to the development and testing of a wide variety of bioaugmentation agents to treat contaminants in soils and waters. Originally, most bioaugmentation efforts focused on fuel hydrocarbons. Until the late 1990s, most of the early bioaugmentation agents failed to show consistent enhancements of biodegradation in controlled field tests when compared to biostimulation alone. Soils and aquifers generally have large microbial populations, and indigenous organisms capable of degrading most contaminants can multiply quickly given favorable environmental conditions.

As a result, bioaugmentation came to be viewed with considerable skepticism. However, over the last decade, bioaugmentation has been particularly successful in treating chlorinated solvents, particularly the chlorinated ethenes such as PCE and TCE. These solvents are widespread recalcitrant groundwater contaminants, and the success of bioaugmentation with cultures containing *Dehalococcoides* species in this application has prompted renewed interest in bioaugmentation for other situations.

If used properly, bioaugmentation can be a very cost- and time-effective way to expedite *in situ* site remediation in a relatively noninvasive manner. The technology often can be applied using injection and monitoring wells, or even by one-time direct injections of solutions containing concentrated cultures. As this volume demonstrates, bioaugmentation has progressed to the point that useful guidance and quality control protocols have been developed. While it already has proven to be a valuable remediation technology for some cases and a profitable commercial practice, there is room for future improvements and exciting new applications as our knowledge of molecular biology and genetics grows.

Bioaugmentation is still a relatively young field, but its history does have some lessons for future research and development. Successful bioaugmentation requires extensive site characterization, informed selection of the type and manner of inoculation and a profound understanding of the way the inoculum will interact with the environment. The *Dehalococcoides* story has shown the value of a firm scientific understanding of the bioaugmentation culture and its genetics, physiology and ecology. Future successes, possibly expanding bioaugmentation techniques to include GEMs and MGEs, will likely rely on a similar strong basis of microbiology, biochemistry and genetics.

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CHAPTER 2

DEHALOCOCCOIDES AND REDUCTIVE DECHLORINATION OF CHLORINATED SOLVENTS

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2.1 INTRODUCTION

2.1.1 The Chlorinated Ethene Problem

Since the beginning of the twentieth century, short-chain C1 to C3 chlorinated aliphatic hydrocarbons (CAHs) have been manufactured in large amounts and extensively used in industrial, military, agricultural and household applications. The widespread use of CAHs is based on their desirable properties including low cost, easy availability, excellence as solvents, chemical stability and fire safety (i.e., most chlorinated solvents are nonflammable and do not form explosive mixtures with air). The widespread use, careless handling and storage, ignorance of health effects and environmental dangers, and the lack of regulations over decades of extensive use led to wide-ranging groundwater contamination.

Exposure to CAHs is of public concern because these chlorinated chemicals are toxic, several are classified as potential human carcinogens and some, such as vinyl chloride (VC), are proven human carcinogens (IARC, 1995; Kielhorn et al., 2000). Today, the United States Environmental Protection Agency (USEPA) and Occupational Safety and Health Administration (OSHA) enforce stringent regulatory standards to protect humans from CAH exposure through contaminated drinking water and other routes (e.g., inhalation). After recognizing the dangers that CAHs pose in the environment and to human health, handling and use practices improved so that uncontrolled release has been eliminated in most countries. Although accidental spills of CAHs remain a risk, the majority of CAH sites were contaminated decades ago. Table 2.1 lists CAHs that were, or still are, being used in industrial, military, agricultural and household applications, and are commonly encountered at contaminated sites.

The total amount of chlorinated solvents used worldwide in 2002 was approximately 764,000 metric tons (www.eurochlor.org/; accessed June 19, 2012); however, exact amounts are difficult to obtain, partly because some solvents are co-produced in a single process or are chemical intermediates, and furthermore not all countries report reliable production numbers. In 1980, about 282,000 tons of perchloroethene (PCE; also termed tetrachloroethene) was used in the United States, but the demand declined to approximately 168,000 tons in 2007 (Table 2.2). This decline reflects the much more efficient use of PCE (e.g., reduced evaporation losses,

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Table 2.1. Common Chlorinated Solvents and Their Major Use(s)

Solvent	Common Acronyms or Trade Names	Main Uses	Comments
Tetrachloroethene	Perchloroethene, perchloroethylene, ethylenetetrachloride, carbon dichloride, PCE, per, perk, perc	Dry cleaning, metal degreasing, chemical intermediate, solvent	Dry cleaning is a multi billion-dollar industry and PCE remains the solvent of choice for the majority of the 27,000–30,000 United States dry cleaners and launderers. United States demand in 2007 was estimated at 168,000 metric tons. European Union (EU) sales in 2005 were 56,000 metric tons.
Trichloroethene	TCE, trichloroethylene, acetylene triloride trethylene, chlorylen, tri, tric, trichlor	Metal cleaning and degreasing, chemical intermediate	Enhanced production of TCE occurred in the 1990s to replace TCA, which was banned by the Montreal Protocol. United States demand in 1998 estimated at 77,700 metric tons. Industrial sales of TCE in the EU declined 89% from 62,000 metric tons in 2001 to 28,000 metric tons in 2005 due to stringent regulations.
Carbon tetrachloride	Carbon tet, tetrachloromethane, perchloromethane, CT	Solvent, chlorofuorocarbon production	Phased out under the Montreal Protocol. Remaining use as essential intermediate or for specialty applications. USEPA banned its use as a grain fumigant in 1985. EU production was 59,691 metric tons in 1996.
Chloroform	Trichloromethane, methyl trichloride, CF	Chlorofuorocarbon production, solvent, chemical intermediate	Mainly used for production of monochlorodifluoromethane (CFC 22). Had an application as inhaled anaesthetic. Total EU capacity is about 316,000 tons.
Dichloromethane	Methylene chloride, methylene dichloride, di-clo, DCM	Solvent for extractions, paint remover, degreaser, aerosol propellant, foam-blowing agent	Ideal paint remover because wood is not harmed in the removal process. DCM is used to decaffeinate coffee and tea. United States demand in 2006 estimated at 83,900 metric tons. EU sales in 2005 were 132,000 metric tons.
1,1,1-Trichloroethane	Methyl chloroform, tri-ethane, TCA	Solvent, metal cleaning and degreasing, chemical intermediate, low-pressure propellant	Clean Air Act Amendments of 1990 regulated TCA as an ozone-depleting chemical. Phased out in 2002 under the Montreal Protocol. Remaining use as intermediate or for specialty applications.

(*continued*)

Table 2.1. (continued)

Solvent	Common Acronyms or Trade Names	Main Uses	Comments
1,2-Dichloroethane	Ethane dichloride, ethylene dichloride, EDC, 1,2-DCA	Chemical intermediate	Major use is for the production of VC, the precursor to manufacture polyvinyl chloride (PVC) resins. The uses of 1,2-DCA as lead scavenger and soil fumigant were discontinued. Annual worldwide production exceeds 1,000,000 tons.
1,2-Dichloropropane	Propylene dichloride, 1,2-D	Solvent, chemical intermediate, soil fumigant	Generated as undesirable by-product of the chlorohydrin process. Use as fumigant to control root parasitic nematodes has been discontinued in the United States and the EU.

Resources: (ATSDR, 1997; Doherty, 2000a, b; Rossberg et al., 2006)
Halogenated Solvents Industry Alliance, Inc. (http://www.hsia.org/)
http://ozone.unep.org/pdfs/Montreal-Protocol2000.pdf
www.eurochlor.org/
www.usdrycleaning.com/
www.epa.gov/air/caa/

increased recycling of spent PCE solutions) rather than a true reduction of PCE use. Dry cleaning is a multibillion dollar industry and PCE remains the solvent of choice for the majority of the 27,000–30,000 United States dry-cleaners and launderers. Recently, some United States states have introduced legislation to phase out PCE in dry cleaner operations over the next decade, and a gradual decline in PCE usage can be expected (Chemical Week, 2007).

Chlorinated solvents are colorless, highly volatile liquids at room temperature, with limited solubility in water, low viscosity and low interfacial tension relative to water. Another unifying feature of chlorinated solvents is their high density relative to water, with densities ranging from 1.1 to 1.7 grams/milliliter (g/mL). The characteristic physical-chemical properties govern chlorinated solvent behavior following release into the environment and profoundly impact their environmental distribution and longevity. Table 2.2 lists some relevant properties of CAHs commonly encountered as groundwater pollutants.

Without clear regulatory guidance in place in the United States until the 92nd United States Congress enacted the Clean Water Act in 1972 (CWA, 1972) chlorinated solvent wastes were commonly disposed by pouring them onto the ground or down a drain. These disposal practices assumed that the solvents would readily volatilize to the atmosphere where photochemical degradation would occur. Unfortunately, the solvents often infiltrated through the subsurface, causing extensive subsurface contamination. Upon release, the poorly water-soluble and denser-than-water chlorinated solvents migrated downward and penetrated the water table, a characteristic that coined the term dense nonaqueous phase liquids (DNAPL).

In aquifer formations, capillary forces retain discontinuous liquid ganglia or droplets of chlorinated solvent within the porous media. Furthermore, low permeability layers (e.g., clay) cause DNAPL to accumulate and spread laterally resulting in the formation of high-saturation

Table 2.2. Properties of Common Chlorinated Solvents

Compound	Formula	CAS Number	MW [g/mol]	Liquid Density at 20°C [g/cm^3]	Vapor Pressure (20°C) [kPa]	Solubility in H_2O (20°C) [mg/L]	Solubility in H_2O (20°C) [mM]	K_h	Air Odor Threshold Concentration [ppm, v/v]	MCL [mg/L]
Tetrachloroethene	C_2Cl_4	127-18-4	165.83	1.62	1.9	200	1.2	0.723	27	0.005
Trichloroethene	C_2HCl_3	79-01-6	131.39	1.46	5.78	1,100	8.4	0.39	28	0.005
cis-1,2-Dichloroethene	$C_2H_2Cl_2$	540-59-0	96.94	1.25	24.0	3,500	36.1	0.17	–	0.07
trans-1,2-Dichloroethene	$C_2H_2Cl_2$	156-60-5	96.94	1.26	35.3	6,300	65.0	0.38	17	0.1
1,1,1-Trichloroethane	$C_2H_3Cl_3$	71-55-6	133.41	1.34	13.3	1,290	9.7	0.70	120	0.2
1,2-Dichloroethane	$C_2H_4Cl_2$	107-06-2	98.96	1.25	8.53	8,600	86.9	0.048	88	0.005
1,2-Dichloropropane	$C_3H_6Cl_2$	78-87-5	112.99	1.16	29.9	2,800	24.8	0.12	0.25	0.005
Carbon tetrachloride	CCl_4	56-23-5	153.82	1.59	11.9	826	5.2	1.244	96	0.005
Chloroform	$CHCl_3$	67-66-3	119.38	1.48	21.3	8,451	66.3	0.15	85	0.08[a]
Dichloromethane	CH_2Cl_2	75-09-2	84.93	1.33	47.3	16,945	153.4	0.09	250	0.005

Note: °C—degree(s) Celsius; CAS—Chemical Abstract Services; g/cm^3—gram(s) per cubic centimeter; g/mol—gram(s) per mole; K_h—Henry's Law Constant; kPa—kilopascal(s); MCL—maximum contaminant level; mg/L—miligram(s) per liter; mM—millimolar; MW—molecular weight; ppm—part(s) per million; v/v—volume per volume

[a]MCL for total trihalomethanes including chloroform, bromodichloromethane, chlorodibromomethane, and bromoform

Data for vapor pressure from Rossberg et al. (2006)

Data for aqueous solubility are from the following sources: Huling and Weaver, 1991; Perry and Green, 2008; Schwarzenbach et al., 2003; Yaws, 1999, http://www.syrres.com/what-we-do/databaseforms.aspx

K_h, Henry's Law constants (dimensionless) of chlorinated ethenes according to Gossett (1987) and http://www.syrres.com/what-we-do/databaseforms.aspx

Air odor threshold concentration values from AIHA, 1989; Amoore and Hautala, 1983; Ruth, 1986. Other sources for odor threshold data include: EPA Reference Guide to Odor Thresholds for Hazardous Air Pollutants Listed in the Clean Air Act Amendments of 1990, March 1992, EPA/600/R-92/047 (www.epa.gov/ttn/atw/publicat.html); www.speclab.com/compound/chemabc.htm; www.osha.gov/SLTC/healthguidelines/index.html

MCL values according to www.epa.gov/safewater/contaminants/index.html

pools (Feenstra et al., 1996; Kueper et al., 1993). Entrapped or pooled DNAPL masses dissolve slowly into flowing groundwater, serving as long-term sources of dissolved groundwater contamination that threaten drinking water supplies (Mackay and Cherry, 1989; Schwille, 1988; Stroo et al., 2003). The high incidence and magnitude of chlorinated solvent groundwater contamination raised public concerns and triggered legislative responses. The USEPA has established a regulatory framework with maximum contaminant levels (MCLs) for many environmental contaminants including commonly used CAHs (http://www.epa.gov/safewater/contaminants/index.html; accessed June 19, 2012) (Table 2.2).

Based on their large production volumes and the numerous incidences of uncontrolled release, PCE and TCE remain the foremost risk drivers at a majority of sites in the United States (Moran et al., 2007) and other countries. Corrective actions are necessary at numerous sites and different remediation technologies are being applied with mixed success. Physical and chemical remedies, including pump-and-treat, excavation, chemical oxidation and reactive iron walls, often are too costly or inefficient to provide general and large-scale solutions. The sheer magnitude of the problem and the quest for innovative remediation technologies triggered efforts to explore the microbiology contributing to the fate and detoxification of chlorinated solvents, in particular PCE and TCE.

2.1.2 Anaerobic Microbial Degradation of Chlorinated Ethenes

Steric hindrance and the highly oxidized nature of the PCE carbons (oxidation state of +2) and the TCE carbons (average oxidation state of +1) hamper the attack of oxygenolytic enzyme systems. Not surprisingly, no naturally occurring microbes have been found that utilize PCE or TCE as a growth substrate under oxic conditions. However, certain non-specific oxygenase enzymes, like methane monooxygenase and toluene dioxygenase, can initiate the cometabolic breakdown of TCE and DCEs (i.e., the microbe does not gain energy) (Arp, 1995). Early attempts at bioremediation of TCE focused on stimulating organisms harboring these enzymes with some success (McCarty et al., 1998).

In contrast to the initial remediation test systems, chlorinated solvent contamination predominantly exists in saturated subsurface environments where oxygen is typically scarce or absent, and anaerobic pathways (i.e., pathways that operate in the absence of oxygen and under reducing conditions) are generally more relevant for PCE and TCE transformation. In anoxic environments, CAHs can undergo reductive dechlorination reactions. Reductive dechlorination (hydrogenolysis) is the replacement of a chlorine substituent with a hydrogen atom. The pathway shown in Figure 2.1 is an example of four subsequent reductive dechlorination steps leading from PCE to environmentally benign ethene. Each dechlorination step

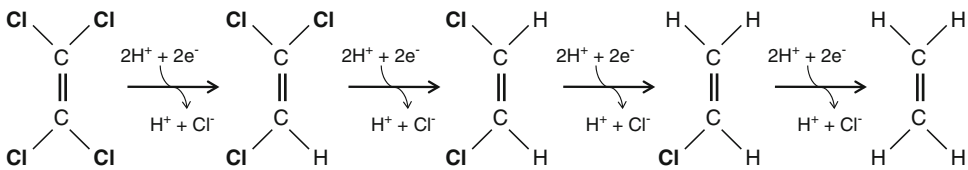

Figure 2.1. Reductive dechlorination pathway leading to detoxification of PCE and TCE. Dichloroethenes (i.e., *cis*-DCE [shown], *trans*-DCE and 1,1-DCE) and vinyl chloride (VC) are also toxic and VC is a proven human carcinogen, so complete dechlorination to ethene is required to achieve detoxification. The depicted dechlorination reactions are catalyzed most efficiently by bacteria capable of organohalide respiration. To date, all bacteria capable of DCE and VC dechlorination to ethene belong to the *Dhc* group.

consumes two electrons and two protons and releases H^+ and Cl^-. The two electrons are added to the organic substrate resulting in a reduced product. The oxidation state of the chlorine substituent is −1 and does not change upon release as chloride (i.e., Cl^-). A special case of reductive dechlorination is vicinal reduction or dichloroelimination, which can occur when aliphatic chloroorganic compounds have chlorine substituents located on adjacent saturated carbon atoms. An example is the reductive dechlorination of 1,2-dichloropropane to propene, which was observed in anaerobic mixed cultures containing *Dehalococcoides* (*Dhc*) (Löffler et al., 1997a; Ritalahti and Löffler, 2004a) and *Dehalobacter* (Schlötelburg et al., 2000, 2002).

As depicted in Figure 2.1, PCE and TCE can be reductively dechlorinated stepwise to less chlorinated ethenes. Cometabolic reductive dechlorination of PCE and TCE was first observed in methanogenic cultures (Bouwer and McCarty, 1983; Fathepure et al., 1987; Vogel and McCarty, 1985). Under conditions of anaerobic growth, methanogens and other microbial groups harbor abundant reduced transition-metal cofactors that fortuitously dechlorinate PCE and TCE. Unfortunately, the cometabolic reductive dechlorination rates decrease by an order of magnitude with each chlorine substituent removed, leading to the accumulation of *cis*-DCE and VC and detoxification (i.e., ethene formation) is not achieved (Gantzer and Wackett, 1991). Reductive dechlorination reactions are thermodynamically favorable and are associated with a considerable change in free energy under standard conditions at pH 7 ($\Delta G^{\circ\prime}$) ranging from approximately −140 to −172 kilojoules (kJ)/reaction (Dolfing and Janssen, 1994; Dolfing, 2000). A fundamental breakthrough was the discovery of *Desulfomonile tiedjei*, a Deltaproteobacterium that derives energy from reductive dechlorination of 3-chlorobenzoate to benzoate (DeWeerd et al., 1991; Suflita et al., 1982). In other words, this bacterium uses 3-chlorobenzoate as a terminal electron acceptor (just like mammals breathe oxygen), captures the energy released in replacing the chlorine substituent with a hydrogen atom and grows via reductive dechlorination as the sole energy source.

This process has been named chlororespiration, dechlororespiration, halorespiration, dehalorespiration, chloridogenesis, catabolic reductive dechlorination, metabolic reductive dechlorination or respiratory reductive dechlorination. All these terms are justified in their own right but this flurry of names is clearly confusing to experts and non-experts. The key information relevant for bioremediation is that the organisms gain energy for maintenance or growth from reductive dechlorination reactions (i.e., the organisms benefit from contaminant transformation) and the term ***organohalide respiration*** accurately describes this process.

The discovery of organohalide-respiring bacteria sparked new dialogue as to how dechlorinating microbes can be obtained. The new strategy provided chlorinated compounds like PCE and TCE as electron acceptors supplied to microcosms with the hope that microbes respiring CAHs would grow and could be enriched. This strategy proved fruitful, and *Dehalobacter restrictus* was the first organism described that grew with PCE as electron acceptor (Holliger et al., 1993, 1998). *Dehalobacter restrictus* dechlorinated PCE to *cis*-DCE as the end product, so detoxification was not achieved. *Dehalobacter* isolates have a highly specialized metabolism and require PCE or TCE as electron acceptors; strain PER23 and strain TEA require hydrogen as electron donor. Subsequently, several PCE-to-*cis*-DCE-dechlorinating bacteria belonging to the Firmicutes and the delta/epsilon subdivisions of the Proteobacteria were isolated and characterized (Table 2.3). The substrate range of *Dehalobacter* spp. is not restricted to chlorinated alkenes and strain TCA1 gains energy from the reductive dechlorination of 1,1,1-trichloroethane to chloroethane (Sun et al., 2002). Recent work demonstrated that a *Dehalobacter* strain present in a 1,1,1-trichloroethane-dechlorinating consortium coupled chloroform-to-dichloromethane reductive dechlorination with growth, providing the first evidence for the existence of microbes capable of organohalide respiration with chlorinated methanes (Grostern et al., 2010).

Table 2.3. Examples of Bacterial Isolates That Partially Dechlorinate PCE to TCE or *cis*-DCE

PCE Dechlorinating Isolate	Dechlorination Activity	Reference
Dehalobacter restrictus	PCE to *cis*-DCE	Holliger et al., 1998; Wild et al., 1996
Desulfuromonas chloroethenica	PCE to *cis*-DCE	Krumholz et al., 1996; Krumholz, 1997
Desulfuromonas michiganensis	PCE to *cis*-DCE	Sung et al., 2003
Sulfurospirillum multivorans	PCE to *cis*-DCE	Luijten et al., 2003; Neumann et al., 1994
Sulfurospirillum halorespirans	PCE to *cis*-DCE	Luijten et al., 2003
Geobacter lovleyi	PCE to *cis*-DCE	Sung et al., 2006a
Desulfitobacterium sp. strain PCE-S	PCE to *cis*-DCE	Miller et al., 1997
Desulfitobacterium hafniense strain TCE1	PCE to *cis*-DCE	Gerritse et al., 1999
Desulfitobacterium hafniense strain Y51	PCE to *cis*-DCE	Suyama et al., 2001
Desulfitobacterium hafniense strain JH1	PCE to *cis*-DCE	Fletcher et al., 2008
Desulfitobacterium sp. strain PCE1	PCE to TCE	Gerritse et al., 1996
Desulfitobacterium sp. strain Viet1	PCE to TCE	Löffler et al., 1997b; Tront et al., 2006

Compared with *Dehalobacter*, these isolates exhibited more versatile metabolisms and used a greater variety of electron acceptors and electron donors for growth. In contrast to *Dehalobacter* strains, PCE-dechlorinating *Desulfuromonas* isolates cannot use hydrogen but use several reduced organic compounds, including acetate as electron donors (Krumholz et al., 1996; Sung et al., 2003). *Geobacter lovleyi* strain SZ uses both acetate and hydrogen as electron donors and was the first PCE-dechlorinating isolate within the *Geobacter* group. Several members of this bacterial group are well known for their ability to reduce metals and radionuclides and *Geobacter lovleyi* reduces PCE and hexavalent uranium simultaneously, suggesting that organisms of this type are promising for remediation of radionuclides and PCE at mixed waste sites (Sung et al., 2006a). *Sulfurospirillum multivorans* (formerly *Dehalospirillum multivorans*) is a well-studied PCE dechlorinator because this organism is easy to culture and high biomass yields facilitate biochemical studies (John et al., 2009; Luijten et al., 2003; Neumann et al., 1996; Scholz-Muramatsu et al., 1995). Several *Desulfitobacterium* isolates were described to dechlorinate PCE, typically to *cis*-DCE as the dechlorination end product, but *Desulfitobacterium* sp. strains PCE1 and Viet1 reduce PCE to TCE. This is an interesting observation because the characterized PCE reductive dehalogenases (RDases) from *Sulfurospirillum*, *Dehalobacter* and *Desulfitobacterium* reduce PCE to *cis*-DCE (Maillard et al., 2003; Miller et al., 1998; Neumann et al., 1996; Suyama et al., 2002), suggesting that strain PCE1 and strain Viet1 possess unique PCE-to-TCE reductive dehalogenase enzyme systems. In any case, a diverse bacterial group contributes to PCE-to-*cis*-DCE dechlorination but the microbes contributing to dechlorination past *cis*-DCE remained elusive.

2.1.3 Discovery of *Dehalococcoides*

In 1989, Freedman and Gossett (Freedman and Gossett, 1989) published a seminal paper that demonstrated that microbes capable of reductive dechlorination past *cis*-DCE to ethene exist. Subsequent studies by DiStefano and Gossett (DiStefano et al., 1991, 1992) showed that an anaerobic PCE-dechlorinating enrichment culture converted high concentrations of PCE to ethene at unprecedented rates and that hydrogen (H_2) served as the electron donor for dechlorination. These results indicated that reductive dehalogenation in these cultures was a catabolic process analogous to 3-chlorobenzoate dehalogenation by *Desulfomonile tiedjei* (Suflita et al., 1982), whereas the conventional wisdom at that time was that reductive dehalogenation of chloroethenes was a slow and cometabolic process carried out by methanogens and some other anaerobes. A few researchers, notably Perry McCarty from Stanford University, were more visionary and encouraged colleagues to search for novel dechlorinators, and subsequent studies by Gossett and Zinder led to the isolation of an unusual bacterium that dechlorinated PCE to VC and ethene (Maymó-Gatell et al., 1997) from Freedman and Gossett's enrichment culture (Freedman and Gossett, 1989). They called this isolate "Dehalococcoides ethenogenes" strain 195 because individual cells were round and the culture produced ethene from PCE. More detailed investigations revealed that strain 195 grew with PCE, TCE, *cis*-DCE and 1,1-DCE but not with VC as electron acceptors. VC was slowly and cometabolically dechlorinated to ethene after utilization of all polychlorinated ethenes (Maymó-Gatell et al., 2001).

Nevertheless, the discovery of strain 195 demonstrated the existence of microbes that can overcome the "DCE stall"; however, the formation of VC remained a major concern for bioremediation applications. Subsequent identification of PCE-to-ethene-dechlorinating enrichment cultures that produced ethene without significant VC accumulation coupled with hydrogen consumption to very low concentrations provided strong evidence for VC-respiring microbes (Löffler et al., 1999). Hydrogen consumption to certain threshold concentrations serves as a measure of the free energy change associated with the hydrogen-consuming oxidation reduction reaction (Löffler and Sanford, 2005). The low consumption concentrations measured in VC-dechlorinating cultures indicated that microbes present in these enrichment cultures gained energy from VC-to-ethene reductive dechlorination (Löffler et al., 1999). A milestone discovery was the isolation of *Dhc* sp. strain BAV1, the first isolate capable of organohalide respiration of VC to ethene (He et al., 2003b). Additional *Dhc* isolates, strain GT (Sung et al., 2006b), and strain VS (Müller et al., 2004), that both grew with VC as electron acceptor were subsequently described, and several research groups obtained *Dhc*-containing mixed cultures that dechlorinate chlorinated ethenes to ethene (e.g., Duhamel et al., 2002; Richardson et al., 2002; Vainberg et al., 2009). Table 2.4 depicts the available *Dhc* isolates and several consortia containing *Dhc* strains capable of chlorinated ethene reductive dechlorination. The genus *Dehalococcoides* has recently been published, and all known *Dhc* strains are classified as members of the same species, *Dehalococcoides mccartyi* (Löffler et al., 2012).

The dechlorination range of *Dhc* is not restricted to chlorinated alkenes and alkanes. *Dhc mccartyi* strain 195 has been demonstrated to dechlorinate several chlorinated aromatic compounds including chlorinated benzenes, chlorinated phenols, polychlorinated-dibenzo-p-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) although growth has not been demonstrated with all of these substrates (Adrian et al., 2007a; Fennell et al., 2004; Liu and Fennell, 2008). *Dhc* strain CBDB1 was isolated with trichlorobenzenes as catabolic electron acceptors, which were dechlorinated to dichlorobenzenes (Adrian et al., 2000b). In addition to polychlorinated chlorobenzenes, this isolate dechlorinates certain polychlorinated phenols and some PCDD and PCB congeners (Adrian et al., 2007a, 2009; Bunge et al., 2003; Jayachandran

Table 2.4. Utilization of Chlorinated Ethenes and Other Chloroorganic Compounds by Different *Dhc* Strains and *Dhc*-Related Bacteria Available as Pure Cultures or Present in Dechlorinating Consortia

Culture	Chlorinated Ethenes Used as Electron Acceptors	Chlorinated Ethenes Co-metabolized	Major End Product(s)	Substrate Range Includes[a]	Reference
Strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE	*trans*-DCE, VC	VC, ethene	1,2-DCA, 1,2-dibromoethane, PCBs, PCDDs, chlorinated naphthalenes, chlorobenzenes	Maymó-Gatell et al., 1997, 1999; Seshadri et al., 2005; Löffler et al. 2012
Strain BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC	PCE, TCE	Ethene	1,2-DCA, vinyl bromide	He et al., 2003b
Strain FL2	TCE, *cis*-DCE, *trans*-DCE	PCE, VC	VC, ethene	ND	He et al., 2005
Strain GT	TCE, *cis*-DCE, 1, 1-DCE, VC	None	Ethene	ND	Sung et al., 2006b
Strain VS[b]	TCE, *cis*-DCE, 1, 1-DCE, VC	None	Ethene	ND	Cupples et al., 2003; Müller et al., 2004
Strain CBDB1	None	PCE, TCE	*trans*-DCE (*cis*-DCE)	Chlorobenzenes, PCDDs, PCBs	Adrian et al., 2000b; Kube et al., 2005; L. Adrian, pers. commun
Dehalobium chlorocoercia strain DF1	PCE, TCE	None	*trans*-DCE, *cis*-DCE	PCBs; HCB, PeCB	May et al., 2008; Miller et al., 2005
Dehalogenimonas lykanthroporepellens strain BL-DC-9	None	None	–	1,2,3-TCP, 1,2-D, 1,1,2,2-TeCA, 1,1, 2-TCA, 1,2-DCA	Yan et al., 2008
***Dhc*-Containing Mixed Cultures**					
Culture RC (containing one *Dhc* strain)	None	None	–	1,2-D	Löffler et al., 1997a; Ritalahti and Löffler, 2004a

(*continued*)

Table 2.4. (continued)

Culture	Chlorinated Ethenes Used as Electron Acceptors	Chlorinated Ethenes Co-metabolized	Major End Product(s)	Substrate Range Includes[a]	Reference
Culture KS (containing one *Dhc* strain)	None	None	–	1,2-D	Löffler et al., 1997a; Ritalahti and Löffler, 2004a
Culture KB-1 (containing two *Dhc* strains)	TCE, *cis*-DCE, VC	ND	Ethene	Some chlorobenzenes	Duhamel and Edwards, 2006; E. Edwards, pers. commun
ANAS (containing two *Dhc* strains)	TCE, *cis*-DCE, VC	ND	Ethene	ND	Holmes et al., 2006; Richardson et al., 2002
SDC-9	PCE, TCE, *cis*-DCE, 1,1- DCE, VC	None	Ethene	CT, CF, DCM, 1,1,1-TCA, 1,1-DCA, CFC-113, CFC-11	Schaefer et al., 2009; Vainberg et al., 2009

Note that growth with the chlorinated electron acceptors has not in all cases been conclusively demonstrated
Note that in older literature, the abbreviation "t" is used to indicate the "total" concentration of DCEs (i.e., *cis*-DCE, *trans*-DCE and 1,1-DCE). With the enhanced resolution of newer capillary column technology the three DCE isomers can be resolved and the term "tDCE" indicates *trans*-DCE
[a]PCBs—polychlorinated biphenyls; PCDDs—polychlorinated dibenzo-*p*-dioxins; HCB—hexachlorobenzene; PeCB—pentachlorobenzene; 1,2,3-TCP—1,2,3-trichloropropane; 1,2-D—1,2-dichloropropane; 1,1,2,2-TeCA—1,1,2,2-tetrachloroethane; 1,1,2-TCA—1,1,2-trichloroethane; 1,1-DCA—1,1-dichlororethane; 1,2-DCA—1,2-dichloroethane; CFC-113—1,1,2-trichloro-1,2,2-trifluoroethane; CFC-11—trichlorofluoromethane
ND—not determined

et al., 2004). Strain CBDB1 appears to be specialized to dechlorinate chlorinated aromatic compounds and no chlorinated aliphatic compound has been found that supports its growth. Since all *Dhc* strains contain multiple predicted reductive dehalogenase (RDase) genes on their genomes (see below), the true substrate spectrum is likely to exceed the range of chlorinated electron acceptors currently described. For example, the DCE- and VC-dechlorinating *Dhc* isolate strain BAV1 has been implicated in debromination of polybrominated diphenyl ethers (He et al., 2006)

2.2 *DEHALOCOCCOIDES* ISOLATION AND CULTIVATION STRATEGIES

2.2.1 General Considerations

Dhc are slow growing, strict anaerobes that require anoxia and reducing conditions for growth. Even brief exposure to air can be detrimental and kill *Dhc* (Adrian et al., 2000b; Amos et al., 2008a). Stringent anoxic techniques are essential and required reducing conditions are established by the addition of chemical reductants to the medium (see below). *Dhc* in mixed cultures, where the presence of oxygen-consuming microbes affords some protection against oxygen, are more robust than pure cultures but still require faithful application of anoxic techniques. Moreover, it is commonly observed that *Dhc* populations grow more slowly and become more fastidious as they are purified from other organisms; only a few laboratories worldwide have succeeded in obtaining and maintaining *Dhc* isolates (Adrian et al., 2000b; He et al., 2003b, 2005; Maymó-Gatell et al., 1997; Müller et al., 2004; Sung et al., 2006b). Specific techniques for *Dhc* cultivation and isolation are available in the aforementioned publications, and a summary by Löffler et al. (2005) provides detailed guidance and protocols. The following section provides an overview of the general principles involved in *Dhc* cultivation.

An enrichment culture for *Dhc* or other dechlorinators is typically initiated by transferring an inoculum (1–10%, vol/vol) from a microcosm that exhibits the desired reductive dehalogenation reaction(s). The mineral composition of the growth medium used should be as similar as possible to that of the original habitat, particularly with regard to dominant salts. Concentrations of the important mineral nutrients ammonium and phosphate need to be higher than present *in situ*, but their concentrations should be maintained below 1 mM. To provide trace metals, chloride salts of minerals should be used rather than sulfate salts because sulfate serves as an electron acceptor for sulfate-reducing bacteria. The described *Dhc* grow best around pH 7 and the pH of the enrichment should be buffered to near neutrality. Commonly, a carbon dioxide/bicarbonate (CO_2/HCO_3^-) buffer system is used. Oxygen removal is essential and the medium must be chemically reduced prior to inoculation with *Dhc*. Culture vessels should be sealed with thick butyl rubber stoppers. Teflon®-coated septa also will work, but require more experience with anoxic cultivation techniques because leakage of gases out of or into the culture vessel is more difficult to control. Ideally, slight positive pressure is maintained in the culture vessels to avoid oxygen contamination during sampling events. Hydrophobic chlorinated compounds, such as PCE and TCE, are notorious for "disappearing" due to sorptive losses. Teflon®-lined rubbers stoppers help to minimize sorptive losses but are more prone to leakage of volatile compounds out of, and oxygen into, the culture vessels. Hence, thick rubber stoppers are more appropriate to cope with the oxygen sensitivity of *Dhc*. In any event, appropriate control vessels are needed, and the measurement of daughter product formation is always a more reliable indicator of dechlorination activity than is substrate (e.g., PCE, TCE) disappearance.

2.2.2 Electron Acceptor

The electron acceptor provided for *Dhc* enrichment is the target halogenated organic compound (e.g., chlorinated ethenes). Ideally, the concentration of the target compound should be as high as possible to maximize growth of dehalogenating organisms while avoiding toxicity. Since toxicity cannot be predicted *a priori*, a good preliminary approach is to add the chlorinated compound at the minimum concentration that is analytically tractable and supply more electron acceptor to the cultures as it is consumed. The low solubility and high toxicity of many chlorinated compounds often indicates that their concentrations will be well below the millimolar concentrations typically used for enrichment and cultivation of microbes using more common electron acceptors such as sulfate or nitrate.

One potentially useful strategy to provide larger amounts of toxic, lipophilic CAHs, is to dissolve them in an inert hydrophobic "carrier" phase. For example, dissolving PCE in hexadecane, which is metabolized slowly, if at all, by most anaerobic cultures, allows addition of larger amounts of chlorinated electron acceptor while keeping the aqueous concentration low (Holliger et al., 1993; Krumholz et al., 1996; Löffler et al., 2005). Although this procedure maintains constant, low chlorinated electron acceptor concentrations in the aqueous phase, utilizing a carrier compound has drawbacks. The addition of a separate organic phase complicates analysis of hydrophobic compounds due to partitioning, the organic phase may sequester aqueous phase micronutrients (e.g., lipophilic vitamins) retarding *Dhc* growth, and the separate phase may interfere with downstream procedures such as biomass collection and deoxyribonucleic acid (DNA) extraction.

Whether provided with an organic carrier phase or directly (i.e., undiluted) into the growth medium, aqueous PCE concentrations above 0.54 mM, about half its solubility limit, inhibit dechlorination and growth of pure cultures (Amos et al., 2007). Although PCE-to-ethene-dechlorinating consortia may tolerate slightly higher PCE concentrations (Amos et al., 2007, 2009), growth of dechlorinators at saturated aqueous phase PCE concentrations has not been demonstrated. Thus, sustained growth in batch cultures without a carrier phase will require repeated feedings when the chlorinated electron acceptor has been consumed. A prerequisite, of course, is that no inhibitory products accumulate. Maintaining elevated concentrations of PCE that are tolerated by the dechlorinators has been shown to inhibit methanogenic archaea, and thus can eliminate certain microorganisms (DiStefano et al., 1991). The Löffler laboratory web site (University of Tennessee, Knoxville, Department of Microbiology) offers an Excel spreadsheet that conveniently calculates the amounts of chlorinated solvent added to the medium undiluted or dissolved in a carrier phase to achieve a target aqueous phase electron acceptor concentration.

2.2.3 Electron Donor

Pure *Dhc* cultures use only hydrogen as an electron donor. Hydrogen also supports growth of methanogens and acetogenic bacteria and hydrogen consumption may result in negative pressure in the culture vessels. Therefore, hydrogen may not be the best electron donor for enrichment. In initial studies of the PCE-dehalogenating enrichment culture from which strain 195 was isolated, methanol appeared to be a suitable electron donor (Freedman and Gossett, 1989). In retrospect, this was probably because methanol-utilizing methanogens and acetogens were supplying *Dhc* with vitamin B_{12}, a required growth factor (see below) and reducing equivalents, presumably as hydrogen. Typically, an electron donor that generates hydrogen upon fermentation, such as lactate, butyrate or benzoate is used. The latter two compounds have an advantage in that their fermentation proceeds slowly under anoxic conditions due to

thermodynamic constraints (Schink, 1997). Hence, the hydrogen concentration is poised at a level too low to be used by carbon dioxide-reducing homoacetogens and is used only slowly by methanogens, while *Dhc*, carrying out thermodynamically more favorable reductive dechlorination reactions, can outcompete the methanogens for electron donor (Fennell et al., 1997; Löffler et al., 1999). A disadvantage of these electron donors is that thermodynamically constrained fermentation processes generally are slow and the organisms oxidizing butyrate or benzoate also grow slowly. This slow growth may result in extended lag times before dechlorination is observed and requires long-term incubation (several weeks to months) and monitoring.

2.2.4 Carbon Source

In addition to the electron donor and acceptor, other nutrients are needed to support *Dhc* growth. Acetate, a carbon source for *Dhc*, is produced from any electron donor supporting reductive dechlorination, including hydrogen, in microcosms and mixed communities (He et al., 2002). Hence, the addition of acetate is not needed, but acetate is commonly included in medium formulations at 0.5–5 mM concentrations. Vitamin mixtures are a useful addition to enrichments, especially vitamin B_{12} (see below). Vitamins are added in trace concentrations, and thus should not support growth of contaminating organisms. Low concentrations (<10 mg/L) of undefined organic substrates like yeast extract provide diverse nutrients (but not vitamin B_{12}, which eukaryotes cannot produce), and may help stabilize the mixed culture by allowing growth of accessory organisms that consume oxygen and/or provide nutrients to *Dhc*; however, these organisms will then become "contaminants" when isolation is attempted, and such additions will then need to be eliminated. The addition of higher concentrations of complex organic nutrients should be avoided because *Dhc* are adapted to oligotrophic (i.e., nutrient-poor) environments with dechlorination and growth inhibited under nutrient-rich conditions (Maymó-Gatell et al., 1997).

2.2.5 Reducing Agent (Reductant)

Another crucial component of growth media for anaerobes is the reducing agent, which removes traces of oxygen from the system as well as poises the redox potential. In mixed cultures, oxygen-consuming organisms can serve these purposes and spare the requirement for a reducing agent, but as cultures are enriched, the reducing agent becomes increasingly important. Commonly used reductants include sodium sulfide, L-cysteine, DL-dithiothreitol, iron sulfide and titanium(III) citrate. These reducing agents can be supplied in combination and will generate redox conditions suitable for *Dhc* activity and growth; however, it is best to determine empirically which reducing agents are most suitable to achieve robust dechlorination activity in a given culture. An important aspect is the supply of sulfur, a required macronutrient for all organisms. Some of the synthetic, defined medium recipes described for *Dhc* pure cultures do not contain any sulfur. Hence, the use of sulfur-containing reducing agents is pertinent unless another suitable sulfur source is provided. Unfortunately, sulfur metabolism in *Dhc* has not been explored and the type and concentration of a sulfur source for optimal growth is unknown.

2.2.6 Incubation Conditions

Enrichments typically are incubated at room temperature (20–25°C) under stationary conditions. Agitation has not been shown to improve growth unless hydrogen is provided as electron donor and mass transfer of the electron donor from the gas to the aqueous phase limits dechlorination. Incubation at 30°C also may increase rates, but this should be determined only after enrichments have been established.

2.2.7 Isolation

After successful enrichment of reductive dehalogenators is achieved, isolation can be considered. Specific isolation techniques for *Dhc* have been described by Löffler et al. (2005). Isolation is essentially a numbers game, in which the chances of success increase as the organisms of interest begin to outnumber other organisms. Thus, the enrichment conditions should be optimized to maximize growth of the organohalide respirers and minimize growth of other "contaminating" microorganisms.

2.3 *DHC* PURE CULTURES

2.3.1 Isolation of *Dhc mccartyi* Strain 195

In the case of the first isolation of *Dhc mccartyi* strain 195, several properties of the dechlorinator facilitated isolation. The ability of strain 195 to withstand high concentrations of PCE that were inhibitory to methanogens allowed the elimination of methanogenic archaea (DiStefano et al., 1991). This was particularly fortuitous, since 2-bromoethane sulfonate, often used as an inhibitor of methanogenesis, also can inhibit reductive dehalogenation of chlorinated ethenes (DiStefano et al., 1992; Löffler et al., 1997b). A feature of *Dhc* that is particularly useful for isolation is the lack of a peptidoglycan cell wall typically found in bacteria. This feature makes *Dhc* resistant to antibiotics that interfere with peptidoglycan biosynthesis, like vancomycin (DiStefano et al., 1992) and ß-lactam antibiotics such as ampicillin (He et al., 2003b; Maymó-Gatell et al., 1997). Treatment with these antibiotics helps eliminate many bacterial contaminants, including acetogens, a group that can convert methanol or hydrogen plus carbon dioxide to acetate, and rapidly outgrow *Dhc*. A final useful attribute of *Dhc* is its small cell size; *Dhc* cells can pass through 0.45-micrometer (μm) membrane filters that retain many other bacteria.

In the original studies leading to the isolation of strain 195, enrichment cultures were grown in medium supplemented with hydrogen, PCE, acetate, vitamins (including high levels of vitamin B_{12}), filter-sterilized extract of anaerobic digestor sludge, and sulfide as a reducing agent (Maymó-Gatell et al., 1995). These cultures dechlorinated PCE when transferred to medium with 1 gram per liter (g/L) ampicillin, an antibiotic that inhibited growth of all contaminating bacteria, but subsequent transfers showed significantly reduced dechlorination activity. Therefore, transfer cultures were supplemented with filter-sterilized extracts from the original mixed culture containing active *Dhc*, which allowed reductive dechlorination of PCE and growth in transfer cultures. This culture underwent serial dilutions in liquid medium and the culture obtained from the 10^{-7} dilution tube only contained tiny, flattened cocci. This disc-shaped morphology was maintained in medium lacking ampicillin, and this pure culture of strain 195 could ultimately be transferred repeatedly in defined mineral salts medium amended with the PCE, acetate, hydrogen and the Wolin vitamin mixture (He et al., 2007; Wolin et al., 1963).

Strain 195 uses PCE, TCE, *cis*-DCE and 1,1-DCE as electron acceptors for growth, dechlorinating them to VC. VC is slowly dechlorinated to ethene, but this process is cometabolic and does not support growth (Maymó-Gatell et al., 1999). Other electron acceptors used by strain 195 include 1,2-dichloroethane, which is dehalogenated mainly to ethene with trace amounts of VC, and some polychlorinated phenols including 2,3-dichlorophenol, from which only the ortho-chlorine is removed (Adrian et al., 2007a). *Dhc* strain 195 also dechlorinates chlorobenzenes with four or more chlorine substituents and some PCB and PCDD congeners (Fennell et al., 2004) (Table 2.4).

2.3.2 Isolation of *Dhc* sp. Strain CBDB1

The next *Dhc* isolate described in the literature was strain CBDB1, which was obtained from microcosms established with sediment from the Saale River, Germany, amended with 1,2,3- and 1,2,4-trichlorobenzene (Adrian et al., 2000b). Strain CBDB1 grows with some polychlorinated benzenes and polychlorinated phenols, and dechlorinates other chlorinated aromatic compounds such as PCDD and PCB congeners, although growth with these compounds has not been conclusively demonstrated (Adrian et al., 2007a, 2009; Bunge et al., 2003) (Table 2.4). Strain CBDB1 grows in synthetic mineral salts medium amended with acetate, a growth-supporting chlorinated benzene, vitamins and titanium(III) citrate as reducing agent. Remarkably, growth occurred without a sulfur source suggesting that traces of sulfur, likely supplied with other medium components, fulfilled *Dhc* requirements for this macronutrient (Adrian et al., 2000a). Isolation was achieved by repeated dilution in low-melting agarose tubes, where tiny colonies formed a few millimeters below the surface, suggestive of inhibition by trace amounts of oxygen in the headspace (Adrian et al., 2000b).

2.3.3 Isolation of *Dhc* sp. Strain FL2

Dhc strain FL2 is the only described *Dhc* isolate to date that was obtained from an uncontaminated sediment (i.e., no reported chlorinated solvent contamination) (He et al., 2005). Initial transfers received PCE as electron acceptor but following ampicillin treatment, PCE dechlorination ceased and only occurred when TCE was present. Strain FL2 dechlorinates TCE, *cis*-DCE and *trans*-DCE to VC and ethene. PCE and VC are co-metabolically dechlorinated in the presence of a growth-supporting electron acceptor, which explains why the enrichment failed to grow with PCE following elimination of a PCE dechlorinator susceptible to the antibiotic treatment. The enrichment process yielded a co-culture consisting of *Dhc* strain FL2 and two unusual spirochetes, which belonged to the novel genus *Sphaerochaeta* and were designated the free-living, pleomorphic spirochetes (FLiPS) (He et al., 2005; Ritalahti and Löffler, 2004b, 2005; Ritalahti et al., 2012). This co-culture exhibited robust TCE dechlorination activity but in axenic strain FL2 cultures, dechlorination rates slightly decreased and lag times following transfers increased noticeably, suggesting the FLiPS provided some service to *Dhc*. Such microbe-microbe interactions are the subject of intense study and will help to manipulate (i.e., enhance) and predict *in situ Dhc* activity.

2.3.4 Isolation of *Dhc* Strains That Respire VC: Strains BAV1, GT and VS

To date, three *Dhc* isolates capable of using VC as an electron acceptor for growth have been obtained. Their isolation proved to be milestone discoveries and suggested that efficient *in situ* bioremediation of chlorinated ethenes in anoxic aquifers is feasible. The isolation of

Dhc strain BAV1 was facilitated by the enrichment culture's ability to generate hydrogen, which is the required electron donor for strain BAV1 to dechlorinate VC to ethene, from acetate. Acetate fermentation is thermodynamically feasible at low hydrogen partial pressures, and hydrogen consumption by strain BAV1 maintained conditions conducive for mesophilic acetate oxidation (He et al., 2002).

Although dechlorination with acetate as the only source of reducing equivalents was slow and required long incubation periods to demonstrate significant VC dechlorination with *Dhc* growth, repeated transfers under these conditions eliminated contaminating organisms and ultimately enabled the isolation of *Dhc* strain BAV1 by ampicillin treatment and dilution-to-extinction series in low melting agarose dilution tubes (He et al., 2003a; Löffler et al., 2005). Unfortunately, the organisms responsible for syntrophic acetate oxidation in this enrichment culture have not been identified. Strain BAV1 grows in a defined medium that, in addition to acetate and vitamins, contains L-cysteine, as well as low amounts of sulfide (0.1–0.2 mM) as reducing agents.

Strain BAV1 grows with all DCE isomers, VC and 1,2-dichloroethane as electron acceptors and generates ethene and inorganic chloride as products. Strain BAV1 does not grow with PCE or TCE but is able to dechlorinate these compounds when growth-supporting DCEs or VC are available, in which case BAV1 converts all chlorinated ethenes to ethene. The cometabolic dechlorination of PCE and TCE generates growth-supporting *cis*-DCE. This represents a unique form of cometabolism because strain BAV1 ultimately benefits from the fortuitous dechlorination of PCE and TCE by generating the growth-supporting electron acceptor *cis*-DCE.

Similar enrichment strategies yielded *Dhc* isolate GT and culture VS (Müller et al., 2004; Sung et al., 2006b). The isolation efforts for strain GT yielded a culture that only contained *Dhc* cells and a single 16S ribosomal ribonucleic acid (rRNA) gene sequence was detected. Interestingly, quantitative real-time polymerase chain reaction (qPCR) monitoring of *Dhc* 16S rRNA genes and the reductive dehalogenase genes *tceA*, *bvcA*, and *vcrA* (see below) revealed that this culture consisted of multiple *Dhc* strains that shared an identical 16S rRNA gene. Consecutive enrichments with different chlorinated ethenes yielded a pure culture that consisted of a single *Dhc* strain designated strain GT (Sung et al., 2006b).

Dhc strains GT and VS generate ethene as dechlorination end product but, in contrast to strain BAV1, use TCE as growth-supporting electron acceptor. Both strains GT and VS harbor the VC RDase VcrA, which is different from the VC-dechlorinating enzyme system BvcA of strain BAV1. Interestingly, both strain GT and strain VS fail to dechlorinate PCE even in the presence of a growth-supporting electron acceptor (e.g., TCE) suggesting that both strains possess a unique TCE RDase that differs from the TCE-dechlorinating enzyme system(s) of strains FL2 and 195.

2.3.5 Isolation of *Dhc* Strain MB

Dhc populations have been implicated in PCE and TCE dechlorination to *trans*-DCE rather than *cis*-DCE (Griffin et al., 2004). The recently described isolate MB produced predominantly *trans*-DCE; however, this organism cannot dechlorinate DCEs and VC to ethene (Cheng and He, 2009). Isolate MB was obtained from a PCE-to-DCE-dechlorinating microcosm established with San Francisco Bay sediment. The isolation process used the dilution-to-extinction principle in a minimal medium amended with acetate, hydrogen and PCE. Sequential transfers in the presence of ampicillin increased the *trans*-DCE to *cis*-DCE ratio, presumably by inhibiting PCE-to-*cis*-DCE dechlorinators, which also were present in the enrichment cultures (Cheng and He, 2009). While the original microcosms dechlorinated PCE to about equal amounts of *trans*-DCE and *cis*-DCE, isolate MB produces about seven times more *trans*-DCE than *cis*-DCE.

2.4 MAINTENANCE OF *DEHALOCOCCOIDES* PURE CULTURES

2.4.1 General Considerations

Growth and maintenance of *Dhc* pure cultures is difficult and requires experience, strictly anoxic techniques and faithful transfers to reduced medium amended with toxic chlorinated compounds as electron acceptors. Cultures should be transferred when dechlorination ceases due to substrate limitations (i.e., electron donor or electron acceptor consumed). *Dhc* in mixed cultures exhibit shorter lag times following transfers, grow faster and exhibit higher dechlorination rates than pure *Dhc* cultures.

Mixed cultures with *Dhc* strains generally outperform pure cultures because other members of the microbial community protect *Dhc* from toxic oxygen or provide required growth factors for which *Dhc* lacks the biosynthetic machinery. For example, *Dhc* cultures require the cobalt-containing corrinoid cofactor vitamin B_{12}, (He et al., 2007). Optimal dechlorination and growth occur at vitamin B_{12} concentrations ranging from 25 to 50 micrograms per liter (μg/L), considerably higher amounts than the 1 μg/L concentration of standard vitamin amendments (Balch et al., 1979; Wolin et al., 1963). Activity has been restored easily in mixed cultures that are not fed, or stored at 4°C for several months. In addition, anoxic freezer stocks (7% dimethylsulfoxide [DMSO]) of 100-fold concentrated biomass prepared in 2-mL cryogenic vials have been used successfully to maintain the bioaugmentation inoculum KB-1 (Day and Stacey, 2007).

Because *Dhc* pure cultures are less robust than their mixed culture counterparts, regular transfers are needed to maintain active cultures. Unfortunately, *Dhc* cannot be grown with alternate substrates and require often toxic halogenated compounds as electron acceptors. Maintaining *Dhc* pure cultures can be a frustrating experience for novice researchers, who are often not successful in their initial attempts to recover activity in transfer cultures. Not surprisingly, deposition of *Dhc* pure cultures in type culture collections has been challenging although recent efforts have been successful (Löffler et al., 2012).

2.4.2 Growth Factors

The vitamin B_{12} requirement of *Dhc* is attributed to the presumptive role of corrinoids as prosthetic groups of RDases (see below). The RDases are enzymes essential to reductive dechlorination and *Dhc* energy conservation, and thus growth. The genomes of *Dhc* strains contain genes encoding proteins involved in the uptake and modification of vitamin B_{12} and/or other corrinoid precursors, but not for their synthesis (Kube et al., 2005; Seshadri et al., 2005). It is curious that an organism would not be capable of synthesizing a compound so essential for growth but relies on other organisms to provide the nutrient(s). To date, a requirement for the addition of vitamin B_{12} to stimulate reductive dehalogenation in microcosms or at contaminated sites has not been demonstrated, indicating that the microbial community supplies this essential growth factor to *Dhc*. The genome sequence of strain 195 suggests that this strain also requires biotin since a gene predicted to encode a biotin transporter is present but genes for biotin synthesis are absent (Seshadri et al., 2005). Although a biotin requirement for *Dhc* has not been demonstrated, biotin is routinely present in vitamin mixtures or yeast extract added to cultures. *Dhc* are difficult to grow in isolation, possibly because current media formulations lack known or other, not yet identified, growth factors and stimulants. Hence, the comprehensive understanding of *Dhc* nutritional requirements and *Dhc* interactions with community members is relevant to enable in-depth laboratory studies and improve predictions of *in situ* dechlorination activity.

2.5 *DEHALOCOCCOIDES* MORPHOLOGY AND PHYSIOLOGY

Dhc are tiny bacteria with a flattened, round morphology approximately 0.5 µm in diameter and 0.1 µm thick. The first isolate was designated *Dehalococcoides*, indicating a spherical morphology, but additional microscopic analysis revealed a disc-shaped morphology with characteristic biconcave indentations on opposite flat sides of the cell. This donut-shape morphology resembles that of a red blood cell, except that the overall dimensions are much smaller (Figure 2.2).

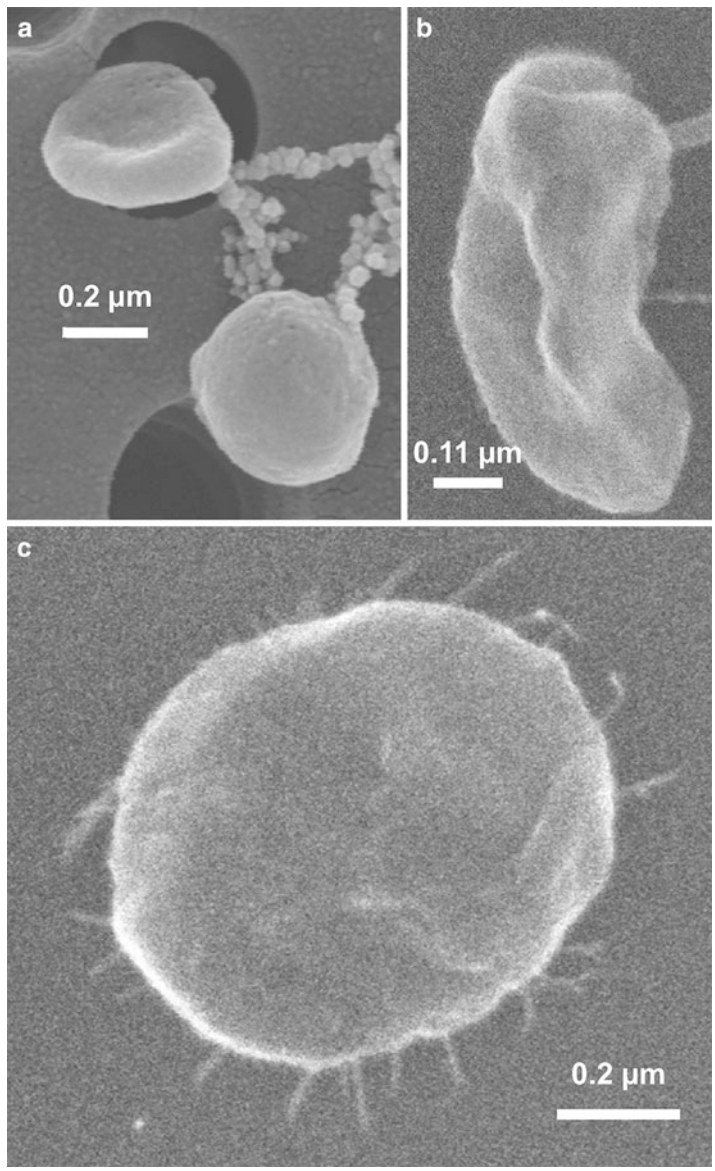

Figure 2.2. Scanning electron micrographs of *Dhc* strain FL2 (a) and BAV1 (b and c) (modified from He et al., 2003b). Micrographs a and b indicate the disc-shaped, indented cell morphology. Visible in the micrograph are the open pores of a 0.2-µm membrane filter demonstrating the small *Dhc* cell size. Micrograph c shows a BAV1 cell displaying peculiar appendages of unknown function.

Dhc cells are among the smallest bacteria described, and the volume of an individual cell is roughly 30-fold lower than that of an average bacterial cell (based on an average *E. coli* cell volume of 0.64 μm^3 and a *Dhc* cell volume of 0.02 μm^3) (Duhamel et al., 2004; Kubitschek, 1990). The small and disc-shaped size has implications for light microscopic observation because cells suspended between the glass slide and the cover slip tumble end over end. The highest resolution a light microscope achieves is 0.2 μm, which explains why *Dhc* cells are visible or "disappear", depending on their orientation in the light path. Inexperienced researchers can get easily frustrated when attempting microscopic observation because *Dhc* cells are difficult to distinguish from debris and some training is required to identify *Dhc* cells. Staining with fluorescent DNA-binding dyes like acridine orange or 4',6-diamidino-2-phenylindole (DAPI) facilitates microscopic observation and enumeration of *Dhc* cells (He et al., 2003b).

The small size and disc shape of *Dhc* cells also serves to maximize the surface area-to-volume ratio, which can aid in scavenging scarce substrates like haloorganics and hydrogen. The small cell size also implies that *Dhc* cell titers can actually be quite high (10^6–10^8 cells per mL), even when little biomass is present. Turbidity of *Dhc* pure cultures is generally very low and optical density measurements are not applicable for monitoring growth. Growth of *Dhc* pure cultures is best monitored by qPCR (see below) or by microscopic counts after staining the cells with a fluorescent dye and filtration onto black polycarbonate membranes (He et al., 2003b; Sanford et al., 2007).

Dhc are strictly hydrogenotrophic (i.e., they require hydrogen as the electron donor) and cannot use organic compounds to derive reducing equivalents for reductive dechlorination. These findings apply to all known *Dhc* and also to the few characterized affiliated dechlorinators included in the phylogenetic tree shown in Figure 2.3. Apparently, all organisms in this particular branch of the *Chloroflexi* are adapted to strictly hydrogenotrophic metabolism, using particular aliphatic and aromatic halogenated organic compounds as electron acceptors. *Dhc* dechlorination in mixed cultures is supported by organic compounds provided as the sole source of reducing equivalents due to the presence of (syntrophic) fermenters that generate hydrogen. In contrast, pure *Dhc* cultures depend on hydrogen as electron donor, which must be supplied to the culture vessels. Reductive dechlorination is a thermodynamically favorable process and accordingly, hydrogen consumption threshold concentrations of <1 nanomolar (nM) were reported (Löffler et al., 1999; Löffler and Sanford, 2005; Smatlak and Gossett, 1996; Yang and McCarty, 1998). It is practical to feed cultures with a 5–10 fold excess of hydrogen required to achieve complete dechlorination of the total amount of chlorinated electron acceptor added.

Each *Dhc* strain has a unique complement of RDase genes and the range of halogenated substrates used is strain-specific. The range of carbon sources has not been established but all described *Dhc* isolates can synthesize their macromolecules from acetate provided in the growth medium. *Dhc* genome analysis suggests that several genes encoding enzymes of the acetyl-coenzyme A pathway for CO_2 fixation are present, but some key components responsible for the reduction of CO_2 to CO are missing (Seshadri et al., 2005). Apparently, *Dhc* are not able to grow autotrophically and require a reduced organic compound (i.e., acetate) as a carbon source. *Dhc* are typically grown in bicarbonate-buffered medium, and a few reports suggest that CO_2 enhances growth (Müller et al., 2004), consistent with the typical pathway for acetate assimilation in which acetyl-CoA is reductively carboxylated to pyruvate.

The RDases, which are the key components of *Dhc* energy metabolism, contain cobalamin (vitamin B_{12}) as a cofactor. As discussed above, *Dhc* cannot synthesize vitamin B_{12}. Instead, *Dhc* possess genes encoding for corrinoid salvage pathways including corrinoid uptake and modification (Seshadri et al., 2005), so vitamin B_{12} must be added to *Dhc* growth media, typically at concentrations of 25–50 μg per liter to support reductive dechlorination and growth (He et al., 2007).

2.6 PHYLOGENY OF *DEHALOCOCCOIDES* AND RELATED BACTERIA

The analysis of the 16S rRNA gene sequence of *Dhc* strain 195 showed that it was affiliated with the domain *Bacteria*, but this phylogenetic marker was distantly related to other sequences deposited in the GenBank database in 1997. Soon after, 16S rRNA gene sequences from uncultured organisms (i.e., environmental clone sequences) appeared in the database and these led to the affiliation of strain 195 with the phylum *Chloroflexi* (green non-sulfur bacteria). This bacterial phylum is poorly characterized and encompasses a phylogenetically diverse collection of 16S rRNA gene sequences, yet it is represented by remarkably few isolates (Hugenholtz et al., 1998b) Figure 2.3 illustrates the two main *Chloroflexi* lineages, one of which includes both the *Thermomicrobia* and the classical *Chloroflexus aurantiacus* isolate, the namesake of the group (Hugenholtz and Stackebrandt, 2004).

"Traditional" *Chloroflexi* in the *Thermomicrobium*/*Chloroflexus* branch are not known to catalyze reductive dechlorination. The other branch leads to three lineages, including the *Anaerolinea* (which includes sludge isolates and environmental clone sequences from subsurface, marine, and aquatic habitats), the SAR202 cluster of marine *Chloroflexi*, and the *Dhc* (and related obligate dechlorinators) (Hugenholtz et al., 1998a; Hugenholtz and Stackebrandt, 2004; Morris et al., 2004). The phylogenetic branch leading to the *Dhc* is deeply rooted, suggesting an ancient origin of this unusual bacterial group and possibly the reductive dechlorination process.

In studies using PCR primers targeting *Dhc* 16S rRNA genes, it soon became evident that *Dhc* were present in microcosms that dechlorinated PCE or TCE to ethene and were associated with sites where reductive dehalogenation of PCE and TCE to VC and ethene was occurring (Fennell et al., 2001; Löffler et al., 2000). An extensive survey of sites contaminated with chlorinated ethenes in North America and Europe corroborated the correlation between the presence of *Dhc* 16S rRNA genes and dehalogenation products past DCE (i.e., VC and ethene) (Hendrickson et al., 2002). These findings have since been observed worldwide and *Dhc* have always been detected when DCE and VC dechlorination to ethene occurred. Apparently, the ability to reductively dechlorinate DCEs and VC is not widespread among the bacterial domain and may in fact be limited to select members of the *Dhc* group.

Members of the *Dhc* group possess highly similar 16S rRNA gene sequences, sharing greater than 98% sequence identity (Cupples, 2008; Ritalahti et al., 2006). Within this tight cluster, Hendrickson et al. (2002) distinguished three phylogenetic subgroups of *Dhc*: the "Cornell" subgroup that includes strain 195, the "Victoria" subgroup that includes strain VS, and the "Pinellas" subgroup, which comprises most of the cultured *Dhc* strains and isolates as well as the majority of the environmental clone sequences. The "Pinellas" cluster includes the chloroethene-dechlorinating *Dhc* strains BAV1, FL2 and GT and KB-1VC (Duhamel and Edwards, 2006; He et al., 2003b, 2005; Sung et al., 2006b), the 1,2-dichloropropane- dechlorinating *Dhc* strains KS1 and RC1 (Ritalahti and Löffler, 2004a) as well as the chlorobenzene-dechlorinating *Dhc* strain CBDB1 (Adrian et al., 2000b), and *Dhc* strains that dechlorinate PCBs (Adrian et al., 2009; Bedard et al., 2006, 2007; Bedard, 2008). Members of the "Pinellas" group share identical or highly similar (1–3 nucleotide differences) 16S rRNA gene sequences, and 14–16 and 23–24 bases distinguish the 16S rRNA genes of members of the Pinellas subgroup from members of the Victoria and Cornell subgroups, respectively (He et al., 2003a; Hendrickson et al., 2002). The phylogenetic grouping into three subgroups has endured as additional *Dhc* 16S rRNA gene sequences have accumulated.

A survey of 117 nearly full-length ($\geq$1,400 bases) *Dhc* 16S rRNA sequences deposited in the GenBank database (April 2009) showed that 77 sequences were affiliated with the Pinellas

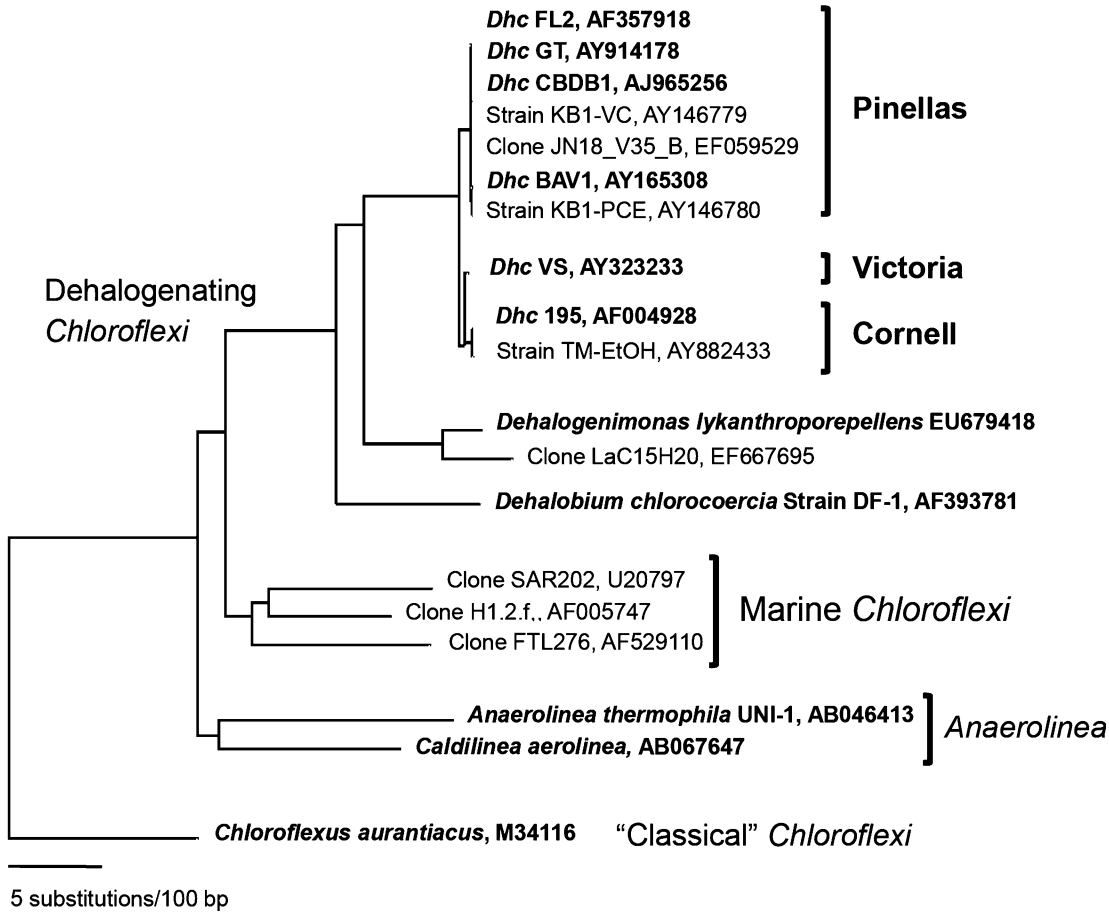

Figure 2.3. Phylogenetic affiliation of *Dhc* and related dechlorinators in the phylum *Chloroflexi* (green non-sulfur bacteria). Represented are the two main branches, the *Thermomicrobia*/*Chloroflexus* branch (represented by *Chloroflexus aurantiacus*) and the branch containing *Dhc* and relatives. The Neighbor-Joining tree was generated using an alignment of 1,350 bases of the 16S rRNA gene of the *Chloroflexi*, with focus on *Dehalococcoides* relatives. At the *top* is the *Dehalococcoides* cluster, followed by 16S rRNA gene sequences of dechlorinating *Chloroflexi* isolates that are not detected with the *Dehalococcoides* targeted PCR assays. The *Anaerolinea* and marine clusters are represented by isolates and environmental clone sequences, respectively. Pure cultures are indicated by bold font. "Classical" *Chloroflexi* isolates form deeper branches, and are in the lineage with *Chloroflexus aurantiacus*. The tree is based on a matrix calculated with maximum likelihood analysis with Juke's-Cantor adjustments. Scale bar represents 5% sequence divergence.

subgroup, 32 with the Cornell subgroup, and 8 with the Victoria subgroup. While this survey is not statistically valid, it suggests that members of the Pinellas subgroup are most commonly encountered in contaminated environments (where most of the sequences were retrieved). It should be noted that this grouping has no bearing on dechlorination activity. Members of all three groups dechlorinate chlorinated ethenes and strain VS (Victoria subgroup) and strains BAV1 and GT (Pinellas subgroup) grow with *cis*-DCE and VC as electron acceptors. An important observation is that some members of the Pinellas subgroup share identical l6S

rRNA gene sequences but exhibit distinct dechlorination activities. For example, *Dhc* strains BAV1 and KS1 share an identical 16S rRNA gene sequence, but strain BAV1 does not dechlorinate 1,2-dichloropropane and strain KS1 cannot grow with chlorinated ethenes. This incongruence between 16S rRNA gene sequence and dechlorination activity indicates that 16S rRNA gene-based analysis is insufficient to infer dechlorination activity, and this fact has triggered a search for process-specific biomarker genes (see below).

Knowledge of *Dhc* nutritional requirements and appropriate cultivation techniques enhances the ability of researchers to grow and enrich for these fastidious dechlorinators. These efforts generated a suite of 16S rRNA gene sequences of *Dhc* relatives within the *Chloroflexi*, and the corresponding organisms also have been associated with reductive dehalogenation (Kittelmann and Friedrich, 2008a, b). Recently, a few novel dechlorinating isolates have been obtained that are only distantly related to the *Dhc* (>10% 16S rRNA gene sequence divergence). These isolates share features with the *Dhc* but represent novel lineages of dechlorinating bacteria within the *Chloroflexi*. A recently described, tiny, disc-shaped organism with 89% 16S rRNA gene sequence similarity to *Dhc* dehalogenates 1,2,3-trichloropropane to allyl chloride (3-chloro-1-propene), which abiotically reacts with water and sulfide (the reducing agent in the medium) to form allyl alcohol and allyl sulfide (Figure 2.3, Table 2.4) (Yan et al., 2008). This organism was named *Dehalogenimonas lykanthroporepellens*, with the species name reminiscent of the alleged ability of garlic, which contains allyl sulfide, to repel werewolves (Moe et al., 2009).

Another distant *Dhc* relative is strain DF-1, tentatively named "Dehalobium chlorocoercia," also a tiny, disc-shaped bacterium that removes doubly-flanked chlorine substituents from PCBs provided as single congeners or as Aroclor 1260 (a commercial PCB mixture) (May et al., 2008). Strain DF-1 gains energy from PCB reductive dechlorination and also dechlorinates highly chlorinated benzenes (Wu et al., 2002) and PCE (Miller et al., 2005). Remarkably, PCE is dechlorinated to predominantly *trans*-DCE and some *cis*-DCE as end products. A similar observation was made in microcosms and enrichment cultures in which *Dhc* populations were implicated in PCE and TCE dechlorination to predominantly *trans*-DCE (Griffin et al., 2004). Strain *o*-17, a bacterium similar to strain DF-1, was identified in a culture that removes *ortho* chlorine substituents from PCBs (Cutter et al., 2001). A considerable diversity of 16S rRNA gene sequences related to the sequences of these PCB-dechlorinating *Chloroflexi* has been detected at anoxic, PCB-contaminated sites (Watts et al., 2005). As mentioned above, PCBs also are dechlorinated by *Dhc,* and quantitative monitoring of cell growth demonstrated that some *Dhc* strains belonging to the Pinellas subgroup grow at the expense of Aroclor 1260 dechlorination (Bedard et al., 2007). Recently, *Dhc* strain CBDB1, a member of the Pinellas subgroup, was shown to dechlorinate a number of congeners present in Aroclor 1260 (Adrian et al., 2009). These findings suggest that the ability to dechlorinate PCBs is not uncommon among members of the *Dhc* group.

Environmental clone sequences related to *Dhc* have been recovered from a variety of environments including sediments (Adrian et al., 2000a; He et al., 2005), aquifers (Sung et al., 2006b), the deep subsurface (Chandler et al., 1998; Inagaki et al., 2003, 2006; Teske, 2006), acid mine drainage biofilms (GenBank # AY082458), anaerobic digestors (Godon et al., 1997) and the open ocean (Morris et al., 2004). Although cultivation and isolation are prerequisites to shed light on the physiology of these elusive bacteria, their apparent diversity suggests a vast untapped reservoir of novel organisms, genes and enzymes for biotechnological applications including bioremediation. Apparently, the ability to perform reductive dechlorination is not uncommon in deeply branching groups within the *Chloroflexi* phylum and the available isolates merely represent the tip of the iceberg while a huge diversity of dechlorinators awaits discovery.

It is interesting that the next most closely related 16S rRNA gene sequences from cultured organisms or environmental clone sequences are only about 90% similar to those of *Dhc*.

Considering that there are presently hundreds of thousands of nearly complete bacterial 16S rRNA gene sequences collected from diverse habitats in publicly accessible databases, it is striking that no "transitional" organisms between *Dhc* and their non-dechlorinating relatives are present in this collection.

2.7 *DEHALOCOCCOIDES* GENETICS

2.7.1 Insights from *Dehalococcoides* Genomes

The genomes of *Dhc* strains 195, CBDB1, BAV1, VS and GT have been sequenced and the genome analysis revealed many interesting features. *Dhc* have circular chromosomes of 1.3 to 1.5 mega (10^6) bases (Mb) in length. These are among the smallest genomes known for free-living organisms (for comparison, *Escherichia coli* genomes are at least three times larger), indicative of genome streamlining and specialization (Giovannoni et al., 2005). Comparative genome analyses demonstrated that *Dhc* share essentially identical sets of core "housekeeping" genes for cellular functions like biosynthesis of amino acids and other cellular components, transcription and translation, nutrient transport, and energy conservation. Further, these core genes are organized in the same order (synteny) in all of the sequenced *Dhc* genomes. This conservation of gene sequence and synteny in *Dhc* genomes of isolates that were obtained from geographically distinct locations is remarkable and differs from the findings with other bacterial groups such as closely related *Shewanella* strains (Konstantinidis et al., 2006).

This highly conserved and stable *Dhc* genome core is interrupted by High Plasticity Regions (HPRs) near the origin of replication (ori) (Figure 2.4). These HPRs differ noticeably from the rest of the genome and show signs of extensive genomic rearrangements including insertions, deletions and inversions. Contained in these HPRs are distinct genomic islands, in which RDase, transposase, and phage integrase as well as hypothetical genes are noticeably overrepresented (Kube et al., 2005; McMurdie et al., 2009).

A total of 96 putative RDase genes are present on the genomes of strains 195 (17 genes), CBDB1 (32 genes), BAV1 (11 genes), and VS (36 genes), and 91 of them are located in HPRs. The reasons for this highly localized genome plasticity, and its consequences, are currently unresolved but obviously of great interest because these HPRs contain the majority of the genomic islands with the (putative) RDase genes. It should be noted that *bvcA*, a gene implicated in VC dechlorination in strain BAV1, is embedded in a genomic island located outside an HPR suggesting that RDase genes implicated in reductive dechlorination are not limited to the HPRs (McMurdie et al., 2009).

The first characterized *Dhc* RDase gene was *tceA* (Magnuson et al., 2000) encoding a TCE-to-VC RDase. The *tceA* gene is located within an integrated genetic element (i.e., a genomic island) on the genome of strain 195, suggesting that this gene had been acquired via horizontal gene transfer from another organism (Seshadri et al., 2005). When chromosome walking was applied to examine the extragenic regions flanking the *tceA* genes in several TCE-dechlorinating *Dhc* strains, similar sequences of variable length and flanked by insertion sequences were found upstream and downstream of the *tceA* genes (Krajmalnik-Brown et al., 2007).

The presence of highly similar RDase genes (e.g., *tceA*) in *Dhc* strains from geographically distinct origins suggests that horizontal gene transfer (HGT) events between *Dhc* are not uncommon and contribute to RDase gene dissemination. The *bvcA* gene of strain BAV1 and the *vcrA* genes of strains VS and GT also reside in regions flanked by mobile genetic elements. Compared to other genes on *Dhc* genomes, these VC RDase genes display an unusual bias towards the nucleotide T in the third position of the codons (triplets of nucleotides that code for specific amino acids) suggestive that these genes were horizontally acquired from a foreign

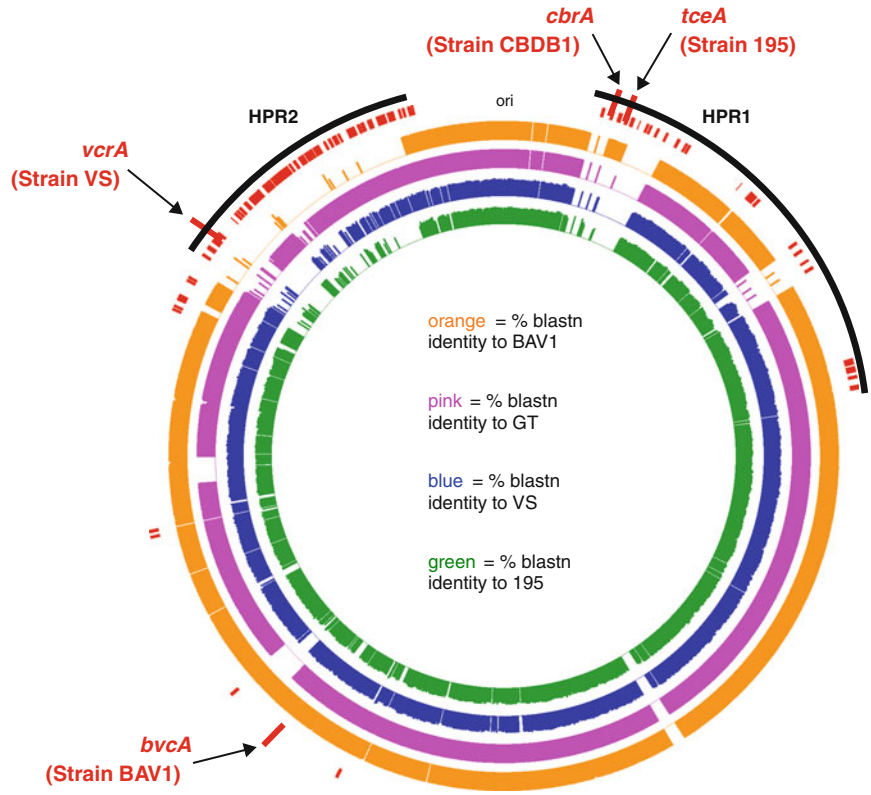

Figure 2.4. Circular maps of *Dhc* genomes of strains BAV1, GT, VS and 195 with the genome of CBDB1 (not depicted) serving as the reference. The non-contiguous red slashes correspond to RDase genes in the genomes of all five *Dhc* strains. The outermost ring represents the blastn alignment of 1,000 bp blocks of strain CBDB1 to strain BAV1. The second, third and fourth rings represents alignments of the genomes of strains GT, VS and 195 with CBDB1, respectively. The height of the bars indicates blastn similarity to the corresponding gene in strain CBDB1 genome. Indicated by the *black* arcs are the High Plasticity Regions (HPRs) on either side of the origin of replication. These HPRs contain the majority of RDase genes and distinguish reductive dechlorination functionality of the different *Dhc* strains. *tceA, bvcA and vcrA* encode chlorinated ethene RDases and *cbrA* encodes a chlorobenzene RDase. Note that *bvcA*, a gene implicated in VC reductive dechlorination, is located in the core genome outside the HPRs in strain BAV1 rdh abbreviates reductive dehalogenase homologous genes.

host (McMurdie et al., 2007). The mechanisms contributing to RDase gene mobilization and transfer are unclear but the genome analysis provides some hints. A prophage is present in the strain 195 genome and microscopic observations suggested the presence of phage (bacterial viruses) in pure cultures of strain BAV1 (Helton et al., 2008; Ritalahti et al., 2007). Phages are important vehicles of gene transfer and may play roles in RDase gene mobilization between and within *Dhc* genomes.

A striking aspect of the sequenced *Dhc* genomes is the presence of multiple RDase genes ranging from 11 in strain BAV1 to 36 in strain VS (Table 2.5), highlighting the specialization towards reductive dechlorination.

Another shared feature of the sequenced *Dhc* genomes is the presence of five distinct hydrogenase gene clusters, which are highly similar in sequence and organization in all sequenced *Dhc* genomes. Hydrogenase enzyme systems catalyze the reversible oxidation of hydrogen to two protons and two electrons. Hydrogen is the required electron donor for *Dhc,*

and the presence of five distinct hydrogenase complexes suggests that *Dhc* have fine-tuned responses to environmental hydrogen concentrations. The small genome size, the presence of multiple RDase and five hydrogenase gene operons (clusters of genes related to a specific metabolic function or pathway), and the absence of genes predicted to encode utilization of other substrates all support the extreme metabolic specialization of *Dhc*.

These findings suggest a model for *Dhc* evolution, in which all *Dhc* genomes contain a highly conserved set of core housekeeping genes, with a few HPRs where most of the variability resides. *Dhc* evolution is discussed further in Section 2.11, but the emerging conceptual model is that the ancestor of these organisms arose early in the history of life on Earth, and modern *Dhc* represent an evolved and highly specialized bacterial group adapted to use a wide variety of halogenated organics by exchange of RDase genes.

2.7.2 *Dehalococcoides* Reductive Dehalogenases Gene Operons

Dhc RDase genes were first identified through reverse genetics approaches and the application of degenerate PCR primers designed using sequences of known RDase genes (Hölscher et al., 2004; Krajmalnik-Brown et al., 2004; Müller et al., 2004). Subsequently, the analysis of *Dhc* genomes revealed a large diversity of RDase genes and the presence of multiple RDase genes on individual genomes (Table 2.5). A generic *Dhc* RDase operon is depicted in Figure 2.5.

Table 2.5. Features of Genomes from *Dhc* Isolates

Dhc strain	Size [bp]	Predicted ORFs	# of putative RDase genes	Accession #	Reference
195	1,469,720	1,591	17	CP000027	Seshadri et al., 2005
CBDB1	1,395,502	1,458	32	AJ965256	Kube et al., 2005
BAV1	1,341,892	1,371	11	CP000688	McMurdie et al., 2009
VS	1,413,474	1,447	36	CP001827	McMurdie et al., 2009
GT	1,360,154	1,417	20	CP001924	Unpublished

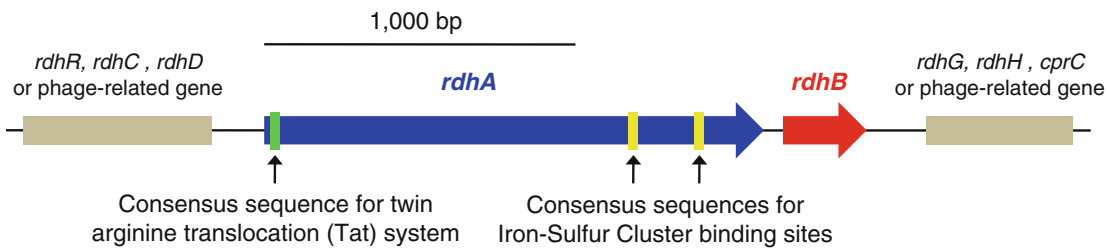

Figure 2.5. Organization of a typical RDase operon in *Dhc*. All *Dhc* RDase gene clusters consist of *rdhA* (blue) encoding the catalytic subunit and *rdhB* (red) encoding a transmembrane anchor, which is located downstream of *rdhA*. Most (putative) *Dhc* RDase gene clusters lack one or all of the accessory genes *rdhC*, *rdhD* and *rdhG*, which can be located on the forward or reverse strands. The functions of the accessory genes are unclear. *Arrows* indicate the direction of the open reading frames (ORFs) in a typical *rdh* operon. *rdh* abbreviates reductive dehalogenase homologous genes.

The *rdhA* genes encoding the RDase catalytic subunits share common features including a twin arginine translocation (Tat) consensus sequence that enables secretion of the mature protein through the cell membrane (see below) and two iron-sulfur cluster binding consensus sequences. The average length of *Dhc rdhA* and *rdhB* genes is 1,491 and 237 bp, respectively. In *Dhc*, the *rdhB* gene is located downstream of *rdhA,* and both genes are transcribed together (Müller et al., 2004). *rdhA* and *rdhB* are separated by an intergenic spacer region that ranges from 2 to 50 bp in length.

The *rdhA*/*rdhB* gene clusters can occur with accessory genes; however, most of the *Dhc rdhA*/*rdhB* gene clusters lack one or all of the accessory genes *rdhC*, *rdhD* and *rdhG*. *rdhC* and *rdhD* are located upstream of *rdhA*. Computational analysis suggests that *rdhC* encodes a PAS/PAC sensor histidine kinase and *rdhD* encodes a putative DNA-binding regulator; however, these functions have not been experimentally demonstrated. Located in variable distance downstream of *rdhB* is a phage-related gene of unknown function, or *rdhG*, which encodes a protein of unknown function. Understanding the details of RDase operon organization and how individual parts function and interact is relevant for the design of biomarkers that not only provide information regarding RDase gene presence but also RDase gene expression (i.e., activity).

2.8 *DEHALOCOCCOIDES* REDUCTIVE DEHALOGENASES (RDASES)

Reductive dechlorination is catalyzed by RDase enzyme systems encoded by *rdhA*. *Dhc* RDases are monomeric enzyme systems with molecular weights ranging from 48.5 to 63 kDa (based on putative RDase genes identified in sequenced *Dhc* genomes). Together with the isomerases and the methyltransferases, RDases represent one of the three currently recognized classes of B_{12} enzyme systems (Banerjee and Ragsdale, 2003). RDase A proteins are very oxygen sensitive and are irreversibly inhibited by exposure to air. Since obtaining large amounts of biomass of a *Dhc* pure culture is impractical, protein purification from *Dhc* cultures is challenging. Hence, heterologous expression of *Dhc* RDase genes in a host that produces biologically active RDase is a major objective as this would allow detailed study of these interesting enzyme systems. Cloning and overexpression of the *pceA* gene from *Sulfurospirillum multivorans* was successful but the recombinant *PceA* protein had no dechlorinating activity, likely because of incorrect protein folding and/or the cloning host was unable to synthesize and incorporate the cobalt-containing corrinoid (i.e., cobalamin) cofactor (Neumann et al., 1996). Only four *Dhc* RDases, PceA, TceA, VcrA and CbrA, have been partially characterized but the limited amount of enzyme recovered prevented detailed mechanistic studies (Table 2.6).

The physiological electron carrier(s) that donates electrons to RDases is unknown but RDase activity can be measured *in vitro* conveniently with artificial, low potential electron donors such as reduced methyl viologen ($E_0' = -446$ millivolts [mV]) (Corbin and Watt, 1990; Neumann et al., 1998). The *Dhc* PCE and TCE RDases also can use reduced benzyl viologen, a weaker reductant (−360 mV) than methyl viologen, as an electron donor (Jayachandran et al., 2004; Nijenhuis and Zinder, 2005) whereas the PCE RDase of *Sulfurospirillum multivorans* cannot, suggesting a difference in reaction mechanism.

Characteristics of the RDase A proteins are a Tat signal sequence and two Fe-S clusters. The Tat signal sequence suggests RDase trafficking into the periplasmic space following RDase maturation in the cytoplasm (Sargent, 2001). The Tat leader peptide has a length of approximately 40 amino acids and the mature RDase A protein is 415–514 amino acids long. Both Fe-S cluster-binding motifs occur in the C-terminal region of RDase A and are likely

Table 2.6. *Dhc* RDase Genes with Assigned Function

Gene	*Dhc* RDase	Reaction Catalyzed	Molecular Mass[b] [kDa]	Reference
pceA	PceA	PCE → TCE	50,800	Magnuson et al., 2000
tceA	TceA	TCE → VC	57,700	Magnuson et al., 2000
vcrA	VcrA	DCEs, VC → ethene	53,100	Müller et al., 2004
bvcA	BvcA[a]	VC → ethene [a]	52,800	Krajmalnik-Brown et al., 2004
cbrA	CbrA	1,2,3,4-TeCB → 1,2,4-TCB	49,700	Adrian et al., 2007b

[a]The function of BvcA has not been biochemically verified and the information shown was drawn from transcriptional data
[b]Molecular masses without the Tat leader peptide deduced from the *rdhA* sequence

involved in electron transfer. *Dhc* RDase B proteins are 76–100 amino acids long with 2–3 predicted transmembrane spanning regions suggesting they are integral membrane proteins (Maillard et al., 2003; Villemur et al., 2002). *Dhc* RDases are associated with the membrane fraction, and the current models suggest that the RdhB proteins anchor the RdhA enzymes to the outside of the cytoplasmic membrane (Müller et al., 2004).

2.9 BIOCHEMISTRY OF REDUCTIVE DECHLORINATION BY *DEHALOCOCCOIDES*

Although the details of electron transport in *Dhc* are not well explored, it is likely that one or more of the five hydrogenase complexes encoded on the *Dhc* genomes is responsible for the initial uptake of electrons from hydrogen. The mechanisms and components involved in electron transfer from the hydrogenase complex(es) to the terminal reductase (i.e., the specific RDase) are unclear and direct electron transfer from the hydrogenase to the RDase (i.e., without intermediate electron carriers) is being discussed.

The reasons why *Dhc* possess five gene clusters encoding potential hydrogenase enzyme complexes to do the simple reaction of removing electrons from protons in hydrogen (i.e., hydrogen oxidation: $H_2 \rightarrow 2\ H^+ + 2e^-$) are unclear, but suggest that *Dhc* have a fine-tuned response to fluctuating environmental hydrogen concentrations. Using proteomic techniques, peptides (i.e., short amino acid chains) from three of these predicted hydrogenases (Figure 2.6) were detected in strain 195 membrane fractions derived from biomass grown in medium amended with PCE and hydrogen (Morris et al., 2006). The highest peptide coverage, which is a semi-quantitative measure of protein abundance, was obtained for the *Hup* hydrogenase, predicted to have its catalytic subunit on the outside the cell membrane. Detected in lower abundance were peptides of the *Vhu* and *Hym* hydrogenases. Thus, these three hydrogenases are candidates for being quantitatively relevant hydrogenases during growth of strain 195 with PCE.

Quinones are well known hydrophobic and membrane-associated organic molecules that participate in electron transport, and high amounts of ubiquinone and lower amounts of

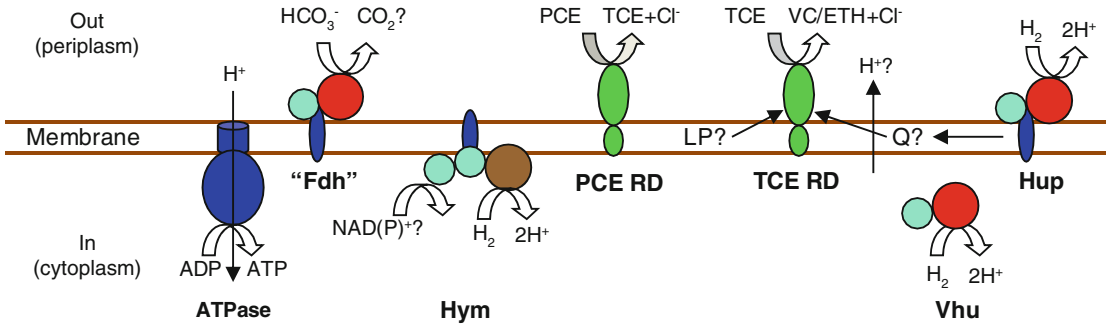

Figure 2.6. Proteins detected with high abundance in proteomics studies and other components potentially involved in organohalide respiration in *Dhc* strain 195 (modified after Morris et al., 2006). Legend: "Fdh", protein complex annotated as formate dehydrogenase; Hym, Vhu, and Hup hydrogenases; PCE and TCE RDases; Q, quinones potentially involved in electron transport; LP, low potential electron carrier potentially involved in electron transport.

menaquinone derivatives were found in the membranes of *Dhc* strains BAV1 and FL2 (White et al., 2005). It is not clear whether these quinones take part in electron transport in *Dhc*, and it has been proposed that their role is to quench radicals that form in the reductive dehalogenation process (White et al., 2005). Moreover, quinones carry electrons at relatively high redox potentials near 0 V, whereas evidence obtained from investigations of the PCE RDase of the PCE-to-*cis*-DCE dechlorinator *Dehalobacter restrictus* indicates that at least one of the two electrons passed on to the RDase must have a redox potential below ~−360 mV to reduce the cobalt in the corrinoid to the +1 oxidation state (Holliger et al., 2003; Schumacher et al., 1997). Thus, quinones are unlikely electron carriers involved in reductive dechlorination in *Dhc* (Figure 2.6).

The exact mechanisms by which *Dhc* obtain energy for growth and maintenance from reductive dechlorination reactions are not understood. It is presumed that somehow the transport of electrons from the hydrogenases to the RDases leads to the generation of a proton motive force that drives a membrane-bound F_1F_o ATPase to generate ATP (Figure 2.6). Preliminary studies with ionophores and other inhibitors in strains 195 and CBDB1 support such a mechanism (Jayachandran et al., 2004; Nijenhuis and Zinder, 2005). Peptides from an F_1F_o ATPase are readily detected in membrane preparations from strain 195 (Morris et al., 2006), but this enzyme occurs in essentially all organisms and its presence does not prove any particular mechanism for energy conservation.

The biochemistry of the reductive dechlorination reaction is not fully understood and the majority of information was generated with the PCE RDases of *Sulfurospirillum multivorans* and *Dehalobacter restrictus* (Holliger et al., 2003). PceA of *Sulfurospirillum multivorans* contains a modified cobalamin called norpseudo-B_{12} (Kräutler et al., 2003). The requirement of *Dhc* for vitamin B_{12} for dechlorination and growth, preliminary *in vitro* biochemical data (i.e., the requirement for a low potential electron donor and reversible inhibition of dechlorination in cell extracts by alkyl iodides), and the presence of corrinoid-binding motifs in some RDases suggest that *Dhc* RDases also contain a cobalamin cofactor (Adrian et al., 2007b; Hölscher et al., 2004; Rosner et al., 1997). Unfortunately, the structure of these *Dhc* cobalamin cofactor(s) has not been resolved. Since only low potential electron donors drive reductive dechlorination, the most plausible pathway involves a Co(I) species in catalysis and the intermediate formation of a radical anion (Banerjee and Ragsdale, 2003). Mechanistic studies of reductive dechlorination have mainly focused on chlorinated alkenes (Banerjee and Ragsdale, 2003; McCauley et al., 2005; Schumacher et al., 1997; Holliger et al., 2003); it remains to be seen if reductive dechlorination of chlorinated alkanes and aromatic compounds involve similar cofactors and mechanisms.

2.10 *DEHALOCOCCOIDES* BIOMARKERS

Dhc-containing consortia can be mass-produced (Vainberg et al., 2009) and have been used successfully at numerous sites to initiate dechlorination or increase dechlorination rates and achieve desired end points (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002). Prognostic site assessment and diagnostic bioremediation monitoring tools are desirable to detect and quantify total *Dhc* and individual *Dhc* strains with specific dechlorination activities of interest. Various laboratories utilize primer sets that target slightly different regions of the *Dhc* 16S rRNA gene (Table 2.7).

Typically, DNA is extracted from a groundwater sample (Ritalahti et al., 2010a) and *Dhc* biomarker genes are either detected with qualitative PCR methods (Hendrickson et al., 2002; Löffler et al., 2000) or quantified using qPCR. Robust qPCR protocols that either use TaqMan or SYBR-Green detection chemistries have been published (Behrens et al., 2008; Holmes et al., 2006; Mackay, 2004; Ritalahti et al., 2006; Hatt and Löffler, 2012). TaqMan detection relies on fluorescently-labeled, linear hybridization probes and the application of probes carrying different fluorophores allows quantification of up to four targets in multiplex format (Mackay, 2007). The 16S rRNA gene serves as an excellent biomarker to detect and quantify *Dhc* in laboratory and environmental samples, although this analysis has its limitations. As more environments are explored and new sequences are added to the GenBank database, the available information expands the diversity and reveals the unexpected breadth of this bacterial group. As a result, some tests designed to specifically assay for *Dhc* later have been found to target a broader range of organisms. For example, several primer pairs designed to be *Dhc*-specific also amplify the 16S rRNA genes of 1,2,3-trichloropropane-dechlorinating *Dehalogenimonas lykanthroporepellens* strains, which are distant *Dhc* relatives (Figure 2.3) (Yan et al., 2008). In other words, the lack of knowledge of the diversity of *Dhc* and related bacteria can result in false positives and lead to erroneous conclusions, possibly limiting the value of the analysis.

Another drawback of 16S rRNA gene targeted analyses is the similarity of 16S rRNA gene sequences among *Dhc* strains that exhibit different dechlorination activities. For example, some members of the Pinellas group share identical 16S rRNA genes but the strains use different chlorinated compounds as electron acceptors (Table 2.4) indicating that the resolution of the 16S rRNA gene is insufficient to infer dechlorination activity. Thus, the 16S rRNA gene analysis provides information about the presence of *Dhc* and related dechlorinators but the analysis falls short of providing insights into specific dechlorination activities (Löffler and Edwards, 2006). Despite this shortcoming, quantitative monitoring of *Dhc* 16S rRNA gene provides useful information, especially when the data are correlated with contaminant transformation over time (i.e., temporal assessment of the same monitoring wells).

To overcome the limitation of the 16S rRNA gene analysis, genes that correlate directly with dechlorination activity are being sought. Specific function has been assigned to few *Dhc* RDase genes (Table 2.6) and a major task is to elucidate the substrate range of each functional RDase represented on the *Dhc* genomes. Knowledge of the full spectrum of haloorganic substrates is pivotal for designing a comprehensive suite of molecular tools for monitoring abundance and expression of individual RDase genes and predicting dechlorination activity. Figure 2.7 shows RDase genes that have been implicated in the reductive dechlorination of chlorinated ethenes. The genes *tceA*, *vcrA* and *bvcA* have served as useful markers to track individual *Dhc* strains relevant for chlorinated ethene detoxification (Behrens et al., 2008; Holmes et al., 2006; Ritalahti et al., 2006; Scheutz et al., 2008).

Although the utility of these targets for monitoring chlorinated ethene reductive dechlorination has been demonstrated, it is clear that only a small fraction of all RDase genes

Table 2.7. Primers and Probes Used for the Specific Detection/Quantification of *Dhc* and Dechlorinating *Dhc* Relatives

Dehalococcoides 16S rRNA Gene – Targeted	Probe/ Primer	Chemistry	Sequence (5′–3′)	Nucleotide Position	Reference
Dehalococcoides strains	*Dhc*1200F	TaqMan	CTGGAGCTAATCCCCAAAGCT	1,200–1,220	He et al., 2003a, b
	*Dhc*1271R	TaqMan	CAACTTCATGCAGGCGGG	1,265–1,248	He et al., 2003a, b
	*Dhc*1235P	TaqMan	TCCTCAGTTCGGATTGCAGGCTGAA	1,222–1,246	He et al., 2003a, b
Dehalococcoides strains	Forward	TaqMan	GGTAATACGTAGGAAGCAAGCG	467–488	Holmes et al., 2006; Rahm, 2006
	Reverse	TaqMan	CCGGTTAAGCCGGGAAATT	564–546	Holmes et al., 2006; Rahm, 2006
	Probe	TaqMan	ACATCCAACTTGAAAGACCACCTAC GCTCACT	543–512	Holmes et al., 2006; Rahm, 2006
Dehalococcoides strains (Dupont primer set 1)	*Dhc*1f	SYBR green	GATGAACGCTAGCGGCG	1–17	Duhamel et al., 2004; Hendrickson et al., 2002; Duhamel and Edwards, 2006; Duhamel and Edwards, 2007; United States Patent US6894156B2 (Hendrickson and Ebersole)
	*Dhc*259r	SYBR green	CAGACCAGCTACCGATCGAA	259–240	Duhamel et al., 2004; Duhamel and Edwards, 2006; Duhamel and Edwards, 2007; Hendrickson et al., 2002; United States Patent US6894156B2 (Hendrickson and Ebersole)
Dehalococcoides strains	Forward	SYBR green	GAAGTAGTGAACCGAAAGG	240–259	Schaefer et al., 2009
	Reverse	SYBR green	TCTGTCCATTGTAGCGTG	1,176–1,159	Schaefer et al., 2009
Dehalogenimonas strains	BL-DC-631f	SYBR Green	GGTCATCTGATACTGTTGGACTTGA GTATG	634–663	Yan et al., 2008
BL-DC-8 and BL-DC-9	BL-DC-796r	SYBR green	ACCCAGTGTTTAGGGCGTGGACTA CCAGG	799–827	Yan et al., 2008

(*continued*)

Table 2.7. (continued)

Dehalococcoides **16S rRNA Gene – Targeted**	**Probe/ Primer**	**Chemistry**	**Sequence (5′–3′)**	**Nucleotide Position**	**Reference**
All known dehalogenating chloroflexi	Chl348F	SYBR green	GAGGCAGCAGCAAGGAA	348–364	(Fagervold et al., 2005) – *Chloroflexi* specific
	Dehal884R	SYBR green	GGCGGGACACTTAAAGCG	867–884	Fagervold et al., 2005: Dehalogenating *Chloroflexi* including *Dehalococcoides* and DF-1
bvcA gene	bvcAF	TaqMan	AAAAGCACTTGGCTATCAAGGAC	1,011–1,033	Krajmalnik-Brown et al., 2004; Ritalahti et al., 2006
	bvcAR	TaqMan	CCAAAAGCACCACCAGGTC	1,076–1,058	Krajmalnik-Brown et al., 2004; Ritalahti et al., 2006
	bvcAP	TaqMan	TGGTGGCGACGTGGCTATGTGG	1,035–1,056	Krajmalnik-Brown et al., 2004; Ritalahti et al., 2006
bvcA gene	Forward	TaqMan	GGTGCCGCGACTTCAGTT	73–90	Holmes et al., 2006; Johnson et al., 2005
	Reverse	TaqMan	TCGGCACTAGCAGCAGAAATT	140–120	Holmes et al., 2006; Johnson et al., 2005
	Probe	TaqMan	TGCCGAATTTTCACGACTTGGAT GAAG	92–118	Holmes et al., 2006; Johnson et al., 2005
vcrA gene	vcrAF	TaqMan	CGGGCGGATGCACTATTTT	400–418	Ritalahti et al., 2006
	vcrAR	TaqMan	GAATAGTCCGTGCCCTTCCTC	471–451	Ritalahti et al., 2006
	vcrAP	TaqMan	CGCAGTAACTCAACCATTTCCTGG TAGTGG	420–449	Ritalahti et al., 2006
vcrA gene	Forward	TaqMan	CTCGGCTACCGAACGGATT	1,303–1,321	Holmes et al., 2006
	Reverse	TaqMan	GGGCAGGAGGATTGACACAT	1,367–1,348	Holmes et al., 2006; Johnson et al., 2005
	Probe	TaqMan	CGCACTGGTTATGGCAACCACTC	1,345–1,323	Holmes et al., 2006
vcrA gene	Forward	SYBR green	TGCTGGTGGCGTTGGTGCTCT	627–647	Müller et al., 2004
	Reverse	SYBR green	TGCCCGTCAAAAGTGGTAAAG	1,067–1,047	Müller et al., 2004

(*continued*)

Table 2.7. (continued)

Dehalococcoides 16S rRNA Gene – Targeted	Probe/ Primer	Chemistry	Sequence (5′–3′)	Nucleotide Position	Reference
tceA gene	tceAF	TaqMan	ATCCAGATTATGACCCTGGTGAA	446–468	Holmes et al., 2006; Ritalahti et al., 2006; Johnson et al., 2005
	tceAR	TaqMan	GCGGCATATATTAGGGCATCTT	512–491	Holmes et al., 2006; Ritalahti et al., 2006; Johnson et al., 2005
	tceAP	TaqMan	TGGGCTATGGCGACCGCAGG	470–489	Holmes et al., 2006; Ritalahti et al., 2006; Johnson et al., 2005
tceA gene	tceA-500 F	SYBR green	TAATATATGCCGCCACGAATGG	500–521	Fung et al., 2007
	tceA-795R	SYBR green	AATCGTATACCAAGGCCCGAGG	816–795	Fung et al., 2007
pceA gene	DET0318-484f	SYBR green	ATGGTGGATTTAGTAGCAGCGGTC	505–528	Fung et al., 2007
	DET0318-664r	SYBR green	ATCATCAAGCTCAAGTGCTCCCAC	708–685	Fung et al., 2007
cbrA gene	cbdbA84_f	SYBR green	CTTATATCCTCAAAGCCTGA	118–137	Wagner et al., 2009
	cbdbA84_r	SYBR green	TGTTGTTGGCAACTGCTTC	319–301	Wagner et al., 2009

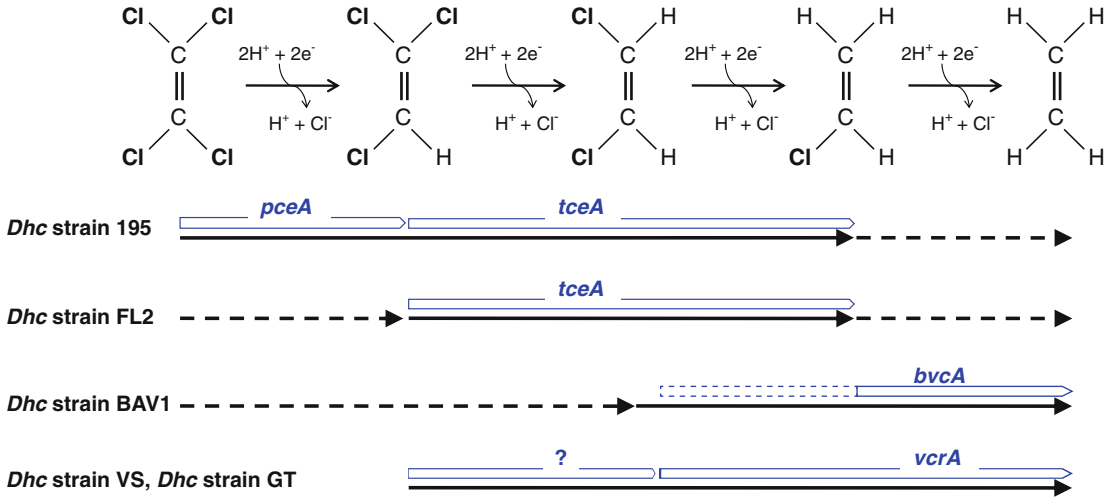

Figure 2.7. *Dhc* RDase genes implicated in reductive dechlorination of chlorinated ethenes. Although the RDase genes identified to date are only a subset of the total number of RDase genes contributing to the reductive dechlorination of chlorinated ethenes, the quantitative assessment of *tceA*, *vcrA*, *bvcA* and *Dhc* 16S rRNA genes has proven useful for prognostic site assessment and bioremediation monitoring. Strains GT and VS dechlorinate TCE but do not possess *tceA* and the gene(s) encoding this function may serve as an additional biomarker for this process. VcrA has been biochemically characterized and dechlorinates all DCE isomers and VC in *in vitro* assays (Müller et al., 2004; Rosner et al., 1997). Transcriptional analysis implicated BvcA in VC dechlorination (Krajmalnik-Brown et al., 2004) but its involvement in DCE dechlorination has yet to be demonstrated.

implicated in chlorinated ethene dechlorination has been identified (Ritalahti et al., 2006). To achieve comprehensive monitoring of the numerous *Dhc* strains with distinct RDase genes contributing to chlorinated ethene detoxification, function must be assigned to the remaining identified RDase genes, and such efforts are underway in several laboratories. In addition, other process-specific *Dhc* biomarker genes are being sought including hydrogenase genes (i.e., *hup*, *hym, hyc, ech and vhu*), as well as other genes indirectly associated with reductive dechlorination. For example, the requirement for corrinoid cofactors to perform reductive dechlorination suggests that monitoring the expression of genes encoding proteins for corrinoid transport or salvage may serve as a proxy for monitoring actively dechlorinating *Dhc* populations.

Table 2.7 shows qPCR primers or probes for commonly utilized gene targets for the analysis of *Dhc* and *Dhc* relatives. The quantitative analysis of biomarker genes (i.e., DNA) provides useful information about the presence and temporal dynamics of the population of interest. Although temporal analysis provides some clues about *Dhc* growth and activity, the DNA-based analysis does not directly inform about activity (i.e., rates) and cannot distinguish live and active cells from dead *Dhc* cells or free DNA released from lysed cells.

Targets that typically correlate more directly with activity are biomarker gene transcripts (i.e., messenger RNA, or mRNA). A few studies have shown that the quantitative assessment of biomarker mRNA provides information about activity under laboratory conditions (Johnson et al., 2005; Rahm and Richardson, 2008). Although promising, this approach has several drawbacks that limit its applicability, in particular when working with field samples. RNA is inherently unstable and prone to degradation. The use of internal standards to quantify RNA loss and RNA stabilizing agents can improve the analysis but uncertainties remain especially when applying these techniques to natural populations (Johnson et al., 2008; Ritalahti et al., 2010b).

The historical basis for correlating transcript abundance with activity is that gene induction and transcription (i.e., mRNA production) occurs specifically in response to the substrate (e.g., a chlorinated electron acceptor) and further, mRNA turnover is rapid and the transcripts are quickly degraded after protein biosynthesis. This model applies to many model microorganisms, such as *E. coli*, and is outlined in every microbiology textbook; however, the classical model may not apply to slow-growing dechlorinating bacteria such as *Dhc*. For example, a single chlorinated substrate can induce the expression of multiple RDase genes, suggesting that the presence of specific mRNA transcripts may not be firmly linked with a specific dechlorination reaction (Johnson et al., 2008). Also, RDase gene transcript turnover in *Dhc* can be slow, so that biomarker transcripts may persist after the chlorinated compound has been dechlorinated. Further, the relative *tceA* transcript levels increase following oxygen exposure, suggesting that RDase gene expression is also a stress response and therefore can be uncoupled from dechlorination activity (Amos et al., 2008a). Although gene expression monitoring is promising, procedural advances are needed and the regulation of *Dhc* transcription must be understood in greater detail before transcript measurements will be useful to infer dechlorination activity and rates.

Recent technological advances in proteomic workflows allow the identification of peptides of biomarker proteins, though quantitative proteomic techniques remain elusive (Aebersold and Mann, 2003; Ram et al., 2005; Werner et al., 2009). The analysis of the catalysts (i.e., specific RDases) is a direct measure of activity. Detection of peptides of *Dhc* biomarker proteins has been accomplished with *Dhc* pure cultures and dechlorinating consortia (Werner et al., 2009); however, the applicability of this approach to complex environmental samples with high microbial diversity and low biomass has yet to be demonstrated. Independent of the technology used, a prerequisite for obtaining defensible results is knowledge of process-specific biomarkers. Further, internal standards are needed to analytically measure biomarker loss during sampling, shipment and storage and during sample processing in the analytical laboratory.

2.11 *DEHALOCOCCOIDES* EVOLUTION AND DISSEMINATION OF REDUCTIVE DEHALOGENASE GENES

Dhc are unique bacteria because of their streamlined genomes and extreme specialization with regard to substrate utilization (i.e., strictly hydrogenotrophic organohalide respirers). Many argue that the evolution of reductive dechlorination of chlorinated ethenes started at the beginning of the twentieth century after the introduction of anthropogenic compounds, which are also called xenobiotics, into the environment. Although humans have introduced large quantities of diverse halogenated compounds into the environment, the assertion that these chemicals have no natural counterparts has been proven wrong. Literally thousands of haloorganics are produced by natural processes (e.g., combustion and geogenic processes, volcanic emissions) dating back to before life originated on Earth (Gribble, 2003, 2005; Häggblom and Bossert, 2003). Furthermore, numerous biological processes, sometimes in concert with abiotic reactions, generate a variety of halogenated compounds, including PCE, TCE and other CAHs (Weissflog et al., 2005). Processes generating haloorganic compounds may have been operational for billions of years, possibly before life originated on Earth. Hence, it is likely that reductive dechlorination evolved early in life's history on Earth and that the capability to perform organohalide respiration arose long before humans released chlorinated compounds into the environment. The small, streamlined genomes and the minimalist, highly specialized lifestyle of *Dhc* (i.e., strictly hydrogenotrophic organohalide respirers) likely reflect

an early evolutionary development rather than a recent adaptation to the anthropogenic release of large amounts of CAHs. This assertion also is consistent with the deep phylogenetic branching of *Dhc* and related bacteria within the *Chloroflexi* (Figure 2.3).

As discussed in Section 2.7.1 above, the known *Dhc* strains possess up to 36 RDase genes and dozens of distinct (putative) *Dhc* RDase genes have been identified (Hölscher et al., 2004; McMurdie et al., 2009). The complement of RDase genes is dynamic and appears to undergo continuous exchange and recombination events, possibly mediated by phage activity, thus allowing evolution of new dehalogenation phenotypes. The staggering diversity of potential RDase genes indicates that the few characterized dehalogenation reactions are just the tip of the iceberg and that the vast dehalogenation potential of *Dhc* has yet to be explored. Emerging lines of evidence, such as the distinct codon usage in the VC RDase genes *vcrA* and *bvcA,* suggest that at least some *Dhc* RDase genes originated from a non-*Dhc* source (i.e., were acquired from a foreign host) (McMurdie et al., 2007) and that *Dhc* RDase genes are subject to HGT events (Krajmalnik-Brown et al., 2007; McMurdie et al., 2009, 2011). Genome sequencing demonstrated that transposable elements flank RDase operons suggesting that intra-chromosomal rearrangements and inter-chromosomal HGT events occur.

The mechanisms and rates (i.e., frequency) of these HGT events are unclear but obviously of great importance for understanding *Dhc* adaptation and possible evolution of dechlorinating ability in response to anthropogenic CAH exposure. For example, if the indigenous *Dhc* population cannot respire VC because it lacks the genes encoding for VC RDases, is it possible that over time a *Dhc* strain with the ability to respire VC will emerge? If so, what are the evolutionary mechanisms promoting this evolution, and how long would it take to establish a VC-respiring phenotype? Knowledge of the rates of RDase gene HGT, adaptation and evolution of new strains could enable researchers to predict the longevity and fate of chloroorganic contaminants. An expression study performed with a *Dhc*-containing consortium revealed that phage-related genes are up-regulated during active TCE dechlorination, but the reason and consequences remain elusive (Johnson et al., 2008). Indeed, phage particles were detected in growing cultures of *Dhc* strain BAV1 and genes located in strain BAV1 HPR regions were identified on the phage genomes suggesting that phage are vehicles for HGT. The involvement of "dehalophage" in HGT of *Dhc* RDase genes is an intriguing hypothesis and is being explored in more detail.

2.12 *DEHALOCOCCOIDES* BIOGEOGRAPHY

Dhc isolates and mixed cultures have been obtained from sludge, contaminated and uncontaminated river sediments, contaminated estuarine harbor sediments, and contaminated aquifers from geographically distinct locations. *Dhc* environmental clone sequences have been recovered from an even broader suite of environments, including the deep subsurface and terrestrial habitats (Futagami et al., 2009; Krzmarzick et al., 2012). *Dhc* sensitivity to oxidizing conditions implies that *Dhc* distribution is limited to habitats where reducing conditions prevail, but anoxic microsites are common even in environments that are considered oxic (e.g., surface soil). *Dhc* strains may respond differently to sulfide but both *Dhc* strain 195 and strain FL2 were not inhibited by 1 mM sulfide (Adrian et al., 2007a; He et al., 2005). However, no dechlorination occurred in strain FL2 cultures at 5 mM sulfide indicating that *Dhc* dechlorination activity cannot be expected in zones where very high dissolved sulfide concentrations exist.

The majority of *Dhc* cultures originated from freshwater environments although *Dhc* and *Dhc*-related bacteria implicated in the dechlorination of PCBs and PCDDs have been detected in and enriched from estuarine sediments (Ahn et al., 2007; Bedard, 2008; Cutter et al., 2001; Fagervold et al., 2007; May et al., 2008). The PCB-dechlorinating *Dhc* relative "Dehalobium chlorocoercia" was isolated from Charleston, South Carolina, harbor sediment but does not

require elevated salt concentrations for growth (May et al., 2008). The high abundance of halogenated organic chemicals produced naturally in marine environments (Gribble, 1998, 2003, 2004) suggest that marine habitats harbor a considerable diversity of dechlorinators, including *Dhc* and *Dhc* relatives. Since drinking water contamination with chlorinated solvents is generally a freshwater problem, efforts to enrich and isolate dechlorinators from marine habitats have been limited. Recent 16S rRNA gene-based surveys indeed suggest that *Dhc* are present in marine habitats including the ocean floor deep subsurface (Chandler et al., 1998; Inagaki et al., 2003, 2006; Rappé and Giovannoni, 2003; Teske, 2006). It should be noted that contamination with chlorinated compounds is not a prerequisite to find and enrich *Dhc*. For example, the TCE-dechlorinating *Dhc* strain FL2 was obtained from presumably pristine river sediments with no reported contamination with chlorinated compounds (He et al., 2005). Therefore, it is likely that marine systems and deep subsurface sediments harbor an unexplored diversity of *Dhc* and *Dhc* relatives that await discovery.

2.13 *DEHALOCOCCOIDES* ECOLOGY

Dhc are unique dechlorinators but have features such as slow growth rates and intricate nutritional requirements that may be characteristic of the uncultured majority of slow-growing, fastidious subsurface bacteria. Despite the difficulties in culturing *Dhc* isolates in the laboratory, *Dhc* perform well in the "wilderness" when introduced back into a contaminated, anoxic aquifer. Typically, environmental microbes maintained and spoiled (e.g., provided with "good food") in the laboratory do not compete when reintroduced into the wilderness (Atlas and Philip, 2005). The situation is different with *Dhc,* and bioaugmentation with *Dhc*-containing consortia has become a viable bioremediation technology (Major et al., 2003; Ritalahti et al., 2005). The increased availability of *Dhc* isolates and related dechlorinators, along with pure cultures of community members that provide essential services to *Dhc*, will enable detailed studies of interspecies interactions and generate new insights into *Dhc* nutritional requirements and ecology.

The reasons for enhanced *Dhc* dechlorination performance in mixed cultures have not been explored in detail but can be attributed to community members that protect *Dhc* from toxic oxygen and supply required substrates and growth factors to *Dhc*. For example, the energy metabolism of methanogens and acetogens involves methyl-group transfer reactions, which are catalyzed by corrinoid enzyme systems. Hence, these microbial groups may play roles in providing crucial cofactors (e.g., corrinoid precursors) that *Dhc* cannot synthesize.

Dhc are strictly hydrogenotrophic and compete with other microbes for hydrogen as electron donor. Secondary fermentation processes (i.e., fermentation of organic acids and alcohols) are the primary source of hydrogen in anoxic subsurface environments. Such fermentations release hydrogen but cease for thermodynamic reasons when the "waste" product hydrogen accumulates (Schink, 1997). To overcome hydrogen-controlled thermodynamic constraints, fermenters associate with hydrogen consumers in so-called syntrophic associations (Schink, 1997). The hydrogen-consuming populations, such as hydrogenotrophic dechlorinators, associate with the fermenters to scavenge hydrogen, thus alleviating thermodynamic constraints on fermentation. This process, called interspecies hydrogen transfer, lessens inhibition of fermentation due to hydrogen accumulation. Indeed, as previously mentioned, reductive dehalogenation is more favorable thermodynamically than methanogenesis, and *Dhc* outcompete methanogens for low levels of hydrogen produced from fermentable substrates like fatty acids and vegetable oil, a substrate often used for *in situ* biostimulation (Fennell et al., 1997; Löffler et al., 1999; Yang and McCarty, 1998). The close physical association (i.e., juxtaposition) between *Dhc* cells and cells of other community members, such as

methanogens, may facilitate exchange of growth factors and/or nutrients including hydrogen (Heimann et al., 2006; Schink, 1997).

Obviously, understanding such microbe-microbe interactions is relevant for managing and predicting dechlorination performance at bioremediation sites. The PCE-to-ethene-dechlorinating bioaugmentation consortium Bio-Dechlor INOCULUM (BDI) harbors multiple *Dhc* strains and rapidly dechlorinates PCE to ethene in the absence of methanogens, indicating that methanogenic archaea are not required for robust dechlorination activity, and other bacteria (not Archaea) fulfill the nutritional requirements of *Dhc* (Amos et al., 2008b; Amos et al., 2009). As mentioned in Section 2.3.3, strain FL2 was isolated from a robustly dechlorinating, defined co-culture that included two unusual spirochete populations (FLiPS) that provide an as-yet unknown benefit to *Dhc* (Ritalahti and Löffler, 2004a, b, 2005; Ritalahti, 2012). Similarly, strain DF-1, a PCB-dechlorinating *Dhc* relative obtained from Charleston Harbor sediment, requires the presence of a *Desulfovibrio* sp. (or the addition of *Desulfovibrio* sp. cell extract) for PCB dechlorination (May et al., 2008; Wu et al., 2002). *Dhc* strain 195 also benefits from the presence of *Desulfovibrio desulfuricans* or *Acetobacterium woodii* (He et al., 2007). The availability of pure cultures of *Dhc* and *Dhc* relatives along with isolates that apparently provide growth factors and/or other services to the dechlorinators provide opportunities for studying the interspecies interactions and *Dhc* ecology in more detail.

Another interesting observation that warrants further exploration is the occurrence of multiple *Dhc* strains in the same environmental sample or enrichment. For example, microcosms established with the same river sediment that yielded the TCE-dechlorinating *Dhc* isolate strain FL2 also harbored *Dhc* strain RC1, which cannot dechlorinate chlorinated ethenes but uses 1,2-dichoropropane as an electron acceptor (Löffler et al., 1997a; Ritalahti and Löffler, 2004a). The PCE-to-ethene-dechlorinating consortia BDI, KB-1, ANAS and SDC-9 each contain at least two *Dhc* strains with distinct dechlorination activities (Amos et al., 2008a, 2009; Duhamel et al., 2004; Holmes et al., 2006; Richardson et al., 2002; Vainberg et al., 2009). During efforts aimed at isolating *Dhc* strain GT, a culture that contained only *Dhc* (and no other microorganisms) was obtained initially. However, this early culture contained multiple *Dhc* strains, each with different RDase gene complements (Sung et al., 2006b). These findings suggest that multiple *Dhc* strains with distinct dechlorination activities commonly coexist in the same habitat and enrichment cultures. The consequences with respect to *Dhc* ecology and bioremediation of an extensive RDase gene pool, contributed to by more than a single *Dhc* strain, remain to be explored.

2.14 OUTLOOK

The availability of pure cultures of *Dhc* and related bacteria, along with an increasing pool of students trained to grow and maintain *Dhc* cultures, will provide new information regarding the phylogenetic and functional diversity of these unique dechlorinating bacteria. Detailed studies with community members that provide essential services to *Dhc* will unravel interspecies interactions and generate new insights into *Dhc* nutritional requirements and ecology. The outcomes will include improved culturability and allow a broader research community to study these interesting organisms and facilitate the production of bioaugmentation consortia.

The expanding information gained from genome sequences will continue to reveal insight into *Dhc* adaptation and evolution, as well as elucidate mechanisms and frequencies of RDase gene mobilization and dissemination. Knowledge of reaction stoichiometries and reaction kinetics will enable metabolomic flux modeling and generate more detailed understanding of how *Dhc* respond to bioremediation treatment (e.g., electron donor addition) (Ahsanul Islam et al., 2010). Combined with a comprehensive suite of molecular biological tools for monitoring

biomarker targets, such integrated approaches will transform bioremediation from an empirical practice to a technology with predictable outcomes.

2.15 IMPLICATIONS FOR BIOREMEDIATION PRACTICE: TAKE HOME MESSAGES

1. *Dehalococcoides* (*Dhc*) are naturally occurring, strictly anaerobic, specialized bacteria that require hydrogen as electron donor and certain halogenated organic compounds as electron acceptors.
2. *Dhc* are often detected in chlorinated solvent-contaminated, anoxic subsurface environments but may be present at low abundances, with prevailing environmental conditions limiting dechlorination activity.
3. Dechlorination activity can be initiated or rates increased by biostimulation, which can be combined with bioaugmentation.
4. Some *Dhc* strains reductively dechlorinate toxic dichloroethenes (*cis*-DCE, *trans*-DCE, 1,1-DCE) and VC to environmentally benign ethene. To date, no other bacteria have been found that detoxify these compounds via reductive dechlorination. Note that not all *Dhc* strains dechlorinate DCEs and VC to ethene and some *Dhc* strains do not dechlorinate chlorinated ethenes at all.
5. *Dhc* isolates are difficult to obtain and grow in pure culture; however, *Dhc* are more easily maintained and exhibit robust dechlorination activity in consortia.
6. *Dhc* have small genomes but possess many (up to 36) reductive dehalogenase genes, whose functions are largely unknown (i.e., the range of chloroorganic compounds that *Dhc* can dechlorinate has not been explored).
7. Quantitative PCR assays that enumerate the *Dhc* 16S rRNA gene are available to monitor the *Dhc* population size in environmental samples.
8. The *bvcA* and *vcrA* genes serve as biomarkers for complete reductive dechlorination of chlorinated ethenes to ethene. Although the knowledge of biomarker genes for monitoring the chlorinated ethene reductive dechlorination process is incomplete, the combined analysis of *Dhc* 16S rRNA genes and the *tceA*, *bvcA* and *vcrA* genes provides valuable information for site assessment and bioremediation implementation.
9. Under conditions of limited hydrogen flux or when the chlorinated contaminants have been consumed, *Dhc* growth and activity cease and the *Dhc* population declines.

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CHAPTER 3

PRODUCTION AND HANDLING OF *DEHALOCOCCOIDES* BIOAUGMENTATION CULTURES

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3.1 INTRODUCTION

Chlorinated ethenes have been used extensively as industrial solvents and cleaning agents, and improper disposal practices and accidental spills have led to them becoming common groundwater contaminants throughout the United States and the world (Moran and Zogorski, 2007; Westrick et al., 1984). Treatment of chlorinated solvent contamination has involved the use of a wide range of technologies including soil vapor extraction, air sparging, chemical oxidation or reduction, *in situ* thermal treatment, and biological oxidation or reduction. Currently, the most common treatment alternative for these compounds is biological degradation facilitated by either stimulating indigenous dechlorinating organisms or adding cultures of exogenous microorganisms enriched especially for this task. Adding exogenous organisms is commonly referred to as bioaugmentation.

Although the use of bioaugmentation has a long history for treating challenging pollutants, overselling of the technology as a panacea for pollutant remediation and underperformance of some commercial products led to a period of low acceptability of this technology for remedial activities. In many cases, the lack of acceptance of the technology was justified because the addition of microbes to contaminated environments did not improve remediation beyond what could be achieved by stimulating indigenous microbial populations (DeFlaun and Steffan, 2002; Unterman et al., 2000). In the case of remediating chlorinated solvent contaminated aquifers, the technology was limited to aerobic cometabolism that was challenged by poor transport of the biocatalysts, an inability of the microbes to use the contaminant as a growth substrate, the need to maintain aerobic conditions, the production of toxic intermediates, and the inability to degrade some important solvents (most notably perchloroethene [PCE]) (Steffan et al., 1999).

The success of early applications of *Dehalococcoides* spp. (*Dhc*) containing consortia for *in situ* remediation of chlorinated solvent contaminated aquifers has led to a renewed interest in bioaugmentation because the added cultures reproduced *in situ*, were transported well through the treated aquifer, and improved bioremediation performance (Ellis et al., 2000; Major et al., 2002). The fact that the cultures did not require oxygen to degrade the contaminants made them easy to transport and apply, and only a fermentable carbon source was needed to support their growth and degradative activity. To date, several hundred bioaugmentation applications have been performed to remediate chlorinated solvent contaminated aquifers.

3.1.1 Microbial Cultures Used for Bioaugmentation

The predominant biodegradation pathway used for chlorinated ethene remediation in contaminated aquifers is anaerobic reductive dehalogenation. During reductive dechlorination,

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chlorinated ethenes are used as electron acceptors by naturally adapted bacteria, and the process results in a chlorine atom on the chlorinated compound being removed and replaced with a hydrogen atom. Sequential dechlorination of PCE most commonly proceeds to trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and finally the desired end product, ethene. In some bacteria, *trans*-1,2-DCE or 1,1-DCE (Zhang et al., 2006) are the predominant TCE dechlorination products.

Although biodegradation of chlorinated ethenes often can be performed by naturally occurring microorganisms that use endogenous resources to support contaminant degradation (i.e., intrinsic bioremediation) or nutrients that are purposefully added to support their activity (i.e., biostimulation), some aquifers lack an indigenous microbial population capable of completely dechlorinating the contaminants. This lack of an adequate microbial population capable of completely dechlorinating PCE and TCE to ethene can sometimes lead to the accumulation of *cis*-DCE and VC (Hendrickson et al., 2002), which are more toxic than the parent compounds. Consequently, the addition of exogenous organisms (i.e., bioaugmentation) is sometimes used to supplement the indigenous microbial population.

While many dechlorinating microorganisms have been identified, only bacteria of the genus *Dhc* have been shown to completely reduce PCE and TCE to ethene (Maymó-Gatell et al., 1997; He et al., 2003a, b). These organisms use molecular hydrogen as an obligate electron donor and halogenated compounds as obligate respiratory electron acceptors. Acetate is typically used by *Dhc* as a carbon source. Studies of field sites have strongly correlated the presence of *Dhc* strains with complete dehalogenation of chlorinated ethenes *in situ* (Hendrickson et al., 2002). Therefore, microbial cultures used to augment chlorinated solvent contaminated groundwater contain at least one strain of the *Dhc*. A list of some known suppliers of bioaugmentation cultures for chlorinated solvents is presented in Table 3.1.

Because of the difficulty of growing *Dhc*-type organisms in pure culture (Maymó-Gatell et al., 1999; He et al., 2003a, b), consortia containing *Dhc*, fermentative bacteria and other microbes that support the growth and activity of the *Dhc* strains are used for remedial applications (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002). The consortia, and the *Dhc* therein, can be grown using a wide range of carbon sources that are fermented to

Table 3.1. List of Known U.S. Vendors of *Dhc*-Containing Bioaugmentation Cultures

Vendor	Culture Name	Contact Information
FMC Corp.	Dechlorination culture	815-235-3503; http://environmental.fmc.com/
BCI, Inc.	BCI-e	617-923-0976; http://www.bcilabs.com
Environmental Bio-Systems, Inc.	Dechlorination culture	415-381-5195; http://www.ebsinfo.com/
EOS Remediation, LLC	BAC-9™	888-873-2204; http://www.eosremediation.com/
JRW Bioremediation, LLC	Dechlorination culture	913-438-5544; http://www.jrwbioremediation.com
Redox Tech, LLC	RTB-1	919-678-0140; http://www.redox-tech.com/
Regenesis	Bio-Dechlor INOCULUM® PLUS(+)	949-366-8000; http://www.regenesis.com
Shaw Environmental, Inc.	SDC-9™, Hawaii-05™, PJKS™	609-895-5350; http://www.shawgrp.com/capabilities/technology/environmental/bioaug
SiREM Labs	KB-1®	519-822-2265; http://www.siremlab.com
Terra Systems, Inc.	TSI DC bioaugmentation culture™	302-798-9553; http://www.terrasystems.net

Web sites in Table 3.1 were last accessed May 2012

hydrogen (H_2) and acetate. Hydrogen serves as an electron donor for dechlorinating bacteria, and a chlorinated ethene (usually PCE or TCE) is added as the electron acceptor to allow growth. The substrate fermentation product, acetate, may serve as a carbon source and in some cases also serves as an electron donor.

3.1.2 Why High Density Microbial Cultures Are Important

One of the significant challenges of performing bioaugmentation at a commercial scale is the large size of contaminant plumes and the large amount of culture that often is needed to facilitate timely and successful remediation. The issue of scale can best be illustrated by a simple hypothetical example. One acre of land (0.4 hectare [ha], equal to 43,560 square feet [ft^2] or 4,047 square meters [m^2]) is slightly smaller than the size of an American football field including the end zones (57,600 ft^2; 5,353 m^2). If we assume that a groundwater plume extends throughout this 1 acre area (300 ft × 145 ft; 91 m × 44 m) within a 10 ft (3 m) saturated thickness, the total volume of the contaminated media would be ~435,000 cubic feet (ft^3) (123,000 cubic meters [m^3]). If the aquifer has an effective porosity of 25%, the total volume of contaminated water in the plume would be 109,000 ft^3 (3,087 m^3; ~3×10^6 liters [L]). To achieve a final *Dhc* concentration of 10^7 *Dhc*/L of groundwater to effectively remediate the site (Lu et al., 2006), 3×10^{13} *Dhc* cells would be required. If the culture growing process produced 10^9 *Dhc*/L (Major et al., 2002), ~30,000 L of *Dhc* culture would be required. At a cost of $150–$300/L, the culture cost for this moderately-sized plume would be $4.5–$9 million. Using a culture with 10^{11} *Dhc*/L would reduce the cost by a factor of 100.

Of course, several factors come into play in actual remediation scenarios (Lee et al., 1998). For example, it may be unrealistic to expect even distribution of the *Dhc* across a contaminated aquifer, so we would expect higher concentrations of culture, and dechlorination activity, near injection points. Practitioners also may consider constructing a series of *in situ* flow-through *Dhc*-seeded barriers or recirculation systems, depending on the remedial goals, to reduce the amount of culture needed. In addition, if conditions are favorable, some growth of the culture can be expected *in situ*. Nonetheless, it is apparent that large volumes of culture may be needed to treat some plumes, and production of high cell density cultures can greatly reduce the volume of culture needed for, and the cost of, bioaugmentation treatment.

If only a small amount of organisms is added and *in situ* growth is anticipated, however, the actual cost of growing these organisms *in situ* under sub-optimal growth conditions also should be considered. For example, typical *in situ* temperatures (12–15 degrees Celsius [°C]) would likely promote significantly slower and less efficient growth of *Dhc* than the optimum temperatures (25–35°C) maintained in reactor systems. Similarly, although substrate feed rates can be carefully controlled in a reactor system, *in situ* substrate feeding, especially electron acceptor feeding, is determined by groundwater flow rates and is likely to result in inefficient growth of *Dhc* and inefficient electron donor utilization. Poor growth and substrate utilization can ultimately result in greater overall treatment costs than adding additional high density culture at the beginning of treatment.

3.2 GROWING INOCULA

3.2.1 Microbial Growth Options: Batch Versus Continuous

Growth of bacterial inocula is a mature science, but in practice it is often as much art as science. Ljungdahl and Wiegel (1986) have provided excellent general guidance for growing anaerobic bacteria. The production of consistent bioaugmentation cultures for chlorinated

solvent remediation, however, presents many unique challenges to practitioners. First, the cultures are consortia, meaning that the success of the culturing process requires maintaining many different bacterial strains, even some that may not be identified. Likewise, growth of dehalogenating organisms, such as members of the genera *Dehalococcoides* and *Dehalobacter*, requires the contribution of other consortium members that provide electron donor (H_2) and other growth nutrients (e.g., corrinoids) for the dehalogenating microbes.

Growth of bacterial inocula can be performed in continuous mode where the culture is continually grown and harvested from a flow-through reactor, in fed-batch mode where a culture is grown in a vessel and harvested and then another culture begun, or in a hybrid of the two whereby the culture is maintained in a reactor until a volume of culture is harvested and then replaced with fresh medium.

The primary advantage of a continuous growth system is that the culture remains at a relatively high cell density and specific activity through the culturing process (Stafford, 1986). This technique is typified by the operation of a chemostat where the growth medium continuously flows into and out of a reactor and the feed rate is balanced against the growth rate of the culture. Theoretically, continuous cultures allow the cell population to grow indefinitely in an unchanging environment. This continuous growth technique is likely rare for the production of bioaugmentation cultures because of the sporadic demand for cultures and because of the need to maintain anaerobic conditions. Use of this method would require that the produced cultures be continuously collected and stored until use, and media fed into the reactor would have to be made anaerobic. In addition, continuous culturing requires a more complicated control system (to balance growth rate and dilution rate) and installation of additional equipment (e.g., tanks and pumps) that can hold and continuously supply anaerobic medium to the reactor and to collect and handle the produced culture.

The continuous culture technique may be useful for some on-site applications where the culture is grown with contaminated groundwater fed into the reactor and the effluent is used as an aquifer inoculum (Fam et al., 2004). The approach would require sufficiently high groundwater contamination to maintain growth of the organisms because adding chlorinated solvents for growth could result in further contamination of the aquifer.

A more likely approach for producing cultures for bioaugmentation is a semi-continuous process whereby the culture is maintained in the reactor until needed and then some of the culture is harvested. The harvested volume would then be replaced with fresh medium and growth would continue. This approach is common in research laboratories that maintain cultures for study. The primary advantage of this technique is that cell growth must replace only the volume of culture removed. For example, if one half of the culture is harvested, a single doubling of the remaining culture will replace the cells removed. This process may be most suitable for cases where demand for the culture is high, and medium is regularly removed from the culture and replaced with fresh medium.

The primary disadvantages of the semi-continuous culture method is that the cultures are typically maintained in a stationary growth phase in the reactor and specific activity of the culture can be reduced relative to that of actively growing and reproducing cells. In addition, long-term continuous growth or prolonged maintenance of a culture in the reactor vessel can lead to the accumulation of toxic metabolites that affect culture activity, survival, or performance. In fact, many semi-continuous microbial growth processes are designed to produce the accumulating toxic product, for example, ethanol. Extended maintenance of cultures in reactors, during either continuous or semi-continuous growth, is rare in industrial applications because it commonly leads to an accumulation of mutations that ultimately results in strain degeneration (Dykhuizen and Hartl, 1983; Harder et al., 1977; Heineken and O'Conner, 1972). Furthermore, long-term maintenance of a mixed culture reactor can result in population

changes that may affect the performance of the consortium during environmental applications if an important member of the population is lost, for example.

During fed batch microbial production, cultures are grown from a low cell density to a high cell density by controlling substrate addition and reactor conditions. This process allows harvesting of cultures during their most active growth states, and minimizes the risk of population changes that can occur during long-term culture maintenance. Likewise, the buildup of recalcitrant toxic metabolic products in the medium is minimized.

The primary limitation of the fed batch growth approach is that cells may have to be harvested before they are needed for field application. As such, it must be possible to store the cultures until needed. The storage of large culture volumes, especially anaerobic cultures that cannot be air dried because of oxygen toxicity, can require a large space or even a large refrigerated space. Concentrating the cultures under strict anaerobic conditions before storage, however, can reduce storage space requirements (see below).

For production of *Dhc*-containing bioaugmentation cultures, the fed batch growth process appears most practical. This approach allows cells to be harvested in late log phase (i.e., when cells are at their greatest growth rate and density) or early stationary phase (i.e., just at the end of the rapid growth phase) to ensure the greatest cell numbers and highest possible activity in the applied cultures, and to maintain culture consistency between batches. Fed batch growth also prevents the accumulation of extracellular metabolic products (e.g., acetate and propionate) which ultimately could affect culture activity. Experimentation has demonstrated that *Dhc* cultures grown by the fed batch process can be concentrated by membrane filtration and can be stored refrigerated for more than 30 days (d) without considerable loss of activity (Vainberg et al., 2009). Inoculum concentration processes and storage studies are presented below.

3.2.2 Culture Growth Protocol

Production of bacterial cultures is typically accomplished in a series of vessels that increase culture volume in a step-wise fashion. That is, an initial starter culture is grown in a vessel, and that culture is used as a seed culture for inoculating a larger culture. For example, for growth of *Dhc*-containing cultures, small serum vial enrichment cultures (160 milliliters [mL]) can be used to inoculate 2–7-L flasks or reactors. Once high *Dhc* levels are achieved, this culture is used to inoculate 10–20 L of culture medium, and so on. It is usually desirable to start a *Dhc* culture at an optical density at 550 nanometers (nm) (OD_{550}) of approximately 0.1. This optical density equates to approximately 10^{11} total cells/L, and approximately 10^9 *Dhc*/L (note, 10^9 *Dhc*/L is equivalent to 10^6 *Dhc*/mL). Thus, it is important to plan seed culture steps to ensure a sufficient inoculum size at each scale-up step. The following sections describe methods used to prepare *Dhc*-containing cultures in Shaw Environmental Inc.'s laboratory and are derived from Vainberg et al. (2009).

3.2.2.1 Seed Cultures

Bench-scale experiments and seed culture production are performed in 3-L or 7-L Applicon reactors (Cole Parmer, Vernon Hills, Illinois) equipped with pH, dissolved oxygen (DO) and mixer controls. Substrate and sodium hydroxide (NaOH) feeds are controlled by using syringe pumps (Harvard Apparatus, Holliston, Massachusetts) and low-flow peristaltic pumps (Cole Parmer, Chicago, Illinois). Larger seed cultures are produced in a similarly equipped 20-L Biolafitte reactor (Pierre Guerin, Inc., Spring Lake Park, Minnesota). Still larger cultures are produced in a 750-L ABEC reactor (ABEC Inc., Bethlehem, Pennsylvania) or a custom built 4,000-L stainless steel reactor. In each case, anaerobic conditions are maintained by pressurizing the vessels with nitrogen. At the end of the growth cycle, cells in the reactor broth are

concentrated by passing the broth over a custom-built cell concentrator constructed with six Kerasep™ tubular ceramic membranes (Novasep, Inc., Boothwyn, Pennsylvania; refer to Figure 3.10 in Section 3.4.3) contained within stainless steel piping to prevent oxygen intrusion. Concentrated cells are stored at 4°C in 18.5-L stainless steel soda kegs (refer to Figure 3.12 in Section 3.5.2) that are pressurized with nitrogen.

For seed culture production, revised anaerobic mineral medium (RAMM) medium (Shelton and Tiedje, 1984) without sodium bicarbonate ($NaHCO_3$) and sodium sulfide (Na_2S) is added to the 20-L reactor and steam sterilized at 121°C and 15 pounds per square inch (psi) for 45 minutes (min). After sterilization, the reactor is connected to a nitrogen tank to maintain a positive pressure of nitrogen in the vessel during cooling to 30°C. After the temperature in the reactor reaches the set point temperature of 28–30°C and anaerobic conditions are achieved (measured DO = 0 mg/L), nitrogen flow is stopped and $NaHCO_3$ solution is added aseptically to the medium. The reactor is then inoculated with 2 L of SDC-9™ or other culture containing approximately 10^{10}–10^{11} *Dhc*/L. The final volume of medium in the 20-L reactor is 16–18 L.

After inoculating the reactor, sterile 10% yeast extract (YE) solution is added to a final concentration of 0.1% YE (weight per volume [w/v]), vitamin B12 (0.03 mg/L) and PCE or TCE is added to a final concentration of 10 mg/L. No lactate is added at the beginning of the culture process because the YE provides sufficient carbon to support the initial cell growth and the addition of lactate at this point in the process results in significant methane formation in the reactor. The reactor is operated at 28–30°C with an agitator speed of 100 revolutions per minute (rpm). pH is maintained at 6.4–7.2 by the addition of an anoxic solution of NaOH (2 N). Alternatively, to increase pH during bacterial growth, the reactor is sparged with nitrogen to remove dissolved carbon dioxide (CO_2). After 1 day of culture growth, sodium lactate (60% solution) is added continuously to the reactor at a flow rate of 0.02–0.04 mL/hour (h) × L of medium. A second addition of PCE or TCE (10 mg/L) is added to the reactor only after complete dechlorination of PCE/TCE, but before complete dechlorination of *cis*-DCE. Typically, PCE/TCE is added to the medium when the concentration of *cis*-DCE in the medium is reduced to 1–3 mg/L. When the culture reaches an OD_{550} of approximately 1.0, it is transferred anaerobically to the 750-L reactor.

3.2.2.2 550-L Scale

Intermediate size batches (to 550 L) of *Dhc* cultures are prepared in a 750-L stainless steel reactor. The 750-L reactor is prepared with 540 L of RAMM medium containing 0.1–0.2% (w/v) YE, but without $NaHCO_3$, and sterilized as previously described. After sterilization and cooling, vitamin B12 (0.03 mg/L) and $NaHCO_3$ (660 grams [g]) dissolved in 10 L of deionized (DI) water is added to the reactor through a sterile filter, and neat PCE/TCE is added to a final concentration of 10 mg/L. The reactor is connected to a nitrogen tank to maintain anoxic conditions, and is operated under the same conditions as described for the 20-L reactor except the agitator speed is set at 60 rpm. The automatic pH control system on the reactor is inactivated to avoid addition of excess sodium ion (as NaOH).

Once the appropriate temperature (28°C) is reached in the reactor, the seed culture is aseptically transferred to the larger reactor while maintaining strict anaerobic conditions. After 1 day of culture growth, a continuous feed of sodium lactate (60% solution) is initiated with a flow rate of 0.02–0.04 mL/h × L. Periodically, samples are taken from the reactor and analyzed for the presence of chlorinated products, volatile fatty acids (VFAs) and *Dhc* concentration. After complete dechlorination of the first addition of PCE/TCE, chlorinated solvent is again added to a final concentration of 10 mg/L. Subsamples (25 mL) of the culture are periodically removed from the reactor to measure cell density and to perform bottle assays

to determine specific activity. When the specific PCE and *cis*-DCE dechlorination activity reaches 1.3–1.7 mg/h × g of dry weight (DWT), a continuous feed of neat PCE/TCE is initiated at a rate of 0.18–0.25 microliter (μL)/h × L. This rate is increased to 0.9–1.2 μL/h × L as the culture cell density and dechlorination activity increases. The culture is grown for 13–15 days until an $OD_{550} \approx 0.7–1.1$ or $10^{10}–10^{11}$ *Dhc*/L is achieved. Higher *Dhc* concentrations can be obtained by extending the growth period for up to 35 days.

3.2.2.3 4,000-L Scale

Growth of the cultures in the 4,000-L reactor (working volume 3,200 L) is performed essentially as described for the 750-L reactor, but because the 4,000-L reactor does not have an impeller, the cells are continuously suspended by using a centrifugal pump that circulates the culture medium. To provide effective distribution of relatively high amounts of added PCE/TCE (up to 40 mL initially and then continuously up to 21 mL/h) in the reactor medium, these chemicals are added directly to the centrifugal pump where they are mixed with a high flow of recirculating medium from the reactor.

The PCE feed is supplied by using either an ISMATEC high precision multichannel pump (Model C.P 78023-02, Cole Parmer, Vernon Hills, Illinois) or a syringe pump and 100-mL gas tight glass syringes. To supply the TCE feed, which has four times higher vapor pressure than PCE (57.9 millimeters of mercury [mm Hg] and 17.8 mm Hg, respectively) and cannot be added accurately by using a syringe pump, the ISMATEC high precision multichannel pump is used. The 4,000-L reactor is chemically sterilized by using NaOH and a clean in place system. The culture medium in the 4,000-L reactor is not sterilized. Substrate feeding and other parameters are as described for the 750-L reactor. The reactor is inoculated with either culture from the 750-L reactor or refrigerated concentrated cell stocks, but in each case under strict anaerobic conditions. The inoculum volume is calculated to achieve an initial *Dhc* concentration of approximately $10^8–10^9$ *Dhc*/L.

During the initial growth phase with continuous or periodic PCE feed, the *cis*-DCE and VC dechlorination rate is lower than the rate of PCE dechlorination; this results in a rapid accumulation of *cis*-DCE and VC in the reactor (Figure 3.1a). After 1–2 days of growth, however, even with continuous PCE feed, the concentrations of *cis*-DCE and VC begin to decline rapidly and continuous feeding of PCE can resume. This may suggest that it takes longer to induce *cis*-DCE and VC degradation genes than PCE degradation genes, that organisms in the consortium that degrade PCE to *cis*-DCE initially grow faster than organisms that degrade *cis*-DCE and VC, or that a combination of both of these factors creates this affect.

3.3 FULL-SCALE PRODUCTION RESULTS

Examples of large-scale production of the SDC-9™ consortium in a 4,000-L reactor (culture volumes of 2,500 L and 3,200 L, respectively) are presented in Figure 3.1b and c. Figure 3.1b shows the growth of a culture inoculated with a culture transferred directly from the 750-L reactor without refrigeration or storage, and Figure 3.1c shows the growth of a culture inoculated with a similar concentrated culture that had been stored for 19 days at 4–6°C. The data show a slight difference in the lag phase observed before the start of log phase growth. The lag phase varied from 2 days for the culture directly inoculated from the 750-L reactor to about 5 days for stored culture. During production-scale and research applications, *Dhc*-containing consortia are typically grown with lactate as an electron donor and PCE as an electron acceptor. Other electron donors or electron donor mixtures, however, have been used successfully, at least for small-scale production. For example, early studies with the KB-1

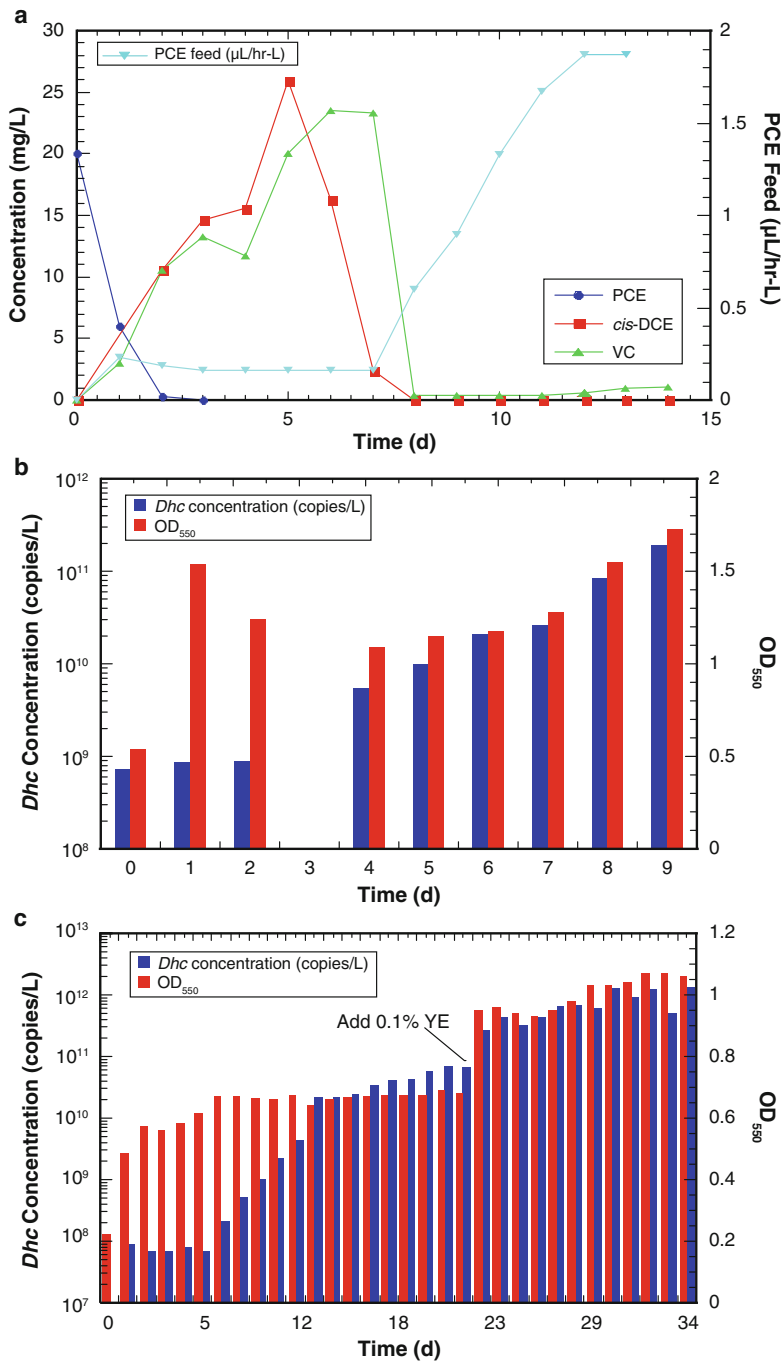

Figure 3.1. Growth of SDC-9™ consortium in a 4,000-L reactor. (a) Changes in concentrations of PCE, *cis*-DCE and VC during growth of SDC-9™ in a 4,000-liter L reactor. Initially, PCE and YE were added to the medium to final concentrations of 20 mg/L and 0.1%, respectively. Sodium lactate feeding (0.03 mL/h × L) was initiated after 1 day of culturing. (b) Reactor inoculated with fresh concentrated culture directly from a 750-L reactor. YE (0.2% w/w) was added at the beginning of the growth process. (c) Reactor inoculated with concentrated culture that had been stored for 19 days at 4–6°C. YE (0.1% w/w) was added at the beginning of bacterial growth and on day 22 of culturing. In each case sodium lactate and PCE were used as electron donor and electron acceptor substrates, respectively. In panels (b) and (c) *blue bars* represent *Dhc* concentration as measured by qPCR, and *red bars* represent OD_{550}.

culture suggested a growth benefit if the culture was grown with a mixture of methanol, ethanol, acetate and lactate (Duhamel et al., 2002) presumably because the electron donors are utilized at different rates or because they support different groups of microbes in the culture.

Testing of different substrates including lactate, ethanol, methanol and citrate revealed that the SDC-9™ consortium was able to utilize all of these substrates, but sufficient degradation activity and bacterial growth rate is achieved with lactate as the electron donor substrate (data not shown). Experience at Shaw Environmental, Inc. (Shaw) has shown that it is easier to control substrate feeding rates by using a single primary electron donor such as sodium lactate. Even with a single electron donor/hydrogen source like lactate, utilization of the substrate by the consortium leads to production of a complex mixture of metabolites, primarily VFAs, which can themselves act as electron donors/hydrogen sources for the culture. Balancing the concentration of a mixture of electron donors and mixtures of electron acceptors (i.e., PCE and PCE daughter products) during large-scale bacterial culturing adds increased complexity to process optimization. Although PCE is used as a primary electron acceptor, similar results, in terms of specific activity and final cell densities, have been obtained when TCE is used as an electron acceptor to grow SDC-9™.

For routine process monitoring, the OD of reactor samples is measured (Figure 3.1b and c). During the initial lag phase, the OD of the consortium increases about threefold due primarily to the rapid growth of non-*Dhc* organisms in the consortium on the added YE. A similar rapid increase in non-*Dhc* organisms, and OD, also is observed if a high concentration of lactate (5–12 millimolar [mM]) is added to the medium at the beginning of the culture process (data not shown), despite the lag in *Dhc* growth. These results demonstrate that, at least during the early stages of cell growth, OD measurements are not a good indicator of *Dhc* concentration in the culture, and more advanced measurements like quantitative polymerase chain reaction (qPCR; Löffler et al., 2000; Ritalahti et al., 2006) are needed to effectively estimate *Dhc* numbers in the culture.

Following the lag phase, and after lactate is fed continuously at a low rate to generate low levels of hydrogen (<20 nanomolar [nM]), the *Dhc* concentration begins to increase exponentially (i.e., log phase growth) and reaches about 10^9–10^{10} cells/L. During this period of growth, the culture OD is correlated with the growth of *Dhc* culture. These results suggest that during certain periods of the cell growth process, measurements of OD may be useful for estimating *Dhc* levels in the reactor and to automate the control of the culturing process.

The OD of the cultures typically stabilizes after approximately 10 days, but exponential growth of *Dhc* continues until approximately day 24 (Figure 3.1b and c). These results suggest that non-*Dhc* microorganisms in the consortium initially grow much faster than *Dhc*. During this early growth period, *Dhc* represent a relatively low proportion of the total bacterial population of the culture, but during extended growth the relative abundance of *Dhc* in the culture increases (Figure 3.2).

During the initial stages of 3,200-L cell culturing (to day 25), a maximum *Dhc* concentration of ~10^{11} *Dhc*/L is achieved in the reactor, even though growth substrates are still present in the culture broth (Figure 3.1a). However, *Dhc* concentrations in the reactor can be increased ~10-fold by the addition of YE as a nutrient source. The exact role of the YE is not known, but its addition also revives the growth of non-*Dhc* organisms in the consortium as reflected in a rapid increase in culture OD (Figure 3.1c). Because the RAMM medium used at Shaw does not contain sodium sulfide or other sulfur-containing salts, it is possible that the YE provides a needed source of sulfur for the cultures. One g/L of YE provides ~5 mg/L sulfur and 0.48 mg/L iron. YE also could provide a needed source of amino acids and/or precursors for the production

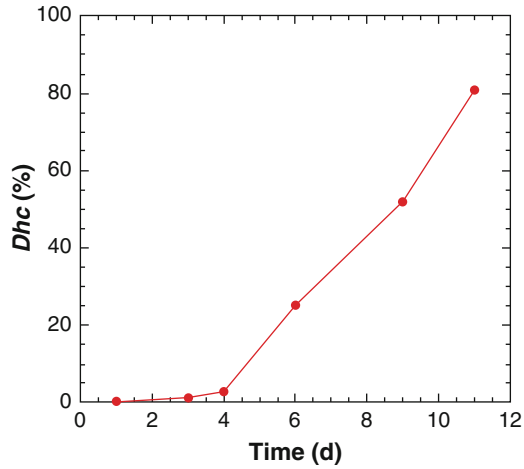

Figure 3.2. *Dhc* concentration relative to total Eubacteria in the SDC-9™ consortium during growth in a 750-L reactor. Both *Dhc* and eubacteria were quantified by using qPCR.

of corrinoid co-factors that are necessary for dehaologenation by *Dhc* strains (Maymó-Gatell et al., 1997). Genome sequencing of *D. ethenogenes* strain 195 (Seshadri et al., 2005) has revealed that this strain does not have all the genes necessary for *de novo* corrinoid synthesis, but it does contain several genes for corrinoid salvage, and He and colleagues (2007) demonstrated that the addition of vitamin B12 allowed for increased growth of *Dhc*.

Analysis of growth parameters from five culture batches (550-L and 3,200-L) has shown that the average *Dhc* specific growth rate for the SDC-9™ culture under the conditions described here was 0.036 h^{-1} with a range of 0.027–0.043 h^{-1}. *Dhc* doubling time averaged 19.3 ± 2.7 h (Vainberg et al., 2009). The described protocol has produced similar results with all three cultures tested (SDC-9™, PJKS™, and Hawaii-05), and in each case the final *Dhc* concentration in the resulting culture is $>10^{11}$ *Dhc*/L (Table 3.2).

Few studies have reported large-scale production of bioaugmentation cultures. Ellis et al. (2000) reported relatively large-scale production of *Dhc* for bioaugmentation, but the study was performed before the widespread use of qPCR methods for specific monitoring of *Dhc*. In that study, batch culturing on lactate and TCE was used to produce about 180 L of the Pinellas culture (Harkness et al., 1999). The resulting culture contained about 2×10^8 total bacteria/mL, a relatively low abundance of *Dhc*, and about 35 g DWT of cells were produced in the culture. The culture had a doubling time of 30–40 h under optimum laboratory conditions. Likewise, Lendvay and colleagues (2003) reported producing 200 L of a *Dhc*-containing culture for field application at a Michigan site. The culture was grown in glass vessels on lactate and PCE, and it contained 1.1×10^{11} total bacteria/L and 1×10^9 *Dhc*/L.

The results presented herein demonstrate that large cultures of *Dhc* can be produced and that high *Dhc* cell densities can be achieved in these cultures. Production of such cultures creates the potential for treating even large contaminated sites by using bioaugmentation. Assuming that 10^7 *Dhc*/L of contaminated groundwater are needed to obtain effective and timely remediation (Lu et al., 2006), 3,200 L of culture with 10^{11} *Dhc*/L could potentially support remediation of 3.2×10^7 L of groundwater, even without further *in situ* growth of the organisms. This equates to a site of about 5.3 acres (2.1 ha) with a 10 ft (3 m) thick plume and soil porosity of 25%.

Table 3.2. Multiple Culture Production Runs with Chlorinated Solvent Dechlorinating Consortia

Culture	Date (month/ year)	Volume (L)	Final OD_{550}	Final *Dhc* (cells/L)[a]	DWT (g/L)	PCE Activity (mg/h/g DWT)	*cis*-DCE Activity (mg/h/g DWT)
SDC-9™	01/2006	550	1.3	1.4 E11	0.51	16	13
SDC-9™	02/2008	550	1.7	2.8 E11	0.66	22	14
SDC-9™	03/2008	3,200	1.6	1.4 E11	0.65	41	37
SDC-9™	05/2008	2,500	1.6	2.4 E12	0.59	42	39
SDC-9™	08/2008	2,000	1.4	1.0 E12	0.51	80	69
SDC-9™	07/2009	2,500	1.5	5.5 E11	0.52	88	82
SDC-9™	08/2009	2,500	1.5	8.6 E11	0.64	101	69
SDC-9™	01/2010	2,500	1.7	7.0 E11	0.57	113	79
SDC-9™	03/2010	2,500	1.4	9.3E11	0.55	126	107
PJKS™	01/2008	2,500	1.1	9.4 E11	0.41	32	14
PJKS™	02/2008	1,700	1.3	1.0 E11	0.50	64	45
Hawaii-05™	11/2007	550	1.2	1.5 E11	0.50	23	16

[a]Based on qPCR assuming 1 16 S rRNA gene copy/cell

3.3.1 Factors Affecting Culture Growth

Several factors can affect the results obtained during growth of *Dhc* cultures, including substrate type and feed rates, pH, and VFA accumulation. Growth of *Dhc* requires the presence of a chlorinated substrate as an electron acceptor, H_2 as an electron donor, and a carbon growth source such as acetate (Cupples et al., 2003; He et al., 2003b; Löffler et al., 2003; Maymó-Gatell et al., 1997). In *Dhc* consortia, the primary growth substrate (e.g., lactate) is fermented by non-*Dhc* members to H_2 and acetate that can be utilized by *Dhc*. The presence of excess H_2, however, can lead to substrate competition with methanogenic bacteria in the consortia that also can use H_2, albeit at a higher substrate threshold than *Dhc* (Löffler et al., 1999; Lu et al., 2001; Yang and McCarty, 1998). Therefore, in developing a cell culturing protocol for the described cultures, attempts were made to maintain consistent low H_2 concentrations within the reactor. The sodium lactate feed rate used during the Shaw culturing process results in a sustained dissolved hydrogen concentration in the reactor of <20 nM. During the initial batch feeding of lactate and YE added prior to inoculation, H_2 concentrations sometimes exceed 100 nM; however, during the extended cell culture process the H_2 concentrations are typically 3–5 nM, which is similar to the half velocity coefficient for hydrogen previously calculated for the VS culture (7 ± 2 nM) (Cupples et al., 2004).

Fermentation of lactate also leads to an accumulation of VFAs (e.g., propionate and acetate; Figure 3.3) that can potentially inhibit dechlorinating organisms in a consortium. Studies with SDC-9™, demonstrated that dehalogenation of chlorinated ethenes by the culture was not inhibited by propionate and acetate concentrations to 6,000 mg/L (82.1 and 101.6 mM, respectively) (data not shown). Figure 3.3a and b show the formation of VFAs during growth of SDC-9™ and PJKS™, respectively. In both cases, the VFA concentrations do not reach inhibitory levels with the culture production protocol described here. Notably, the SDC-9™ culture accumulates much less propionate than the PJKS™ culture grown under the same conditions. Although the reason for this lower accumulation of propionate is not certain, it is

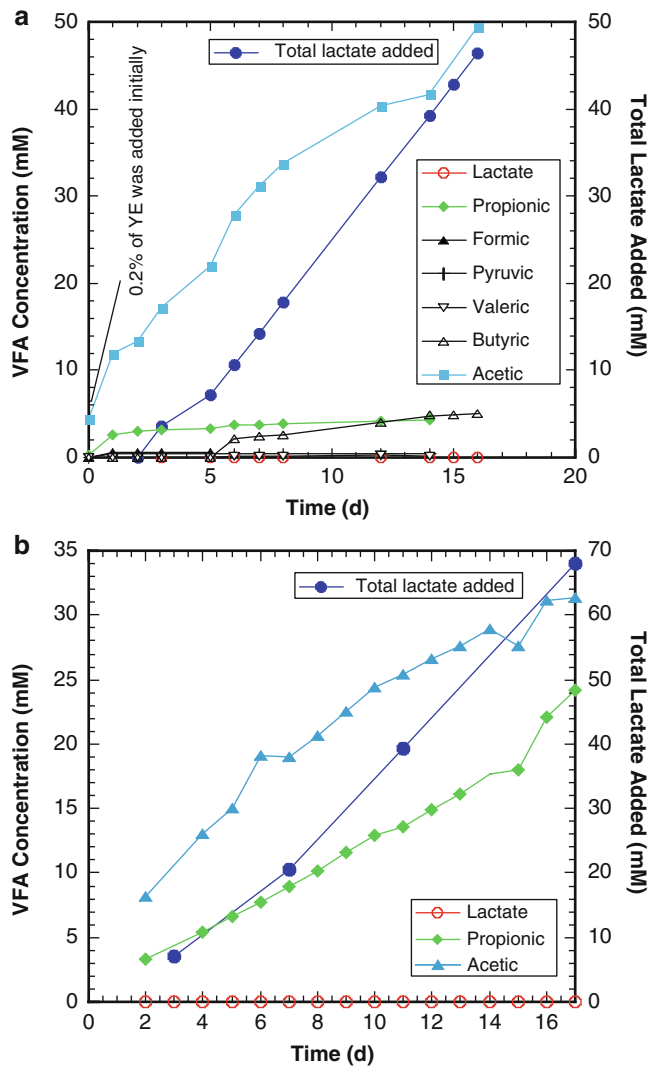

Figure 3.3. Accumulation of VFAs during growth of SDC-9™ (a) or PJKS™ (b) cultures in a 750-L reactor. YE (0.2%) was added as a nutrient at the beginning of the culture process.

likely due to evolution of the SDC-9™ culture to more efficiently ferment propionate during several years of maintenance on lactate as a primary growth substrate.

To optimize the growth of the SDC-9™ consortium, it has been necessary to determine a relationship between PCE feed rate and *Dhc* cell concentration (Schaefer et al., 2009). The culture production process is complicated by the fact that the cultures are mixtures and likely contain multiple populations of dehalogenating microbes. The primary concern is to maintain the VC-reducing population(s) in the consortium, because VC reduction is less energetically favorable than the other dehalogenating reactions. Hence, PCE and TCE dehalogenating populations potentially can outcompete VC reducers if the higher chlorinated substrates are maintained in excess. Furthermore, Cupples and colleagues (2004) observed that net decay in dechlorinating microorganisms could occur in the VS culture if DCE plus VC concentrations are below 0.7 micromolar (μM). In addition, with SDC-9™, based on many bottle assays, the VC dechlorination rate is 28–35% of the PCE dechlorination rate. Therefore, there is a tendency for VC to accumulate in the reactor during high rate PCE feeding. Consequently, PCE feed

rates are adjusted to prevent accumulation of PCE, TCE or *cis*-DCE while maintaining a residual VC concentration in the medium of ~1 mg/L (16 μM). The PCE feed rates for the SDC-9™ consortium have been optimized after evaluating the results from multiple large-scale production runs and bottle assays. The relationship between *Dhc* cell numbers and PCE feed rate is best described by the following equation: *Dhc* concentration (cells/L) = $-6.77 \times 10^{11} + [8.40 \times 10^{11} \times$ PCE feed rate (mg/h × L)] ($R^2 = 0.999$) (Vainberg et al., 2009).

Dehalogenation of chloroethenes by SDC-9™ also is affected by culture pH, with little or no dehalogenation below pH 5.6 and above pH 9.0 (Figure 3.4; Vainberg et al., 2009). In another experiment, the effect of elevated pH on TCE dechlorination activity was studied by incubating SDC-9™ culture in groundwater at pH 9.9 for 1 day at 15°C, and then reducing the pH to 7.0 before measuring TCE degradation activity. This short incubation at pH 9.9 resulted in the loss of 99% of TCE degradation activity of the SDC-9™ consortium. Both reductive dehalogenation and fermentation of the growth substrates used to grow the cells consumes considerable amounts of alkalinity (McCarty et al., 2007). The pH of the medium in the Shaw 4,000-L reactor decreases from an initial pH of 7.4 to approximately 6.1 during the first 30 days of cell growth (Figure 3.5). Because the culture is fed sodium lactate, however, adding NaOH to control pH could reduce growth rates due to an excess of sodium ions in the reactor. Analysis of PCE dechlorination with added sodium chloride (NaCl) to RAMM medium to a final total dissolved solid (TDS) concentration of 1,000 mg/L showed that elevated level of TDS reduced dechlorination rates, especially for *cis*-DCE and VC (Figure 3.6). Therefore, instead of adding NaOH to control pH, the reactors are sparged periodically with nitrogen (N_2) to remove dissolved CO_2 from the culture medium. This approach sufficiently regulates the medium pH to allow completion of the culture production (Figure 3.7). The duration of sparging affects the extent of pH increase and typically sparging for 10–15 min allows the pH to increase 0.3–0.4 Standard Units (SU) (Figure 3.8). Sufficiently high rates of growth and substrate dehalogenation are sustainable in the reactors, provided the pH is maintained above 6 SU.

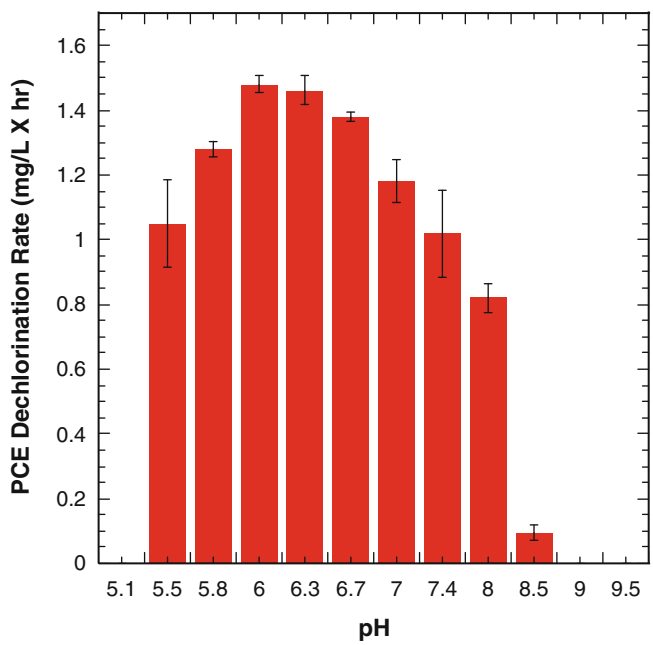

Figure 3.4. Effect of pH on PCE dehalogenation by SDC-9™. There was no measurable activity at pH 5.1. Values represent the mean of triplicate samples, and error bars represent one standard error of the mean.

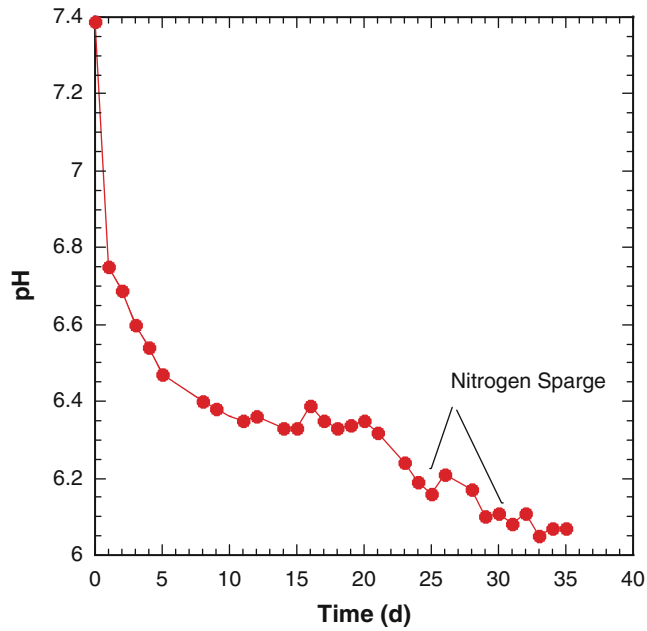

Figure 3.5. Change of pH during growth of SDC-9™ in a 4,000-L reactor. YE (0.1%) was added at the beginning of the fermentation and again on day 22. Nitrogen sparging was conducted on days 25, 31, 32 and 33 to increase culture pH.

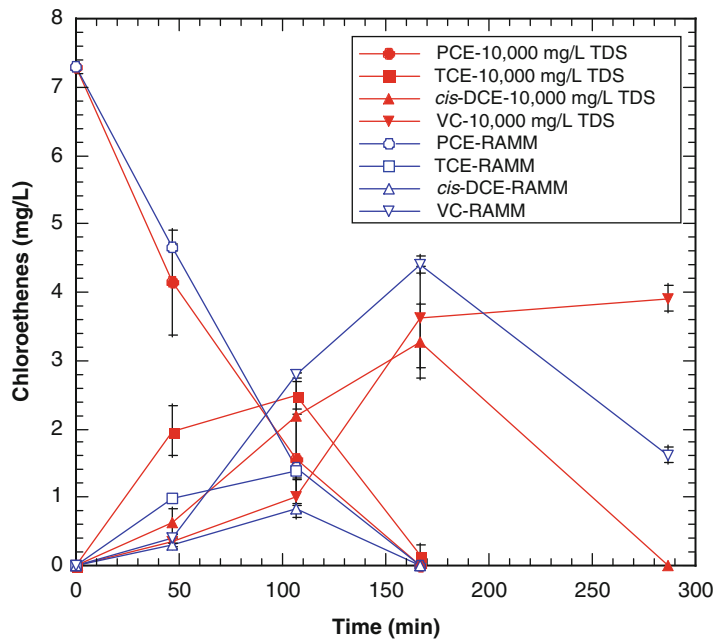

Figure 3.6. Effect of elevated concentration of TDS on SDC-9™ dechlorination rates. NaCl was added to RAMM medium to a final TDS concentration of 10,000 mg/L. The control samples were prepared with only RAMM medium.

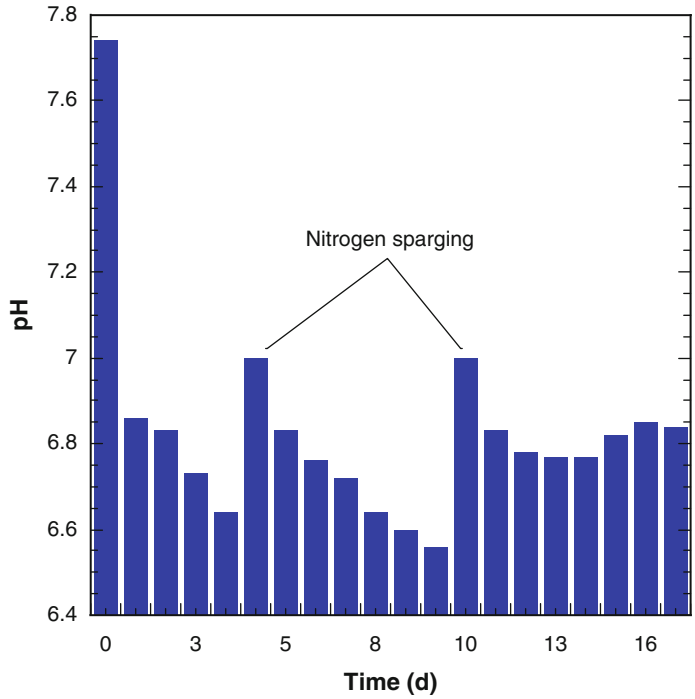

Figure 3.7. Effect of nitrogen sparging on pH during growth of the PJKS™ culture. YE (0.1%) was added at the beginning of the fermentation, and again on day 16 (0.05%). Sodium lactate (60% solution) feeding began on day 2 at a rate of 21.6 μL/h × L, and it was increased to 24 μL/h × L on day 3 and to 28.8 μL/h × L on day 11.

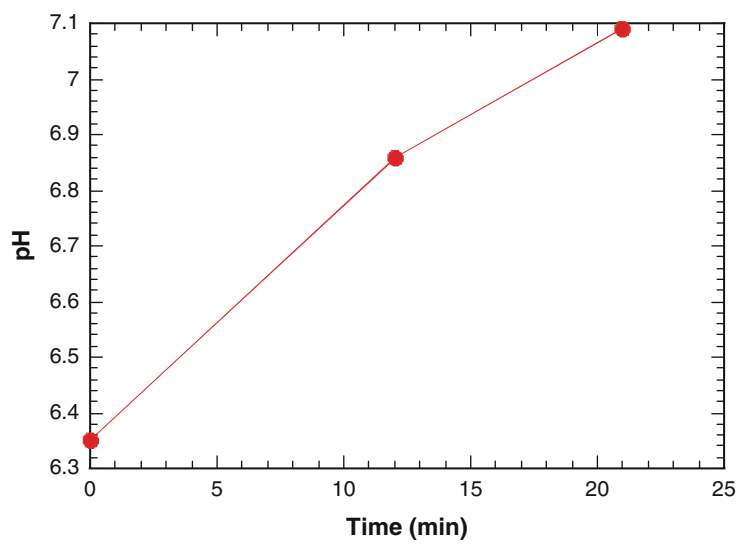

Figure 3.8. Effect of nitrogen sparging on the pH of an SDC-9™ culture in a 750-L reactor at 28°C. These tests were performed to evaluate the duration of nitrogen sparging required to maintain a desirable pH for *Dhc* growth. The OD_{550} of the culture at the time of sparging was 1.1, and nitrogen was added at the rate of 12–14 L/min or 4.4–4.8 mL/min × L medium.

3.4 QUALITY ASSURANCE/QUALITY CONTROL CONSIDERATIONS

Quality assurance and quality control (QA/QC) procedures play several important roles in the commercial production of microbial inocula. They ensure that the growth process is efficient, they confirm the integrity of the produced culture, and they provide customers with assurance that the purchased culture is active, safe and functions as promised. QA/QC procedures also assure regulatory authorities that a culture added to an environment is safe and meets local regulatory requirements. Although no industry standards exist for production of cultures used for bioaugmentation, most producers adopt their own procedures to meet their own requirements and those of their clients. Below are some considerations for the development of a QA/QC program for producing bacterial cultures for remediation of chlorinated solvent-contaminated aquifers.

3.4.1 Pathogen Analysis

Pathogen analysis is commonly performed to assess the safety of bacterial cultures. An early study suggested that some indicator organisms (e.g., coliforms and fecal coliforms) may survive in early-stage enrichment cultures grown for bioaugmentation, especially if the enrichment inoculum is derived from sewage sludge (Skramstad et al., 2003). Furthermore, such analyses are sometimes required by regulatory authorities to evaluate the suitability of a culture for injection into an aquifer.

Pathogen analysis is available through a number of commercial vendors. A common readily available battery of pathogen tests includes assays for the following: (1) *Salmonella* spp. (enteric pathogens), (2) *Listeria monocytogenes* (food borne pathogen), (3) *Vibrio* spp. (enteric pathogen, causative agent of cholera and other infections), (4) *Clostridium perfringens* (causative agent of gas gangrene; food poisoning and flesh-eating infections), (5) *Pseudomonas* spp. (many plant and animal infections), (6) yeast (multiple infections), (7) *Escherichia coli* (enteric pathogen; indicator of fecal contamination), total coliforms (indicators of fecal contamination or enteric pathogens), (8) *Bacillus* spp. (causative agent of anthrax and some food poisonings), (9) *Yersinia* spp. (causative agent of plague), (10) *Streptococci* (multiple infections), (11) *Campylobacter jejuni* (food poisoning agent usually associated with poultry) and mold (multiple infections).

The cost of such a battery of tests is reasonable, but the actual utility of these tests for assessing the safety of bioaugmentation cultures is questionable. For example, many of the organisms identified are members of diverse bacterial families that contain multiple species, strains or pathovars, many of which are non-pathogenic. Also, many strains in these families are common soil bacteria that could reasonably be expected to test positively in a culture isolated from an environmental sample (e.g., *Pseudomonas, Bacillus, Yersinia,* mold and yeast). Thus, a positive test for one of these potential pathogens could raise unnecessary concerns about the safety of a culture. Furthermore, because all *Dhc* bioaugmentation cultures are grown under strict anaerobic conditions, a greater focus on potential anaerobic pathogens may be more suitable for assessing culture safety, but assays for such infective agents are less readily available.

3.4.2 *Dhc* Concentrations

Knowledge of the *Dhc* concentrations in bioaugmentation cultures is critical for planning and for determining the relative value of commercially available cultures. Because the

Dhc-containing cultures are consortia and *Dhc* are difficult to grow in pure culture, enumerating *Dhc* in mixed cultures typically requires use of qPCR methods. Several *Dhc*-specific qPCR assays and PCR primer sequences have been described (Hendrickson et al., 2002; Ritalahti et al., 2006). Molecular tools for quantifying *Dhc* are described elsewhere in this volume (Chapter 6), and the methods used at Shaw are described in Vainberg et al. (2009). qPCR assays should be performed on each batch of culture produced, but for routine microbial growth monitoring, OD can sometimes be used, provided enough preliminary work is performed to understand the relationship between total cell density and *Dhc* concentration at different stages of culture growth (Vainberg et al, 2009). Typical final *Dhc* concentrations in some cultures produced by Shaw are shown in Table 3.2.

3.4.3 Specific Activity

Specific activity is a measure of the amount of target contaminant that can be degraded per unit of culture within a given time. Measuring the specific activity of both PCE (Figure 3.9a) and *cis*-DCE (Figure 3.9b) degradation is important because most cultures have multiple dechlorinator populations, some of which can degrade DCE to ethene and others that degrade PCE to only TCE or *cis*-DCE. Furthermore, qPCR analysis does not allow differentiation between live and dead *Dhc* cells, so even with high *Dhc* numbers, the degradative activity of a culture could be low.

Specific activity can be measured in terms of *Dhc* numbers or protein concentration, but using the total DWT of washed cells as a standard is preferable for commercial production of SDC-9^{TM}. Because the cultures are mixtures, DWT measurements allow assessments of the ratio of *Dhc* to non-*Dhc* organisms. For example, low *Dhc* numbers with high DWT can indicate that culture production has led to an imbalance in the relative amount of *Dhc* to non-*Dhc* organisms in the culture. Likewise, high DWT-based specific activity indicates that the culture

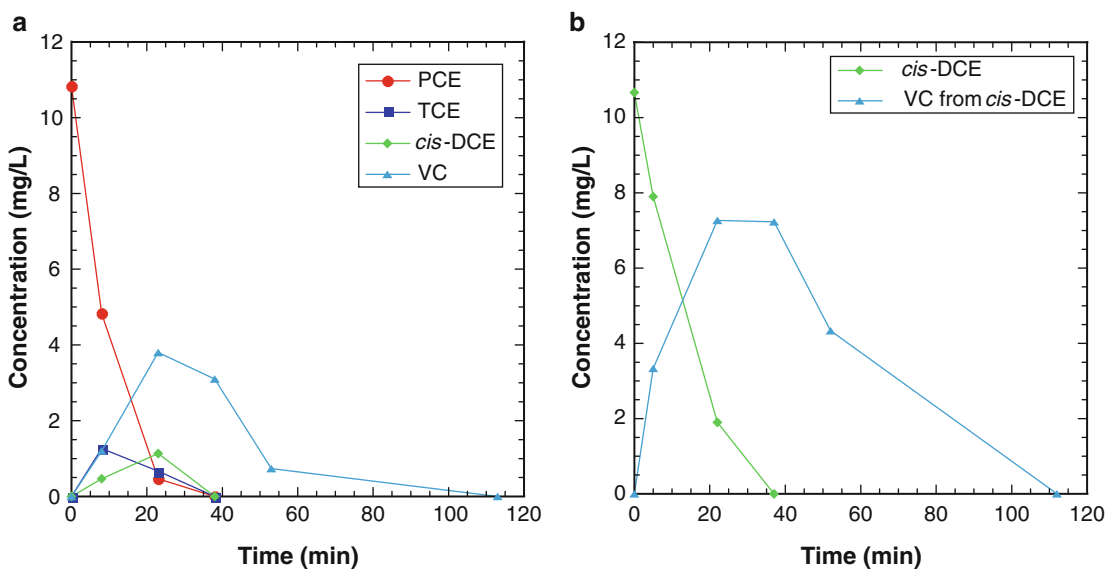

Figure 3.9. Dechlorination assay to monitor the specific activity of an SDC-9^{TM} culture. The PCE (a) and *cis*-DCE (b) degradation rates are measured by using bottle assays to evaluate the specific activity of the cultures for QA/QC. The incubation temperature was 28 °C, the *Dhc* concentration was 1.4 × 10^{12}/L, the OD$_{(550)}$ was 1.6, and the DWT was 0.65 g/L.

has a high *Dhc* concentration relative to non-*Dhc* organisms. Finally, DWT measurements eliminate variability in qPCR results that can occur when the culture is diluted several orders of magnitude to have the *Dhc* concentration range suitable for qPCR analysis. Results of typical PCE and *cis*-DCE bottle assays used for evaluating specific activity are shown in Figure 3.9.

The timing of specific activity measurements is an important consideration for QA/QC assessment. These measurements can be made before harvesting culture, before or after packaging, or after they arrive at a site for injection. Prior experience has shown that removing a small quantity of the packaged culture prior to shipping provides an adequate assessment of the specific activity of cultures delivered by overnight courier. However, if the cultures are shipped by other methods that require several days of transport, or if they are stored at a site for a few or several days prior to injection, it may be prudent to collect samples for specific activity measurements just prior to injection.

3.4.4 Other QA/QC Considerations

An often overlooked aspect of QA/QC is the presence of potential groundwater pollutants in injected cultures. As shown in Figure 3.2a and b, the finished fermentation broth can contain relatively high concentrations of VFAs that can be injected with the bacterial culture. Although these components likely will not affect water quality in an aquifer, especially an aquifer undergoing biological treatment facilitated by electron donor injection, injection of this material may violate groundwater injection regulations. As such, QA/QC monitoring of culture broth composition may be prudent for addressing such concerns or to allow full disclosure of the solution characteristics for injection permit applications. More importantly, some bacterial growth broths may contain residual levels of chlorinated solvents or daughter products such as *cis*-DCE and VC, the injection of which would certainly violate groundwater injection regulations. This is of particular concern if PCE or TCE are added to shipping containers to maintain activity during shipment. Again, careful analysis for these compounds prior to culture injection may be warranted. Concentrating cultures (Section 3.5.1) can reduce the amount of fermentation byproducts remaining in the culture and allow for overnight shipment of large culture volumes, thereby minimizing some of the above concerns.

3.5 CONCENTRATING AND STORING INOCULA

The use of relatively large volumes of bioaugmentation cultures presents several challenges for culture producers and users. For example, the timing of bioaugmentation injection events is usually controlled by other field activities including weather events and the availability of field staff or drilling equipment. As a result, culture injection schedules can be uncertain and delays are commonplace, and culture producers often must unexpectedly extend culture preparation activities or delay culture shipments. Such delays can disrupt scheduling of upcoming deliveries or force producers to keep a culture in the reactor beyond its optimal growth and activity period. Likewise, production of large *Dhc* cultures requires considerable time (Figure 3.1) and shipping delays can reduce the amount of time available to produce consistent cultures, especially for short-lead orders. In addition, injection of large culture volumes may take several days in the field depending on the injection method and site conditions. As a result of these challenges, the ability to store cultures, or at least understand the stability of stored cultures, becomes an important consideration.

Another significant cost consideration for use of large culture volumes is transportation to the treatment location. Ground-based shipping of cultures to distant areas can require several days and often refrigeration is necessary to maintain the stability of large cultures. Similarly,

overnight shipment of large culture volumes can be costly and/or impractical. There also exists a concern that injecting large volumes of culture that may be contaminated with fermentation byproducts (e.g., VFAs, as shown in Figure 3.3) or residual growth substrates (e.g., PCE, TCE, DCE or VC) could lead to further contamination of the site or, at the very least, legal implications (see above).

One approach for addressing the issues of culture storage and shipping is to concentrate the cells for storage and shipping. Cell concentration reduces shipping and storage volumes, and it removes the bulk of the bacterial culture broth and its potential byproducts or contaminants. The suitability of cell concentration depends on the robustness of the cultured cells, however, and the potential for losing an important member of a consortium during the concentration process should be evaluated.

3.5.1 Concentrating Cultures

Several techniques including vacuum evaporation, spray evaporation, continuous centrifugation and ultra- or cross-flow filtration have been used in biotechnological applications to concentrate bacterial cells. Many of these, however, are difficult to apply while maintaining strict anaerobic conditions (Ljungdahl and Wiegel, 1986). Therefore the SDC-9™ culture is concentrated by cross-flow filtration over a custom-built concentrator constructed with six Kerasep™ KBX tubular ceramic membrane units (Novasep, Inc., Boothwyn, Pennsylvania) operated in series (Figure 3.10). Each filter unit contains seven BX-7c ceramic elements containing seven flow channels each, all of which are contained within stainless steel shells. The filters represent 72 ft^2 (6.6 m^2) of membrane surface area with an effective pore size of 0.2 micrometers (μm). The ceramic membranes are chemically cleaned by circulating a solution of 0.5% NaOH through the system for 8 h prior to cell concentration activities. All manipulations are performed under strict anaerobic conditions facilitated by charging the entire system with N_2 prior to introducing the cells, and by connecting the concentrator directly to the reactors so that liquid does not have to be removed from the system for concentration activities.

The culture from the 4,000-L reactor is passed over the membranes at a pressure of 50–55 psi and returned to the reactor by using a two-pump system. The first pump is the reactor circulation pump (G&L SSH-S 2 × 2.5-8; A Gould Pump Co., Seneca, New York) that is capable of transferring 100 gallons (gal)/min (378 L/min), and the second is a high pressure pump (G&L NPE 1-1/4 × 1 – ½-1: A Gould Pump Co., Seneca, New York) with a capacity of 50 gal/min (189 L/min). The culture from the 750-L reactor is concentrated by using a separate lower capacity (24 gal/min; 91 L/min) pump (Model CHI-4–50; Grundfos Pump Corp. USA, Olathe, Kansas). The system is designed to remove ~400–500 L of liquid/h at an initial cell concentration 1.0–1.2 g/L of biomass (DWT), or in the case of the 750-L reactor, to remove 80–85 L/h. The culture from the 4,000-L reactor can be concentrated to ~120 L within the large reactor vessel (i.e., ~26 fold), or subsequently transferred to the 750-L vessel and concentrated to ~50 L (i.e., 64-fold). The culture in the 750-L reactor (550-L of broth) can be concentrated to ~50 L (i.e., ~10-fold). The concentration process also can be stopped at any time during the process to generate a culture with a desired *Dhc* concentration. Concentrated cells are transferred to N_2-charged 18.5-L stainless steel soda kegs (refer to Figure 3.12), pressurized to 15 psi with N_2, and stored at 4°C.

Figure 3.10 shows a photo of the cell concentration system connected to the 4,000-L reactor, and Figure 3.11 shows the results of the concentration of a 3,500-L SDC-9™ culture in the ceramic membrane concentrator system. The cell culture is chilled during concentration to ensure maintenance of cell viability. Analyses of the specific activity of the cells before and

Figure 3.10. Stainless steel cell filtration system for concentrating *Dhc* cultures. The concentration system consists of tubular ceramic membranes contained within stainless steel shells. The individual membrane units are connected in series and water from the culture passes through the membranes and is captured in the shell and disposed. Concentrated culture is transferred back to the fermentor (*background*) and the culture recirculated through the system until the desired concentration factor is achieved.

after concentration demonstrated only small changes in activity during concentration. For example, specific activity of two cultures tested were 24 and 16 mg PCE/h × g DWT before concentration and 23 and 15 mg PCE/h × g DWT after concentration, respectively. Because the concentration process results in approximately 90% reduction in culture volume, it also removes ~90% of any metabolic byproducts remaining in the culture broth.

Cell concentration also allows standardization of *Dhc* concentrations and activity of culture batches. That is, the concentrated cultures can be diluted to a predetermined *Dhc* concentration, allowing producers to deliver consistent cultures and allowing users to develop more reliable estimates of the volume of culture needed for field applications. It is worth noting that some culture biomass will be unrecoverable from the ceramic membranes, because some cells will be trapped in any retained liquid or will adhere to the membranes. However, given the

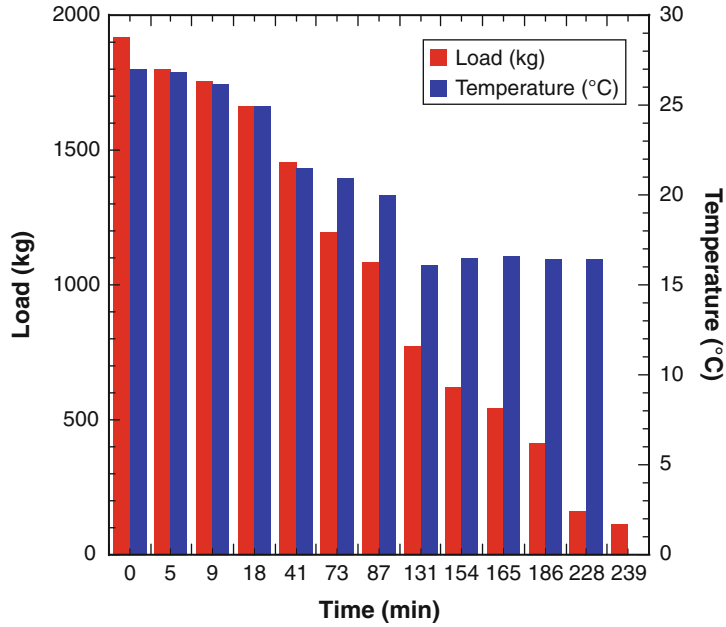

Figure 3.11. Membrane concentration of a 3,500-L SDC-9™ culture using a ceramic membrane cell concentration system. Cell concentration is monitored by using load cells to measure changes in the weight of the reactor as water is removed. The culture is chilled during concentration to ensure its viability.

Table 3.3. Effect of Storage Temperature on PCE Degradation Activity of SDC-9™

Time (days)	PCE Dechlorination Rate (mg/L × h)[a]			
	4 °C	13 °C	22 °C	28 °C
0	6.45 ± 0.29	6.45 ± 0.29	6.45 ± 0.29	6.45 ± 0.29
7	ND	ND	1.20 ± 0.11	0.33 ± 0.10
14	7.30 ± 0.13	4.10 ± 0.57	ND	ND
35	8.20 ± 0.70	2.28 ± 0.10	0.70 ± 0.03	0.23 ± 0.05
82	4.20 ± 0.19	0.57 ± 0.38	ND	ND

[a] Values represent mean ± SE of triplicate samples; ND - not determined

large volumes and high *Dhc* concentrations obtained during large-scale production, this loss generally is insignificant relative to the benefits of cell concentration.

3.5.2 Culture Stability and Storage

Storage of bacterial cultures is critical for allowing timely delivery of cultures to contaminated sites, to coordinate culture injection with the availability of field personnel and equipment (e.g., drilling rigs), and also to allow cultures to be injected over several days of field-scale injection. To evaluate storage longevity, 10X-concentrated SDC-9™ cultures were incubated for up to 82 days at either 4°C, 13°C or 28°C in stainless steel containers. Periodically, samples of the stored cultures were removed and assayed for their ability to degrade PCE and *cis*-DCE. Activity of the culture decreased rapidly if stored at 13 or 28°C, but SDC-9™ could be stored at 4°C for >35 days without loss of activity (Table 3.3). Cultures stored in this

manner should be suitable for field application. In fact, a concentrated SDC-9™ culture stored for 7 months at 4°C still retained 58% of its original PCE degradation activity and 68% of its *cis*-DCE degradation activity. Initial PCE and *cis*-DCE degradation activities were 22.2 and 14.4 mg/h × g DWT, respectively, and after storage of the culture PCE and *cis*-DCE degradation activity were 12.9 and 9.8 mg/h × g DWT, suggesting that cultures stored for very long periods, although perhaps not optimum for field application, should still be suitable for seeding reactors for further culture production.

The storage results presented demonstrate that care must be taken to keep cultures refrigerated during shipment to sites and during storage during injection events. Cultures that are not adequately refrigerated could lose considerable activity during overland shipment to sites or during onsite storage. But the results also show that cultures can be stored refrigerated for several days during application in the field without significant loss of activity.

Shipping costs are not insignificant, especially when shipping large volumes of culture and when overnight delivery is preferred to retain the greatest culture viability and specific activity. Cell concentration can significantly reduce these costs. As an example of the potential shipping cost savings, overnight roundtrip shipping within the United States of an 18.5-L (5 gal) soda keg full of culture in a suitably sized cooler with sufficient ice packs to retain refrigerated temperatures costs approximately US $200 at today's shipping rates. Shipping 185 L of unconcentrated culture requires ten kegs and coolers and costs approximately US $2,000 for roundtrip shipping. Thus, being able to concentrate 185 L of culture tenfold so it can be shipped in a single keg can result in a savings of US $1,800 in shipping costs alone. Furthermore, transporting and handling ten large coolers, for example from a delivery point to the injection site, creates ground transportation challenges, whereas a single cooler can be transported easily in the back of a pickup truck or even a car.

The greatest challenge when working with concentrated cultures is measuring and delivering the required volume of concentrated culture for a given injection application. Whereas adding 10 L of unconcentrated culture directly into an injection point can usually be done easily either by using a sight glass in the keg or by weighing the keg, measuring and delivering 0.1–1 L can be more challenging. Consequently, vendors supplying concentrated cultures have developed and supply simple anaerobic measuring devices (Figure 3.12) that now simplify measuring and injecting small volumes of culture.

3.6 SHIPPING CULTURES

Cultures should be transported to treatment sites in a manner that maintains strict anaerobic conditions to ensure *Dhc* viability. The cultures also should be refrigerated during transport, especially if the culture will not be applied until several days after production. It also is important to ensure that the culture to be injected into aquifers is free of chlorinated contaminants that could cause additional site contamination and/or result in injection permit violations.

One option that has been used successfully is to ship the cultures in 5-gal (18.5-L) stainless steel soda kegs. This approach has now been adopted by most culture vendors. An example of the kegs is presented in Figure 3.12. These kegs are readily available (e.g., from home brewing suppliers), inexpensive and durable. They also can be chemically or steam cleaned and autoclaved. Furthermore, they fit well within coolers that can be readily shipped with included cold packs via overnight carrier, and they can be modified as needed to suit specialized culture injection requirements. Each keg contains an internal drop tube that extends to near the bottom of the keg. Liquid is removed from the kegs by attaching quick connect ball lock (or pin lock depending on the manufacturer) devices to "gas in" and "liquid out" (drop tube) ports on the

Figure 3.12. Stainless steel soda keg (5 gal/18.5 L) with attached measuring device, used to deliver and inject *Dhc*-containing cultures. Most bioaugmentation vendors now use these, or similar, containers to maintain anaerobic conditions during transport and injection of bioaugmentation cultures. The attached measuring device is calibrated to allow accurate delivery of a desired culture volume while maintaining anaerobic conditions.

top of the kegs. As gas (nitrogen or argon) is added through the "gas in" port, the culture is expelled from the "liquid out" port. Flow of the culture from the keg can be controlled by simple valves.

3.7 ONSITE HANDLING

Dehalogenating bacteria are strict anaerobes (He et al., 2003b; Löffler et al., 2003; Maymó-Gatell et al., 1997), and therefore exposure to oxygen should be prevented during any handling. Most culture distributors now deliver cultures in containers, like those described above, that allow the cultures to be anaerobically injected into aquifers with no exposure to oxygen.

3.7.1 Direct Injection

Bioaugmentation cultures can be injected directly into aquifers using direct push technologies, by adding them to injection wells or by adding them to recirculation systems. Prior to injecting the cells, aquifers are often preconditioned to remove oxygen and lower the oxidation reduction potential. Preconditioning is typically accomplished by injecting the planned electron donor into the aquifer several days, weeks or months prior to injecting the culture.

The amount of time required to achieve suitable conditions and the amount of electron donor needed depends on conditions at the site, including oxygen levels and the presence of other bacterial electron acceptors (e.g., nitrate, sulfate, ferric oxide [Fe^{3+}]). Some bioaugmentation treatments, however, have been performed without extensive aquifer pretreatment. Because the consortia used for bioaugmentation contain fermentative and other facultative aerobic microorganisms able to use oxygen, it is likely that these organisms rapidly use low levels of DO, thereby protecting the oxygen sensitive *Dhc* organisms. In most cases of direct injection, *in situ* distribution of the culture is aided by injecting anaerobic water following culture injection.

3.7.2 Dilution

An alternative to direct injection of bacterial cultures into aquifers is to dilute them first and inject over a long period of time or into many injection wells. In most cases, groundwater or potable water is made anaerobic by adding electron donor to the water in a closed container and incubating it until the bacteria in the water consume the DO. In the case of groundwater, the number of bacteria present is sufficient to remove the oxygen in a few days. Potable water, however, because of its low bacterial numbers can take quite long to become anaerobic. The process can be accelerated by adding an inoculum of oxygen consuming bacteria. The inoculum can be a commercially available culture. Injection water also can be made anaerobic by adding soil, compost, or other readily available microbe rich material if necessary. Experience has shown that free residual chlorine from chlorination in most potable waters is not inhibitory to *Dhc* cultures (data not shown), but because of the potential variability of free chlorine in drinking waters, testing treated potable water with the selected bioaugmentation culture is recommended.

An alternative to using bacteria to remove residual DO from dilution water is to sparge the containerized dilution water with nitrogen or argon. This approach can remove DO to below 1 mg/L, but the ease of using this method depends on the volume of water being treated. Argon may have an advantage over nitrogen because it is heavier than air and forms an anaerobic gas blanket on top of the treated water, thereby preventing further dissolution of oxygen into the water. This sparging approach also reduces levels of free chlorine in potable water.

3.7.3 Mixing with Other Reagents Before Injection

Field personnel often desire to mix bioaugmentation cultures directly with electron donors and/or reducing agents such as L-cysteine so that both can be injected simultaneously. The compatibility of the bioaugmentation culture with high concentrations of electron donors and reducing agents must be evaluated before using this approach. Initial testing has shown that L-cysteine does not inhibit the SDC-9™ consortium at concentrations up to 0.69 g/L (data not shown). However, the pH of some electron donors is extreme (to avoid spoilage) and high concentrations of some electron donors may be directly toxic to *Dhc*. For example, even typical injection concentrations (10% as carbon) of five tested commercially available electron donors inhibited SDC-9™ in laboratory testing even after pH adjustment (data not shown). In addition to direct inhibition, rapid fermentation of electron donor substrates can result in the production of metabolic acids and CO_2, that reduce the pH of the mixture or recipient groundwater to levels that can inhibit *Dhc* (McCarty et al., 2007; Vainberg et al., 2009). Therefore, if cultures

are to be mixed directly into electron donor solutions, the compatibility of the compounds and cultures should be evaluated.

3.8 SUMMARY

Large-scale production of *Dhc*-containing cultures for *in situ* bioaugmentation of chlorinated ethene contaminated aquifers can be performed economically and result in reproducible high specific activity and high cell density cultures. Successful large-scale inoculum production depends on the electron donor (i.e., lactate) and acceptor (PCE) feed rates, and the addition of YE greatly improves cell yield.

The initial stages of inoculum preparation are characterized by rapid growth of non-*Dhc* organisms in the culture, while the growth of *Dhc* exhibits a short lag period and then is relatively constant to final *Dhc* concentrations of $>10^{11}$/L. The inoculum preparation protocol presented here is scalable to 550-L and 3,200-L batches, and produces comparable results with consortia enriched from three different sites. The cultures produced by this protocol still can completely dehalogenate PCE to ethene, suggesting that the protocol retains organisms capable of degrading all of the chlorinated PCE daughter products including VC.

The results summarized in this chapter also demonstrate that *Dhc*-containing cultures designed for bioaugmentation can be concentrated by cross-flow filtration to reduce shipping volumes, and that the concentrated cultures can be stored under refrigeration for >40 days to allow for injection schedule flexibility. The use of inexpensive soda kegs provides a simple method for delivering and injecting the concentrated cultures.

With the increased use of bioaugmentation to treat challenging chlorinated ethene contaminated sites, the ability to produce large volumes of high density cultures is becoming increasingly important. This chapter provides information needed to produce *Dhc* cultures for bioaugmentation, including at scales suitable for treating large contaminant plumes. However, additional culture-specific process optimization may be required for reproducible and reliable large-scale production of other bioaugmentation cultures.

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CHAPTER 4

BIOAUGMENTATION WITH *DEHALOCOCCOIDES*: A DECISION GUIDE

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4.1 INTRODUCTION

Many bacterial species are capable of the initial steps in reductive dechlorination, i.e., the conversion of perchloroethene (PCE; also termed tetrachloroethene) and trichloroethene (TCE) to *cis*-1,2-dichloroethene (*cis*-DCE). However, the only anaerobic organisms known so far that are able to efficiently dechlorinate *cis*-DCE to vinyl chloride (VC), and VC to the innocuous product ethene, are strains of *Dehalococcoides* spp. (Maymó-Gatell et al., 1997; He et al., 2003; Cupples et al., 2004a). These organisms are widespread, but not ubiquitous (Hendrickson et al., 2002), and often are present in low numbers in contaminated groundwaters (van der Zaan et al., 2010). Therefore, introducing cultures containing competent *Dehalococcoides* (*Dhc*) strains into the saturated zone of contaminated aquifers (bioaugmentation) often improves the performance of *in situ* bioremediation systems (SERDP and ESTCP, 2005; Ritalahti et al., 2005; Stroo et al., 2010). However, it can be difficult to decide if bioaugmentation with *Dhc* is needed or will be beneficial at a specific site.

There is no doubt that bioaugmentation can lead to complete reductive dechlorination at some sites where dechlorination is stalled at *cis*-DCE or VC (Ellis et al., 2000; Major et al., 2002; ESTCP, 2007). Incomplete dechlorination is of concern not only because it leaves contaminants of regulatory concern in place, but it also may result in plume expansion, increased vapor intrusion risks, inefficient use of added electron donors and increased methane and sulfide generation (Hood et al., 2008). At such persistently stalled sites, bioaugmentation may be essential for effective *in situ* bioremediation.

More commonly, sites have low numbers of competent *Dhc* present in the subsurface, and these numbers typically increase after additions of electron donor sources without the addition of organisms (i.e., biostimulation). After biostimulation, the *Dhc* numbers usually increase over time, resulting in complete dechlorination to ethene after a lag period until there is sufficient growth and dispersal throughout the treatment area (Koenigsberg et al., 2003). This lag period may be several months to more than a year (Morse et al., 1998; AFCEE et al., 2004), and bioaugmentation at the start of treatment can reduce the lag period considerably (Adamson et al., 2003; Lendvay et al., 2003).

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The cost for bioaugmentation includes the culture itself, as well as the labor and equipment needed to inject the culture into the subsurface. It is important to realize that the cost is often lower to bioaugment at the same time that electron donors are added, rather than to bioaugment at a later date when it is proven to be necessary, particularly if separate mobilizations and drilling time are needed. Bioaugmentation itself (i.e., the supplemental addition of dechlorinators) is not usually a large cost item relative to the overall site remediation cost (Chapter 11), and bioaugmenting done concurrently with biostimulating often can reduce the overall life cycle cost. However, project managers need to justify the additional costs, particularly if there is a perception that bioaugmentation is not truly required or is being used just as an insurance policy.

The difficulty in justifying the costs results from the uncertainty in predicting the benefits. Biostimulation may be sufficient, and it requires less initial cost. So managers often are faced with a choice between biostimulation only, relying on "more time and more electrons" to eventually attain optimal performance (Koenigsberg et al., 2003), or bioaugmentation for faster results and greater certainty, even if it is not absolutely required.

This chapter is intended to help site managers in the decision-making process regarding the use of biostimulation or bioaugmentation at a given site. The discussion is structured around a decision flowchart based on a series of diagnostic questions and parameters critical in determining whether to biostimulate and/or bioaugment. After introducing the decision guidance and the key sources of uncertainty involved in the decision making, the remainder of the chapter describes these diagnostic questions, parameters to be considered/monitored and key sources of information useful in answering them. The final section summarizes key issues for managers when deciding whether to bioaugment a specific site.

4.2 NEED FOR DECISION GUIDANCE

Bioaugmentation with *Dhc* has been successfully implemented at hundreds of sites (see Chapter 1). Many times the decision has been an easy one, but at other sites it has been more difficult because of the uncertainties involved. The uncertainties ultimately stem from the low numbers, slow growth, patchy distribution and genetic variability of the *Dhc* strains in the subsurface. As a result, it can be difficult to be certain that bioaugmentation is necessary at a particular site, or to predict the lag time before measurable ethene production occurs.

Even persistent stalling at *cis*-DCE or VC after biostimulation is not conclusive proof that bioaugmentation is needed. There are several other possible explanations for stalling. These include: (1) unknown sources providing a constant feed of parent compounds (TCE and/or PCE); (2) the parent compounds (PCE and TCE) being dechlorinated faster than the daughter products (DCE and VC) causing a temporary daughter product accumulation (Cupples et al., 2004b); (3) the differences in water solubility (VC > DCE > TCE > PCE) making the daughter products more prevalent in the dissolved phase; (4) unfavorable geochemical conditions, such as acidic pH inhibiting complete dechlorination (Vainberg et al., 2006); and (5) an excess of iron shunting electrons away from DCE and therefore inhibiting later stage dechlorination (Koenigsberg et al., 2002).

One of the most useful diagnostic analyses for bioaugmentation decisions is the direct measurement of *Dhc* numbers in the groundwater. But these measurements may not provide definitive answers in every case. For example, the lack of detectable *Dhc* or key biomarkers may be misleading because the organisms can be sparsely distributed and the samples analyzed may not be representative (Koenigsberg et al., 2003). On the other hand, detection of such biomarkers is not necessarily proof that biostimulation alone will be effective, because *Dhc* capable of VC reduction can be eliminated by early stage dechlorinators that outcompete *Dhc* for hydrogen (Becker, 2006; Huang and Becker, 2009).

Another uncertainty is the frequency of incompetent sites – the probability that a given site will require bioaugmentation for complete dechlorination in a reasonable timeframe. Practitioners estimate that roughly 5–10% of all sites contaminated with chlorinated ethenes do not have the microbial capacity for complete dechlorination (e.g., Koenigsberg et al., 2002), although there has been no thorough study of the frequency of incompetent sites. However, recent surveys indicate that complete dechlorination will eventually occur under favorable conditions at most, but not all, solvent contaminated sites (Lu et al., 2009; van der Zaan et al., 2010).

While such uncertainties complicate bioaugmentation feasibility assessments, the scientific foundation and experience base has increased dramatically in recent years (ESTCP, 2005). Researchers and practitioners have learned enough to provide useful guidance for those facing bioaugmentation decisions at specific sites. The decision guidance presented in this chapter builds on that existing knowledge and experience, provides practical recommendations and discusses the rationale for them.

4.3 DECISION GUIDANCE OVERVIEW

This guidance is not intended to be prescriptive in nature. Rather it presents a systematic approach to making a decision that generally requires both technical information on specific site conditions, as well as non-technical judgments regarding risk tolerance and economic assumptions. The guidance is depicted in Figure 4.1 as a flow chart, and begins with questions intended as "off ramps," to allow rapid screening of sites where the decision is relatively easy. Later questions require more detailed information and testing, and the final questions require consideration of management objectives and development of comparative cost estimates.

The guidance assumes that *in situ* bioremediation has been selected as a feasible technology based on remedial investigations and feasibility studies. Although presented as a decision flow, most or all of the following questions will have to be addressed at most sites.

4.4 IS COMPLETE DECHLORINATION OCCURRING?

The initial question in evaluating bioaugmentation is to determine whether complete dechlorination is already occurring. Even if the rate and extent are not optimal, if complete dechlorination (production of innocuous nonchlorinated compounds, notably ethene and ethane) is already occurring, bioaugmentation is unlikely to be beneficial. Several parameters deserve attention when assessing this issue, notably:

Oxidation-Reduction Potential (ORP). At some sites, there will be electron donors present already (naturally or due to prior deliberate additions) and the ORP will be sufficiently reducing to promote reductive dechlorination. Examples may include landfill sites or mixed-waste sites with chlorinated solvents as well as reduced organic compounds. Biodegradation of these reduced compounds can deplete any available oxygen as well as other electron acceptors and cause the subsurface to become sufficiently anaerobic for reductive dechlorination to occur (i.e., < -100 millivolts [mV], or preferably < -200 mV).

The influx of oxygen and other electron acceptors also should be considered when evaluating inhibition. The electron acceptor influx may be so great that sufficiently reducing conditions cannot be established, or cannot be sustained at reasonable expense. Maintaining an ORP below about -100 mV is necessary for complete dechlorination, and below -200 mV is preferable (i.e., the geochemistry should indicate that the subsurface is at least sulfate-reducing, and ideally some methane will be generated).

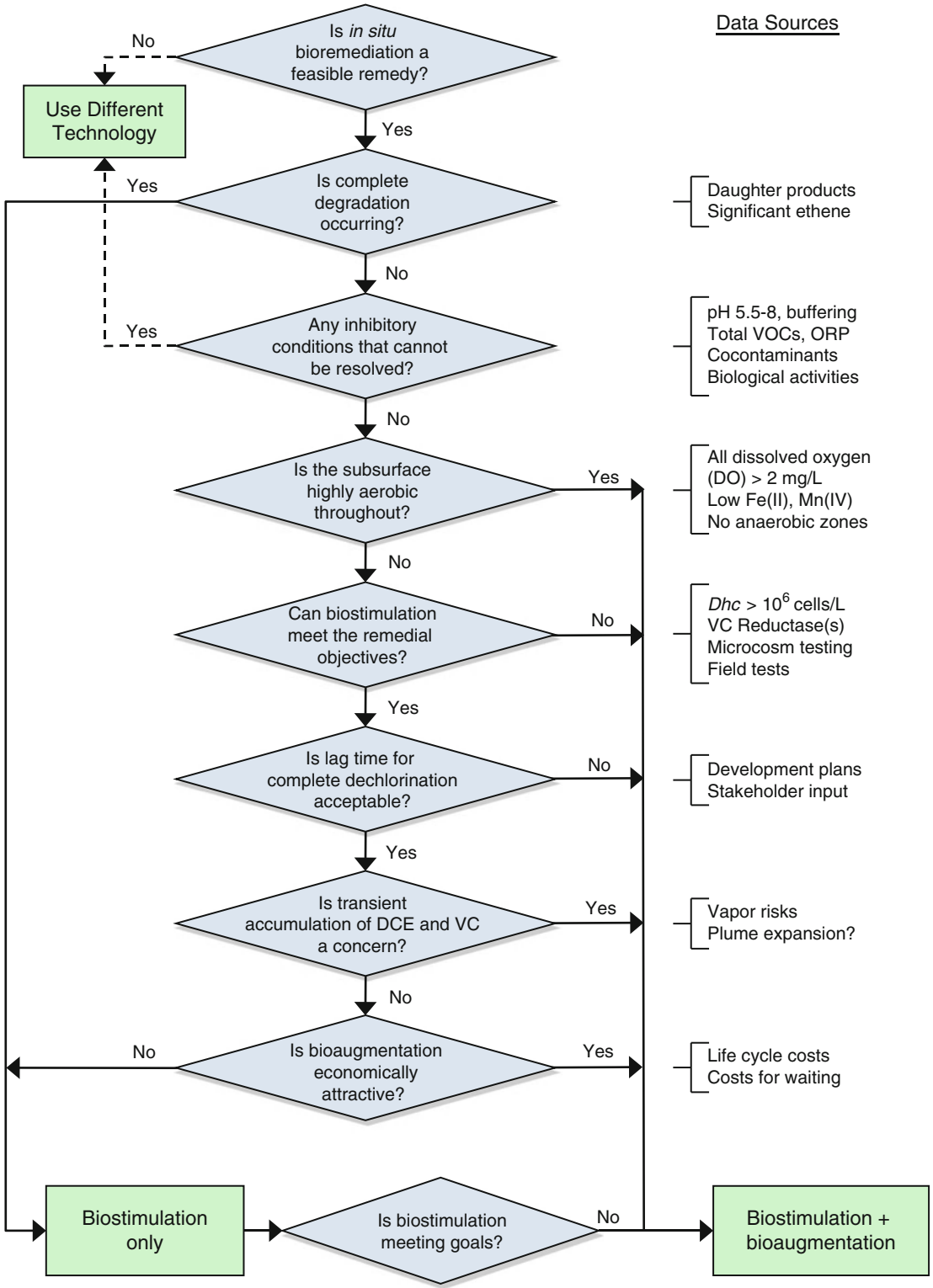

Figure 4.1. Decision guide flowchart for bioaugmentation.

Ethene, Ethane or Carbon Dioxide (CO_2) Formation. If the environmental conditions are favorable and competent microbial strains are present, reductive dechlorination can proceed through *cis*-DCE and VC to ethene or ethane. The presence of significant ethene generally is conclusive evidence for complete microbial dechlorination (Major et al., 2002; Bradley and Chapelle, 2010). Although trace levels of ethene may occur naturally or as a result of relatively inefficient cometabolic processes (Bradley and Chapelle, 2010), if ethene or ethane represent a significant fraction (>10%) of the total volatile organic compounds (VOCs), bioaugmentation should not be needed.

At other sites, reductive dechlorination may not proceed all the way to ethene, but the resulting byproducts (*cis*-DCE and VC) may be further degraded to CO_2 under aerobic conditions. Aerobic biodegradation of these partial degradation products can occur in aerobic groundwaters downgradient of the anaerobic zone, or may occur in an aerobic vadose zone if the compounds are volatilized. Aerobic biodegradation of VC may occur even if the dissolved oxygen (DO) concentration is far below typical detection limits of roughly 1 milligram per liter (mg/L), in environments that typically would be considered "anaerobic" (Gossett, 2010). If such aerobic polishing has been observed and can be relied upon to protect potential receptors, even if the production of *cis*-DCE and VC is increased as a result of biostimulation, then bioaugmentation should not be needed.

Contaminant Concentrations. Contaminant data need to be viewed with some caution. Mass balances from chlorinated solvent sites are rarely, if ever, complete. There are several reasons for the difficulty in determining an accurate mass balance. In particular, some of the later products (VC and ethene) can be quickly degraded under aerobic conditions, and probably under anaerobic conditions as well (Davis et al., 2008; Bradley and Chapelle, 1998; Klier et al., 1999). These byproducts also can be volatilized during sampling, handling or analysis. Also, abiotic degradation can lead to rapid losses of chloroethenes (Lee and Batchelor, 2002), and can be an important degradation mechanism (Ferrey et al., 2004). However, abiotic degradation is rarely evaluated; the key intermediate (acetylene) can be quantified from the analyses used for ethene and ethane, but acetylene may be degraded so quickly that it is not a reliable indicator (AFCEE et al., 2008). Guidance on recognizing and quantifying abiotic degradation could significantly improve mass balances and degradation rate estimates.

4.5 ARE THE SITE CONDITIONS INHIBITORY?

If contaminant data indicate that reductive dechlorination is not occurring or is not complete, it may be due to process-specific inhibitory conditions that are sometimes overlooked when screening technologies. An exhaustive investigation of potential inhibitory factors is not needed at this stage, but a few common problems can be evaluated by examining the existing site data. In some cases, a brief screening of conditions that often inhibit reductive dechlorinating bacteria can be enough to reject *in situ* bioremediation from further consideration. Some of the most common inhibitory parameters include:

pH. The most common site-specific issue is pH. Bacteria capable of dechlorinating DCE and VC are sensitive to even mildly acidic conditions. They are at least partially inhibited below pH 6.0, and a pH below 5.5 is clearly a concern (Vainberg et al., 2006; Fogel et al., 2009). Even if the pH is favorable before biostimulation, acidification during fermentation of added electron donors can cause pH values to decrease below 5.5, at least temporarily inhibiting reductive dechlorination. Understanding the buffering capacity of an aquifer, therefore, can be as important as knowing the current pH and alkalinity. On the upper end, the pH should not be greater than 8.0 for dechlorinators to function effectively.

Contaminant Concentrations. The contaminant concentration also may be inhibitory to dechlorinators, and toxicity may be a concern at relatively high dissolved phase concentrations. For example, Amos et al. (2007) measured complete inhibition of reductive dechlorination at about 540 micromolar (μM) PCE (90 mg/L), or roughly half the maximum solubility in water, though others have demonstrated that dechlorination can proceed at near-solubility PCE concentrations (Carr et al., 2000). The contaminant concentrations also can be too low for effective reductive dechlorination. Populations of *Dhc* can die off over time at a certian threshold concentration of total electron acceptors, and this threshold can be above typical regulatory cleanup levels (Cupples et al., 2004b). As a general rule, *Dhc* will die off below a total VOC concentration of 50 micrograms per liter (μg/L), and repeated bioaugmentation events will be required to sustain complete dechlorination.

Presence of Inhibitory Cocontaminants. It is also important to evaluate the presence of cocontaminants that may be inhibitory, such as trichloroethane (TCA) or chloroform. These compounds can inhibit dechlorination at concentrations that have been observed in chlorinated ethene plumes (Duhamel et al., 2002; Grostern and Edwards, 2006). Other substances present in contaminated aquifers also may be inhibitory. For example, Freon from nearby sites may inhibit TCE dechlorination in commingled plumes (Figgins et al., 2007). The presence of potential inhibitors may not necessarily disqualify a site for bioaugmentation, but it may require adjustments to the design, or even inclusion of additional bioaugmentation cultures such as TCA-degrading anaerobic bacteria (e.g., Fung et al., 2007).

Sulfate/Sulfide. Sulfate concentrations also may be inhibitory, although this issue has been a confusing one. Common guidance is that sulfate levels >1,000 mg/L can be problematic because sulfate-reducing bacteria outcompete dechlorinators for electrons. But sulfate actually may not be a serious problem, as long as excess electron donor is added (Heimann et al., 2005).

Sulfides also can be potent inhibitors. Sulfides that are naturally present, or formed during sulfate reduction, can be toxic to dechlorinators. However, sulfide toxicity can be alleviated by precipitating the sulfide into unavailable mineral forms, for example by natural or added iron (Jeong and Hayes, 2003). Ferric iron levels may be inhibitory to dechlorination at some sites (Koenigsberg et al., 2002).

Temperature. In rare cases, temperature also may be an inhibitory factor to consider, although complete dechlorination has been measured at groundwater temperatures as low as 10 degrees Celsius (°C), and it still can occur at temperatures up to approximately 40–45°C (Holliger et al., 1993; Friis et al., 2007).

4.6 IS THE SITE HIGHLY AEROBIC?

The presence of oxygen is fatal to *Dhc* and the other organisms on which they depend. However, *Dhc* and other obligate anaerobic bacteria are found in most aquifers, even those considered aerobic, though they often are restricted to anaerobic microsites or low permeability zones. These reservoirs of indigenous *Dhc* can disperse and colonize the aquifer after biostimulation, eventually leading to complete dechlorination without bioaugmentation (e.g., Suthersan et al., 2002).

However, there are sites that are so aerobic that they should be considered "functionally incompetent." It is likely that *Dhc* strains capable of complete dechlorination will be absent from highly aerobic sites, or present in low numbers at widely-separated locations, resulting in unreasonable lag times before effective treatment is achieved. Therefore, bioaugmentation is recommended for highly aerobic sites, after biostimulation has established a sufficiently

reducing environment. Highly aerobic sites are defined as sites with DO >2 mg/L throughout, low levels of reduced metals (particularly dissolved iron and manganese), and no evidence of significant anaerobic zones within the target treatment volume that can serve as reservoirs of competent *Dhc*.

4.7 WILL BIOSTIMULATION WORK?

The testing used to evaluate biostimulation includes both laboratory diagnostic tests and field testing methods. The available laboratory analyses and tests are summarized in Table 4.1 and in the following section. Field testing techniques are discussed in Section 4.7.2.

Table 4.1 Key Diagnostic Analyses Available to Assess the Need for Bioaugmentation

Analysis	Advantages	Limitations	Interpretations
Dhc 16S rRNA	Specific for *Dhc* Rapid results Relatively low cost	Some *Dhc* are not capable of complete dechlorination Possible sample bias or inhibitory materials	$>10^6$ *Dhc*/L – Bioaugmentation likely not needed
VC reductase genes	Specific for key detoxifying step in reductive dechlorination Rapid results Relatively low costs	Not all VC reductive dehalogenase genes (RDases) have been identified	$>10^5$ *vcrA* or *bvcA*/L – Bioaugmentation likely not needed
Ethene/ethane	Proof of complete dechlorination	Can be degraded anaerobically or aerobically Low levels may be generated by inefficient cometabolism	>10% of original VOCs – Bioaugmentation likely not needed
Compound specific isotope analysis (CSIA)	Can provide conclusive proof that biodegradation is occurring *in situ*	Limited availability May require added cost, time and significant volumes of water for analysis	Fractionation changes in VC prove complete biodegradation Biodegradation rates estimated from changes in fractionation along a plume
Laboratory microcosms	Very strong evidence for or against the need to bioaugment in the field	Requires additional cost and time (6–12 months) May require aquifer solids and groundwater for accuracy	Ethene production within 12 months – Bioaugmentation likely not needed Lack of ethene and *Dhc* $<10^5$/L – Bioaugmentation likely necessary
In situ microcosms	Very strong evidence for or against the need to bioaugment in the field	Requires additional cost and time (usually 1–2 months) Well microenvironment may not reflect *in situ* conditions	Ethene production within 2 months – Bioaugmentation likely not needed Lack of ethene and *Dhc* $<10^5$/L – Bioaugmentation likely necessary

Note: rRNA-ribosomal ribonucleic acid

4.7.1 Laboratory Diagnostic Tests

Laboratory testing is by nature conducted at relatively small scales under controlled conditions so information can be gathered with greater precision and lower costs than in the field. However, there can be laboratory artifacts and difficulties in extrapolating to field scale. The laboratory diagnostic tests useful for bioaugmentation decisions include molecular biological analyses, compound specific isotope analyses and laboratory microcosms. The advantages, limitations and interpretations of the most commonly used diagnostic analyses are summarized in Table 4.1.

4.7.1.1 Molecular Biological Tools

The least costly and quickest option for additional site testing is to perform specific molecular diagnostic analyses, which are collectively described as molecular biological tools (MBTs). These analyses have been applied to several environmental problems in recent years (SERDP and ESTCP, 2005). These types of molecular analyses are discussed in greater detail in Chapter 6, so they are described only briefly below.

MBTs target key biomarkers (e.g., specific nucleic acid sequences, proteins or lipids) that provide information about organisms and processes important for site characterization and remediation. These methods have great potential to improve environmental characterization and remediation (Lovley, 2003; Koenigsberg et al., 2005) and in particular they have the potential to determine whether bioaugmentation will be needed or beneficial at a site (Ritalahti et al., 2005).

The method that has proven most useful to date is the quantitative polymerase chain reaction (qPCR), especially as the range of genes analyzed is broadened and the technique is extended to messenger ribonucleic acid (mRNA) (Stroo et al., 2006). A method that may help in future bioaugmentation decisions is fluorescence *in situ* hybridization (FISH), which allows direct visualization of cells with specific gene sequences of interest (Yang and Zeyer, 2003).

Dehalococcoides Biomarkers. The difficulties in isolating and studying *Dhc* by conventional microbiological methods have spurred the effort to develop meaningful MBTs for these important microorganisms (Cupples, 2007). MBTs can measure specific microbial capabilities within a site and may be useful to quantify the current degradation potential and to identify environmental conditions that are limiting the current potential.

The qPCR method can quantify several key gene sequences important in reductive dechlorination of chlorinated ethenes (Figure 4.2) including:

1. 16S rRNA characteristic of *Dhc* (Löffler et al., 2000; Fennel et al., 2001; Hendrickson et al., 2002),
2. *pceA*, a sequence from gene (PCE reductive dehalogenase) capable of dechlorinating PCE to form TCE,
3. *tceA*, a sequence from gene (TCE reductive dehalogenase) capable of dechlorinating TCE and other chlorinated aliphatic hydrocarbons (Magnuson et al., 2000),
4. *vcrA*, a gene sequence from the first VC reductase gene identified (Müller et al., 2004), and
5. *bvcA*, a separate sequence from a VC reductase found in a different *Dhc* strain that also reduces VC to ethene (Krajmalnik-Brown et al., 2004).

To date, the most useful of these biomarkers has been the 16S rRNA probe, which is now routinely used to quantify *Dhc* in a sample by qPCR. This analysis has made it possible to

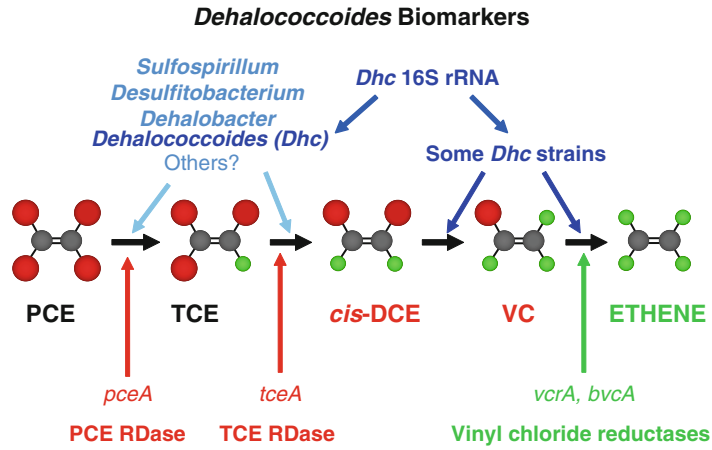

Figure 4.2. Pathway for reductive dechlorination and identification of the key *Dehalococcoides* biomarkers. Biomarkers include the 16S subunit of the ribosomal RNA that is characteristic of *Dehalococcoides* spp. as well as portions of reductive dehalogenase genes (RDases) acting on each of the chloroethenes along the metabolic pathway.

detect and quantify *Dhc* populations with low detection limits (typically 10^2–10^3 cells/L in groundwater). Although it cannot discriminate between *Dhc* strains that do or do not have the ability to gain energy from VC reductive dechlorination, it seems reasonable that the numbers should correlate with both the rate and extent of dechlorination.

Probes for VC reductase genes could provide more specific information on the ability to completely dechlorinate chloroethenes. However, there are numerous VC reductase sequences (Hölscher et al., 2004) and so far there are probes for only two (*vcrA* and *bvcA*). These probes have proven very useful for tracking introduced cultures and for detecting indigenous VC dechlorinators at some sites. Not surprisingly, VC reductase levels can be a better indicator of the dechlorination capacity than the *Dhc* numbers (van der Zaan et al., 2010). But at this point, false negatives are certainly possible (i.e., neither *vcr* or *bvc* may be present, but reductive dechlorination of VC can still occur). The *tceA* levels do not correlate well with dechlorination rate or extent (Da Silva and Alvarez, 2008).

There are other biomarkers and techniques that may improve decision making in the future. For example, it also may be possible to measure the *in situ* activity, and not just the genetic potential, by measuring the mRNA levels directly (Johnson et al., 2005; Lee et al., 2008; Rahm and Richardson, 2008). Direct measurement of key proteins such as VC reductases also is possible, and may prove to be a powerful monitoring technique in the future (Morris et al., 2007; Werner et al., 2009). Finally, deoxyribonucleic acid (DNA) microarrays make it possible to characterize the entire microbial community at a site in extraordinary detail (Löffler and Edwards, 2006; Johnson et al., 2008).

Interpreting MBT Results. MBT analyses can be powerful tools for characterizing and monitoring sites. For example, Lu et al. (2006) showed that the 16S rRNA gene analysis for *Dhc* could be used to decide if monitored natural attenuation (MNA) is a viable option. Specifically, *Dhc* numbers $\geq 10^7$ cells/L groundwater were correlated to "generally useful" rates of reductive dechlorination. In another example, Scheutz et al. (2008) demonstrated the value of a VC reductase biomarker (*vcrA*) to track the growth of a bioaugmentation culture and the onset of ethene generation at a site previously stalled at *cis*-DCE.

With respect to the need for bioaugmentation, Lu et al. (2009) showed that high levels of *Dhc* ($\geq 10^7$ cells/L) were found only at sites where complete dechlorination to ethene was detected. Some sites with detectable but lower numbers of *Dhc* did not exhibit complete dechlorination. Lu et al. (2009) concluded that detection of *Dhc* DNA in a groundwater sample was strong evidence that dechlorination to ethene or VC would occur, but "a failure to detect *Dhc* DNA in a sample of groundwater should not be taken to mean that dechlorination will stop at the level of dichloroethenes."

MBTs also can provide insight into the ecology of *Dhc* spp., which may prove useful in assessing bioaugmentation. For example, Behrens et al. (2008) showed that *Dhc* strains with VC reductases (*bvcA* and *vcrA*) were spatially separated from the early stage PCE- and TCE-dechlorinating bacteria, with the VC dechlorinators further downgradient. Similar separation in space or time seems likely in many situations, because *Dhc* do not compete well for hydrogen against other PCE and TCE dehalogenating bacteria (Flynn et al., 2000; Becker, 2006). It is therefore reasonable to expect that *Dhc* populations will be most numerous at locations where late stage dechlorination (*cis*-DCE and VC reduction) is occurring and *Dhc* spp. have little or no competition for that niche.

Monitoring after biostimulation or bioaugmentation confirms that *Dhc* numbers (and VC reductase genes) increase over time, and levels $>10^7$ cells/L appear characteristic of optimized systems (Lee et al., 2008; Scheutz et al., 2008; Amos et al., 2009; van der Zaan et al., 2010). At such population densities, *Dhc* must constitute a dominant member of the community, considering that groundwaters typically have total bacterial numbers of 10^7–10^9 cells/L, based on recoverable bacteria (Hirsch and Rades-Rohkohl, 1988). Consequently it is not surprising that such numbers would be found only at sites where complete dechlorination is occurring.

Establishing a specific trigger for whether bioaugmentation is needed involves some judgment, but it is reasonable that if the *Dhc* numbers exceed 10^6 cells/L across most of the target treatment zone, then complete dechlorination is either already measurable or is likely to be evident soon. In interpreting *Dhc* numbers, generally it will be important to analyze several samples from across the site to understand the spatial distribution and ensure that adequate numbers of *Dhc* are present throughout the majority of the site.

4.7.1.2 Compound Specific Isotope Analysis (CSIA)

Compound specific isotope analysis is a diagnostic chemical analysis with many potential environmental uses (Hunkeler et al., 2008; Schmidt et al., 2004). CSIA measures the naturally occurring isotopic composition of the chemicals of concern. Because the relative abundance of different stable isotopes can reflect the influence of different processes acting on the contaminants, these results can be useful in many assessments, including:

1. Elucidating key biotic and abiotic reaction processes and separating these degradation losses from those due to physical attenuation processes, such as dilution, sorption and volatilization.
2. Providing a powerful line of unequivocal evidence for *in situ* degradation, and estimating degradation rates.
3. Predicting the progress and extent of plume migration, in conjunction with groundwater models.

For chlorinated solvents in groundwater, CSIA has proven to be a sensitive tool for determining that natural biodegradation is occurring (Sherwood Lollar et al., 2001; Nijenhuis et al., 2007) or for assessing and monitoring enhanced reductive dechlorination

(Hirschorn et al., 2007). CSIA also can provide a sensitive method to identify the effects of bioaugmentation, since the isotopic fractionation in the daughter compounds, DCE and VC, is powerful evidence regarding the nature and rate of biodegradation processes (Morrill et al., 2005). CSIA can provide powerful evidence that bioaugmentation is not needed at a site if the chloride fractionation in the VC shows evidence of further biodegradation.

CSIA has not been used as a method for testing field samples to determine if bioaugmentation is needed at a specific site. The main reasons are that CSIA is more costly and less available than MBTs, but also the results may not be as definitive. For example, low levels of VC-degrading *Dehalococcoides* may be present and detectable using qPCR, but their effects may not yet be measurable using CSIA. Because carbon uptake from the chlorinated solvents does not occur, carbon isotope analyses of the biomass cannot be used to monitor biodegradation after addition of labeled carbon, as has been done for hydrocarbon degraders (Geyer et al., 2005).

4.7.1.3 Microcosm Testing

Laboratory testing using microcosms is an established method to obtain strong evidence regarding the need for bioaugmentation. Microcosms using site groundwater (and aquifer solids from the site, if possible) can be amended with electron donors (and bioaugmentation cultures, if desired) and incubated under anaerobic conditions following established methods. Guidance has been developed specifically for testing biostimulation to treat chlorinated ethenes (the Reductive Anaerobic Biological *In Situ* Treatment Technology [RABITT] test protocol) (ESTCP, 2003).

The RABITT protocol includes both microcosm and field testing methods, with results from several sites that showed complete dechlorination with biostimulation only. These methods may be modified or streamlined to address the need for bioaugmentation. In most cases, bioaugmentation testing can be done by using replicate anaerobic serum bottles (e.g., Lu et al., 2009). Many replicates can be established and analyzed for relatively little cost, and the sampling schedule can be modified easily as interim results are obtained. Although serum bottle tests will be best suited for most purposes, larger and more costly column testing still may be useful in some cases (Schaefer et al., 2009).

Any anaerobic incubation, particularly from a site that has been aerobic and/or donor-limited, has to continue long enough to allow growth of the native microbial community to sufficient numbers to biodegrade the chloroethenes to a meaningful extent. It may take several months to achieve complete dechlorination if populations of the key organisms must increase by several orders of magnitude, particularly if environmental conditions such as pH or temperature are suboptimal. Further, genetic transfer within the *Dhc* population may be partly responsible for the spread of the genes needed for complete dechlorination (Regeard et al., 2005) and this process may require time.

Results from one site-specific microcosm test are provided in Figure 4.3 to illustrate the types of information that can be gained. In this case, samples of groundwater and solids from a TCE-contaminated site (Hunter Army Air Field, Georgia) were incubated in anaerobic microcosms. Test microcosms were bioaugmented with a commercially available culture containing *Dhc* strains capable of complete dechlorination (SDC-9™, Shaw Environmental, Inc.) 59 days after the incubations began. The results show a classic *cis*-DCE stall without bioaugmentation. However, after bioaugmentation, complete dechlorination to ethene was observed, if the pH was adjusted to neutral.

Importantly, microcosms are not necessarily accurate predictors of field performance. Conditions within a closed incubator differ from the open real-world environment, which is

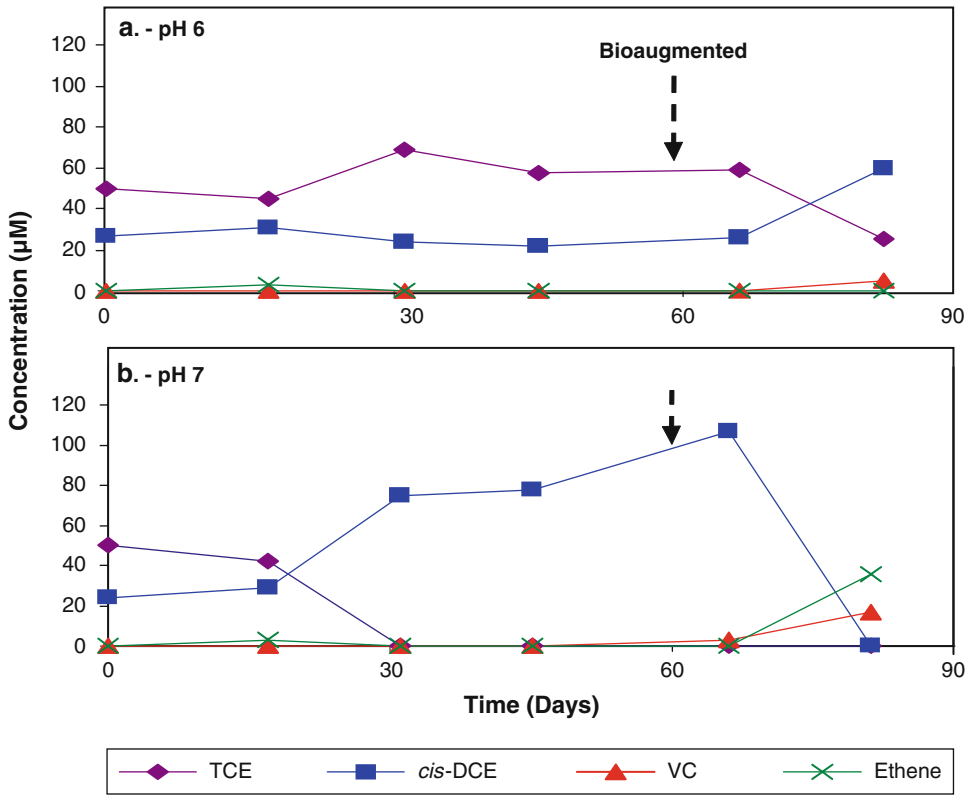

Figure 4.3. Results from microcosm testing performed with solids and groundwater from a TCE-contaminated site (Hunter Army Air Force Base, Georgia). Half of the microcosms were incubated at the initial pH of the site groundwater (pH 6.0), and half were adjusted to neutral pH (pH 7.0). A commercial bioaugmentation culture (SDC-9™, Shaw Environmental, Inc., Lawrenceville, New Jersey) was added to both sets of microcosms after 59 days. Results showed no dechlorination in the pH 6.0 microcosms until bioaugmentation (Panel A), with slow development of complete dechlorination ability afterwards. In the pH-adjusted microcosms (Panel B), partial dechlorination was rapid before bioaugmentation, and complete dechlorination was observed soon after bioaugmentation.

characterized by important heterogeneities, greater scales and dynamic features that are difficult to reproduce in the laboratory. As a result, researchers have long realized that biodegradation rates in the field may differ from microcosm results (e.g., Spain et al., 1984), but also microcosms may not always predict the need for bioaugmentation accurately. For example, Lu et al. (2009) showed that microcosms did not always exhibit complete dechlorination, even when the samples were taken from wells with high concentrations of ethene in the groundwater.

These results illustrate the strengths and limitations of microcosm testing. Under controlled conditions, it is possible to demonstrate with reasonable certainty whether bioaugmentation is necessary, and to diagnose the reasons for disappointing performance. But microcosm testing requires time and money, and it may not provide accurate rate estimates.

4.7.2 Field Testing

Field testing is obviously preferable for many purposes, e.g., estimating degradation rates under real-world conditions. A variety of field test methods have been developed and used to assist in evaluating biostimulation. These are described in the following sections.

4.7.2.1 *In Situ* Sampling Devices

An increasingly popular technique for field testing is to use *in situ* sampling devices, such as Bio-Traps® or Bio-Flo® devices (Microbial Insights, Inc., Knoxville, Tennessee). Bio-Traps®, which can be deployed in monitoring wells (generally for 1–3 months), contain inert beads that provide a large surface area for microorganisms to colonize. The microorganisms present in the groundwater then form biofilms on and within the pores of the beads, similar to biofilm formation on native aquifer materials. Once recovered from the well, DNA, RNA or phospholipids can be readily extracted from the Bio-Trap® beads for analysis. This approach can be used to characterize the microbial ecology in terms of classes of organisms (for example, aerobic hydrocarbon degraders, nitrate reducers, sulfate reducers and reductive dechlorinators) as well as specific degraders of importance, such as *Dhc*, and the presence of key genes.

These sampling devices also can be used to perform *in situ* bioaugmentation testing. Devices can be amended with electron donors and/or bioaugmentation cultures and suspended in monitoring wells, often with passive diffusion samplers to provide samples for analysis of the contaminants and geochemical conditions (Figure 4.4). After incubation in place – generally for a few weeks – the samplers are recovered and analyzed for *Dhc* as well as specific biomarkers.

This type of *in situ* testing can provide very valuable information for relatively little cost. For example, if sampling an unamended or biostimulated site shows that *Dhc* and VC reductase

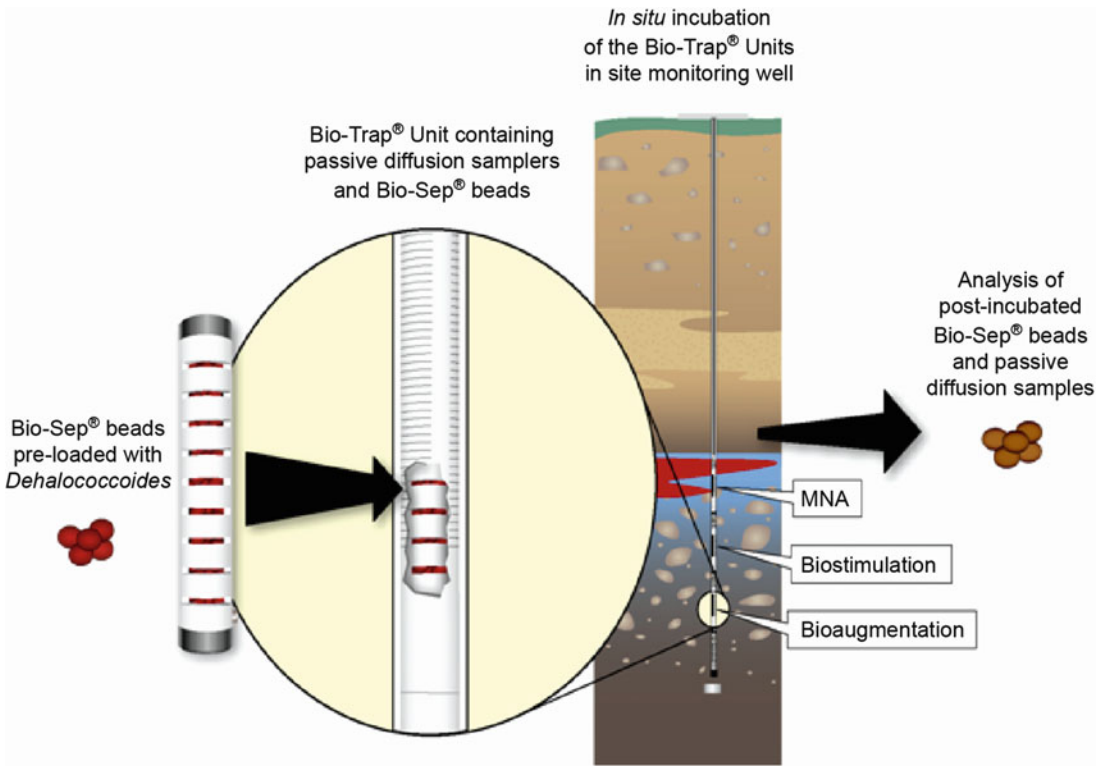

Figure 4.4. Example of *in situ* sampling device (Bio-Trap® Unit) used to test benefits of bioaugmentation. Bioaugmentation cultures can be added on a solid matrix (Bio-Sep® beads) and left in place for weeks to months before chemical and molecular biological analysis. Figure courtesy of Greg Davis, Microbial Insights.

genes are present and represent a large fraction of the total bacterial numbers, bioaugmentation is almost certainly not necessary. Conversely, the presence of such biomarkers only in bioaugmented samplers is powerful evidence that they are needed.

There are some caveats to consider when using this method and interpreting the data. Notably, the environment inside a well can differ in important ways from the surrounding aquifer. Oxygen and ORP levels may differ, pH and geochemistry may be modified within the well environment and the biological community also may be different. The time of incubation also may be an important consideration. Incubation for roughly 30 days is typical, and should be sufficient for many sites. However, lag times can extend to several months in some cases, possibly yielding false negative results.

4.7.2.2 Push-Pull Tests

One relatively rapid and inexpensive field method to assess microbial activities is the so-called "push-pull" test procedure. Push-pull tests involve injection through a well into the surrounding aquifer, and later extraction of water from the same well (Istok et al., 1997). The injected solution contains one or more tracers, as well as reactive solutes such as electron donors. After some incubation time – referred to as drift time, as the injected solution moves downgradient and unamended groundwater moves into the capture zone from upgradient – the groundwater is extracted and analyzed. The method can be used for *in situ* testing of several biological and chemical processes (e.g., Istok et al., 2004). Of course, the drift time must be sufficient to allow *in situ* growth and measurable dechlorination without allowing too much of the injected solution to move beyond the well's capture zone.

The push-pull test has been proposed as a method to directly assess the need for bioaugmentation by adding augmentation cultures to some wells or portions of an aquifer with comparisons to biostimulated controls (Lee, 2006). Inclusion of a reactive tracer such as trichlorofluoroethene that is not generally found in groundwater can increase the power of the technique and allow accurate *in situ* dechlorination rate measurements (Field et al., 2005). This technique is still considered innovative and can require considerable planning and data analysis, but it may have specialized applications. In addition to measuring dechlorination rates, this method also may allow measurements of the *in situ* rates of consumption of other amendments (e.g., electron donor).

4.7.2.3 Field Plots

More robust data can be obtained from field test plots. However, field testing is expensive and the heterogeneity at the field scale can lead to relatively high variability and extensive sampling networks. Although the results can be the most conclusive evidence that bioaugmentation is or is not beneficial, the cost and effort may be difficult to justify given the development and demonstrated value of less costly diagnostic tests. Field tests can make sense, however, if project managers are committed to using *in situ* bioremediation, if the site is large or has a complex hydrogeology or contaminant mixture and if the managers have the time to optimize the design and operations by using field testing as a first phase of full-scale treatment.

For example, in one case study (ESTCP, 2003), significant ethene concentrations were detected in the groundwater from a TCE- and VC-contaminated area at Cape Canaveral Air Station, Florida, but not until approximately 3 months after continuous lactate injection was initiated (Figure 4.5). In this case, bioaugmentation was not tested, but the results clearly showed that it would not be essential for treatment, and that the biostimulation lag time would be roughly 3 months.

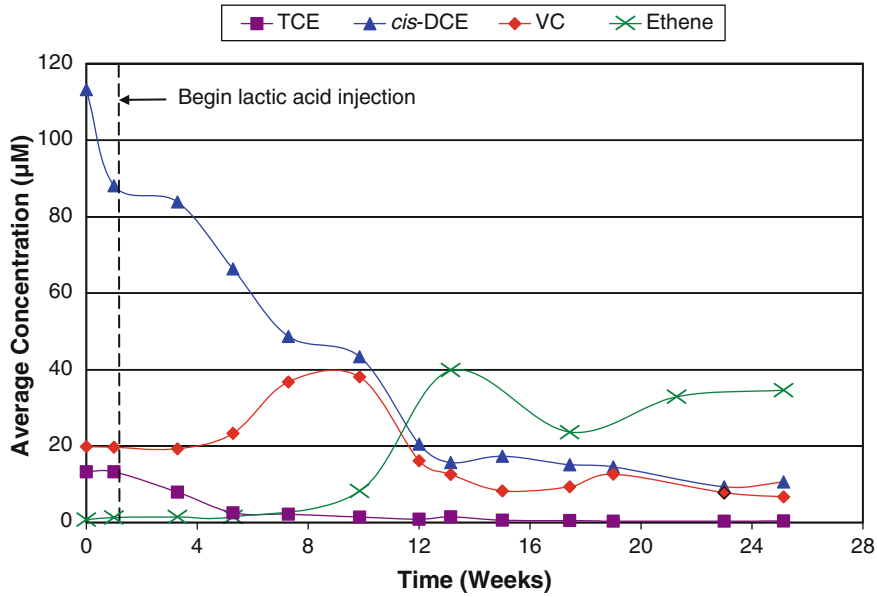

Figure 4.5. Results from a field test measuring biostimulation performance, showing molar concentrations of TCE and daughter products in groundwater from Cape Canaveral Air Station, Florida (Facility 1381) (from Morse et al., 1998). Lactate was added into the subsurface using two injection wells, and several monitoring wells within the treatment zone were sampled over time. The results shown represent the average concentrations from 49 sampling points located within the treatment zone.

There are several well-monitored field tests that have shown the need for bioaugmentation and can provide examples of typical dosages and monitoring methods. These demonstrations include tests at Dover Air Force Base (AFB), Delaware (Ellis et al., 2000), Kelly AFB, Texas (Major et al., 2002), and Bachman Road, Minnesota (Lendvay et al., 2003). These tests have used closed-loop active recirculation systems. Such systems allow continuous extraction and injection of electron donors, improved distribution of the bioaugmentation culture, and hydraulic isolation of the test plots to some extent. Further, these field tests relied on relatively dense monitoring networks and frequent sampling intervals, as compared to typical full-scale remediation designs. Although such active and relatively costly operations may not be needed in many full-scale remediation projects, this approach is reasonable for field tests of the benefits of bioaugmentation. The results of these field tests suggest that 3–6 months of operation may be needed before conclusive results are available.

4.8 HOW VALUABLE IS TIME?

Faster treatment is probably the most common reason for bioaugmenting a site. Bioaugmentation can reduce the duration of active treatment, and it also can reduce the lag time before complete dechlorination is observed. The value of faster treatment can vary tremendously, depending on several factors. At some sites, or for some responsible parties, time is critical. Rapid treatment can be important for economic or regulatory reasons, and reaching remediation objectives as quickly as possible can be far more important than the costs of bioaugmenting, making it a compelling insurance measure. For example, property transactions or redevelopment projects can make it extremely important to prevent any delays in the time before active treatment is over or remedial objectives are met. In such cases, the costs of bioaugmentation may be minor compared to the costs of several months of suboptimal performance.

Stakeholder input and concerns often are important when determining whether the time required for optimal performance will be acceptable. In the case of bioaugmentation decisions, the fundamental point is that if adaptive management is relatively easy, biostimulation is a more attractive alternative. If managers have an effective relationship with the regulators and public, adaptive management can be easier to implement. For example, there may be much less need for certainty regarding the eventual performance if there is an atmosphere of trust. Conversely, at sites where the relationship has deteriorated, it may be more costly to wait for several months prior to bioaugmentation because of the additional monitoring and testing that may be required.

At many sites, the lag time will be a relatively unimportant factor. Waiting 12–18 months after the first electron donor injection before the onset of measurable complete dechlorination may be of little concern and less expensive than an unneeded bioaugmentation treatment. There are many long-term, relatively passive *in situ* bioremediation systems that will need very infrequent reinjections, such as edible oil biobarriers injected into low-strength plumes at sites that pose little risk or public concern. In such cases, it may be prudent to bioaugment only after the monitoring data demonstrate a failure to attain remedial objectives despite otherwise favorable conditions for long periods of time.

4.9 IS THE RISK OF EXPOSURE TO TOXIC INTERMEDIATES UNACCEPTABLE?

Human health and environmental risks can play a critical role in bioaugmentation decisions. The key question is whether the risks of a temporary or long-lasting accumulation of more toxic byproducts – particularly VC – are acceptable. Incomplete dechlorination for some period of time may be of little concern if the concentrations are sufficiently low, or if there is no open exposure pathway. The exposure pathways of potential concern include public contact with groundwater, surface water exposures, indoor air vapor intrusion and even outdoor air exposure from shallow groundwater with little vapor attenuation.

Biostimulation, particularly when used for source zone remediation, is designed to enhance the dissolution of chlorinated compounds. Treatment therefore can lead to an increase in the mass discharge from the source zone and possible plume expansion (ITRC, 2005). It also will alter the contaminant distribution so that the plume may pose a greater risk to receptors exposed to the groundwater or vapors released from the groundwater.

If accumulation of partial dechlorination daughter products represents a potential risk to human health or the environment, then bioaugmentation as a method to reduce the potential or duration of such accumulations becomes a more attractive alternative. In some cases, temporary accumulations of daughter products may not increase the health hazards or environmental risks, but still may be unacceptable from a regulatory or public perception perspective. Therefore, addressing the risks of partial dechlorination will sometimes require considering the interests of other stakeholders as well as technical factors.

4.10 ECONOMIC ASSESSMENTS OF BIOAUGMENTATION

An economic assessment of the benefits of bioaugmentation is worth doing at any site, unless it is clear from the existing data that bioaugmentation is not necessary. As a first approximation, the detailed cost assessments presented in Chapter 11 should be consulted. These assessments, done for three template site scenarios, concluded that bioaugmentation increased the capital costs for an *in situ* bioremediation system by roughly 10–12%, which represented an increase of roughly 1–3% in the life cycle costs of the remedy. For example, Krug et al. (Chapter 11) calculated an additional cost of $8,000 to bioaugment a source zone 250

square meters (m^2) in area (roughly 0.06 acre), over a 3-m (10-foot [ft]) deep interval. This additional cost estimate includes the costs for both the culture as delivered to the site (assuming a requirement for 20 L of culture) and injection into existing wells. The added cost of bioaugmenting amounted to \$10.67/m^3, or \$8.20 per cubic yard (cy), which represented 11% of the capital cost for the treatment system, and 2.5% of the total Net Present Value (NPV) cost of the overall remedy.

This template site is relatively small, and it is likely that some unit costs will be lower at larger sites or under differing conditions. Further, the amount of culture that has to be injected can differ significantly. A separate cost evaluation was completed for this chapter, assuming a larger model site and lower volumes of culture per unit treated aquifer volume, based on the detailed cost analyses developed by Steffan et al. (2008). This cost analysis was based on a review of actual costs for 40 sites that had been bioaugmented by Shaw Environmental, Inc. The average cost of the bioaugmentation culture for the 40 sites was \$0.30/m^3, or \$0.23/cy. The model site used for this analysis was 0.4 hectare (ha) (1 acre) in area, with a 3-m (10-ft) thick target treatment interval. The analysis assumed a maximum culture cost of \$300/L, a requirement for roughly 50 L of culture and approximately \$3,000 for the labor required to inject the culture in existing wells. The resulting upper estimate of the total additional cost to bioaugment the model site (12,000 m^3, or roughly 15,700 cy) was \$12,000 (an additional cost of \$1.00/m^3, or \$0.76/cy).

As these separate cost evaluations demonstrate, the costs at specific sites can vary greatly depending on several factors. Because the costs are so variable, any economic assessment will require a preliminary site-specific design. These preliminary designs should consider the wide variety of bioaugmentation options that are available to optimize the technology for a given set of objectives and site conditions (see Chapter 5, Methods of Bioaugmentation). Also, it can be important to consider some potential economic impacts that are often overlooked. For example, the evaluations done in Chapter 11 do not take into account some expenses that proactive bioaugmentation may prevent, such as additional monitoring events or project meetings.

The most critical site characteristics that will impact the decision to bioaugment are those that control the volume of inoculum needed, the spacing needed between injection points and the need for active recirculation to improve distribution within the treatment zone. These characteristics include the depth and thickness of the contaminated zone, the hydraulic conductivity, the degree of heterogeneity and the spatial distribution of the contamination within the subsurface.

The type of bioremediation strategy used also can impact the cost comparison. For example, source zone bioremediation often is an active remediation process, involving frequent or even continuous injections of electron donors. In this case, the costs of waiting for optimal performance can be much higher than the cost of bioaugmentation. On the other hand, bioaugmentation may be less attractive when using more passive, long-term bioremediation approaches (e.g., use of longer lasting donors, such as edible oils, to treat sources or plumes, or to establish biobarriers).

Economic assumptions and requirements can affect bioaugmentation decisions in many ways. In essence, bioaugmentation becomes more attractive as the long-term life cycle costs become a more important consideration, and it becomes less attractive as minimizing the initial capital costs becomes more crucial. For example, the discount rate used in assessing the NPV of expenses incurred often is much higher for private industries than for the public sector (typical discount rates may be 7% for the private sector, as opposed to approximately 3% for government agencies). The result is that in the private sector it may be more attractive to avoid expenses early in a project (e.g., bioaugmenting at the start of treatment), especially if there is a reasonable chance that the expense may not be needed later.

Finally, there can be some less obvious economic drivers. Some responsible parties may have financial constraints that make deferring expenditures more attractive, even if the life

cycle costs may be higher. Conversely, some parties may prefer to spend more money early in the process for greater certainty. For example, some parties put a high premium on public perception, so may prefer bioaugmentation in order to avoid negative publicity related to even temporary failures of a treatment system. Similarly, when transferring a property it may be viewed as preferable to bioaugment prior to transfer to reduce the uncertainty regarding future expenses for site management.

4.11 SUMMARY AND RECOMMENDATIONS

Bioaugmentation with *Dehalococcoides* can improve bioremediation performance. However there is a cost, both for the bioaugmentation cultures and for the labor and equipment needed. Whether it is worth the cost can be a difficult decision because initial characterization data are rarely conclusive regarding the need or benefits. It is also important to recognize that there can be a significant cost for not bioaugmenting, resulting from the additional time and uncertainty associated with the lag time during biostimulation.

In many cases, bioaugmentation decisions can be made on the basis of existing geochemical data and limited site-specific groundwater analyses for *Dhc* (using 16S rRNA qPCR methods). Further testing should be needed only if these results are inconclusive and bioaugmenting is particularly costly or contentious. The decision guidance provided in this chapter can be used to systematically evaluate the likely benefits of bioaugmentation at a specific site.

Although the scientific basis is incomplete and many bioaugmentation decisions will require consideration of management priorities and risk tolerance, some technical guidance is possible. The following recommendations are incorporated in the decision guidance and are summarized below:

1. If *Dhc* are $>10^6$ cells/L groundwater and ethene is present, bioaugmentation is probably not necessary.
2. If speed is a critical consideration (i.e., considerable ethene production in <6 months) and *Dhc* numbers are $<10^5$ cells/L groundwater, bioaugmentation should be performed as soon as conditions are suitable (i.e., pH 5.5–8.0 and ORP < about −100 mV).
3. If an extended period of suboptimal performance (as much as 1–2 years) is acceptable and *Dhc* are $<10^5$ cells/L groundwater, do not bioaugment initially, but monitor for *Dhc* and all parent and product compounds every 1–2 months. Increases in *Dhc* numbers of at least 1–2 orders of magnitude should be apparent within 6 months after biostimulation, and VOC analyses should show evidence of increasing late-stage daughter products (DCE and preferably VC and ethene) within the same timeframe.
4. If a biostimulation system has been operated for 12 months under suitable conditions (pH 5.5–8.0 and ORP < -100 mV) but complete dechlorination (ethene/ethane production) has not occurred and *Dhc* remain $<10^6$ cell/L, bioaugmentation should be performed (assuming other causes have been ruled out, such as inhibition by cocontaminants or sulfides).
5. If site-specific testing is necessary, the best approach generally is to establish anaerobic microcosms containing solids and groundwater from the site, or deploy *in situ* microcosms if anaerobic conditions can be maintained in the test well. Test both biostimulation and bioaugmentation in laboratory or field microcosms, and incubate for 2 months. Bioaugment if significant ethene (>10% of the starting contaminant molar concentration) is generated in the bioaugmentation microcosms and not in the biostimulation-only microcosms.

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CHAPTER 5

BIOAUGMENTATION CONSIDERATIONS

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5.1 INTRODUCTION

Bioaugmentation can be employed in bioremediation systems to improve the rate and extent of solvent remediation. This chapter is intended to assist practitioners by summarizing the factors that should be considered when selecting and designing a bioremediation approach employing bioaugmentation.

Several factors can influence the effectiveness of bioaugmentation. These factors include the environmental site conditions, inoculum volumes and densities, and the overall bioremediation strategy to be used at the site. In short, practitioners have to make several decisions when implementing bioaugmentation that can affect the success of the effort, the time required for effective treatment, or the costs for remediation.

This chapter begins by describing the effects of site conditions on the effectiveness of bioaugmentation (Section 5.2). Section 5.3 (Field Methods) discusses electron donor, buffer and culture requirements, along with culture injection and initial distribution techniques. The final section, Section 5.4, describes typical bioremediation system configurations and the implications and considerations for bioaugmentation.

5.2 EFFECT OF SITE CONDITIONS ON EFFECTIVENESS OF BIOAUGMENTATION

Several factors influence the establishment and performance of bioaugmentation cultures introduced into groundwater environments. These include exposure to oxygen, temperature and pH, competition for electron donor, chlorinated solvent concentration, the presence of other chlorinated solvents, and site hydrogeology. These factors are briefly discussed below.

5.2.1 Exposure to Oxygen

Dehalococcoides spp. (*Dhc*) are strict anaerobic microorganisms, and oxygen toxicity can significantly impact culture viability (Maymó-Gatell et al., 1997). To prevent oxygen exposure, delivery of several of the *Dhc* – containing commercial cultures (such as KB-1® and SDC-9™) has been accomplished using shipping canisters that maintain anaerobic culture conditions, and the culture is typically added to aquifers after anaerobic and reducing conditions have been achieved. For example, one vendor recommends that groundwater have an oxidation–reduction potential (ORP) below −75 millivolts (mV) and a dissolved oxygen concentration of less than 0.2 milligrams/liter (mg/L) prior to the injection of the *Dhc*-containing culture. Other important ORP indicators prior to bioaugmentation are evidence of nitrate and sulfate reduction.

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5.2.2 Temperature and pH

Temperature and pH are two other factors that affect the growth and activity of *Dhc*. Temperature will affect the rates of *Dhc* growth and solvent biodegradation. Complete dechlorination from trichloroethene (TCE) to ethene has been observed between 10°C and 30°C for commercial dechlorinating cultures (with the exception of a site located in Alaska where the complete dechlorination of TCE to ethene was observed at groundwater temperatures between 6–8°C). Dechlorination stalled at *cis*-1,2-dichloroethene (*cis*-DCE) at temperatures less than 4–10°C (depending on the electron donor added) and above 40°C (Friis et al., 2007). Maximum growth rates (μ) and zero order degradation rates were highest for TCE dechlorination at 30°C with lactate as a substrate (μ_{TCE} of 7.00 ± 0.14 $days^{-1}$). In general, maximum growth rates and TCE dechlorination rates were up to an order of magnitude higher than rates for utilization of *cis*-DCE and vinyl chloride (VC). Temperature dependence of maximum growth rates and degradation rates of *cis*-DCE and VC were similar and highest at 15–30°C (Friis et al., 2007). Therefore, *Dhc* growth and dechlorination rates will be slower in regions where groundwater temperatures are lower and faster in regions where groundwater temperatures are higher. To mitigate for colder temperatures, additional *Dhc* culture can be added.

Like most microbial processes, dechlorination activity is affected by pH and is highest near neutral. The optimal pH for the growth of the KB-1® bacterial culture is between pH 6.0 and 8.3. Complete degradation to ethene occurs within this pH range, while partial degradation of TCE to *cis*-DCE and VC occurs between the 5–6 and 8.6–10 pH ranges. Dechlorination was not observed to occur below pH 5 and above pH 10 (Rowlands, 2004). At pH values less than 5, dehalogenation of perchloroethene (PCE; also termed tetrachloroethene) was found to be completely inhibited in *Dhc*-containing cultures (Vainberg et al., 2009). Others have found VC dehalogenation to be more sensitive to pH than TCE dechlorination, with strong inhibition occurring at a pH less than 6 (Eaddy, 2008). Buffers can be used to adjust the native groundwater pH to near neutral conditions to improve rates, as discussed in Section 5.3.2.2.

5.2.3 Competition for Electron Donor/Geochemical Conditions

Successful bioaugmentation and TCE dechlorination to ethene requires sufficient electron donor to drive the process. When other electron acceptors, such as nitrate, dissolved iron, dissolved manganese and sulfate are present, bacteria utilizing these alternate electron acceptors will require electron donor to drive their reduction, resulting in competition for electron donor. Generally this competition can be overcome by adding sufficient electron donor to meet the demand of these other processes.

The presence of high background sulfate concentrations appears to adversely impact bioaugmentation and dehalorespiration in some cases but not in others. For example, complete dechlorination of TCE to ethene was not observed in bioaugmented microcosms (Pinellas culture) containing high concentrations of sulfate (3,000–6,000 mg/L), despite stimulating active sulfate reduction to remove sulfate, repeated reamendments with the Pinellas culture, and the application of different electron donors (Battelle, 2004).

However, groundwater at the site of origin for KB-1® contained more than 1,000 mg/L of sulfate, and as such, the KB-1® culture appears to have adapted to high concentrations of sulfate. As part of the Source Area Bioremediation (SABRE) project, the effect of high sulfate concentrations (e.g., 1,250 mg/L) on reductive dechlorination using the KB-1® culture was studied. At these concentrations, sulfate reduction was concurrent with *cis*-DCE reduction. The transformation of VC to ethene occurred once sulfate concentrations were reduced below

~50 mg/L (Dworatzek et al., 2006). Treatability studies may be warranted to assess the effects of high sulfate concentration given that it enhances reductive dechlorination at some sites while inhibiting it at others.

One possible problem with high levels of sulfate is the potential for the production of excessive sulfide concentrations as a result of sulfate reduction, which may inhibit anaerobic dechlorination as well as some fermentation reactions (Maillacheruvu and Parkin, 1996). The sulfide levels that may potentially inhibit dechlorinating microorganisms are not well documented. One study that investigated sulfide inhibition indicated that 5.0 millimolar (mM) (161 mg/L) sulfide stopped all dechlorination activity, but no inhibition was observed at 1 mM (32.1 mg/L) (He et al., 2005). In general, dissolved sulfide and hydrogen sulfide are rapidly co-precipitated with ferrous iron (a byproduct of ferric iron reduction), providing sufficient iron is present to react with the sulfides.

The role of iron- and manganese-reduction in inhibiting bioaugmentation performance is largely uninvestigated. At some sites, high dissolved iron or manganese concentrations are thought to adversely affect dechlorination in a manner similar to other competing electron acceptors. The concentrations of dissolved iron and manganese that may inhibit anaerobic dechlorination have not been well documented or defined (Parsons Corporation, 2004).

5.2.4 Volatile Organic Compound (VOC) Concentration

It was once thought that bioremediation processes were ineffective for treating high concentrations of chlorinated ethenes such as those found in dense nonaqueous phase liquid (DNAPL) source areas, which historically limited the application of the technology to dissolved plume treatment or containment. However, data collected over the last several years demonstrate that dechlorinating microorganisms are active over a wide range of chloroethene concentrations. Duhamel et al. (2002) reported dechlorination of PCE, TCE, *cis*-DCE and VC at initial concentrations of 132, 197, 77 and 87 mg/L, respectively, in microcosm studies. In microcosms conducted for the SABRE project, complete dechlorination of TCE to ethene occurred at 400 mg/L and, in some cases, as high as 800 mg/L (Harkness et al., 2006). Similar results were reported by Yang and McCarty (2000), who observed PCE dechlorination in the presence of *cis*-DCE and ethene at concentrations of 0.66 and 1.05 mM (64 and 29 mg/L, respectively). The presence of such high concentrations of PCE, *cis*-DCE and ethene can be inhibitory to methanogenesis (Yang and McCarty, 2000), improving electron donor availability for dehalorespiration. The occurrence of dechlorinating activity at very high chlorinated solvent concentrations has permitted bioremediation/bioaugmentation to be used for DNAPL source remediation (Schaefer et al., 2010b).

It is also important to recognize that there is a lower volatile organic compound (VOC) concentration limit for sustaining reductive dechlorination. This level has not been well-defined in the field. However, in laboratory studies, Cupples et al. (2004) indicate that concentrations of *cis*-DCE and VC must be above 0.7 micromolar (μM) (44–68 μg/L) so that growth of *Dhc* outpaces its decay.

5.2.5 Inhibitory Constituents

While chloroethenes appear to be inhibitory only at extremely high aqueous concentrations, several VOCs have been demonstrated or are suspected to exert inhibitory effects at much lower concentrations. Both chloroform (CF) and 1,1,1-trichloroethane (TCA) slowed rates of VC dechlorination to ethene by *Dhc*, with complete inhibition at concentrations of 450 micrograms per liter (μg/L) (3.8 μM) and 700 μg/L (5.2 μM), respectively (Duhamel et al., 2002). Comparable results have been reported for other chloroethenes, including inhibition of *cis*-DCE

dechlorination by 190 µg/L (1.6 µM) chloroform (Maymó-Gatell et al., 2001), and of PCE dechlorination by 1,000 µg/L (8,400 µM) chloroform (Maymó-Gatell et al., 2001). To overcome inhibition, bioaugmentation cultures such as SDC-9™ or KB-1® Plus, which both contain *Dehalobactor* species that are capable of dechlorination of 1,1,1-TCA and CF, can be used.

5.2.6 Hydrogeology

The hydrogeology of a site also can influence the success of bioaugmentation. If the hydraulic conductivity is very low, it will be more difficult and time-consuming to deliver both electron donor and bacteria to the targeted treatment zone. Likewise, if the subsurface is heterogeneous, it will be more challenging to achieve a uniform distribution of electron donor and inoculum. Consequently in low permeability formations, injection wells will need to be spaced closer together than in more permeable aquifers.

5.3 FIELD METHODS

5.3.1 Injection Infrastructure Considerations

Dhc culture is typically delivered to the subsurface via injection wells, which permit the subsequent addition of electron donor, buffer or inoculum should these be required (Figure 5.1). Another commonly used method to deliver culture is via direct push injection (Figure 5.2), whereby culture is injected into the subsurface directly without the use of wells. The choice of culture delivery method will hinge on both technical and economic considerations, which will be a function of the depth of the target contaminant zone and the number of anticipated electron donor injections. If the contamination is deep (>~100 feet (ft) [30 meters (m)]) and/or multiple injections of electron donor are likely to be required, then the installation of wells will probably

Figure 5.1. Photo of injection of bioaugmentation culture in injection well.

Figure 5.2. Photo of direct push injection of bioaugmentation culture.

be the most feasible option. Shallower plumes or plumes in tight formations may favor the use of direct injection of electron donor and culture.

Ideally, the spacing of injection wells/points should incorporate the expected transport distances of the bioaugmentation culture to permit good distribution of the culture. However, the transport of bioaugmentation cultures is still under study. Research using pure cultures suggests that introduced microorganisms will be removed as a result of straining and filtration processes within a short distance (inches to feet) from the point of introduction (Mawdsley et al., 1996; Emelko et al., 2006). However, pilot and field studies have documented that dehalorespiring bacteria can move through aquifer materials greater than 100 ft (30 m) from the point of introduction (Major et al., 2002), suggesting that downgradient movement is due to growth and detachment processes.

5.3.2 Preconditioning Requirements

Prior to injecting *Dhc*, it is important that the ORP and pH are suitable for growth and that there is sufficient electron donor present. In the following sections, the selection and addition of electron donors and buffers are discussed.

5.3.2.1 Selection and Addition of Electron Donors

Given the diversity of organisms contained within mixed consortia, most bioaugmentation cultures can utilize a wide variety of electron donors in fermentative processes, typically resulting in the conversion of the electron donor into molecular hydrogen and acetate. *Dhc* depend primarily on hydrogen as the electron donor for dechlorination (Löffler et al., 2003) although some dechlorinating populations also can utilize acetate (He et al., 2002). Non-*Dhc* microorganisms present in mixed consortia play a significant role in supporting dechlorinating activity through the production of hydrogen through fermentative processes.

There are many organic substrates that can be naturally degraded and fermented in the subsurface to generate hydrogen. Examples of easily fermentable organic substrates include alcohols, low-molecular-weight fatty acids (e.g., lactate), carbohydrates (e.g., sugars), vegetable oils and plant debris (e.g., mulch). The substrates most commonly added for enhanced anaerobic bioremediation include lactate, molasses, Hydrogen Release Compound (HRC®), mulch and emulsified vegetable oils. Substrates used less frequently include ethanol, methanol, benzoate, butyrate, high-fructose corn syrup (HFCS), whey, chitin and gaseous hydrogen (Parsons Corporation, 2004). The physical nature of the substrate (i.e., liquid, solid or gas) will influence the frequency of addition, the addition technique, and the potential system configurations.

The *Dhc*-containing culture KB-1® has been demonstrated to work with most commonly used electron donors, including sugars (e.g., glucose, molasses), alcohols (e.g., methanol, ethanol), organic acids (e.g., lactate), vegetable oils (canola), emulsified oils (e.g., EOS®, Newman Zone© and SRS™) and slow release compounds (e.g., HRC®) (http://www.siremlab.com/products/kb-1, accessed June 19, 2012; Duhamel et al., 2002). *Dhc* sp. BAV1 readily utilizes hydrogen but not formate, acetate, lactate, pyruvate, propionate, glucose, ethanol or yeast extract as an electron donor (He et al., 2003). SDC-9™ also has been applied with a wide variety of electron donors. Although hydrogen is used by methanogenic populations, several studies suggest that *Dhc* microorganisms competitively utilize hydrogen at concentrations below those supporting methanogenesis (Smatlak et al., 1996; Yang and McCarty, 1998; Löffler et al., 1999).

The choice of electron donor will depend on the method of application and cost considerations. For example, biobarriers typically lend themselves to the use of emulsified vegetable oil or mulch whereas active recirculation systems favor soluble electron donors, such as lactate. Regardless of the electron donor selected, sufficient electron donor must be provided to meet the demand of the competing electron acceptors (most notably sulfate) so that sufficient electron donor is available for dechlorination reactions. The addition of electron donor is often required to reduce the ORP of the aquifer to the desired range for complete reductive dechlorination (generally less than −75 to −100 mV).

Electron donor is typically added prior to or during bioaugmentation to provide a source of fermentable substrate and lower the ORP of the groundwater. Depending on the bioremediation system configuration, the electron donor can be added using either extracted groundwater or municipal water. If either is oxygenated, it is generally recommended to wait for reducing conditions to be established *in situ* after the electron donor is injected before bioaugmenting. The time required for aquifer conditions to be appropriate for bioaugmentation after electron donor addition varies from site to site; however, typical lag times are 4–8 weeks. Extracted water can be reduced *ex situ* by amending a tank of water with a soluble electron donor and allowing time (typically days to weeks) for the biomass to consume oxygen and lower the ORP to below −75 mV. Chemical reductants, such as sodium sulfite, also may be used to reduce the extracted water. It is recommended that small-scale tests be conducted prior to field implementation to better estimate the time required to produce anaerobic water. Another approach involves the addition of the bioaugmentation culture in a "donut" of anaerobic water part way through the injection of the electron donor as discussed further in Section 5.3.4.

5.3.2.2 Selection and Addition of Buffers

As mentioned earlier, *Dhc* is most active between pH 6 and 8.3. Prior to bioaugmentation, it is important to establish the aquifer pH in this range. To maintain growth and activity of *Dhc* following bioaugmentation, it is also necessary to maintain the pH of the groundwater in

this range. However, volatile fatty acids and carbonic acid are produced during electron donor fermentation and hydrochloric acid (HCl) is generated through reductive dechlorination; both of these processes can cause a decrease in pH. Thus, reductive dechlorination in source zones with high VOC concentrations can be associated with extensive localized dechlorination and production of HCl, whereby the soil's natural buffering capacity can be exceeded (Robinson and Barry, 2009). In these instances, it is important to supply enough buffer to compensate for acid production to maintain suitable conditions for *Dhc*. It should be borne in mind that other processes can act to increase the pH of the aquifer. These processes include sulfate reduction, iron (III) oxide reduction, acetate fermentation, calcite dissolution and proton exchange on clays (Robinson et al., 2009).

If the natural aquifer pH is not in the 6–8.3 range, then the aquifer pH can be adjusted through the addition of buffers. Three commonly used buffers or buffer-containing products include sodium bicarbonate, AquaBupH™ (EOS Remediation, Raleigh, NC, USA), and Neutral Zone© (RNAS, Brooklyn Center, MN, USA). These products are discussed below.

Sodium Bicarbonate is an inexpensive, readily available buffer, which has been widely used to buffer groundwater for bioremediation/bioaugmentation applications. The disadvantage of using sodium bicarbonate is that it is soluble and can wash out of the treatment area, thereby requiring multiple, often time-consuming, applications. For recirculating systems, sodium bicarbonate can be metered in and distributed throughout the target area. For passive systems, the buffer solution can either be made up in tap water (which may need to be pre-reduced prior to injection and may cause localized plume dilution) or the contaminated groundwater can be extracted, buffered and re-injected, which may be subject to permitting or regulatory barriers and is time-consuming, especially for low permeability aquifers. The addition of sodium bicarbonate also can increase the sodium concentration of the groundwater significantly, and may alter the geochemical conditions enough to cause some mineral species to precipitate, which can lead to aquifer clogging.

AquaBupH™ is a mixture of an "alkaline buffering" suspension, emulsified vegetable oil, and a proprietary food additive. It is designed to provide a long-term source of buffer through the distribution of particulate buffer (median particle size of 2.5 micrometer [μm]) in the vicinity of the injection point and has a low sodium content (<0.4%). This product can be used for bioremediation/bioaugmentation applications requiring the addition of both buffer and electron donor. The vendor recommends that the acid demand of the aquifer matrix and groundwater be determined and then the amount of AquaBupH™ needed to achieve the desired pH can be calculated. This approach may mean that additional injections of emulsified vegetable oil (without buffer) may be required initially and subsequently to provide sufficient electron donor. AquaBupH™ is injected like an emulsified vegetable oil, with the product metered into an injection line. EOS Remediation provides a design spreadsheet to assist in estimating the appropriate dose of AquaBupH™ (www.eosremediation.com). The spreadsheet accounts for the HCl produced from dechlorination, the carbonic acid produced through substrate fermentation, and the hydroxyl released from electron acceptor reduction.

Neutral Zone© is a colloidal suspension of calcium carbonate and is designed to remain in place and not wash out of the targeted treatment zone. According to the vendor, it will not cause adversely high pH because the base is liberated by the acid it will neutralize and not by dissolution in water. The advantage of Neutral Zone© is that it can be added independently of the amount of electron donor added. Neutral Zone© is significantly more viscous and dense than emulsified vegetable oil. Therefore, the vendor recommends that the product be diluted 5–10 fold with water and then injected with a proportional feed system into multiple points (permanent wells or direct-push points) simultaneously.

Buffer capacity testing of the groundwater and soil or determination of the acid demand of the aquifer material and acidity of the groundwater by Standard Method 2310 should be conducted to determine the appropriate dose of the selected buffer (in the absence of biological reactions). Design tools can be employed to estimate buffer dose (e.g., EOS Remediation design spreadsheet for AquaBupH^{TM} or spreadsheet tool and equations for sodium bicarbonate [Robinson and Barry, 2009; Robinson et al., 2009]). Geochemical models, such as PHREEQC and MINTEQ, and bench-scale biotreatability tests also can be employed to estimate buffer requirements.

5.3.3 Culture Requirements

Even though bioaugmentation for chlorinated VOC remediation is widely used, no rigorous model has been generated to estimate the amount of inoculum needed for a given site. Most practitioners appear to rely on the guidance of Lu et al. (2006) and attempt to achieve a minimum *in situ* concentration in the range of 10^7 *Dhc*/L, where complete degradation of TCE to ethene is often observed.

The simplest approach to estimating the amount of culture required is to estimate the pore volume of water within a targeted treatment zone and then multiply the treatment volume by 10^7 *Dhc*/L. The volume of culture needed to achieve 10^7 *Dhc*/L is dependent on the *Dhc* concentration in the bioaugmentation culture, which varies from vendor to vendor. Also, it generally is preferable to include a lag time to achieve the desired culture density, not only to reduce the culture costs but also because it is difficult and costly to distribute the cells throughout the target treatment zone without relying on growth and migration *in situ*. In any case, some amount of time (weeks to months) will be required for the culture to grow and spread throughout the treatment area.

The use of this simple approach does not account for the effects of potentially important factors, notably the VOC concentrations in the target aquifer and the actual hydrogeology of the site. Both factors can have a significant effect on the distribution and growth of the added culture. In cases with relatively high concentrations of VOCs, the model may overestimate the amount of culture needed, provided other geochemical conditions are appropriate for *Dhc* growth and transport. The model may underestimate the amount of culture needed in aquifers where VOC concentrations are low or where other geochemical factors may limit growth or transport of *Dhc* cells *in situ*. Fine tuning the model may be difficult in many cases, and it will likely require extensive laboratory microcosm and column studies and the application of more complex models like those provided by Schaefer et al. (2009).

Ultimately, the decision may be made by comparing the cost or risk of adding too much or too little culture with the cost of performing extensive laboratory testing. To aid in evaluating the amount of culture to apply, Table 5.1 provides data from several pilot and field-scale bioaugmentation projects to treat a variety of aquifers with varying chemical and hydrogeological characteristics. Further information on the relationship between inoculum density and degradation rates can be found in Appendix A.

5.3.4 Injection Techniques

Prior to adding the *Dhc* bioaugmentation culture, the well or drive point is purged with certified 100% argon or nitrogen to remove any oxygen from the well casing and to maintain an inert gas blanket in the well headspace. All of the required tubing also is purged to remove oxygen. Following purging, the compressed gas is used to pressurize the culture vessel and push the culture out of the vessel and down the pre-purged tubing positioned within the well screen at

Table 5.1. Amount of Culture Used in Various Field Pilot and Full-Scale Bioaugmentation Applications

Site Designation	Total VOCs (ppb)	Geology	Approx. Surface Area (ft^2)	Approx. Saturated Thickness (ft)	Volume Culture Added (L)	Approx. Conc. *Dhc* Added (cells/L)	Approx. Time to Significant Degradation	Electron Donor	Other Treatments
Aerojet NPL (Area 20)/ Rancho Cordova, CA	2,000	Sand/gravel	3,640	20	50	1×10^{10}	<1 month	Ethanol	Recirculation
Manufacturing Facility Portland, OR	>100,000	Silty sand	300	50	60	1×10^{11}	1–3 months	EHC©	Passive barrier; 12 injection points
IR Site 40, Seal Beach, CA	<1,000	Coastal aquifer	1,368	40	40	1×10^{11}	<1 month	Lactate	Semi-passive; 2 injection points
Former Manufacturing Facility, IN	~3,000	Sand/gravel	25,000	45	340	1×10^{11}	1–3 months	EVO	Passive barrier; 60 injection points
Moody AFB, SS-39, GA	>500	Sand/silt	360,000	10	360	1×10^{10}	<3 months	Sodium lactate	Recirculation; 10 injection wells, 9 extraction wells
Moody AFB SS-38, GA	>1,000	Sand/silt	400,000	10	925	1×10^{10}	<90 days	Sodium lactate	Recirculation; 700-ft horizontal wells: 1 injection, 1 extraction
Moody AFB, SD-16 (west), GA	~ 1,000	Sand/silt	100,000	10	2,900	1×10^{10}	9–12 months	Veg oil	Passive; 110 injection points; sodium bicarbonate
Moody AFB, SD-16 (east), GA	>100	Sand/silt	156,000	10	177	1×10^{10}	3–6 months	Veg oil	Passive; 175 injection points
Moody AFB, FT-07, GA	~10,000	Sand/silt	8,000	20	285	1×10^{10}	3–6 months	Sodium lactate	Passive; 57 gravity feed injection points
Columbus AFB, SS32, MS	>50	Sand/gravel	248,000	20	539	1×10^{10}	3–6 months	Sodium lacate/ veg oil mix	Passive; 442 injection points

(*continued*)

Table 5.1. (continued)

Site Designation	Total VOCs (ppb)	Geology	Approx. Surface Area (ft^2)	Approx. Saturated Thickness (ft)	Volume Culture Added (L)	Approx. Conc. *Dhc* Added (cells/L)	Approx. Time to Significant Degradation	Electron Donor	Other Treatments
Myrtle Beach AFB, FT-11, SC	70–900	Sand/silt	78,000	5	276	1×10^{10}	6 months	Sodium lactate	Passive; 337 injection points
AFP-4, Building 181, TX	50,000	Silt and clay	8,000	10	75	1×10^{10}	3 months	Veg oil	Passive; 12 injection wells
Camp Bullis, TX	~ 2,000	Karst	47,250	5	15	5×10^{10}	6 months	Veg oil	Passive; 5 horizontal wells (~240 ft in length)
Naval Station Treasure Island, Site 24, CA	~25,000	Sand, silty sand and clay	10,500	25	60	1×10^{10}	75 days[a]	Sodium lactate	Recirculation; 3 injection, 3 extraction wells.
Naval Station Treasure Island, Site 24, CA	~ 35,000	Sand, silty sand and clay	350,000	25	60	1×10^{11}	90 days	Lactic acid	Recirculation; 19 injection, 27 extraction wells. 105 biobarrier injection points
Naval Station Treasure Island, Site 21, CA	~ 1,500	Sand, silty sand and clay	37,500	20	60	1×10^{11}	90 days	Lactic acid	Passive; 45 1″ diameter direct injection points
Vandenberg AFB, Site 9, CA	~1,000	Sand and silty sand	10,000	5	60	1×10^{10}	6 months	Sodium lactate	Passive; injected in 10 monitoring wells.
Hickam AFB, Site LF-05, HI	~100,000	Calcium carbonate sand	100	10	60	1×10^{10}	60 days	Sodium lactate	Passive; 2 injection wells
Hickam AFB Site, CG-110, HI	~800	Volcanic sand and silt	10,600	15	60	1×10^{11}	<34 days[b]	Sodium lactate	Passive; 15 direct injection locations
Pearl Harbor Naval Base, Former Aiea Laundry Facility, HI	~100	Volcanic sand and silt	100	10	60	1×10^{10}	<60 days	Sodium lactate and veg oil and vitamin B	Passive; 2 injection wells

(*continued*)

Table 5.1. (continued)

Site Designation	Total VOCs (ppb)	Geology	Approx. Surface Area (ft^2)	Approx. Saturated Thickness (ft)	Volume Culture Added (L)	Approx. Conc. *Dhc* Added (cells/L)	Approx. Time to Significant Degradation	Electron Donor	Other Treatments
Pearl Harbor Naval Base, Former Aiea Laundry Facility, HI	~100	Volcanic sand and silt	100	10	60	1×10^{11}	< 60 days	Sodium lactate and veg oil and vitamin B	Passive; 2 injection wells
PJKS EPL Pilot Study, CO	~3,600	Sandstone	500	40	56	1×10^{11}	1 month	Sodium lactate and Restore 375©	Passive; 3 injection wells
PJKS EPL Full Scale, CO	~8,600	Sandstone	60,000	40	189	1×10^{11}	Ongoing	Sodium lactate and Restore 375©	Passive; 4 horizontal wells
PJKS D-4, CO	~700	Crystalline fractured bedrock, gneiss	20,000	40	38	1×10^{11}	15 months	Sodium lactate and Restore 375©	Passive; 6 injection wells
PJKS SCA North Full Scale, CO	~1,600	Sandstone	40,000	40	75	1×10^{11}	4 months	Sodium lactate and Restore 375©	Passive; 2 horizontal wells
PJKS SCA South Full Scale, CO	~9,100	Sandstone	35,000	40	151	1×10^{11}	Ongoing	Sodium lactate and Restore 375©	Passive; 4 horizontal wells
Cape Canaveral AFS, FL	155,000	Sand, crushed shells	400	10	39	not reported	<1 month	Ethanol	Recirculation
Bachman Road, Pilot, MI	50–1,200	Fine grain sand	272	6	200	1×10^{9}	15 days	Sodium lactate	Recirculation
Dover AFB, DE	1,200–4,800	Sand/silt	1,102	10	180	2×10^{11}	90 days	Sodium lactate	Recirculation
Kelly AFB, TX	800	Gravel, sand, silt, clay	614	20	13	1×10^{9}	100 days	Methanol/ acetate	Recirculation

Note: AFB – Air Force Base; AFS – Air Force Station; L – liter(s); ppb – parts per billion; EPL – Engineering Propulsion Laboratory; EVO – Emulsified Vegetable Oil; NPL – National Priorities List

[a]Complete conversion of TCE to ethene by day 75

[b]Complete conversion of TCE to ethene by day 34

the desired depth. A digital scale or other measuring device provided by vendors can be used to determine the mass or volume of culture added to each well/drive point.

For injections requiring smaller volumes (0.3–1 L) of culture per well/drive point, metering devices have been developed that allow for the accurate injection of a smaller target culture volume. For deeper injections in direct-push points, anaerobic water can be used to push the culture into the formation. In these cases, the tubing from the culture vessel is not positioned to the desired depth in the drive point, but rather is connected at the surface to the tubing carrying the anaerobic water to the desired depth.

5.3.5 Distribution Techniques

There are a number of techniques that can be used to distribute the culture *in situ*. The first traditional approach is to "push" in or chase the culture with an electron donor solution to distribute the culture further into the formation. While a small fraction of the culture may be pushed further into the formation, it appears (based on column studies) that most of the culture will stay close to the injection well initially (as discussed in Section 5.3.1 and Appendix A). It is unclear how much further the culture will be transported under this scenario versus injecting the *Dhc* at the end of the electron donor injection, as most *Dhc* transport is expected to occur as a result of *in situ* growth and cell detachment.

A similar approach, which has been used to distribute *Dhc* in an aquifer prior to it being fully reduced, is to sandwich *Dhc* between electron donor additions by injecting reduced anaerobic water before and after the *Dhc*. In other words, a portion of electron donor mixed with aerobic water is injected, then anaerobic water, then culture, followed by more anaerobic water and the remaining electron donor mixed with aerobic water (as illustrated in Figure 5.3). This technique is referred to as the "donut" approach, and it was developed to permit bioaugmentation and electron donor addition in one mobilization and to allow for a large portion of the electron donor to be introduced with aerobic water from an easily-obtained hydrant source. The disadvantage of this approach is the possibility of *Dhc* being exposed to oxygen in the electron donor injectate or aquifer. Therefore, the onset of reductive dechlorination may be slower if a fraction of the *Dhc* is inactivated. However, bioaugmentation and electron donor addition can be accomplished in one mobilization, lowering field labor costs.

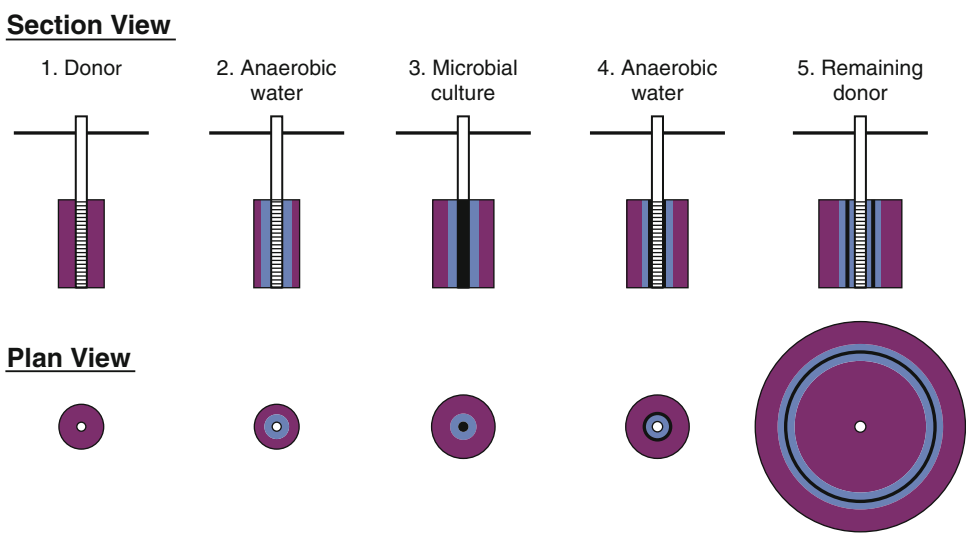

Figure 5.3. Schematic of "donut" approach to bioaugmentation culture injection.

Two other techniques have been used to improve the distribution of *Dhc* after injection. One method is to use a forced gradient or recirculation system (discussed in Section 5.4). The other technique is to directly inject the culture in a large number of locations to achieve a better initial distribution of *Dhc*. This technique permits the *Dhc* to be injected away from oxidized areas (e.g., near an injection well or plume fringes) or downgradient in a number of locations to minimize the time required for the *Dhc* to be transported downgradient naturally through growth and detachment. The downside of this approach may be the additional cost of a direct-push rig to accomplish the injections.

5.4 BIOREMEDIATION CONFIGURATIONS EMPLOYING BIOAUGMENTATION

Because almost all bioaugmentation applications require the addition of an electron donor, it is important to consider the electron donor delivery method and bioremediation system configuration.

The typical electron donor emplacement methodologies used for bioremediation include (adapted from ITRC [2005]):

- Conventional injection wells: Electron donors are injected into an existing or newly-installed well. Typically a network of wells is used to inject relatively large volumes of liquids containing a soluble electron donor. Conventional wells often are used for moderate to high permeability aquifers or treatment zones.
- Direct-push injection points: A network of relatively closely spaced points is usually used, with injections of a relatively small volume of a soluble electron donor at each injection point. Direct-push injection is most applicable for shallow sites with relatively homogeneous conditions with a moderate to high permeability, although it also can be used in sandy clays or silty sands.
- Trenching: Trenches are usually backfilled with a large mass of a solid electron donor (e.g., mulch or chitin) and/or a long-lived liquid electron donor such as vegetable oil, often mixed with sand. Trenches can be used in aquifers with any degree of permeability, as long as the permeability of the trench is at least as high as the formation.
- Hydraulic or pneumatic fracturing: Fracturing of low permeability regions creates zones in which electron donor (or culture) may be injected over greater distances. Either solid or liquid electron donors may be emplaced during or immediately after fracturing. Fracturing is generally used in low permeability conditions or at highly heterogeneous sites where the low permeability zones require treatment.

Various bioremediation approaches (classified as active, semi-passive or passive), can be used in conjunction with bioaugmentation. In the following subsections, these approaches are described. The advantages and disadvantages of each approach for achieving and maintaining optimal conditions for bacterial growth (biostimulation) are considered, as these also impact bioaugmentation. Afterwards, the practical implications of each approach for successful bioaugmentation are discussed.

5.4.1 Active Recirculation Approach

Active recirculation uses pumping (extraction) and reinjection of groundwater and electron donor across a treatment area. Often, the goal of a recirculation system is to control groundwater flow and donor distribution across the target treatment area in a more precise manner

than is generally achievable in either the passive or semi-passive approach. Active recirculation systems use conventional injection wells for electron donor delivery and usually also use injection wells for culture injection (Table 5.2).

Active recirculation systems generally pump groundwater continuously. Electron donor is usually blended with the extracted water prior to its reinjection and can be added continuously to the extracted water (generally at low concentrations), or it can be pulsed into the extracted water periodically (generally at higher concentrations). Soluble electron donor frequently is used in recirculation systems, because it is easiest to mix and pump and allows for the distribution over the largest distances.

The type and amount of equipment can vary significantly depending on the size of the site, the desired extraction/injection flow rate, and the amount of automation required. A simple recirculation system may involve extraction wells, injection wells and temporary conveyance (i.e., hoses) to transport water between them. More complex systems may have permanent piping, surge tanks, flow meters, valves, transfer pumps and process instrumentation/controls such as level switches, alarms and a programmable logic controller (PLC). Amendment dosing can be accomplished by manually mixing batches at the desired concentration, using proportional flow mixers, or by using metering pumps. An example of a recirculation system is shown in Figure 5.4.

5.4.1.1 Advantages/Disadvantages for Biostimulation

In general, recirculation is most appropriate for biostimulation at sites that have moderate to high hydraulic conductivity. It has been used for biostimulation (with bioaugmentation in some cases) at many sites (e.g., Lee et al., 2008; Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002; Hood et al., 2008; Wymore et al., 2009; Brown et al., 2009). The recirculation approach provides the greatest engineering control for biostimulation because of the ability to manipulate hydraulic gradients using the injection/extraction system. Compared to passive and semi-passive approaches, other advantages include:

- Rapid onset of reducing conditions because of the use of soluble donors;
- Largest electron donor distribution from an individual injection point (i.e., largest radius of influence during injection); and
- Ability to add large amounts of amendments over a relatively short timeframe.

The most significant disadvantage for active recirculation is that it generally has the highest capital costs and O&M requirements of any approach. Continual system monitoring, either by automated instrumentation or by onsite staff, is needed to ensure upset conditions are not encountered and that all above ground equipment is operating as designed. Besides requiring more O&M, other disadvantages of active recirculation approaches include:

- Logistical constraints at active facilities may impact placement of above ground infrastructure;
- Active systems are more prone to biofouling; and
- While good donor distribution can be achieved from individual injection points, multiple recirculation loops may be required to cover larger treatment areas.

5.4.1.2 Implications for Bioaugmentation

Active recirculation systems are costly and rely on frequent pumping. As a result, bioaugmentation may be relatively desirable for several reasons: (1) the relative cost of the

Table 5.2. Electron Donor Delivery Methods for Biostimulation/Bioaugmentation

	Injection Wells	Direct Push	Trenching	Fracturing
Applicable delivery type	• Passive • Semi-passive • Active recirculation	• Passive	• Passive • Active recirculation	• Passive
Electron donor type	• Liquid donors or liquid donors with very small scale suspended solids	• Liquid donors or liquid donors with very small scale suspended solids	• All electron donor types can be used	• All electron donor types can be used
Geology	• Applicable for all geologies	• Applicable for unconsolidated media without large gravel or cobbles	• Applicable for all geologies	• Applicable for low permeability media
Areal extent of treatment zone	• Achieve larger radius of influence (ROI) with low pressure injections than direct-push, but incremental ROI improvement decreases with volume	• ROI generally small because inefficiency of injection points (flow rate per unit head) unless injections are pressurized • Pressurized injections achieve larger ROI, but vertical distribution generally becomes erratic due to preferential flow	• Limited to the trench footprint	• Large ROI
Thickness of treatment zone	• Well screens can be installed for any thickness; however, longer screens will not achieve good vertical distribution in heterogeneous systems (nested wells can be used to overcome this, but capital costs are increased)	• Can be used for any thickness (subject to depth limitations) • Conventional “bottom up” injection approach can lead to erratic vertical distribution due to preferential flow in permeable strata	• Limited to the trench depth	• Treatment zone limited to the fracture thickness • Multiple fractures generally required

(*continued*)

Table 5.2. (continued)

	Injection Wells	Direct Push	Trenching	Fracturing
Depth of treatment zone	• Can be used for any depth	• Depth limited by size of rig used and is very dependent upon formation properties • Depths up to 60–70 ft are often achievable in unconsolidated media, though some rigs are capable of going deeper	• Limited to the trench depth. Depths greater than 80 ft are generally cost prohibitive	• Limited to drilling equipment used
Hydraulic conductivity of treatment zone	• Most effective in moderate to high permeability conditions	• Most effective in high permeability conditions • Injection point inefficiency can make flow rates low even in moderate permeability zones unless injections are pressurized	• Generally trench is filled with high hydraulic conductivity media	• Fracture zones have high hydraulic conductivity
Heterogeneity of treatment zone	• Most effective for low to moderate heterogeneity • Well efficiency (flow rate per unit head) often much higher than direct-push points, which allows larger volume injections with less pressure, leading to less preferential flow during injection	• Most effective for low heterogeneity • Injection point inefficiency leads to a higher degree of preferential flow for a given flow rate in heterogeneous formations relative to injection wells	• Treatment zone homogenized by emplacing homogenous media in trench	• Homogenous media emplaced in fractures
Groundwater velocity	• Low groundwater velocities will minimize downgradient impact of electron donor emplacement • High groundwater velocities will limit lateral impact of electron donor emplacement	• Low groundwater velocities will minimize downgradient impact of electron donor emplacement • High groundwater velocities will limit lateral impact of electron donor emplacement	• Trench can be designed to allow for adequate treatment time depending on groundwater velocity	• Low groundwater velocities will minimize downgradient impact of electron donor emplacement • High groundwater velocities will limit lateral impact of electron donor emplacement

(*continued*)

Table 5.2. (continued)

	Injection Wells	Direct Push	Trenching	Fracturing
Capital cost	• Capital cost is moderate relative to other passive approaches • Costs increase substantially when nested wells are used to treat large contaminated thicknesses	• Lowest capital cost for passive approaches	• Highest capital cost of passive approaches	• High capital cost due to hydraulic fracturing technology required
Operations and maintenance (O&M)	• O&M is fairly minimal in passive applications because injections are infrequent • Some well rehabilitation due to biofouling might be required when injections occur over several years	• O&M is minimal unless many injections are required over the life of the project; as the number of injection events increases, O&M of direct-push injection will approach, and eventually surpass injection wells	• Low to moderate O&M required. Recirculation systems may require O&M to prevent fouling	• No O&M required • Generally fracturing is only performed one time
Implications for bioaugmentation	• Inoculation into injection wells is preferred and most commonly used method	• Inoculation into direct-push points is generally not preferred • Few examples of direct-push inoculation in literature	• Direct inoculation during trench installation not recommended because of likely exposure to oxygen • Inoculation generally performed through wells directly in or just downgradient of trench	• Inoculation during fracturing generally not performed because high pressures and high volumes would create unpredictable distribution of low volume inoculum • Inoculation generally performed following fracturing

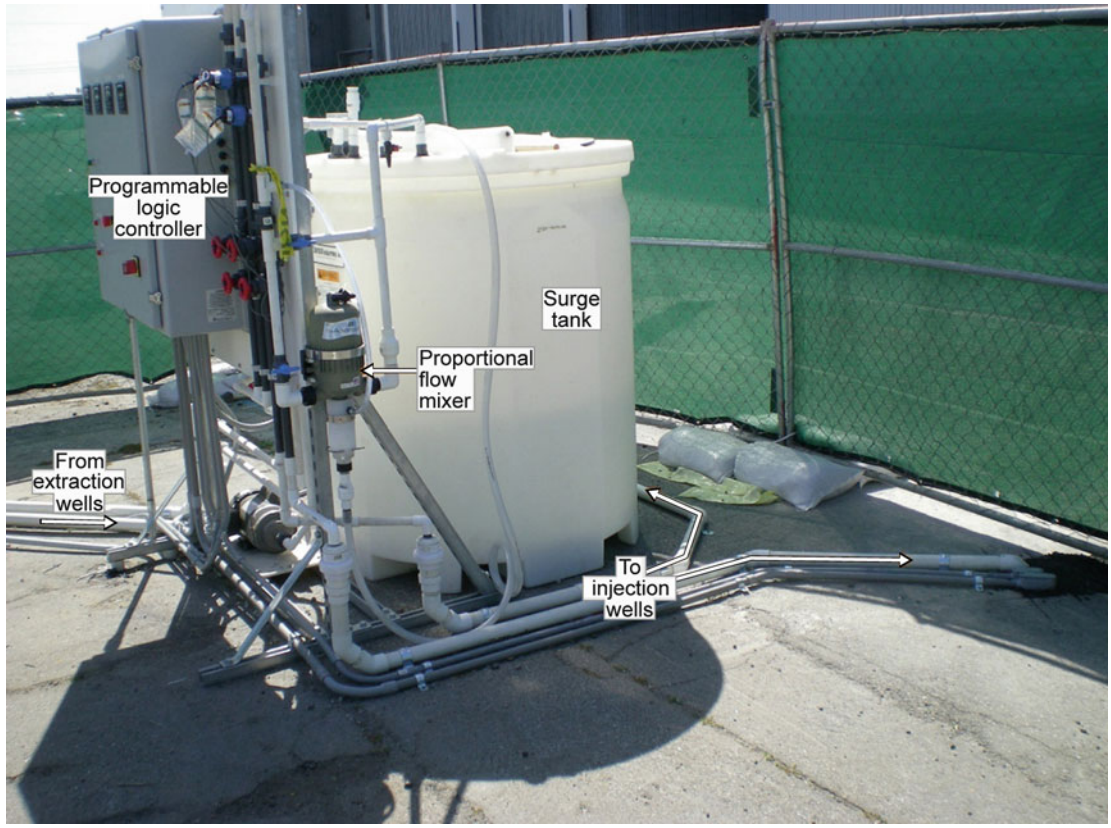

Figure 5.4. Photo of an active recirculation bioremediation system.

bioaugmentation culture is low, compared to the capital and operating costs for biostimulation alone; (2) if bioaugmentation can reduce the time that active recirculation is required, it can reduce the overall remediation costs significantly; (3) the infrastructure needed for bioaugmentation is already present so there is usually little additional cost to inject cultures; and (4) the active recirculation may act to distribute the bioaugmentation culture.

The use of active recirculation to distribute bacteria and induce complete dechlorination is well documented at the pilot scale (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002; Hood et al., 2008; Schaefer et al., 2010a), although sufficient sampling was not performed in all cases in order to provide a full assessment of bacterial growth and distribution. A study at Kelly AFB, TX (Major et al., 2002) was one for which *Dehalococcoides* transport times could be reasonably estimated and compared to conservative transport times. Travel times for *Dhc* were between 61 and 176 times longer than for conservative transport (i.e., the average groundwater velocity, based on the average rate of movement of a conservative tracer that is not destroyed or retarded during transport). The Bachman Road, MI site study suggested that the *Dhc* transport time was only about 2.3 times greater than groundwater transport times (Lendvay et al., 2003).

However, performing inoculations can be complicated if the system is already constructed and operational, and bioaugmentation was not considered in the initial design. For example, the recirculation system for ESTCP project ER-200513 was constructed and operated to "pre-condition" the aquifer to establish reducing conditions prior to inoculation. Subsequently,

the injection wellheads had to be disassembled to perform the inoculation to ensure that exposure to air was minimized. The addition of bioaugmentation culture directly to the reinjection line during recirculation operations is not advised as this approach can risk exposing the culture to oxygen if the pipes/hoses are not flowing full or if the extracted groundwater is not sufficiently reduced (Trotsky et al., 2010).

5.4.2 Semi-Passive Approach

The semi-passive approach refers to pulsed injection of amendment solution to achieve a large radius of influence around each injection point, and then allow amendments to drift along with the groundwater. Semi-passive also can refer to systems employing lateral mixing of electron donor perpendicular to groundwater flow to minimize the number of required injection wells. Inoculation is generally performed as a single injection event after establishing reducing conditions that are suitable for bioaugmentation. Recent studies have shown that reducing conditions adequate for bioaugmentation can be achieved 50 ft (~15 m) or more downgradient from an injection point, depending on the site's hydrogeologic conditions (Mora et al., 2008).

To perform the injections, electron donor amendment is blended above ground to achieve the desired injection concentration. The amendment used in this approach can be either a soluble or a slow-release electron donor, although generally semi-passive systems use soluble donors such as sodium lactate or ethanol, which are immediately bioavailable upon injection into the subsurface. The frequency of injections is dependent on many factors, including the electron donor type used, hydrogeologic conditions, competing electron acceptors, and the concentrations of the target contaminants.

5.4.2.1 Advantages/Disadvantages for Biostimulation

Semi-passive systems are generally favorable for biostimulation at sites that have moderate to high hydraulic conductivity and moderate hydraulic gradient, allowing ambient groundwater flow and/or lateral mixing to distribute electron donor. The semi-passive technique has been successfully applied at many sites (Mora et al., 2008) and during the recent ESTCP project ER-200513 (Trotsky et al., 2010).

The primary advantage to the semi-passive technique is that it is a flexible approach that allows for frequent applications of electron donor, while keeping the operational requirements and costs low.

Compared to passive and active recirculation approaches, other advantages include:

- Ability to distribute and maintain high concentrations of electron donor in a large radius of influence from individual injection points;
- Ability to perform frequent (i.e., monthly to quarterly) amendment injections cost effectively (on smaller scales);
- Large areas can be treated effectively with fewer injection points compared to passive systems; and
- Less O&M and capital requirements compared to active recirculation.

The semi-passive technique does have disadvantages compared to the passive and active recirculation approaches, including:

- Individual injections can take multiple days depending on subsurface conditions;
- Vertical mixing may be inconsistent and more dependent on ambient flow conditions compared to active recirculation.

5.4.2.2 Implications for Bioaugmentation

Similar to the active recirculation strategy, bioaugmentation is not generally a large incremental cost for semi-passive systems, because the infrastructure is already in place. Inoculation in a semi-passive approach is most often performed using the existing electron donor injection well locations. Generally, only a single inoculation for bioaugmentation is required after sufficient electron donor injections have been performed to create an appropriate reducing environment for the bacterial culture. Recent studies have shown that semi-passive bioaugmentation can be successfully applied to sites to achieve complete dechlorination under a variety of subsurface conditions (Lee et al., 2008; Mora et al., 2008). If intermittent groundwater recirculation is used between wells, the potential exists for more uniform culture distribution transverse to groundwater flow.

5.4.3 Passive Approach

A passive approach refers to a system where the electron donor is emplaced once or on an infrequent basis (i.e., annually or less) and where no forced gradient is applied. Bioaugmentation may be conducted either during or following the electron donor addition. Both electron donor and bacterial transport occur primarily under the influence of ambient groundwater flow. Example applications include "biobarriers", where a treatment zone is created perpendicular to groundwater flow (using injection wells, direct push injection points, or trenches) to reduce the flux of contaminants downgradient and large-scale inundation of source areas or plumes with a long-lived, or "slow-release," electron donor with the goal of achieving substantial treatment during the lifetime of the electron donor.

5.4.3.1 Advantages/Disadvantages for Biostimulation

Given that several methods for passive biostimulation/bioaugmentation exist, the advantages and disadvantages of the methods cannot be generalized, but must be considered individually. Table 5.2 discusses each delivery method relative to several key considerations for design and implementation. While advantages and disadvantages differ among the passive approaches, some generalizations can be made for passive approaches relative to semi-passive and active biostimulation/bioaugmentation strategies. In general, passive approaches require less O&M and have lower capital costs at small scales, but can have higher capital costs at large scales.

5.4.3.2 Implications for Bioaugmentation

The means of inoculation in a passive approach can depend largely on the methodology used for electron donor emplacement. When conventional injection wells or direct-push injection points are used, bioaugmentation is most often performed using the injection locations. For trenching, inoculation can be performed using a pipe laid in the trench during installation or using wells installed in, or immediately downgradient, of the trench. For fracturing applications, the boreholes used to initiate fractures can be used to inoculate groundwater once fractures have been installed. Several studies have been performed showing that bioaugmentation with hydraulic fracturing has significantly better results than standard bioaugmentation, especially in low permeability subsurface environments (Bjerg et al., 2006).

Although it is common to inject electron donors via single-use direct-push points, few cases of inoculations in direct-push injection points have been documented in the literature. One reason direct-push points are not used more often may be that they are temporary points

installed at the time of injection, and therefore it can be difficult to know if reducing conditions are present before (and after) the inoculation.

Inoculation in passive systems is generally performed in the same injection locations used for electron donor addition. Because survival and effectiveness of the inocula requires highly reducing conditions, bioaugmentation may have to occur after electron donor addition has resulted in appropriate conditions (Ellis et al., 2000; Major et al., 2002). However, if the aquifer is sufficiently reducing prior to initiation of biostimulation, inoculation can be performed simultaneously with the initial electron donor addition. In most cases, only one inoculation is required.

A passive approach can be a successful method for bioaugmentation (Dybas et al., 1998; Lendvay et al., 2003). Kovacich et al. (2007) showed that *Dhc* moved significant distances downgradient under ambient groundwater flow, reaching levels of 10^6–10^7 cells/L at wells 45 ft (~15 m) downgradient within only 8 months. Similarly, a recent field-scale comparison of "active" and "passive" bioaugmentation (ESTCP Project ER-200513; Trotsky et al., 2010) showed that *Dhc* could spread over considerable distances following passive bioaugmentation (i.e., a one-time injection, relying on migration with groundwater for further distribution). The passively-injected *Dhc* cells migrated at least 30 ft (10 m) downgradient, causing complete dechlorination to ethene over that distance. The measured travel time for the *Dhc* was only 1.5–3 times longer than the conservative tracer (bromide).

5.5 CONCLUSIONS

Bioaugmentation will be most successful if exposure to oxygen is minimized, the aquifer pH is near neutral, and sufficient electron donor is provided to meet the demand of the chlorinated solvents and competing alternate electron acceptors. Many different types of fermentable carbon substrates can be employed to provide a source of hydrogen to the *Dhc* bacteria to drive reductive dechlorination. The choice of substrate will largely depend on the system configuration and cost. If aquifer conditions are not immediately suitable, preconditioning through the addition of electron donor and/or buffer is often required to lower the ORP to less than −75 mV and change the pH to near neutral. Electron donor and buffer selection will be influenced by the system configuration and cost considerations.

Currently there are no firm guidelines with respect to the amount of culture to inject. Typically, practitioners aim to achieve a minimum of 10^7 *Dhc*/L *in situ* for a target treatment volume, as empirically this density has generally corresponded with the presence of ethene. A more complex model for estimating dosage effects has been developed (Schaefer et al., 2009) and verified in the field (Schaefer et al., 2010a), and it is expected to be available soon as a module for a widely-used groundwater transport model (Torlapati et al., 2012).

There are various techniques for distributing the culture, including using the electron donor solution to push the culture following injection, recirculation, or multiple direct push injections of culture. The relative effectiveness of these various approaches is still under study, but all of the techniques have been widely used. Laboratory column studies have shown that most *Dhc* remain near the point of injection, and the more mobile cells have been shown to have the same or better activity that the original inoculum. However, several field-scale studies have demonstrated that *Dhc* can travel hundreds of feet from the injection well over time, presumably through growth and detachment processes.

There are a variety of bioremediation system configurations (i.e., active, semi-passive and passive) where bioaugmentation can be employed. All three general approaches have been successful for bioaugmentation applications. Time is required for *Dhc* to migrate throughout

and colonize the treatment zone, and this fact should be borne in mind during the design and performance monitoring of bioremediation systems employing bioaugmentation.

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APPENDIX 5A

BACKGROUND ON INNOCULUM DENSITY AND DECHLORINATION RATES

For any bioaugmentation application, a key question is how many organisms must be added to a site. The amount of organisms needed directly affects the cost of the technology, and it can

be affected by several factors, including: (1) the concentration of contaminant present; (2) hydrogeochemical conditions at the site; (3) competition by indigenous organisms; (4) the number and relative activity of dehalogenating organisms (i.e., *Dhc*) in the added consortium; (5) *in situ* growth or death of the added organisms; and (6) filtration, attachment and detachment of the added culture.

Although several hundred bioaugmentation projects have now been performed around the world, most have relied on simple assumptions, simple models, cost barriers, a sense of feel, or wild guesses to determine the amount of culture to add. Because relatively few of these applications have been described in published literature, and because the published studies often do not describe how many organisms were used or how the amount of culture was selected, the task of selecting the "correct" amount of culture for a given application remains challenging.

Some guidance on the amount of culture needed for successful remediation was provided by Lu et al. (2006) who evaluated eight sites to determine the amount of *Dhc* needed to achieve reasonable rates of remediation at field scale. They concluded that sites with a "generally useful" rate of dechlorination of *cis*-DCE and VC (rate constant $\geq$0.3/year) had *Dhc* densities greater than 10^7 cells/L of groundwater. Although this data set was small, the results are consistent with field-scale results where successful bioaugmentation was associated with *Dhc* numbers $>10^7$/L (Hood et al., 2008; Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002; Ritalahti et al., 2005).

However, Röling (2007) analyzed the data provided by Lu et al. by using "metabolic control analysis" (MCA) and concluded that the flux reported by Lu et al. was not regulated by population size, but rather it was regulated at the cellular level (e.g., the specific activity of the cells). The key point is that effective bioaugmentation requires not only an adequate number, but the organisms also must be in an appropriate physiological condition. A recent study by Schaefer et al. (2009) provides support for this conclusion, and suggests that *in situ* treatment of VOCs can select for *Dhc* populations with faster dechlorination rates. Unfortunately, these findings complicate the challenge of predicting the amount of *Dhc* organisms that must be added to a target aquifer to achieve timely and cost effective remediation.

Laboratory Studies

Several laboratory microcosm, column and model aquifer studies have been performed to evaluate bioaugmentation for chlorinated solvent remediation. Although these studies are useful for evaluating the efficacy of a bioaugmentation remedy for a chosen site, they rarely compare the effect of different cell dosages on remedial efficiency. In fact, the amount of *Dhc* cells added to microcosms is often not reported. In one case, 2 milliliters (mL) of a culture presumably containing 10^6 *Dhc*/mL was added to microcosms containing 60 g of soil and 150 mL of groundwater and a TCE concentration of approximately 800 μg/L TCE (Major et al., 2002). This inoculum density is equal to approximately 1×10^7 *Dhc*/L. TCE degradation began after about 30 days of incubation and all of the TCE was converted to *cis*-DCE by about day 42. When a 100-fold higher concentration of TCE was added, TCE degradation began at about day 42 and was complete by about day 90.

In another laboratory study (Sleep et al., 2006), 30 mL of a *Dhc* culture containing 2×10^7 *Dhc*/mL was added to a bench-scale flow cell with a pore volume of approximately 2 L of water and a PCE DNAPL. This inoculum density represented a *Dhc* concentration of $\sim 3 \times 10^8$ *Dhc*/L. *cis*-DCE was detected in the flow cell effluent 13 days after bioaugmentation, and the *Dhc* concentrations in the effluent increased corresponding to increased concentrations of *cis*-DCE in the effluent. Over the course of the study, approximately

Table 5A.1 Measured *Dhc* Concentrations in Soil and Water Samples from a Laboratory Bioaugmentation Column Constructed with Fort Dix, New Jersey Aquifer Materials (derived from Schaefer et al., 2009).

Distance from Column Influent (centimeter(s))	Soil (*Dhc*/g)	Water (*Dhc*/L)
0–3.5	1×10^3	
3.5–7	84	8×10^7
7–10.5	<80	
10.5–14	<80	5×10^7
14–17.5	<80	
17.5–20	<80	1×10^7

30 times more *Dhc* was recovered from the box than the amount added, indicating significant growth of the organisms in the flow cell, although the highest concentration of *Dhc* was found near the injection point.

Column studies are especially useful because they allow assessment of bacterial transport and degradation. Several column studies have been performed to evaluate microbial transport through saturated soils. Straining, filtration processes and the formation of biofilms often control microbial transport in soils (Ginn et al., 2002; Yang and McCarty, 2000). The ultimate effect of these processes on transport is dependent upon microbial characteristics, porous media properties, flow rates, and aqueous geochemical properties. For example, Fuller et al. (2000) showed that subpopulations of a microbial consortium may have a range of adhesion properties, resulting in variable transport distances of each microbial population in soil.

Azizian et al. (2008) inoculated a continuous-flow anaerobic column constructed with Hanford site soils to evaluate reductive dechlorination of PCE. The influent PCE (0.09 mM) was transformed to VC and ethene within a hydraulic residence time of 1.3 days, and the *Dhc* concentrations in the column ranged from about 4×10^6 cells/g near the column influent to $\sim 2 \times 10^6$ cells/g near the column effluent (Behrens et al., 2008). In work by Schaefer et al. (2009) with dechlorinating bioaugmentation cultures, Monod kinetic parameters were determined for batch cultures used to inoculate aquifers and bacteria that passed through a saturated soil column (Schaefer et al., 2009). The *Dhc* concentrations within the column increased from nondetectable before inoculation (at a detection limit of approximately 5×10^4 cells/L) to $\sim 10^8$ cells/L after inoculation and electron donor addition, and the *Dhc* concentrations in the aqueous phase were within an order of magnitude throughout the length of the column (Table 5A.1). The Schaefer et al. observations also were consistent with the results of Yolcubal et al. (2002) who observed that actively growing bacteria are more prevalent in the aqueous phase than associated with the solid phase.

These microcosm, tank and column studies provide useful insights to aid those planning field bioaugmentation activities. The microcosm studies demonstrate that in well-mixed systems high inoculation densities (e.g., $>10^7$ *Dhc*/L) result in fairly rapid rates of VOC dechlorination as was predicted by Lu et al. (2006). The *Dhc*-containing cultures grow well under ambient environmental conditions, provided sufficient electron donor and VOC electron acceptor are present. Column and tank studies, however, suggest that the distribution of *Dhc* can vary based on VOC composition and electron donor availability, and that a large proportion of an inoculum applied may be filtered out by soils near an injection point (Behrens et al., 2008; Schaefer et al., 2009; Sleep et al., 2006). Nonetheless, the *Dhc* are highly mobile in some aquifer

materials and they can be readily transported in the aqueous phase (Schaefer et al., 2009), which is consistent with early field observations (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002). Further, the *Dhc* subpopulations that move through the aquifer may be compositionally different than the original inoculum (Behrens et al., 2008), and they may have a greater specific activity that the original inoculum (Schaefer et al., 2009).

Correlations Between Inoculum Density and *In Situ* Dechlorination Rates

Despite the extensive laboratory research performed to evaluate bioaugmentation with *Dhc*-containing consortia, and the application of the technology at several hundred sites, no clear correlations between inoculum density and *in situ* dechlorination rates have emerged. The only study focused on correlating *in situ Dhc* levels in the field to degradation activities are those discussed above by Lu et al. (2006) and that study focused on natural *Dhc* populations rather than those added as a bioaugmentation remedy.

Only a few field-scale bioaugmentation projects have been described in detail, and many of these were pilot studies performed early in the development of *Dhc*-containing cultures for VOC remediation before quantitative polymerase chain reaction (qPCR) techniques were available to quantify the numbers of *Dhc* present in the aquifers. In a recent study, Lee et al. (2008) measured *Dhc* numbers and reductive dehalogenase gene transcripts to evaluate *Dhc* performance and activity at a bioaugmentation site. The site, located at Fort Lewis, WA, USA, contained TCE at DNAPL concentrations, and bioaugmentation was performed in two plots containing a recirculation system. During the 3 months after culture injection, VC reductase gene (*vcrA*) copy numbers increased by as much as 2 orders of magnitude in the plots, but no attempts were made to correlate *Dhc* or reductive dehalogenase numbers to *in situ* dechlorination rates. In a similar recent study (Scheutz et al., 2008), successfully bioaugmented a site with *Dhc* and found that the *Dhc* numbers increased to approximately 10^8 cells/L after 76 days, and remained relatively constant throughout most of the remainder of the demonstration.

Most recently, Schaefer et al. (2010a) evaluated the effect of inoculation density on dechlorination rates in a silty sand aquifer at Fort Dix, NJ, USA (Steffan et al., 2010). The aquifer at the site had native *Dhc*, but the indigenous strains were unable to dechlorinate DCE or VC, resulting in a DCE stall at the site. Furthermore, the aquifer had a low natural pH (pH <5) that required pH adjustment in order to achieve complete dechlorination, even with an added inoculum. The study used four recirculation loops initially designed to achieve a 30-day travel time between the injection and extraction wells. Three loops were inoculated with 10X, 1X, or 0.1 X of the predicted necessary inoculum. The predicted inoculum density was based on an even distribution of the added cells within the treatment zone to an aqueous *Dhc* concentration of 10^7 *Dhc*/L. Figure 5A.1 shows the VOC concentrations in the second row of monitoring wells (20 ft [6 m] from the injection well) in the three inoculated recirculation loops. Most notably, ethene was produced in all three inoculated recirculation loops, including loop three (BMW 6) which received the lowest inoculum volume (i.e., 100-fold less than loop 1 [BMW2]). No ethene was produced in loop 4 which was not inoculated but did contain native *Dhc* strains. The study concluded that many factors affect the amount of culture needed for effective treatment and that selecting the amount of culture needed cannot reliably be based solely on the amount of groundwater to be treated. A 1-dimensional model has been developed to aid practitioners in determining the amount of culture needed (Schaefer et al., 2009), and the utility of the model for

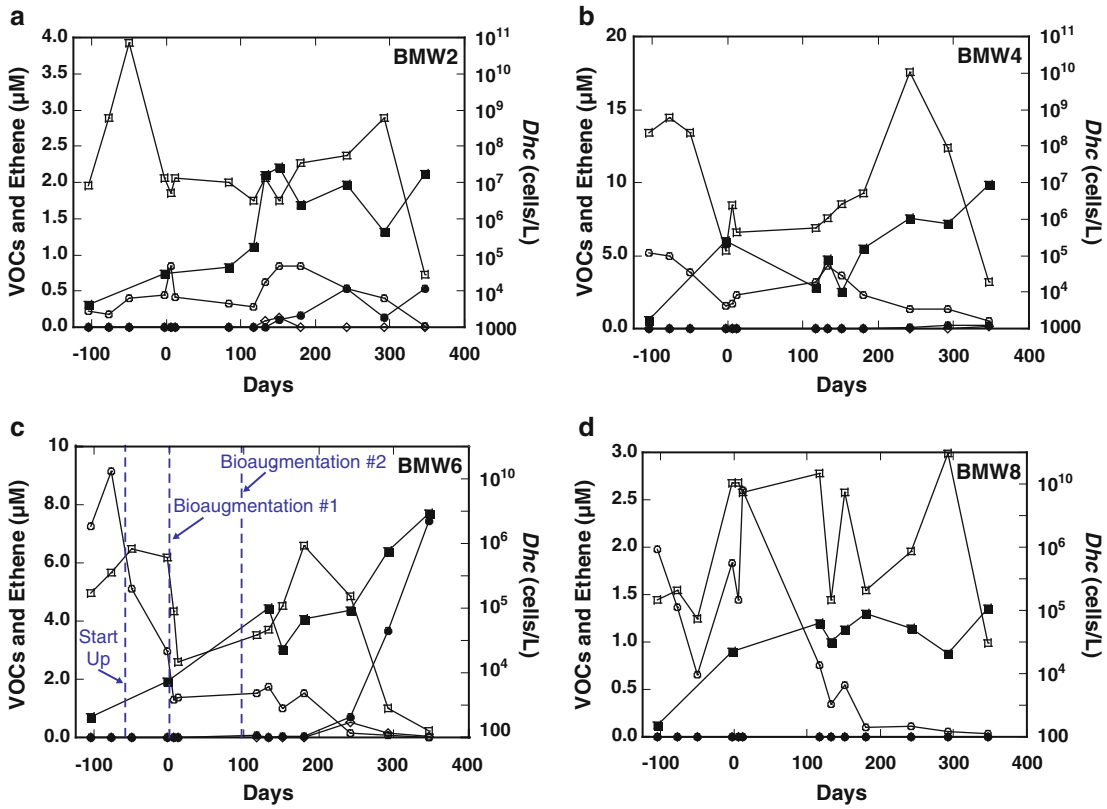

Figure 5A.1. Concentration of VOCs and *Dhc* at the second row of monitoring wells of a field demonstration system at Fort Dix, New Jersey. The monitoring wells were located 20 ft downgradient of the recirculation system injection wells. The system employed four recirculation loops that were inoculated with enough *Dhc* culture to theoretically achieve 10^8 *Dhc*/L (BMW2), 10^7 *Dhc*/L (BMW4), 10^6 *Dhc*/L (BMW6), and native *Dhc* only (BMW8). Bioaugmentation events were as identified for BMW6. Shortly after the first bioaugmentation event, pH in the aquifer exceeded pH 9 due to a system failure, so a second inoculum was applied to the site as indicated. Symbols represent TCE (○), DCE (□), VC (◇), Ethene (●), and *Dhc* (■).

predicting remediation performance with different inoculum amounts was verified during the field study (Schaefer et al., 2010a).

Also notable from the Fort Dix field experiment data is significant transport and apparent growth of *Dhc in situ*. These results are consistent with previously described field results and the results of laboratory studies as described above. *Dhc* levels in the actively dechlorinating treatment loops were in the range of 10^7–10^9 *Dhc*/L. The results from this demonstration indicate that there is no simple relationship between numbers and activity. Rather, the migration of injected *Dhc* cultures, and subsequent treatment of dissolved chlorinated ethenes, is highly dependent upon a relatively small fraction of the *Dhc* population that is mobile in the subsurface (because they readily detach from the solid surfaces). The rate at which these mobile *Dhc* increase is dependent upon the growth rate of the injected (and primarily immobile) *Dhc*, which do not migrate a substantial distance into the soil. In addition, based on the model simulations and sensitivity, the *Dhc* detachment and growth rate may be more important for determining overall bioaugmentation success than initial *Dhc* dosage or the utilization rate (Schaefer et al., 2010a; Torlapati et al., 2012).

CHAPTER 6

MICROBIAL MONITORING DURING BIOAUGMENTATION WITH *DEHALOCOCCOIDES*

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6.1 INTRODUCTION

The use of bioremediation has increased steadily as scientists and engineers have recognized the importance of microbes in the transformation and degradation of toxic compounds (Lebrón et al., 2011a). Over the past few decades, the use of bioremediation in aerobic and anaerobic subsurface environments has permitted closure of hundreds of impacted sites (USEPA, 2009; ESTCP, 2002; Regenesis, 2011). Guidance documents published by organizations such as the U.S. Environmental Protection Agency (USEPA), the Interstate Technology & Regulatory Council (ITRC), the Environmental Security Technology Certification Program (ESTCP) and the Strategic Environmental Research and Development Program (SERDP), involving some aspect of microbial degradation of recalcitrant compounds now number in the dozens. The widespread use of bioremediation has resulted in a broader and more diverse practitioner group, and so a more thorough understanding of the complexities, strengths, limitations and difficulties of this remedial option is required. In particular, accurately assessing the potential metabolic ability of microbial communities (and specific organisms) at contaminated sites provides an opportunity for significant cost- and efficiency-savings (on the order of tens to hundreds of thousands of dollars per site).

Traditional culture-based methods, useful in assessing microbial activities, are commonly used in environmental systems such as wastewater treatment plants or for determining the contribution of stormwater run-off into aquatic systems (e.g., total maximum daily loads). These techniques, while highly developed, are less useful when dealing with the subsurface environments encountered during environmental remediation. Methods such as direct plate counts and most-probable number (MPN) dilution series provide cell concentration estimates for somewhat generic growth conditions, such as the number of cells present that are capable of aerobic heterotrophic growth (e.g., growth using oxygen and organic compounds like glucose).

These methods, while useful in some instances, suffer from biases, low resolution and, in the case of *Dehalococccoides* (*Dhc*) and many other microbes relevant for bioremediation, an inability to selectively screen for these organisms based solely on culture conditions. Additional issues, such as the differential ability of individual *Dhc* strains for complete reductive dechlorination to ethene, further complicate the interpretation of these culture-based enumeration techniques. These methods are analogous to the use of historical photographs for delineating the entire extent of contamination at a site; while useful, on their own the photographs provide limited information on the specific contaminant type, its concentration and its distribution.

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Just as the examination of soil borings and groundwater hydrogeology augment historical document evaluation in determining the appropriateness of traditional treatment technologies such as pump-and-treat, the use of molecular biological tools (MBTs) now permit a more accurate assessment of potential bioremediation options.

Even more so than groundwater or soil sampling for chemical analyses, MBT use can suffer from careless planning, sloppy field sampling protocols and misinformed interpretation. The great power of MBTs is countered by their extreme sensitivity, which is capable of compounding any errors in sample collection and rendering the acquired data useless or (even worse) nonrepresentative of site conditions. Coupled with their relatively high cost when compared with traditional chemical analyses, MBTs often are neglected as useful and cost-effective tools for site characterization and remedial design. This chapter provides a practical introduction to the science behind several MBTs, as well as their value to remedial practitioners for site characterization, remedial technology screening and performance monitoring.

MBTs measure specific biomarkers (e.g., deoxyribonucleic acid [DNA] or ribonucleic acid [RNA] sequences, peptides, proteins or lipids) that are indicators of the ability to degrade specific contaminants. A comprehensive summary of MBT techniques, applications, issues, questions and associated research needs was presented in a SERDP/ESTCP workshop report (Stroo et al., 2006). Among all MBTs, application of nucleic acid-based tools is the most advanced, and specific tests for the presence and abundance of key dechlorinating (i.e., *Dhc* bacteria) as well as petroleum degrading bacteria are commercially available. Peptide, protein and lipid biomarkers also are useful for monitoring target bacterial populations; however, the techniques targeting these biomarkers are not as specific as nucleic acid-based tools for site assessment and bioremediation monitoring. Hence, the current focus is on nucleic acid-based tools.

Used in conjunction with contaminant and geochemical data, nucleic acid-based MBTs can be utilized to: (1) confirm the presence of naturally occurring bacterial populations capable of biodegradation; (2) identify the need for bioaugmentation at a site; and (3) monitor the performance of a bioremediation treatment. Quantitative real-time polymerase chain reaction (qPCR) techniques have been most widely used to quantify key bacteria and the functional genes (e.g., *tceA*, *vcrA* and *bvc*A) responsible for reductive dechlorination processes (Figure 6.1). Other nucleic acid-based techniques (e.g., fluorescent *in situ* hybridization [FISH], denaturing gradient gel electrophoresis [DGGE] and terminal restriction fragment length polymorphism [T-RFLP]) have been developed, but their use for site assessment and bioremediation monitoring is currently limited. Additional techniques such as reverse-transcriptase qPCR (RT-qPCR) of RNA, qPCR of messenger ribonucleic acid (mRNA) and compound specific isotope analysis (CSIA) have considerable promise but require further research for field-scale application (Stroo et al., 2006).

This chapter discusses monitoring strategies and sampling procedures for groundwater sampling, as well as data interpretation for qPCR, FISH and community profiling techniques. These procedures are currently applied to *Dhc* biomarker analysis in support of bioremediation at chlorinated solvent sites, but the protocols are universal and should be applicable for monitoring other groundwater bacterial populations of interest. The sampling process is generally the greatest source of variability in the use of MBTs (Stroo et al., 2006; Lebrón et al., 2011a; SERDP and ESTCP, 2005). Although not standardized like sampling procedures for chemical analysis, the procedure described herein is common in the remediation field and can be considered a current best practice (Lebrón et al., 2011b; Ritalahti et al., 2010).

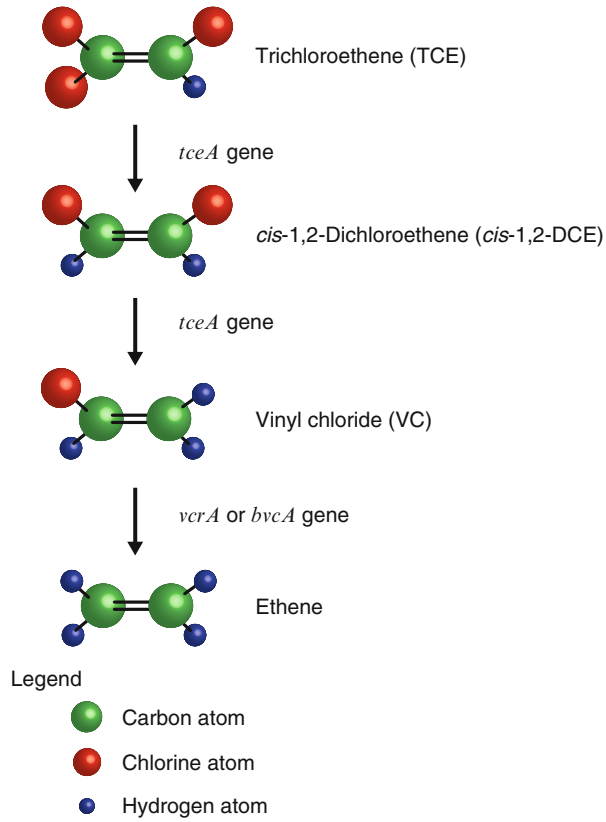

Figure 6.1. *Dehalococcoides* reductive dechlorinase genes.

6.2 MBTS FOR CHLORINATED ETHENE BIODEGRADATION

The key microbes with the ability to efficiently dechlorinate chlorinated ethenes, including dichloroethenes (DCEs) and vinyl chloride (VC), to ethene are *Dhc*, and a link between *Dhc* presence and complete dechlorination to ethene has been established (Ellis et al., 2000; He et al., 2003; Hendrickson et al., 2002; Lu et al., 2006). Bioremediation of contaminated aquifers using both biostimulation (i.e., electron donor additions) and bioaugmentation with *Dhc*-containing consortia or groundwater have been successfully implemented (Lendvay et al., 2003; Lookman et al., 2007; Major et al., 2002; Ritalahti et al., 2005).

Biomarkers have been developed to indicate both *Dhc* presence and abundance, and *Dhc*-specific prognostic and diagnostic tool kits have been designed (Cupples, 2008; Holmes et al., 2006; Löffler et al., 2000; Regeard et al., 2004; Ritalahti et al., 2005, 2006). Although the available tools have been rigorously tested in laboratory settings and are commercially available, the applications of these tools are still being refined. The correlation between *Dhc* biomarker concentrations and evidence of reductive dechlorination in the field has been explored (Lu et al., 2006; Da Silva and Alvarez, 2008; Lebrón et al., 2011a). Studies have identified a consistent relationship between *Dhc* cell titers and potential for complete dechlorination to ethene (Lebrón et al., 2011a).

6.3 DEVELOPING A MONITORING STRATEGY

Sampling aquifers for microbial and geochemical analyses in support of bioremediation applications faces several constraints and the ideal sampling regime with regard to sampling locations, sampling frequency and sample type is rarely achieved. For example, site access limitations, obstruction by existing infrastructure, lack of sampling wells, and limitations on well installation and sample collection may constrain sampling efforts. However, the importance of thoroughly considering monitoring strategies at the pre-design phases of a project cannot be overstated. A monitoring strategy for assessing geochemical and microbiological conditions before, during and after bioaugmentation can help to improve treatment efficiency and allow changes in site conditions to be better anticipated and contingencies implemented. The following sections provide an overview of key considerations for developing and implementing a monitoring strategy for bioaugmentation.

6.3.1 Defining Monitoring Objectives

Prior to sampling, the expected outcomes of the analysis should be clearly defined. Some key questions to consider when defining monitoring objectives include:

- What information should the analysis provide?
- Are data objectives qualitative (i.e., *Dhc* presence) or quantitative (i.e., *Dhc* abundance)?
- At what frequency will samples be collected?
- Will samples be collected from all monitoring wells or a subset?

6.3.2 Temporal Considerations

Dhc cell titer data are valuable throughout the remedial investigation and feasibility study process, and samples for *Dhc* analysis should certainly be collected prior to the design of a bioremediation system (Stroo et al., 2006). These data, in conjunction with chlorinated ethene and geochemical information, are crucial in deciding whether to proceed with bioaugmentation. A one-time sampling event is sufficient for pre-design purposes and this may serve as the baseline data for subsequent sampling events. Following bioaugmentation, *Dhc* analyses should be conducted for at least 12–18 months, or until the system performance has stabilized. Temporal *Dhc* data from a treated aquifer zone are critical for evaluating and managing the system's performance. Monitoring strategies for assessing *Dhc* populations following aquifer treatment are dependent upon site-specific conditions; however, a flexible strategy that can be adjusted based on the monitoring results (e.g., changes in geochemistry or *Dhc* populations), is recommended. As a general rule of thumb, the period of time between monitoring events should be relatively short immediately following bioaugmentation (a few weeks), but may increase as changes in the geochemistry and microbial activity decrease.

6.3.3 Selection of Sampling Wells

For practical reasons, MBT sampling focuses on groundwater. Solid aquifer samples are typically not included in routine analyses, due to the cost and heterogeneity of aquifer core samples. The distribution between solid and aqueous phases in different aquifer matrices is poorly understood for most microbial populations. Until further information is available

regarding the phase distribution of bacterial populations of interest, sampling of aquifer solids is optional.

Groundwater samples for *Dhc* analysis should be collected from the source area(s) and downgradient plume locations where biodegradation products may have been observed or are anticipated, and where geochemical conditions are favorable for anaerobic bioremediation. Wells should produce sufficient water for adequate purging and appropriate sample quantities. Well screen depths and lengths should be considered when selecting a sampling location. Utilize wells installed for establishing vertical profiles of contaminant (i.e., chlorinated ethenes) concentrations to establish a similar profile for *Dhc* distribution. Discrete sampling zones in intervals with dechlorination daughter products (i.e., DCEs, VC) are preferred for *Dhc* analysis. However, sampling in zones without dechlorination daughter products can be useful to establish baseline conditions prior to bioaugmentation. Wells with extended screens used for injection of substrates (e.g., electron donor) or bioaugmentation culture may be considered for sampling immediately following bioaugmentation to confirm the presence and abundance of the injected culture, but should not be used for monitoring performance of the bioremediation system because there may be artificially elevated concentrations of *Dhc* surrounding these injection points.

6.4 MBT SAMPLING METHODS

6.4.1 General Sampling Considerations

Groundwater sampling methods for microbial analysis typically are the same as those established for evaluating groundwater chemistry. Selecting a sampling method for MBT analyses depends on a number of site-specific conditions including sampling depth, well construction, and aquifer permeability, as well as historic site data and regulatory requirements. Groundwater sampling for subsequent biological analysis can be done using a variety of purging and sampling devices, and applicable procedures have been described (reviewed in Yeskis and Zavala, 2002). The ultimate goal of these procedures, as with sampling approaches for chemical analyses, is to generate a sample representative of the formation groundwater in the vicinity of the well.

Geochemical and contaminant stratification within a screened interval is affected by seasonal changes (e.g., rain events, temperature changes) and can lead to variations in biomarker abundance, which in turn may confound data interpretation (Stroo et al., 2006). Such environmental influences on sample quality should be considered when developing a sampling strategy and when evaluating MBT data. Appropriate cover is recommended to protect sample equipment, samples and field personnel from influences such as direct sunlight or rain.

This section describes two different procedures for sample collection following well preparation (e.g., purging and surging). The first procedure relies on collecting groundwater for off-site laboratory filtration and biomass collection, whereas the second involves on-site collection of biomass by field filtration or retrievable media devices (RMDs). For analyzing DNA biomarkers, each of these approaches provide valuable information (Ritalahti et al., 2010); however, on-site filtration using Sterivex™ cartridges has several advantages. The cartridges (Figure 6.2) are easy to ship, it is easy to add nucleic acid preservatives to stabilize biomarkers, and depending on the aquifer characteristics, larger volumes of groundwater can be collected and filtered. In addition, bottle breakage and disposal of contaminated groundwater in the analytical laboratory are avoided. The primary advantage of laboratory filtration is the reduced effort in the field.

Figure 6.2. Sterivex™ filter used for groundwater sampling for MBT analysis (photo courtesy of MO BIO Laboratories, Carlsbad, CA).

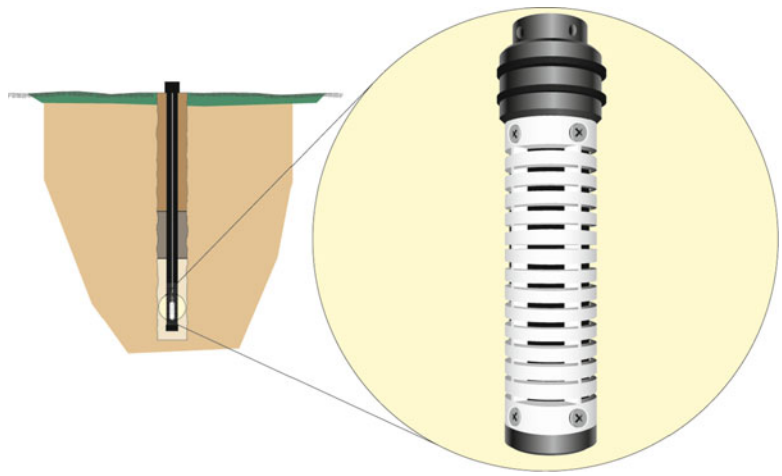

Figure 6.3. In-well retrievable media device (Bio-Trap®) used for groundwater sampling for MBT analysis (courtesy of Microbial Insights, Rockford, TN).

RMDs facilitate the colonization of microorganisms onto a matrix that can be retrieved for off-site laboratory analysis (Figure 6.3). The advantages of RMDs over groundwater collection include ease of storage and transport, and the potential for RMD arrays to provide a more accurate representation of the microbial community surrounding a sampling well (Peacock et al., 2004). RMDs may be more appropriate for analysis of microbes that live as biofilms. RMDs supplemented with substrates also can serve as *in situ* microcosms. However, due to biases resulting from the physical and chemical composition of the matrix, and the fact that the devices are only exposed to standing water in wells, there is significant uncertainty regarding

how well RMD data represent the natural microbial populations. Ultimately, the selection of sample collection procedure is dependent on site-specific data objectives.

Traditional "well volume" groundwater sampling methods involve bailers or high speed pumps (>500 mL/min) to purge 3–5 well casing volumes prior to collecting groundwater samples. Alternatively, low-flow purging methods (100–500 mL/min) with a peristaltic or submersible bladder pump are generally recommended to collect groundwater samples for volatile organic compounds (VOCs) and/or geochemical analysis (Puls and Barcelona, 1996). Whether using traditional "well volume" methods or "low-flow" methods, it is imperative that the same protocol be applied for every sampling event and for each sampling location in an event. These approaches also apply to microbial (e.g., *Dhc*) sampling, which should occur after geochemical parameters have stabilized.

Surging the monitoring well with a surge block or disposable bailer can increase particulate matter in the sample and recovery of associated (i.e., attached) biomass. However, if field filtration is used for on-site biomass collection, excess sediment in the filter material can impact the DNA extraction process in the laboratory and inhibit subsequent analytical techniques. Additionally, the presence of excess sediment can restrict the flow of groundwater through the filter and may clog a filter prior to collection of adequate sample volume.

Low-flow purging and sampling methods typically can be completed in 1–2 h per well, and field filtration using Sterivex™ cartridges will add approximately 30 min per well. Volume requirements for MBT analysis of a single sample are generally about 1 liter (L) for laboratory filtration and 1–3 L for field filtration.

Since the stability of microbial biomarkers is of concern, it is important to maintain sample integrity through all of the sample handling and shipping procedures. Aseptic techniques should be employed to the extent possible when handling groundwater samples destined for biological analysis. Preservation of samples through use of chemical additives can help minimize chemical and biological changes within a sample; however, current laboratory-recommended procedures typically specify no chemical preservative additions. Collected samples (i.e., bottles filled with groundwater and/or Sterivex™ cartridges) should be maintained at 4 degrees Celsius (°C) and shipped, with appropriate packaging to prevent breakage, for overnight delivery to the analytical laboratory.

Innovative sampling techniques are being developed, such as cryogenic collection of "complete" subsurface core samples (i.e., water plus aquifer solids). By collecting a complete core with direct push technology, this technique promises to improve the accuracy of MBT data for planktonic and attached microbes and provide an opportunity to evaluate small scale features in subsurface samples (Johnson, 2007).

Microbial surrogates have been developed and tested for use as quality control methods. Such surrogates could be added to samples at the time of sampling, or prior to nucleic acid extraction in the field or laboratory (Lebrón et al., 2008). Adding appropriate surrogates can allow for quantitative assessments of potential biases throughout the process from sampling, shipping, storage and analysis. Such biases may include microbial growth due to prolonged storage at warm temperatures prior to analysis.

6.4.2 Groundwater Sampling Protocol

The following protocol provides a step-by-step approach that can be used for groundwater sampling during bioaugmentation monitoring (also see Figure 6.4). Methods may vary according to site-specific conditions; however, it is crucial that the sampling protocol for a given well (or site) be defined and maintained for the duration of the monitoring efforts. Changes to the protocol during monitoring will complicate data interpretation and should be avoided.

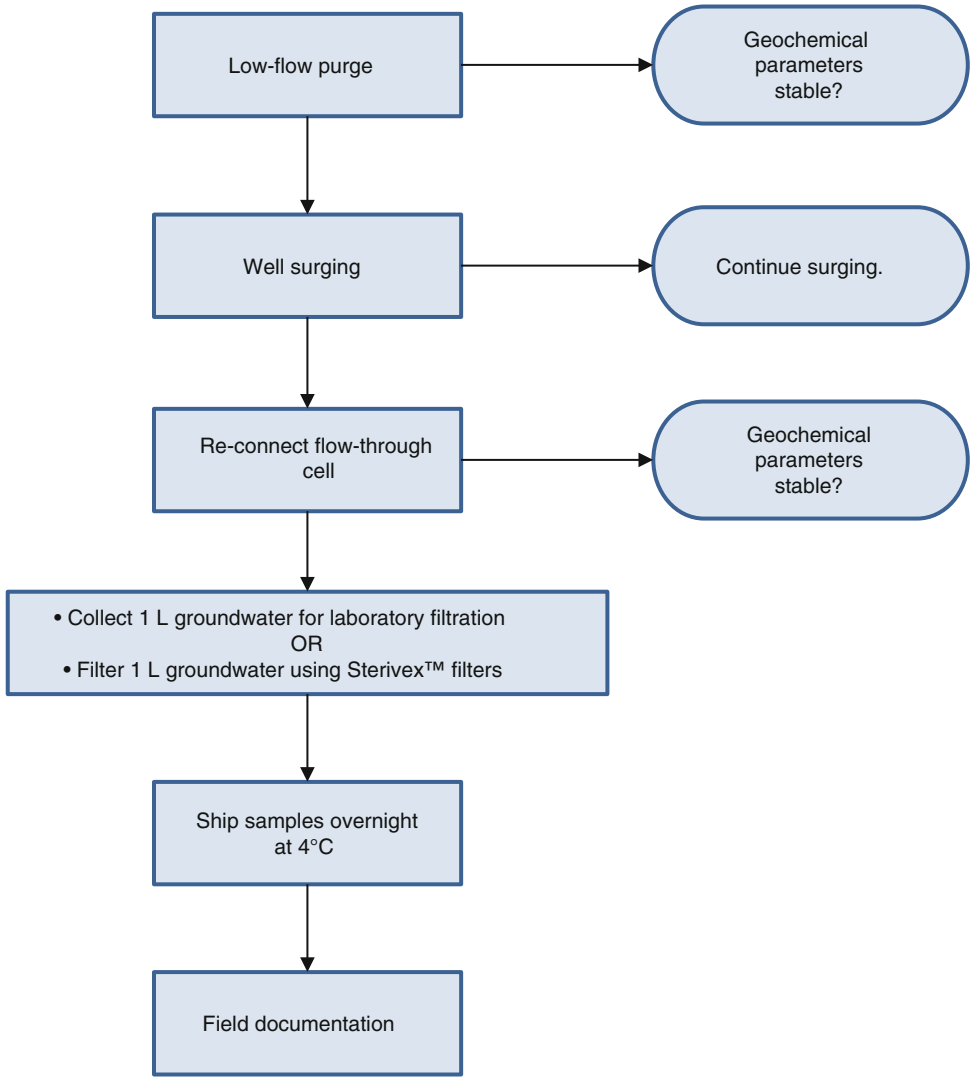

Figure 6.4. Flowchart of groundwater sampling for MBT analysis.

1. Connect a flow-through cell and hand held multiparameter instrument to a low-flow pump (e.g., peristaltic pump) and begin purging. Record the start time and field measurements for pH, oxidation-reduction potential (ORP), specific conductance, temperature, dissolved oxygen and turbidity.
2. Disconnect the flow-through cell after parameter stabilization. Collect samples as appropriate for VOC, natural attenuation or other project-required parameters. Continue to purge the well after collection of samples for chemical/geochemical analyses is complete. At this time, samples for MBT analysis can be collected. However, based on site-specific planning documents and knowledge of historical turbidity levels, well surging may or may not be appropriate prior to MBT sample collection. If surging is appropriate, proceed to step 3. If surging is not needed, proceed to step 5 (a) or (b).
3. Lower a disposable polyethylene bailer into the well to the midpoint of the screen and move the bailer up and down within the water column to surge the well. It is important

to agitate at the midpoint of the well screen to avoid stirring up sediment in the sump and/or at the bottom of the well.

4. While continuing to surge the well with the bailer, reconnect the flow-through cell and record the field measurements for pH, ORP, specific conductance, temperature, dissolved oxygen and turbidity until stabilization of geochemical parameters is achieved.
5. Disconnect the flow-through cell but continue to surge the well with the bailer through the sample collection process.
   (a) In order to sample groundwater for off-site biomass collection, fill the appropriate sample containers (e.g., clean, sterile 1-L amber glass or plastic bottles with Teflon©-lined caps, no preservatives added) directly from the effluent end of the pump. The bottles should be filled with groundwater from tubing that has already been used to withdraw one to two well volumes of groundwater to ensure that a representative sample of aquifer water, rather than well water, is collected. The bottles should be filled to capacity (i.e., minimal headspace) to minimize air exposure. Apply the Teflon©-lined caps and ensure a tight seal.
   (b) For on-site biomass collection, use sterile Sterivex™-GP 0.22-micrometer (μm) membrane filter cartridges. Attach ¼–5/16-inch polyethylene tubing to the inlet of the Sterivex™ cartridge and secure with a clamp. Place the cartridge over a graduated cylinder that can accurately measure the volume of water filtered. Ideally, 0.5–2 L of water should be collected; however, depending on groundwater characteristics, up to 10 L may be filtered, and as little as 10 mL may be sufficient for subsequent biomarker analysis. Close the inlet and the outlet of the Sterivex™ cartridge with male and female Luer-Lock plugs, respectively. If needed, replicate samples should be collected consecutively without flow interruption. Record the volume of filtered groundwater on the chain-of-custody form and on the Sterivex™ cartridge barrel with a black permanent marker, and transfer each capped Sterivex™ cartridge to a separate, new 50 mL Falcon conical plastic tube.
6. Immediately after sampling, transfer samples to coolers with ice packs and/or blue ice (in Ziploc© bags) to ensure refrigeration at 4°C until arrival at the analytical laboratory. Falcon tubes (50 mL) or equivalent containers are used to protect Sterivex™ cartridges during shipping and storage. Use additional packing material, as appropriate, to prevent movement and breakage during shipping, and place each sample in separate Ziploc© plastic bags. The coolers with samples should be shipped for next day delivery to the analytical laboratory. It is important to notify analytical laboratories when samples are shipped to avoid delays in handling and processing that could affect biomarker integrity.
7. Immediately following sample collection, record the sampling well location, the well name, notes on individual samples (e.g., the volume of water that passed through each Sterivex™ cartridge), date and time of sampling, and the type of analyses requested. Standard chain-of-custody forms must accompany each sample shipment.

A variety of analytical techniques can be used to obtain qualitative and quantitative microbiological data for environmental samples. The monitoring objectives for a particular sample (or site) will largely dictate the choice of analytical approach. Descriptions of these techniques are provided in the following section.

6.5 QUANTITATIVE-PCR

qPCR is a powerful molecular tool used to determine the concentration of specific genes of interest in environmental samples. Both qPCR and its predecessor, PCR, are now routinely used in laboratory analyses of DNA extracted from a wide variety of live or dead organisms. Fields as diverse as environmental remediation, forensics, medicine, paleontology and archaeology now use data acquired through PCR on a regular basis. These techniques also are commonly referenced in film, television programming, litigation trials and print media. While the origins of the PCR process extend back into the early 1970s, with qPCR developed in the 1990s, both still are undergoing rapid advancement.

Before any analysis and interpretation of qPCR data can be performed, an understanding of the basic principles and limitations of PCR is required. The following section introduces common terminology, provides a summary of the underlying science and a general example of qPCR use in environmental remediation. This section focuses solely on the use of qPCR for microbial DNA analysis, although the science and techniques are generally the same irrespective of the source of DNA.

6.5.1 Description and General Methodology

PCR techniques use one of several purified microbial enzymes to produce large numbers of nearly identical copies of specific pieces of DNA (Figure 6.5). These enzymes are known

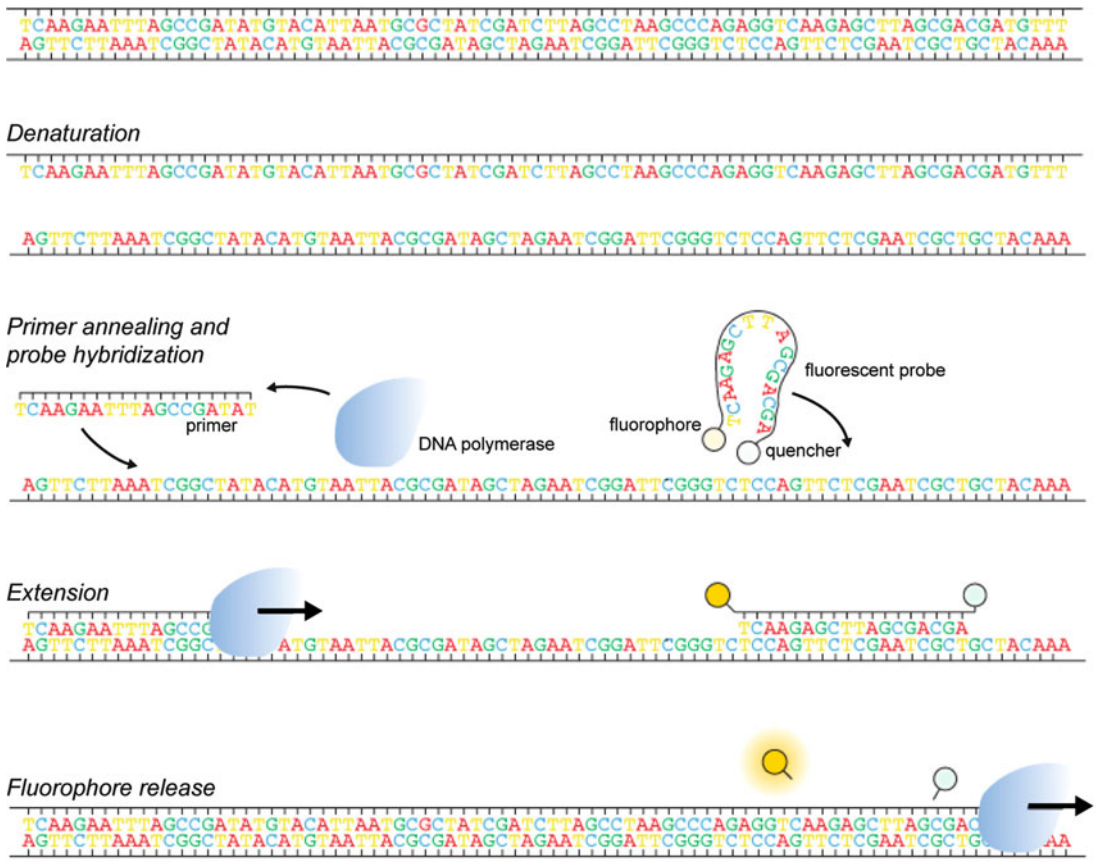

Figure 6.5. Schematic diagram of qPCR.

generally as DNA polymerases and they function during replication of microbial DNA. Replication using DNA polymerases occurs rapidly and reasonably accurately, with error rates less than 1 in 10,000 basepairs. In addition to the DNA polymerase, PCR requires small fragments of DNA called oligonucleotide primers (or simply just "primers") in order to target specific DNA sections of interest within a microbial genome. These primers are complementary to short sections of DNA, are usually 20–30 basepairs in length, and used in pairs to amplify genes of interest. These genes range from species-specific indicators, such as 16S ribosomal RNA (rRNA) genes (found in all microbes), to functional genes such as those responsible for specific metabolic reactions like ammonia-oxidation (e.g., the *amoA* gene) or VC reduction (e.g., *vcrA*).

Some genes, like the 16S rRNA, contain highly conserved portions of DNA (i.e., common among a large group of different microbes) interspersed with highly variable sections of DNA. The conserved regions allow the design of primers to amplify the DNA, while the variable regions permit molecular analyses useful in identifying closely related microbes. Genes such as *vcrA*, which codes for the catalytic subunit of the enzyme responsible for the reductive dechlorination of vinyl chloride, are less conserved, but are found in only a limited number of microorganisms. As described below, this conserved and variable nature of various DNA sections may affect data interpretation, especially when analyzing environmental samples.

PCR consists of a number of cycles (commonly 25–50), each composed of three steps, with each step occurring at a different temperature. A device named a thermocycler is used to automatically adjust the temperatures required for each step. The three steps are: (1) an initial denaturing step, (2) an annealing step, and (3) an extension step. The denaturing step uses elevated temperatures to separate double-stranded DNA into two single-stranded pieces. Once the DNA is denatured, the temperature is rapidly decreased and the two primers anneal along complementary matches on the single-stranded DNA. The primers guide the DNA polymerase towards the targeted sequence, permitting the beginning of DNA replication, which occurs in the extension step. During the extension step, the temperature is increased and the DNA polymerase adds complementary nucleotides, which are added as part of the reaction mixture, based upon the sequence of the original DNA strand. These newly synthesized fragments serve as the DNA templates for the next round of reactions. At the end of each three-step cycle, the number of synthesized DNA fragments is double the previous quantity, producing extremely large working concentrations of DNA from relatively small starting concentrations. Thus, PCR is an extremely sensitive technique for detecting small numbers of genes.

qPCR functions in a manner similar to PCR, but through the use of fluorescent probes, qPCR allows quantification of the amplified DNA sequence. A qPCR probe is similar to the primers described above, except that it contains a fluorescent reporter at one end and a quencher at the opposite end. As with the primers, this probe binds along complementary DNA during the annealing step. When the fluorescent reporter and the quencher are in close proximity, such as at the probe ends, no signal is emitted. However, as the DNA polymerase adds nucleotides and moves along the DNA strand during replication, the fluorescent reporter and quencher are cleaved from the probe and separate. The reporter subsequently emits a fluorescent signal, which is detected and quantified in the thermocycler at the end of every cycle. A larger fluorescent signal corresponds to a greater number of cleaved fluorescent reporters. Since one probe containing a single fluorescent reporter hybridizes with a single copy of DNA, the resulting signal strength after the reporter releases from the probe is directly proportional to the number of DNA copies present at the end of a cycle. A comparison of the fluorescent signal strength against standard curves permits the quantification of specific DNA sequences within complex samples.

Over the course of the qPCR cycles, the fluorescent signal strength typically appears sigmoidal, with an exponential increase, followed by a linear increase, an exponential decrease and finally a plateau. A point is selected from this curve where the fluorescent signal strength is

greater than the background signal intensity – above this threshold the signal intensity increases exponentially for a period. The cycle number associated with this intensity threshold is referred to as the "cycle threshold" (C_T) and is commonly determined by finding the maximum for a plot of the derivative of the signal intensity versus cycle number (Figure 6.6). Once determined, the C_T is compared against a set of standard curves and a gene concentration is then calculated. As a result of increases in sensitivity, the lower quantification limit of a particular target gene is approximately 100–1,000 copies per L. Using traditional PCR, the sensitivity of detection is lower, at 1–10 copies per L; however, this number is highly variable and should only be used as an estimate. Conventional PCR is not a quantitative method.

6.5.2 Standards

Since environmental samples are compared against a standard curve, the choice of DNA used for qPCR standard curves greatly affects any results. An appropriate reference DNA sample or target gene(s) must be selected based on the qPCR analysis performed. The kinetic reactions resulting from the fluctuating temperatures used in PCR differ depending on such factors as DNA nucleotide content and the length of the targeted gene. Since PCR exponentially increases the number of DNA copies, small biases in reaction kinetics can produce large differences in target gene concentrations. Thus, if the DNA (or target gene) used during generation of standard curves differs markedly from the target gene being analyzed in environmental samples with respect to nucleotide content or gene length, the C_T values corresponding with a given concentration may not accurately represent target gene copy numbers.

Many targeted genes of interest are present only as a single copy per genome, thus a 1:1 ratio exists between gene and cell concentrations. However, in some instances, microbes contain greater than 10 copies of a single gene per genome, and this must be accounted for in either the standard curve preparation or the comparison of sample C_T values with the standard curve. In environmental applications of qPCR for chlorinated ethene bioremediation, remediation practitioners should not encounter this problem, as known *Dhc* biomarkers are present as a single copy per genome. However, as both the use of molecular biological tools and the understanding of environmental microbiology increases, attention should be paid to this point to ensure analyses are not underestimating actual cell concentrations.

6.5.3 Limitations

The tremendous power and sensitivity of qPCR (and PCR) sometimes conceal its limitations. Any qPCR analysis provides concentration data only on the DNA in the environment from which it was extracted. Additionally, qPCR analysis of DNA only indicates the presence and abundance of a particular gene of interest. Variances in sampling strategies may result in DNA obtained from an unrepresentative portion of the subsurface microbial community. For example, microbes survive both as attached biofilms and as free-swimming, planktonic organisms. While improvements in groundwater sampling permit the relative ease in collection of a sample containing planktonic microbes (as described above), nontrivial complexities exist when obtaining samples of microbes growing as biofilms.

Additionally, DNA obtained from environmental samples may contain compounds (e.g., humics) which are inhibitory to PCR and qPCR, resulting in decreased sensitivity and inaccurate target gene concentration data. Inhibition can result from interference of the primers and/or probe annealing with the targeted DNA, or compounds may continue quenching the fluorescent reporter even after its release during the elongation step. Improved DNA extraction and purification techniques have recently been developed; however, it is important to recognize that PCR inhibition does occur, and to attempt to minimize the presence of inhibitory compounds.

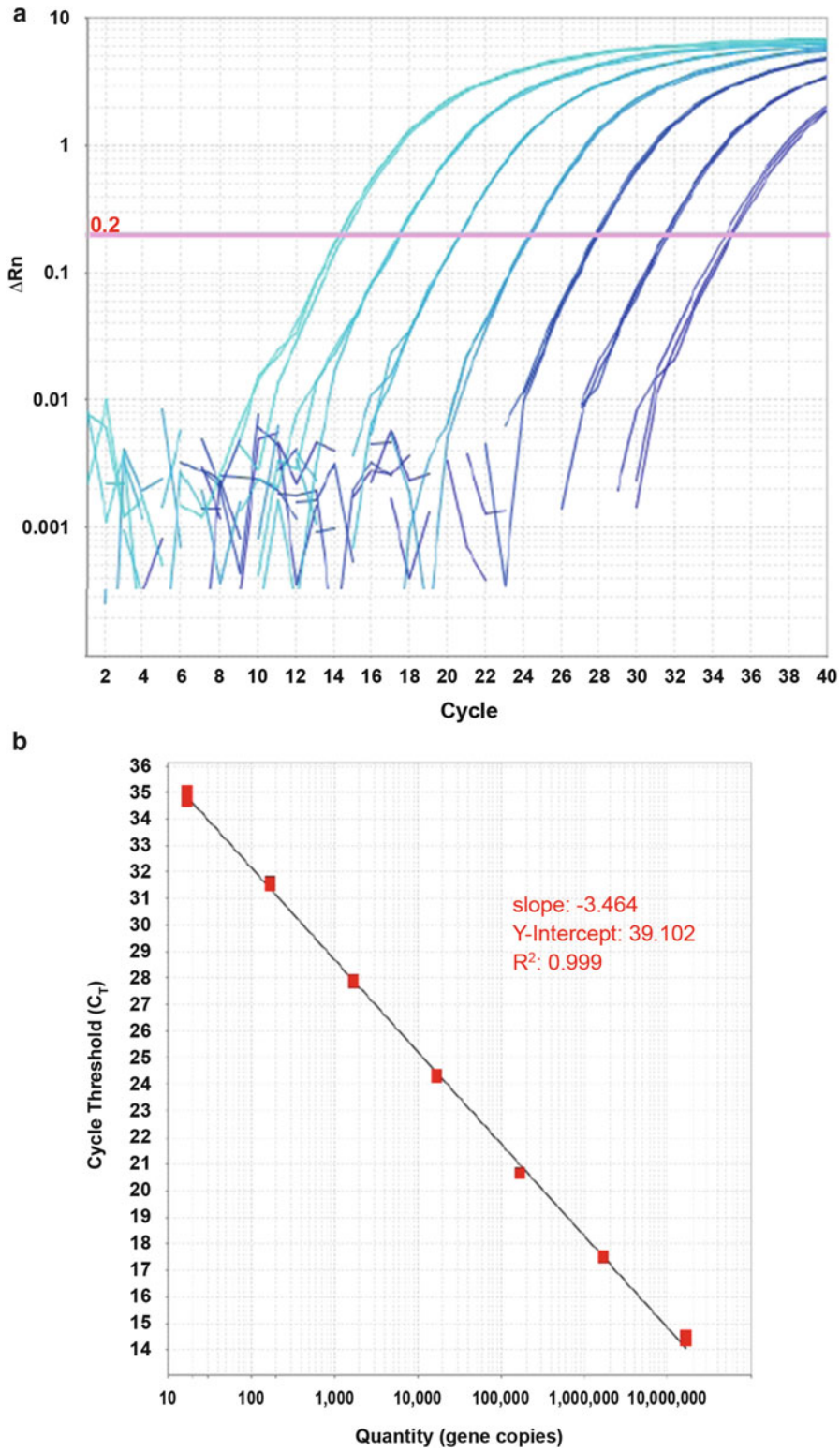

Figure 6.6. (a) Increase in fluorescence intensity (ΔRn) at each cycle number. The cycle threshold is indicated by the line at 0.2. (b) The cycle at which the fluorescence intensity crosses the cycle threshold is plotted against the gene copies for each standard, to generate a standard curve for use in quantification of a gene target. Courtesy of Kirsti Ritalahti and Frank Löffler, University of Tennessee.

Even when DNA extraction occurs from a representative environmental source and contains no inhibitory compounds, qPCR requires a solid understanding of the distribution and diversity of any genes being measured. Poorly designed or selected primers may result in qPCR-determined gene concentrations not reflective of actual environmental conditions. Appropriate selection of target genes must reflect whether they are conserved, variable, or a combination of both. Measuring a broadly distributed target gene may result in cellular quantification errors resulting from differing gene copies being present in differing microbes. Careful primer development, target gene selection and use of standard curve quantification methods can overcome this limitation. Commercially available and well validated primers and probes can be readily attained, specifically for analysis of *Dhc*-related genes.

PCR bias is a commonly reported limitation of these techniques (Reysenbach et al., 1992) and refers to the preferential amplification of one target sequence over another. For example, in a groundwater sample containing equal concentrations of different species of bacteria, PCR may preferentially amplify the 16S rRNA gene of one species, leading to an overestimate of the relative abundance of that species. These preferential amplifications may be due to the nucleotide content, secondary structure or even degradation of the target sequences. The effect of these biases may be reduced by performing replicate PCR analysis on multiple DNA extracts and pooling the replicates. Practical solutions to PCR bias under high biomass and high copy-number amplifications also are described elsewhere (Polz and Cavanaugh, 1998).

Finally, it also must be remembered that the presence of a given gene does not guarantee activity of that gene, but instead informs of its *potential* activity. In response to stimuli, DNA is transcribed to messenger RNA that is then translated to a protein (enzyme). Many environmental influences may result in inhibition or cessation of messenger RNA synthesis, so that even though a gene is present in a microbial genome, it may not be transcribed or translated. In these instances, the use of other molecular techniques capable of measuring actual activity may be required. RT-qPCR is one such technique, which was developed to measure gene expression. Assays of messenger RNA for *Dhc* activity should be interpreted with caution, as transcript abundance may not correlate with activity (Fletcher et al., 2011).

The use of 16S rRNA genes as qPCR target genes carries with it an explicit assumption that all members of the target population exhibit the same potential metabolic activity. As discussed in the general example below, this is not always the case and care must be taken when choosing the appropriate target gene of interest. Quantification of functional gene targets, in addition to rRNA genes, can help to provide a more complete description for the metabolic capabilities of a microbial community.

6.5.4 *Dhc* Analysis

Bioaugmentation of aquifers impacted by chlorinated ethenes is now commonly accepted by regulatory agencies and practiced by remediation experts. Bioaugmentation cultures containing *Dhc* species with the ability to respire chlorinated ethenes are commercially available. Using qPCR, remedial design engineers may assess the need (or lack thereof) for augmentation of native microbial populations with *Dhc*. Typically, groundwater samples are collected for *Dhc* analysis, followed by extraction and purification of microbial DNA. The extracted DNA undergoes qPCR analysis using primers specific for the 16S rRNA gene of the genus *Dhc*. The genomes of all known *Dhc* species (like many microbes) contain a single copy of this 16S rRNA gene that encodes for 16S rRNA, and ultimately relates to a protein subunit involved in biosynthetic reactions. Thus, one copy of this gene corresponds with a single *Dhc* cell and the greater number of this gene present in a sample, the higher the likelihood reductive dechlorination will occur.

Table 6.1. *Dehalococcoides* qPCR Analysis Rules of Thumb

Dhc 16S rRNA (gene copies per L)	Interpretation
10^3 or lower	Suboptimal *Dhc* to sustain dechlorination rates
10^4–10^6	May sustain appreciable dechlorination rates
10^7 or greater	Usually associated with high rates of dechlorination and ethene production

There is some uncertainty regarding the numbers of *Dhc* that are needed for acceptable rates of dechlorination, or that would indicate complete biodegradation to ethene is occurring. However, some rules of thumb have been developed for interpreting *Dhc* results (Lebrón et al., 2011a, b). In general, *Dhc* concentrations less than 10^3 per L indicate suboptimal concentrations of *Dhc* cells in groundwater, and such levels may not be capable of sustaining complete dechlorination (van der Zaan et al., 2010; Lebrón et al., 2011a). Concentrations of 10^4–10^6 per L may provide acceptable dechlorination rates, while concentrations greater than 10^6 per L often achieve high rates suitable for effective bioremediation (van der Zaan et al., 2010; Lu et al., 2006, 2009) and are considered the threshold concentration. These rules of thumb for *Dhc* data interpretation also are shown in Table 6.1.

As noted above, the limitations of qPCR must be considered when analyzing data obtained from environmental samples. For example, even when sufficient numbers of *Dhc* cells are present, complete reductive dechlorination may not occur, because the *Dhc* group contains a diverse number of species and strains, with some capable of transforming TCE through to ethene, and others capable of transformation only to DCE or VC. Research studies discovered a correlation between particular genes (named "dehalogenases") and the varied ability to transform these compounds. One example is the *vcrA* gene mentioned in the introduction, which is responsible for the final dehalogenation step reducing VC to ethene, and is not found in the genomes of all known *Dhc*. Although qPCR analysis of the *Dhc* 16S rRNA gene may indicate greater than 10^8 cells per L (a quantity likely capable of sustained and vigorous reductive dechlorination), if a sufficient quantity of these cells are *Dhc* species lacking the *vcrA* gene, VC will accumulate. Thus, analysis using qPCR for determining the concentration of the *vcrA* gene (and/or other VC reductase genes) may be required.

6.5.5 Conclusion

The development of qPCR as a viable molecular biological tool for environmental analyses provides a tremendous opportunity for collecting data in support of bioaugmentation design, implementation and performance monitoring. However, as with chemical data obtained from the environment, careful planning and a consistent approach are critical to ensure an accurate assessment of site conditions is made. This is especially true given the nature of qPCR, where sampling variability may result in reported concentrations orders of magnitude different from actual concentrations.

6.6 FLUORESCENT *IN SITU* HYBRIDIZATION

6.6.1 Introduction

Fluorescent *in situ* hybridization (FISH) is a molecular technique used to detect genes within microbes of interest in environmental samples, including *Dhc* (Madigan et al., 2003; Yang and Zeyer, 2003). Through the use of fluorescently-labeled molecular probes, microbial

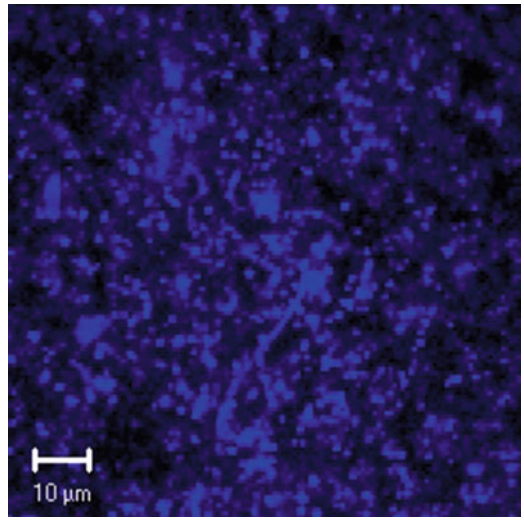

Figure 6.7. Determination of target cell concentrations using fluorescent *in situ* hybridization (FISH) (courtesy of Natuschka Lee, Technische Universität München, Germany).

cells can be screened using fluorescent microscopy for genes of interest (Figure 6.7). This target specificity is the principal difference between FISH and general fluorescent staining using DNA intercalators. Although more labor intensive than molecular techniques such as qPCR, FISH permits investigation of specific target genes or activities in their native environment without the need for DNA extraction or amplification. FISH originally was developed as a tool for clinical diagnostics in medical research but it has been routinely used in environmental microbiology since the 1990s. The following section provides a brief description of FISH in the context of environmental microbiology and bioremediation.

6.6.2 Description and General Methodology

FISH analysis uses fluorescently-labeled molecular probes in conjunction with a series of techniques permitting transmittance of the probes into cells. Direct examination of the FISH-labeled cells using fluorescent microscopy permits the determination of target cell concentrations. Much like the qPCR probes described earlier, FISH probes are short (approximately 20–30 nucleotides), complementary pieces of DNA that contain a fluorophore (fluorescent molecule) at one end. They differ from qPCR fluorescent probes in two key aspects: (1) FISH probes lack the quenching molecules at the terminal end, and thus will continually fluoresce, and (2) FISH probes used in environmental microbiology generally bind with RNA, not DNA. The use of RNA (versus DNA) as a target binding-site stems from the higher RNA concentrations present within a cell. These higher concentrations result in greater signal intensity as a larger number of probes are able to bind with target sites. The ability to hybridize a sample with multiple probes (each of which with a specific fluorescence) targeting different sequences, also adds to the power of this technique.

FISH probes generally fall into two categories: group-specific and functional probes. Group-specific probes bind to rRNA with specificities ranging from domain- (e.g., bacterial and archaeal) to strain-level (e.g., *Dhc* strain BAV1 and *Dhc* strain VS). These probes are useful in delineating the types of microbes present, such as determining the *Dhc* concentration in a groundwater sample. Functional probes bind to RNA coding for enzymes involved in specific metabolic processes, such as sulfate-reduction or ammonia-oxidation. The use of functional

probes permits evaluation of specific metabolic features that may be shared by unrelated organisms, where group-specific probes cannot capture the breadth of metabolic diversity. In general, RNA coding for specific metabolic processes is present at lower concentrations than ribosomal RNA. These lower concentrations result in decreased fluorescent signal intensity and often complicate functional FISH analyses.

The use of FISH for direct examination via fluorescent microscopy requires several steps: (1) fixation of cells on a filter or microscope slide, (2) hybridization of probes with target genes, (3) washing of excess probe material, and (4) microscopic evaluation. Slight variations in the specifics associated with these steps occur depending if the analysis is performed on planktonic or biofilm communities, or if concentration of cells (by filtration or centrifugation) from larger sample volumes is required. However, the general principles and processes remain consistent regardless of the origin of the sample. The initial fixation step utilizes a number of chemicals for attaching the cells onto a glass slide, then drying them in place. Commonly, dilute paraformaldehyde is used for "fixing" the cells to the slide, followed by baths in successively higher concentrations of ethanol for the drying step. This process kills the cells, but preserves the enzymes, RNA and DNA, thus providing a "snapshot" of current metabolic activity. After fixation, the cell walls and membranes become more permeable and diffusion of small molecules (such as FISH probes) becomes possible. During the hybridization step, the cells are bathed in a solution containing a buffering reagent, the probe (or mixture of several different probes), and variable concentrations of formamide. Formamide affects the stability of probe-RNA duplexes and allows incubation at elevated temperatures (35–50°C), thereby improving the hybridization reaction kinetics. The formamide concentration used depends upon the nucleotide composition of the probe and the probe hybridization stringency desired. During this hybridization step, FISH probes diffuse through the cell membrane and bind with complementary sections of RNA. The temperatures, although elevated, are not significant enough to denature DNA, thus limited binding occurs. Nonspecific fluorescent counter-stains that intercalate within DNA, such as 4′,6-diamidino-2-phenylindole (DAPI), are sometimes added during this step to provide a method of determining total cell numbers versus those with the targeted gene of interest. After hybridization and incubation, the cells undergo a washing step using a dilute salt solution to remove unbound probe and disrupt any nonspecific hybridization. Varying the salt concentration and wash temperature also provides another control over the stringency of hybridization.

Examination of the fluorescently labeled cells requires a microscope equipped with an ultraviolet bulb or lasers emitting specific wavelengths. Since different fluorophores adsorb and emit at a variety of wavelengths, multiple probes may be used on a single sample and a variety of target cells screened at once (e.g., *Dhc* cells and sulfate-reducing microbes). Cell concentrations are determined through manual or automated cell counting, then back-calculated to a cells per unit volume (or mass, in the case of biofilms). Thus, the native concentration of cells containing the targeted gene of interest can be estimated for environmental samples.

6.6.3 Limitations

The limitations of FISH reflect those discussed for qPCR in the previous section for many of the same reasons. Sample selection must be representative, although since no amplification step occurs, this is less critical for FISH than for qPCR. Instead, probe selection and an understanding of probe specificity becomes even more crucial when using FISH. However, sample variability (e.g., differing matrices) can result in higher natural background fluorescence, which makes the sensitivity of FISH difficult to assess. Probe-RNA hybridization

depends not only on the stringencies described above, but also on the location of targeted sequences within the RNA strand. In fact, some probes theoretically hybridize based on sequence comparisons with target RNA, but in reality, poor signal intensities result from such physical problems as steric hindrance; the probe simply cannot reach the targeted region within the folded RNA. Additionally, hybridization of multiple different probes within a single cell can be problematic. Thus, FISH allows determination of cell identity (e.g., a *Dhc*) using group-specific probes, or metabolic activity (e.g., sulfate-reduction) using functional probes, but rarely both at the same time.

Non-specific and mismatched binding present problems when using FISH to analyze environmental samples. Non-specific binding results from probes adsorbing or hybridizing with background material such as humics or organic debris. Since non-specific binding generally appears visually different from fluorescently-labeled cells, careful visual observation by personnel skilled in fluorescent microscopy can usually distinguish and account for this. Discriminating between cells labeled as a result of mismatched binding is much more difficult. Mismatched binding occurs when probes hybridize with less than perfectly complementary RNA sequences. While software has been developed to predict the overall quality of a probe design and to estimate the possibility of mismatches, the enormous genetic variability within microbes almost guarantees some mismatched hybridizations will occur in environmental samples. Careful selection of thoroughly tested probes currently remains the best solution for overcoming this interference. In addition, mineral matrices often autofluoresce under various wavelengths of light, and may add to the background 'noise' of a given sample, leading to under- or over-estimates during quantification.

6.6.4 Conjunctive Technologies

A number of technologies used in conjunction with FISH have arisen over the past decade. These technologies attempt to link the identity of organisms (as determined by FISH) with their physiological activity. One such method utilizes radiolabeled compounds (e.g., ^{14}C-lactate, ^{32}P-phosphate, etc.) that become incorporated into DNA by microbes capable of consuming or incorporating those substrates. The cells are screened using microautoradiography to determine the metabolically active cells and FISH to determine identity. This process, termed MAR-FISH, possesses its own limitations, but generally combines the strengths of two technologies in examining microbial populations.

Another technology rapidly gaining acceptance uses FISH to fluorescently label target cells, followed by rapid sorting and cell counting using flow cytometry. Flow cytometry can screen thousands of microbial cells per minute, permitting rapid quantification and/or identification of microbes from environmental samples in a substantially more accurate manner than manual enumeration. Cost is the principal limitation of this technology at this time, preventing its routine use in environmental sampling.

6.6.5 Conclusion

FISH provides a generally unbiased method of analyzing microbial populations in their natural conditions. Although not explicitly mentioned within this text, numerous variations on FISH exist, with most emphasizing methods for improving the signal intensity and/or quality. These methods include the use of longer polynucleotide probes that target 10–100× longer portions of RNA and fluorescent signal enhancements using catalyzed reporters. As these technologies develop, they will undoubtedly affect the ability of remediation practitioners to properly assess the need and/or effect of bioaugmentation at contaminated sites.

6.7 COMMUNITY PROFILING

The advent of molecular techniques, PCR in particular, provided the first opportunity for the broad investigation of environmental microbial communities. Links between the community population structure and metabolic functions in native environments are being determined as a result of many of the techniques described below. The majority of the technologies described below require PCR amplification of environmental DNA, making them subject to the PCR biases and limitations described in an earlier section. The analysis of PCR amplicons (the pool of amplified target gene DNA) provides relative information only for the timepoint at which the environmental sample was taken. The true power of these techniques comes not from a single snapshot, but through comparing multiple timepoints and determining changes in community structure resulting from varying environmental conditions (e.g., diurnal patterns, saline/freshwater interfaces, aerobic slugs of groundwater, etc.).

6.7.1 Gel Electrophoresis

Conventional gel electrophoresis, as a stand-alone technique, does not result in community profile data. However, it is a commonly used DNA separation technique that is often used in conjunction with other DNA separation and profiling methods, and thus a brief overview is warranted. Gel electrophoresis relies on the differential migration of DNA with different sizes or secondary structures through a semi-solid, but porous, gel matrix. DNA, being negatively charged, will migrate along a gel toward the positive electrode if subjected to an electric field. In gels of a uniform nature, the larger a fragment of DNA, the more the gel retards movement and the shorter the distance that fragment travels in a given time. Similarly, in gradient gels, DNA becomes denatured and occupies more physical space, subsequently slowing movement. Thus, much like chromatography permits separation of mixed gases or chemicals in solution, electrophoresis separates mixed fragments of DNA. The DNA is visualized using a variety of staining techniques, with individual blocks of DNA referred to as "bands." The intensity of these bands roughly corresponds with the concentration of DNA.

6.7.2 Cloning and Sequencing

Cloning and sequencing of target genes of interest offer the most informative (although not necessarily the most economical) method of determining microbial community composition. This technique uses PCR-amplified target genes of interest, with both group-specific genes (e.g., 16S rRNA) and functional genes (e.g., *vcrA*) used with great frequency. Individual PCR amplicons from the pooled mixture are ligated into plasmids (small circular pieces of extrachromosomal DNA), which in turn are inserted into *Escherichia coli* host cells. This process, termed "transformation", results in each *E. coli* host cell containing a single plasmid with a single PCR amplicon. Thus, when cells are grown on media plates, each colony (derived from a single *E. coli* cell) contains a DNA fragment representative of a single target gene. Extracted DNA from these colonies is analyzed using sequencing technologies, producing hundreds (or thousands) of DNA sequences from the initial PCR amplicon pool. Comparison of the generated sequences with molecular databases provides information on the identity of the organism from which the target gene was derived. Phylogenetic and ecological comparisons are commonly used for determining the complexity of the microbial communities sampled, as well as for inferring some degree of metabolic functionality.

6.7.3 Terminal-Restriction Fragment Length Polymorphism

Terminal-restriction fragment length polymorphism (T-RFLP) analyzes microbial communities using PCR-amplified DNA for the gene of interest subjected to digestion with a restriction enzyme (Liu et al., 1997). Restriction enzymes cut DNA at very specific basepair sequences, typically 4–6 nucleotides in length. Since each amplified PCR product from a different species is unique, the restriction enzymes cut the DNA at different points. For example, one restriction enzyme might digest DNA from a species into two 600-basepair fragments, while cutting DNA from a second species into a 300- and 900-basepair fragment.

Although functional genes are sometimes used for T-RFLP, its most common application is on 16S rRNA genes. PCR-amplification occurs as described in the previous section; however, one primer contains a fluorophore (much like the probes discussed in the earlier sections). After PCR-amplification and digestion using a restriction enzyme, one terminal fragment of each digested PCR amplicon contains the fluorophore, while the rest are unlabeled. The fragments are separated by size using electrophoresis, with smaller fragments migrating faster. As the terminal restriction fragments (TRFs) migrate past a sensor, those fragments with a fluorophore are detected, with larger concentrations of fragments producing greater signal intensities. Those fragments lacking the fluorophore pass through undetected (hence, only terminal fragments are measured). Through the proper selection of restriction enzymes, each TRF represents one (or a small portion) of the microbial community members. Shifts in the community composition result in varied numbers and signal intensities for the TRFs present, permitting semi-quantitative interpretation between samples. In general, a greater number of TRFs indicates greater community complexity. Determining the exact species associated with a given peak requires prior sequence knowledge, usually obtained through cloning and sequencing.

6.7.4 Denaturing Gel Gradient Electrophoresis

Denaturing gel gradient electrophoresis (DGGE) uses PCR-amplified target genes of interest and a gel composed of a gradient of denaturing agents, commonly formamide and urea (Madigan et al., 2003). The amplicon pool (all of approximately equal size) is separated by electrophoresis based on the melting profiles unique to their nucleotide sequences. As the double-stranded PCR-amplified DNA migrates through the gradient into increasingly higher concentrations of denaturing agent, the amplicons melt (denature) and cease migration. While the PCR amplicons are approximately the same size, the differences in melting properties result from the varied nucleotide sequences located between the ends selected by the primers. Visualization and comparison of the DNA bands after separation permits an evaluation of the community complexity (Figure 6.8). A larger number of bands generally represents a more complex and diverse microbial community. Once the fragments are separated, individual DNA bands can be excised and sequenced to determine the species present within a community.

6.7.5 Temperature Gel Gradient Electrophoresis

Temperature gel gradient electrophoresis (TGGE) is very similar to DGGE, but instead of using a gradient of denaturing agents, a temperature gradient is employed. As the DNA fragments migrate through the gel and up a temperature gradient, they will denature and further migration is retarded. Depending upon the nucleotide composition of the PCR amplicons, they will denature at varying temperatures. As with DGGE, differences in band intensity

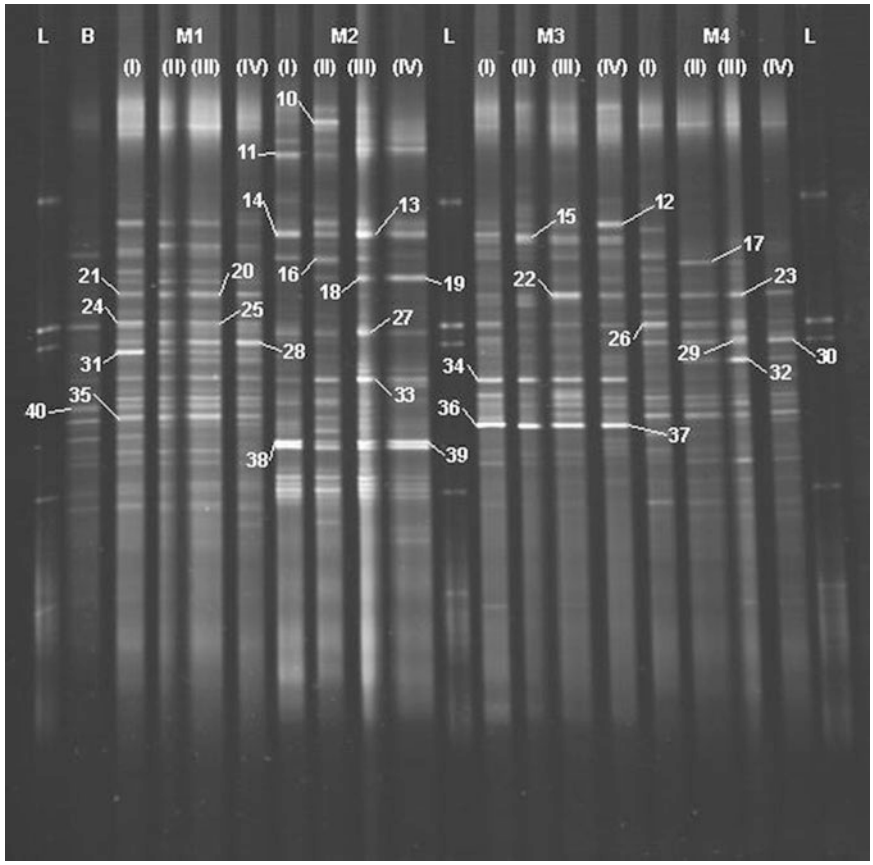

Figure 6.8. Community profile analysis of electron donor study using denaturing gel gradient electrophoresis (DGGE).

and location correspond with shifts in the community structure. Individual bands of interest may be excised from the gel and sequenced to determine the target gene identity.

6.7.6 Microarrays and High-Throughput Sequencing

Both microarrays and advances in sequencing technology have recently revolutionized the field of environmental microbiology (Alvarez-Cohen, 2007). Microarrays, consisting of thousands of probes on a single chip the size of a microscope slide, permit the rapid and comprehensive analysis of microbial communities (Figure 6.9). The probes consist of short sections of DNA (20–100 nucleotides) representative of a specific gene (either group- or function-specific), and affixed onto a chip or slide. DNA hybridizes with the target probes, with signal intensity proportional to the concentration of DNA. Shifts in the overall population structure thereby result in different intensity patterns. Microarrays typically consist of genes representative of a single (or several) complete genome for a given organism, group-specific genes (e.g., 16S rRNA) or functional genes from a broad distribution of organisms. The major limitation of microarrays is that a predetermined selection of probes is required, and so it is only possible to detect the genes that the array is designed to target, thereby lending a bias to the approach.

High-throughput sequencing technologies, such as pyrosequencing, now permit the rapid, high-quality sequencing of entire environmental populations. This technique therefore creates the most

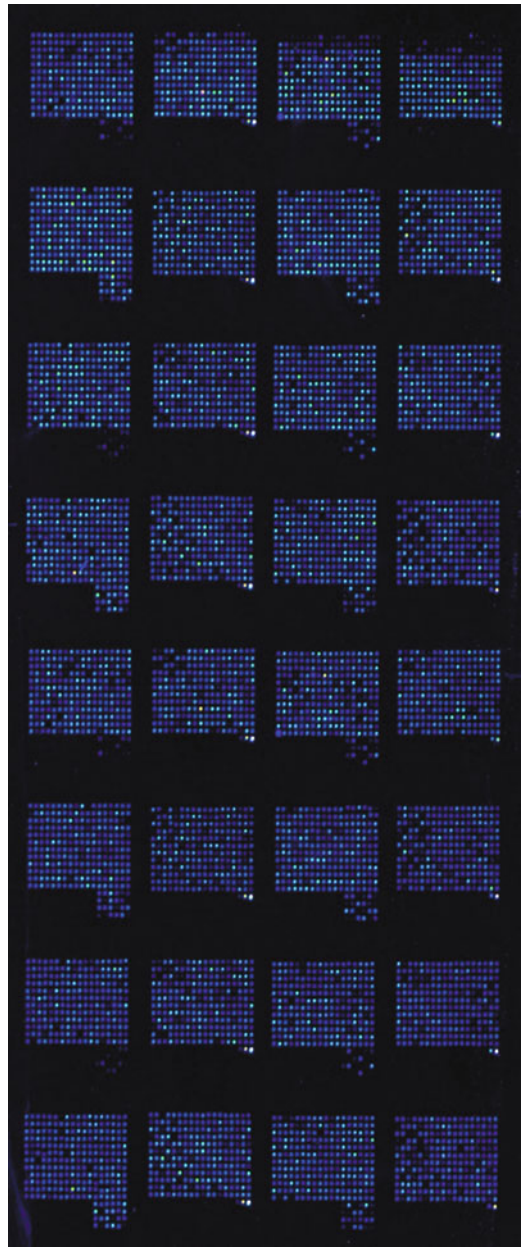

Figure 6.9. Microarray for high throughput screening of microbial communities.

unbiased and representative snapshot of microbial communities. However, significant computational analyses are required for analyzing the approximately 500 million basepairs sequenced during a 1-day typical operation period, leading to increased cost and analytical complexity.

6.7.7 Conclusion

Microbial communities govern biogeochemical cycling of nutrients in our environment, with the breakdown of most toxic compounds occurring as a result of microbial interactions

within these communities. Understanding the microbial community shifts associated with fluctuating environmental conditions provides remediation engineers an opportunity to manipulate microbial populations towards more beneficial metabolic activities. Monitoring the health of these communities through the techniques outlined above provides information useful in determining if nutrient addition or bioaugmentation is required for the continued degradation or sequestration of harmful compounds.

6.8 DATA EVALUATION AND INTERPRETATION OF MBTS

The evaluation of data obtained through use of MBTs necessitates a broader examination of site-wide characteristics for effective site management. Combined with traditional monitoring approaches, a more holistic picture can be obtained rather than relying solely on MBTs for assessing the performance of bioremediation activities. At a minimum, monitoring of geochemical parameters, such as pH, ORP and dissolved oxygen, should be conducted in parallel with MBT analysis, verifying appropriate conditions exist for effective bioremediation. Measuring concentrations and valence state (or inferred valence state through the use of filtered and unfiltered samples) of iron, nitrate, sulfate and sulfide can provide additional information on the conditions that may affect bioaugmentation performance. Along with geochemical parameters, measuring concentrations of both the parent contaminant and applicable daughter products assists in verifying sufficient activity is coupled with the presence of the appropriate microbes. Other chemical parameters, such as volatile fatty acids and total organic carbon, are useful indicators of electron donor abundance and distribution, and also may provide further indirect evidence of microbial biomass and activity.

Sampling using MBTs (and associated chemical parameters) should be conducted at a sufficient number of locations such that conditions are understood throughout the treatment zone. Sampling should confirm that favorable conditions exist throughout the targeted treatment zone, and that appropriate distribution and abundance of the necessary microorganisms and other amendments, as appropriate, has been achieved, and is maintained until treatment objectives are met. If inappropriate conditions and/or insufficient concentrations of microorganisms exist, corrective actions should be taken. These may include injection of additional microbes, electron donor or acceptor, or redistribution using hydraulic controls (e.g., groundwater recirculation systems).

As an example of a comprehensive site strategy for assessing and interpreting MBTs, consider a TCE-contaminated site bioaugmented with a *Dhc*-containing consortium. The site-wide monitoring approach should confirm the following:

- General anaerobic conditions within the targeted bioaugmentation distribution area.
- An increase in the concentration of *Dhc* (as determined through qPCR) after injection.
- A steady or increasing concentration of *Dhc* genes, along with a concomitant decrease in TCE concentrations and rise in daughter product and ethene concentrations during subsequent temporal sampling.
- A transient increase (and subsequent decrease) of DCE and VC concentrations likely will be observed (depending upon sampling frequency and the level of microbial activity), further confirming the effectiveness of the bioaugmentation remedial approach.
- The *Dhc* cell titers should remain elevated until TCE (and daughter product) concentrations fall below cleanup levels. If *Dhc* concentrations begin decreasing, or if anaerobic conditions begin to deteriorate (i.e., become more aerobic), then additional amendments (e.g., electron donor) may be required.

This broader examination of both the MBT and chemical parameters will provide a more complete interpretation of site data, and increase the likelihood of achieving remedial objectives.

6.9 FUTURE RESEARCH NEEDS

Design and performance monitoring of bioaugmentation systems requires analysis of the presence, abundance, activity and spatial and temporal dynamics of process-specific biomarkers such as *Dhc* and VC reductase targets. The tools of molecular biology, particularly qPCR, have allowed researchers to overcome the limitations imposed by the difficulties in culturing *Dhc* from environmental samples, and have greatly improved the ability to evaluate the need for bioaugmentation at specific sites, and to monitor the performance of sites after biostimulation or bioaugmentation.

To further advance groundwater monitoring, additional process-specific biomarkers must be identified. In the case of chlorinated ethenes, *Dhc* bacteria have been identified as key players to achieve complete reductive dechlorination to nontoxic end products, and currently several reductive dechlorinase genes have been identified. However, other bacteria, such as *Geobacter, Dehalobacter, Desulfitobacterium and Desulfuromonas* species, may play just as important a role in remediating chlorinated solvent-impacted sites. For example, a study by Amos et al. (2009) identified genes in a *Geobacter* strain that are implicated in the reductive dechlorination of chlorinated ethenes, but their potential value as MBT targets in the field is not well understood.

To reduce analysis costs, even with a broader suite of biomarker targets, a tiered approach to evaluating microbial communities is recommended. Initial characterization of a site should focus on a wider range of available *Dhc* biomarkers. These results then can be used to tailor qPCR assays that only enumerate biomarkers that are informative for a given site. Groundwater analysis for monitoring microbial processes in the subsurface requires that the target microbes are, at least partly, planktonic. The factors controlling the switch from sessile (e.g., biofilm growth) to planktonic lifestyle are largely unknown for most subsurface bacteria, which confounds quantitative assessment of groundwater samples and data interpretation.

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CHAPTER 7

BIOAUGMENTATION FOR AEROBIC DEGRADATION OF *CIS*-1,2-DICHLOROETHENE

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7.1 INTRODUCTION

Incomplete reductive dechlorination of perchloroethene (PCE) or trichloroethene (TCE) in the subsurface often results in the accumulation of the suspected carcinogen, *cis*-1,2-dichloroethene (*cis*-DCE) and the known carcinogen, vinyl chloride (VC) (Adamson et al., 2003; Ellis et al., 2000; Hendrickson et al., 2002; Singh et al., 2004). Persistence of VC and *cis*-DCE due to incomplete reductive dechlorination can be attributed to the absence of bacteria capable of complete reductive dechlorination or to environmental conditions that are not conducive to reductive dechlorination. Whereas bacteria that can partially dechlorinate PCE or TCE to *cis*-DCE [e.g., *Dehalobacter restrictus* (Holliger et al., 1998) and *Dehalospirillum multivorans* (Scholz-Muramatsu et al., 1995)] are common, only *Dehalococcoides* spp. completely dechlorinate PCE to ethene (Maymó-Gatell et al., 1997; Seshadri et al., 2005). Bioaugmentation with *Dehalococcoides* spp. has been shown to stimulate reductive dechlorination in the laboratory (Schaefer et al., 2009; Sleep et al., 2006) and at the field scale (Ellis et al., 2000; Hood et al., 2008; Lendvay et al., 2003; Major et al., 2002).

Insufficient electron donor or the presence of oxygen can inhibit reductive dehalogenation even when appropriate bacteria are present. VC and *cis*-DCE that accumulate from incomplete reductive dechlorination can migrate downgradient into aerobic zones. Under these circumstances, it may be more effective to oxidize the compound aerobically, rather than try to create subsurface conditions suitable for reductive dechlorination. Another possibility is to create or enlarge an aerobic zone downgradient, to facilitate aerobic oxidation. Organisms that oxidize VC appear to be widespread, and as a result VC often degrades quickly under aerobic conditions (Coleman et al., 2002b).

In contrast, reports of bacteria able to degrade *cis*-DCE aerobically are very rare. Several organisms can catalyze the cometabolic oxidation of *cis*-DCE (Hopkins and McCarty, 1995; Semprini, 1995; Semprini et al., 1990). They transform the chlorinated solvent, but do not obtain any energy or growth from the process. Consequently, the addition of a growth substrate such as methane, phenol, propane or toluene is required for transformation (Hopkins and McCarty, 1995; Bradley and Chapelle, 2000). Bioaugmentation to stimulate cometabolic oxidation is limited by the requirement for cosubstrate, which can cause competitive inhibition since both the contaminant and the primary substrate are often transformed by the same enzyme. Other problems with cometabolism include excessive growth, resulting from the high dose of primary substrate, leading to clogging of injection wells and aquifer, as well as oxygen demands that are difficult to satisfy (McCarty et al., 1998). Furthermore, the production of reactive intermediates during the cometabolic oxidation of chlorinated solvents can damage enzymes and cells, causing loss of degradation activity and cell viability (Alvarez-Cohen and Speitel, 2001).

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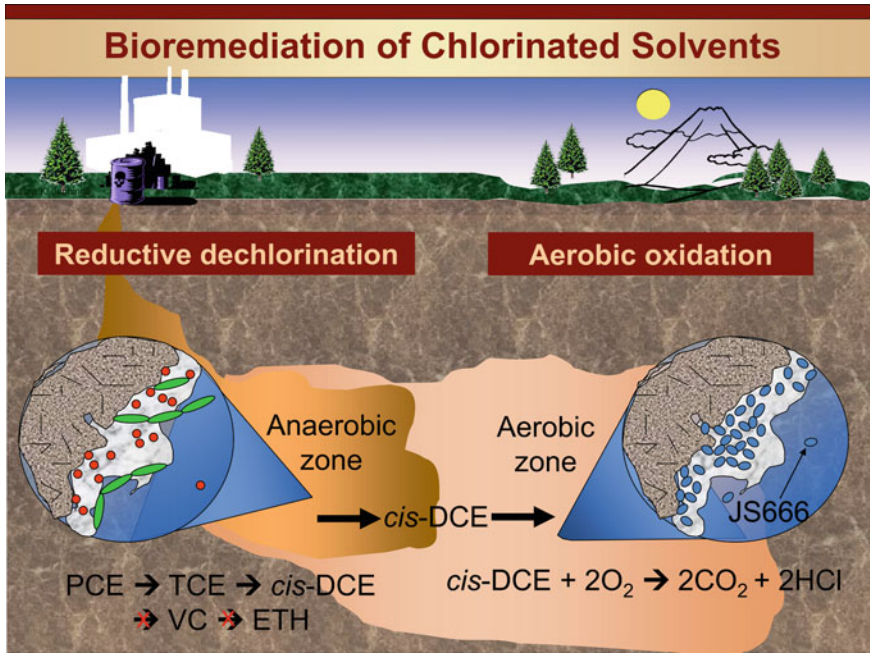

Figure 7.1. *cis*-DCE that accumulates from incomplete reductive dechlorination can be completely mineralized by *Polaromonas* sp. JS666.

Despite such obstacles, the cometabolic oxidation of *cis*-DCE has been demonstrated in the field (Azizian et al., 2005, 2007; Semprini et al., 1990, 2007).

Polaromonas sp. strain JS666 is the first isolate capable of using *cis*-DCE as its sole carbon and energy source under aerobic conditions (Coleman et al., 2002a). It is a promising candidate for bioaugmentation at *cis*-DCE-contaminated sites where *cis*-DCE has migrated downgradient into an aerobic zone (Figure 7.1). Addition of the strain can circumvent the problems associated with cometabolic oxidation as a bioremediation strategy because it catalyzes rapid degradation without the addition of a cosubstrate, and the requirements for oxygen are much lower than for cometabolic transformations. The metabolic capabilities of JS666, development of a molecular probe for process monitoring, microcosm assessment of site suitability and the preliminary results of a field-scale study are discussed in this chapter.

7.2 *POLAROMONAS* SP. STRAIN JS666

Preliminary evidence for the aerobic oxidation of *cis*-DCE was noted in stream-bed sediments (Bradley and Chapelle, 1998a, b), and the microbial mineralization of *cis*-DCE was confirmed by the sequential transfer of the sediment from microcosms to defined medium (Bradley and Chapelle, 2000). The organism(s) responsible for *cis*-DCE transformation in the sediment were not identified, however, because *cis*-DCE concentrations were too low to support significant growth (Bradley and Chapelle, 2000). The results provided the first evidence that *cis*-DCE could serve as a primary substrate in aerobic metabolism.

7.2.1 Isolation

Polaromonas sp. strain JS666, the first bacterium capable of coupling growth to the aerobic oxidation of *cis*-DCE, was isolated from activated carbon used for treating water contaminated with PCE, TCE and *cis*-DCE in Dortmund, Germany (Coleman et al., 2002a). Microcosms containing *cis*-DCE as the sole carbon source were inoculated with activated carbon from the pump-and-treat plant. A pure culture was obtained by sequential transfers to minimal salts medium (MSM) [modified from that used by Hartmans et al. (1992)], with *cis*-DCE as a sole source of carbon and energy (Coleman et al., 2002a). Biodegradation of *cis*-DCE in enrichments began after 50 days (Coleman et al., 2002a). Similar samples from 18 other solvent contaminated sites did not yield *cis*-DCE degraders, whereas 23 of 27 field samples yielded VC degraders (Coleman et al., 2002b). Others also have isolated VC degraders from the environment (Verce et al., 2000). The results indicate that VC oxidizing bacteria are more common than *cis*-DCE oxidizers and that VC may be more readily degraded via natural attenuation than *cis*-DCE. It is also possible that the small fraction of microcosms with *cis*-DCE oxidation reflects difficulties in cultivating DCE degraders. Therefore, future research is necessary to determine the distribution and abundance of *cis*-DCE oxidizers. However, the large number of sites where *cis*-DCE accumulates suggests that *cis*-DCE oxidizers are not widespread in the environment.

Phylogenetic analysis of the 16S ribosomal ribonucleic acid (rRNA) gene indicated that strain JS666 is a β-Proteobacterium closely related to the Antarctic marine isolate, *Polaromonas vacuolata,* (97% sequence identity). Strain JS666 shares a 98% sequence identity to the 16S rRNA gene of *Polaromonas* sp. GM1, a psychrotolerant arsenite-oxidizing bacterium (Osborne et al., 2010), and a 97% 16S rRNA nucleic acid identity with *Polaromonas naphthalenivorans* CJ2, which has been implicated in naphthalene degradation at sites contaminated with coal-tar waste (Jeon et al., 2006). Neither organism possesses the ability to degrade *cis*-DCE. There is increasing evidence that bacteria of the *Polaromonas* genus may play an important role in the biodegradation of contaminants (Mattes et al., 2008; Yagi et al., 2009).

7.2.2 Kinetics, Thresholds and Tolerances to *cis*-DCE and Oxygen

JS666 will grow in a minimal medium supplemented with *cis*-DCE as the sole carbon source (Figure 7.2). Successive additions of *cis*-DCE were administered in a manner preventing its complete depletion because JS666 experiences long lags before *cis*-DCE degradation resumes if cultures are deprived of *cis*-DCE for even short periods (Jennings, 2005). The slowing of *cis*-DCE degradation after several additions is something often observed with JS666, and can result from pH decline or accumulation of chloride. The growth yield was 6.1 ± 0.4 gram (g) protein/mole *cis*-DCE, and at 20 degrees Celsius (°C), the doubling time was 74 ± 8 h (Coleman et al., 2002a). Complete mineralization of *cis*-DCE was indicated by the release of 1.94 moles chloride per mole of *cis*-DCE degraded (Coleman et al., 2002a).

In batch cultures at 20°C, the half-velocity constant (K_s) was 1.6 ± 0.2 micromolar (μM), and the maximum specific substrate utilization rate (k) ranged from 12.6 to 16.8 nanomoles/minute/milligram (nmol/min/mg) of protein (Coleman et al., 2002a). The high k and low K_s indicate that JS666 is capable of degrading *cis*-DCE to extremely low levels without kinetic limitations, which is important if the organism is to degrade *cis*-DCE completely at contaminated sites. In the laboratory, JS666 was routinely able to degrade *cis*-DCE to below detection limits of 0.03 micrograms per liter (μg/L) (Coleman et al., 2002a), which is well below the drinking water standard for *cis*-DCE of 70 μg/L (www.epa.gov).

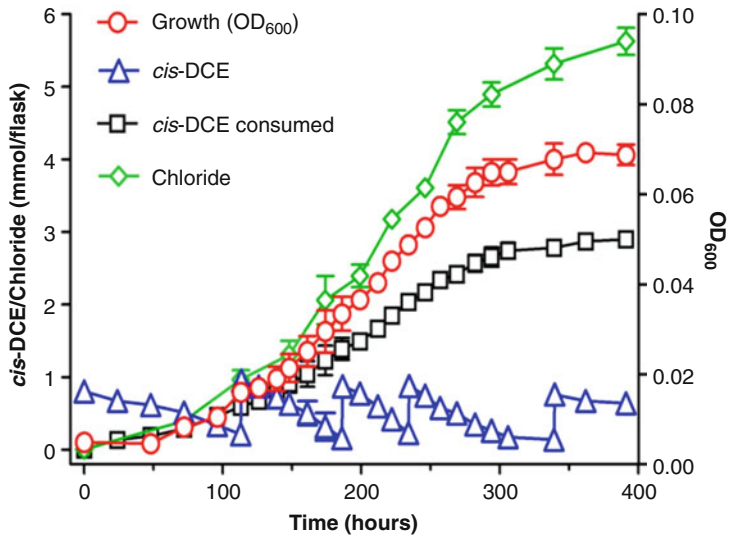

Figure 7.2. Mineralization of *cis*-DCE by JS666 showing amount of *cis*-DCE (*triangles*) and optical density (OD_{600}, *circles*) (adapted from Coleman et al., 2002a).

JS666 can tolerate and transform *cis*-DCE at aqueous concentrations of 0.5–0.9 millimolar (mM), but concentrations above 1 mM are inhibitory (Jennings, 2009). The optimum growth temperature is 22°C, with no growth at 30°C, suggesting no potential for human pathogenicity – always a concern with cultures proposed for bioaugmentation. Degradation is inhibited at pH below 6.5 with an optimum around 7.2. The sensitivity of JS666 to low pH may require the addition of buffer for *in situ* bioaugmentation. Phosphate buffer (20–40 mM) is currently being using in laboratory studies to maintain neutral pH. JS666 tolerates higher equivalents/liter (eq/L) of orthophosphates than carbonates. JS666 cultures challenged with high oxygen levels (partial pressure of oxygen = 0.34 and 0.77 atmospheres [atm]) degrade less *cis*-DCE than cultures with levels below 0.21 atm (Jennings, 2009). JS666 is capable of using oxygen to levels below analytical detection (ca. 0.01 milligrams per liter [mg/L]). The results indicate that oxygen levels at 0.21 atm or lower are optimum for *cis*-DCE degradation by JS666; addition of excess oxygen to stimulate degradation is not necessary and may even hinder degradation in the field.

JS666 was observed to prefer low-ionic-strength environments (conductivity <15 millisiemens per centimeter [mS/cm]), and high chlorides accumulated through dechlorination of repeated additions of *cis*-DCE can be problematic (S. Nishino and J.C. Spain, Georgia Institute of Technology, unpublished). For culture maintenance, a strategy of periodic transfer of inocula to fresh media has normally been employed. Alternatively, growth to high densities involved biomass separation and exchange of medium.

7.2.3 Insight About Metabolic Pathways from Genomics and Proteomics

cis-DCE degradation pathways in JS666 have not been determined. The genome of JS666 has been completely sequenced, but genomic analysis did not reveal any obvious *cis*-DCE degradation operon (Mattes et al., 2008). Genes potentially involved in *cis*-DCE degradation (e.g., flavin-containing monooxygenase, cytochrome P450, glutathione S-transferase, and haloacid dehalogenase) are scattered among the chromosome and two large plasmids. It seems likely that the *cis*-DCE degradation pathway in JS666 was assembled recently by

recruitment of genes that encode degradative enzymes from other pathways. Thus, pathway prediction using bioinformatics is complicated by degradation genes that are not in a single operon or are surrounded by genes that are not involved in degradation.

An integrated "omics" approach including proteomics and transcriptomics provided some insight about *cis*-DCE degradation pathways in JS666. The approach was based on the premise that proteins or messenger RNA (mRNA) transcripts that are upregulated by growth on *cis*-DCE compared to growth on a reference substrate are more likely to be involved in *cis*-DCE degradation pathways. Proteomics using two-dimensional (2-D) gel electrophoresis revealed that genes annotated as a cyclohexanone monooxygenase (CMO), glutathione S-transferase (GST), and haloacid dehalogenase (HAD) were upregulated during growth on *cis*-DCE (Jennings et al., 2009). Transcriptomics experiments using complementary deoxyribonucleic acid (cDNA) microarrays confirmed that the above genes were among the most highly upregulated genes, while identifying many others that could play an important role in the initial attack on *cis*-DCE (e.g., cytochrome P450) (Jennings et al., 2009).

Comparative genomics also can aid in predicting degradation pathways. Genes that are present in JS666 but not in the closely related *P. napthalenivorans* are more likely to be involved with *cis*-DCE degradation in JS666. Selected genes upregulated by *cis*-DCE in JS666 were compared to similar genes in *P. napthalenivorans* using the BLASTP algorithm. The CMO, GST, and HAD seem not to be present in *P. napthalenivorans* – supporting the hypothesis that the enzymes are important in *cis*-DCE degradation (Jennings et al., 2009).

Monooxygenases can catalyze the addition of oxygen to a double bond and form an epoxide. The oxidation of halogenated alkenes in bacteria is thought to occur primarily by monooxygenase-catalyzed epoxidation (Figure 7.3) (Ensign, 2001; Van Hylckama Vlieg and Janssen, 2001). The upregulation of a monooxygenase and the ability to convert ethene and propene to the corresponding epoxides supports the hypothesis that a monooxygenase is involved in *cis*-DCE degradation in JS666 (Coleman et al., 2002a). However, compound specific isotope analysis (CSIA) indicates that the first step in the primary *cis*-DCE degradation pathway in JS666 does not involve a monooxygenase (Jennings et al., 2009). The results suggest that there may be two *cis*-DCE degradation pathways in JS666.

CSIA can be used to discriminate between biodegradation and abiotic losses of contaminants. It also can reveal the initial mechanism of degradation since the degree of fractionation depends on the type of bond being broken in the first step (Hirschorn et al., 2007). The degree of fractionation can be described by the isotopic enrichment factor, ε. A large fractionation (larger negative number) can be expected from the cleavage of bonds between heavy atoms (e.g., carbon-chloride bond, C–Cl). A small fractionation (smaller negative number) can be expected from the cleavage of bonds between small atoms (e.g., carbon-carbon double bond, C=C).

In JS666, the carbon isotopic fractionation associated with aerobic *cis*-DCE degradation is −17.4 to −22.4‰ (Jennings et al., 2009), which is consistent with theoretical predictions for C–Cl cleavage (Glod et al., 1997) and experimental fractionation values for C–Cl cleavage in chloroethenes (Bloom et al., 2000; Hunkeler et al., 2002; Lee et al., 2007; Slater et al., 2001) and

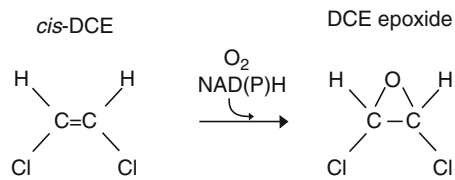

Figure 7.3. Monooxygenase-catalyzed formation of *cis*-DCE epoxide from *cis*-DCE in *Rhodococcus* sp. strain AD45 (adapted from Van Hylckama Vlieg and Janssen, 2001).

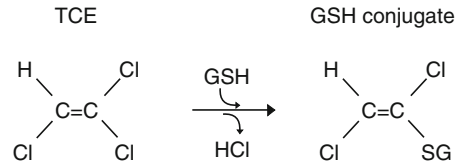

Figure 7.4. GST-catalyzed dehalogenation of trichloroethene (TCE) in mammals forming a glutathione conjugate (adapted from Dekant et al., 1990). Note: GSH - glutathione.

chloroethanes (Elsner et al., 2007; Hirschorn et al., 2004). If the first step in the primary degradation pathway of *cis*-DCE by JS666 involved an epoxidation by a monooxygenase (C=C cleavage), much smaller fractionation factors would be expected – consistent with those reported for aerobic VC assimilation (−8.2 to −7.0‰) (Chartrand et al., 2005; Chu et al., 2004) or cometabolic *cis*-DCE oxidation, which were below detection (Chu et al., 2004) or very small (−7.1 to −9.8‰) (Tiehm et al., 2008).

Bacteria have not been reported to catalyze an initial C–Cl cleavage in aerobic haloalkene degradation, which suggests that *cis*-DCE degradation pathways may involve a novel mechanism. In mammalian systems, GST-catalyzed dehalogenation of TCE or PCE involves a C–Cl cleavage in the first step (Figure 7.4) (Anders and Dekant, 1998; Dekant et al., 1986, 1990). In bacteria, GSTs act as dehalogenases in dichloromethane metabolism (Kohler-Staub and Leisinger, 1985), although an analogous reaction for two-carbon compounds has never been reported. When degradation proceeds simultaneously in one organism by two distinct pathways, the observed carbon isotopic fractionation represents a weighted average of the two pathways (Elsner et al., 2008). When one pathway is dominant, the fractionation effect of the minor pathway may not be observed. Thus, CSIA results in JS666 indicate that the first step in the major degradation pathway involves a C–Cl cleavage that is consistent with a first step involving an enzyme other than a monooxygenase (e.g., GST- or P450-catalyzed dehalogenation), but does not rule out the contribution of a monooxygenase to a minor pathway.

Collectively, the results indicate that there may be two *cis*-DCE degradation pathways in JS666. Work is currently underway to confirm the function of upregulated enzymes and clarify their role in *cis*-DCE degradation pathways. The presence of two *cis*-DCE degradation pathways in JS666 could explain the observation of two degradation phenotypes – one characterized by growth-coupled *cis*-DCE degradation and the other characterized by cometabolic degradation, with low rates and specific activity 10–20% of previously reported values (Jennings, 2005). The elucidation of degradation pathways in JS666 might allow for the development of strategies to promote the growth-coupled pathway during bioaugmentation. For example, once pathway-regulation is better understood, biodegradation could be improved with the addition of appropriate inducers to stimulate the growth-coupled pathway or by avoiding substrates and factors that might promote the undesirable, co-metabolic behavior.

7.2.4 Cometabolism of Other Chlorinated Solvents

JS666 is capable of transforming *trans*-1,2-dichloroethene (*trans*-DCE), TCE and VC, but does not obtain energy from such processes. Previous studies indicated that JS666 does not grow on 800 μM (40 μmol/bottle) 1,2-dichloroethane (DCA) (Coleman et al., 2002a). More recent studies suggest that JS666 is capable of growth on DCA at lower concentrations (400 μM) (S. Nishino and J.C. Spain, Georgia Institute of Technology, unpublished). The degradation of mixtures of contaminants would be particularly important for bioremediation applications at contaminated sites.

The mutual effects of binary mixtures of TCE, VC, DCA and *cis*-DCE on the kinetics of degradation by JS666 were investigated (F. Liu, E. Wood, and J. M. Gossett, unpublished). Although the presence of TCE, VC or DCA reduced the degradation rate of *cis*-DCE, both *cis*-DCE and the cocontaminant can be degraded to completion. Thus, JS666 may be able to completely degrade mixtures of chlorinated solvents in the field. However, it is unknown how the presence of the non-*cis*-DCE substrate might have affected growth because these were short-term, batch experiments designed in such a manner that biomass would remain constant during the assays. Observation of approximately stoichiometric chloride release from VC and TCE degradation indicates their mineralization by JS666 (F. Liu and J. M. Gossett, unpublished).

With *cis*-DCE at 40 μM (ca. 4 mg/L), presence of VC or TCE over a wide range of concentrations (up to 10 μM) caused maximum *cis*-DCE-degradation rates to decrease by as much as half. There was no evidence that one compound was being preferentially degraded, since the fraction of compounds remaining was essentially the same for each of the binary mixtures. Degradation of VC was enhanced by the presence of *cis*-DCE, while TCE degradation was unchanged. Preliminary results suggest that TCE and VC may be degraded by different enzymes or pathways than *cis*-DCE. The effect of the presence of DCA on *cis*-DCE degradation differs from that of VC or TCE. *cis*-DCE and DCA degradation rates are inversely correlated with each others' concentrations – i.e., the highest concentration of DCA caused the slowest *cis*-DCE degradation, and vice-versa (F. Liu and J. M. Gossett, unpublished). Preliminary results thus suggest that DCA competitively inhibits *cis*-DCE degradation in JS666, which indicates that they may be transformed by the same enzyme or pathway. Future studies of the degradation of mixtures of contaminants in JS666 can be expected to provide insight into the mechanisms of degradation.

7.2.5 Development of a Molecular Probe for Process Monitoring

The ability to track the presence of the bioaugmented organism in the subsurface is important for monitoring the progress of *in situ* bioaugmentation. A quantitative polymerase chain reaction PCR (qPCR) assay was developed for the specific detection of JS666 in soil (Giddings et al., 2010a). The qPCR assay targeting the JS666 isocitrate lyase gene is specific, accurate and reproducible in soil (Giddings et al., 2010a). The molecular probe was used in a field bioaugmentation study with JS666 (Section 7.4.4). One objective of the field study was to combine the qPCR assay with microcosm studies to determine whether the presence of JS666 can be correlated with degradation activity.

There are a number of different techniques available to quantify microorganisms in soil, including culture-based techniques (e.g., most probable number or selective plating), fluorescent *in situ* hybridization (FISH) and qPCR. Techniques based on cultivation are tedious and cannot be used to quantify the vast majority of microbes that have not yet been cultured. Techniques such as FISH (Amann et al., 1995), do not require cultivation, but can be difficult to apply to soil or to cultures with low cell concentrations. qPCR is quickly becoming the method of choice for quantifying microbes in mixed communities because of the ability to detect extremely low concentrations of cells without the need for cultivation (Sharma et al., 2007; Smith et al., 2006). qPCR has been applied to the quantification of genes or microbes associated with the biodegradation of chlorinated ethenes (Cupples, 2008; He et al., 2003; Lendvay et al., 2003; Smits et al., 2004) and ethanes (Van Raemdonck et al., 2006).

A qPCR assay for JS666 was developed using strain-specific primers (Giddings et al., 2010a) and SYBR Green reagent (Van Raemdonck et al., 2006). Primers were designed to target the chromosomal gene that encodes isocitrate lyase, because the 16S ribosomal gene in JS666

was not sufficiently unique to facilitate the design of strain-specific primers. Plasmid-encoded genes were avoided as targets because they are less stable than chromosomal genes, and targeting plasmid-encoded genes in qPCR assays can result in errors in quantification (Wang et al., 2004). Primers were designed to avoid similarity with known sequences in the NCBI database. In addition, the annealing temperature of the primers was optimized to avoid non-specific amplification.

The absolute quantification of JS666 in soil was verified by comparison with plate counts and direct-microscopic counts. Samples of serially diluted cultures were applied to 0.5 g of soil obtained from the Savannah River Site (SRS) in Aiken, South Carolina. SRS soil was selected from an aerobic plume in which *cis*-DCE had accumulated and persisted – presumably due to incomplete reductive dechlorination. DNA was extracted from duplicate soil samples amended with JS666, and the copy number of target genes (isocitrate lyase) was quantified using duplicate qPCR reactions. Copy number from qPCR positively correlated with cell counts from plating and viability staining. The largest source of variability in the qPCR assay was between the different soil extractions. The optimum assay conditions require 100-fold dilution to minimize the effect of inhibitors and provide a conservative minimum detection limit of 10^5 cells per gram of soil. Minimum detection limits could be improved by using a 50-fold dilution of DNA extract.

In addition to monitoring the progress of bioaugmentation by tracking the bioaugmentation agent in the subsurface, the qPCR assay for JS666 could be combined with assays for other dechlorinators to evaluate the potential for natural attenuation via aerobic *cis*-DCE oxidation or reductive dechlorination at contaminated sites.

7.2.6 Development of Strategy for Growth of Inocula

Microcosm studies indicated that 10^5 cells/milliliter (mL) is an effective inoculum level to stimulate *cis*-DCE degradation by JS666 (Giddings et al., 2010b). This cell density is similar to the range of recommended inoculum levels for anaerobic bioaugmentation with *Dehalococcoides* spp. cultures – ca. 10^4–10^6 cells/mL (Steffan et al., 2010). Therefore, bioaugmentation with JS666 in the field will require generating sufficient biomass to treat large contaminated sites.

To achieve high biomass levels necessary for inoculation in the field, it is desirable to grow JS666 on a substrate other than *cis*-DCE. *cis*-DCE in relatively pure form (without impurities such as chloroform) is expensive, and *cis*-DCE's toxicity to JS666 limits the concentrations to which the cultures can be exposed. It is imperative, however, that the ability to grow on *cis*-DCE is not lost during growth on the alternative substrate. Several potential alternative substrates were screened for their ability to generate biomass and maintain induction of *cis*-DCE degradation enzymes (S. Nishino and J.C. Spain, Georgia Institute of Technology, unpublished). 2-Chloroethanol did not support growth. Cells grown on succinate or acetate that were subsequently amended with *cis*-DCE showed more growth than *cis*-DCE-only controls, but reduced specific activity toward *cis*-DCE.

The use of ethanol as a cosubstrate was evaluated by growing dense suspensions of cells at different ratios of *cis*-DCE to ethanol. After the degradation of *cis*-DCE began, cells were harvested by centrifugation and suspended into cultures with *cis*-DCE as the only carbon source. A 3:2 (volume to volume [v:v]) ratio of *cis*-DCE to ethanol resulted in the generation of the most biomass and the fastest *cis*-DCE degradation. However, when rates were normalized to biomass, the *cis*-DCE control without ethanol had the highest specific activity for *cis*-DCE degradation. The results indicate that the use of ethanol, succinate, and acetate as cosubstrates increases the amount of biomass but causes a reduction in *cis*-DCE degradation performance. They are also more likely to support growth of contaminants.

Acetonitrile (MeCN) is a good growth substrate for JS666. Rates of *cis*-DCE degradation were slower in cultures containing MeCN and *cis*-DCE. However, after cells were harvested and transferred to fresh medium with *cis*-DCE as the only carbon source, the specific activity of MeCN − *cis*-DCE-grown cultures was the same as that of *cis*-DCE only cultures. In contrast, cells grown on MeCN without *cis*-DCE were only half as active when transferred to medium with *cis*-DCE as the sole carbon source. Thus, MeCN can be used as a cosubstrate to generate large amounts of JS666 biomass without sacrificing specific activity. Cyclohexanone also supported growth of JS666 without the loss of ability to degrade *cis*-DCE. The growth strategy using cyclohexanone alternating with *cis*-DCE routinely yielded cultures with OD_{600} >1.0 in a 66-L reactor. Both substrates supported less growth of contaminants in the cultures than did succinate or ethanol. To attain such high densities, it was necessary to periodically exchange the medium (using cross-flow filtration), because the accumulation of chloride otherwise inhibits JS666.

Several methods were investigated for long-term storage of cells, including use of cryoprotectants such as glycerol or DMSO, lyophilization, and flash-freezing in liquid nitrogen. *cis*-DCE degradation activity was best restored from cell pellets stored at −80°C without cryoprotectant. Liquid cultures also could be stored with *cis*-DCE as the sole carbon source at 4°C for up to 14 days without significant loss of activity.

7.3 MICROCOSM ASSESSMENT OF SITE-SUITABILITY

Bioaugmentation with JS666 for aerobic degradation of *cis*-DCE is a technology in its nascent stage. Therefore, the relative lack of experience with JS666 under different site conditions makes it prudent to conduct microcosm assessment of site suitability. With greater experience, this step may become less important, and decisions regarding suitability may be reasonably made based on site physical and biogeochemical parameters.

7.3.1 Microcosm Preparation

Microcosm studies should be conducted in 160-mL serum bottles with ambient-air headspace, sealed with Teflon™-lined, butyl-rubber septa and aluminum crimps. It is best if microcosms are incubated at the subsurface temperature of the prospective site, under agitation (to enhance aeration from the headspace), and in the dark. Where possible, microcosms should be prepared with both soil (50 g dry mass) and groundwater (in volumes such that groundwater − soil water − any liquid amendments = 50 mL) from the prospective site. If soil samples are not available, microcosms can be conducted with groundwater only. Care should be taken to avoid cross-contamination of microcosms with materials from different site locations, otherwise interpretation could be compromised.

Microcosms of the following types should be included: (1) native (i.e., neither pH-adjusted, nor amended with buffer or nutrients); (2) pH-adjusted (to neutral pH with either HCl or sodium hydroxide [NaOH]; but not buffered); and (3) buffer/nutrient amended (i.e., with 5 mL of 10X-concentrated MSM). Each of the above should be done in both inoculated and uninoculated (control) versions.

After the microcosms are prepared (but before inoculation), they should be allowed to equilibrate under agitation for at least 6 hours (h) prior to volatile organic compound (VOC) analysis in headspace samples. *cis*-DCE should be added to achieve concentrations similar to levels at the site; however, concentrations above 0.5 mg/L are recommended for analytical precision, and below 50 mg/L for toxicity avoidance.

JS666 inoculum should come from an active culture grown on *cis*-DCE as sole carbon/energy source. Where possible, the use of several inoculum levels (10^7, 10^6, and 10^5 cells/mL) is recommended in microcosm studies, to assist in determining necessary bioaugmentation levels in the field. Minimum inoculum levels should become standardized after more field experience with the technology.

7.3.2 Previous Experiences with Microcosm Assessment

The potential of JS666 as a bioaugmentation agent was initially assayed in microcosm studies (Giddings et al., 2010b). JS666 survival and activity were assessed using subsurface materials from five sites: Savannah River Site (SRS), South Carolina; Robins Air Force Base (AFB), Georgia; Hill AFB, Utah; Fort Lewis, Washington; and an Aerojet facility near Sacramento, California. Microcosm experiments were performed under what could be considered ideal conditions (i.e., pH buffered and amended with nutrients), and then systematically challenged with inhospitable conditions and other potential barriers. *cis*-DCE degradation was monitored, and because the organism would later be used in field tests of bioaugmentation, the molecular probe (based on the isocitrate-lyase gene of JS666) was applied to track JS666 within some microcosms as a test of the probe's efficacy as well as survival of JS666. Additionally, microcosms were constructed using two dilutions of primary sewage effluent – unautoclaved (contributing both complex organic substrates as well as competing and/or predatory microbes), and autoclaved (thus contributing only complex organics).

In buffered, neutral pH microcosms constructed from all five site materials, *cis*-DCE at high concentration (ca. 60 mg/L) was degraded completely within 10–15 days when inoculated with JS666 at 7×10^6 cells/mL. Without inoculation of JS666, no significant *cis*-DCE degradation was observed. Studies were undertaken to determine effective inoculum size, using three levels (1X, 0.1X, and 0.01X) where 1X corresponds to the 7×10^6 cells/mL concentration with SRS soil. In microcosms constructed of SRS soil − MSM, *cis*-DCE was depleted in about 20 days at 1X, and was about 50% depleted in 60 days at both 0.1X and 0.01X inoculum levels. With a more realistic initial *cis*-DCE concentration (0.6 mg/L), complete degradation was observed in about 5 days at 1X and 0.1X, and in about 20 days at the 0.01X inoculum level. The results suggest that 10^5 cells/mL is a reasonable minimum inoculum level for field application.

As a rigorous test of both microbial competition/predation, and of the presence of alternative substrates, studies were conducted in which municipal primary sewage effluent was added to SRS-soil microcosms along with JS666. Without JS666 addition, no significant degradation of *cis*-DCE occurred. All JS666-inoculated microcosms prepared with either 1% or 10% primary effluent were able to degrade 60 mg/L *cis*-DCE, regardless of the initial inoculation level. The lower inoculum level required more time to degrade the *cis*-DCE. This demonstrates that even in the presence of a mixture of alternative (and most likely preferable) carbon sources and competing/predatory microbes, JS666 is able to degrade large amounts of *cis*-DCE.

7.4 FIELD DEMONSTRATION

7.4.1 Test Site Selection

The *ideal* site for bioaugmentation with JS666 would have the following characteristics:

- Concentrations of *cis*-DCE in ground water greater than 300 µg/L to serve as a growth substrate for JS666;

- Relatively low concentrations of TCE or VC in groundwater (i.e., less than 500 μg/L) to prevent inhibition of JS666;
- Relatively shallow depth (<50 feet below ground surface [ft bgs] [15.2 m]) to minimize well installation costs;
- Neutral pH (6.5–8) to provide optimal growth conditions;
- Aerobic groundwater (oxidation-reduction potential [ORP] >0 millivolts [mV] and dissolved oxygen [DO] >1 mg/L and <8 mg/L) (Note: dissolved oxygen at low concentration is difficult to measure accurately in the field. It is easily over-estimated by introducing air into samples or, more commonly, by not waiting sufficiently for readings to stabilize. DO readings only asymptotically approach zero with standard membrane probes);
- Low groundwater ionic strength (conductivity <15 mS/cm);
- Groundwater seepage velocity of 70–180 feet per year (ft/yr) (21.3–54.9 m/yr);
- Reasonable access to utilities; and
- Reasonable site access, both in terms of proximity to a major commercial airport and ability of technical staff to work on site without escort.

Given the lack of field experience with JS666, it is not yet clear how far from the above ideal conditions one can stray. Certainly the most challenging of the criteria is that of circumneutral pH. Many groundwaters have pH < 6.5. However, this limitation may not necessarily persist. For the present, the only source of JS666 inoculum is a culture that has been grown at pH 7.2 and does not function well below pH 6.5. There is a reasonable chance of adapting JS666 to lower pH through selection approaches.

For the first pilot-scale field demonstration of JS666 as a bioaugmentation agent, a site was selected at St. Julien's Creek Annex (SJCA), Chesapeake, Virginia. For details of the field demonstration the reader is referred to the project's final report (Major et al., 2010). The location, in the Columbia aquifer, is a "trough" in two groupings of TCE isopleths where *cis*-DCE has accumulated between what are thought to be two different TCE sources. Reductive dechlorination has apparently caused formation of *cis*-DCE and lesser amounts of VC in this intermediate area, with the daughter products persisting perhaps because of presence of aerobic conditions and/or depletion of suitable electron donors (hydrogen [H_2] sources). Groundwater analyses prior to the field study showed TCE, *cis*-DCE and VC concentrations ca. <10, 800 and 2 μg/L, respectively. Groundwater pH, however, was only about 5.6–5.9 – necessitating the addition of buffer in the bioaugmentation field study. On the other hand, DO was 1.6 mg/L and ORP 79 mV; though values reflected integrated conditions across the 10 ft (3 m) depth of well screens. Depth to groundwater was 5–7 ft (1.5–2.1 m) bgs, with depth to the confining layer about 20 ft (6.1 m) bgs. Groundwater temperature was about 17°C.

7.4.2 Preliminary Microcosm Study

Before the field bioaugmentation study was conducted, access was available for collecting groundwater, but not soil in the vicinity of the planned test site and from only one well (MW-04 S) at what was to be the most upgradient, central location of the test plot.

Microcosms from the SJCA site were prepared in the following manner in 160 mL serum bottles, all bioaugmented with JS666:

1. Pure-culture controls: 50 mL MSM medium.
2. Buffered and nutrient-amended SJCA water: 5 mL 10X MSM – 45 mL SJCA groundwater.

3. pH-neutralized SJCA water: 50 mL of SJCA groundwater adjusted to pH 7.0–7.2 with NaOH. [The initial pH of SJCA groundwater was 5.65 – judged too low for JS666.]

After microcosms were prepared in duplicate, all were dosed with *cis*-DCE at a nominal concentration of 11 mg/L (5.9 μmol/bottle). Each treatment (except pure-culture controls) was matched with an uninoculated control. Microcosms were inoculated with JS666 to achieve roughly either "1X" = 7×10^6 cells/mL or "0.1X" = 7×10^5cells/mL.

All 1X- and 0.1X-inoculated microcosms with groundwater, whether buffered or not, degraded all of the *cis*-DCE within 2 and 4 days, respectively (Figure 7.5). The GW − MSM and MSM-only controls actually required *more* time to completely degrade the *cis*-DCE (data not shown). There was no degradation in any of the uninoculated controls.

7.4.3 Titration Studies with SJCA Groundwater

Given the low pH of the native SJCA groundwater (ca. 5.6–5.9), buffer addition was deemed necessary in the field study. Though carbonates are less expensive than phosphates, our studies indicate that JS666 tolerates much higher concentrations (eq/L basis) of orthophosphates than of carbonates. Testing indicated that 10 mM phosphate buffer (equimolar mixture of mono-basic and dibasic forms of orthophosphate) was required to titrate SJCA groundwater to pH 7.0. However, 20–30 mM phosphate buffer was used in the bioaugmentation field study. Such levels are consistent with typical laboratory culture conditions and should adequately adjust the native pH. Furthermore, the effect of the subsurface soil on the amount of phosphate buffer that would be necessary to achieve neutral pH could not be determined; therefore, excess buffer addition was deemed prudent.

7.4.4 Field Test

The test-plot area was installed as a pattern consisting of a 4 in. (10.2 cm) diameter injection well (which would receive JS666 inoculation − buffer), surrounded by 2 in. (5.1 cm) diameter monitoring wells spaced at 2–4-week travel-time intervals between adjacent wells (Figure 7.6). Considerably upgradient (and laterally distant also) was a control plot, consisting of a similar injection well (which would receive buffer only), with two downgradient monitoring wells.

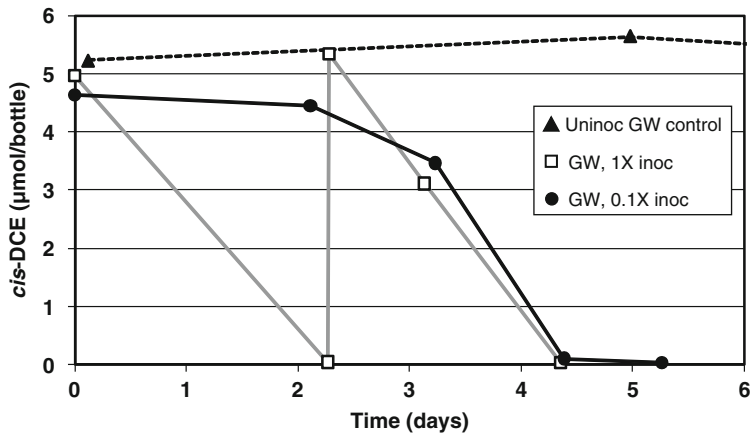

Figure 7.5. *cis*-DCE degradation in microcosms conducted on SJCA groundwater (MW-04 S) prior to start of field bioaugmentation study (adapted from Giddings et al., 2010b). Results are for pH-neutralized (NaOH) groundwater; 1X inoculation level corresponds to 7×10^6 cells/mL.

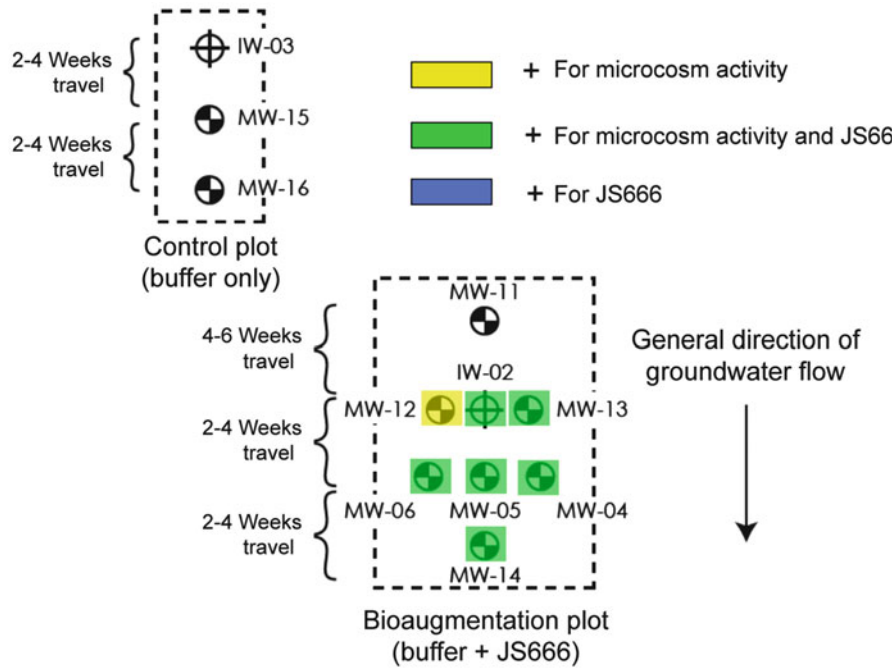

Figure 7.6. Depiction of control and bioaugmentation plots at SJCA field site.

All wells were fully screened (8–18 ft [2.4–5.5 m] bgs). A bromide-tracer study (1,000 mg/L bromide achieved in IW-02) preceded the actual bioaugmentation study.

Prior to inoculation, baseline groundwater samples were taken from all wells in the test area (control and bioaugmentation plots). Microcosms were prepared from them in triplicate; over 3 weeks of monitoring, none showed *cis*-DCE degradation activity prior to bioaugmentation. The JS666 probe (based on its isocitrate lyase gene) was employed with quantitative, real-time PCR to test for background levels (copies/mL). None was detected (limit of detection $= 10^3$ copies/mL) in any test area wells.

Groundwater was extracted (2,000 L) from each of the injection wells; buffer was added (equimolar mixture of monobasic and dibasic potassium phosphate to achieve 25 mM P). In the inoculated test plot, about 500 L of the buffered water was re-injected, followed by 20 L of concentrated JS666 inoculum, followed by the remainder of the buffered groundwater. The control plot received buffered groundwater only. A qPCR analysis of the inoculum showed that it contained JS666 at about 10^8 copies/mL. The objective was to attain about 10^6 copies/mL, when diluted by the 2,000 L of buffered groundwater. The radius of influence of the 2,000 L injection was estimated to be 3 ft (0.9 m).

The field test was run for 7 months. Unfortunately, assessment of the degree of biodegradation achieved in the field through monitoring *cis*-DCE concentrations was compromised by an almost two-fold increase in *cis*-DCE flowing into the plot during the course of the study, coupled with what turned out to be insufficient dissolved oxygen in the bioaugmentation plot. The main control on *cis*-DCE concentrations in the test area was not biodegradation but fluctuations due to pumping and/or groundwater transport processes.

Groundwater samples were acquired six times over the 7 month period following bioaugmentation. In addition to the aforementioned *cis*-DCE concentrations, these samples also were analyzed via qPCR for the presence of JS666. Microcosms also were prepared from all groundwater samples and assessed for potential *cis*-DCE degradation activity (defined as

positive if 1 mg/L added *cis*-DCE was degraded in them within 3 weeks). At the beginning, of course, results were negative for both JS666 presence and microcosm activity in downgradient samples, since the added JS666 had not yet been transported far from IW-02. On the other hand, by the end of the study, results were negative for both JS666 and microcosm activity near the injection well, as JS666 had been transported away from the site of injection over the 7 months since pulse-bioaugmentation.

Representative results (from sampling about 5 months after bioaugmentation) are summarized in Figure 7.6. Samples that were positive only for *cis*-DCE degradation in microcosms are coded in yellow; samples positive for presence of JS666, but negative for microcosm activity are coded in blue (but there were none); and samples positive for both JS666 presence and microcosm activity are coded in green. It is apparent from all of the green-coded wells downgradient of bioaugmentation well IW-02 that JS666 was successfully transported through the bioaugmentation plot and was capable of effecting *cis*-DCE biodegradation. In the first 2 months of operation, *cis*-DCE also degraded in microcosms prepared from upgradient well MW-11, though qPCR analysis of water samples and post-run microcosms showed no detectable JS666 associated with this activity. It appears that extraction/injection and buffering activities might have stimulated some short-lived, aerobic, perhaps cometabolic, degradation. The microcosm activity from downgradient samples is undoubtedly from JS666 because increases in isocitrate lyase genes of JS666 were confirmed at the end of the 3-week microcosm tests compared to levels in the water samples from which the microcosms were constructed.

Microcosm results demonstrated *in situ* survival and activity of JS666 over the course of the study in the bioaugmentation plots. Though the levels of JS666 were low (i.e., 3×10^3 to 10^4 colony forming units [CFUs]/mL), they were adequate to effect *cis*-DCE degradation if suitable environmental conditions (adequate oxygen, pH and absence of inhibitory levels of TCE) were present as was the case in microcosms constructed from samples of site material, but not in the test plot itself. Positive microcosm activity was generally correlated with detectable (by qPCR) JS666.

7.5 SUMMARY AND FUTURE PROSPECTS

The isolation of JS666 allowed the growth-coupled aerobic oxidation of *cis*-DCE to be explored as a remediation strategy at *cis*-DCE contaminated sites. Progress has been made in laboratory studies to characterize the metabolic capabilities of the organism including its ability to (1) completely mineralize high concentrations of *cis*-DCE to levels well below drinking water standards, and (2) transform mixtures of chlorinated solvents including TCE, VC, DCA, *trans*-DCE and *cis*-DCE. Knowledge from fundamental microbiological and biochemical studies was used to develop an effective protocol for growth of JS666 to high densities appropriate for bioaugmentation.

Microcosm studies demonstrated that the bioaugmentation of JS666 stimulated complete *cis*-DCE degradation in a variety of soil types. Molecular tools were developed to track JS666 in the subsurface to monitor the progress of bioaugmentation in the field. A pilot-scale field test was successful in demonstrating the spread and stability of the JS666 organisms in the bioaugmented plot, though interpretation of the field results was compromised by fluctuations in incoming *cis*-DCE concentrations and low DO. Results demonstrated that the JS666 cells maintained their potential for *cis*-DCE degradation, even when field conditions precluded activity.

Future work is needed on several fronts if the technology is to become widely applicable:

- Elucidate the *cis*-DCE degradation pathways in JS666 to enable the optimization of bioaugmentation and the ability to search for other *cis*-DCE degraders that use similar

enzymes for degradation. Determination of the pathway is the subject of a Strategic Environmental Research and Development Program (SERDP)-funded project that has yielded substantial insight, but the details of the reactions and their regulation remain to be worked out.

- Validate and optimize the molecular probe at multiple field sites to determine its ability to track JS666 in the field. Once the pathway is known, additional probes should be developed to evaluate the *in situ* activity of the key enzymes, perhaps by reverse-transcription qPCR to reveal the active genes.
- Test the efficacy of bioaugmentation with JS666 to stimulate aerobic oxidation of *cis*-DCE at multiple field sites to fully evaluate the remediation strategy and the range of conditions under which it can operate in the field.
- Determine the potential for adapting JS666 to different conditions by acclimation at the bench scale. If future work continues to support the current view that bacteria able to aerobically degrade *cis*-DCE are rare, the ability of JS666 to adapt or evolve to function under different environmental conditions of pH, temperature, ionic strength, and contaminant concentrations should be investigated in the laboratory.
- Confirm JS666 degradation enzymes and elucidate *cis*-DCE degradation pathways to develop molecular tools to search for other bacteria capable of *cis*-DCE mineralization based on sequence similarity to JS666 degradative enzymes. JS666 is the first isolate capable of coupling growth to aerobic *cis*-DCE oxidation, and results indicate that the first step in *cis*-DCE oxidation may involve a novel mechanism other than monooxygenase-catalyzed epoxidation. The molecular approach should be done in conjunction with classical strategies of selective enrichment.

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CHAPTER 8

BIOAUGMENTATION FOR THE *IN SITU* AEROBIC COMETABOLISM OF CHLORINATED SOLVENTS

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8.1 INTRODUCTION

In situ aerobic cometabolism is a method for remediating groundwater contaminated with chlorinated aliphatic hydrocarbons (CAHs) (Semprini et al., 1990; Hopkins and McCarty, 1995; McCarty et al., 1998a). The process relies on the fortuitous transformation of the CAHs by nonspecific oxygenase enzymes. The process most commonly applied in the field is to stimulate the indigenous microorganisms through additions of an appropriate cometabolic growth substrate as the electron donor, and oxygen as the electron acceptor. Field studies of this biostimulation strategy have been performed at the pilot scale and at the full scale, as described by Semprini (1997).

A few field trials also have evaluated different approaches to enhancing *in situ* aerobic cometabolism through bioaugmentation. To date, bioaugmentation has provided little benefit in these field trials, although a great deal has been learned about the reasons for its limited success so far. An earlier review of the older bioaugmentation field trials was provided by ESTCP (2005). Presented here is a review of these and other more recent field trials, as well as support work performed in microcosms and column studies. The process of cometabolism also is discussed, to provide insight into the potential benefits of bioaugmentation for aerobic cometabolism, the challenges faced when attempting to bioaugment for this process, and the reasons that several different approaches to bioaugmentation have been developed.

8.2 AEROBIC COMETABOLIC PROCESSES

A detailed review of the kinetics of aerobic cometabolism of CAHs is provided by Alvarez-Cohen and Speitel (2001), while Arp et al. (2001) review the complex biochemical processes. McCarty (1997) and Semprini (1997) review processes and results of field evaluations of the stimulation of indigenous microorganisms for enhanced aerobic cometabolism. Reviews of modeling approaches are presented by Goltz et al. (2001) and Semprini et al. (1998). The key concepts are presented in this chapter, but the reader should refer to these reviews for more detail.

Aerobic cometabolic transformation results from nonspecific enzymes fortuitously initiating the oxidation of a CAH. Presented in Figure 8.1 is a simplified representation of the process of aerobic cometabolism. The initial oxidation of a substrate such as methane, propane or butane requires a monooxygenase enzyme that also initiates the oxidation of the molecule. A common feature of the enzymes shown capable of CAH cometabolism is that they are used at the beginning of the pathway to harvest a growth substrate (Arp et al., 2001). Metabolism of the hydrocarbon to carbon dioxide and water provides the energy and carbon for cell growth. The monooxygenase enzyme initiates the oxidation of a chlorinated ethene, for example

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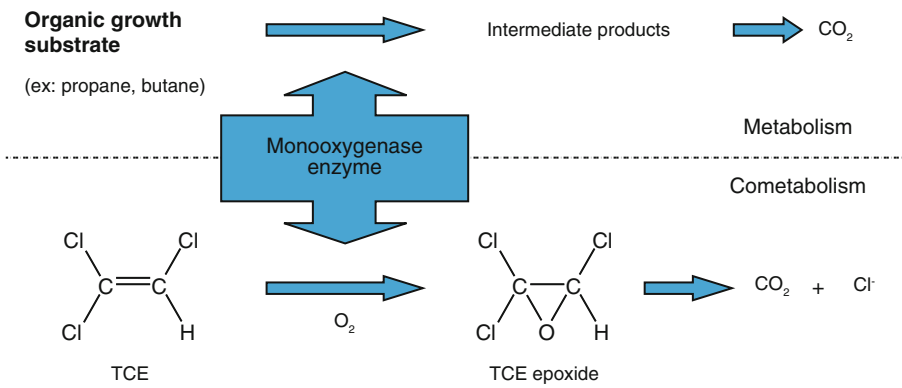

Figure 8.1. Conceptual model of the cometabolism of TCE by microbes expressing monooxygenase enzymes.

trichoroethene (TCE), by forming a TCE epoxide. The unstable epoxide breaks down and forms a range of transformation products described by Little et al. (1988) and Fox et al. (1990) and others (see review of Arp et al., 2001). In an effective cometabolic system, the end products are carbon dioxide, water and chloride ion.

Cometabolic transformations do not provide energy or carbon for organism growth, so a primary substrate must be supplied to stimulate growth of the cometabolizing microorganisms. In oxidative cometabolism, the microbes usually require the presence of the growth substrate to induce the monooxygenase enzymes, although there are constitutive systems (i.e., the enzyme is continuously produced without induction or repression) that do not require the presence of a growth substrate (Shields and Reagin, 1992).

Cometabolic biotransformation is a complex process at the whole cell and enzyme level. Potential challenges include substrate inhibition, transformation product toxicity and energy limitations (see detailed reviews of these processes in McCarty [1997], Arp et al. [2001] and Alvarez-Cohen and Speitel [2001]). Substrate inhibition is a common concern, because the growth substrate and the CAH must compete for the same enzyme site. This competition leads to inhibition of the rates of utilization of the growth substrate in the presence of the CAH, and inhibition of the transformation of the CAH in the presence of the growth substrate.

During transformation reactions, products may form that pose toxic threats to cells or enzymes, thereby inactivating them. This phenomenon, termed transformation product toxicity, may be assigned one of several parameters to account for cell/enzyme death in mathematical models. Transformation capacity (T_c) represents one such parameter, defined as the quantity of a compound that a specific mass of microorganisms can degrade before they are inactivated by toxicity from transformation products. Units of transformation capacity are typically mass of degrading substrate per mass of cells (Alvarez-Cohen and Speitel, 2001). When cultures are bioaugmented for their catalytic transformation potential, having a high transformation capacity is an important parameter (Duba et al., 1996; Steffan et al., 1999).

Finally, energy is required to sustain the organisms responsible for the desired cometabolic reactions. Oxygenase enzymes consume molecular oxygen and reductants such as NAD(P)H (reduced form of nicotinamide adenine dinucleotide phosphate [$NADP^+$]) during oxidation of the energy generating and cometabolic substrate (see review of Alvarez-Cohen and Speitel, [2001]). In the subsequent metabolism steps the reductant is regenerated. Thus, the rate and extent of the cometabolic transformation is limited in the absence of growth substrate. Some aerobic cometabolic cultures can regenerate reductant using alternate energy substrates, such as formate, or internal storage polymers such as poly-β-hydroxybutyrate (PHB). Duba et al.

(1996), for example, developed a culture of methanotrophic bacteria for bioaugmenation that had a high PHB content.

Along with physicochemical, geological and hydrological parameters, the feasibility of *in situ* bioremediation at any given contaminated site is dependent upon the capacity of the indigenous microbial population to degrade the compound(s) of interest (Jenal-Wanner and McCarty, 1997). When the indigenous microbial population is not effective at a given site, bioaugmentation might be used (Steffan et al., 1999). Bioaugmentation involves injections of desired exogenous microorganisms along with required nutrients directly into the contaminated zone. For bioaugmentation, there are two distinct methods (Steffan et al., 1999). The first method is to add the microorganisms to complement or replace the native microbial population. The goal of this approach is to achieve prolonged survival and growth of the added organisms and sustained degradation of the target pollutants. The second bioaugmentation method is to add large numbers of degradative bacteria to a contaminated site as biocatalysts to degrade a significant amount of target contaminant before becoming inactive or perishing, in which case the long-term survival and growth of an active microbial population are not required.

The most widely studied microbial process used for aerobic cometabolism is the oxidation of CAHs by methanotrophs, which are microorganisms that grow on methane and which require methane monooxygenase enzyme (MMO) for the initial transformation of methane to methanol (Arp et al., 2001). MMO also initiates oxidation of CAHs such as TCE. TCE oxidation is initiated through the formation of an epoxide, with an oxygen inserted across the carbon-carbon double bond. Epoxides are unstable in aqueous solutions and break down rapidly. In the subsurface, it is possible to stimulate microorganisms possessing soluble MMO (sMMO) and particulate MMO (pMMO). Microbes expressing sMMO are typically stimulated under conditions of limited copper. Studies of CAH transformation typically show faster rates of transformation are achieved when sMMO is expressed compared to pMMO (Alvarez-Cohen and Speitel, 2001). When type I methanotrophs that possess pMMO are predominately stimulated *in situ*, limited TCE transformation has been observed (Baker et al., 2001).

The other microbial system that has been studied in great detail is microorganisms that are stimulated on phenol or toluene where toluene monooxygenase (TMO) or toluene dioxygenase (TDO) is expressed. The TMO enzymes are responsible for the addition of a hydroxyl group to the ring structure of toluene or phenol or the methyl group to initiate the oxidation of toluene. Microbes can possess *o*-, *p*- or *m*-TMOs depending on the location at which the hydroxyl substitution occurs. Different TMOs exhibit different rates of TCE cometabolism (see reviews of Alvarez-Cohen and Speitel [2001] and Arp et al. [2001]).

Aerobic cometabolism is best suited for *in situ* remediation for CAH contamination at concentrations of approximately 1 milligram per liter (mg/L) or less, but well above the drinking water standard for most of the contaminants. Compounds for which aerobic cometabolism has been evaluated in laboratory and field studies include the chlorinated ethenes (TCE, *cis*-1,2-dichloroethene [*cis*-DCE], *trans*-1,2-dichloroethene [*trans*-DCE], 1,1-dichlorothene [1,1-DCE], and vinyl chloride [VC]); the chlorinated ethanes (1,1,1-trichloroethane [1,1,1-TCA] and the lower chlorinated ethane isomers); and the chlorinated methanes (chloroform [CF] and the lower chlorinated methanes) (Semprini, 1997). Perchloroethene (PCE) is not susceptible to aerobic cometabolic transformation (Semprini, 1997).

Stimulating indigenous microorganisms through primary substrate addition has been the most commonly applied form of *in situ* aerobic cometabolism. Pilot-scale field studies have demonstrated the potential for stimulating indigenous methane-utilizing microorganisms (Semprini et al., 1990, 1991), phenol-utilizing organisms (Hopkins et al., 1993a, b) and toluene-utilizing organisms (Hopkins and McCarty, 1995). Large-scale demonstrations of the

stimulation of indigenous organisms were conducted with methane-utilizing microorganisms at the Savannah River Department of Energy (DOE) Site, South Carolina (Hazen et al., 1994) and at Edwards Air Force Base (AFB), California with toluene-utilizing microorganisms (McCarty et al., 1998a). The results of microcosm and field studies of biostimulation for aerobic cometabolism are reviewed briefly in the following section to provide a background for understanding the potential benefits and challenges for the different bioaugmentation approaches that have been developed.

8.3 AEROBIC COMETABOLISM BY INDIGENOUS MICROORGANISMS

8.3.1 Microcosm Studies with Indigenous Microorganisms

Microcosm studies using aquifer solids and groundwater from sites have proven useful for predicting the performance of *in situ* cometabolic treatment where indigenous microbial populations were stimulated. Packed column studies with aquifer solids and groundwater from the Moffett Test Facility, California performed by Lanzarone and McCarty (1990) showed that stimulation of indigenous methane utilizers did not effectively transform TCE, which was consistent with the results observed in the pilot-scale field demonstration (Semprini et al., 1990). Hopkins et al. (1993a, b) also found good agreement between microcosm results and the *in situ* pilot-scale studies where TCE was effectively cometabolized by indigenous microcosms stimulated through either phenol or toluene addition. The transformation yields (mg TCE/mg substrate) achieved in the *in situ* field tests agreed with those obtained in microcosms. Jenal-Wanner and McCarty (1997) showed TCE removal (93–94%) in their microcosms, which was close to the field-scale results from the Moffett Test Facility when similar amounts of phenol and toluene were supplied (Hopkins and McCarty, 1995). Microcosm studies with aquifer material and groundwater from Edwards AFB (Jenal-Wanner and McCarty, 1997) also agreed with the TCE removals of 87–100% measured after toluene additions in the large-scale field test performed at the site (McCarty et al. 1998a).

The results from batch/slurry microcosms that were supplied with propane (Timmins et al., 2001) also agreed with observations from a pilot-scale field demonstration of cometabolic sparging performed at McClellan AFB, California (Tovanabootr et al., 2001; Connon et al., 2005). Lag times in the microcosms of several weeks were similar to those observed in the field. In addition, *cis*-DCE was transformed more rapidly than TCE, which was consistent with the results from the field tests. The agreement between microcosm and field tests when testing biostimulation for aerobic cometabolism led to the use of similar column and microcosm tests for evaluating bioaugmentation approaches, as will be discussed Section 8.4.

8.3.2 Field Studies with Indigenous Microorganisms

The most detailed field studies evaluating *in situ* aerobic cometabolism by indigenous microorganisms have been performed at the Moffett Test Facility and at Edwards AFB, both located in California. Studies at the Moffett Test Facility were conducted at the pilot scale in a shallow alluvial aquifer under induced gradient conditions of injection and extraction. Since indigenous microorganisms were stimulated on a broad range of substrates under a consistent set of test conditions, the results illustrate why bioaugmentation has been considered for specific cases. All of the tests were performed using the same experimental approach, in which the growth substrates and oxygen were added as dissolved components in the injected groundwater. The CAHs of interest also were added at known concentrations to

the injected groundwater, along with conservative tracers, so that accurate estimates of the degree of removal could be performed.

Table 8.1 provides a summary of the tests performed at the Moffett Test Facility, the substrates used, the CAH tested, the extent of treatment achieved and some key observations. A broad range of growth substrates were tested including methane, phenol, toluene and butane. Oxygen was added as pure oxygen dissolved in water or as hydrogen peroxide (H_2O_2). A range of chlorinated ethenes (TCE, *trans*-DCE, *cis*-DCE, 1,1-DCE and VC) and chlorinated ethanes (1,1,1-TCA and 1,1-dichloroethane [1,1-DCA]) were evaluated.

Studies were first performed using methane as a growth substrate and the transformation of mixtures of chlorinated ethenes (TCE, *trans*-DCE, *cis*-DCE and VC) was evaluated along with 1,1,1-TCA as a background contaminant. Methane utilization was observed after about 10 days of addition. In successive seasons of testing, methane utilization was much more rapid, indicating the indigenous microorganisms stimulated in the previous season were still present in the test zone. The degrees of treatment achieved were compound specific with very effective removal of VC and *trans*-DCE, followed by *cis*-DCE, with limited removal of TCE. 1,1,1-TCA was not transformed. The tests demonstrated that treatment to drinking water standards of VC (less that 2 μg/L) could be achieved. Inhibition of the rates of cometabolic treatment with methane as the primary substrate was observed and the addition of energy yielding substrates, such as formate and methanol that were non-inhibitory, resulted in temporary enhanced transformation. Cometabolism was strongly linked to methane utilization, demonstrating that the continuous addition of substrate was needed to promote cometabolism. Microbial growth and cometabolic treatment were achieved close to the injection well. The results obtained with methane also were consistent with observations from laboratory microcosms and columns. The pattern of contaminant transformation, of limited TCE and no 1,1,1-TCA removal, and *trans*-DCE transformed to a greater extent than *cis*-DCE, also suggested that microorganisms that express pMMO likely were stimulated since conditions of copper limitation required for the expression of sMMO likely did not exist (Semprini, 1997).

Studies conducted at the Moffett Test Facility with indigenous microorganisms grown on phenol showed greater potential for the treatment of TCE and *cis*-DCE with up to 90% removal achieved. VC also was very effectively removed. Concentrations up to 1,000 μg/L of TCE could be effectively transformed and greater extents of transformation could be achieved through the addition of more phenol. The maximum transformation yield reached 0.06 g TCE/g phenol, indicating that effective cometabolic treatment could be achieved. This value compared with a value of 0.11 g TCE/g phenol observed with a mixed phenol utilizing culture derived from the field site (Hopkins et al., 1993b). Like the methane tests, about 10 days were required for effective phenol utilization and cometabolism to be achieved. The results demonstrated effective utilization of TCE, *cis*-DCE and VC. In addition, *cis*-DCE and VC, which are often present as anaerobic transformation products, also could be effectively transformed. The results of laboratory studies in microcosms using mixed cultures enriched from the site groundwater were consistent with those obtained in the field with respect to the transformation potential of the compounds tested.

A later study at the Moffett Test Facility (Hopkins and McCarty, 1995) demonstrated that indigenous microorganisms stimulated on toluene were as effective as those stimulated on phenol in promoting TCE, *cis*-DCE and VC transformation. When toluene was transformed, transient evidence of the formation of *o*-cresol, and not *m*- or *p*-cresol, indicated that *o*-toluene monooxygenase (*o*TOM) was expressed. *o*TOM is the same oxygenase used by *Pseudomonas cepacia* G4 for phenol and toluene oxidation, and this microorganism is one of the most effective in transforming TCE with respect to transformation yield and rates (Alvarez-Cohen and Speitel, 2001). 1,1-DCE also was transformed, but its transformation resulted in transformation product toxicity, thereby decreasing the removal of TCE from over 90% to around 50%.

Table 8.1. Pilot Studies Performed at the Moffett Test Facility, California for Aerobic Cometabolism of CAHs Using Indigenous Microorganisms

Study	Growth Substrates – Average Injected Concentration (mg/L)	CAH	Injected Concentration (μg/L)	Percent Removal (%)	Key Findings
Semprini et al. (1990)	Methane (5.3) Oxygen (23.4)	TCE *trans*-DCE *cis*-DCE VC	42–51 52–112 91–136 44	20–30 80–90 45–55 90–95	• Cometabolism was concurrent with methane utilization • Less halogenated ethenes were transformed to a greater extent • TCE removal was limited • 1,1,1-TCA was not transformed
Semprini et al. (1991)	Methane (6.6) Oxygen (21.3)	TCE *trans*-DCE *cis*-DCE VC	46 52 100 34	20 90 50 95	• Results were consistent with previous tests • Methane inhibition of CAH transformation • Rates were temporally increased when a non-competing substrate was added
Hopkins et al. (1993a)	Phenol (6.2–12.4) DO (35)	TCE *cis*-DCE	40 45	63–90 90	• Microorganisms stimulated on phenol more effectively transformed TCE and *cis*-DCE than those on methane • Greater removals were observed as phenol concentrations were increased
Hopkins et al. (1993b)	Phenol (12.5–25) DO (35)	TCE	62–1,000	88–90	• Effective first-order removal was achieved over a broad range of TCE concentrations • A maximum transformation yield of 0.062 g TCE/g phenol was achieved
Hopkins and McCarty (1995)	Toluene (9) Phenol (12.5) DO (32) H_2O_2 (97)	TCE *trans*-DCE *cis*-DCE 1,1-DCE	250 125 125 65	50–90 74 90 50	• Toluene was as effective as phenol in achieving TCE and *cis*-DCE treatment • 1,1-DCE transformation produced transformation toxicity that lowered TCE removal efficiency • 1,1,1-TCA as a background contaminant was not transformed
Semprini et al. (2007b)	Butane (3.5–8.5) DO (20)	1,1-DCE 1,1-DCA 1,1,1-TCA	65 200 140	86 0 0	• 1,1-DCE removal as observed • 1,1,1-TCA and 1,1-DCA were not transformed

[a]TCA was present as a background contaminant at the Moffett Test Facility at a concentration of 46 μg/L. 1,1,1-TCA was not transformed to a significant extent in any of the tests cited in Table 8.1

Note: μg/L–microgram(s) per liter; DO–dissolved oxygen; g–gram(s)

Hopkins and McCarty (1995) noted that 1,1-DCE toxicity also was observed in laboratory studies with methane utilizing microorganisms. 1,1,1-TCA was not transformed by additions of either phenol or toluene.

A recent study at the Moffett Test Facility using butane as a growth substrate evaluated the potential for 1,1-DCE, 1,1-DCA and 1,1,1-TCA cometabolism (Semprini et al., 2007a, b; 2009). Consistent with earlier observations, the time required for observable stimulation of indigenous butane utilizers was about 2 weeks, while in the second season of testing this time was reduced to just a few days, indicating that microorganisms stimulated in the first season of testing were still present in the test zone. Approximately 80% of 1,1-DCE was transformed, but no evidence for 1,1-DCA or 1,1,1-TCA was indicated. The results suggest transformation product toxicity likely resulted from 1,1-DCE cometabolism.

Larger scale demonstrations of *in situ* TCE treatment also have been conducted at Edwards AFB through the stimulation of indigenous toluene utilizers (McCarty et al., 1998a). Groundwater contaminated with 500–1,200 µg/L TCE was treated *in situ* through the pulsed injection of pure toluene and concentrated hydrogen peroxide to give time average concentrations of 3.8–13.4 mg/L toluene and 29–44 mg/L DO. Groundwater was circulated between two contaminated aquifers through two treatment wells located 10 meters (m) (30 feet [ft]) apart (Figure 8.2). Each well was screened at two depths, with a submersible pump installed in between. An *in situ* bioactive zone was created in the aquifer around the discharge screen of each treatment well. The demonstration showed that effective long-term treatment within the contaminant plume could be achieved with removal of 97–98% of the TCE.

Despite the successful demonstrations at both the Moffett Test Facility and Edwards AFB, cometabolic treatment of TCE through toluene- or phenol-induced cometabolism has not been

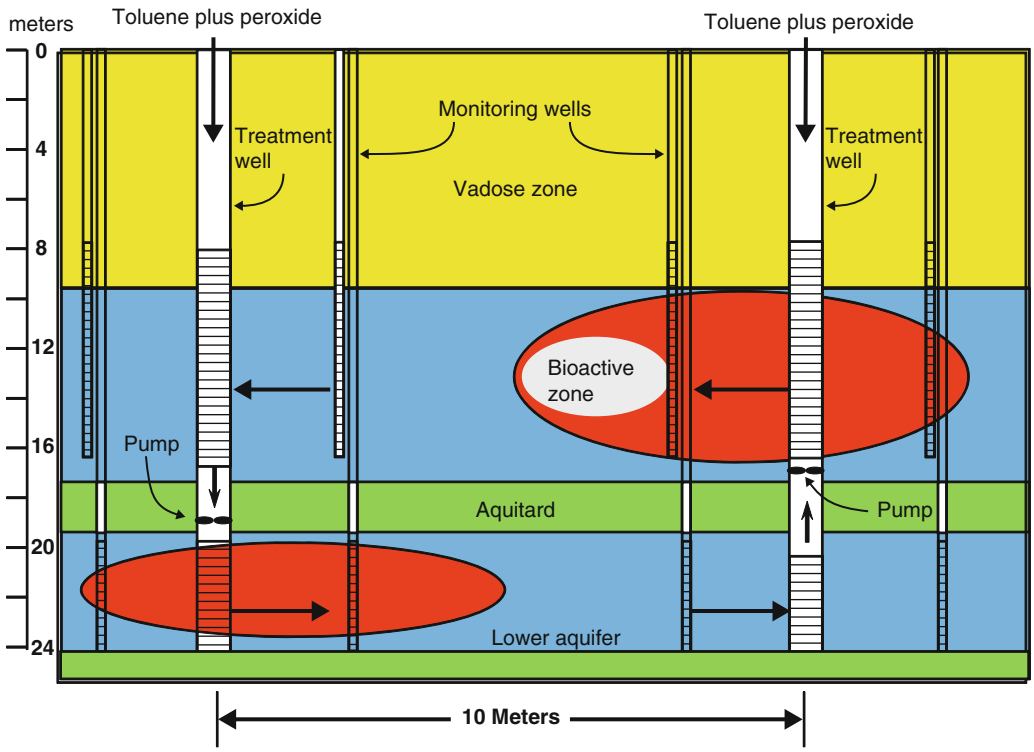

Figure 8.2. Well recirculation system that can be applied to bioaugmentation systems for aerobic cometabolism (from McCarty et al., 1998a).

widely used by practitioners. One likely reason is the requirement for adding regulated compounds, or compounds that could cause taste and odor problems. Toluene is a regulated compound with a drinking water standard of 1,000 µg/L and has a taste and odor threshold of 40 µg/L (Pontius, 1992). There is no maximum contaminant limit (MCL) or maximum contaminant limit goal (MCLG) for phenol, but as discussed by Hopkins and McCarty (1995), the 1962 U.S. Public Health Service (PHS) standard was 1 µg/L based on taste and odor threshold and there is potential for the formation of chlorinated phenols upon chlorination. The other reason is the potential complexity of the process that requires (1) both primary substrate and oxygen addition and (2) having effective means for mixing the contaminated groundwater with the biostimulated zones that are created in the subsurface.

The following key observations from the Moffett Test Facility pilot studies and Edwards AFB demonstration illustrate some possible roles bioaugmentation can play to enhance aerobic cometabolic treatment:

1. Several weeks of substrate and DO addition were required to stimulate the indigenous cometabolic population.
2. Chlorinated ethanes, including 1,1,1-TCA and 1,1-DCA, were not effectively transformed by indigenous microorganisms stimulated on methane, phenol, toluene or butane. Phenol and toluene utilizing microorganisms were most effective for TCE treatment.
3. Competitive inhibition of CAH transformation by the growth substrate was observed.
4. Treatment performance was strongly tied to the consumption of the growth substrate, since energy is required to drive the cometabolic transformation.
5. Methane utilizers were not effective at transforming TCE, which likely resulted from pMMO being expressed.
6. Transformation product toxicity resulted from the oxidation of 1,1-DCE, and likely from TCE oxidation as well.
7. Bioactive zones were created close to the injection location, thus bioaugmentation cultures need not be transported long distances for effective treatment to be achieved.

Based on these observations, bioaugmentation might be used to:

1. Shorten the lag times observed during biostimulation.
2. Achieve effective treatment of chlorinated ethanes through addition of effective strains.
3. Eliminate the need to add phenol or toluene by adding strains that constitutively express TMO.
4. Limit inhibition by adding strains that constitutively express the oxygenase of interest when grown on non-inhibiting substrates.
5. Eliminate the need to add primary growth substrates through bioaugmentation with strains selected for their biocatalytic potential.
6. Provide controlled geochemical conditions during *ex situ* growth in order to add strains that have high biocatalytic potential.
7. Improve treatment by adding strains that are less susceptible to transformation product toxicity.
8. Create biobarriers and recirculation systems (Figure 8.2) where the effective strains are close to the location of substrate addition, but achieve very effective contaminant treatment.

Table 8.2. Characteristics of Cometabolism and Their Importance to Treatment Using Indigenous and Bioaugmented Microorganisms

Aerobic Cometabolic Property	Importance for the Indigenous Process	Importance for Bioaugmentation
Aerobic cometabolism is often initiated by oxygenase enzymes, which are most often induced by the growth substrate	Contaminant and growth substrate usually compete for same enzyme resulting in competitive inhibition and the reduction in the rates of transformations	Competitive inhibition might be prevented if microorganisms are added that can grow constitutively on non-inhibiting substrates
Cometabolic transformation requires the presence of oxygen	Continuous addition of oxygen is required	Injection of high cell mass for enzyme activity may deplete the needed oxygen
Cometabolic transformation usually drains energy from the microorganism	Cometabolic transformation is not maintained for long periods after growth substrate addition is stopped	Microbial strains that effectively store energy may have potential advantages over indigenous strains
Rates of transformation can vary widely among different microbial strains and different oxygenase systems	Performance is highly dependent on the indigenous microbial populations stimulated under field conditions	Cultures that effectively transform the contaminants of interest might be bioaugmented
Transformation product toxicity can occur where the products formed from the transformation can be toxic to the cells	Provides potential advantages to indigenous microorganisms that consume the growth substrate but do not transform the contaminant(s) of interest	Cultures might be added that effectively transform the contaminants of interest but are more resistant to transformation product toxicity
Transformation yields (ratio of mass of contaminant transformed to mass growth substrate consumed) can depend on the contaminant cometabolized and the microbial cultures	Performance depends greatly on the microbial populations stimulated under subsurface conditions and can vary greatly from site to site	Microbial cultures might be added that have high transformation capacities for the contaminants of interest.
Primary growth substrates are often not present in the subsurface	Low indigenous microbial populations are present resulting in long lag times before substrate consumption is observed	Bioaugmentation of the desired strains would shorten the lag period for biostimulation and cometabolic transformation
Geochemical conditions play an important role in the communities stimulated and types of oxygenases expressed	Indigenous populations stimulated through substrate addition may not be effective in transforming CAHs of interest	Subsurface microbial strains that are effective under the site's geochemical conditions might be added

Table 8.2 lists these and other important characteristics of the aerobic cometabolic process, as indicated by the Moffett Test Facility and Edward AFB demonstrations as well as supporting laboratory studies with microcosms and mixed and pure culture studies. Some of the challenges related to aerobic cometabolism that may be overcome through bioaugmentation also are presented in Table 8.2 as well as some of the those for which bioaugmentation is problematic.

8.4 BIOAUGMENTATION APPROACHES

In order to overcome some of the problems associated with the stimulation of indigenous microorganisms for aerobic cometabolism, four bioaugmentation approaches have been tested:

Approach I. Bioaugmentation with strains selected for their biocatalytic transformation ability and grown in aboveground bioreactors prior to injection

Approach II. Bioaugmentation with strains that express the desired oxygenase enzyme constitutively while maintained on a benign and non-inhibiting growth substrate

Approach III. Bioaugmenation with strains that are more capable of cometabolizing the contaminants when grown on an inducing growth substrate than the indigenous strains

Approach IV. Bioaugmentation with indigenous strains through injections of groundwater from active areas into other areas of a site

These approaches range from the fairly complicated method in Approach I, where strains are grown under very controlled conditions in aboveground reactors and are injected for their biocatalytic potential, to the very simple method in Approach IV of adding microbes from one aquifer or section of an aquifer to another to seed an indigenous strain. Approaches II and III involve addition of selected strains that have specific advantages over the indigenous strains. Approach II would permit adding non-inhibiting substrates, or would eliminate the need to add toxic substrates such as phenol or toluene. Approach III involves adding strains that would be effective for resistant CAHs such as 1,1,1-TCA and 1,1-DCE that cannot be treated effectively by stimulating the indigenous microorganisms. Laboratory and field scale examples of each of these approaches are presented in the following sections.

8.4.1 Bioaugmentation Approach I

Approach I tries to overcome the complexities of competitive inhibition and subsurface environmental conditions by growing the microorganisms in surface bioreactors for their biocatalytic potential. Cultures are selected for their high transformation capacity, ability to remain active for long periods after injection, ease of growth to high cell densities on inexpensive substrates, and for their specific transport properties. Transport properties may include both good adhesion (to form a biobarrier) or poor adhesion (to permit cells to be transported further after injection). In the following sections, the results of microcosm, column and field studies of this approach are presented.

8.4.1.1 Bioaugmention with *Methylosinus trichosporium* OB3b

Duba et al. (1996) evaluated bioaugmentation with *Methylosinus trichosporium* OB3b, a methanotrophic bacterium that expresses sMMO, in both column and field tests. For the field test, a large number of bacteria were grown aboveground and then injected along a transect to create an *in situ* biofilter at a TCE-contaminated site. They concluded that the performance of the biofilter of resting cells depended on several factors, including the transformation capacity of the cells (g CAH/g cell), longevity of the enzyme system to maintain the transformation ability, and the attachment and detachment of bacteria to the biofilter matrix.

The bacterial strain was selected because it is naturally occurring and nonpathogenic, it had high initial transformation rates of TCE and high resting cell transformation capacities of ~0.25 mg of TCE per mg dry weight of cells. The strain had adequate attachment/detachment properties to create an *in situ* biofilter and could be grown effectively *ex situ* in bioreactors to high cell densities. Laboratory studies also indicated that the cells maintained TCE

transformation activity for up to 6–7 weeks when stored as cell suspensions and sustained 70% of their activity for up to 21 days (Taylor et al., 1993; Shah et al., 1996).

8.4.1.2 Column Studies with *Methylosinus trichosporium* OB3b

Cells having favorable attachment characteristics (i.e., they attach readily and are slow to detach) are desirable for developing a biofilter of resting cells for aerobic cometabolism (Hanna and Taylor, 1996). The attachment/detachment of a rosette-dominated form of *Methylosinus trichosporium* OB3b and the longevity of the resting cells' ability to transform TCE was evaluated in small 1 centimeter (cm) × 10 cm columns packed with a quartz-sand (Hanna and Taylor, 1996) (Table 8.3). Because the chemical composition of the solution affects the attachment/detachment, a medium containing phosphate buffer at pH 7 was employed. Initial attachment resulted in a cell concentration of 7 to 8 × 10^8 cells/g of dry sand. Including1.0 millimolar (mM) magnesium chloride ($MgCl_2$), 100 micromolar (μM) ferrous sulfate ($FeSO_4$) and 0.025% agar resulted in an increase in the attached cell concentration to 1.5 × 10^9 cell/g dry sand. These additions increased the time to reach 50% detachment from 5 days to ~ 45 days. About 34% of the cells were retained on the biofilter for about 15 weeks. Studies of TCE cometabolism showed weekly pulses of 250 μg/L of TCE were transformed to below the drinking water standard of 5 μg/L for a period of 8 weeks. These tests demonstrated that a biofilter could be operated for up to 8 weeks before needing replenishment, and the replenishment was needed primarily because the sMMO enzymes were slowly inactivated over time.

Tompson et al. (1994) also evaluated the addition of *Methylosinus trichosporium* OB3b expressing sMMO for its resting cell cometabolic transformation potential (Table 8.3). A 10-cm thick biofilter was created by injecting bacteria into a sand pack through five wells. A steady flow of groundwater at a velocity of 1.5 cm/hour (h) was established through the filter and TCE was added as 4 mg/L pulse input every 13 days. TCE cometabolism was found to be complete from 0 to 5 days and then became increasingly limited from 5 to 15 days. The results of the test were simulated using an advective-dispersive transport model that included Michaelis-Menton kinetics and a limited transformation capacity model for TCE transformation. Based on simulation fits to the observed breakthrough of TCE at the downstream monitoring wells, the transformation capacity was estimated to be 0.30 g TCE per g cells. The authors indicated that this capacity would not limit the application of this technology in the field.

8.4.1.3 Field Study: Resting Cell Biofilter Using *Methylosinus trichosporium* OB3b

The field test conducted at the Chico Municipal Airport, California, involved injection of about 5.4 kilograms (kg) (dry weight) of cells suspended in 1,800 L of groundwater, to achieve a cell injection concentration of 5.4 × 10^9 cells/mL (Duba et al., 1996) (Table 8.4). The cells were delivered to the site as a cold paste and diluted into site groundwater and injected at a rate of 3.8 L/min for about 8 h, followed by the addition of groundwater without cells to help spread the cells further into the formation. At the end of the injection process, the injection well was turned into an extraction well and groundwater was extracted for 30 h at a rate of 3.8 L/min, followed by a rate of 2 L/min of 38 days.

Measurements of the suspended cells in the extracted groundwater indicated that the bioaugmentation culture became attached to the aquifer solids. As shown in Figure 8.3, background concentrations of TCE in the groundwater of 425 μg/L decreased to less than 10 μg/L during the first 50 h of extraction and then gradually increased to the background level

Table 8.3. Microcosm and Column Studies of the Bioaugmentation Process

Study	Bioaugmentation Process	Type of Study	Culture Added	Contaminant Added	Substrate or Cells Added
Hanna and Taylor (1996)	Approach I Resting cell biocatalysis activity	Continuous flow column	*Methylosinus trichosporium* OB3b	TCE	Resting cells (7×10^8–1.5×10^9 cell/g dry sand)
Tompson et al. (1994)	Approach I Resting cell biocatalysis activity	Continuous flow column	*Methylosinus trichosporium* OB3b	TCE (4 mg/L)	Resting cells (2×10^8 cell/g dry sand)
Munakata-Marr et al. (1996, 1997)	Approaches I and II Biocatalysis activity Strain with constitutively expressed oxygenase	Batch exchanged column Aquifer/groundwater	*Burkholderia cepacia* G4 *B. cepacia* $PR1_{23}$ and $PR1_{301}$	TCE (250 μg/L)	Lactate (15 mg/L) Phenol (6.5 mg/L) 70 μg of cells
Synder et al. (2000)	Approach I and II Resting cell and substrate	Continuous flow column	*Burkholderia cepacia* G4	TCE (66–100 μg/L)	6.3–6.6 $\times 10^7$cells/mL Phthalate (4 mM)
Komlos et al. (2004)	Approach II Biofilm cometabolism	Continuous flow column	*Burkholderia cepacia* PR1 and *Klebsiella oxytoca*	TCE (1.1–1.2 mg/L)	*B. cepacia* 5.7×10^{10} CFU *K. oxytoca* 1.6×10^{11} CFU 30 to 70 mg/L DOC
Jitnuyanont et al. (2001)	Approach III Strain with enhanced transformation abilities	Batch slurry Aquifer/groundwater	Mixed culture	1,1,1-TCA (50–250 μg)	Butane (5 mg)

(*continued*)

Table 8.3 (continued)

Study	Bioaugmentation Process	Type of Study	Culture Added	Contaminant Added	Substrate or Cells Added
Semprini et al. (2007a, b)	Approach III Strain with enhanced transformation abilities	Batch slurry Aquifer/groundwater	*Rhodococcus sp.*	1,1,1-TCA (60 μg) 1,1-DCE (60 μg) 1,1-DCA (60 μg)	Butane (9 mg)
Frascari et al. (2007)	Approach III Strain with enhanced transformation abilities	Batch slurry Aquifer/groundwater	Mixed culture *Rhodococcus aetherovorans* BCP1	CF (1.0–1.12 mg/L)	Butane (1.5–1.8 mg/L)
Frascari et al. (2006) Gualandi et al. (2007)	Approach III Strain with enhanced transformation abilities	Batch slurry Aquifer/groundwater	Mixed culture	VC (1,567 μg/L) *trans*-DCE (331 μg/L) *cis*-DCE (302 μg/L) 1,1,2-TCA (40 μg/L) TCE (250 μg/L) 1,1,2,2-TeCA (25.3 μg/L)	Methane (0.5–2.3 mg/L) Propane (0.48–2.2 mg/L)
Semprini et al. (2005)	Approach III Strain with enhanced transformation abilities	Continuous flow column	*Rhodococcus sp.*	1,1,1-TCA (200–400 μg/L) 1,1-DCE (130 μg/L)	Butane (3–5 mg/L)
Takeuchi et al. (2005)	Approach IV Priming with groundwater from a different aquifer	Batch groundwater microcosms	Mixed culture	*cis*-DCE (10–12 μg/L)	Methane (0.2–1 mg/L)

Note: 1,1,2-TCA – 1,1,2-trichloroethane; 1,1,2,2-TeCA – 1,1,2,2–tetrachloroethane; CF – chloroform; CFU – colony forming unit(s); DOC – dissolved organic carbon; mL – milliliter

Table 8.4. Field Studies Evaluating Bioaugmentation for Enhanced *In Situ* Aerobic Cometabolism

Study	Bioaugmentation Approach	Microbe Bioaugmented Oxygenase	Remediation Method	Contaminant Concentration (μg/L)	Substrates Added
Duba et al. (1996)	Approach I Biocatalysis activity	*Methylosinus trichosporium* OB3b sMMO Methane	*In situ* biofilter around a single well	TCE (425)	None
Steffan et al. (1999)	Approach I Biocatalysis activity	*Burkholderia cepacia* ENV435 *o*-TMO constitutive	Distributed injection of culture	TCE, *cis*-DCE and VC Sum (2,200)	None
Walsh et al. (2000)	Approach I and II Biocatalysis activity	*Burkholderia cepacia* ENVE435 *o*-TMO constitutive	Injection combined with pneumatic fracturing	TCE (5,000–10,000)	Liquid carbon source
Tani et al. (2002)	Approach I Biocatalysis activity	*Ralstonia eutropha KT1*	Injection at a single well	TCE (200)	None
Bourquin et al. (1997)	Approach I + II Resting cell biocatalysis activity and substrate addition	*Burkholderia cepacia* $PR1_{301}$ *o*-TMO constitutive	Continuous injection injection/extraction well	TCE (125) *cis*-DCE (95) *trans*-DCE (4)	None Glucose

(*continued*)

Table 8.4. (continued)

Study	Bioaugmentation Approach	Microbe Bioaugmented Oxygenase	Remediation Method	Contaminant Concentration (μg/L)	Substrates Added
McCarty et al. (1998b)	Approach II Strain with constitutively expressed oxygenase	*Burkholderia cepacia* $PR1_{301}$ *o*-TMO constitutive	Daily culture addition and continuous substrate addition	TCE (80)	Lactate Phenol
Semprini et al. (2007a, b)	Approach III Strain with enhanced transformation abilities	*Rhodococcus* sp. BMO	Single culture addition with continuous substrate addition	1,1-DCE (65) 1,1,1-TCA (140) 1,1-DCA (130)	Butane
Semprini et al. (2009)	Approach III Strain with enhanced transformation abilities	*Rhodococcus* sp. BMO	Single culture addition with continuous substrate addition	1,1,1-TCA (80–140) 1,1-DCE (4–12)	Butane
Takeuchi et al. (2004; 2005)	Approach IV Groundwater containing methane utilizers	Indigenous methanotrophs	Methane addition and addition of indigenous microorganisms	TCE (128) *cis*-DCE (35)	Methane

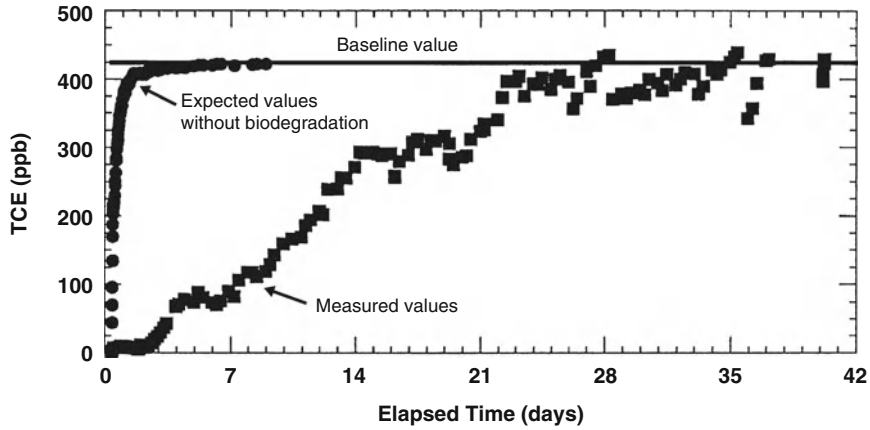

Figure 8.3. Long-term performance of the biofilter inferred from TCE measurements on the extracted fluid with bioaugmentation of *Methylosinus trichosporium* OB3b for its biocatalytic potential (from Duba et al., 1996).

of 425 μg/L. About 20 g of TCE was transformed, which represented about 40% of the TCE that passed through the biofilter. Based on laboratory studies, the injected biomass had the potential to transform 420 g of TCE.

DO concentrations remained at about 5 mg/L, but at some locations decreased to about 1 mg/L, which was likely problematic for TCE transformation. Reasons for the reduced performance were not identified, but were likely associated with reduced enzyme activity, a reduction in the attached bacterial population, reduced transformation capacity in the natural environment, or the low DO levels. The authors did not believe that it was related to grazing by protozoans based on their enumeration, which represented about 2% of the total numbers on day 38. No attempt was made to enhance oxygen addition to the subsurface, which may have helped performance. Although TCE transformation of 98% was demonstrated, TCE was removed to below regulatory levels for only 2 days, although in the laboratory testing removal to below these levels continued for several weeks (Tompson et al., 1994; Hanna and Taylor, 1996).

8.4.1.4 Bioagumentation with *Burkholderia cepacia* ENV435

Another field study evaluated a biofilter of resting cells capable of cometabolic transformation of TCE. In this case, an adhesion-deficient strain of *Burkholderia cepacia* ENV435 was used. ENV435 has the ability to transform chlorinated ethenes in the absence of an inducing cosubstrate (Steffan et al., 1999) because it produces toluene *o*-TMO constitutively (Munakata-Marr et al., 1996). The variant selected was adhesion deficient, facilitating transport further into the aquifer after injections (DeFlaun et al., 1998).

The adhesion-deficient mutant strain ENV435 was obtained by passing cells of strain *Burkholderia cepacia* G4 PR1$_{301}$ through columns packed with sediment from Savannah River or Roseland aquifer sediment (DeFlaun et al., 1998). After passing the cells through the sand columns six times, the percentage of PR1$_{301}$ retained on the column decreased from 100% to 34%, while 80 to 93% of the wild type G4 or the parent strain of G4 were retained. Transport studies with the selected strain in sand and sediment columns showed the wild type strain of G4 was retained or highly retarded, while ENV435 was transported like the conservative chloride tracer. Using hydrophobic interaction chromatography, DeFlaun et al. (1998) found that G4 was significantly more hydrophobic than ENV435. The highly active ENV435 was grown to have storage polymers (high-energy polyalkanoate) to drive the cometabolic

transformation. Such polymers can provide a long-term supply of reducing energy (i.e., NADH) to drive the cometabolic process, without added growth substrates (Henrysson and McCarty, 1993).

For the field trials, the bioaugmented culture was grown in a 750-L fermentor in 550 L of basal salt media that contained (1.6% w/w) sucrose as a substrate, and fed alternating batches of sucrose and phenol as carbon sources. Bacterial storage polymers were produced naturally, as the ammonium in the reactor became depleted.

The field test was conducted at an industrial facility in Pennsauken, New Jersey, in a heterogeneous aquifer consisting of silty fine to medium sand interspersed with thin lenses of clay. The site was contaminated with a mixture of chlorinated ethenes including PCE, TCE, DCE and VC, and chlorinated ethanes including DCA and TCA. The test plot consisted of a treatment plot that was bioaugmented with ENV435 and a control plot that did not received ENV435. The plots measured 4.6 m wide and 12 m long, and were separated by 9.2 m and contained six injection wells, a recovery well located 12 m downgradient from the injection well, and nine monitoring wells located in between the injection and monitoring wells.

Oxygen was delivered either by adding pure oxygen to extracted groundwater prior to reinjection, or by directly adding gaseous oxygen to the injection wells. In the first trial, ENV435 was added to upgradient injection wells at concentrations of 1.2×10^{11} CFUs/mL. The second trial involved injecting ENV435 under pressure into selected monitoring wells to distribute cells throughout the test zone. Prior to and after the inoculum injection, compressed oxygen was used to force the culture into the formation. Oxygen was added periodically in this manner to maintain DO concentrations above 8 mg/L.

Upon bioaugmentation, ENV435 followed the path of the bromide tracer in the test zone. The highest concentration measured was 1.9×10^{8} CFU/mL at a well located 2 m downgradient of the injection well. The several log reductions in concentration indicated significant numbers of cells were being filtered out in the aquifer. First-order decay estimates indicated the half-life for the ENV435 cells in the aquifer was only 1 to 2 days.

DO concentrations decreased significantly, with measureable DO levels occurring only 2 m from the injection well. DO levels in the control plot usually exceeded 20 mg/L, indicating significant DO consumption in the bioaugmented plot. The direct injection of oxygen in the second test trial resulted in higher DO concentrations, often above 20 mg/L after injection and up to 2 mg/L 3–5 days after the injection.

The total concentration of TCE, DCE and VC decreased from 2,200 μg/L initially to below 500 μg/L at most locations in the bioaugmented test plot. Concentrations tended to rebound several days after the tests. Greater removal spatially was observed in the second test where ENV435 was added throughout the test zone and pure oxygen was injected into the well. Mass balance estimates indicated about 141 g of VOC were removed in the treatment process as a result of the addition of 38.5 kg of culture, corresponding to a transformation capacity of 0.004 g VOC/g biomass. This value represents about 10% of the transformation capacity measured by Chang and Alvarez-Cohen (1995) for TCE by phenol degraders (0.03 TCE/g biomass).

The adhesion-resistant strain of ENV435 also was evaluated in a bedrock aquifer at a former chemical manufacturing facility (Walsh et al., 2000). To facilitate transport of the strain, pneumatic fracturing was used to expand the fracture network in the bedrock aquifer. Bottle tests performed prior to bioaugmentation showed that, to achieve the objective of a 90% reduction in TCE from the initial concentration of 5–10 mg/L, it would require a concentration of ENV435 of 5×10^{8} CFU/mL. During the fracturing processes, approximately 676 L of solution was injected into the aquifer that contained strain ENV435 at a cell optical density of 50, and groundwater containing a liquid form of organic carbon. The inoculation occurred in batches of 114–160 L with pressurized air. To supply needed oxygen, the inoculation was

followed by the injection of air for 28 days at a rate of 28 L per minute. The inoculation volume of 676 L was small compared to the estimated volume of TCE-contaminated water of 3.8×10^4 to 5.6×10^4 L within a 7.6 m radius of the injection well.

Following injection, plate count analysis demonstrated that the microorganisms were distributed 6.1–7.6 m from the injection well (fracture well), and cell numbers in groundwater from monitoring wells ranged from 10^7 to 10^8 CFU/mL, below the target level of 5×10^8 CFU/mL. The microbial concentration decreased with radial distance from the injection well. Average TCE concentrations decreased from 20 to 30 mg/L to less than 5 mg/L within several days of injection. Rates of TCE transformation decreased over a 2 week period and were consistent with a decrease in ENV435 numbers. An estimated 825 g of TCE was transformed by approximately 46,000 g of wet ENV435 cells, corresponding to a transformation capacity on a wet cell basis of 0.018 mg TCE/mg cells. This value is in the range of the transformation capacity reported by Chang and Alvarez-Cohen (1995). The study demonstrated a novel approach of combining bioaugmentation with pneumatic fracturing.

8.4.1.5 Bioaugmentation with *Ralstonia eutropha* KT1

A field study was conducted of the bioaugmentation of *Ralstonia eutropha* KT1 in a TCE-contaminated aquifer in Kururi, Kimitsu City, Chibe, Japan (Tani et al., 2002). Groundwater at the site was contaminated with TCE at a concentration of approximately 200 μg/L. The bioaugmented bacteria were monitored using *in situ* PCR targeting the phenol hydroxylase and by fluorescent *in situ* hybridization (FISH) using 16S ribosomal ribonucleic acid (rRNA). Bioaugmentation was carried out by injecting 7,000 L of cell suspension at an optical density of 1.0 at 600 nm. Prior to bioaugmentation, the total cell concentration in the groundwater was 3×10^5 cells/mL, and the amount carrying the phenol hydroxylase gene of *R. eutropha* was about 0.1% of the total bacteria. The concentration of bacteria carrying the phenol hydroxylase gene in groundwater samples taken 1 h after injection was approximately 3×10^7 cells/mL using *in situ* PCR and was similar using FISH (Figure 8.4). The numbers of bacteria with the phenol

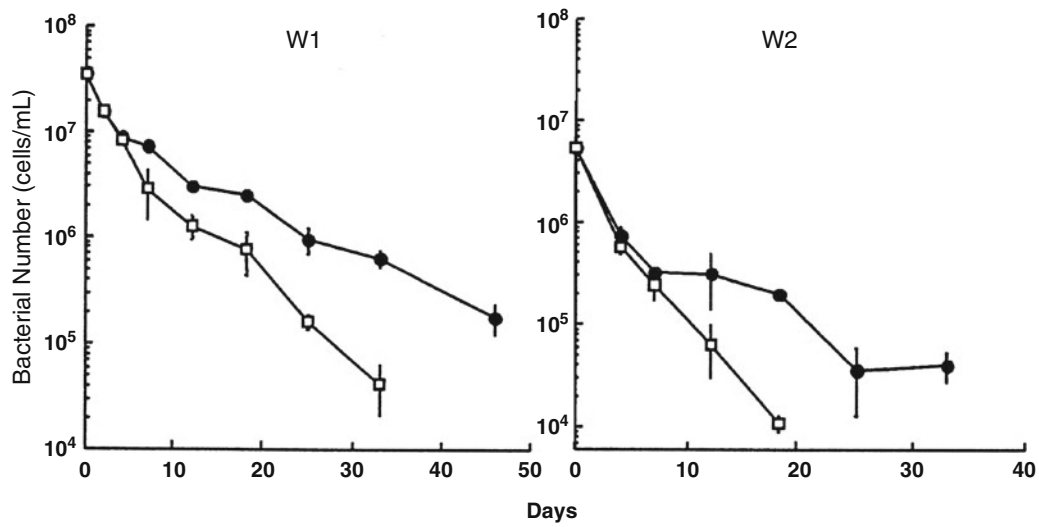

Figure 8.4. Changes in the number of bacteria carrying the phenol hydroxylase gene (●) detected by *in situ* polymerase chain reaction (PCR) and *Ralstonia eutropha* KT1 (□) detected by fluorescence *in situ* hybidization (FISH) in W1 and W2 during *in situ* bioaugmenation of *Ralstonia eutropha* KT1 (from Tani et al., 2002).

hydroxylase gene decreased by several orders of magnitude after 33 days of monitoring with FISH, yielding lower numbers than *in situ* PCR. The authors indicated that in the rRNA-targeted FISH, the fluorescence intensity was dependent on the copy number of the rRNA in an individual cell, which is not the case for *in situ* PCR. *R. eutropha* probably did not have ribosomal content high enough for detection by FISH because it was not synthesizing protein or growing actively under the oligotrophic conditions of the aquifer. The study provides a good example of how molecular methods can be applied to monitor the survival of bioaugmented microorganisms.

8.4.2 Bioaugmentation Approach II

Approach II uses an effective constitutive strain that can transform TCE or other CAHs while being maintained *in situ* on a non-inhibiting growth substrate. A mutant strain of *Burkholderia cepacia* G4 ($PR1_{301}$) that constitutively expresses TMO was developed for this purpose, and tested in microcosms (Munakata-Marr et al., 1996, 1997) and in the field (Bourquin et al., 1997; McCarty et al., 1998b).

8.4.2.1 Bioaugmentation with a Constitutive *Burkholderia cepacia* G4: Microcosm Tests

Munakata-Marr et al. (1996) evaluated TCE cometabolism in small laboratory columns packed with aquifer material from the Moffett Test Facility. The columns were bioaugmented with a wild type strain of *Burkholderia (Pseudomonas) cepacia* G4 and a nonrecombinant mutant of G4 ($PR1_{301}$) that is capable of constitutive degradation of TCE in the absence of toluene or phenol. The bacterial strain used in the bioaugmentation was the wild type strain *Burkholderia (Pseudomonas) cepacia* G4 that was isolated by Nelson et al. (1986). Strain G4 cometabolizes TCE using the *o*TOM enzyme, and is induced by either phenol or toluene. Two mutants of G4 also were evaluated: *B. cepacia* $PR1_{23}$ (Shields et al., 1995) and $PR1_{301}$ developed by Munakata-Marr et al. (1996). Both mutants express *o*TOM constitutively when grown on substrates such as lactate.

The columns were periodically exchanged with groundwater amended with TCE (250 μg/L), DO (31 mg/L) and either lactate (15 mg/L) or phenol (6.5 mg/L). Two types of tests were performed: (1) a high density single bioaugmentation (1–11 mg of cells) to sterile and nonsterile aquifer columns; and (2) low density semicontinuous bioaugmentation (70 μg of cells were added with each exchange).

In the high density tests, dissolved oxygen was completely consumed in all the phenol- and lactate-fed microcosms when higher primary substrate concentrations were used. Even when the substrate concentrations were lowered, TCE degradation was limited. The high density bioaugmentation with $PR1_{301}$ did not successfully degrade TCE.

In the low density tests, the microcosm that was fed G4 with no substrate achieved the same degree of TCE removal as the microcosm fed only phenol and not bioaugmented. This result indicated the successful bioaugmentation of an induced culture for its transformation potential alone (Approach I). The addition of $PR1_{301}$ culture when grown on lactate, but fed phenol, was effective in transforming TCE. The authors indicated that this result has practical implications for field scale bioaugmentation, in that large quantities of cells for bioaugmentation can be grown on a non-toxic compound, such as lactate, and then induced on concentrations of phenol as low as 1 mg/L. Lactate-grown G4 and $PR1_{301}$, when fed phenol, transformed TCE. Bioaugmentation with cultures known to degrade TCE more than doubled the extent of TCE degradation compared with microcosms fed phenol only and that stimulated indigenous strains.

The poor performance of the lactate-fed PR1 culture compared to the phenol-fed PR1 indicated lactate could not replace phenol in the field, which was later confirmed in field experiments (Approach II). In addition, the microcosm results indicated problems that were likely associated with oxygen limitations. The results also demonstrated that lag periods could be reduced through bioaugmentation, and that additions of strains that effectively transform TCE could improve transformation as compared to indigenous strains. Finally, this testing showed that it was feasible to bioaugment groundwater with an induced strain selected for its catalytic biotransformation potential.

Munakata-Marr et al. (1997) studied the long-term (300 days) cometabolism potential of these aquifer microcosms and measured the presence of G4 or $PR1_{301}$ in the column effluent using molecular methods. Indigenous phenol utilizers transformed about 100 μg/L of the 250 μg/L of TCE added for about the first 100 days, but the transformation ability gradually decreased. The authors suggested that this decreasing effectiveness over time may have occurred because the introduced microorganisms had a competitive disadvantage due to TCE transformation product toxicity. In the microcosm, continuously bioaugmented strain G4 grown on phenol, but with no substrate added, transformed TCE to a similar extent as the indigenous fed phenol. However, after 70 days of operation, DO levels decreased to below detection. Upon stopping bioaugmentation, DO levels increased but TCE concentrations also increased to levels of the control, indicating TCE removal had stopped. In the microcosm that was bioaugmented with phenol-grown G4, but fed lactate as a substrate, TCE removal was enhanced for the first 100 days, but DO became limiting and TCE eventually reached the same levels as the control. The microcosms that degraded TCE most effectively were ones that were fed phenol and bioaugmented with phenol or lactate-grown G4 or $PR1_{301}$. However, while G4 and $PR1_{301}$ were detected in the column effluents during the bioaugmented periods, they generally were not detected once bioaugmentation was stopped. Thus, the enhanced TCE transformation at a later time could not be attributed to the bioaugmented bacteria.

These microcosm tests demonstrated the importance of maintaining the DO concentrations to achieve effective TCE removal. Continuous addition of microorganisms likely generated a large DO demand as a result of the slow biomass decay. This conclusion was supported by mass balance calculations. Lactate fed microcosms were observed to have an even greater DO demand than phenol-fed microcosms. The authors speculated that bioaugmentation may be one means of maintaining TCE transformation activity when indigenous activity declines. The results also demonstrated that G4 and PR1 strains were lost from the microcosms once bioaugmentation was stopped, even in the substrate amended microcosms, which is unfavorable for bioaugmentation. The constitutive strain also transformed less TCE when stimulated on lactate compared to phenol.

Matheson et al. (1997) developed strain-specific DNA probes for determining the persistence of *Burkholderia cepacia* G4 used in bioaugmentation. For one of the probes with a 650-base-pair (bp) fragment, as few as 10 CFU of *B. cepacia* could be detected. The method was applied to the long-term microcosm studies of Munakata-Marr et al. (1997) discussed above. In non-bioaugmented microcosms, G4 and PR1 were never detected, but in the bioaugmented microcosms, they were detected for periods of up about 83 days, and their presence was lost several column exchanges after bioaugmentation was stopped.

8.4.2.2 Bioaugmentation with *Burkholderia cepacia* PR1: Column Studies

Snyder et al. (2000) performed continuous flow studies of the addition of *Burkholderia cepacia* PR1 to columns packed with sediments from the Borden aquifer. The goal of this study was to evaluate the impact of bacterivorous protists on the bioaugmented culture and TCE

remediation. A single column was used for a series of experiments including culture addition, culture addition with TCE, and culture addition with TCE and substrate. The PR1 culture was added at concentrations ranging from 6.3 to 6.6 $\times$ 10^7 CFU/mL. Bacterivores increased with repeated additions of PR1 to the column, resulting in a decrease in the half-life of PR1. The addition of TCE and growth substrate (phthalate, 4 mM) resulted in prolonged survival of PR1 and TCE transformation. The results indicated significant and predictable losses of PR1 in the column due to native bacterivores.

In aquifer sediment column studies with *B. cepacia* G4 5225-PR1, Winkler at al. (1995) used highly specific monoclonal antibody techniques to track its survival. The microorganisms were continuously added to sterilized sediments and non-sterile sediments. 5225-PR1 survived well in the sterilized sediments but rapidly decreased in the non-sterile sediments. The loss in the non-sterile sediment was presumed to be due to predation by bacteriovorous protists. Their study showed the utility of monoclonal antibody tracking methods and the importance of biotic interaction in the persistence of introduced microorganisms.

Komlos et al. (2004) performed column studies to investigate the concept of developing biofilm barriers to control migration of TCE through the creation of a thick biofilm capable of treating influent TCE. The studies were performed with two cultures to form a dual species TCE degrading/reduced permeability biofilm barrier using *Burkholderia cepacia* PR1-pTOM31c and *Klebsiella oxytoca*. *B. cepacia* was used for its ability to transform TCE and to constitutively express its oxygenase enzyme, and *K. oxytoca* was selected for its ability to form thick biofilms. Studies were performed in columns packed with 1 mm glass beads. The columns studies were conducted with the pure culture of *B. cepacia* and a column inoculated with the combined cultures. The columns were continuously fed TCE, dissolved oxygen and diluted Luria-Bertani (LBG) medium as a growth substrate.

TCE removal was most effective in the column inoculated with the pure culture of *B. cepacia* (79% removal), compared to 49% removal in the dual culture column fed the same concentration of substrate (30 mg/L chemical oxygen demand [COD]). This greater removal corresponded to a higher population of *B. cepacia* in the single culture column. The presence of *K. oxytoca* had a negative effect on TCE removal performance. As the concentration of COD was increased, TCE removal in the dual species column decreased with only 27% achieved at 70 mg/L COD, and no removal achieved at 700 mg/L COD. Dissolved oxygen limitations in the columns fed higher concentrations of COD were likely responsible for the lower TCE removals. Permeability reductions were correlated with the higher COD concentrations, and corresponded with the *K. oxytoca* population density. The COD was reduced by as much as a factor of 5 as compared to the initial conditions. The study provides a good example of how the competition for a benign substrate between TCE transforming and non-TCE transforming microorganisms can result in decreased remediation performance.

8.4.2.3 Bioaugmentation with *Burkholderia cepacia* G4 with Glucose Addition: Field Study

Bourquin et al. (1997) performed a feasibility study evaluating bioaugmentation with *Burkholderia cepacia* G4 $PR1_{301}$ in a shallow aquifer in Wichita, Kansas, USA. Strain G4 $PR1_{301}$ is highly effective in transforming TCE, but requires neither phenol nor toluene to induce the oxygenase activity (McCarty et al., 1998b). The sandy aquifer was contaminated with TCE (125 µg/L), *cis*-DCE (95 µg/L) and *trans*-DCE (4 µg/L). The remediation system consisted of an injection well, extraction well and multi-depth multiport monitoring wells. The PR1 culture was grown in a 16 L stirred-tank bioreactor on site using a drain-and-fill protocol with glucose (7.2 g/L) as a growth substrate to achieve a cell density of 10^{12} cells/mL

for bioaugmentation. The culture was added to the injected groundwater to achieve a concentration of 10^9 cells/mL. TCE and *cis*-DCE concentrations were reduced to below detectable levels after 24 h of injection, and the levels did not increase until PR1 injection was stopped. PR1 was observed 30 cm from the injection well 8 days following injection, which coincided with a decrease in DO concentrations at the same monitoring well.

In a second phase of testing, the cells were added at different concentrations to determine the minimum concentration for effective TCE and *cis*-DCE removal and to avoid plugging. The addition of 10^7 cells/mL achieved partial CAH removal, while increasing the concentration to 10^8 cells/mL brought contaminant concentrations to non-detectable levels. Glucose and nutrients were then added, but the population could not be maintained through glucose addition. The researchers concluded that bioaugmentation with PR1 could effectively degrade TCE and *cis*-DCE at cell injection concentrations of 10^8 cells/mL. PR1 was not effectively transported in the aquifer, and plugging was evident. Oxygen transport problems were also an issue at the site.

8.4.2.4 Bioaugmentation with *Burkholderia cepacia* G4 with Lactate Addition: Field Study

A pilot-scale field study at the Moffett Test Facility also was conducted to evaluate bioaugmentation with $PR1_{301}$ grown *in situ* through lactate addition. Three seasons of field studies were conducted where $PR1_{301}$ was bioaugmented on a daily basis into the test plot, with doses in the range of 3.5–5.0 g/day in years 1 and 2, and 10.5 g/day in year 3. Lactate was fed as the primary substrate three times a day (every 8 h) with a high concentration pulse over a period of 15–30 min, which resulted in an average injection concentration of 13 mg/L. In year 1, lactate was the primary substrate, while in years 2 and 3 there were periods when either lactate or phenol were fed. In all the tests, TCE was continuously added at concentrations ranging from 80 to 100 μg/L.

The first season of testing showed lactate was consumed below non-detectable levels at all monitoring locations after 103 h of injection. Even though lactate was consumed by the first monitoring well (1 m from the injection well), there was no evidence of excessive bioclogging, based on injection pressure measurements. Most of the decrease in dissolved oxygen (about 16 mg/L) occurred between the injection well and the first monitoring wells, and was in good agreement with the 15 mg/L of DO calculated to be required for the complete oxidation of 13 mg/L of injected lactate. Over a period of 280–450 h, TCE concentrations increased to about 80% of the injection concentrations. At the other monitoring locations, TCE concentration reached about 50% of the injection concentration over the 600 h of the test, indicating partial removal of the TCE. Using molecular methods, $PR1_{301}$ was detected at the first monitoring well during the first 6 days after bioaugmentation, but not at the second or third monitoring wells. Detection of $PR1_{301}$ was very limited throughout the test.

In the second season of testing, the primary substrate was varied between lactate, then phenol, and back to lactate. The results of the second season of testing are shown in Figure 8.5. During the early period with lactate fed, TCE concentrations at the monitoring locations approached about 50% of the injection concentration. At 125 h, the substrate was changed, and phenol was injected at a pulse-averaged concentration 6 mg/L. The approach here was to determine if bioaugmentation would permit the rapid development of a biostimulated zone so that if phenol or toluene were used in field scale systems they would not migrate. About 70% removal of TCE was achieved and removal remained effective during the 250 h period phenol was added. The degree of treatment was similar to that achieved when indigenous phenol utilizers were stimulated at the site on 6 mg/L of phenol (Hopkins et al., 1993b). By the second day of addition, phenol was reduced to below the detection limit of 1 μg/L. The test

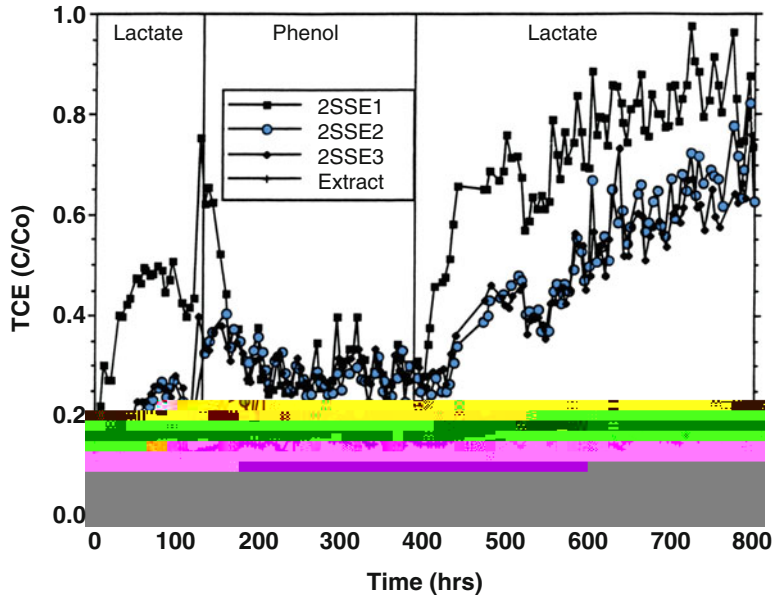

Figure 8.5. TCE concentration history during the second season of testing with the bioaugmentation of mutant PR1$_{301}$ of *Burkholderia cepacia* G4 that expressed TOM constitutively (from McCarty et al., 1998b). Periods of lactate to phenol and back to lactate additions are shown.

demonstrated that bioaugmentation was successful in achieving rapid utilization of phenol to limit its spread during *in situ* biostimulation. In previous studies where indigenous phenol utilizers were stimulated, it took over 2 weeks before phenol was reduced below the detection limit.

After 390 h, the substrate was switched from phenol back to lactate. As shown in Figure 8.5, TCE concentrations gradually increased to the injection concentration, indicating treatment was no longer effective. DO concentrations suggest lactate was being consumed and residual DO levels remained above 8 mg/L, which was an ample amount to maintain TCE cometabolism. Analysis of the presence of PR1$_{301}$ using molecular methods indicated that its detection in the test zone was rapidly lost. It was detected in the first monitoring well for the first 4 days, and then was only detected two times during the phenol injection and was not detected in the later period of lactate injection.

In the third season of testing, the PR1$_{301}$ was grown under more controlled conditions in the laboratory, the amount injected was doubled, and lactate was continuously fed. Initially TCE concentrations decreased, showing 80–90% removal, but as time progressed, TCE concentrations increased toward the injection concentration, indicating that TCE transformation ceased. After about 830 h, the feed was switched from lactate to phenol (6 mg/L). TCE concentration decreased and about 50% removal was achieved.

In general, the results showed good TCE removal early in the three studies, but TCE transformation could not be sustained when lactate was fed as a substrate. The researchers suggested two possibilities for the loss in TCE transformation: (1) predation of the introduced population; or (2) an inability of the PR1$_{301}$ strain to compete for the added lactate (although both processes may have contributed). The researchers concluded that in order for TCE cometabolism to be successful, methods are needed to avoid the competition for lactate.

The molecular probes described by Matheson et al. (1997) were used to detect *B. cepacia* and the mutant strain PR1$_{301}$ in the field tests. In the first season of testing, PR1$_{301}$ was detected at the first monitoring well (1 m from the injection well) during the first 6 days of

bioaugmentation, but not at the further monitoring wells. After 6 days, PR 1_{301} was no longer detected through most of the field season, and was not detected on glass bead coupon samples removed at the end of the field season. Similar observations were obtained in the second season of testing. The monitoring for PR 1_{301} in the field tests was generally consistent with the field observations. When the culture was initially present, effective TCE removal was achieved. The rapid loss of transformation activity coincided with the loss of detection of PR 1_{301}, and likely resulted from the lack of survival of PR 1_{301} in the subsurface environment present.

8.4.3 Bioaugmentation Approach III

Bioaugmentation Approach III focuses on developing effective strains to transform contaminants that were not effectively transformed by indigenous microorganisms when fed a cometabolic substrate such as methane. Strain selection has focused on microbes that grow on butane and are able to transform chlorinated ethanes, chlorinated methanes and 1,1-DCE (Kim et al., 2000, 2002). Isolated strains often were found to be in the *Rhodococcus* sp. group. A number of microcosm studies have been performed to evaluate bioaugmentation using this approach (Table 8.3) along with field demonstrations (Semprini et al., 2007a, b, 2009) where butane was added as the growth substrate (Table 8.4).

8.4.3.1 Bioaugmentation with Butane Utilizers

A microcosm study with the butane enrichment culture of Kim et al. (2002) was conducted by Jitnuyanont et al. (2001) to study the transformation of 1,1,1-TCA in bioaugmented and non-augmented microcosms constructed with aquifer and groundwater from the Moffett Test Facility. The non-bioaugmented microcosm required 80 days of incubation before butane utilization was observed while the bioaugmented microcosm required only 3 days. Initially the augmented microcosms were effective in transforming 1,1,1-TCA, but their transformation ability decreased with prolonged incubation (400 days). The non-augmented microcosms showed limited transformation ability in the beginning, but improvement occurred after 400 days of incubation. After 400 days, both the non-bioaugmented microcosms and the bioaugmented had similar transformation yields of 0.04 mg 1,1,1-TCA/mg butane. DNA fingerprints showed the microbial composition after 400 days was similar in the bioaugmented and non-bioaugmented microcosms. The microcosms with the bioaugmented culture and 50% mineral media effectively utilized butane and transformed 1,1,1-TCA, while those with only 5% mineral media in groundwater lost their 1,1,1-TCA transformation ability. Microbial fingerprints indicated shifts in the microbial population with the different media combinations. The authors indicated that the most successful bioaugmentation was achieved by enriching butane utilizers from the Moffett Test Facility microcosms that were effective in groundwater with no mineral media added. The authors suggested that *in situ* bioremediation might be achieved by adding enriched cultures that perform well under the subsurface nutrient conditions of the site.

These microcosm studies were continued by Semprini et al. (2007a, b) where a butane-grown culture was enriched from previously bioaugmented Moffett Test Facility microcosms that performed well. The enrichment consisted of *Rhodococcus sp.* microorganisms that transformed mixtures of 1,1,1-TCA, 1,1-DCA and 1,1-DCE under the groundwater nutrient conditions of the Moffett Test Facility. Microcosm and modeling studies showed rapid transformation of 1,1-DCE with high transformation product toxicity and weak inhibition by butane, while 1,1,1-TCA was much more slowly transformed and was strongly inhibited by butane. The microcosms were repeatedly stimulated on butane and transformed the mixture of CAHs over a period of 100 days. More rapid uptake and transformation of the butane and the

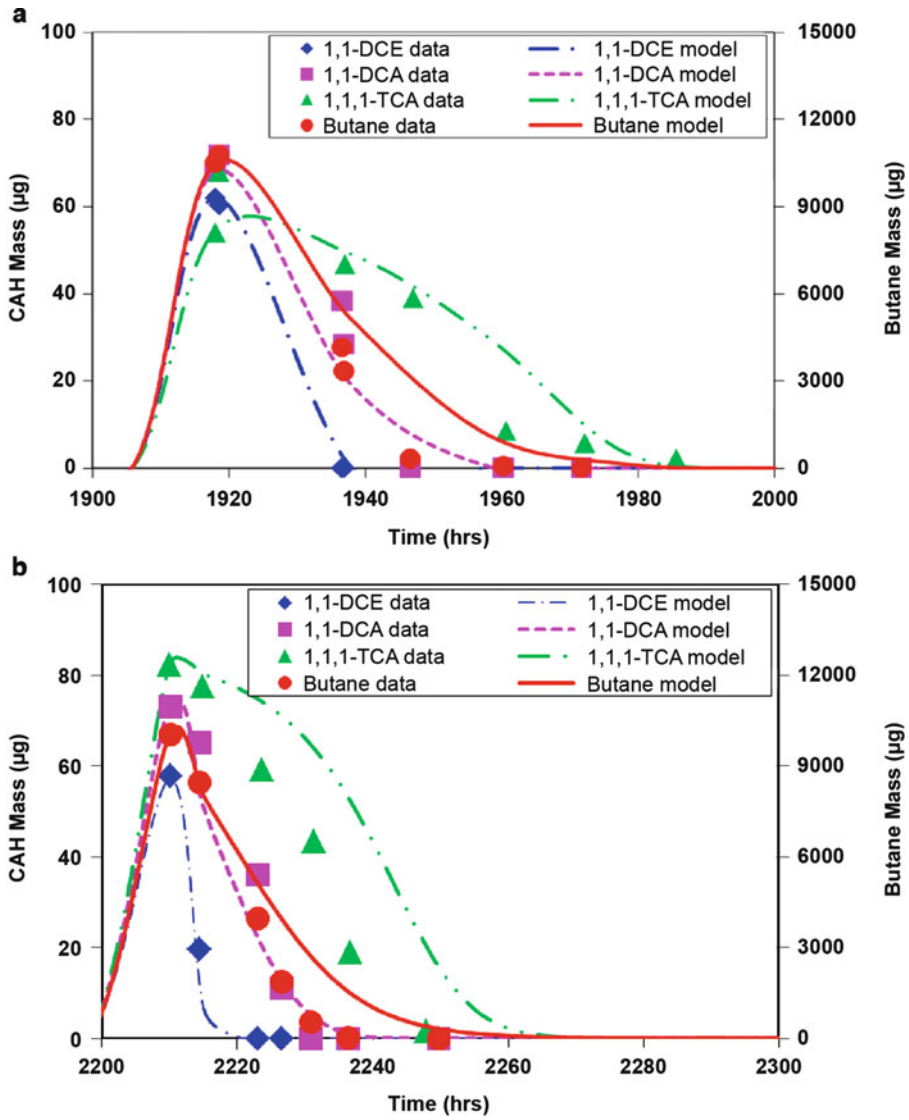

Figure 8.6. Results of an aquifer solids/groundwater slurry microcosm test and modeling analysis where a butane enrichment culture (*Rhodococcus* sp.) was added to promote the transformation of a mixture of 1,1-DCE, 1,1-DCA and 1,1,1-TCA (adapted from Semprini et al., 2007b).

mixtures was observed as indicated in Figure 8.6. Model simulations of the microcosms indicated that the microbial populations increased from less than 1 mg/L to over 20 mg/L over the 100 days of incubation (Semprini et al., 2007b). The simulations of the results of the microcosm tests (Figure 8.6) showed that butane inhibition of CAH transformation (especially transformation of 1,1,1-TCA) and transformation product toxicity (especially on 1,1-DCE) were important processes. The microcosm tests yielded trends similar to the field tests (described in Section 8.4.3.3), where 1,1-DCE was observed to be rapidly transformed while 1,1,1-TCA was the most slowly transformed and was the most inhibited by the presence of butane.

Microcosm studies of bioaugmentation with butane-utilizing bacteria for the aerobic cometabolism of chloroform (CF) were performed by Frascari et al. (2007). Bioaugmentation

was performed with butane enrichments that were effective in CF cometabolism along with an isolate *Rhodococcus aetherovorans* BCP1. The lag time for stimulation of butane utilizing organisms was strongly affected by temperature, with less than 2 weeks required in all cases, and the shortest lags were observed when CF was absent. CF cometabolism by indigenous butane utilizers was not observed even after several weeks of incubation at groundwater temperatures of 15°C. Decreases in the lag period were observed in treatments performed with the two different butane-utilizing inocula even at the lowest concentration of the augmented culture (3.5×10^3 CFU/mL). Sustained CF cometabolism was maintained in bioaugmented microcosms at a butane/CF molar ratio of 2.0–3.1. The results showed the potential of both decreasing the lag phase and promoting more effective cometabolism of CF through bioaugmentation.

Microcosm studies also were performed on mixtures of CAHs that included VC, *trans*-DCE, *cis*-DCE, TCE, 1,1,2-TCA and 1,1,2,2-TeCA using aquifer material and groundwater from a contaminated site (Frascari et al., 2006) and methane or propane as cometabolic substrates. Lag times for the onset of methane or propane utilization were 36–264 days in the non-bioaugmented microcosms. In microcosms inoculated with cultures directly sampled from the non-bioaugmented microcosms, the lag period was significantly shortened to 0–15 days and transformation abilities were maintained. The biodegradation and cometabolism of the mixture of six CAHs was maintained for up to 410 days, with the less chlorinated CAHs transformed at the fastest rates. These tests again showed the potential of decreasing lag times and transferring effective cometabolic potential. Gualandi et al. (2007) showed that a dual-culture fed both methane and propane was most effective in transforming this mixture of CAHs.

The ability to enhance CF transformation and decrease lag time also was demonstrated with a butane-enriched culture that was selected for its CF transformation abilities (Frascari et al., 2005). The culture was a *Rhodococcus* strain that was later identified as *Rhodococcus aetherovorans* BCP1 (Frascari et al., 2007). Introducing this strain into autoclaved soil slurry microcosms eliminated any lag time, and produced effective CF cometabolism (a transformation capacity of 0.031 mg CF/mg protein).

8.4.3.2 Bioaugmentation with Butane-Utilizers: Continuous Column Studies

Continuous flow column studies evaluated the potential of adding a highly enriched butane-utilizing culture containing *Rhodococcus sp*. microorganisms to promote effective transformation of 1,1,1-TCA (Semprini et al., 2005) through butane addition (Approach III). The column was packed with aquifer core material from the Moffett Test Facility. The bioaugmentation approach was to add the culture and then continuously add dissolved butane (3 to 5 mg/L) and oxygen (20–30 mg/L). A flow rate of 0.2 mL/min resulted in a fluid residence time in the column of about 1.5 days. The column (2.5 cm diameter; 30 cm length; volume = 150 mL) was bioaugmented at the column influent with a small mass (0.5 mg) of culture and pulse fed butane, dissolved oxygen and 1,1,1-TCA. Butane was effectively utilized in the column and about 80% of the added 1,1,1-TCA (200 μg/L) was effectively transformed. When the 1,1,1-TCA concentration was increased to 400 μg/L, less 1,1,1-TCA was transformed, and upon lowering the concentration about 60–70%, it was again transformed. 1,1,1-TCA transformation was maintained in the column for a period of 120 days. When 1,1-DCE (130 μg/L) was added along with 1,1,1-TCA, concentrations of 1,1,1-TCA, oxygen and butane increased, while about 50% of the 1,1-DCE was transformed. The results indicated that 1,1-DCE transformation product toxicity was occurring, and that effective 1,1,1-TCA transformation was difficult to maintain in the presence of 1,1-DCE.

8.4.3.3 Bioaugmentation with Butane Utilizers: Field Studies

A pilot-scale study was performed at the Moffett Test Facility to evaluate the potential to bioaugment a butane-utilizing culture that had better transformation abilities than the indigenous microorganisms. Environmental isolates of *Rhodococcus* sp. strains that were capable of cometabolizing a broad range of chlorinated ethenes and ethanes were evaluated (Semprini et al., 2007a, b, 2009). A mixture of CAHs including 1,1,1-TCA, 1,1-DCA and 1,1-DCE was tested, since the abiotic and biotic transformations of 1,1,1-TCA produce 1,1-DCE and 1,1-DCA, respectively (Vogel and McCarty, 1987). The cometabolism resulted from the transformation by a butane monooxygenase enzyme (BMO) which has been shown to be able to transform a broad range of CAHs (Kim et al., 2000) including this CAH mixture (Kim et al., 2002).

Field studies were conducted in the saturated zone at the Moffett Test Facility following the protocols of previous tests (Roberts et al., 1990; Semprini et al., 1990). The tests were conducted in two experimental legs, a bioaugmented test leg and a control leg operated under the same conditions as the test leg, but not bioaugmented. Dissolved butane and oxygen were both added to the treatment legs, with butane continuously added as a high concentration pulse of about 15 min followed by 45 min of dissolved oxygen, resulting in time averaged injection concentrations for butane ranging from 3.5 to 8.8 mg/L and oxygen of 20 mg/L. In order to obtain pseudo-steady-state concentrations in the test legs, 1,1-DCE, 1,1-DCA, and 1,1,1-TCA were added to the injected groundwater for 9 days prior to bioaugmentation and the addition of butane and oxygen.

The concentration history of the CAHs at the S1 monitoring well in the bioaugmented leg is shown in Figure 8.7 (top). The increase in CAH concentrations over the first 10 days of tests as a result of their injection is evident. The CAH concentrations reached their injection concentration, indicating little transformation during this stage of the test. Approximately 5 g of culture was bioaugmented into the test leg after day 8.5, and butane and oxygen addition was initiated. 1,1-DCE concentration began to decrease soon after the bioaugmentation and butane addition. Butane and oxygen concentrations are shown in Figure 8.7 (bottom). Butane concentrations reached levels of about 3 mg/L. Around day 15, the butane concentration decreased dramatically, coinciding with decreases in the 1,1-DCE, 1,1-DCA and 1,1,1-TCA concentrations.

Butane concentrations increased over the period of 16–17 days, which coincided with increases in 1,1-DCE, 1,1-DCA and 1,1,1-TCA concentrations. When butane concentrations were reduced to low levels as a result of biostimulation, from 19 to 20 days, 1,1-DCE was effectively removed to very low concentrations, and 1,1-DCA also was reduced significantly in concentration. 1,1,1-TCA was the least effectively transformed. The increases in butane concentrations at 17 days and 21 days both caused increases in the concentrations of all three CAHs, possibly from strong inhibition by butane of 1,1-DCA and 1,1,1-TCA transformation, as was observed and simulated in microcosm tests (Figure 8.6). During the period from days 18 to 20, the CAHs were removed to the following extent: 1,1-DCE (97%); 1,1-DCA (77%); and 1,1,1-TCA (36%).

In the control, non-bioaugmented west leg, there was little evidence of CAH transformation over the same time period (data not shown). 1,1-DCE concentrations increased to essentially constant levels that approached injection concentrations at the monitoring wells, indicating essentially no transformation. Butane concentrations reached higher levels in the indigenous leg compared to the bioaugmented leg and then decreased more slowly. By day 25 the butane concentrations were significantly reduced, indicating that biostimulation of indigenous butane utilizers had likely occurred. Despite indications of butane utilization, 1,1-DCE concentrations did not decrease, suggesting that 1,1-DCE was not being transformed.

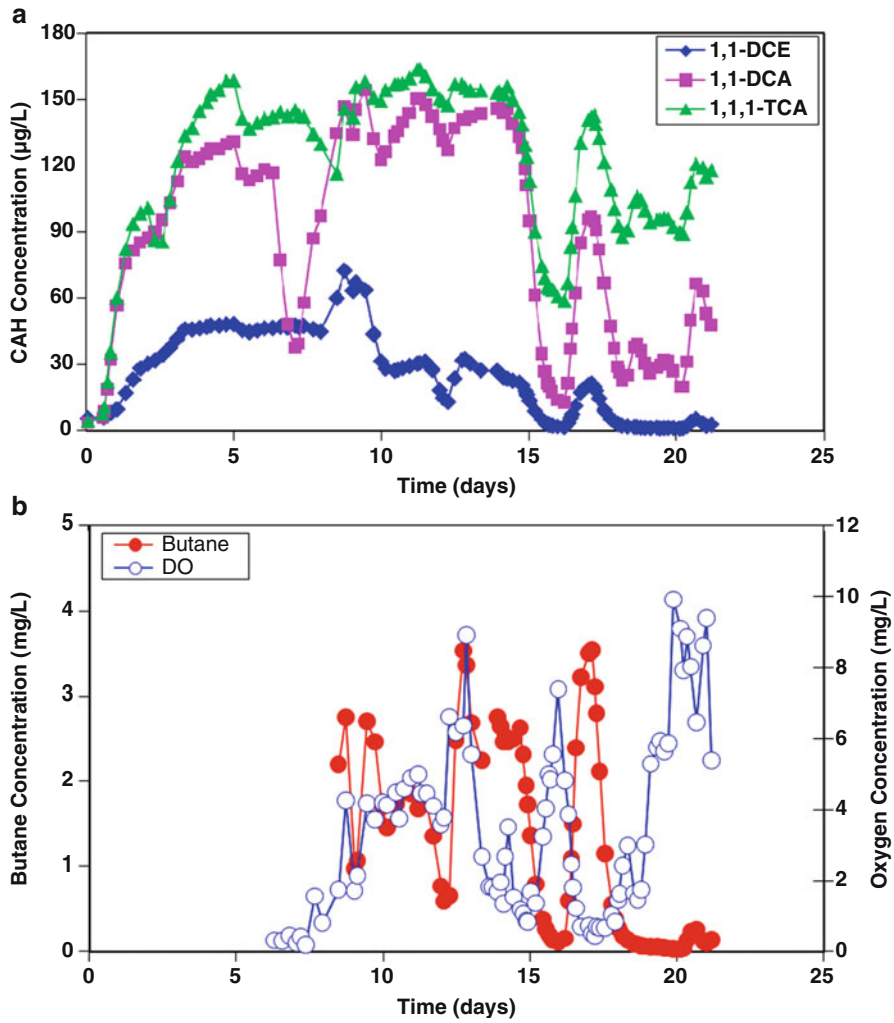

Figure 8.7. Concentrations of CAHs (*top*) and butane and DO (*bottom*) at the east S1 monitoring well during the first bioaugmentation and biostimulation experiment with the *Rhodococcus* sp. butane enrichment culture at Moffett Test Facility (adapted from Semprini et al., 2007a). Bioaugmentation occurred on day 9 of the experiment.

The degree of treatment decreased as the experiment progressed. A modeling analysis of the test results suggested that the loss of transformation performance was associated with 1,1-DCE transformation toxicity and insufficient levels of the cometabolic substrate (butane) (Semprini et al., 2007b). 1,1-DCE removal was restored when more butane was added to the treatment zone. At the end of the 70-day trial, removals of about 94%, 8% and 0% were observed for 1,1-DCE, 1,1-DCA and 1,1,1-TCA, respectively. In the control leg, prolonged biostimulation removals of 86%, 5% and 0% were observed for 1-DCE, 1,1-DCA and 1,1,1-TCA, respectively. The test results and associated modeling analysis indicated that the biostimulated microorganisms were gradually replaced by indigenous microorganisms that could transform 1,1-DCE, but had limited ability to transform 1,1-DCA and essentially no ability to transform 1,1,1-TCA.

The results of the second season of field testing are provided by Semprini et al. (2009). The study evaluated potential for cometabolism of 1,1,1-TCA through bioaugmentation with a butane enrichment culture containing predominantly two *Rhodococcus* sp. strains

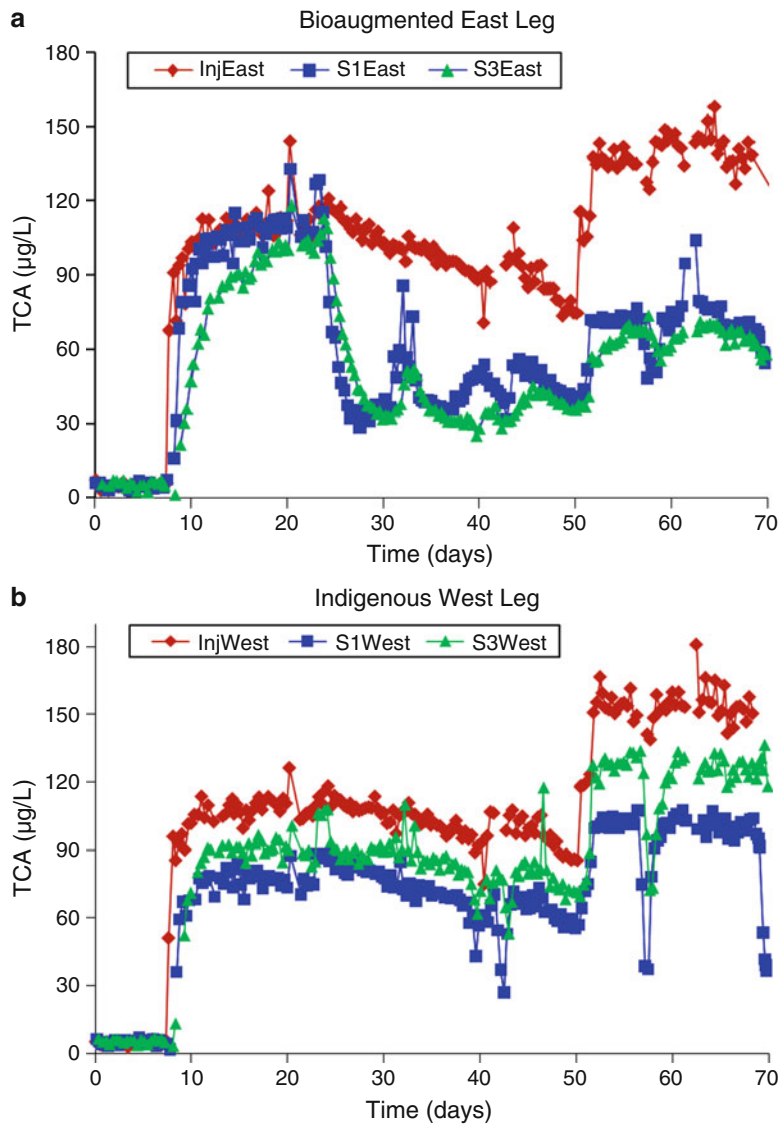

Figure 8.8. 1,1,1-TCA concentrations in the second season of testing with the butane-utilizing *Rhodococcus* sp. culture (adapted from Semprini et al., 2009). The east leg was bioaugmented on day 24, while both legs were fed butane and dissolved oxygen.

(named 179BP and 183BP) that could cometabolize 1,1,1-TCA and 1,1-DCE. Batch tests indicated that 1,1-DCE was more rapidly transformed than 1,1,1-TCA by both strains, with 183BP being the most effective organism (Semprini et al., 2009). Tests were repeated in the two test legs used previously in the first season of testing using a similar protocol. 1,1,1-TCA was injected at concentrations ranging from 80 to 140 µg/L in both legs and 1,1-DCE was present as a background contaminant at concentrations ranging from 4 to 12 µg/L. Hydrogen peroxide also was added to increase the dissolved oxygen concentration in the treatment zone and to permit more butane to be added. The pulsed averaged injected butane concentration ranged from 4 to 8 mg/L.

Figure 8.8 shows the results from the monitoring wells for the bioaugmented leg (top) and indigenous leg (bottom) for 1,1,1-TCA. Butane (not shown) was observed to increase to

maximum concentrations of about 4 mg/L in the west leg, and about 3 mg/L in the bioaugmented east leg. The butane concentration decreased to below detection by day 25 in the bioaugmented east leg, and decreases in DO were observed in response to butane utilization. Upon bioaugmentation and biostimulation, with continuous addition of butane and dissolved oxygen and/or hydrogen peroxide as sources of dissolved oxygen, there was about 70% removal of 1,,1,1-TCA (Figure 8.8, top). In contrast, there was no removal of 1,1,1-TCA in the non-augmented test leg (Figure 8.8, bottom), although butane and oxygen consumption by the indigenous population was similar to that in the bioaugmented test leg. Some 1,1-DCE removal, about 40%, was observed in the control leg, while 80% removal was observed in the bioaugmented leg (data not shown). With prolonged treatment, removal of 1,1,1-TCA in the bioaugmented leg decreased to 50–60%. Hydrogen peroxide (H_2O_2) injection increased dissolved oxygen concentration, thus permitting more butane addition into the test zone, but more effective 1,1,1-TCA treatment did not result.

The results showed that bioaugmentation with the enrichment cultures was effective in enhancing cometabolic treatment of both the 1,1,1-TCA and the low concentrations of 1,1-DCE over the entire period of the 50-day test. Compared to the first season of testing, cometabolic treatment of 1,1,1-TCA was not lost. The better performance achieved in the second season of testing may be attributed to less 1,1-DCE transformation product toxicity, more effective addition of butane, and bioaugmentation with the highly enriched dual culture. The results showed that the addition of an enrichment culture improved the performance of cometabolic treatment of 1,1,1-TCA, while no treatment was achieved in the control leg, even though the indigenous butane utilizers were successfully stimulated.

8.4.4 Bioaugmentation Approach IV

An interesting bioaugmentation approach is to add indigenous CAH-cometabolizing microorganisms by injecting groundwater that contains the organisms of interest into target areas where the desired organisms are absent or present in low numbers. For example, microbes present in one aquifer might be added to another aquifer for their bioremediation potential. This approach has been called the "primed" method of bioaugmentation (Singer et al., 2005). An example of this approach is the field study conducted by Takeuchi et al. (2004) for a TCE contaminated aquifer in Mobara, Japan. Groundwater along with methanotrophic bacteria from an aquifer rich in naturally occurring methane, which seeped into the aquifer from a methane gas formation, was injected into a nearby TCE-contaminated site. Groundwater containing methane at 0.016 mg/L, along with methanotrophic bacteria at 1.1×10^4 cells/mL, was injected into a shallower aquifer through an injection pit.

Field observations showed that the initial concentration of about 128 µg/L of TCE was reduced to below the detection limit. The authors concluded that removal was due to methanotrophs present in the amended groundwater, and not in the indigenous groundwater. They reached this conclusion based on microcosm tests that supported the field experiments. Most of the methanotrophs that were added became attached because the numbers of methanotrophs in the remediated groundwater were lower than numbers in the injected groundwater and the highest activity occurred close to the injection pit. The added methanotrophs expressed sMMO, which is effective in transforming TCE. The authors concluded that the methane-enriched groundwater from the natural gas area was valuable both for its supply of methane and also for its microbial content.

At another site in Chikura, Chiba, Japan, bioremediation of *cis*-DCE was accomplished by injecting uncontaminated water containing dissolved oxygen and methanotrophic bacteria into another aquifer that contained *cis*-DCE and higher concentrations of dissolved oxygen

(Takeuchi et al., 2005). The *cis*-DCE was present as a result of the anaerobic transformation of TCE. Groundwater from a third aquifer containing 0.16 to 32 mg/L methane, 3.9 to 30 $\times$ 10^3 cells/mL of methanotrophs and low DO was added to aquifer 2 that contained less methane (0.032 mg/L), fewer methanotrophs (0.088 to 1 $\times$ 10^3 cells/mL) and higher DO. Water from a first aquifer that contained low methane (0.008 mg/L) and 0.044 to 33 $\times$ 10^3 methanotrophs was added as a control to the second aquifer. The *cis*-DCE concentrations decreased by about 50% with injection of the methane-rich water, while no significant decrease was observed with the same rate of injection of the control water. Dissolved methane concentrations also decreased when groundwater from the third aquifer was injected. The concentration of methanotrophs was lower at the observation well (2.9 $\times$ 10^4 cells/mL) compared to the injection well (1.1 $\times$ 10^5 cells/mL), and those expressing sMMO numbered around 1.4 $\times$ 10^4 cells/mL. It was not determined whether methanotrophs carried in the injected groundwater improved the performance of treatment. However, the methodology used here is consistent with the previous study demonstrating bacteria in the injected groundwater can help prime the aquifer with a transforming culture. Injecting groundwater from the uncontaminated aquifer also provides a cheap continuous seed of culture and may help overcome effects of transformation product toxicity.

8.5 SUMMARY

The results of the field demonstrations performed to date indicate bioaugmentation to promote effective cometabolic transformation still should be considered experimental in nature. Although some benefits have been demonstrated, many of the studies have shown that performance enhancements cannot be maintained over long periods. In addition, performance demonstrated under laboratory conditions of microcosms and columns often cannot be achieved under field conditions.

Shown in Figure 8.9 is a general overview of the observations from the field tests and supporting laboratory and microcosm studies, ranging from successful to unsuccessful.

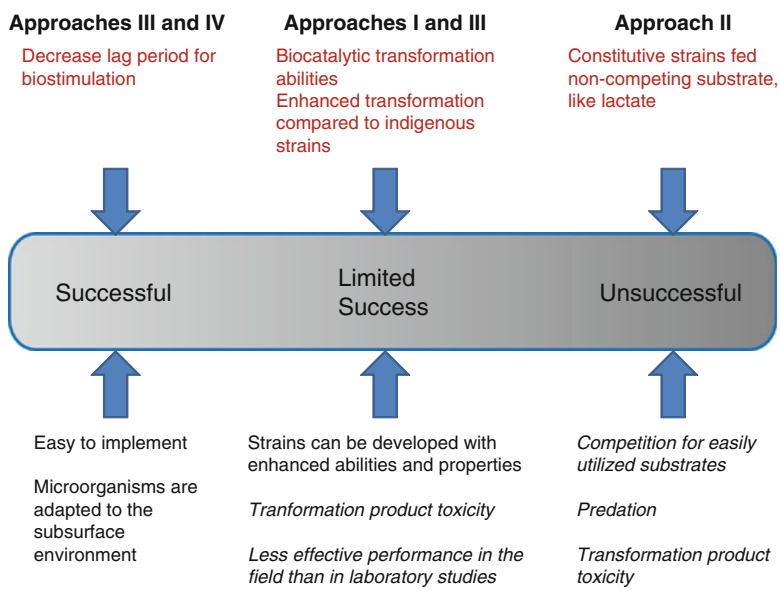

Figure 8.9. Likelihood of success of processes based on the bioaugmentation studies for aerobic cometabolism performed to date. Italicized text represents processes that are problematic for the approach taken.

Bioaugmentation can be used successfully to decrease the lag period for cometabolic treatment *in situ* using Approaches II, III and IV. Field studies of McCarty et al. (1998b) showed that the stimulation of phenol or toluene utilizing microorganisms could be greatly reduced through the addition of PR1$_{301}$ (Approach II). This finding has practical implications when the spread of toluene or phenol are of concern. Semprini et al. (2007b, 2009) also showed that adding a butane-utilizing culture reduced the lag time before effective cometabolism was measured (Approach III). This process is easy to implement because large quantities of microorganisms are not needed. In addition, strains can be enriched from the subsurface of the site, increasing the likelihood that the introduced bacteria will survive *in situ.*

Approach I, adding strains for their biocatalytic transformation potential, has had some success but has not proceeded to large-scale implementation. Field evaluations reviewed here stopped at the pilot-scale stage. One of the limitations of Approach I is the need for effective oxygen delivery when high concentrations of cells are added to the subsurface. Both studies with *Methylosinus trichosporium* OB3b (Duba et al., 1996) and *Burkholderia cepacia* ENV435 (Steffan et al., 1999) resulted in lower transformation yields than demonstrated under laboratory conditions. It is possible that oxygen limitations can partly be responsible. It is not clear from the demonstrations performed to date that this approach, and the situations where this technology has potential, have been well established, or whether there is a niche where it can be practically applied. One of the most interesting applications thus far was the test in which cultures were added during pneumatic fracturing. The approach of adding microbes during the fracturing process is a novel and interesting application of the process.

Research needs include determining how best to apply Approach I and whether there are situations in which the process might have both application and cost advantages over competing processes, such as *in situ* chemical oxidation (ISCO). An excellent review of ISCO is provided by Huling and Pivetz (2006) and Seigrist et al. (2011). Reviews such as this one are helpful when evaluating competing processes. For example, it would be valuable to know the cost of delivering cells compared to Fentons Reagent to achieve the same degree of treatment.

Approach II, the least successful thus far, involves adding strains that express TMO constitutively, with biostimulation accomplished by adding a benign substrate such as lactate. The mutant PR1$_{301}$ of *Burkholderia cepacia* G4 has been the most studied for this purpose. Studies performed by McCarty et al. (1998b) showed that enhanced cometabolism of TCE could not be maintained for extended periods, even with the continuous addition of microorganisms (Figure 8.5). Competition for the easily utilized substrate by other organisms, predation and transformation product toxicity potentially contributed to the poor performance.

Research on Approach II might focus on protecting the bioaugmented strains from predation and competition for substrate utilization. As reviewed by Gentry et al. (2004), one potential method is to encapsulate the bacteria in a material that creates a non-toxic environment through which gases and liquids can diffuse. Materials such as alginate, agarose, and gellan gums might be used. The benign substrate also could be incorporated into the encapsulant. Substrates, like phenol, that can be potentially formed slowly via a hydrolysis reaction as a slow release substrate, also might be useful for maintaining the introduced bacteria for longer times.

There are few peer-reviewed publications on Approach III, bioaugmentation with strains selected for their enhanced cometabolic abilities. This approach requires a lower mass of cells than Approach I and II, because growth occurs *in situ* through substrate addition. Recently, Semprini et al. (2009) showed that transformation of 1,1,1-TCA could be enhanced by bioaugmentation with a *Rhodococcus* sp. butane-utilizing strain. The approach could be implemented with previously demonstrated approaches for remediation, such as the recirculation well technology used by McCarty et al. (1998a) for the stimulation of indigenous toluene utilizers

at Edwards AFB (Figure 8.2). Transformation product toxicity appeared to play an important role in this process, since results were less impressive in the field and in column tests when 1,1-DCE was present. Here microbes that do not transform the contaminant, but consume the growth substrate, will likely outcompete the introduced microbes that effectively transform the contaminant. Additional research is needed to document strains that have effective transformation abilities for contaminants, such as 1,1-DCE and 1,1,1-TCA. In addition the application of molecular and chemical detection methods that are monooxygenase specific would be useful in determining the effectiveness of added strains.

Microcosm and column studies have been successful at predicting the performance of bioaugmentation in the field. Studies of Munakata-Marr et al. (1996, 1997) showed that Approach II with PR1_{301} was likely to fail in the field test, and this outcome was later observed (McCarty et al., 1998b). Column studies and microcosm studies of Approach III (Semprini et al., 2005, 2007b) showed the potential for enhanced 1,1,1-TCA degradation that was later achieved in the field when an effective strain was added. The column test also revealed the problem associated with transformation product toxicity when 1,1-DCE was present. Since microcosm and column studies are much cheaper than field tests, future research should take advantage of these methods in evaluating bioaugmentation approaches.

Approach IV, priming the target aquifer by adding microbes with desirable cometabolic activities from another aquifer, is one of the simplest approaches to implement. Although limited in applicability, this approach may be beneficial in aquifers undergoing intrinsic remediation where the aerobic processes might be occurring. For example, methanotrophs might be stimulated in an aerobic zone downgradient from an anaerobic zone where methane is being produced. Groundwater from this aerobic zone could be injected into areas where enhanced methanotrophic treatment is desired. A similar approach may be used to add ethene-utilizing microorganisms that have been naturally stimulated by ethene produced from the anaerobic transformation of PCE and TCE. Research in this area could provide a better understanding of whether microorganisms obtained from spatially different areas actually become established and improve treatment as compared to biostimulation of the indigenous bacteria. In addition, the potential value of continuous priming of a treatment zone deserves research. For example, recirculating groundwater from a treatment zone that contains indigenous microorganisms might be used as a means of overcoming the effects of transformation toxicity.

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CHAPTER 9

BIOAUGMENTATION WITH *PSEUDOMONAS STUTZERI* KC FOR CARBON TETRACHLORIDE REMEDIATION

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9.1 INTRODUCTION AND RATIONALE

Until the late 1980s, carbon tetrachloride (CT) was produced in large quantities for use as a dry cleaning agent, grain silo fumigant, degreasing agent and feedstock for refrigerant synthesis (ATDSR, 2005). In 1987, production and consumption of CT was banned under the terms of the Montreal protocol due to its role as an ozone-depleting agent. However CT is still used in some industrial applications and is a regulated groundwater contaminant, present at 201 of the 1,264 National Priorities List sites in the United States (USEPA, 2009). At high levels, CT can damage the liver, kidneys and nervous system, and it can cause cancer in animals (ATSDR, 2005). Its fate in the environment and within cells is a consequence of its structure; its fully oxidized carbon atom and tetrahedral chlorine shield confer resistance to oxidation and hydrolysis, but susceptibility to reduction (Vogel et al., 1987).

Many reducing agents found within living cells, including vitamin B12 (Hashsham et al., 1995), iron porphyrin-based proteins such as cytochromes (Picardal et al., 1993) and menaquinones (Fu et al., 2009), fortuitously reduce CT through one-electron transfers producing CCl_3 free radicals. The radical is implicated in membrane lipid peroxidation (Trimble, 2000), with hydrogen abstraction and concomitant formation of chloroform (CF), another contaminant of human health concern. It is tempting to speculate that indiscriminate intracellular reactivity of CT and CCl_3 explains why no microorganism has yet been isolated that can couple growth and oxidation of an electron donor to CT respiration. It also explains why pathway control, and in particular avoiding the formation of CF, is a critical challenge for CT remediation.

This chapter focuses on aquifer bioremediation by bioaugmentation with denitrifying *Pseudomonas stutzeri* KC, a field-demonstrated strategy that enables pathway control and degradation of CT to levels below the U.S. Environmental Protection Agency (USEPA) maximum contaminant level (MCL) of five parts per billion (ppb, or micrograms per liter [μg/L]). Strain KC is a highly motile (chemotactic toward nitrate) facultative aerobe capable of complete denitrification. Most denitrifying microorganisms convert CT to CF, but strain KC mediates dechlorination through an extracellular process and pathway that does not produce CF and does so at rates that exceed the rates at which other microbial populations can generate CF, thus minimizing or avoiding CF formation. The key to this bioaugmentation strategy is that

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strain KC secretes a compound (pyridine-2,6-bis-thiocarboxylate, or PDTC) that promotes chemical dechlorination of CT outside the cell.

An alternative to bioaugmentation with strain KC is biostimulation, where sufficient organic carbon is added to drive the system into sulfate-reducing or methanogenic conditions (i.e., low pE environments). Under these conditions, CF produced from CT can be sequentially reduced and dechlorinated to dichloromethane**,** which is susceptible to oxidation and hydrolysis (Vogel et al., 1987). For high pE (i.e., oxidized) aquifers, however, use of such a process requires addition of sufficient organic carbon to remove oxygen, nitrate, and ferric iron, with a potential for accumulation of volatile fatty acids, hydrogen sulfide (H_2S), ferrous iron, carbon disulfide, ammonium, methane, and biomass in the resulting low pE environment. Strain KC bioaugmentation avoids these drawbacks: its growth occurs under high pE conditions (aerobic and denitrifying) and even at relatively low cell concentrations, it can sustain adequate rates of CT transformation. As a result, bioremediation can be accomplished with relatively low levels of added organic carbon and without the unwanted byproducts typical of reduced environments.

The following sections detail the chemistry of CT transformation by the secreted agent PDTC, its structure and reactivity, the genes required for its synthesis, and a cellular mechanism proposed for its regeneration. The chapter then describes the transport and ecology of strain KC for bioaugmentation applications, and concludes with a case study.

9.2 PHYSIOLOGICAL FUNCTION OF PDTC PRODUCTION

Early observations associated with the discovery of CT transformation by strain KC determined that it is an iron-regulated process (Criddle et al., 1990). That original work employed a medium in which metals were precipitated by autoclaving with calcium, magnesium and phosphate salts. Adding iron increased the growth yield of the organism, but precluded CT transformation. This finding led Criddle and coauthors to postulate that the physiological function of the components responsible for CT transformation was to scavenge iron. Later work identified the agent of CT transformation as PDTC (Lee et al., 1999), shown in Figure 9.1.

PDTC had been discovered previously by natural products chemists investigating extracellular products of pseudomonads excreted in response to iron stress (Hildebrand et al., 1983). PDTC had thus been assumed to be a siderophore (iron-regulated, extracellular ferric iron chelator with cognate receptor transport) since its discovery almost 30 years ago. An accurate understanding of its function was important because efforts to promote its optimal production typically required some manipulation of site conditions. Different sets of environmental signals

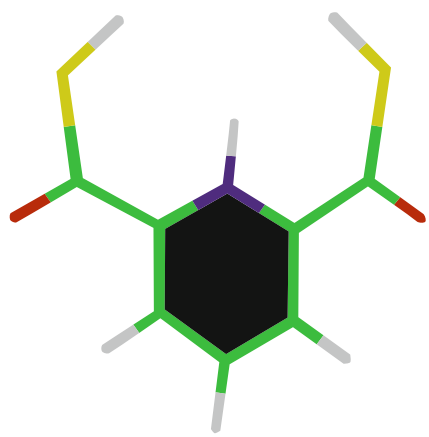

Figure 9.1. Structure of the PDTC.

may have positive or negative effects on PDTC production depending on the PDTC function. For example, if PDTC was excreted simply for its antimicrobial/antagonistic effect, site conditions would have to be manipulated to trigger an antimicrobial response (Cortese et al., 2002).

PDTC was established as a siderophore through experiments with a PDTC-producing strain of *P. putida* (DSM3601) (Lewis et al., 2004). Those experiments showed that: (1) exogenously added PDTC 'rescued' a mutant, unable to produce PDTC, from the growth inhibitory effects of synthetic metal chelators, and (2) an outer membrane protein required for such a rescue effect and for transport of radiolabeled-Fe:PDTC, is encoded within the '*pdt* gene cluster' (Leach and Lewis, 2006). An additional regulation unique to siderophores and superimposed upon iron regulation is coordination among alternative siderophores produced by an individual organism. Current explanations for this coordination in pseudomonads are based on the fact that individual siderophore systems are positively regulated by their own product (i.e., they show positive autoregulation); under iron-limited conditions, no negative regulatory mechanisms are known to repress siderophore gene expression. Therefore, the siderophore that is most successful at procuring iron becomes the most predominant, because the expression of its respective biosynthetic and transport component genes is stimulated more than the genes for less effective siderophores (Poole and McKay, 2003).

Both organisms known to produce PDTC also produce another siderophore: ferrioxamine E for strain KC and pyoverdine for *P. putida* (Lewis et al., 2004). Coordination was observed for the PDTC system of *P. putida* as PDTC production decreased in response to added pyoverdine, while pyoverdine production decreased with added PDTC (Lewis et al., 2004). It is not yet clear what environmental conditions might favor PDTC production over pyoverdine production, but PDTC had a greater role in allowing growth in the presence of a chelator with greater affinity for divalent metals, while pyoverdine had a greater role in resistance to a trivalent metal chelator (Lewis et al., 2004). This finding suggests that the concentrations of a range of metals (Leach and Lewis, 2006) may affect the physiological 'choice' of the siderophore produced.

A fundamental and critical difference between the two PDTC-producing organisms that have been characterized to date is that *P. stutzeri* KC is a denitrifier, capable of anaerobic growth in the presence of nitrate, whereas *P. putida* is an obligate aerobe. Given that nitrate is a highly soluble electron acceptor and is often already present in aquifers or easily added, *P. stutzeri* KC would be the preferred choice for *in situ* bioaugmentation and production of PDTC.

9.3 CT TRANSFORMATION BY *P. STUTZERI* KC AS A NOVEL DECHLORINATION REACTION

The initial description of the CT dechlorination process carried out by *P. stutzeri* KC (Criddle et al., 1990) was of particular interest not because it represented the first of such biological transformations, but because it presented a potentially novel mechanism of dechlorination promoted by a physiologically versatile organism (a denitrifier). Transformations of CT by strictly anaerobic organisms and biomolecules had been described (Gantzer and Wackett, 1991; Vogel et al., 1987). Examples of biomolecule-catalyzed processes include those involving transition metal-containing coenzymes (B_{12}, heme, F_{430}) that promote reductive mechanisms.

The best described of those processes is 'hydrogenolysis', a process in which chlorine atoms are sequentially replaced with hydrogen atoms. The finding that strain KC brought about a net hydrolysis ($CT \rightarrow CO_2$) and that CF was not a product (Criddle et al., 1990), indicated that hydrogenolysis was not involved in that transformation pathway. This property presented the potential advantage of avoiding the accumulation of partially-dechlorinated products, and instead achieving complete dechlorination with a single process. The initial mass balance studies

using $^{14}CCl_4$ also were encouraging, with approximately 55% conversion to carbon dioxide (CO_2) and the balance present as uncharacterized nonvolatile material. The precise chemical mechanism of activation and the resulting transformation pathway was of interest in that it could give insight into the chemical nature of the nonvolatile fraction and the identities of any intermediates produced, and the impacts of local site geochemistry on the products that would be formed *in situ*.

The chemistry of the dechlorination carried out by strain KC was expected to entail an initial mechanism of activating carbon-chlorine bonds, followed by decomposition of reactive intermediates to stable products. To understand the overall process, it was necessary to identify the active agent responsible for the initiating step and the type of decomposition pathway that ensued. A reductive mechanism was suspected due to the fact that CT is largely unreactive toward nucleophiles, as a result of its symmetrical and enveloping arrangement of electronegative chlorine atoms (Jeffers et al., 1989; Kriegman-King and Reinhard, 1992; Schaik, 1983).

An important early finding regarding the relevant chemistry was that copper (Cu) was required in trace amounts for bacterial transformation (Tatara et al., 1993). That data indicated a transition metal-dependent process, but exactly how copper was involved (i.e., whether it affected the production of the active agent or was an integral part of it) was not evident until the active agent was identified. Studies of the decomposition pathway were the first to shed light on the overall process. Those data came from experiments aimed at determining whether one- or two-electron initiating mechanisms were involved.

9.3.1 Pathway of PDTC-Promoted CT Dechlorination

Either a one- or a two-electron mechanism would produce reactive intermediates that could decompose to CO_2, either spontaneously or through a combination of spontaneous and enzymatic steps. A one-electron mechanism was expected to result in trichloromethyl radical, and perhaps phosgene intermediates (Asmus et al., 1985), whereas a two-electron mechanism was expected to produce dichlorocarbene (Asmus et al., 1985; Criddle et al., 1990). Experiments aimed at trapping electrophilic intermediates such as phosgene utilized nucleophilic agents (cysteine, N,N′-dimethylethylenediamine). Those experiments succeeded in substantially altering the products of CT transformation whereas an agent known to react with dichlorocarbene (dimethylbutene) did not (Lewis and Crawford, 1995). The CT carbon atom was bound to a sulfur atom in the resulting products indicating that a sulfur-containing compound played a role in the reaction. Once PDTC was identified as the active agent, its chemical synthesis became possible, as well as its use in cell-free pathway characterization experiments.

The observation that trace amounts of copper were required for optimal CT transformation by bacterial cultures (Tatara et al., 1993) was explained by the fact that the Cu:PDTC complex is the active agent of dechlorination (Lewis et al., 2001). Nanomolar concentrations of copper were sufficient for effective dechlorination, indicating that Cu(II) is catalytic and effective at concentrations likely to be found within contaminated aquifers. Additional experiments allowed trapping of trichloromethyl radical derived from CT when exposed to the Cu-PDTC complex (Lewis et al., 2001).

The proposed pathway (Figure 9.2) includes the following steps: (1) one-electron reductive elimination of chlorine (as chloride) to produce trichloromethyl radical and a sulfur-centered PDTC radical, (2) condensation of trichloromethyl radical and a sulfur-centered PDTC radical to form the first carbon-sulfur bond involving the CT carbon atom (trichloromethyl thioester intermediate), (3) hydrolysis of the thioester intermediate to give trichloromethanethiol and a carboxylate derived from PDTC, (4) rearrangement (*gem* elimination) of trichlromethanethiol

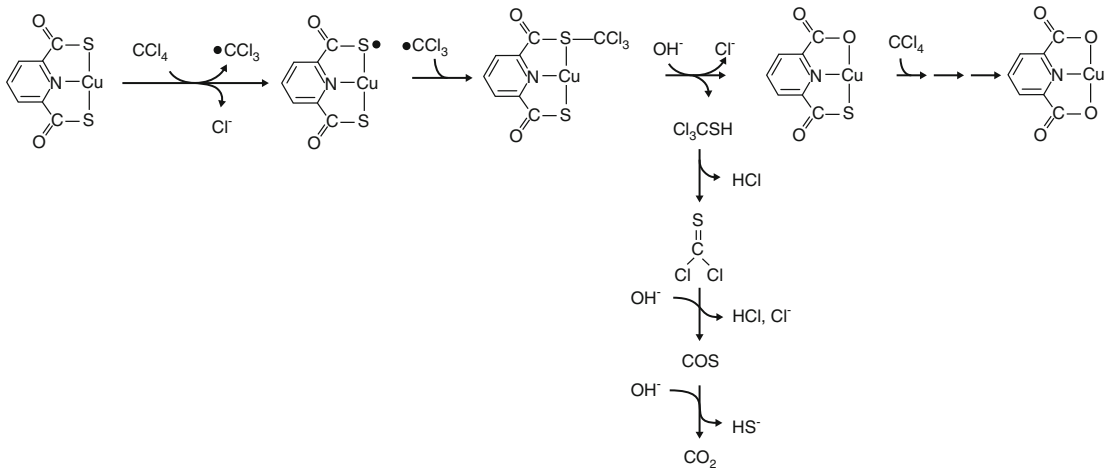

Figure 9.2. Pathway for Cu:PDTC-promoted decomposition of CT. All intermediates have been detected through direct or indirect analytical procedures.

to give thiophosgene, and (5) hydrolysis of thiophosgene via carbonyl sulfide to give CO_2. Intermediates of all steps except (2) were identified directly or indirectly in that earlier work with bacterial cultures or synthetic PDTC (Lewis et al., 2001; Lewis and Crawford, 1995).

Indirect evidence for the trichloromethyl thioester, or at least an electrophilic PDTC derivative, was subsequently obtained using ethylamine as a competing nucleophile, because an ethylamido pyridine derivative was detected rather than the carboxylate derivative expected by hydrolytic attack (Figure 9.3). These data could explain the nonvolatile fraction observed in the initial mass balance experiments as adducts produced from thiophosgene condensation with any of a variety of nucleophiles present in the bacterial cultures. These results also indicate that dechlorination by this process will be quantitative because carbon-chlorine bonds are labilized through this pathway such that nucleophiles present in any aqueous environment will lead to quantitative release of chloride.

The data also indicate that the process is stoichiometric rather than catalytic. Without additional electron donor, the stoichiometry of CT transformation per PDTC approached 2:1, corresponding to both thiocarboxylate sulfur atoms. Though synthetic PDTC might be considered for direct application in a remediation scenario, its oxidative and hydrolytic lability may reduce its cost-effectiveness relative to *in situ* bacterial production, unless other microflora are used for regeneration of synthesized PDTC activity (see Section 9.5).

9.3.2 Transition Metal Chelation of PDTC

The fact that the Cu:PDTC complex is required for CT transformation suggests that other transition metals also might be effective, or could be inhibitory through their ability to form competing PDTC complexes. Accordingly, experiments were undertaken to understand the role of PDTC in potential metal sequestration and detoxification reactions. PDTC complexes of iron (III) (Fe[III]), nickel (II) (Ni[II]), and cobalt (II) (Co[II]) were tested for CT transformation, but none of these showed any significant activity in the absence of added reducing agents (Lewis et al., 2001). Even when iron (III) was added in a 50-fold molar excess to Cu(II) it did not effectively inhibit CT dechlorination (Lewis et al., 2001). The stability constant for the Fe(III):PDTC complex was later determined as 10^{33} (Stolworthy et al., 2001). The lack of effective competition by Fe(III) suggests that the stability constant for the Cu(II):PDTC complex may be

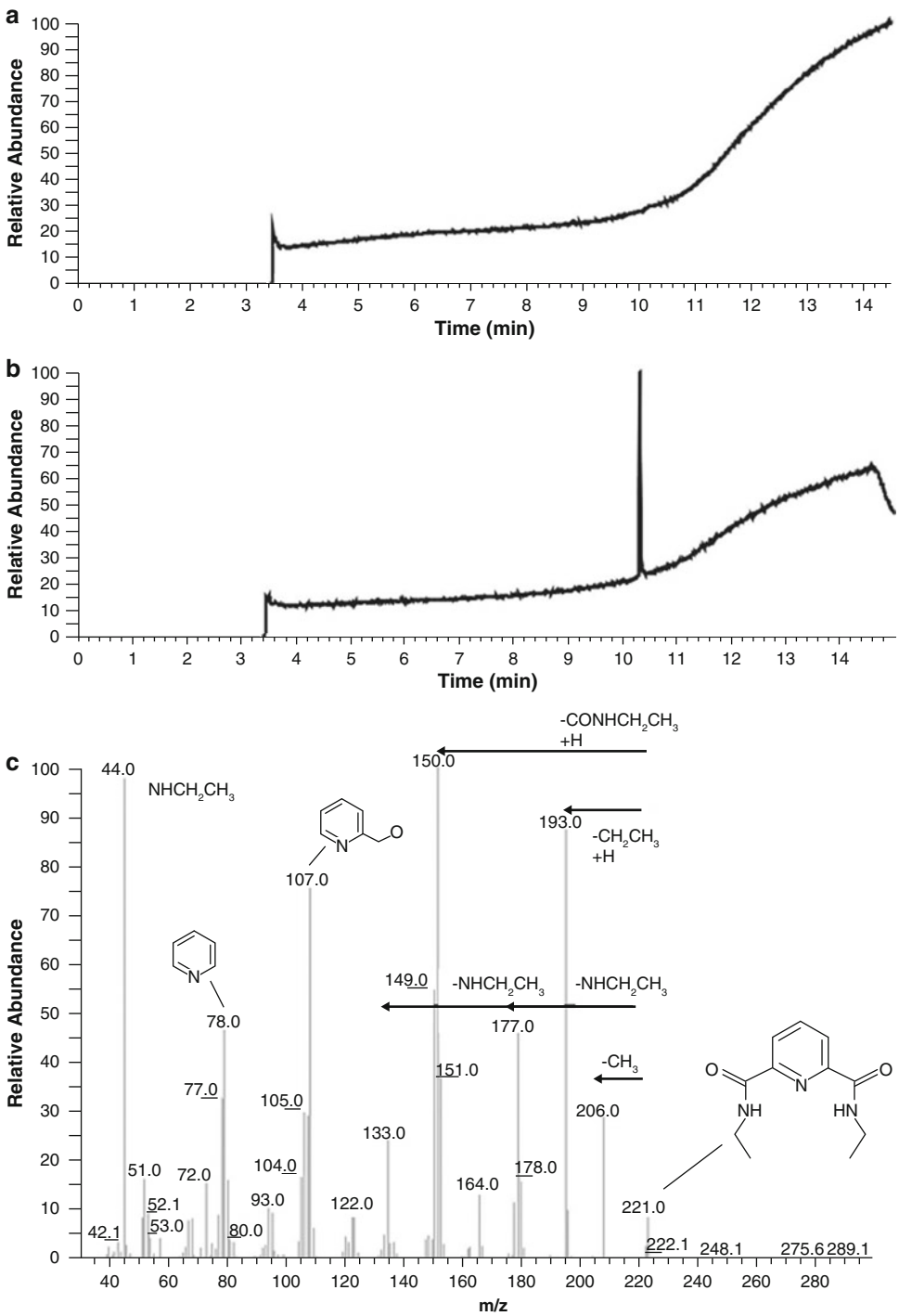

Figure 9.3. Solvent-extractable product formed from Cu:PDTC in the presence of ethylamine. (a) GC chromatogram of extract from reaction mixture including ethylamine and Cu:PDTC. (b) chromatogram of extract from reaction mixture including ethylamine, Cu:PDTC and CCl_4. (c) EI mass spectrum of peak eluting at 10.35 min in b.

even higher. A stability constant of that magnitude suggests that Cu(II) augmentation would not normally be necessary for effective *in situ* remediation.

In addition to Cu(II) and Fe(III), PDTC forms complexes with a variety of elements, including naturally occurring and synthetic transition metals, actinides, and lanthanides (Cortese et al., 2002; Neu et al., 2000, 2001). In several cases, the PDTC:metal complexes of toxic elements were less inhibitory than the metals alone (Cortese et al., 2002). This finding, and the reactivity of PDTC with other toxic elements suggests a possible function of PDTC in detoxification (Zawadzka et al., 2009). Whether this type of selection has influenced evolution of the synthesis and use of PDTC is unknown.

9.4 GENETIC REQUIREMENTS FOR PDTC PRODUCTION

By characterizing genetic requirements of a particular biochemical system, information can be provided for use in genetic 'improvement' or optimization above naturally-evolved levels of expression. Also, insight may be gained into the biosynthetic mechanisms and pathways, which can allow greater predictive capability with regard to which nutrients and/or cofactors may become limiting during sustained operation of a bioprocess. Genes necessary for CT transformation were first identified independently by two groups, using either a spontaneously-arisen mutant (Lewis et al., 2000), or transposon-derived mutants (Sepulveda-Torres et al., 1999). Deoxyribonucleic acid (DNA) capable of complementing the CT transformation/PDTC production phenotype of those mutants was obtained and sequenced, allowing further delineation of a gene cluster sufficient to confer PDTC production upon non-producing pseudomonads (Lewis et al., 2000).

That gene cluster was denoted the *pdt* gene cluster and the alignments of the amino acid sequences of proteins encoded within the *pdt* gene cluster with annotated proteins allowed assignment of putative biosynthetic, transport (uptake and export) and regulatory functions to individual genes (Lewis et al., 2000; Sepulveda-Torres et al., 2002). The sequence of a second *pdt* gene cluster (that of *P. putida* DSM 3601) has since been obtained (Genbank accession AY319946) and shown to be capable of conferring PDTC production upon other pseudomonads (T. A. Lewis, Montana State University, unpublished). In addition, genes from different organisms required for synthesis of a second thiocarboxylic acid-containing siderophore, thioquinolobactin, have been described (*qbs* genes; Matthijs et al., 2004).

The alignment of the two *pdt* gene clusters is shown in Figure 9.4. The minimum set of genes sufficient for PDTC production has not yet been established, and it is possible that some genes within the clusters are superfluous or have modulating effects as opposed to providing an absolutely required function. However, comparisons of *pdt* gene clusters can contribute to our understanding of minimal requirements for conferring a selective benefit, assuming that sufficient time has elapsed during their evolution to allow deletion of random, intervening

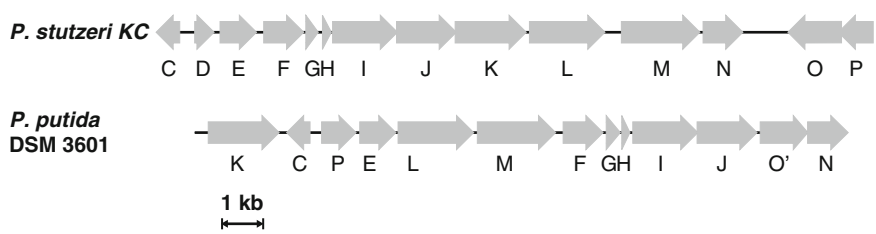

Figure 9.4. Gene clusters encoding PDTC biosynthesis and utilization functions in *P. stutzeri* KC and *P. putida* DSM 3601. Sequences used for open reading frame identification are GenBank accession #s AF196567and AY319946.

DNA (Lawrence and Roth, 1996). Comparisons that include the *qbs* gene cluster also may help elucidate the genes required for the biosynthesis of thiocarboxylate groups on siderophore molecules.

The two *pdt* clusters show strong conservation in terms of gene content, with significant differences in terms of synteny (the co-location of genes along the chromosome). Two genes are not conserved among the *pdt* clusters: (1) *pdt*D, encoding a putative methyltransferase, is found only in the strain KC cluster; and (2) *pdt*O, a putative acylCoA dehydrogenase (ACAD), also has no clear homolog in the DSM 3601 *pdt* cluster. PdtO' of the DSM 3601 cluster is also a putative ACAD but with only approximately 28% sequence identity to PdtO, whereas the other apparent orthologs show between 46% and 79% identity. This suggests that PdtO and PdtO' encode a common function (presumably biosynthetic) for PDTC-producing organisms, but may have been independently 'recruited' into their respective gene clusters.

The similarity of PDTC to dipicolinic acid (DPA) led Hildebrand et al. (1986) to assume its intermediacy in the PDTC biosynthetic pathway. In this view, PDTC biosynthesis could be envisioned as a branch off the lysine and diaminopimelate pathway of bacteria (and plants) which includes 4-hydroxytetrahyrodipicolinate (Blickling et al., 1997). Conversion into DPA could be accomplished by a dehydratase, and a flavin adenine dinucleotide (FAD)-type dehydrogenase, potentially encoded by *pdt*I. Apparent confirmation of DPA as a precursor of PDTC was obtained from experiments that showed incorporation of a deuterium label from DPA into PDTC (Hildebrand et al., 1986). Mass spectrometry of other labeled compounds identified sulfenic acids, and led the authors to propose a pathway including an acylsulfenic acid intermediate, hydrolysis of the acylsulfenic acid, and reduction to thiocarboxylic acid. A monooxygenase would seem critical to such a pathway (for oxygen addition to a thioester) but is not found among the *pdt* genes. The sequence data have instead suggested a different route, related to the well-characterized production of thiocarboxylic acids at the C-termini of certain proteins (e.g., MoeD, a small subunit of *E. coli* molybdopterin synthase or ThiS [Begley et al., 1999; Godert et al., 2007; Leimkühler and Rajagopalan, 2001; Taylor et al., 1998]). Homologs of *pdt*F, G, H, and J are found in the *qbs* gene cluster (Matthijs et al., 2004).

The pathway suggested by the putative enzymatic activities includes adenylate activation of a carboxylic acid precursor by PdtJ, followed by sulfur transfer from cysteine. A cysteine desulfurylase (PdtF) would pass the sulfur atom from the cysteine via a cysteine persulfide modification. This would be transferred to a sulfur transferase enzyme (PdtGH) to form a C-terminal thiocarboxylic acid. How a protein thiocarboxylate is used to form a small-molecule thiocarboxylate like PDTC or quinolobactin is not clear. Resolution of the actual PDTC biosynthetic pathway awaits biochemical characterization of the relevant enzymatic activities encoded in *pdt* clusters.

Expression of *pdt* genes is under transcriptional regulation involving the ferric uptake regulator (*fur*; Sepulveda-Torres et al., 2002) and the product of the *pdt*C gene (Morales and Lewis, 2006). This regulation is ostensibly quite similar to that seen for the siderophore pyochelin, characterized in *P. aeruginosa* (Michel et al., 2005). *fur* regulation explains the original observation of iron regulation of CT transformation activity; the *fur* repressor binds intracellular ferrous iron when supplies are adequate and forms a DNA-binding complex that blocks transcription downstream of its cognate recognition elements ('*fur* box' sequences). *fur* box elements have been identified upstream of *pdt*F in both *pdt* gene clusters, and upstream of *pdt*K and within the *pdt*C/P intergenic region of DSM 3601 (Sepulveda-Torres et al., 2002); Gen Bank AF196567, AF149851, AY319946). The PdtC protein is an AraC-type transcriptional regulator homologous to PchR, a regulator of pyochelin biosynthetic and transport genes dependent upon pyochelin for DNA-binding activity (Michel et al., 2005). Maximal expression from the *pdt*F promoter requires the *pdt*C gene and the siderophore (PDTC) itself, as well as

sequences upstream from the promoter (Morales and Lewis, 2006). This system limits maximal PDTC production to instances in which the siderophore accumulates in the immediate environment of the producing organisms and thus may avoid wasteful investment in the system when the siderophore diffuses away or has a limited half-life.

The concentration threshold for activation of *pdt* gene expression is apparently quite low. PDTC transcriptional activation could be reconstituted in a strain of *P. putida* (KT2440) lacking *pdt* genes. That organism could not be rescued from chelator-induced inhibition by PDTC and is actually inhibited by PDTC (Leach and Lewis, 2006; Sebat et al., 2001). Deletion of genes for the outer membrane receptor (*pdt*K) or the inner membrane transporter (*pdt*E) of DSM 3601 did not produce appreciable negative effects on the transcriptional response to exogenous PDTC, even though the respective strains could no longer use PDTC for iron acquisition (Leach and Lewis, 2006). Together these data indicate that PDTC can enter cells through a non-specific mechanism where it can then bind to and activate PdtC for transcriptional activation.

The functions of other genes found in *pdt* clusters are less clear either because they have not been tested experimentally (*pdt*N, *pdt*P), because their inferred functions do not fit within current models of biosynthesis or transport (*pdt*M), or because alignment searches in existing databases have not yielded informative hits (*pdt*L). PdtN is a putative ABC transporter of the major facilitator superfamily (MFS). It has similarity to efflux permeases such as AraJ, leading to speculation that it may be involved in export of PDTC. A truncated *pdt* gene cluster that lacked *pdt*N, O and P genes showed no CT transformation, whereas deletion of *pdt*O and *pdt*P led to decreased but detectable CT transformation (Lewis et al., 2000). It was not determined whether PDTC had accumulated intracellularly in the *pdt*N deletion-containing strain.

PdtP is a putative S-adenosylmethionine-dependent O-methyltransferase. Since deletion of *pdt*P (and *pdt*O) led to a decrease rather than complete stop in PDTC production, it can be inferred as having an accessory function, perhaps enhancing gene expression or biosynthetic activity/pathway efficiency. No O-methyltransferase activity is required in either of the hypothesized PDTC biosynthetic pathways presented thus far (described above), but a potential role in regulation via post-translational modification has been presented (Sepulveda-Torres et al., 2002). To achieve increased PDTC production, the target of this modification could be a biosynthetic enzyme or a transcriptional regulator.

It is not known whether *pdt*M is required for PDTC production; no *pdt*M mutants have been tested. PdtM is a putative pyridoxal phosphate-containing enzyme with similarity to several aminotransferases. Again, the suggested biochemical function (functional group transfers involving pyridoxal phosphate) has not been incorporated in current hypotheses regarding PDTC biosynthesis.

PdtL is perhaps the most enigmatic gene product of the *pdt* cluster. No tests of its necessity for PDTC biosynthesis have been described to date. It is a protein of 87,538 daltons (Da) and sequence alignment searches identified as homologs only hypothetical proteins (proteins found through *in silico* translation of genome sequences but as yet not known to be expressed in the respective organisms).

9.5 PDTC-MEDIATED CT TRANSFORMATION

PDTC is produced by aerobic or denitrifying *P. stutzeri* KC cells growing under iron-limited conditions. The following sections summarize major factors affecting PDTC production and CT transformation activity.

9.5.1 Trace Metals

As noted previously, the Cu:PDTC complex is the agent of transformation, therefore trace Cu is required for CT transformation. Induction of CT transformation also requires iron limitation. This is because synthesis of PDTC is under *fur* control (Sepulveda-Torres et al., 2002). The *fur* regulator is activated at soluble Fe levels of about 1 micromolar (μM) or less. These low levels can be achieved by adjusting the pH of the growth medium to around 8 (Criddle et al., 1990; Tatara et al., 1993), to cause precipitation of Fe(III) hydroxide, or by adding iron chelating agents (Lewis and Crawford, 1995). To date, field efforts involving bioaugmentation with strain KC have relied upon adjusting the pH to around 8 prior to strain KC introduction. Adjustment of pH to 8 triggers *fur* genes and also confers an ecological advantage for strain KC. Adjusting the pH requires careful attention to the potential for calcium carbonate precipitation, given that many groundwaters are close to saturated with respect to calcite.

9.5.2 Cell and CT Concentration

Before PDTC was identified as the secreted agent responsible for CT transformation, Tatara et al. (1993) found that the initial rate of reaction was first-order with respect to cell concentration and first-order with respect to CT concentration. They described the initial rate of degradation using a second-order rate expression: $-dC/dt = k'CX$, where C is CT concentration, X is biomass concentration, and k' is a pseudo second-order rate coefficient. Initial values for k' are high, in the range of 4 liters/milligram protein/day (L/mg protein/d).

Dybas et al. (1995a) traced CT-degradation activity to the supernatant of strain KC culture medium, but in the absence of actively respiring cells, both the rates and extent of CT transformation were limited. These and other experiments led to the conclusion that transformation of CT involved a cell-free component, later identified as PDTC, as well as one or more cell-associated components. In the absence of cells, the high CT transformation rates initially observed were not sustained, and the extent of CT transformation was limited. By contrast, in the presence of living cells, a period of rapid CT transformation was followed by a period of sustained, but slower CT transformation. The slower sustained rate of transformation enabled a greater extent of transformation, permitting removal of CT at levels of up to 5 mg/L.

The combined results of Tatara et al. (1993) and Dybas et al. (1995a) suggest that CT transformation is initially limited by the concentration of the reduced PDTC-copper complex, and subsequently becomes limited by the rate of synthesis and/or regeneration of the reduced PDTC-Cu complex. Values for k' used in transport models reflect the slower rates of transformation of the slower transformation period, and are in the range of 0.04–0.19 L/mg protein/d (Phanikumar et al., 2002a, b; Vidal-Gavilan, 2000).

To determine whether specific cell types are required for regeneration of the transformation activity, Tatara et al. (1995) combined partially purified cell-free supernatants from strain KC, now known to contain PDTC, with cells that were incapable of CT transformation or that transform it slowly. Table 9.1 summarizes the first-order rate coefficient (k'') and the half-lives obtained. Very rapid CT transformation was obtained when culture supernatant was combined with other cultures, including: other *Pseudomonads* (*P. stutzeri* strains other than KC, *P. fluorescens*); another gram-negative organism (*Escherichia coli* K-12); a gram-positive organism (*Bacillus subtilus*); a consortium (SC-1) derived from CT-contaminated groundwater at Schoolcraft, Michigan; a consortium (HC-14) derived from CT-contaminated groundwater at Hanford, Washington; and yeast (*Saccharomyces cerevisiae*). The results clearly indicated that specific cell types are not required for regeneration of the CT transformation activity.

Table 9.1. Apparent First-Order Rates of Carbon Tetrachloride Transformation (k″) and Half-Lives of Carbon Tetrachloride in Mixtures Containing Diverse Cell Types (washed) and Purified Supernatant Produced by *P. stutzeri* KC (after Tatara et al., 1995). The CT active agent within the purified supernatant was later identified as PDTC.

Cell Type	Growth Conditions[a]	Secreted PDTC Added?[b]	k″ (minute^{-1})[c]	CT Half-life (minute)	Number of Samples
Secreted PDTC alone (No cells present)	Medium D, aerobic or anoxic	NA	0.03 ± 0.03	67 ± 65	13
Pseudomonas sp. strain KC	Medium D, anoxic	Yes	0.18 ± 0.05	4.0 ± 1.1	6
Pseudomonas fluorescens	Medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.20 ± 0.01	3.4 ± 0.1	3
	Nutrient broth	No	0.00 ± 0.00	NA	3
		Yes	0.10 ± 0.00	7.2 ± 0.2	3
Pseudomonas stutzeri	Medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.15 ± 0.00	4.5 ± 0.0	3
Escherichia coli K-12	Medium D, glucose, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.06 ± 0.01	12 ± 1	3
Bacillus subtilus	Nutrient broth	No	0.00 ± 0.00	NA	3
		Yes	0.04 ± 0.01	19 ± 3	3
Schoolcraft consortium (SC-1)	Medium D, aerobic	No	0.00 ± 0.00	NA	3
		Yes	0.25 ± 0.01	2.8 ± 0.1	3
	Medium D, pH 7, 10 μM Fe, aerobic	No	0.00 ± 0.0	NA	3
		Yes	0.28 ± 0.03	2.5 ± 0.3	3
Hanford consortium (HC-14)	SGW pH 7.5 anoxic	No	0.00 ± 0.00	NA	3
		Yes[d]	0.25 ± 0.02	2.7 ± 0.2	3
Saccharomyces cerevisiae	Yeast medium	No	0.00 ± 0.00	NA	3
		Yes[d]	0.12 ± 0.01	5.8 ± 0.5	3

[a]All cells were grown at pH 8.2 unless otherwise noted
[b]Secreted PDTC added as 500 molecular weight (MW) filtrate unless otherwise noted
[c]± Represents the standard deviation of the designated n number of samples
[d]Secreted PDTC added as 10,000 MW filtrate

After Tatara (1996) realized that different cell types could be combined with partially purified cell-free supernatants from strain KC to regenerate PDTC, he developed a bioassay for the then unknown secreted activity. In his bioassay, which evidently assays the PDTC-Cu complex, Tatara (1996) added washed *P. fluorescens* cells to supernatant samples produced by strain KC, added CT, and monitored the rate of CT degradation. These assays confirmed that the rate of CT degradation is directly proportional to the concentration of added cells, up to a saturation cell concentration of ~10^8 colony forming units/milliliter (cfu/mL). Additional studies are needed to determine rate constants for the pure PDTC-Cu complex in the presence and absence of cells.

9.5.3 Cell Membrane Components

While testing different cells for the capacity to regenerate secreted CT transformation activity (Table 9.1), Tatara (1996) discovered one cell type, *Lacobacillus acidophilus*, that was unable to regenerate the secreted PDTC. *L. acidophilus* is a strictly fermenting organism, and lacks electron transport chains, suggesting a possible role of electron transport chains in activation of the secreted PDTC for CT transformation (later identified as the Cu:PDTC complex). To investigate this possibility, Tatara prepared crude cell membrane and cytoplasmic fractions from strain KC cells, then combined them with the secreted PDTC to determine whether membrane bound or cytoplasmic proteins enhance CT transformation. Reduced nicotinamide adenine dinucleotide (NADH) also was added to determine whether it enhances transformation and, if so, whether it does so in combination with an additional mediator protein. Freshly prepared membrane and cytoplasmic fractions were combined with purified supernatant with and without NADH. As shown in Figure 9.5, rapid CT transformation occurred when secreted PDTC (labeled here as SF for secreted factor) was combined with crude cell membranes in the presence of 20 μM NADH. Transformation of CT also occurred when secreted PDTC was combined with crude cell membranes without NADH, but rates were not as fast as with NADH. No CT transformation was observed when secreted PDTC was combined with the cytoplasm of stain KC cells with or without NADH present. The secreted PDTC did not mediate significant CT transformation when combined with NADH, indicating that rapid transformation required a membrane-associated protein and transfer of reducing equivalents from NADH.

Tatara (1996) also tested the effects of inhibitors of respiration, including antimycin and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and the protonophore, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). HQNO inhibits respiration by blocking electron transport between coenzyme Q and cytochrome b, and antimycin inhibits electron transport between cytochrome b and cytochrome c or cytochrome o. No inhibition of CT transformation was found in the presence of antimycin, HQNO or CCCP. This result indicates that coupling via a proton gradient does not directly drive CT transformation, and implies that any electron

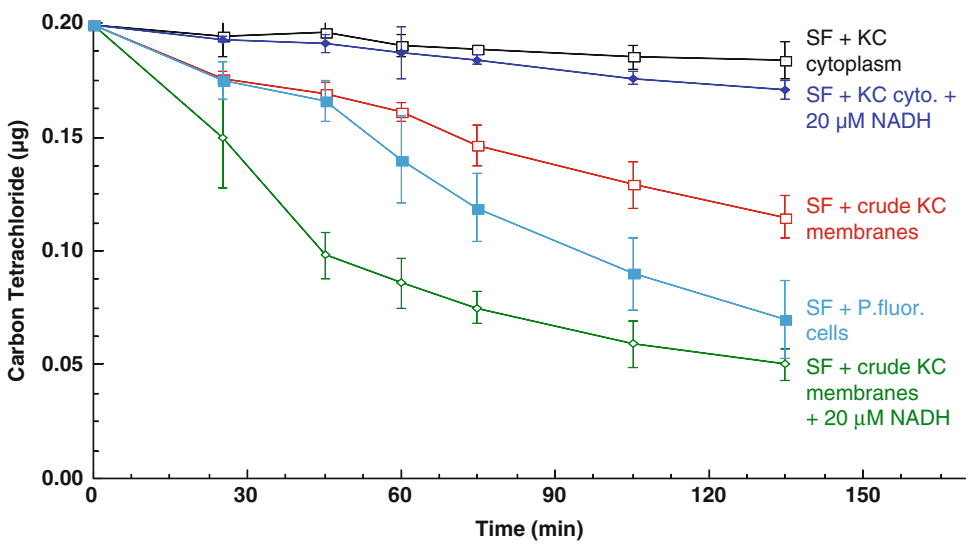

Figure 9.5. Transformation of CT by PDTC secreted by strain KC (SF) in the presence of crude cell membrane and cytoplasm lysate. The ß-form of NADH was used in this experiment. Error bars represent the standard deviation of triplicate sample.

transport chain linkage occurs at a level above cytochrome b. Alternatively, electron transfer may be achieved through a non-respiratory electron transport chain.

9.5.4 An Overall Model for CT Transformation by *Pseudomonas stutzeri* KC

Figure 9.6 illustrates an overall model for PDTC synthesis, activation/regeneration, and sustained CT transformation. Requirements are induction of the *fur* response by the establishment of iron-limiting conditions, presence of actively respiring cells and membrane-linked regeneration of PDTC activity.

9.6 BIOAUGMENTATION WITH *P. STUTZERI* KC: TRANSPORT, GROWTH AND COMPETITION

Bioaugmentation with *P. stutzeri* KC is a three-step process involving: (1) preparation of the treatment zone for the introduction of strain KC; (2) growth of a viable inoculum and injection of strain KC into the treatment zone; and (3) intermittent addition of chemicals to the treatment zone to maintain strain KC concentrations and to induce genes that regulate production and secretion of PDTC.

Because *P. stutzeri* KC does not obtain energy from CT dechlorination (i.e., the reaction is cometabolic), strategies are needed to ensure its growth and survival over the time period

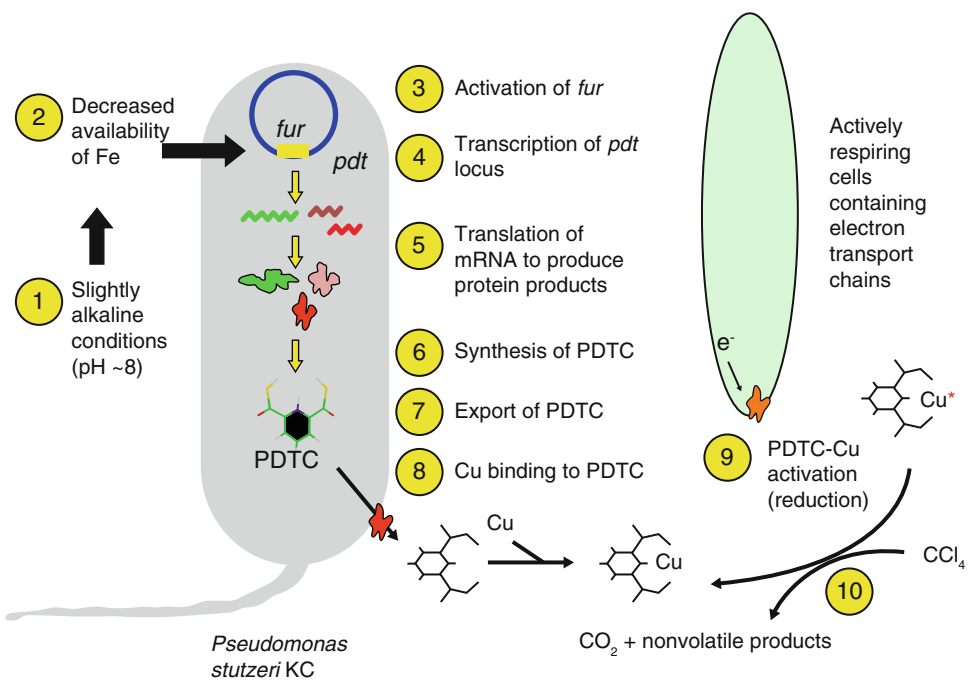

Figure 9.6. Model of CT transformation by *P. stutzeri* KC in the presence of actively respiring cells: a slightly alkaline pH (step 1) causes a decrease in iron bioavailability (step 2), activating *fur* response (step 3), transcription of pdt genes (step 4), translation of mRNA transcripts (step 5), synthesis of PDTC (step 6), secretion of PDTC (step 7). Secreted PDTC interacts with transition metals, such as Cu (step 8). When it binds Cu and is reduced at the cell membrane of an actively respiring cell (step 9), the complex is activated for CT transformation (step 10).

needed for remediation. This time period may be lengthy, on the order of years. One strategy that has proven effective is adjustment of the pH of the environment targeted for remediation to favor strain KC. Also important is delivery of the organism itself. Tests with aquifer solids from Schoolcraft, Michigan, indicated that a minimum concentration of cells required for solids colonization and CT degradation activity was 3×10^4 cells/mL groundwater at pH 8 (S. Hashsham, Michigan State University, East Lansing, MI, personal communication). The requirement for such a "minimum invasion force" is similar to the concept of minimum infectious dose in the field of public health microbiology, but in this case, gives a target for minimal cell delivery to the treatment zone. Achieving this target depends upon factors affecting cell transport, while long-term maintenance of cells and their CT-degradation activity depends upon the properties of the indigenous microflora and chemical delivery. These issues are discussed in the following sections.

9.6.1 Inoculation and Transport

Biomass injected during an inoculation event is transported with the fluid and deposited by adhesion to solid particles. Breakthrough of biomass during cell inoculation is therefore retarded relative to the transport of an ideal tracer. Several authors (Harvey, 1991; Martin et al., 1992; Rijnaarts et al., 1996a, b) have applied clean-bed filtration theory to describe deposition of bacteria in porous media, and modeled this process in terms of collector efficiency. The percentage of biomass that attaches to solid particles is a function of the collision efficiency and a site-specific "blocking factor". These parameters in turn depend upon the solid matrix characteristics, the ionic strength of the solution, the cell physiology and the cell concentration. Radabaugh (1998) used clean-bed filtration theory to determine the basic filtration parameters for strain KC in one-dimensional columns containing solids from the Schoolcraft site. These solids become saturated at a biomass concentration of 3×10^7 cfu/g. Once this concentration was achieved, 100% breakthrough of biomass occurred in the liquid phase. Vidal-Gavilan (2000) successfully used the transport parameters measured by Radabaugh (1998) to model the distribution of strain KC following inoculation in a three-dimensional (3-D) model filled with Schoolcraft aquifer solids.

In addition to adherence, other factors also influence transport of *P. stutzeri* KC, including a tendency to flocculate under nutrient-limited conditions and chemotactic motility in response to nitrate gradients. Flocculent cells are transported poorly, and may necessitate a reinoculation (Dybas et al., 2002), but effective colonization still may be possible if nitrate is present in the background groundwater. Witt et al. (1999) demonstrated use of strain KC for remediation of a 0.5 meter (m) static sediment column filled with of CT- and nitrate-contaminated groundwater. Strain KC cells and acetate were added to the middle of the column. Migration of cells away from the point of inoculation was monitored, along with concentrations of nitrate and CT throughout the column. Introduction of excess acetate quickly caused nitrate-limited conditions at the center of the column, and formation of a nitrate gradient. The nitrate gradient triggered a chemotactic response. As strain KC migrated away from the center of the column and towards the ends of the column, nitrate and CT levels fell. The column was remediated without flow of groundwater.

In the field-scale inoculations performed to date (Dybas et al., 1998, 2002), nitrate was present in the background groundwater. Addition of high levels of acetate along with strain KC likely facilitated a chemotactic response and more rapid colonization of the targeted treatment zones.

9.6.2 Growth and Competition

P. stutzeri KC can be grown aerobically or under denitrifying conditions with several electron donors, including complex organics, such as nutrient agar, acetate, glycerol and vegetable oils (soybean and canola). Remediation efforts to date have focused on the use of acetate. A growth curve typical of acetate-fed denitrifying cells is shown in Figure 9.7. Like other denitrifying *P. stutzeri*, strain KC stoichiometrically converts nitrate to nitrite followed by sequential reduction of nitrite to NO, N_2O, and nitrogen gas.

As already noted, an important consideration in aquifer bioaugmentation with strain KC is the niche that the added cells will occupy after introduction. In order for PDTC-mediated CT degradation to occur, the added strain KC cells must compete successfully with the native microflora. A difficulty is that the native microbial community are adapted to the extant biogeochemical conditions. This advantage can be overcome by altering conditions to favor growth of added strain KC. Because strain KC is an efficient iron-scavenger and capable of rapid growth under iron-limited conditions, it can be given a competitive advantage by adjusting the pH to near 8 prior to inoculation (Dybas et al., 1995b). Figure 9.8 illustrates the maximum specific growth rates for strain KC and indigenous microflora in the Schoolcraft, Michigan aquifer.

From Figure 9.8, it is clear that *P stutzeri* KC has a competitive advantage at pH values above 7.8 in competition with denitrifying microflora from the Schoolcraft, Michigan, aquifer. This may in part be due to the production of PDTC itself since PDTC behaves as an antagonist for competing populations (Sebat et al., 2001).

Table 9.2 gives additional insight into the nature of the competitive advantage afforded strain KC by pH adjustment at the Schoolcraft site, comparing maximum specific growth rate

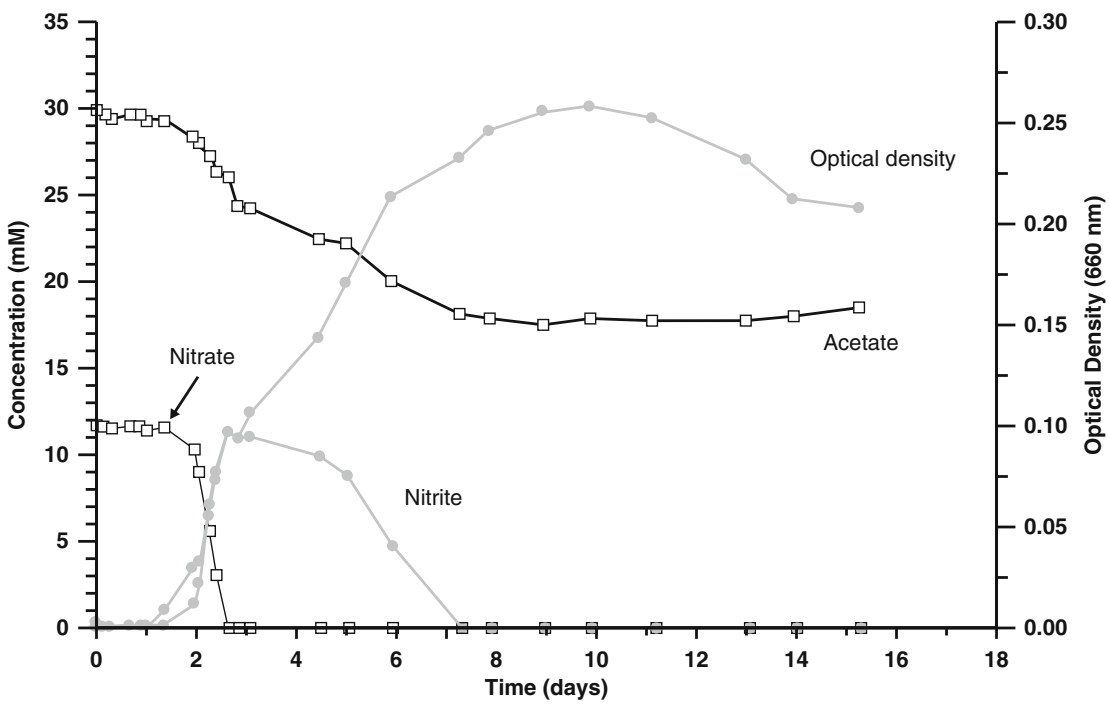

Figure 9.7. Growth curve for denitrifying *P. stutzeri* KC: acetate is the electron donor; nitrate is the electron acceptor; initial pH = 8 (Knoll, 1994).

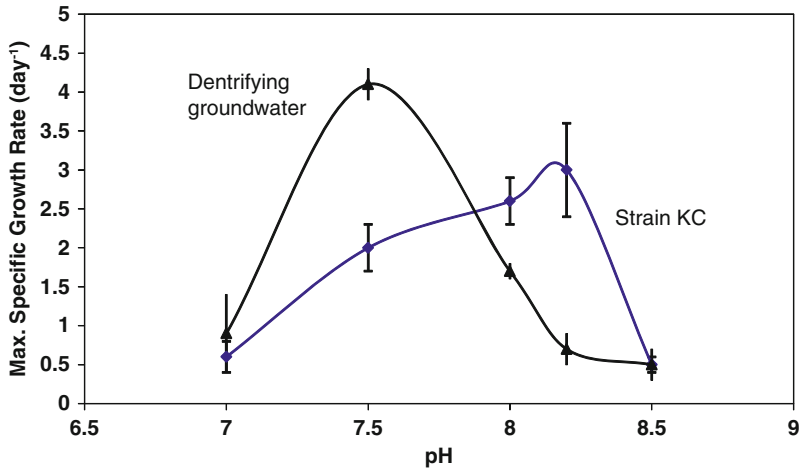

Figure 9.8. Maximum specific growth rates for *P. stutzeri* KC versus native microflora in Schoolcraft, Michigan aquifer groundwater (from Sneathen, 1996).

Table 9.2. Kinetic Parameters for Nitrate Conversion to Nitrite and for Nitrite Conversion to Nitrogen for *P. stutzeri* KC and Denitrifying Microflora Native to the Schoolcraft, Michigan Aquifer (adapted from Knoll, 1994)[a].

	Cultures Grown at pH 8.2	
	Strain KC	**Schoolcraft Site Microflora**
NO_3^- to NO_2^-		
μ_{max}, maximum specific growth rate, d^{-1}	3.1 ± 0.7	0.8 ± 0.2
q_{max} maximum specific rate of acetate utilization, mg acetate/mg vss-d	6.3 ± 1.4	7.8 ± 2.0
Observed yield, mg vss/mg acetate	0.5 ± 0.1	0.1 ± 0.0
Moles nitrate consumed per mole acetate consumed	2.7 ± 0.5	1.6 ± 0.2
Moles nitrite produced per mole NO_3^- consumed	1.0 + 0.0	0.6 ± 0.1
NO_2^- to N_2O and/or N_2		
μ_{max}, maximum specific growth rate, d^{-1}	0.2 ± 0.1	0.7 ± 0.1
q_{max}, maximum specific rate of acetate utilization, mg acetate/mg cell dry weight-d	0.5 ± 0.2	3.3 ± 0.4
Observed yield, mg vss/mg acetate	0.4 ± 0.0	0.2 ± 0.1
Moles nitrite consumed per mole acetate consumed	1.4 ± 0.2	2.2 ± 1.0
Overall		
Observed yield, mg vss per mg acetate	0.41 ± 0.16	0.14 ± 0.06
Moles nitrate removed per mole acetate consumed	1.9 ± 0.2	1.8 ± 0.3

[a]Volatile suspended solids (vss) are assumed to be 90% of the dry cell mass. All cultures were grown at pH 8.2 and T = 21 degrees Celsius (°C)

parameters for the two initial steps of denitrification at pH 8. The data suggest that strain KC has a competitive advantage over native microflora in reducing nitrate to nitrite (higher specific growth rate), while native microflora have a competitive advantage over strain KC in reducing nitrite to nitrogen gas. The expected ecological niche occupied by strain KC at the Schoolcraft site would thus be the first step in denitrification, while native denitrifiers would occupy subsequent steps.

9.7 pH ADJUSTMENT

Adjusting the pH of CT-contaminated soil and groundwater to a value of ~8 creates conditions that enhance the competitiveness of strain KC and promotes CT degradation. Specifically, iron solubility is decreased to low levels, the *fur* gene is expressed, PDTC is produced and strain KC becomes more competitive. A challenge in the execution of this step in the field is the potential for clogging well screens due to the formation of calcium carbonate ($CaCO_3$) scale, especially given that groundwater is often at or near saturation with respect to calcium carbonate. To prevent $CaCO_3$ formation, care must be taken to ensure well development, minimize supersaturation and prevent excessively high pH levels.

Figure 9.9 illustrates a pC-pH diagram for a closed system using data for Schoolcraft groundwater (Warnick, 1998), and thermodynamic data that is adjusted to 15°C. The plot provides understanding of solution chemistry prior to pH adjustment.

As shown in Figure 9.9, for Schoolcraft groundwater at a pH of 7.25 and temperature of 15°C, the equilibrium concentration of Ca^{2+} is $10^{-2.6}$ molar (M) (where the line "*Groundwater pH*" intersects the line labeled "Ca"). Because this equilibrium value is close to the measured

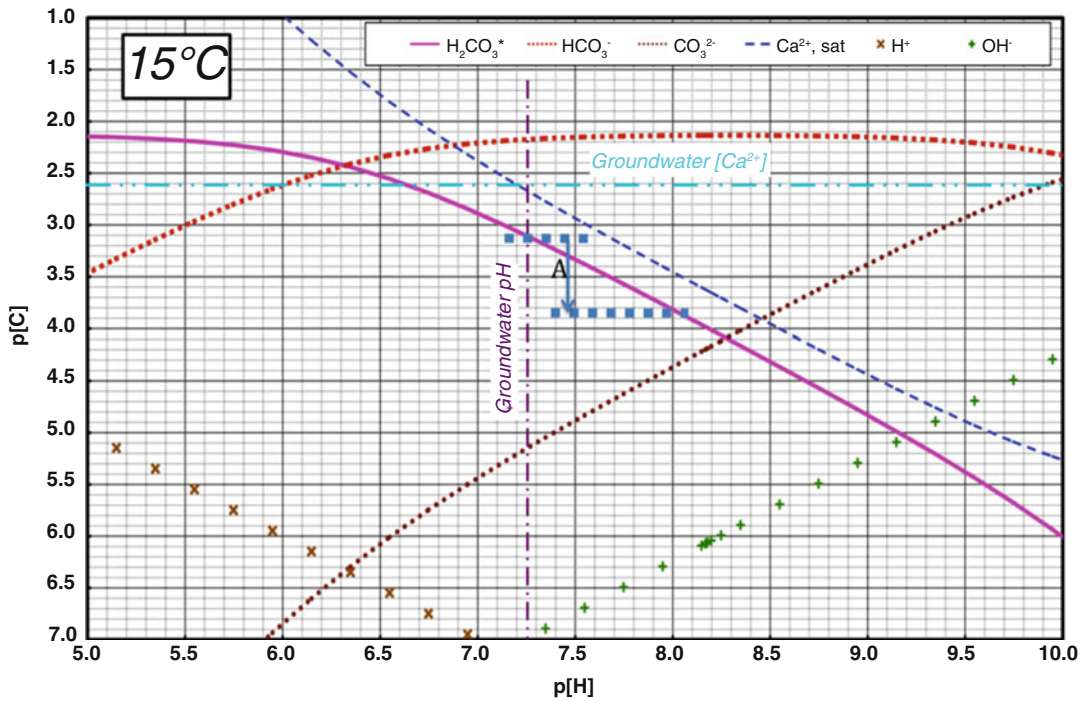

Figure 9.9. pC-pH diagram for Schoolcraft groundwater at 15°C in a closed system. $Ca^{2+} = 10^{-2.6}$ M. *Line A* represents the base addition needed to increase pH from the initial value of 7.25 to a final value of 8.0.

Ca^{2+} concentration of $10^{-2.6}$ M (dashed line labeled "*Groundwater* [Ca^{2+}]"), this groundwater can be considered saturated with respect to $CaCO_3$ at a temperature of 15°C. However, the actual temperature of Schoolcraft groundwater is 13.1°C. At this temperature, the groundwater will be slightly undersaturated.

In order to increase pH to 8, strong base can be added, for example as NaOH, according to the reaction $OH^- + H_2CO_3 \rightarrow HCO_3^-$. Graphically, the NaOH to be added can be determined from the change in H_2CO_3 levels as pH increases from 7.25 to 8: $10^{-3.1}$–$10^{-3.8} = 6 \times 10^{-4}$ M NaOH (line **A**). How this base is introduced into the aquifer becomes critical. It would be unwise to add it directly into the wells as a high pH solution near the well screen would result and scaling would ensue. Extraction of groundwater, with aboveground mixing of base to achieve the target pH allows a gradual titration of subsurface solids. Such a strategy was successfully implemented at Schoolcraft, Michigan (Dybas et al., 2002).

In theory, pH adjustment could be accomplished by allowing aboveground degassing of carbon dioxide. Figure 9.10 illustrates the expected situation for Schoolcraft groundwater pumped to the surface and allowed to equilibrate with the atmosphere.

When groundwater is allowed to equilibrate with the atmosphere, outgassing of CO_2 can be expected because carbonic acid and dissolved levels of CO_2 far exceed the levels that would be in equilibrium with atmospheric CO_2. At the Schoolcraft site, CO_2 levels in the groundwater

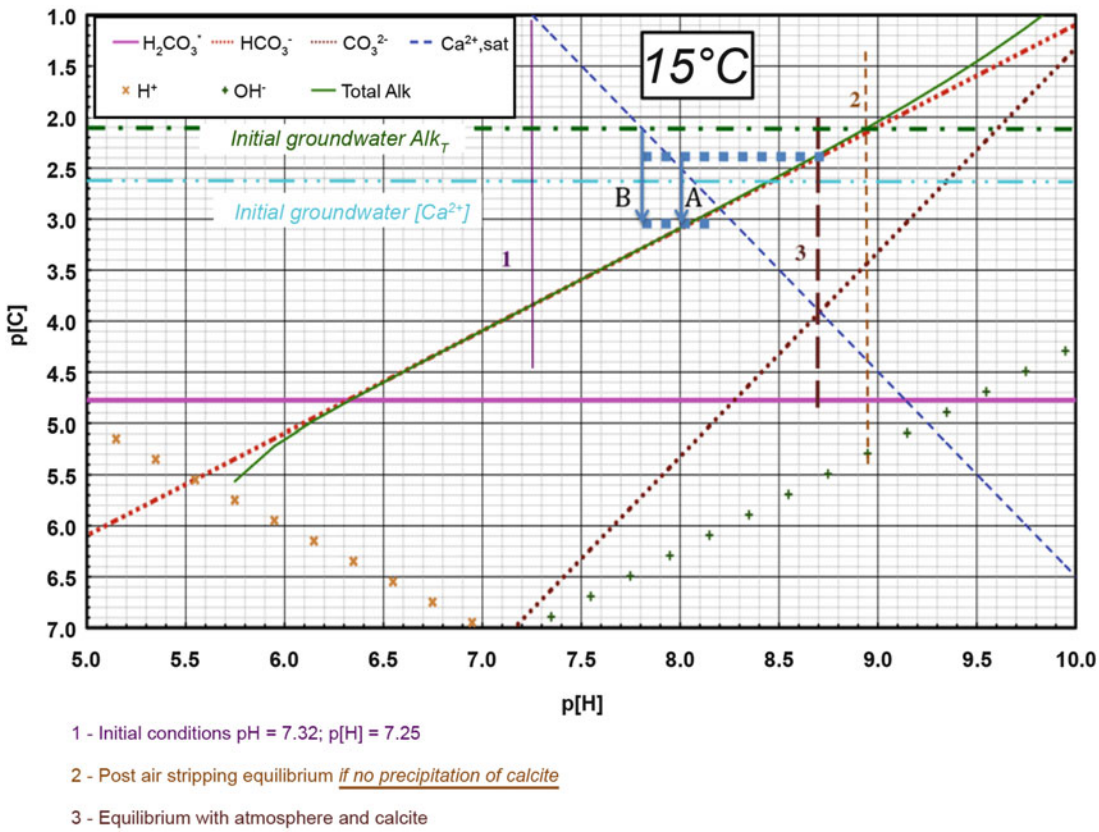

Figure 9.10. pC-pH diagram for Schoolcraft groundwater at 15°C in an open system. *Line 1* represents initial pH. *Line 2* indicates conditions after degassing of CO_2, with calcite still present. *Line 3* indicates conditions after degassing of CO_2 and calcite precipitation. *Line A* represents acid added to decrease pH to 8 if precipitate is removed from solution. *Line B* represents the acid added to decrease pH to 8 if precipitate is not removed from solution.

are more than 50 times those of the atmosphere. If groundwater is pumped to the surface and allowed to equilibrate with the atmosphere, the concentration of dissolved CO_2 and carbonic acid species (i.e., H_2CO_3*) becomes fixed at $10^{-4.8}$ M due to equilibrium with atmospheric CO_2. If no $CaCO_3$ precipitation occurs, alkalinity remains unchanged, and the pH increases from 7.25 (line 1 in Figure 9.10) to 8.95 (line 2). At pH 8.95, however, the water is severely oversaturated: the initial groundwater Ca^{2+} concentration of $10^{-2.6}$ M greatly exceeds the equilibrium value of $10^{-4.4}$ M at pH 8.9. Precipitation is therefore expected. As it occurs, the alkalinity of the water falls to $10^{-2.4}$ M and pH decreases to 8.7 (line 3), and the equilibrium Ca concentration falls from the initial value of $10^{-2.6}$ M to a final value of $10^{-3.9}$ M. If the precipitate resulting from this step is removed, strong acid could be added, for example as HCl, to achieve a pH of 8, per the reaction $H^+ + HCO_3^- -> H_2CO_3$. Carbon dioxide would be removed as bubbles to the atmosphere. Such a protocol would entail significant CO_2 outgassing. Graphically, the amount of HCl to be added for this case is given by the change in HCO_3^-(line A): $10^{-2.4}$–$10^{-3.1} = 3 \times 10^{-3}$ M HCl. On the other hand, if precipitated $CaCO_3$ solids are not removed, even more acid must be added (7×10^{-3} M HCl, line B) to convert the carbonate in the precipitate into CO_2.

In tests of the hydrodynamic factors affecting $CaCO_3$ precipitation, Hammer et al. (2008) reported: "Calcite was . . .observed to grow on the gas-water interface of gas bubbles produced from degassing of N_2 or CO_2. Such bubbles increase the area of the gas-water interface and thereby the total precipitation rate." Based on this information and observations of well screen clogging at the Schoolcraft site when an outgassing strategy was attempted, base addition to a closed system is recommended. Moreover, since interfaces provide nucleation sites for precipitation, the importance of careful well development before pH adjustment steps are implemented cannot be overemphasized.

9.8 FIELD EXPERIENCE: PILOT- AND DEMONSTRATION-SCALE TESTING

9.8.1 Design and Site Characterization

Field tests of bioaugmentation with *P stutzeri* KC were performed at pilot-scale (Dybas et al., 1998) and demonstration-scale (Dybas et al., 2002) in a CT- and nitrate-impacted region of the St. Joseph aquifer located in southwest Michigan. Schoolcraft Plume A is a region of CT contamination approximately 1.6 kilometers (km) long and 160 m wide (Figure 9.11). Solute transport simulations, site geology and a cost analysis are described in detail elsewhere (Hyndman et al., 2000). The aquifer consists of approximately 27 m of glacial outwash sediments, with a water table approximately 5 m below ground surface (bgs). Average groundwater velocity is 15 centimeters per day (cm/day). The formation is underlain by a nearly impermeable clay unit, which appears to be lacustrine in origin. The aquifer sediments can be roughly classified into three different zones (Table 9.3). In addition to these three broad zones, a narrow (5–10 cm) gravel lens was located just above the confining clay unit.

9.8.2 pH Adjustment, Inoculation and Biocurtain Colonization

In the initial pilot-scale experiment, chemicals (base and substrates) and *P. stutzeri* KC cells were mixed, and then introduced directly into the aquifer as slug injections (Dybas et al., 1998). Two key findings were obtained: (1) injection without recirculation resulted in non-uniform delivery of chemicals and organisms, limited CT removal (60–65%), and failure to reliably achieve groundwater CT levels below the MCL of 5 ppb; and (2) *P. stutzeri* KC could be

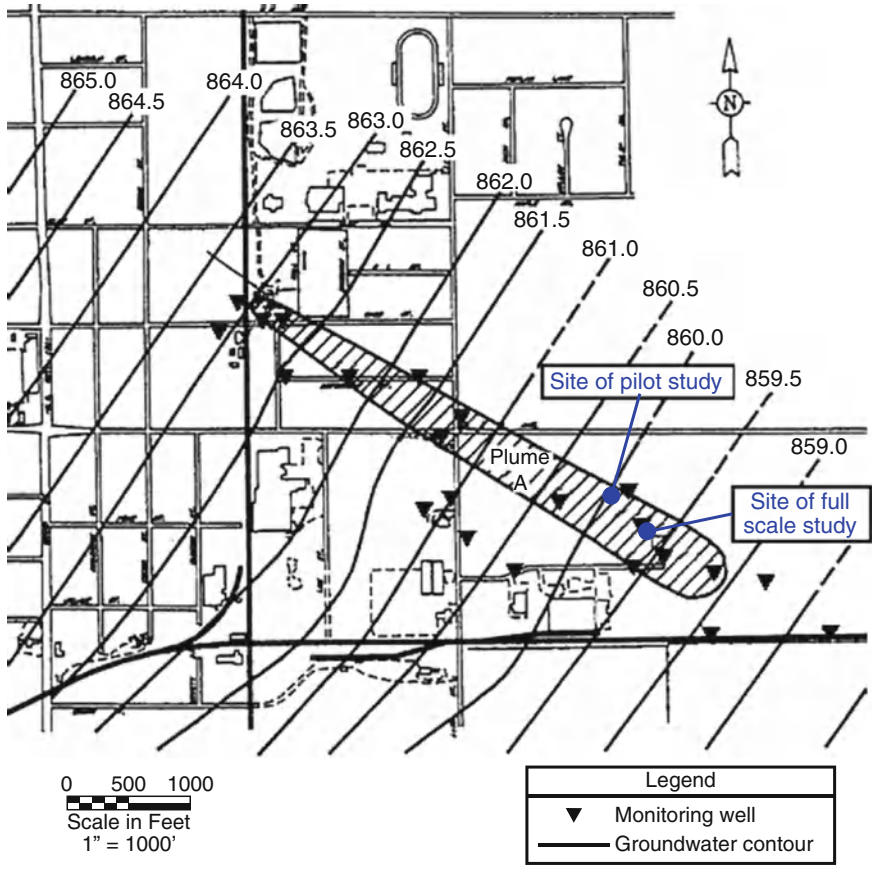

Figure 9.11. Schoolcraft Plume A: site of pilot- and demonstration-scale tests of bioaugmentation with *P. stutzeri* KC.

Table 9.3. Chemical and Physical Characteristics of Aquifer Sediments at the Schoolcraft, Michigan Site {Min-Max (*Median*)}

	Shallow Zone	Middle Zone	Deep Zone
Depth bgs (m)	9–15	15–21	21–27
Sediment class[a]	Fine sand	Medium sand	Coarse sand, gravel, coal
Hydraulic conductivity[a] (cm/second [s])	0.0040–0.038 (*0.012*)	0.0011–0.063 (*0.027*)	0.019–0.11 (*0.046*)
Velocity[b] (cm/day)	6.45	14.53	24.74
Sorbed phase CT[c] (μg/kilogram [kg])	Nd^2-15.7 (*3.39*)	Nd^2-24.1 (*6.50*)	Nd^2-47.6 (*12.7*)
Aqueous phase CT[d] (μg/L)	2.30–25.6 (*5.6*)	4.89–27.6 (*8.08*)	6.46–46.5 (*18.0*)
K_d (L/kg)	0.012–0.529 (*0.145*)	0.015–1.17 (*0.165*)	0.080–3.44 (*0.353*)
Retardation factor[e] (CT)	1.06–3.56 (*1.70*)	1.07–6.67 (*1.80*)	1.39–17.68 (*2.70*)
Aqueous phase nitrate[d] (mg/L)	13.4–57.3 (*40.2*)	22.9–58.8 (*42.0*)	1.87–63.7 (*41.0*)

[a]The sediment class, hydraulic conductivity, and sorbed phase CT values are based on analysis of the cores collected from the even numbered delivery wells (D2-D14)
[b]Calculated from average K in each layer. Bulk velocity is 15 cm/day
[c]Nd = below detection limit (Detection limit (LD) = 0.083 μg/kg, critical level (LC) = 0.043 μg/kg)
[d]The aqueous phase concentration statistics are based on all aqueous phase samples collected from the observation well grid prior to system operation (November 7, 1997)
[e]Calculated from K_d assuming porosity of 0.33 and bulk density of 1.6

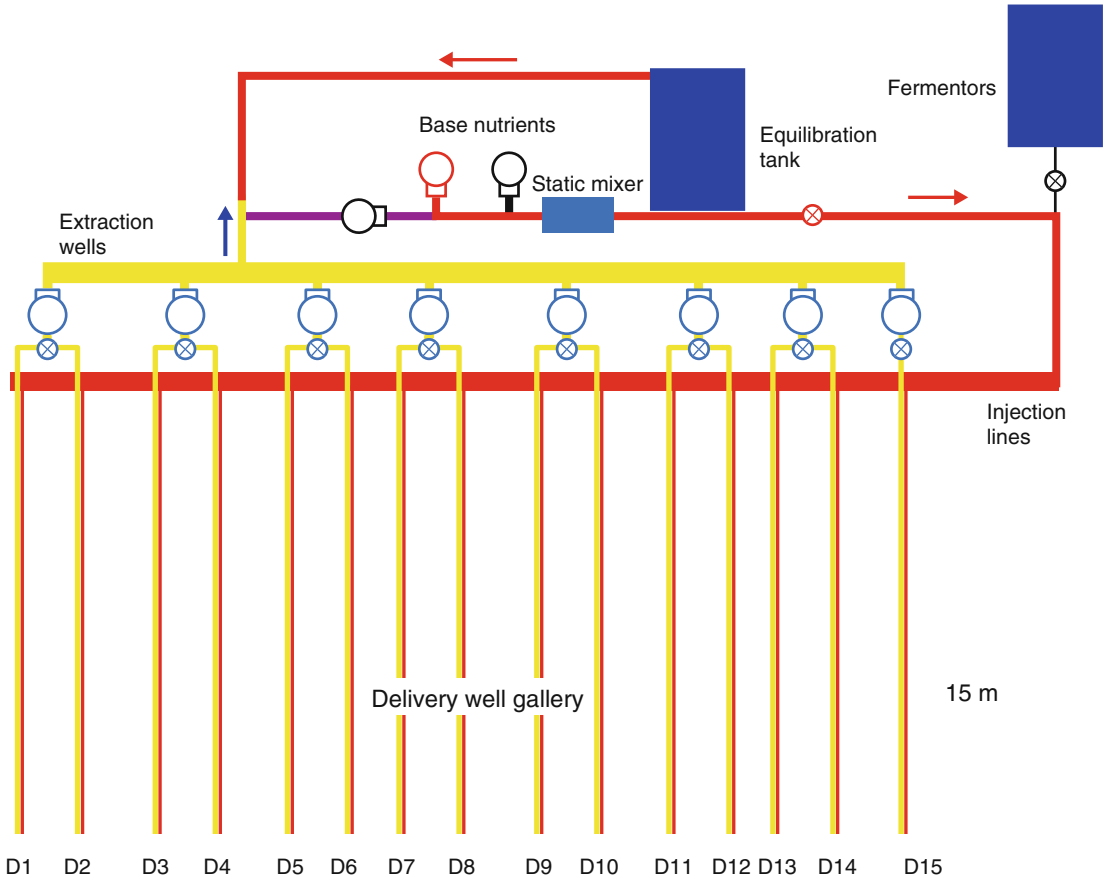

Figure 9.12. Delivery well gallery and aboveground system used to create a biocurtain in the demonstration-scale experiment conducted at Schoolcraft, Michigan.

transported from an injection well to a monitoring well located 1 m away. Both findings impacted the design and operation of the demonstration-scale experiment.

The primary focus of the demonstration-scale experiment was to improve chemical and organism delivery to achieve uniform and reliable CT removal to levels below the MCL. As shown in Figures 9.12 and 9.13, this was accomplished using a "picket fence" of extraction/injection wells located orthogonal to the direction of groundwater flow (transect C in Figure 9.13), spaced 1 m apart, and coupled to an aboveground system for chemical and organism delivery (Dybas et al., 2002). Weekly operation of this system made it possible to colonize a large region of the subsurface with *P. stutzeri* KC and to sustain long-term CT degradation to levels below the MCL.

For the pH adjustment phase, groundwater was extracted from the aquifer, adjusted to pH 8 using sodium hydroxide and injected back into the aquifer. This process was repeated until the pH at monitoring wells located 1-m downgradient from the delivery well gallery was deemed high enough for inoculation with *P. stutzeri* KC.

Three inoculations were evaluated in the two field studies conducted to date, all using aerobically-grown *P. stutzeri* KC. The inoculation strategies included: (1) a single inoculation without recirculation in the pilot-scale experiment; (2) a single inoculation with recirculation in the demonstration-scale experiment; and (3) a reinoculation in the demonstration-scale

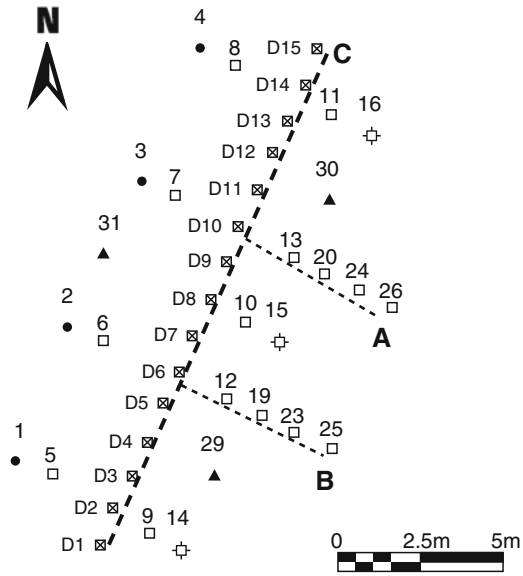

Figure 9.13. Plan view layout of delivery wells (Transect C) and monitoring wells (Transects A and B) in the demonstration-scale experiment conducted at Schoolcraft, Michigan. Reprinted with permission from Dybas et al., 2002. Copyright 2002, American Chemical Society.

experiment with recirculation at four times greater volume and supplemented with trace nutrients to minimize flocculation (Dybas et al., 1998, 2002). The delivery of microorganisms, as well as solutes, was more uniform and occurred over greater distances when groundwater was recirculated by coupled injection/extraction (second and third injections) compared to injection alone (first injection).

In all three inoculations, *P. stutzeri* KC was grown on site in site groundwater, amended with acetate (800–1,600 mg/L) and phosphate (10 parts per million [ppm]), and then maintained at pH 8.2 during the growth phase by the addition of base and acid. In the first two inoculations, flocculation occurred; in the third injection, flocculation was prevented by addition of the trace minerals Fe, Cu, zinc (Zn), Ni and manganese (Mn). Figure 9.14 illustrates the steps used for the first inoculation in the demonstration-scale experiment.

Within 16 days of inoculation, *P. stutzeri* KC was detected in groundwater samples from three monitoring locations located 1 and 2 m downgradient from the delivery well gallery. Within 6 weeks, strain KC was detected at 50% of the monitoring locations (Table 9.4). Inoculation of all delivery wells occurred on day 117, and reinoculation of the Northeast delivery wells (D8-D15) occurred on days 200–201.

As shown in Table 9.4, the once-inoculated and twice-inoculated sections of the treatment zone showed only a transient difference in strain KC levels in downstream monitoring wells. Because *P. stutzeri* KC has a unique "fried-egg" colony morphology when grown on R2A agar, quantitative estimates of strain KC levels on sediment samples were subsequently obtained from plate counts of extracted sediment cores (confirmed by PCR) as per Witt et al. (1999). An analysis of sediment colonization on days 336–342 revealed no difference in strain KC levels between the once- and the twice-inoculated sections of the aquifer (Table 9.5).

The results of this demonstration support the conclusion that reinoculation was not necessary for adequate sediment colonization. Nevertheless, addition of trace metals to the inoculum growth medium is still recommended as it decreased culture flocculation in the reinoculation event.

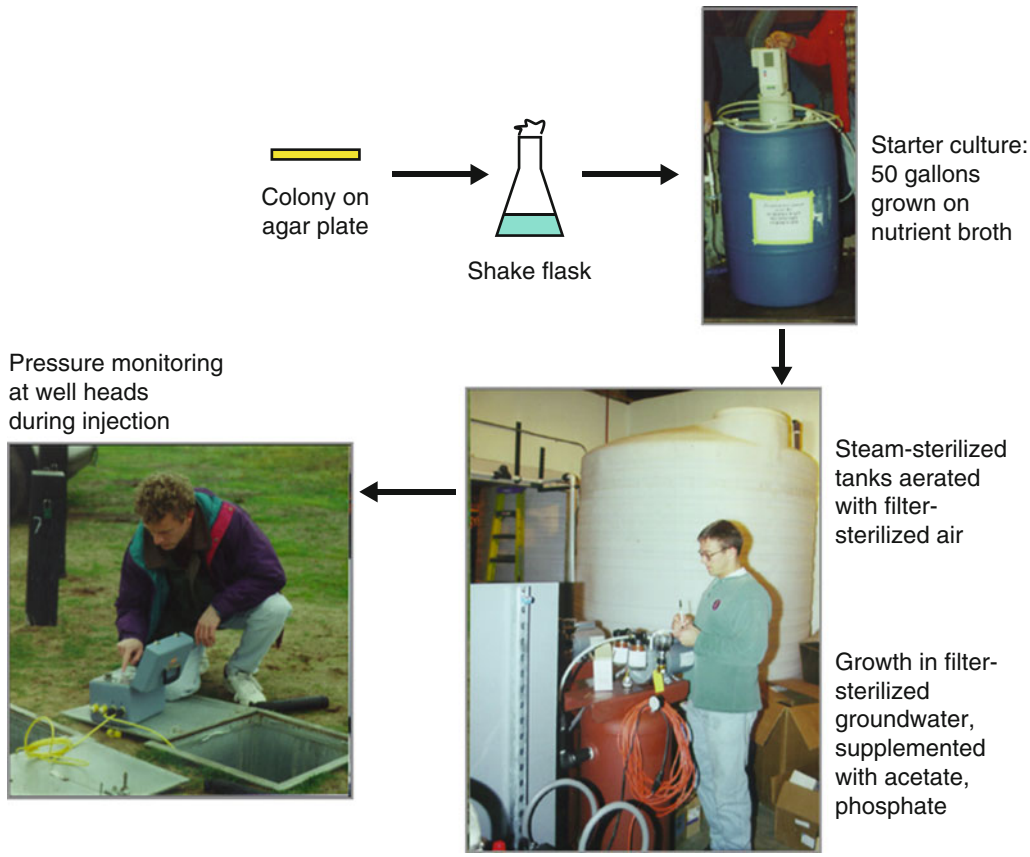

Figure 9.14. Steps in growth of *P. stutzeri* KC for use as inoculum and the inoculation during the demonstration-scale experiment at Schoolcraft, Michigan.

9.8.3 Long-Term Maintenance of the Biocurtain

Key elements of the demonstration-scale design were enhanced delivery via recirculation, periodic vs. continuous nutrient delivery to avoid formation clogging, and enhanced process efficiency by creating a biodegradation/sorption treatment train within the biocurtain zone. Improvements in chemical delivery significantly improved CT removal compared to that achieved during the pilot-scale study (98–99% vs. 60–65%), with CT concentrations in the demonstration scale study falling to levels less than the MCL (5 ppb) throughout the treatment zone (Figure 9.15) (Dybas et al., 2002). Chemical delivery was accomplished in two distinct phases: a short phase (6 hours [h]) of nutrient and substrate addition through the delivery well recirculation, followed by a much longer passive phase (~1 week) in which pumps were shut off and contaminated water entered the treatment zone under the influence of the natural hydraulic gradient.

During a typical chemical delivery event, groundwater was extracted from alternating (odd or even numbered) delivery wells, circulated through the aboveground chemical addition/mixing system, and then injected into adjacent delivery wells. Each extraction/injection cycle (ex. odd to even wells) ended with a 1-h period of flow reversal (e.g., even to odd wells) to ensure more uniform delivery of substrate around the initial extraction wells (the odd numbered wells, in this example). The 5-h pumping assignments (extraction or injection) of well sets

Table 9.4. Strain KC Colonization Pattern During the Demonstration-scale Experiment. Groundwater samples were analyzed for the presence of strain KC by extraction of total community DNA, addition of a primer pair with primary structures 5′-TGGCATGGGTCTGGGCTC TAT-3′ and 5′-CCTGATGACCGATTACGACCA-3′, PCR amplification, and gel separation. Samples were scored positive if the 787 bp fragment was observed. The downgradient distance and depth of multilevel monitoring wells are indicated. Well coordinates are shown in Figure 9.13.

	Southwest Wells				Northeast Wells								
Monitoring Well No.	12		19		11		16		13		20		30
Distance (m)	1.5		2.5		1		2		1.5		2.5		2
Depth (m)	16.8	22.9	16.8	22.9	16.8	22.9	21.3	24.4	16.8	22.9	16.8	22.9	25.9
Day 117	Inoculation				Inoculation								
126													
133													
140													
154													
168													
190													
196													
200–201					Reinoculation								
211													
218													
225													
232													
253													
288													
323													
384													

Strong signal Weak signal

Table 9.5. *P. stutzeri* KC Concentrations in the Sediment (Days 336–342). See Figure 9.13 for delivery well locations.

Sediment Boring Location	Center of Grid, 1.5 m Downgradient from Well D8		Center of Grid, 3.0 m Downgradient from Well D8		Northeast Section, 1.5 m Downgradient from Well D11		Southwest Section, 1.5 m Downgradient from Well D3	
Depth (m)	*P. KC* (cfu/g)	Native flora (cfu/g)	*P. KC* (cfu/g)	Native flora (cfu/g)	*P. KC* (cfu/g)	Native flora (cfu/g)	*P. KC* (cfu/g)	Native flora (cfu/g)
10.7	2.1×10^5	5.8×10^5	9.2×10^4	1.2×10^6	8.1×10^4	4.9×10^5	8.8×10^4	4.1×10^5
13.8	Nd	9.5×10^4	2.5×10^5	6.5×10^5	8.8×10^4	2.7×10^5	7.6×10^4	4.1×10^5
16.8	3.0×10^4	2.1×10^5	4.1×10^4	3.3×10^5	1.3×10^5	2.9×10^5	4.3×10^4	1.6×10^5
19.8	1.6×10^5	3.8×10^5	1.6×10^5	3.7×10^5	6.0×10^4	2.3×10^5	1.1×10^5	2.2×10^5
22.9	1.7×10^5	5.2×10^5	1.8×10^4	1.4×10^5	5.9×10^4	2.7×10^5	1.3×10^5	1.8×10^5
25.9	1.3×10^5	4.1×10^4	Nd	7.1×10^4	1.5×10^4	4.4×10^4	2.2×10^4	5.4×10^4

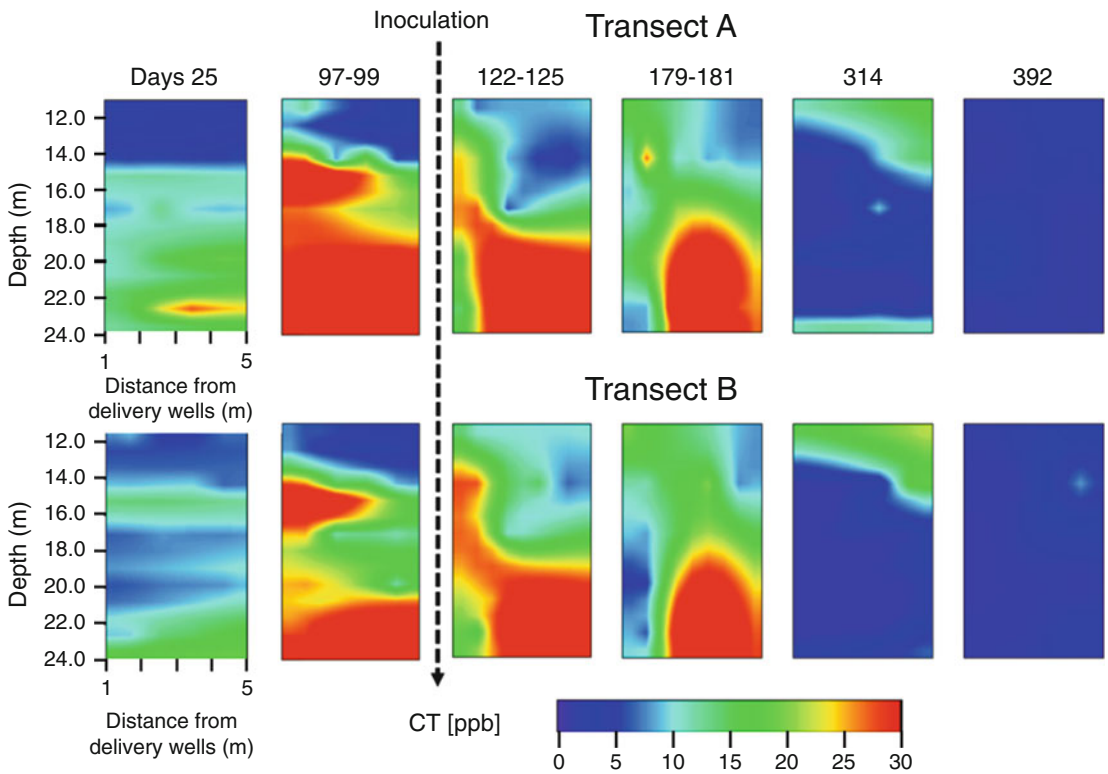

Figure 9.15. Krieged images showing changes over time in CT concentration in the monitoring wells downgradient from the delivery well gallery. Transects A and B are shown in Figure 9.13. The biocurtain was first detected at lower depths were flow was fastest, and in wells closest to the delivery well gallery. Reprinted with permission from Dybas et al., 2002. Copyright 2002, American Chemical Society.

also were switched weekly. A significant benefit of the intermittent pumping strategy employed is the low pumping volumes and short duration of pumping. The modeling used to establish time periods and pumping rates for these operational decisions is summarized in Hyndman et al. (2000).

The use of intermittent pumping for chemical delivery, as developed for the demonstration-scale study, enabled testing of a "trap-and-treat" strategy for sorption/biodegradation (Dybas et al., 2002). The basic concept is that contaminant degradation, stimulated in this case by weekly feeding of *P. stutzeri* KC, enabled removal of solution phase CT, leading to its desorption from the solid phase and cleansing of the solids. Then, in the week-long intervals between feeding events, new CT-contaminated groundwater is allowed to enter the treatment zone, recontaminating the solid phase. CT presumably moves into the treatment zone at a rate equal to the average linear velocity of the groundwater (15 cm/d at Schoolcraft) divided by the contaminant retardation factor (2.7 based on the data for CT shown in Table 9.3), or 5.6 cm/d. Therefore, in a 1-week period between pumping episodes, the CT did not break through the treatment zone, having traveled only $15/2.7 \times 7 = 39$ cm into the treatment zone through which groundwater was intermittently re-circulated. This continuous cycling between transformation/desorption and sorption resulted in a "trap-and-treat" sequence.

This type of cycling between periods of intermittent degradation and transport by natural gradient flow may provide an efficient option for semi-passive removal of contaminants. In this mode of operation, the time required for pumping was only that required to reactivate transformation, and the interval between pumping becomes a function of the sorption capacity of the solids. For details of the modeling used for design and simulation of the strain KC biocurtain, see Hyndman et al. (2000) and Phanikumar et al. (2002a, b, 2005).

In addition to CT degradation, two additional contaminants, CF and nitrate were followed over the course of the demonstration-scale experiment. CF appeared transiently when acetate was added in excess or the amount needed for nitrate removal, thereby stimulating sulfate reduction (Figure 9.16), a condition known to result in CF formation. When the added acetate concentrations were decreased to levels that allowed for some surplus nitrate, CF production ceased, and nitrate levels still remained below the regulatory limit of (37 mg/L or ppm) (Figure 9.17).

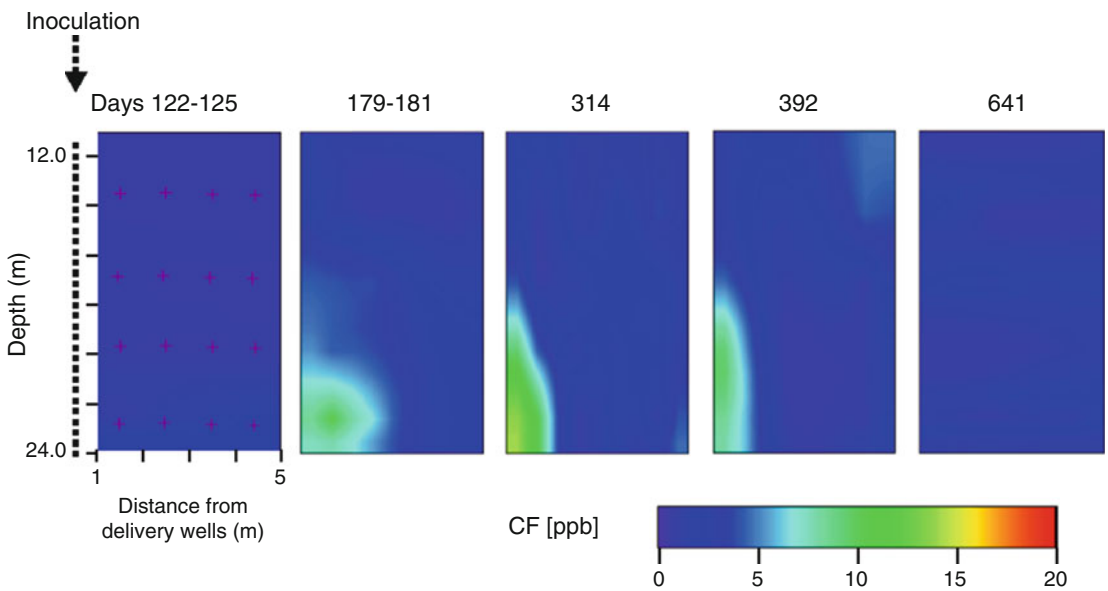

Figure 9.16. Krieged images showing changes over time in chloroform concentrations in the monitoring wells downgradient from the delivery well gallery. Results are shown for Transect A in Figure 9.13. CF was detected during a period of sulfate-reduction. Decreasing acetate feed concentration prevented CF formation, and led to an excess of nitrate (Figure 9.17). Reprinted with permission from Dybas et al., 2002. Copyright 2002, American Chemical Society.

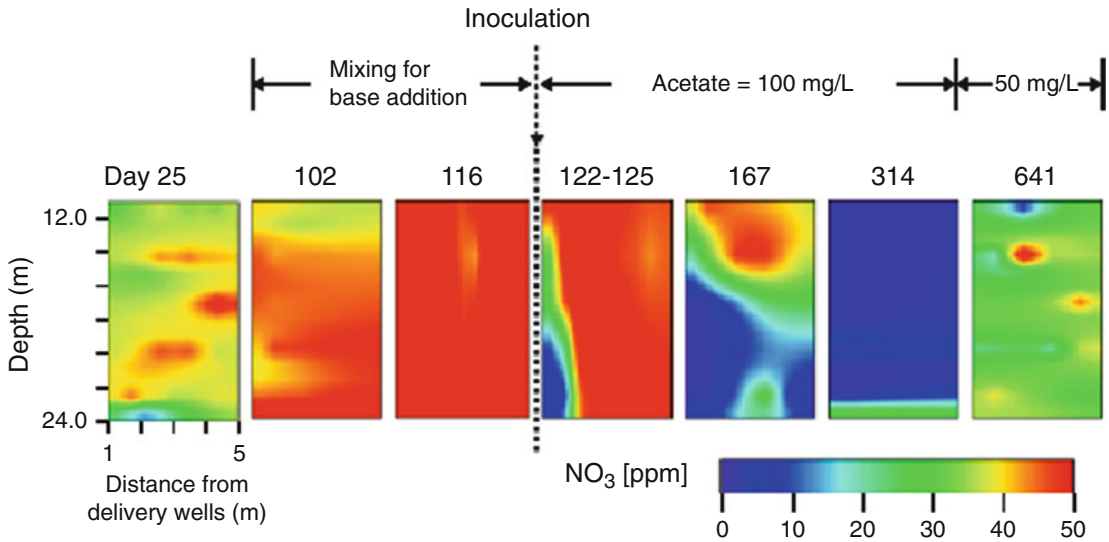

Figure 9.17. Krieged images showing changes over time in nitrate concentrations in the monitoring wells downgradient from the delivery well gallery. Results are shown for Transect A in Figure 9.13. CF was detected during a period of sulfate-reduction. Prevention of CF formation by decreasing acetate addition, led to an increase in nitrate levels, as illustrated for day 641. Reprinted with permission from Dybas et al., 2002. Copyright 2002, American Chemical Society.

9.9 FUTURE USE OF *PSEUDOMONAS STUTZERI* KC AND PDTC

A potential exists for genetic engineering of *P. stutzeri* KC, with introduction of genes encoding PDTC synthesis into other organisms. Gene 'cassettes' are now available that can be used to transfer PDTC synthesis capability using a variety of vectors, with potential for increased expression and broad host range compatibility. The ability to produce PDTC may confer competitive advantages for such genetically-engineered strains. Physiological data indicate that any organism that can produce PDTC must have the components needed to make use of it. Without those components, the organisms would likely be subject to PDTC-imposed growth inhibition (Leach and Lewis, 2006; Sebat et al., 2001). However, the minimal set of genes required for PDTC production are not yet known, and the biosynthetic pathway and central metabolic pathways that supply starting materials for PDTC production are not yet fully elucidated.

Initial observations with recombinant *E. coli* (another gamma proteobacterium) or *Sinorhizobium meliloti* (an alpha proteobacterium) containing plasmid-borne *pdt* gene clusters have not shown PDTC production (Lewis et al., 2000). The reasons for this apparently phylogenetically-restricted capability to support PDTC production are not known. It is possible that components resulting from *Pseudomonas*-specific gene expression are necessary, or that metabolites unique to pseudomonads are used as feedstock for PDTC biosynthesis. Data of Sepulveda-Torres et al. (2002) support the second possibility in that they showed at least modest transcription from the *pdt* F_{KC} promoter by *E. coli*.

Though strategies that may lead to increased transcription of *pdt* genes could be engineered, for example by removing *fur*-binding sites or replacing *pdt* promoters with more active alternative promoters, it is not known whether this would truly affect greater PDTC production

Table 9.6. Protocol for Chemical Synthesis of PDTC

Reactions						
1.	Dipicolinic acid (pyridine-2,6-dicarboxylic acid) + thionyl chloride- → pyridine-2,6-dicarboxylic acid chloride					
2.	Acid chloride + hydrogen sulfide (in pyridine) → pyridine-2,6-dithiocarboxylate (pyridine salt)					
Ingredients						
	Chemical	**FW**	**Mass (g)**	**Mol**	**Density**	**Vol (mL)**
Use	Dipicolinic acid	167.1	5	0.03		
Use	Thionyl chloride	119	71.2	0.598 (10× as much)	1.631	43.7
Expect	Acid chloride	204.1	61.123	0.03		
Expect	PDTC salt	278	8.34	0.03		
Expect	PDTC salt	199.1	5.973	0.03		
Procedure:						
1.	Clean all glassware with soap, deionized water, and acetone. Weigh flasks. Mark flasks for easy solvent addition (45 mL and 75 mL for the 100 mL round bottomed flask with a stir bar; 300 mL for the three necked 500 mL round bottomed flask). Bake glassware overnight at 150°C. List of glassware to be baked: 100 mL round bottomed flasks (one with stir bar), 50 mL round bottomed flasks, 50 mL pipets, a reflux tube, distillation apparatus, three necked flask with a stir bar, a 125 mL addition funnel. Get dry ice and store it at −78°C freezer. Weigh 5 g dipicolinic acid into a 100 mL round bottomed flask. Be sure that there are no chunks. Add 45 mL thionyl chloride into flask. Connect flask, tubes for water circulation, and gas lines to reflux tube. Repeat vacuuming and purging with nitrogen into system. Emerge part of the flask into an oil bath with a paper clip. Under nitrogen, heat and stir until all dipicolinic acid dissolves.					
2.	Distillation: Connect the reaction flask to a distillation setup, which contains water circulation and is under nitrogen. Thionyl chloride is distilled into another round-bottomed flask. Thionyl chloride is very acidic. To dispose of it, neutralize it with base gradually while stirring. The addition of base causes boiling.					
3.	Remove residual thionyl chloride with a pump, operated overnight. There should be a clean trap in isopropanol/dry ice bath for the pump. Acid chloride can be stored at −78°C.					
4.	Saturate 300 mL pyridine with H_2S in the 500 mL three necked round bottomed flask. All three necks are sealed with rubber septa. Hydrogen sulfide gas is bubbled into pyridine with a long needle. Another neck is connected to a nitrogen line (through a needle) that is connected to a bubbler. Dissolve acid chloride in 75 mL acetone (water free). Add acid chloride in acetone into hydrogen sulfide saturated pyridine drop by drop with an addition funnel. This funnel is connected to nitrogen line with a bubbler. Mixture should turn orange.					
5.	Filter out precipitate (acid chloride). Rotoevaporate off pyridine. Crystallize product (pyridine salt of PDTC) with chloroform using a heat gun. If crystals do not form, place flask into refrigerator. Filter to separate crystals and wash crystals with ice cold ethyl acetate. Dry crystals with an aspirator.					
6.	To make the free acid of PDTC, dissolve 500 mg pyridine salt in 100 mL water and bring pH to 1.5 with H_2SO_4. At once, extract five times with 20 mL methylene chloride. Rotoevaporate off solvent.					

since other levels of regulation (e.g., translational, post-translational) may be superimposed (T. A. Lewis, Montana State University, unpublished). Genes that lie outside the *pdt* cluster play roles in PDTC production as well (T. A. Lewis, Montana State University, unpublished) and a thorough analysis of those functions may help to answer some of the remaining questions regarding additional levels of control of PDTC production, and the PDTC biosynthetic pathway.

Ultimately, bioaugmentation with *P. stutzeri* KC may not be needed to obtain the benefits accrued from PDTC production. PDTC is a small molecule capable of passing through an ultrafiltration membrane. It may thus be possible to produce PDTC aboveground in a membrane bioreactor operated under ideal conditions for PDTC production or using strains genetically engineered to overproduce PDTC. Circulation of the filtered supernatant through the subsurface could then be used for remediation, especially if combined with biostimulation of indigenous microflora.

Tatara (1996) demonstrated that the CT-transformation activity secreted by strain KC passed through a column of Schoolcraft aquifer solids without retardation. Thus, circulation of PDTC through a region in which electron donor has been added for biostimulation could presumably enable controlled CT dechlorination while avoiding the need for pH adjustment and ecological issues associated with maintaining the activity of *P. stutzeri* KC in the subsurface over long-time periods.

Finally, if cost-effective, even chemically synthesized PDTC (Table 9.6) conceivably could be added as a biostimulation supplement. Such a strategy might be particularly valuable for environments containing CT at levels above the growth limit of strain KC (~5 mg/L).

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CHAPTER 10

BIOAUGMENTATION FOR MTBE REMEDIATION

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10.1 INTRODUCTION

Bioaugmentation for methyl *tertiary* butyl ether (MTBE; also known as methyl *tert*-butyl ether) remediation has been of interest since the early 1990s (Novak et al., 1992). Research was active for several years and several cultures were developed, tested and commercialized. Interest has waned in recent years as it became apparent that bioaugmentation can be costly, and generally is not needed for effective *in situ* treatment.

Bioaugmentation was initially considered because MTBE, and its cocontaminant and primary daughter product *tertiary* butyl alcohol (TBA; also known as *tert*-butyl alcohol), appeared recalcitrant to biodegradation in the subsurface. Biodegradation was slow and often could not be demonstrated, at least in the early studies (Deeb et al., 2000; Schmidt et al., 2004). MTBE has been reported to be biodegradable under a range of oxidation-reduction potential (ORP) conditions. However TBA may not be biodegraded and therefore may accumulate in some cases (McKelvie et al., 2007), though complete biodegradation of MTBE and TBA has been observed in both aerobic and anaerobic studies (Finneran and Lovley, 2001). Because MTBE generally has been found associated with petroleum hydrocarbons, which were commonly treated by natural or enhanced aerobic *in situ* biodegradation, research focused on developing cultures capable of complete aerobic biodegradation (i.e., mineralization) of MTBE to carbon dioxide and water.

Given the widespread occurrence of MTBE in groundwater, research on bioaugmentation cultures advanced quickly in different groups. Promising cultures were developed, and field demonstrations were performed to test these aerobic cultures. In general, these demonstrations indicated biostimulation alone was sufficient to treat MTBE contamination aerobically, as the indigenous microorganisms adapted and were able to biodegrade MTBE under favorable conditions. These results have pointed out valuable lessons for future bioaugmentation scenarios for MTBE as well as for other compounds.

This chapter describes the scientific basis for bioaugmentation for MTBE remediation, and some of the well documented field experiences with this strategy. In addition, it evaluates the current status and future prospects for bioaugmentation for MTBE remediation.

10.2 MTBE USE AND OCCURRENCE IN GROUNDWATER

MTBE has been added to gasoline as an octane enhancer in the United States since about 1979, originally developed as an additive to replace tetra-ethyl lead. Ironically, its use was promoted by regulators as a method to reduce air pollution due to gasoline emissions (McGarity, 2004). A 1990 Clean Air Act amendment mandated gasoline oxygen concentrations of 2.7% weight to volume (w/v) fuel during cold months in areas with elevated levels of carbon monoxide

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(Winter Oxyfuel Program, implemented in 1992) and oxygen concentrations of at least 2% w/v fuel in cities with the worst ground-level smog (Year-round Reformulated Gasoline Program, implemented in 1995) (Franklin et al., 2000).

Soon after MTBE's introduction into motor fuels, it began appearing in groundwater wells downstream of subsurface gasoline storage tanks and rapidly became a widespread groundwater contaminant (Deeb et al., 2003). MTBE was the third most frequently detected volatile organic compound (VOC) in samples from aquifers studied by the National Water-Quality Assessment Program of the United States Geological Survey (Zogorski et al., 2006) in 1993–2002. MTBE is persistent in groundwater due to its high water solubility (48,000 milligrams per liter [mg/L]), low sorption to aquifer sediments (log K_{oc} [soil organic carbon/water partition coefficient] = 1.1), and slow biodegradation rates (0.001–0.1/day [d]) (Wilson et al., 2000; Kane et al., 2001; Zoeckler et al., 2003; Schirmer et al., 2003). Although typically found with petroleum hydrocarbons, MTBE is sufficiently mobile and recalcitrant in the subsurface that it can move well ahead of benzene and related petroleum constituents (Squillace et al., 1997; Landmeyer et al., 1998). The distinctive, solvent-like odor of MTBE is detectable in very low concentrations (10s of micrograms per liter [μg/L]) in water, rendering MTBE migration in groundwater a significant aesthetic risk to surficial and deeper potable water supplies.

Although MTBE and TBA are biodegradable under some conditions, the loss rates are relatively slow compared to the volatile petroleum hydrocarbons. Further, because they are highly water soluble and poorly sorbed, they can move almost as fast as the groundwater. As a result, MTBE and TBA can persist after the petroleum constituents are removed, and MTBE plumes can be much larger than the petroleum plumes. The apparent mass flux losses of MTBE due to dispersion, dilution and biodegradation in aquifers are much slower than those for most petroleum compounds, with reported first-order decay rate estimates ranging from 0.1 to 0.001/d (aerobic) to 0.007/d (anaerobic) (Borden et al., 1997; Schirmer et al., 2003; Wilson et al., 2000). It is also important to note that the field anaerobic decay rate represented the transformation of MTBE to TBA only. Assuming a conservative aquifer decay rate of 0.001/d and MTBE-only plume concentrations of 100 to ≤10,000 μg/L, Wilson (2003) calculated that it would require 10–25 years to decrease ether levels to ≤20 μg/L solely by natural attenuation at most sites. MTBE plumes, therefore, are likely to persist in aquifers for at least 10 years after fuel spills occur. Consequently, treatment measures, such as enhancing biodegradation, appeared likely to be necessary at many MTBE/TBA sites, and bioaugmentation was originally viewed as a potentially important component of an effective *in situ* bioremediation treatment approach.

MTBE has a federal drinking water advisory level of 20 μg/L, and several states have enforceable cleanup levels that range from 13–202,000 μg/L (ITRC, 2005). Statewide phaseouts of MTBE were instituted in order to decrease the likelihood of drinking water impacts starting in 2000 (USEPA, 2004). These phaseouts resulted in a sharp decline in MTBE production in the United States after 2003 (Figure 10.1), and an apparent stabilization in the prevalence of MTBE in groundwater. However, there are still numerous MTBE/TBA plumes, and these continue to be difficult to remediate (ITRC, 2005).

10.3 SCIENTIFIC BASIS FOR BIOAUGMENTATION OF MTBE AND TBA

10.3.1 MTBE Degrading Bacteria

Since the early 1990s, several MTBE-degrading bacterial cultures have been isolated or enriched from subsoils, sediments and biosolids. Some organisms use MTBE as the sole carbon and energy source (e.g., *Rhodococcus aetherivorans* SC100, *Methylibium petroleiphilum* PM1,

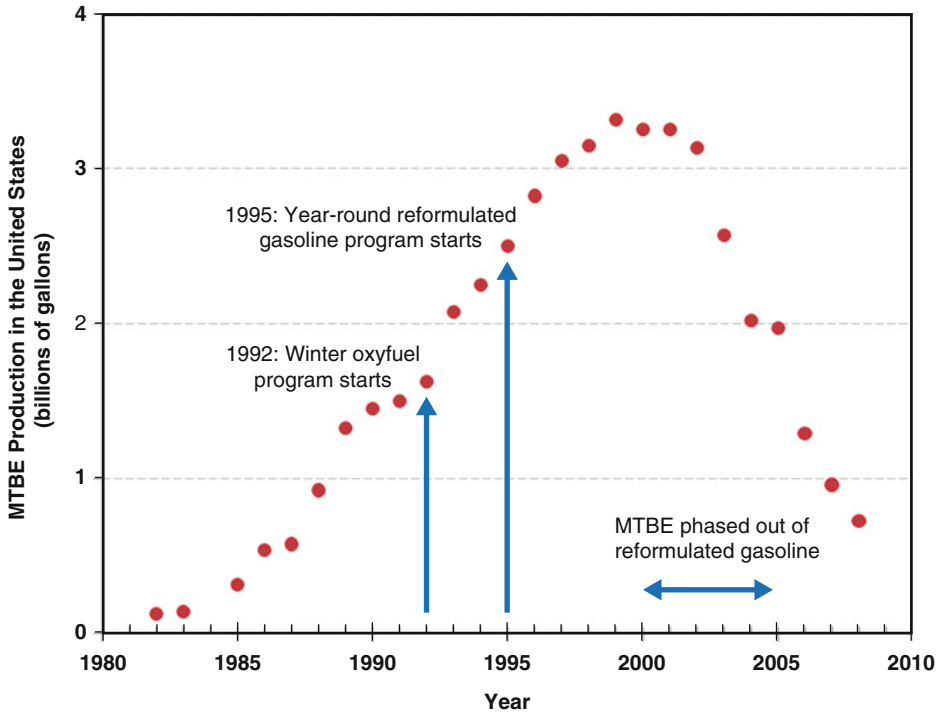

Figure 10.1. MTBE production rates in the United States from 1982–2008 (EIA, 2008).

Aquincola tertiaricarbonis L108, *Mycobacterium austroafricanum* IFP 2012 and *Hydrogenophaga flava* ENV735), while others cometabolically oxidize MTBE using alkanes or alcohols as cosubstrates or fortuitous inducers of ether metabolism (e.g., *Mycobacterium vaccae* JOB5, *Pseudomonas mendocina* KR-1). Direct MTBE degraders exhibit low biomass yields (0.09–0.44 grams (g)-biomass/g-MTBE) and slow specific growth rates (0.2–0.5/d) (Fortin et al., 2001; Fiorenza and Rifai, 2003; Davis and Erickson, 2004) compared to higher energy substrates like alkanes, alcohols, ketones and sugars (with apparent yields of 1–1.5 g-biomass/g-carbon utilized) (Salanitro et al., 1998).

Cometabolic degradation initially appeared attractive because it decouples the biodegradation of contaminants from microbial growth (i.e., the organisms can be grown on a relatively harmless substrate such as propane, while fortuitously degrading MTBE). However, cometabolism was found to lead to the accumulation of TBA, which is also a compound of concern at low µg/L concentrations (Nava et al., 2007). Some direct degraders are associated with the buildup of TBA, while others appear capable of complete mineralization (McKelvie et al., 2007).

The slow growth rates and low yields of ether-degrading organisms compared to benzene, toluene, ethylbenzene and total xylenes (BTEX) degraders, with biomass yields of 0.5–1 g-biomass/g-BTEX and growth rates of 1–6/d (Yerushalmi and Guiot, 1998; Kelly et al., 1996; Salanitro, 1993; Wiedemeier et al., 1995) explain why MTBE plumes tend to outlive those of their co-occurring fuel components. The low cell yields on MTBE and other tertiary butyl ethers like *tertiary* amyl methyl ether (TAME) and ethyl *tertiary* butyl ether (ETBE) is a biochemical limitation potentially affecting the consistency and completeness of ether degradation in groundwater plumes. The slow growth rate and low yields of indigenous degraders in aquifer sediment can make it difficult to sustain natural bioattenuation processes for long term plume management, perhaps even with sufficient nutrient addition and oxygenation.

In addition, calculations of heats of combustion (kilocalorie [Kcal]/g-carbon) of various substrates and associated cell yields (g-biomass/g-carbon) indicate that MTBE and TBA support the growth of only 20–30% of the yields measured when using high energy substrates. Recent calculations by Müller et al. (2007) accounting for half-maximum substrate concentrations (K_s), maintenance and decay coefficients, and the likely metabolic pathway for MTBE, indicate that cell yields should be 0.87 g-cells/g-MTBE utilized, significantly higher than the values actually measured.

Aerobic MTBE oxidation is thought to proceed via an unstable hemiacetal (Figure 10.2) that can be further oxidized to *tertiary*-butyl formate (TBF) or that decomposes abiotically to TBA (Steffan et al., 1997; François et al., 2002; Salanitro, 2002; Fiorenza and Rifai, 2003; Fayolle et al., 2003; Müller et al., 2007). TBA can be further degraded to form 2-hydroxyisobutyrate (HIBA). TBA and HIBA metabolism, as well as initial ether-cleavage to TBA, represent the main enzymatic impediments to the ready metabolism of MTBE. Thus, these steps are likely responsible for the low cell yields in aerobic systems, as well as the difficulty that has been experienced in demonstrating metabolism of MTBE beyond TBA in both anaerobic enrichment cultures and in environmental samples adjusted to low redox potentials. Although the actual reason for this biochemical enigma has not been determined, two mechanisms have been proposed: (1) microbial dehydrogenases and oxygenases have weak affinities for ethers and alcohols with tertiary-carbon structures (MTBE and TBA), and (2) low-level activation of HIBA by a coenzyme A system or slow oxidative attack by an unknown enzyme or free-radical mechanism (Müller et al., 2007) may be needed for the transformation of HIBA to isopropanol or acetone.

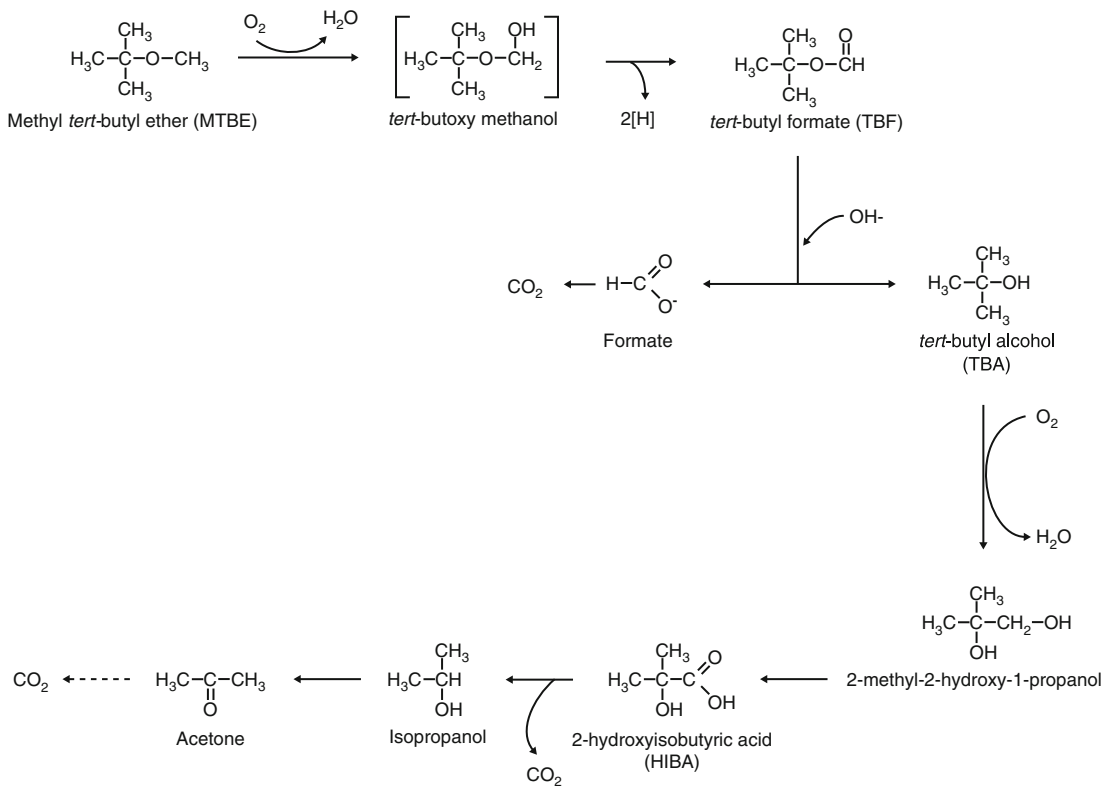

Figure 10.2. Proposed aerobic MTBE biodegradation pathway in *Mycobacterium austroafricanum* IFP 2012 (modified from Fayolle et al., 2003).

The net effect of these cellular impediments is that MTBE degraders have low growth rates (μ_{max}), high substrate levels for removal (K_s), and relatively high threshold concentrations (S_{min} – the concentration below which adequate cell growth (Y_g) cannot be sustained). The K_s values that have been reported for single (pure) aerobic cultures of MTBE-degraders are relatively high, at 45–50 mg/L for the PM1 and L108 strains (Hanson et al., 1999; Müller et al., 2007), 80–130 mg/L for *M. vaccae* JOB 5 (Smith et al., 2003), and 175–350 mg/L for *Ps. putida* G Pol (Smith and Hyman, 2004). It is evident that some naturally occurring microbes which have been described (high K_s, slow growth and low cell yield) may not be able to sustain MTBE decay in plumes containing typical concentrations of 10–10,000 µg/L, as these levels are below the observed cell culture kinetic coefficients. These results suggest that intrinsic or nutrient amended bioremediation may be limited in MTBE plumes.

10.3.2 MTBE and TBA Biodegradation in Microcosms

MTBE generally has been found to be readily biodegraded in microcosms containing aquifer sediments and groundwater from contaminated sites, when incubated under aerobic conditions. A review of available data indicates that MTBE concentrations ranging from 0.05 to 15 mg/L were at least 90% metabolized in 14–65 days, with initial lag times ranging from 5 to 30 days, and apparent first-order degradation rates ranging from 0.02 to 0.16/d (Salanitro et al., 2000; Kane et al., 2001; Wilson et al., 2002; Magar et al., 2002; Schirmer et al., 2003; Zoeckler et al., 2003). The fate of TBA is less consistent. In several microcosm studies, TBA did not degrade, or was degraded slower than MTBE, whereas in other studies TBA was metabolized at rates (0.12/d) comparable to MTBE (Schirmer et al., 2003).

Experimental evidence for the consistent and complete biodegradation of MTBE under anoxic conditions (including nitrate-reducing [NR], iron-reducing [IR], sulfate-reducing [SR], methanogenic conditions [MC], or mixed electron acceptor conditions [MX]) in aquifer sediment microcosms is not entirely convincing. Laboratory data have yielded a wide range of results, including: (1) varying degrees of degradation (10–100% – IR, SR, MC) of 1.5–100 mg/L ether in 130–600 days; (2) little or no degradation (NR, IR, SR, MC, MX) of <5% of the ether (50–75 mg/L) in 250–500 days; (3) very long lag intervals (few to several months) before metabolism is observed; (4) inconsistent degradation among replicate microcosms or different sediment site samples; or (5) TBA accumulating as a MTBE metabolite, with little or no apparent TBA biodegradation (MC, MX) (see reviews by Schmidt et al., 2004; Häggblom et al., 2007; Wilson et al., 2005). Also, no single or mixed cultures of anaerobic species have been isolated and identified to verify anaerobic metabolism of MTBE.

Soil and groundwater microcosms used to evaluate the presence of indigenous MTBE-degrading microorganisms often show no clear correlation between the presence or absence of MTBE-degrading activity and current or historic exposure to MTBE or dissolved oxygen levels (Salanitro et al., 2000; Kane et al., 2001; Lesser et al., 2010). Further, microcosm tests performed with field core samples taken on one-meter (m) (3.3 feet [ft]) spacing suggest there are large variations in degradation rates, lag times and completeness of MTBE metabolism, and that there are large variations in the abundance of MTBE-degrading populations over short distances.

10.3.3 Evaluating MTBE and TBA Biodegradation

MTBE and TBA degraders are difficult to study using conventional microbiological methods (such as plate counts). Fortunately, several functional and molecular methods have become available that do not require bacterial culturing, allowing effective monitoring of biodegradation in the field (Table 10.1). Several oligonucleotide probes have been isolated

Table 10.1. Functional and Molecular Techniques to Evaluate Bacterial Bioaugmentation for MTBE Remediation in Aquifer Samples (Aquifer Sediment and Groundwater)

Method	Feature	Advantages	Disadvantages
1. Biodegradation potential assay	Lab microcosm Aerobic/anaerobic Test period (days, weeks or months)	Estimate decay coefficients (k_1, K_s, S_{min}) Assess aerobic/ anaerobic metabolism	[a]See footnote
2. Oligonucleotide probes and polymerase chain reaction (PCR) for specific MTBE-degraders: *Methylibrium petroleiphilum* (PM1) L108 *Mycobacterium austroafrican* (IFP2012) *Hydrogenophaga flava* (ENV735) *Rhodococcus aetherivorans* (SC-100)	DNA extraction, amplification and matching to database of type species Genus and species specific	Sequences of known MTBE degraders available Enumerate degraders Number of degraders may correlate with decay rates *in situ*	Presence of other unknown MTBE-degraders in a sample are not detected Low levels of degraders may not be detectable (e.g., 10–100's per g soil) Presence of a specific MTBE- degrader may not correlate with metabolic activity *in situ*
3. Oligonucleotide probes and PCR for MTBE degrading enzymes	Amplification of DNA coded enzyme sequences Available sequences for: MTBE monooxygenase, isobutyl CoA[b] mutase, *tert*-butyl alcohol dioxygenase)	Estimate level of specific metabolic genes Copy numbers may correlate to MTBE decay rate *in situ*	Specific enzyme sequence may not be present in sample and in all environments Enzyme levels in soil may not correlate with degradative activity
4. Compound specific stable isotope analysis $\delta^{13/12}$ C-MTBE $\delta^{2/1}$ H-MTBE	MTBE degradation observed by enrichment of heavier isotopes Compare source with downgradient plume for isotope signatures	3-dimensional (3-D) mass balance of aquifer levels of ether not required Aerobic and anaerobic metabolism determined Estimate fractional amount of ether present in an aquifer	Aerobic degradation shows little isotope enrichment Isotope enrichment in anaerobic conditions shows metabolism only to TBA Expensive gas chromatography (GC)/ mass spectrometer (MS) and isotope ratio spectrometer required Confounding mixed fractionation may occur in aerobic/anaerobic zones

(*continued*)

Table 10.1. (continued)

Method	Feature	Advantages	Disadvantages
5. Selective isotope probing techniques	*In situ* method for demonstrating substrate specific biodegradation ^{13}C incorporation into phospholipid fatty acids, dissolved organic carbon or DNA	*In situ* analysis reflects actual site conditions Unambiguous demonstration of biodegradation	Expensive analyses required Requires 30–45 days incubation time Requires substrate addition to wells

Note: CoA–coenzyme A

[a]Other pitfalls of any method given is the analysis of small and few sediment/groundwater samples and the spatial variability (horizontal and vertical) affecting the assessment of microbial function, activity, presence of degraders and enzymes in aquifer plumes

from MTBE-degraders (Table 10.1), and these can be used to amplify and identify specific sequences of MTBE-degraders from environmental samples. Such probes include those for *Methylibrium petroleiphilum* PM1 (Nakatsu et al., 2006), *Rhodococcus aetherivorans* (Goodfellow et al., 2004), *Mycobacterium austroafricanum* IFP 2012 (Francois et al., 2002) and *Hydrogenophaga flava* ENV735 (Hatzinger et al., 2001). These probes also may be used to estimate MTBE decay rates from their copy numbers in deoxyribonucleic acid (DNA) extracts of soil samples. Serious drawbacks of this method are: (1) these specific organisms may not be present in all aquifer sediments where MTBE may be degrading; (2) bacterial cell numbers may be too low for amplification; and (3) other unknown ether-degraders may be present and responsible for the bioattenuation.

More recently, oligonucleotide probes have become available for identifying enzymes responsible for MTBE and TBA metabolism in pure cultures (Method 5, Table 10.1). For example, transcriptosome microarray analysis of enzyme expression in PM1 cells grown on MTBE (enzyme-induced) and ethanol (non-inducing substrate) by Hristova et al. (2003) suggests that: (1) an MTBE monoxygenase is involved in the initial oxidation of the $-O-CH_3$ group of MTBE to TBA, and (2) a TBA hydroxylase converts TBA to HIBA. Rohwerder et al. (2006) have shown that in strain L108, a HIBA cobalamin-dependent mutase carries out the transformation of HIBA to 3-hydroxybutyrate. These three major enzymes represent key transformation steps required for complete mineralization of MTBE to carbon dioxide (CO_2). Again, the presence of these enzyme genes in aquifer sediment samples may correlate with decay rates observed in bioaugmented and biostimulated active zones; however, the mere presence of these genes may not indicate that they are fully functional in the metabolism of MTBE.

Another diagnostic analytical tool used for assessing MTBE biodegradation in sediment microcosms, monitored aquifers and bioactive zones of biobarriers is compound specific stable isotope analysis (CSIA) (Method 6, Table 10.1). This technique measures the abundance of $^{13}C/^{12}C$ and $^{2}H/^{1}H$ in MTBE molecules relative to those in the international standards for carbonate ($^{13/12}C$) and ocean water ($^{2/1}H$), respectively, and are expressed as $\delta^{13}C$ and $\delta^{2}H$ per mil ($\frac{o}{oo}$ or parts per thousand). In principle, the lighter atoms (^{12}C and ^{1}H) are enzymatically attacked preferentially at some rate relative to the heavier atoms ($^{13}C/^{2}H$). Differences in the enrichment of $^{13/12}C/^{2/1}H$ in the remaining MTBE (e.g., residual ether) after significant biodegradation in microcosms or groundwater samples from bioactive zones in an aquifer, would be indicative of metabolism. The same isotope differences can be applied to the MTBE plumes where "lighter" sources of $\delta^{13}C$ and $\delta^{2}H$ are different from the "heavier" isotopes remaining downstream where

natural biodegradation may be occurring. The overall fraction (F) of MTBE remaining (assuming fractionation is constant) can be approximated by the equations for $\delta^{13}C$ and $\delta^{2}H$:

$$\delta^{13}C_{plume} - \delta^{13}C_{source} = \varepsilon_C \ln(F_C) \quad \text{(Eq. 10.1)}$$

$$\delta^{2}H_{plume} - \delta^{2}H_{source} = \varepsilon_H \ln(F_H) \quad \text{(Eq. 10.2)}$$

where ε_C and ε_H refer to the enrichment factors for ^{13}C and ^{2}H.

"Source" and "plume" isotopes ratios can be substituted for bioactive zones and inactive zones in biobarriers or initial and final values, respectively, in microcosm tests. In studies on MTBE isotope fractionation, a difference in enrichment of $\delta^{13}C$ and $\delta^{2}H$ has been observed under aerobic and anaerobic conditions. Aerobic pure cultures of the PM1 organism (Gray et al., 2002) or aquifer sediment microcosms from different sites (Gray et al., 2002; Hunkeler et al., 2001; Lesser et al., 2008) have shown that ε_C varied little from –1.5 to –2.4 while ε_H varied from –29 to –66.

In a field study of an aerobic biobarrier, Lesser et al. (2008) found little significant difference in the enrichment of either $\delta^{13}C$ or $\delta^{2}H$ –MTBE in bioaugmented zones compared to those from a zone unaffected by biostimulation (oxygen addition) or bioaugmentation. In contrast, anaerobic studies of MTBE biodegradation indicated that ε_C varied from –8.1 to 15.6 for sediment microcosms and anaerobic aquifers (Kolhatkar et al., 2002; Kuder et al., 2005; Somsamak et al., 2005; Zwank et al., 2005). It is important to note that under these anaerobic conditions, MTBE is transformed to TBA. In this respect, Zwank et al. (2005) showed the apparent large enrichment observed for MTBE in $\delta^{13}C$ is due to a kinetic isotope positional effect of the enzymatic cleavage of the O–CH_3 group of MTBE. Under aerobic conditions, the ether is essentially degraded beyond TBA and little or no carbon enrichment is observed.

Stable isotopes also can be used to identify whether labeled carbon from MTBE or TBA is being incorporated into microbial biomass within the subsurface (Busch-Harris et al., 2008). In this method, "biotraps" are labeled with ^{13}C-MTBE or TBA, and then suspended in a well. The traps also contain activated carbon beads, and the indigenous bacteria colonize these beads, and are exposed to the labeled compound(s). After some period of time, the traps are retrieved and the cells in the biotraps are analyzed for ^{13}C that has been incorporated into the cell lipids, providing evidence that *in situ* biodegradation is occurring under the prevailing environmental conditions. Of course, there are potential artifacts that should be considered when using such methods, notably that the microenvironment within a well may differ from that within the aquifer.

In summary, laboratory and field data based on stable isotope analyses, including $\delta^{13}C$ fractionation of MTBE and stable isotope analyses of lipids from bacteria recovered from biotraps, may provide powerful evidence for extensive natural and enhanced bioattenuation, both in "aerobic" or "anaerobic" aquifers. However, such data should be used with appropriate caution.

10.4 BIOAUGMENTATION PILOT TESTING

10.4.1 Bioaugmentation with Direct Degraders (MC-100 and SC-100)

MTBE biotreatment technologies have been in development since the early 1990s. Shell Development (now Shell Global Solutions) researchers identified and enriched a mixed culture (MC-100) capable of completely degrading MTBE to carbon dioxide and water (Salanitro et al., 2000). In the late 1990s, a single MTBE-degrading organism (SC-100) was isolated from the mixed culture. The bench-scale studies and ability to produce the MTBE-degrading cultures at a large enough scale led to pilot-scale studies conducted collaboratively between Shell Global Solutions (then Equilon Enterprises, LLC), Arizona State University (ASU) and the Naval

Facilities Engineering Service Center (NAVFAC ESC), Port Hueneme, California. Six pilot-test plots were installed at the Naval Base Ventura County (NBVC) at Port Hueneme, California. These plots examined the performance of 20-ft (6-m) wide biobarriers employing various combinations of oxygen and air injection and the mixed- and single-cultures MC-100 and SC-100. All were placed far downgradient of the source zone where groundwater contained only MTBE and TBA.

Oxygen-rich biologically reactive treatment zones (the "biobarriers") were established *in situ* and downgradient of the source of dissolved MTBE contamination. The system was designed so that groundwater containing dissolved MTBE flowed to, and through, the biobarrier treatment zone. The use of natural flow conditions is generally preferred, but one can imagine groundwater pumping schemes directing impacted groundwater to a treatment zone. As the groundwater passed through the biobarrier, microorganisms converted the MTBE (presumably to CO_2). Ideally, groundwater leaving the downgradient edge of the treatment zone contains MTBE at concentrations less than or equal to the target treatment levels. A system designed to treat MTBE also will very likely reduce concentrations of other aerobically biodegradable chemicals dissolved in the groundwater (e.g., BTEX and TBA). It should be noted that: (1) other contaminants will likely represent competitive oxygen sinks, (2) some organisms like strain PM1 are known to be inhibited by certain BTEX compounds, and (3) the presence of other organic compounds allows the possibility of cometabolic degradation.

Oxygenation of the aquifer was accomplished through periodic oxygen (or air) injection via a line of gas injection wells spanning the width of the biobarrier. In this approach, gas injection is at high pressure, but low volume (e.g., averaged gas flows of >10 cubic feet per minute [cfm]) for durations of about a minute and periodic (e.g., daily) to achieve sufficient gas distribution while not altering the natural groundwater flow through the treatment zone. While there are a number of ways to deliver oxygen to groundwater (e.g., in-well oxygenation systems and oxygen releasing compounds), this section focuses on gas injection because that was the approach successfully demonstrated at full-scale (Miller et al., 2003).

Other than groundwater monitoring wells, the only process components required for this technology are associated with the oxygen delivery system. These might typically include an oxygen generator (or air compressor), oxygen or air storage tanks, gas injection wells, and a series of timers and solenoids to control and direct the oxygen to the gas injection wells. In some cases, oxygen addition will stimulate the growth of indigenous MTBE-degrading organisms, and the growth rate and activity of these organisms will be sufficient to effect the desired reduction in concentration. At other sites, the microbial community may not contain the necessary organisms, or the growth rate and activity may be too low to achieve the desired concentration reduction within time frames acceptable to local regulating agencies. In those cases, it may be necessary to bioaugment the aquifer with MTBE-degrading cultures.

Figure 10.3 shows a plot plan of the original test cells: a control cell, a biostimulation cell, and a bioaugmentation cell. The control cell had 14 paired shallow and deep monitoring wells. The biostimulation cell had 20 paired shallow and deep monitoring wells, and several lines of oxygen injection wells installed transverse to the understood groundwater flow direction. This oxygen delivery system was intended to provide a flow-through cell with at least a week of elevated oxygen contact time. The bioaugmentation cell had 17 paired shallow and deep monitoring wells. This test cell was set as a similarly robust oxygenated flow through cell, with the addition of high concentrations of MC-100 injected on 1-ft centers over 21 ft (6.4 m). There was only a single biomass injection event. Injections took place using a direct-push rig, a grout pump, and several hundred gallons of MC-100 diluted to a total suspended solids (TSS) of about 2.5 g/L. This concentration was found to be the best balance between distribution and loading. A point was pushed to the bottom of the contaminated aquifer (20 ft [6 m] below ground surface [bgs]) and 5 gallons (20 L) was injected at between 50 and 60 pounds per square

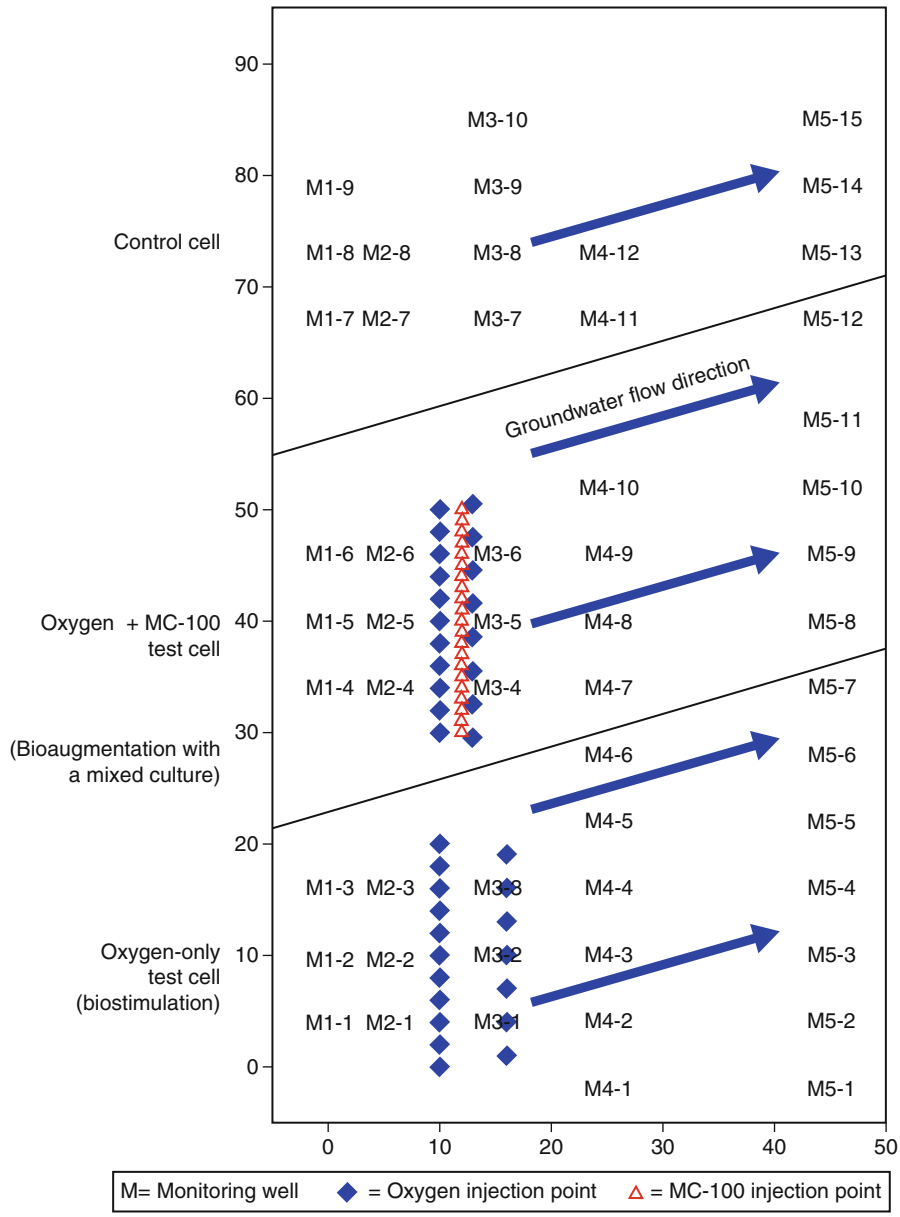

Figure 10.3. Plot plan of MTBE bioaugmentation and biostimulation plots (modified from Salanitro et al., 1999).

inch (psi). The point was lifted in 1-ft increments, with 5 gallons (20 L) of inoculum injected at each interval, to the top of the aquifer (9 ft [3.5 m] bgs). Injection points were spaced a foot apart over 21 locations installed transverse to the understood direction of groundwater flow.

Figure 10.4 illustrates the MTBE distribution in shallow test cells as a series of snapshots before (up to 94 days before) and after (up to 1,007 days after) the bioaugmentation event. The average MTBE concentration in the shallow aquifer prior to remediation was just over 5 mg-MTBE/L-groundwater. Concentrations in the downgradient wells fell to <5 µg/L (the laboratory detection limit) within 2 months in the bioaugmentation test cell. In the

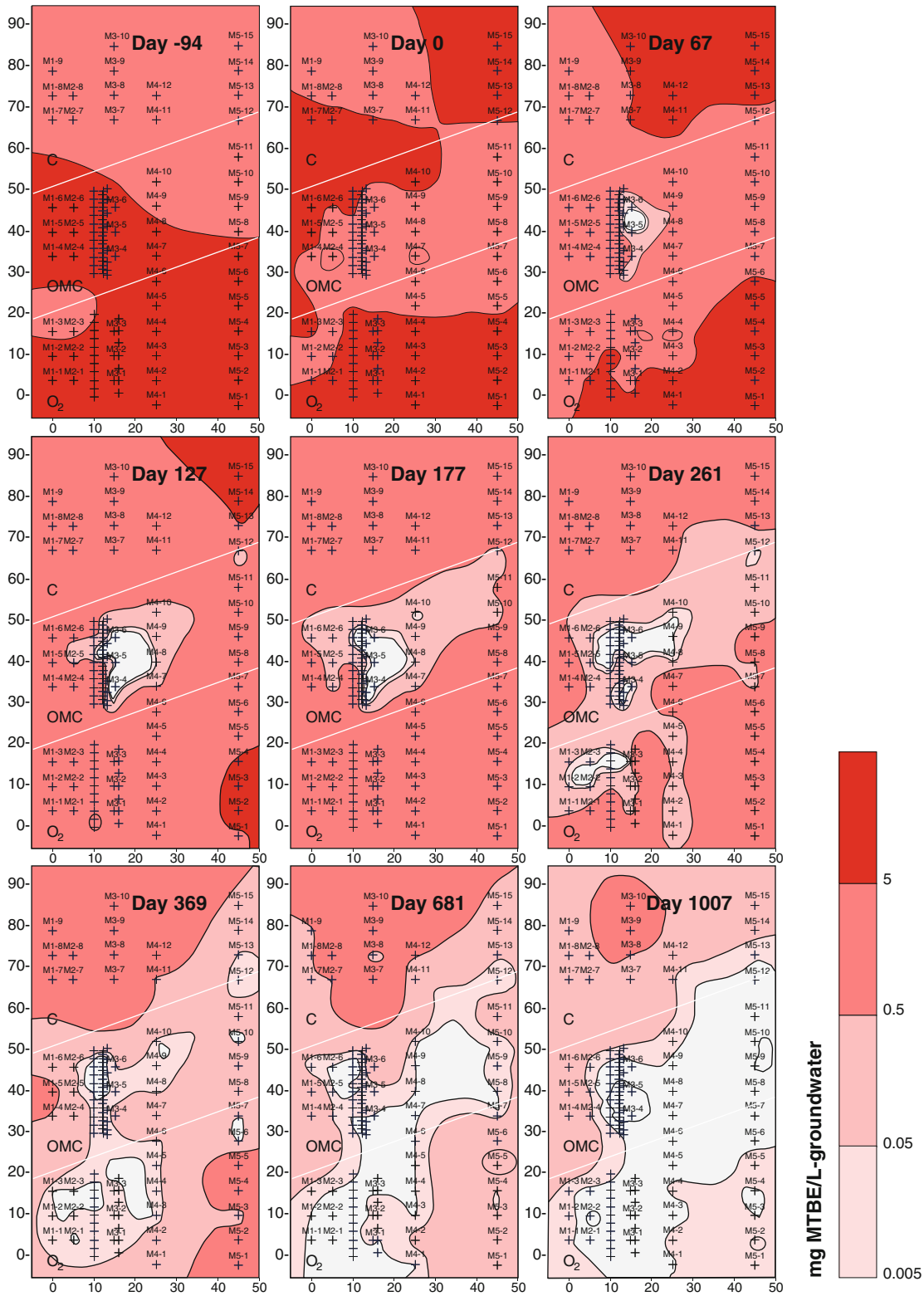

Figure 10.4. Time lapse MTBE concentration snapshots illustrating bioaugmentation and biostimulation behavior (modified from Salanitro et al., 1999). Note that bioattenuation observed only in the bioaugmented cell for the first 6 months.

biostimulation test cell, little impact was seen until 8.5 months had passed. After this lag period, concentrations also fell to the same levels in the biostimulation test cell.

Figure 10.5 shows a plot plan of the next three biobarrier pilot test cells. These test cells were designed to test the utility of bioaugmentation cells fed by air instead of pure oxygen and to evaluate a single culture bioaugmentation versus a consortium. First, a stable zone of oxygenation was established with air, and then MC-100 was injected in a manner similar to

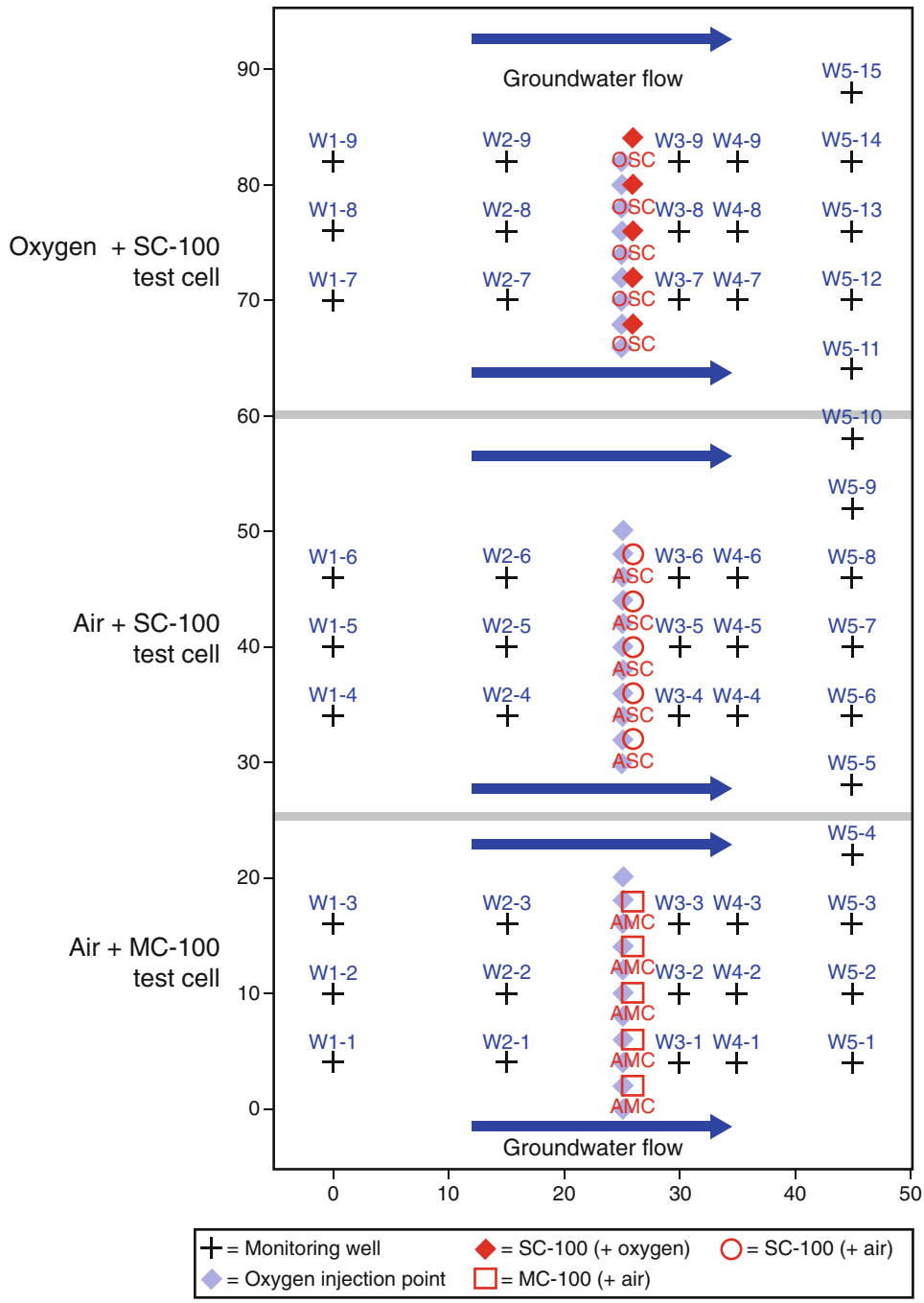

Figure 10.5. Plot plan of MTBE bioaugmentation plots using mixtures of oxygen and air, mixed culture (MC) and single organism (SC, later identified as *R. aetherivorans*).

the original bioaugmentation cell (designated here as AMC). Single culture (SC-1) was diluted to a final TSS of about 2.5 g/L and injected into test cells oxygenated with air (ASC), and oxygen (OSC), respectively. Figure 10.6 illustrates the MTBE distribution in shallow and deep wells before (40 days before) and after (up to 275 days after) injection. No evidence of lessening of MTBE degradation potential was observed over the time periods monitored for any test cell.

In brief, the pilot tests confirmed that the aquifer could be successfully bioaugmented with no loss of biobarrier activity over time. Concentrations of MTBE were reduced from roughly

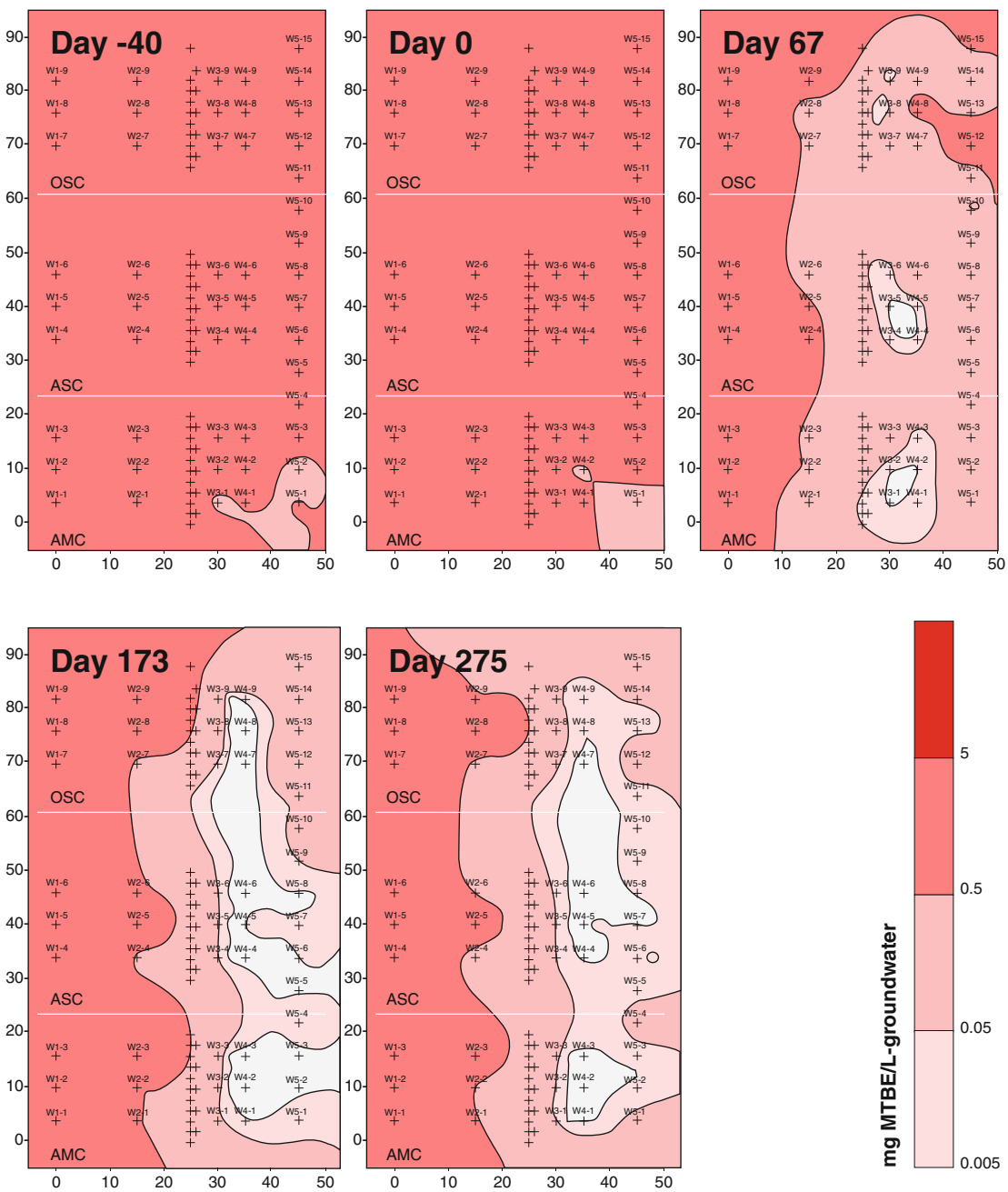

Figure 10.6. Time lapse MTBE concentration snapshots illustrating bioaugmentation behavior. Note that no real difference in bioattenuation observed in all test cells until 6 months have passed.

5,000 μg/L to non-detect concentrations (<5 μg/L MTBE) and TBA also was reduced to non-detect levels (<50 μg/L TBA). These studies demonstrated that overall activity of naturally-occurring MTBE degraders could be stimulated by increasing the dissolved oxygen levels in groundwater, albeit more slowly than the bioaugmented plots. The activity in the biostimulated plot became comparable to the bioaugmented plot after about 240 d of operation. The short-term performance of MC-100 bioaugmented and the biostimulated test plots are reported in Salanitro et al. (2000).

10.4.2 Bioaugmentation with Direct Degraders (PM1)

Another bioaugmentation study at the NBVC was performed by researchers from the University of California Davis (Smith et al., 2005). These researchers generated a stable oxygen-rich environment in two test cells, and then inoculated one of the test cells with the PM1 isolate. They then monitored MTBE concentrations in both systems for several months. The manner and volume of bacterial introduction was not detailed in available literature. Surprisingly, stronger attenuation of MTBE was observed in the control (biostimulation) cell over the 240 days that the test cells were monitored.

One important finding from this test was the discovery of naturally-occurring "PM1-like" activity at this site. Similar sequences were found in samples from aquifers at Vandenberg Air Force Base (approximately 90 miles north of NBVC) and in two other MTBE-impacted sites in northern California (Kane et al., 2001). These results suggested that bacteria similar to PM1 are in fact more common than initially thought, and may be present in low numbers at many sites. Such findings may account for the common observation that MTBE biodegradation does occur in biostumulated sites, albeit after an initial lag period, as long as the dissolved oxygen concentrations are relatively high.

10.4.3 Bioaugmentation with Propane Oxidizers (ENV 425)

A bioaugmentation study performed by Envirogen (now Shaw Environmental, Inc.) at the NBVC attempted the seeding of a test cell similar to those described above with bacteria capable of cometabolic oxidation of MTBE when grown on propane. The pilot test was conducted to determine the effectiveness of using propane and oxygen biostimulation and bioaugmentation (by addition of a propane oxidizing strain of *Rhodococcus ruber*, strain ENV425). The seeding process was a single event for this test, though no description of the introduction method is available. Although MTBE concentrations upgradient and downgradient of the treatment system were evaluated, the concentrations of added deuterated MTBE (d-MTBE) and iodide (a conservative groundwater tracer) were measured to evaluate biotic and abiotic attenuation (due to dispersion) as the contaminant passed through the biological barrier. The ratio of the groundwater tracers between downgradient transects provided evidence concerning the relative losses of MTBE resulting from dispersion and degradation. The use of d-MTBE provided evidence of biodegradation by tracking the generation of deuterated daughter products. Test results showed the downgradient concentrations of d-MTBE consistently increased over the 8 months of the test, and that the control plot (biostimulation) showed an average half life of d-MTBE four times greater than the test plot (bioaugmentation) (USEPA, 2002).

10.5 FULL SCALE BIOAUGMENTATION

10.5.1 MC-100 and SC-100 (Port Hueneme, California, USA)

ASU, Equilon and the NAVFAC ESC collaborated on a full-scale system encompassing the 500-ft (152-m) wide MTBE plume at the Port Hueneme NBVC. The system was constructed immediately downgradient of the source zone (Figure 10.7) and operated and monitored for

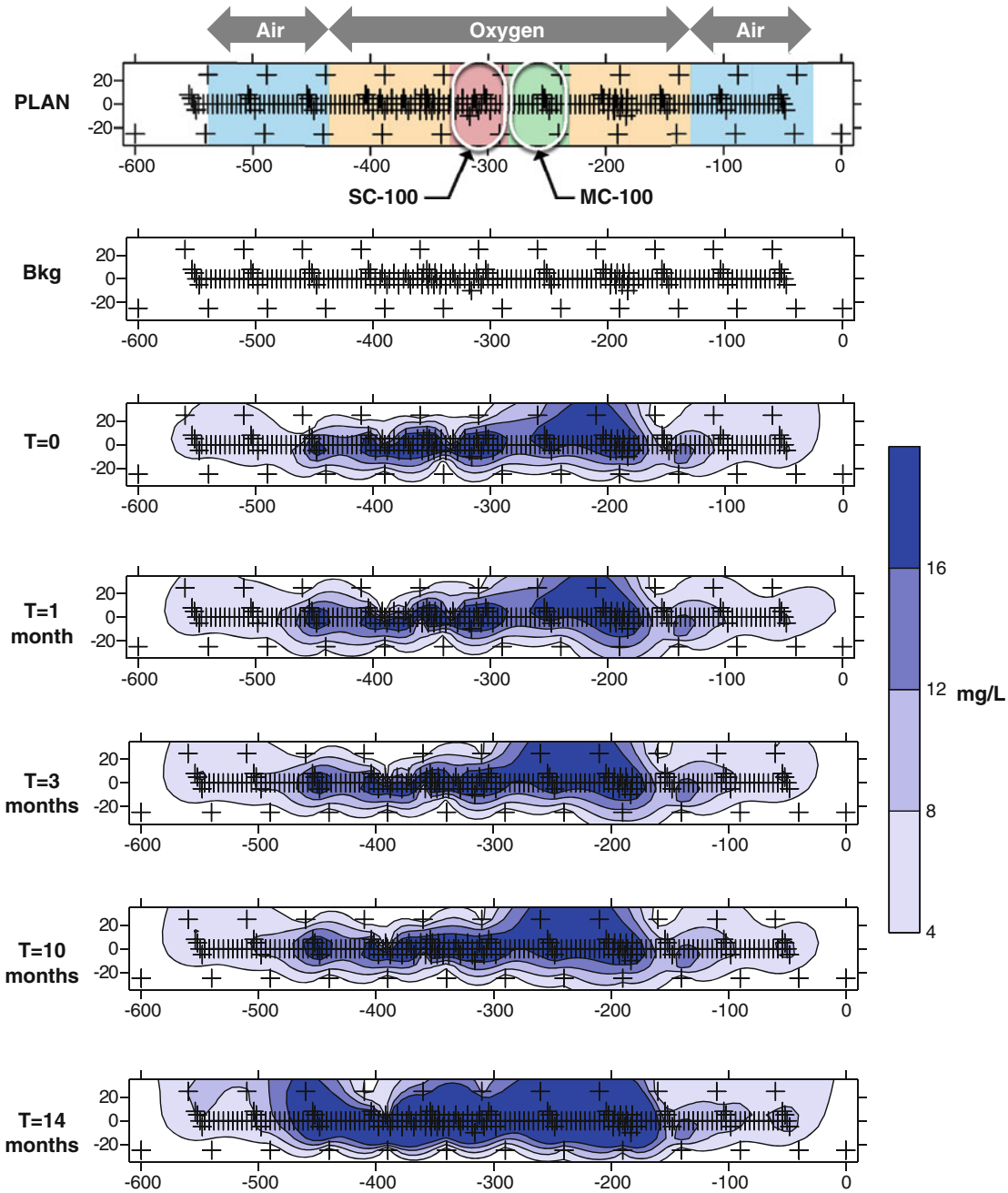

Figure 10.7. Plot plan of site layout and snapshots of the Port Hueneme full-scale oxygen plume over the evaluation period (from Johnson et al., 2003).

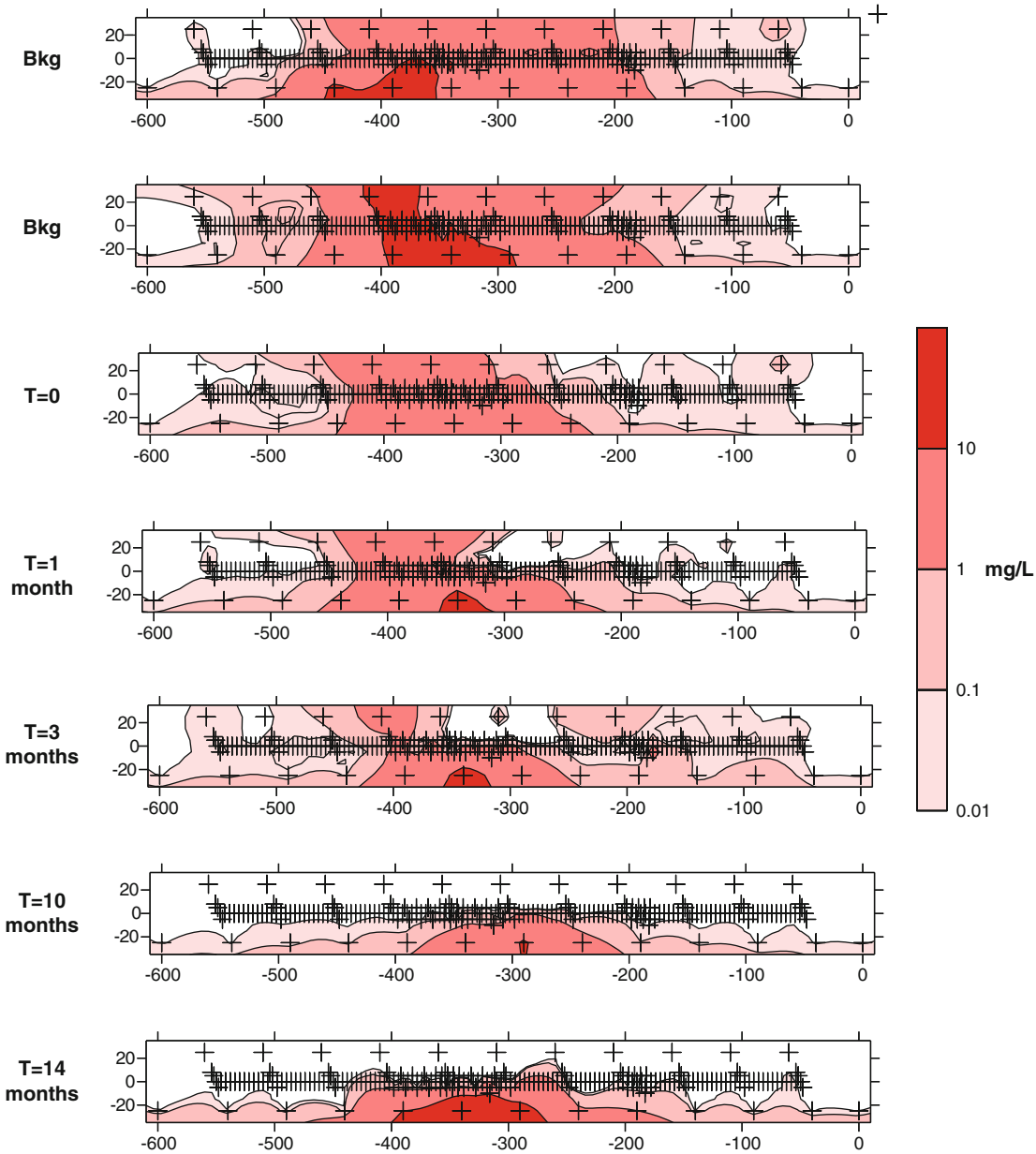

Figure 10.8. Snapshots of MTBE distribution in Port Hueneme's full-scale biobarrier over the evaluation period (from Johnson et al., 2003).

approximately 18 months. The system was comprised of sections relying on biostimulation (air injection and oxygen injection only), and bioaugmentation (oxygen injection plus bioaugmentation with either the MC-100 or SC-100 cultures). Air injection occurred in the lower concentration (<100 µg/L) plume fringes, while oxygen gas addition and bioaugmentation sections were aligned with the central core of the plume where combined concentrations of MTBE, TBA, and BTEX components were in excess of 10,000 µg/L. Figure 10.8 illustrates how this full-scale system effected MTBE concentration reductions to non-detect levels (<5–10 µg/L).

10.5.2 MC-100 (Connecticut, USA)

Equilon reported a full scale bioaugmented biobarrier installation at a retail service station in western Connecticut (Spinnler et al., 2001). For 2 years prior to the biobarrier installation, the average MTBE groundwater concentrations ranged from 7,000–40,000 µg/L, with a target MTBE cleanup level of 70 µg/L. Low concentrations of BTEX also were observed, with average total concentrations less than 1,000 µg/L. The dissolved oxygen concentrations in the plume were less than 1 mg/L prior to bioaugmentation. Oxygen was introduced by pulsed injection. MC-100 injections were spaced approximately 2 ft (0.7 m) laterally and over the vertical range of 12–30 ft (3.7–9.1 m) bgs. Approximately 100 gallons (380 L) of suspension was injected into each boring. The total mass of MC-100 injected at the site was 175 kilograms (kg) dry wt (385 pounds [lb]).

The mean MTBE concentration from monitoring wells in the vicinity of the biobarrier before the system was operational was 12,000 µg/L. After approximately 16 months of operation, the mean concentration was 8 µg/L, a 99.9% reduction. Mean concentrations of MTBE in the "upgradient" wells not affected by the activity of the biobarrier remained relatively constant over the observation period, with a mean concentration of 11,000 µg/L (Spinnler et al., 2001).

10.5.3 MC-100 (California, USA)

Equilon reported a full-scale treatment system at a retail service station site in California, using a biobarrier that was 150 ft (46 m) long. This application used pulsed oxygen injections in order to create an aerobic zone. Five gallons (19 L) of microbe suspension (MC-100) was injected at 3, 5, 7 and 9 ft bgs. Injection points were located at 2-ft (0.7-m) intervals. Difficulties in homogeneous distribution of oxygen and organisms were encountered due to tight site soils, and additional injections were necessary in some locations (Gaarder, 2001).

Evaluation of the mean of biobarrier well concentrations revealed ~99% reduction of MTBE concentrations within 6 months. Although there was no control, the project demonstrated that MTBE degraders could be readily injected and a successful MTBE biobarrier could be established even in relatively tight subsurface materials.

10.5.4 Propane Oxidizing Bacteria (Camden, New Jersey, USA)

A full scale propane-oxidizing bacteria augmentation study was performed by Shaw Environmental, Inc. at a retail gas station in Camden, New Jersey. This technology incorporated the introduction of constant-sparge air (at 13 cfm), propane (0.5 lbs per day, or approximately 0.2 kg per day), propane-oxidizing bacteria (a single seeding of 17 L at ~10^{11} cells/mL) *R. ruber* ENV425), and additions of 120 gallons, or roughly 450 L of sodium bicarbonate into the impacted zone. MTBE concentration reductions of 85% (to below 70 µg/L) were reported over 5 months of operation by the vendor.

10.6 LESSONS LEARNED

The results from the bioaugmentation applications performed between 1998 and 2004 have provided several valuable lessons for MTBE bioaugmentation, as well as for other potential bioaugmentation scenarios. These key lessons include:

1. **Delineation is critical**: The downgradient edge of the source zone is often poorly understood, and careful characterization should be conducted before barrier implementation.
2. **Sufficient oxygen delivery is a common limitation**: A stable, robust zone of oxygenation is required for barriers to stay effective. Pure oxygen delivers higher equilibrium dissolved oxygen levels (about 40 mg/L for pure oxygen gas versus 8 mg/L for air) and may offset the spatially non-uniform nature of gas distributions in aquifers. Anoxic field conditions tend to be associated with low MTBE degradation rates.
3. **Biostimulation is typically sufficient, though a time lag may be experienced**: In many instances, native microbial populations contain the necessary degraders, and increased dissolved oxygen levels result in increased biodegradation rates. This increase in activity may be sufficient to achieve the desired concentration reductions. If it occurs, the success of biostimulation (e.g., as measured by reduced MTBE concentrations) may not be immediately evident. A lag time of 6–12 months before degradation rates are equivalent to bioaugmented plots has been observed in some field studies. Given the age of current MTBE plumes, and the fact that few new MTBE plumes are likely to arise, this timeframe is likely to be acceptable if a receptor is not immediately at risk.
4. **Bioaugmentation cultures can survive and be active *in situ***: Supplementing indigenous communities with high mass loads of MTBE-degrading cultures may result in enhanced degradation rates with little to no lag time, if coupled with a stable oxygen delivery system. However, it is important to note that several side-by-side field applications have reported better results from biostimulation than bioaugmentation.
5. **Typical cocontaminants must be considered**: Other aerobically biodegradable fuel-related chemicals (e.g., BTEX) may affect system performance, depending on culture composition. MTBE was reported to be effectively degraded in a full-scale demonstration treating a mixed MTBE/BTEX/TBA plume (Salanitro et al., 2000) and in some microcosms (Kane et al., 2001). Raynal and Pruden (2008) reported that culture composition is a key factor in determining the success of MTBE-degraders in the presence of BTEX, with more diverse populations being capable of degrading both MTBE and BTEX, while the MTBE-degrading potential of populations dominated by PM1-like strains was severely retarded in the presence of soluble BTEX. Deeb et al. (2001) observed severe inhibition of MTBE degradation in consortia dominated by PM1-like strains in the presence of ethylbenzene, *m*-xylene and *p*-xylene (no degradation in 4 months), strong inhibition in the presence of *o*-xylene (degradation after a lag period of 2 months), and slight inhibition in the presence of benzene and toluene (degradation after a lag period of several hours). In the presence of alkanes (hexane, isopentane), MTBE-cometabolizers may dominate.
6. **Effective treatment typically requires 6–12 months**: Based on experience, the effects of oxygen addition on dissolved oxygen concentrations in the target treatment zone generally occur over a few weeks to a few months. Corresponding increases in biodegradation activity and concentration reductions in the target treatment zone might not be observed for a few months, but are generally observed within 6–12 months (if there is MTBE biodegradation occurring). MTBE degraders are generally regarded to be slow-growing low-yield bacteria, so slow response times in some settings are to be expected. As mentioned above, an 8 month period was necessary to achieve significant activity in one of the biostimulated biobarrier pilot test plots at NBVC, while another pilot test plot nearby showed significant activity after 3 months.

7. **Large numbers of bacteria may be needed for effective treatment**: Several of the pilot- and large-scale demonstrations of this technology have emphasized inoculation with relatively high concentrations of biomass, because MTBE-degrading organisms tend to be relatively slow growing and have low cell yields when grown on MTBE as a sole carbon source (Salanitro et al., 1994). It might be possible to introduce MTBE-degraders into the subsurface in more dilute solutions and then grow them to higher cell densities *in situ* on alternate carbon sources (Smith et al., 2003; Steffan et al., 1997); however, it should be recognized that bacteria from dilute solutions tend to be filtered out within a short distance of an injection well, and the non-native MTBE degraders introduced have to compete with indigenous organisms for the alternate carbon source. Another point of concern associated with high density cell cultures (2.5 g-total suspended solids/L) injected into the subsurface is that these TSS concentrations are high enough to plug most conventional well screens, so delivery to the aquifer through conventional wells is not feasible. In addition, even if the well screens did not filter the suspended solids, the formation would filter the bacterial flocs over a short distance if the infiltration rate was slow (Streger et al., 2002). For more insight into inoculum distribution, the reader is referred to the two-dimensional laboratory scale visualization studies conducted by Braunschneider (2000), where the relationship between aquifer characteristics and distributions of bacteria resulting from different delivery methods were examined. Braunschneider's work includes photos of bacteria distributions with time during injection into a number of idealized geologies. In brief, that work shows that: (1) distributions are roughly spherical and localized in coarse-grained sediments (sands and gravels); (2) the injection causes fracturing and distribution of culture in the fractures for fine-grained silts and clays; and (3) the culture will travel through fractures in fine-grained sediments to the more permeable layers in layered settings.

10.7 CURRENT STATUS

Bioaugmentation is rarely used to enhance MTBE degradation. The key physical limitations include the difficulties in distributing microorganisms (and oxygen) effectively, especially in lower-permeability formations and the difficulty (and therefore expense) of seeding aquifers more than 25 ft (7.6 m) deep. These limitations have largely restricted the application of bioaugmentation for MTBE remediation to shallow contamination in sandy to silty-sand aquifers. In addition, the high cell densities needed to bioaugment a site effectively have resulted in relatively high costs for the culture volumes needed, a practical limitation to routine use of bioaugmentation for MTBE remediation.

Ultimately, the major limitation to bioaugmentation for MTBE and TBA remediation is that it has not been demonstrated to be necessary, since bacteria have proven capable of adapting to these contaminants under most conditions. *In situ* bioremediation of MTBE and TBA has proven effective, most commonly in aerobic biobarriers, without the need for augmentation. A few field tests show that bioaugmentation can drastically minimize the lag time required to achieve measurable or optimal MTBE degradation rates, but biodegradation does occur in time after biostimulation by only oxygen addition. Several side-by-side field tests have resulted in essentially equivalent MTBE reduction in plots treated by biostimulation only or by biostimulation with bioaugmentation.

Although several cultures have been developed for aerobic bioaugmentation, few are commercially available currently. For example, the mixed and single isolate cultures used in the Environmental Security Technology Certification Program (ESTCP)-sponsored large-scale biobarrier demonstration (Miller et al., 2003) were supplied by Shell Global Solutions, but these are no longer commercially available. A survey of vendors and consulting firms found only one

vendor that is currently providing MTBE-degrading cultures with well-documented activity for use in bioaugmentation for MTBE remediation applications.

In cases where MTBE-degrading cultures are not commercially available or are too costly, it is possible to obtain MTBE-degrading organisms from sites where biodegradation is known to occur naturally, and then to grow sufficient quantities of the culture. Sediment and/or groundwater from the site with known MTBE-degrading activity can be seeded into a properly designed reactor (high solids retention time is critical) which is then fed with MTBE and nutrients (Salanitro et al., 2000; Wilson et al., 2002).

10.8 FUTURE PROSPECTS FOR MTBE BIOAUGMENTATION

Bioaugmentation with MTBE degrading organisms is likely to remain a minor field of endeavor. The costs and time associated with generating enough biomass to seed a barrier system has proven to be prohibitive for most applications, and generally not necessary for effective aerobic treatment. Homogeneous microbial distributions can be achieved only in homogeneous materials. Stable and robust zones of oxygenation, however, are achievable in somewhat more complex lithologic systems, making biostimulation a feasible alternative for many MTBE-impacted sites.

It still can be difficult to evaluate if bioaugmentation may, in fact, be beneficial at a specific site. Although indigenous MTBE degraders can be detected at many sites, this information alone is not sufficient to ensure the success of biostimulation. The native microbial population's degrading activity also must increase to a level that is sufficient to achieve the desired MTBE concentration reduction. At this time, it is not clear how to determine if this will be the case on a site-specific basis except through trial and error in the field.

The potential need for bioaugmentation of MTBE and TBA sites led to a burst of research activity. This research has led to valuable improvements in the tools available to monitor and diagnose MTBE biodegradation. These tools can be helpful at MTBE sites because MTBE/TBA biodegradation can be difficult to demonstrate and quantify, although it is vital to natural attenuation decisions. For example, molecular tools and genetic analyses of MTBE and TBA biodegraders offer the potential to quickly detect and quantify specific bacterial strains known to degrade MTBE (Hristova et al., 2003; Kane et al., 2007). However, to be useful on a routine basis, molecular probes still need to be developed for more strains of MTBE-degrading organisms, or for targeted regions that are conserved in most or all MTBE and TBA degraders. Another powerful technique is CSIA. CSIA can be used to demonstrate MTBE and TBA biodegradation and the potential need for augmentation or engineered bioremediation (Wilson et al., 2005).

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CHAPTER 11

ECONOMICS AND VALUATION OF BIOAUGMENTATION

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11.1 INTRODUCTION

Bioaugmentation costs as a component of enhanced *in situ* bioremediation (EISB) generally are small relative to the overall costs of implementing EISB. The additional costs to include bioaugmentation typically represent less than 3% of the total costs for an EISB system. Given the small relative cost and the potential benefits (discussed in Chapter 5), bioaugmentation can be an important enhancement to EISB.

The key potential economic benefits of bioaugmentation are: (1) reduction in the time required to achieve complete dechlorination of chlorinated solvents (or complete degradation of other target compounds), thereby reducing both the monitoring costs and the overall costs for the electron donor (or capturing more of the value of the electron donor initially injected); (2) reduction in regulatory oversight by achieving treatment objectives sooner; (3) reduction in the time required to return the groundwater to beneficial use by achieving treatment goals in a shorter period of time; and (4) ability to apply EISB at sites where this approach would otherwise not be effective and where other more expensive approaches would be required. These benefits will be realized only at sites where suitable microorganisms are not present or are present at low initial concentrations. There are no drawbacks to conducting bioaugmentation even when suitable microorganisms are present, other than the additional cost.

This chapter discusses the primary cost drivers for bioaugmentation (Section 11.2), costs and benefits associated with bioaugmentation (Section 11.3), and the economics of alternative approaches for bioaugmentation (Section 11.4). The chapter then presents example scenarios and detailed costs for bioaugmentation for these scenarios (Section 11.5). Much of the discussion relates to the use of bioaugmentation cultures containing *Dehalococcoides* (*Dhc*) for the degradation of chlorinated ethenes such as perchloroethene (PCE), trichloroethene (TCE) and degradation products of these compounds, because *Dhc* bioaugmentation cultures are well established commercially. However, the cost drivers and general economic considerations will be relevant to other bioaugmentation cultures as well.

11.2 PRIMARY COST DRIVERS

The technical benefits of bioaugmentation as a component of EISB are well established (Major et al., 2002; Lendvay et al., 2003). However, the costs and cost benefits or value of bioaugmentation are dictated by various design parameters and remediation objectives that affect the amount and distribution of the culture, as well as requirements that the EISB system meet stakeholder objectives and expectations.

If the remediation objectives for a site dictate that biodegradation must be initiated in a short timeframe, bioaugmentation can provide greater assurance that the objectives will be

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achieved. The design of the EISB system and bioaugmentation also can be made more robust in order to achieve remediation objectives more quickly. The additional costs of bioaugmentation must be balanced against the potential for negative outcomes, possibly with additional costs of their own, if biodegradation is slow to be initiated and stakeholder expectations and objectives are not met. Stakeholder needs that may require rapid treatment include property transfers, redevelopment schedules, regulatory pressures and concerns over risks from temporary accumulations of intermediates, such as vinyl chloride (VC). A more robust design could include adding more culture at more discrete locations, which can reduce the time to achieve performance expectations of an EISB system but increase bioaugmentation costs. However, if the expectations are lower (i.e., time to achieve complete degradation is not considered critical), then the costs of bioaugmentation may not be warranted.

The remainder of this section discusses the economics of conducting site-specific testing followed by a discussion of the components and design of a bioaugmentation program. In addition, this section will address the impact of remedial objectives and design parameters on the costs and value of bioaugmentation.

11.2.1 Site Specific Testing to Evaluate Bioaugmentation

Testing for the presence and/or activity of microorganisms capable of degrading target contaminants can help evaluate the likely impacts of bioaugmentation. The presence and/or activity of dechlorinating bacteria can be determined in several ways. Genetic testing can be performed on soil and groundwater samples to look for specific organisms (e.g., *Dhc*) or functional genes (e.g., vinyl chloride reductive dehalogenases including *vcrA* and *bvcA*) that are associated with the complete dechlorination of chlorinated ethenes (Müller et al., 2004; Krajmalnik-Brown et al., 2004). Alternatively, laboratory-scale or *in situ* microcosm treatability testing can be conducted to evaluate the need for and potential benefits of bioaugmentation (AFCEE, 2004). The costs and value of each type of testing are discussed below.

Genetic testing can take several forms, but is most commonly performed using quantitative polymerase chain reaction (qPCR) analysis by commercial laboratories. This analysis involves removing microorganisms from soil or groundwater samples (e.g., by filtering groundwater), rupturing the microorganisms to release their deoxyribonucleic acid (DNA), and adding "primers" that bind to targeted gene sequences of interest. These primers act as a starting point for DNA copying enzymes (Taq polymerase) that replicate the target DNA sequence. A qPCR machine is used to facilitate the exponential replication of the targeted gene sequences through a thermo-cycling process that incorporates fluorescent dyes and which reads the fluorescence as it increases using digital optics. The initial number of targeted gene copies in a sample is quantified by how many PCR cycles are required to cross a threshold level of fluorescence; samples with high concentrations of the target gene cross the threshold in fewer cycles while more dilute samples require more cycles (Mackay, 2007).

Analysis by qPCR is available commercially and can be conducted using groundwater or sediment from a specific site. Although soil samples can be assayed by qPCR, groundwater is typically the preferred medium because groundwater samples and sampling techniques are less expensive. This approach allows testing of a larger subsurface volume than soil samples and increases the likelihood of detecting key dechlorinating microorganisms. The Strategic Environmental Research and Development Program (SERDP) Project ER-1561 (project descriptions and documents available at www.serdp-estcp.org; last accessed June 18, 2012) is currently funding efforts to develop standardized qPCR methods, which should further improve the reliability and interpretability of these tests (e.g., Ritalahti et al., 2010).

The cost of qPCR analysis ranges between $250–300[1] for a single target, including DNA extraction from groundwater; additional targets on the same sample adds approximately $100 per target. qPCR analysis provides information on the number of gene copies in a sample and allows tracking microbial population growth during treatment. The results of qPCR analysis can answer questions such as whether the indigenous or bioaugmented microbial populations are growing after addition of electron donor, or if the bioaugmentation cultures are growing and spreading through the treatment area. However, the results provide limited information on the rate of microbial activity under various conditions.

In contrast, simple microcosm (bottle) studies can be conducted to evaluate the nutrient and microbial requirements for biodegradation of target compounds in soil and groundwater from a particular site. The tests typically consist of a number of treatments designed to evaluate the effect of electron donor amendments with and without bioaugmentation relative to controls. A small microcosm study might include three treatments (sterile control, biostimulation, and biostimulation and bioaugmentation), each run in triplicate and cost in the range of $10,000. Microcosm studies also allow for the collection of data pertaining to the nature of specific microbial activities (e.g., sulfate reduction, methanogenesis, reductive dechlorination), the effect of different electron donors and donor loading rates, nutrient requirements, reaction rates, and the factors that inhibit reductive dechlorination. More complex studies will usually require additional microcosms. Statistical methods (fractional factorial experimental designs) can be used to reduce the number of bottles required to evaluate multiple variables. However, these expanded studies typically cost from $20,000 to $30,000.

If dechlorination is not achieved or is slow to occur in microcosms without bioaugmentation, but occurs quickly with bioaugmentation, then it is likely that bioaugmentation will be of benefit in the field. Microcosm studies also are quite effective at determining relative degradation rates with different amendments and are therefore useful for designing the EISB system. However, since microcosm tests represent ideal conditions of mixing and amendment distribution, caution should be used when using the results to predict the rate that biodegradation will occur under field conditions.

In situ microcosms and samplers also may be used to evaluate the potential benefits of bioaugmentation. Since these tests are still considered innovative, they are not covered in this chapter, but are discussed in Chapter 4.

It should be noted that variations in contaminant concentrations and geochemical or hydrogeological characteristics across the site would affect the ability to transfer laboratory results to the field. Therefore, the location and number of sampling locations should be carefully considered in the decision-making process, regardless of the method used to assess the need for bioaugmentation.

11.2.2 Amount and Distribution of Active Organisms

In most cases, the goal of bioaugmentation is to reduce the time needed for effective treatment. The effectiveness of bioaugmentation in meeting this goal is dictated by three primary factors: (1) the volume of culture required; (2) the distribution of the culture during injection; and (3) the growth rate of the culture subsequent to its injection. These factors are discussed in the following subsections.

[1]All costs presented in this chapter are in U.S. dollars.

11.2.2.1 Volume of Active Culture

The volume of active culture required for effective inoculation is a function of the population density of viable cells in the culture shipped to the site, and the number of microbial cells that remain viable in the subsurface following injection of the culture. In the case of *Dhc*, research suggests that "generally useful" rates of biodegradation will occur when the density of viable *Dhc* cells has increased to a concentration of 10^7 cells per liter (cells/L) or greater (Lu et al., 2006). This target can be used to estimate the volume of culture needed for a specific site.

For example, assuming that a bioaugmentation culture contains approximately 10^{11} cells/L (typical for *Dhc* inocula), and that it is uniformly delivered within a target volume of the aquifer, then 1 L of culture could be used to inoculate approximately 10,000 L of groundwater. The onset of degradation should be observed within that volume of groundwater almost immediately provided geochemical conditions are appropriate to support microbial activity. Obviously, if less culture is added then more time will be required before degradation activity is observed (the time required will be a function of the growth rate of the introduced microorganisms in the field). Similarly, faster degradation rates will be observed if more culture is initially added.

From a practical perspective, a minimum of 1 L of culture is typically added to a single injection location or well, although smaller amounts have been used (Philip Dennis, SiREM Laboratories, personal communication, 2010). In many cases, a typical application rate for culture is 1 L of culture for every 35,000 L of groundwater. This inoculation rate is based on significant field experience and results in an initial cell density slightly less than 10^7 cells/L. This cell density is adequate because of the impact of the growth of *Dhc* cells following injection. The inoculation concentration may be increased in situations where there is a potential that geochemical or other factors may result in slower than typical growth rates following injection or where a faster response time is required.

Given the impact of the initial cell density of a bioaugmentation culture on the time before the observed onset of degradation, the number of viable cells that can be delivered to the subsurface is a key parameter. The supplier must provide cultures containing appropriate cell densities, and equipment and procedures for culture transport and injection. Factors such as exposure to oxygen during transport, storage times and injection procedures affect the viable number of *Dhc* cells that are actually injected at a site (Vainberg et al., 2009). Containers used for transport and storage must be carefully designed to prevent exposure to air and to allow the culture to be added to the subsurface under anaerobic conditions. If these conditions are not met, cell death may occur and additional culture or additional time will be required to meet project objectives, both of which involve additional project cost.

11.2.2.2 Distribution of Introduced Organisms

The distribution of the introduced culture is a function of: (1) the distance between injection points; (2) how the culture is injected (e.g., the volume of anaerobic water used to chase or carry the culture from the injection point into the formation); (3) the transport properties or "stickiness" of the microorganisms; and (4) site-specific factors such as the geology and geochemistry of the groundwater. Effective distribution of the culture is one of the key considerations in a bioaugmentation plan.

Injection of the culture may be performed several days or weeks following injection of the electron donor (typical for an active or semi-passive approach to adding electron donors) or may be conducted at the same time (typical for a passive approach of electron donor addition). Injection is accomplished by displacing the culture from the shipping containers into the injection well using an inert gas while maintaining the culture under anaerobic conditions.

Typically, the culture then is disbursed further from the well by injecting additional "chase" water. The water injected immediately following addition of the culture normally is made anaerobic to avoid exposing the culture to oxygen that could reduce the number of viable cells in the injected culture.

Assuming that a bioaugmentation culture contains 10^{11} cells/L, then 1 L could theoretically inoculate an aquifer volume of approximately 33 cubic meters (m^3) of the subsurface, equivalent to 1,177 cubic feet (ft^3), assuming a porosity of 30% and a target concentration of 10^7 cells/L of groundwater. If 1 L of culture is injected over a 3 m (10 ft) screened well located within a homogeneous, isotropic aquifer such that it is uniformly distributed in the water, then the calculated average cell density could be achieved out to a radial distance around the well of 1.9 m (6.2 ft).

However, to achieve the theoretical distribution of the inoculum requires that: (1) sufficient water be injected with the culture to push or carry it out to this radius; and (2) the microorganisms will be transported like a conservative tracer. In reality, water volumes are often insufficient to push the culture to this radius and microorganisms will "stick" to the aquifer solids and be retarded in their migration from the injection point. The degree of stickiness is a function of various physical and chemical properties of the cells and the aquifer. Generally, injected cell density will fall off logarithmically with distance from the injection point and cells will be transported further in higher permeability zones than in low permeability zones. The initial distribution of the culture is important, but as discussed later in this section, active growth of the microorganisms *in situ* generally will be needed to achieve the distribution required to meet the remedial objectives (Hood et al., 2006).

Given this fact, achieving observable degradation rates within short time frames often requires that the culture be injected at a significant number of discrete locations or at closely spaced locations. Dechlorination will be achieved more quickly because a sufficient population of microorganisms will be established at more locations and the time required for the population to spread to areas between injection locations will be shorter. However, injection at more discrete locations will increase the implementation costs for bioaugmentation.

The water used to push the microorganisms out into the formation should be anaerobic to preclude exposing dechlorinating bacteria to oxygen. However, the time to collect and modify (if required) the groundwater (or municipal tap water) used as chase water will increase costs. Bioaugmentation culture often is added with the electron donor solution to simplify the injection process. Experience with numerous injections has demonstrated that *Dhc* cultures are not negatively impacted by high concentration electron donor solutions in the subsurface near the injection point. This approach has been conducted successfully at many sites (Jeff Roberts, SiREM Laboratories, personal communication, 2010).

11.2.2.3 Growth Following Injection

The growth rate of the culture directly affects the time needed to achieve meaningful results following bioaugmentation, since actively growing cultures distribute throughout the subsurface much more quickly than slow growing cultures. After injection, bioaugmented microorganisms may migrate only a short distance from the injection point before they are filtered out by the aquifer matrix. Consequently, microbial growth in the subsurface is generally necessary for effective bioaugmentation, since the progeny of introduced microbes must move with groundwater and colonize downgradient regions to bioaugment a reasonable volume of the aquifer.

Dhc have been shown to grow and spread downgradient relatively quickly under favorable conditions (Schaefer et al., 2009). Growth of *Dhc in situ* requires several key conditions,

including: (1) an adequate supply of electron donor; (2) an adequate supply of electron acceptor (i.e., chlorinated ethenes for *Dhc*) (Cupples et al., 2004); (3) appropriate geochemical conditions (AFCEE, 2004); (4) the absence of inhibitory compounds in the groundwater (Grostern et al., 2010); and (5) an appropriate groundwater temperature (Friis et al., 2007).

A well-designed EISB program will provide for an adequate supply of electron donor to ensure reducing conditions are maintained and to promote rapid growth of native or bioaugmented microorganisms. Sufficient electron donor is required for effective treatment, but adding too much electron donor should be avoided because it can cause excess production of undesirable byproducts such as methane, and it represents a nonbeneficial cost to the project.

In EISB systems where there are adequate amounts of donor supplied, it is possible that concentrations of chlorinated ethenes may be too low to support *Dhc* growth. *Dhc* populations require chlorinated ethene concentrations greater than about 0.05 milligrams per liter (mg/L) to grow at significant rates (Cupples et al., 2004; Schaefer et al., 2009), since these compounds are required for respiration. If the concentrations of the chlorinated ethenes are too low, growth rates and subsequent spread of the microorganisms may be slow, requiring considerably more culture initially and tighter spacing of inoculation points to achieve the required distribution of cells and reasonable degradation rates. At sites with chlorinated ethene concentrations at or near these lower limits, higher volumes of culture and repeated injections may be required to maintain biodegradation activity.

Adverse geochemical conditions in groundwater in the vicinity of the injection point also can inhibit active growth of bioaugmented microorganisms. In situations where the pH of the groundwater is outside the neutral range (6.8–7.8) optimal for dechlorinating bacteria (Middledorp et al., 1999) there is likely to be a benefit to adjusting the pH of the groundwater. Non-reduced groundwater also can be problematic. It may be necessary to delay bioaugmentation until after the electron donor has been added in order for anaerobic conditions to develop in the aquifer. An oxidation reduction potential (ORP) of -100 millivolts (mV) or less is considered ideal for *Dhc* growth and survival (Dennis, 2005).

Finally, the presence of certain inhibitory compounds (such as chloroform) can slow or prevent growth of bioaugmentation cultures (Grostern et al., 2010). If the concentrations of inhibitory compounds are high, consideration may be given to implementing pretreatment of the groundwater prior to bioaugmentation to reduce the concentrations of inhibitory compounds, or to bioaugmentation with other cultures that target these inhibitory compounds (Grostern and Edwards, 2006).

The effort required to create an environment that is favorable for the growth and activity of bioaugmented or even indigenous microorganisms can be substantial. For example, highly acidic and buffered aquifers may require large amounts of alkaline amendments before *Dhc* can grow *in situ*. In such cases, EISB with bioaugmentation may not be economically attractive.

11.3 COSTS, VALUE AND BENEFITS OF BIOAUGMENTATION

11.3.1 Costs for Bioaugmentation Culture and Injection

Bioaugmentation cultures containing *Dhc* range in price from approximately $100 to several hundred dollars per liter. However, there can be a wide range in the *Dhc* cell density, with concentrations in the range of 10^{11} up to 10^{12} cells/L (Vainberg et al., 2009). Given the impact of cell density on the time to achieve results after injection, a purchasing decision should not solely be based on the cost per liter of culture. The cell density and ability of a bioaugmentation culture to degrade different contaminants concurrently also must be considered. Bioaugmentation cultures containing *Dhc* and *Dehalobacter* (*Dhb*) are currently available that can degrade chlorinated ethenes and chlorinated ethanes (Grostern and Edwards, 2006).

Additional costs associated with injection of a bioaugmentation culture include delivery of the culture vessels to the site, purchase or rental of additional equipment required for the bioaugmentation process, and labor for the field technicians. These costs are generally small relative to costs for injection of electron donor. The cost of injection equipment and related activities will vary depending on the volume of culture being injected, but for a typical, medium sized application of 80 L of culture, these costs may add $50–$100 per liter to the cost of bioaugmentation (Jeff Roberts, SiREM Laboratories, personal communication, 2010).

A rough planning level cost estimate for bioaugmentation can be developed based on the information presented in this chapter. Assuming that 1 L of culture is used to bioaugment 35,000 L of groundwater, the culture costs $200 per liter to purchase and $100 per liter to inject, and the porosity is 30%, the cost to bioaugment a site would be $2.60/$m^3$ of aquifer (roughly $2.00/cubic yard).

11.3.2 Value of Bioaugmentation Relative to a "Wait and See" Approach to Degradation of DCE and VC

As stated earlier, the key potential economic benefits or value of bioaugmentation are: (1) reduction in the time required to achieve complete dechlorination of chlorinated solvents (or complete degradation of other target compounds), thereby reducing the overall costs for injection of electron donor (or capturing more of the value of the electron donor initially injected) and groundwater monitoring; (2) reduction in regulatory oversight by achieving treatment objectives sooner; (3) reduction in the time required to return the groundwater to beneficial use by achieving treatment goals in a shorter period of time; and (4) the ability to apply EISB at sites where bioremediation would not be effective otherwise, and where other more expensive approaches would be required.

It is difficult to quantify the actual cost savings or value of these benefits, but the magnitude can be significant. Reducing the time to achieve degradation also has the potential to reduce other costs. These costs may be associated with increased monitoring or evaluation of risks that may be considered necessary by site owners, regulators or other stakeholders if EISB is not meeting goals in a timely fashion. Every site will be different and it is not possible to predict what these additional costs may be, but it is clear that site owners, regulators, and other stakeholders are likely to be more confident that the EISB application will be successful if they see data showing complete degradation soon after EISB is implemented.

The "Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents" (AFCEE et al., 2004) states that, "it has been observed at numerous locations that dechlorination species require as long as 12–36 months of substrate addition to grow to concentrations that provide timely and complete dechlorination of dichloroethene (DCE) and VC to ethene." The document later states that, "bioaugmentation can shorten lag times or improve the rate of dechlorination in environments where native dechlorinating species are poorly distributed, present at low population densities, or not an ideal strain."

There are no definitive tools that can quantitatively predict how much a specific remediation time frame can be reduced through the use of bioaugmentation. However, given that the time to achieve remedial objectives with bioaugmentation can be on the order of months to several years (AFCEE, 2004), significant cost savings can result from shortening the duration of active treatment (Dennis et al., 2009). The potential cost savings to consider when evaluating bioaugmentation include:

1. **Reducing the cost to purchase and inject electron donor**. If aggressive treatment of a targeted key area is being conducted and it is expected that 5 years of active addition

of electron donor will be required to achieve remedial goals, and if bioaugmentation will allow remedial goals to be achieved even 12 months sooner than they would be without bioaugmentation, then the costs to purchase electron donor and operate the bioremediation system could be reduced by 20% with bioaugmentation. This cost saving also may be viewed as maximizing the value of the electron donor and system operating costs during the first year of operation, when these activities would otherwise not contribute effectively to the treatment of the target contaminants. As can be seen in the examples presented later in this chapter, the cost of bioaugmentation is very small (typically less than 3% of other costs) relative to a potential savings of 20% in other costs. In the case of a biobarrier that may be operated for a much longer period of time, the savings in electron donor and system operation actually may not be realized until the end of the operating period sometime in the future, but the use of bioaugmentation will still capture the value of the electron donor added and system operation in the early period of operation.

2. **Reducing the monitoring costs**. Reducing the operating time for an EISB program also reduces the costs for monitoring. As discussed in the example above, if bioaugmentation reduces the operating time by 20%, then a 20% reduction in associated operational costs also may be achieved. Bioaugmentation also may result in further reductions in monitoring costs (or avoid escalations in monitoring costs) relative to situations where complete dechlorination is not achieved for some period of time (e.g., 12–36 months). It is quite possible that responsible parties or regulators will have an increased level of concern regarding the success of an EISB program until complete dechlorination can be demonstrated. Further, delayed reductions in the concentrations of intermediate degradation products such as VC may result in potential exposures that need to be addressed. These factors may result in requirements for additional monitoring that would not be required if bioaugmentation was implemented and dechlorination was demonstrated sooner.
3. **Reducing costs associated with reporting and discussions with stakeholders**. If bioaugmentation is implemented and dechlorination is achieved, it is likely that costs associated with reporting and discussions with stakeholders (i.e., property owners, regulators and neighbors) will be reduced relative to situations where remedial objectives are not being achieved for some period of time. In more extreme cases, the use of bioaugmentation may avoid the need to implement contingent remedies that may be required if remedial objectives are not achieved within a time period expected by stakeholders.

11.4 ECONOMICS OF ALTERNATIVE APPROACHES

11.4.1 Costs for Purchase and Injection of Concentrate Versus *In Situ* Growth and Distribution

It may be possible at some sites to accomplish bioaugmentation by pumping groundwater from specific areas of the site where elevated concentrations of *Dhc* are already established either by bioaugmentation or by natural processes, and inoculating other areas where *Dhc* is absent or less abundant. This approach can work, but consideration must be given to the time and effort required relative to the costs to purchase culture. The concentrations of *Dhc* in natural groundwater, even under optimal conditions, are typically in the range of 10^8 cells/L, while a typical bioaugmentation culture contains 10^{10}–10^{11} cells/L. Thus, to establish active

biological degradation in a short time frame (i.e., before 6–12 months), significant volumes of water must be pumped. For example, to achieve the same number of cells that would be injected with 20 L of a commercially available culture, roughly 2,000–20,000 L of groundwater would have to be extracted from the active area, transported to the new location, and injected into the subsurface. These steps would need to be conducted while taking measures to maintain anoxic conditions in the groundwater and prevent significant die-off of *Dhc* cells during the transfer process. The process must be conducted in a way that completely eliminates contacting air at any step in the process.

The cost of 20 L of culture may be in the range of $4,000. Recirculation of 2,000–20,000 L of water would require installation and operation of a pump for some period of time, transfer piping between the wells, or the rental and use of a tanker truck. The process also would require labor and equipment to see that air is excluded from the operation. The cost for such an operation easily could exceed the cost for a bioaugmentation culture with an equivalent number of cells.

11.5 ESTIMATED COSTS FOR TEMPLATE SCENARIOS

The economic analysis of remedial options is a key activity in any remedy selection process. It is typically employed in the feasibility study phase of remedy selection to aid in selecting the optimal remedial option for a site, in concert with a number of other criteria. In an earlier volume in this series (Stroo and Ward, 2010) on remedial technologies to treat dissolved phase chlorinated aliphatic compounds in groundwater, an evaluation of costs for several remedial technologies, including EISB was presented. The EISB costs presented in this earlier evaluation assumed that bioaugmentation would not be conducted as part of the implementation of the technology. In this chapter we look at the cost to include bioaugmentation as a component of the EISB scenarios previously evaluated to provide three examples of what bioaugmentation might cost.

This section presents a basic approach to generating cost information for EISB and for the bioaugmentation component of EISB at three example sites where this approach could be used to treat dissolved phase chlorinated aliphatic compounds in groundwater. Cost information is broken down into design elements, capital expenditures, operation and maintenance (O&M), and monitoring costs to help understand the primary cost drivers. The intent of this section is not to provide definitive cost information for EISB and bioaugmentation technology, since these are highly site-specific. Rather, the purpose is to give the design engineer a general process to use in estimating costs when considering EISB and bioaugmentation at specific sites.

11.5.1 Template Site Descriptions

The economic analysis presented here involves detailed costing for hypothetical template sites and will be used in this section to cost the application of EISB with bioaugmentation for a residual source area and two downgradient groundwater plumes containing TCE and its daughter products. The template sites have been divided in this manner because source areas and plumes are typically addressed independently in many feasibility studies.

The template residual source area (Case 1) consists of an area of 250 m^2 (2,690 ft^2). This source area contains 200 kilograms (kg), or 440 pounds (lb) of TCE that is either adsorbed to or present as a dispersed residual phase in the aquifer matrix. This situation is typical of many small TCE source areas. The depth to groundwater is 1.5 m (4.9 ft), and it is 4.5 m (14.8 ft) to a

Table 11.1. Description of Template Site Cases

Parameter	Case 1	Case 2	Case 3
Location	Source Area	Plume	Plume
Area (m^2)	250	–	–
Width (m)	–	75	75
Depth to Groundwater (m)	1.5	1.5	3.0
Depth to Aquitard (m)	4.5	4.5	12.0
Saturated Thickness (m)	3.0	3.0	9.0
Hydraulic Conductivity (cm/s)	1×10^{-4}	1×10^{-4}	1×10^{-4}
Groundwater Gradient (m/m)	3×10^{-3}	3×10^{-3}	9×10^{-3}
Groundwater Velocity (m/year)	10	10	30
Porosity	0.3	0.3	0.3
Effective Porosity	0.2	0.2	0.2
Injection Rate (L/min)	10	10	10
EVO[a] Adsorption (g/kg solids)	1	1	1
Residual TCE (kg)	200	–	–
Groundwater concentrations (mg/L)			
TCE	20	0.1	0.1
cis-DCE	20	2.0	2.0
VC	–	0.2	0.2
Dissolved Oxygen	–	–	–
Nitrate	–	–	–
Dissolved Iron	5	5	5
Sulfate	100	100	100

[a]EVO – emulsified vegetable oil

clay aquitard that underlies the saturated zone, providing a saturated thickness of 3 m (9.8 ft). The groundwater velocity across this area is 10 m (32.8 ft) per year. The groundwater in this area contains 20 mg/L of both TCE and *cis*-dichloroethene (*cis*-DCE), along with 5 mg/L dissolved iron and 100 mg/L sulfate. These and other characteristics of the subsurface matrix and groundwater are summarized in Table 11.1 for all three cases considered.

Characteristics of the template sites for Case 2 and Case 3 also are presented in Table 11.1. Case 2 consists of a plume that extends off-site. The width of the plume at the site boundary is 75 m (246 ft). The depth to groundwater is 1.5 m (4.9 ft), and it is 4.5 m (14.8 ft) to a clay aquitard that underlies the saturated zone, providing a saturated thickness of 3.0 m (9.8 ft). The groundwater velocity across this area is 10 m (32.8 ft) per year. The groundwater in this area contains 0.1 mg/L of TCE, 2 mg/L *cis*-DCE, and 0.2 mg/L VC, along with 5 mg/L dissolved iron and 100 mg/L sulfate.

Case 3 consists of a plume of similar width that also extends off-site. In this case the depth to groundwater is 3 m (9.8 ft), and it is 12 m (39.4 ft) to a clay aquitard that underlies the saturated zone, providing a saturated thickness of 9 m (29.5 ft). The groundwater velocity across this area is 30 m (98.4 ft) per year. The groundwater in this area contains 0.1 mg/L of TCE, 2 mg/L *cis*-DCE and 0.2 mg/L VC, along with 5 mg/L dissolved iron and 100 mg/L sulfate.

This combination of contaminant concentration, saturated thickness, and groundwater velocity gives a contaminant groundwater flux that is nine times higher than the previous scenario (Case 2). The greater flux increases the costs for EISB. The greater depth interval for treatment increases costs for electron donor additional and the volume of bioaugmentation culture needed.

11.5.2 Costs Categories and Components

Cost estimates for EISB at each of the three template sites have been developed. The costs for EISB were divided into general categories, including design, capital, operation and maintenance, and monitoring. It has been assumed that basic site data derived from remedial investigation activities are available prior to the design phase. Specific cost components in each cost category are listed in Table 11.2.

Operation and maintenance activities include all labor, materials, replacement parts, waste management and disposal, electricity usage and reporting required to keep the remedy operating as designed. EISB will require multiple amendment applications to be effective. These applications have been included as O&M costs. An average O&M cost is calculated by averaging the cost of periodic amendments and annual O&M expenses over time. It has been assumed that the contractors performing periodic maintenance are located near the site, so that travel costs are not significant relative to the time spent on-site.

Monitoring costs include labor, equipment, analytical and reporting costs associated with monitoring the performance of the remedy. The assumed monitoring network consists of five monitoring wells for the source area (Case 1) and shallow plume (Case 2) and seven monitoring wells for the deeper plume (Case 3). It has been assumed that groundwater sampling will occur quarterly for the first 5 years that the remedy is active and semi-annually thereafter.

The net present value (NPV) of future O&M and monitoring also has been calculated, assuming that cleanup is achieved for Case 1 in 5 years and monitoring continues for another 5 years and that the total lifetime for O&M and monitoring of the biobarriers (Case 2 and

Table 11.2. Components of Cost Analysis

Design Phase	Capital
• Pre-design sampling and utility survey • Laboratory studies • Groundwater modeling • In-field hydraulic testing • Detailed design • Permitting and reports	• Site preparation • Mobilization and demobilization of equipment • Injection and monitoring well installation and development • Delivery equipment • Materials • Labor • Waste management and disposal • Reports
O&M (per event)	**Monitoring (per year)**
• Labor • Electricity • Amendment re-addition • Replacement parts and materials • Waste management and disposal • Reports	• Labor • Analytical • Reports

Case 3) is 30 years. NPV calculations were conducted using a discount rate of 3% for annual costs. This discount rate is derived from Department of Defense (DoD) guidance on using economic analyses in decision-making (DoD, 1995). This discount rate is based on current Office of Management and Budget (OMB) data and assumes a 5% nominal return is available on U.S. Treasury Notes and Bonds and an annual inflation rate of roughly 2%. This real discount rate is appropriate for government entities, while a higher discount rate may be more appropriate for private parties who can obtain higher returns on investments.

11.5.3 EISB Remediation Technology Description

For the purposes of this analysis, emulsified vegetable oil (EVO) will be used as the electron donor to promote reductive dechlorination of TCE to ethene in the residual source area (Case 1) and in the barrier configurations (Cases 2 and 3). The advantages of this donor include longevity and ease of distribution in the subsurface relative to other slow-release donors. The costs for laboratory treatability testing to verify that EISB will be effective at the site and to ensure that EVO is a suitable donor have been included in the design phase.

It is possible to implement EISB applications by circulating groundwater between permanent wells while injecting a soluble electron donor in a semi-passive approach rather than using direct push of EVO in a passive approach. The different approaches to adding electron donors are discussed in more detail in an earlier monograph in this series on *in situ* bioremediation of perchlorate (Stroo and Ward, 2009). The semi-passive approach would require more frequent addition of electron donor (e.g., every 6–8 months) but fewer injection locations are required and the costs for electron donor are typically less, so the overall costs are generally similar to the passive approach. The overall costs of the passive (EVO injection) and semi-passive approaches are similar and the choice between one versus the other is unlikely to significantly impact the cost analysis of bioaugmentation presented below.

In Case 1, the EVO will be applied through a series of 15 injection wells spaced on 5-m (16.4 ft) centers distributed across the 250 m^2 (2,690 ft^2) source area. A groundwater model will be used in the design phase to help specify the injection sequence and ensure the amendment will contact the entire source zone. The 5-cm (2-inch) diameter injection wells will be screened across the saturated zone and developed prior to EVO injection. Pre-design injection testing is included in the cost analysis to establish the injection rate, which is assumed to be 10 L/min (2.6 gal per minute [gpm]). The costs include the addition of 185 kg (407 lb) of a commercial EVO solution to each injection point, along with 9,000 L (2,376 gallons [gal]) of fluid (dilute EVO and chase water) to ensure complete distribution of the EVO. EVO will be metered through a dosimeter pump connected to a nearby water source, and a manifold will be used to allow injection into as many as five injection wells simultaneously. Each injection line will contain a pressure gauge and flow totalizer, to allow accurate measurements of the fluid injections into each well. The injections will be performed by a two-person crew requiring 11 days of labor, including set up and breakdown.

Bioaugmentation will be conducted along with the first injection of electron donor to provide maximum distribution of the initial culture and minimize additional costs for injection. Culture will be delivered to the site in stainless steel vessels and one liter of culture will be injected into each of the 15 injection points based on a cell density of 10^{10}–10^{11} cells/L. The culture medium will be transferred to the injection point using argon gas to pressurize the culture vessel and displace the media into the injection point. Because the injection of culture will be conducted along with the injection of electron donor, the additional labor for culture injection will be minimal. Cost for the culture medium and the additional costs for labor and equipment to inject the culture are included in the cost estimate.

It is assumed that it will take 5 years to reach remedial goals using EISB. Each application of EVO will support dechlorination for 2–3 years, so a second application of EVO will be required part way through the third year. The second application will deliver 100% of the initial EVO loading using the same injection well network. No bioaugmentation will be necessary during the second injection. No other operation and maintenance is required with this remedial option. Quarterly monitoring will be performed for the first 5 years, followed by semi-annual monitoring for another 5 years. The quarterly monitoring will include parameters specific to the remedy, such as total organic carbon (TOC) and other relevant monitored natural attenuation (MNA) analytes. The semi-annual sampling will include only volatile organic compounds (VOCs).

In Case 2, EISB will be applied as a barrier, with the objective of reducing the contaminant concentrations by 90–95% before leaving the site. It is assumed the barrier will need to operate for 30 years. Five 5-cm (2-inch) diameter monitoring wells will be installed. EVO will be used as the electron donor to promote reductive dechlorination of chlorinated VOCs within the zone of influence of the shallow barrier. The EVO will be applied through a single row of 15 injection wells spaced on 5-m (16.4 ft) centers along the length of the shallow barrier. The 5-cm (2-inch) diameter injection wells will be screened across the saturated zone and developed prior to EVO injection. Pre-design injection testing will be used to verify the injection rate, which is assumed to be 10 L/min (2.6 gpm). EVO (185 kg [407 lb]) will be added to each injection point, along with 9,000 L (2,376 gal) of fluid (dilute EVO and chase water) to ensure complete distribution of the EVO. EVO will be added in the same manner described in Case 1. The injections will be performed by a two-person crew and will require 11 days, including set-up and breakdown.

Bioaugmentation will be conducted along with the first injection of electron donor to provide maximum distribution of the initial culture and minimize additional costs for injection. The injection will be conducted following the same procedure as for Case 1.

Each application of EVO will support dechlorination for 2–3 years, so that an additional application of EVO will be conducted every 3 years. These subsequent applications will deliver 100% of the initial EVO loading utilizing the same injection well network. No bioaugmentation will be necessary after the first injection. No other O&M is required with this remedial option. Quarterly monitoring will be performed for the first 5 years, followed by semi-annual monitoring thereafter. Both the quarterly and semi-annual monitoring will include parameters specific to the remedy, such as TOC and other relevant MNA analytes.

In Case 3, EISB also will be applied as a barrier using EVO with the objective of reducing the contaminant concentrations by 90–95% before leaving the site, but the barrier depth will be greater than in Case 2. The EVO will be applied through a single row of 15 paired injection wells spaced on 5 m (16.4 ft) centers along the length of the deep barrier. A groundwater model will be utilized in the design phase to verify well spacing and ensure flow will pass through the barrier. Each set of paired wells will consist of two 5-cm (2-inch) diameter injection wells, one screened across the upper 4.5 m (14.8 ft) of the saturated zone and the second screened across the lower 4.5 m (14.8 ft). All the wells will be developed prior to EVO injection. Pre-design injection testing will be used to verify the injection rate, which is assumed to be 10 L/min (2.6 gpm). Commercial EVO solution (280 kg or 616 lb) will be added to each injection point, along with 13,500 L (3,564 gal) of fluid (dilute EVO and anaerobic chase water) to ensure complete distribution of the EVO. EVO will be added in the same manner described in Case 1. The injections will be performed by a two-person crew and will require 24 days, including set-up and breakdown.

Bioaugmentation will be conducted along with the first injection of electron donor to provide maximum distribution of the initial culture and minimize additional costs for injection. The injection will be conducted following the same procedure as for Case 1 and 2.

It is expected that each application of EVO will support dechlorination for 2–3 years; therefore, an additional application of EVO will be conducted every 3 years. These subsequent applications

will deliver 100% of the initial EVO loading utilizing the same injection well network. No bioaugmentation will be necessary after the first injection. No other operation and maintenance is required with this remedial option. Quarterly monitoring will be performed for the first 5 years, followed by semi-annual monitoring thereafter. Both the quarterly and semi-annual monitoring will include parameters specific to the remedy, such as TOC and other relevant MNA analytes.

11.5.4 EISB Remediation Technology Costs

This section presents the costs for EISB for each of the three template sites.

11.5.4.1 Cost for Case 1: Residual Source Area

The cost analyses for EISB for Case 1 are presented in Table 11.3. A summary of the costs is presented in Table 11.4.

Monitoring costs constitute 45% of the total cost for this remedy, reflecting the need for long-term monitoring after source treatment is completed. This analysis highlights the magnitude of one of the potential benefits of bioaugmentation, which is that it has the potential to reduce the time required to achieve remedial objectives and thereby reduce the duration and cost of the monitoring program. Given the assumption that only one additional injection of electron donor will be required to achieve remedial objectives, the O&M costs in this case are

Table 11.3. EISB Cost for Case 1

Cost Element	EISB Cost ($)
Design	
Pre-design sampling and utility survey	15,000
Laboratory studies	20,000
Groundwater modeling	20,000
In-field hydraulic testing	2,000
Detailed design	10,000
Permitting and reports (including bioaugmentation related)	13,000
Total design	*80,000*
Capital	
Site preparation	2,000
Injection/monitoring well installation and development	23,500
Process equipment	9,000
Materials	12,000
Labor	13,000
Cost for bioaugmentation culture	4,000
Additional cost for injection of culture	4,000
Waste management and disposal	1,000
Reports	5,000
Total capital	*73,500*

(*continued*)

Table 11.3. (continued)

Cost Element	EISB Cost ($)
Operation and Maintenance (per one event)	
Amendment re-addition (per event)	25,000
Reports	2,500
Average annual O&M (average for 5 year)	*5,500*
Annual Monitoring Costs (for first 5 years)	
Annual monitoring labor	5,000
Annual analytical, groundwater	7,000
Annual monitoring reports	10,000
Total monitoring (per year)	*22,000*
Annual Monitoring Costs (for second 5 years)	
Annual monitoring labor	2,500
Annual analytical, groundwater	3,500
Annual monitoring reports	5,000
Total monitoring (per year)	*11,000*

Table 11.4. EISB Cost Summary for Case 1

Cost Element	EISB Cost
Total design costs	$80,000
Total capital costs without bioaugmentation	$65,500
Design and capital cost for bioaugmentation	$11,000
NPV of total O&M costs (assumed for 5 years)	$25,000
NPV of total monitoring costs (assumed 10 years)	$144,000
NPV of total cost (5 year O&M and 10 year monitoring)	$323,000
Bioaugmentation costs as a percentage of NPV total cost	3.4%

quite small (only about 8% of the total cost). The cost to include bioaugmentation as part of the implementation adds only 3.4% to the total cost of the remedy.

11.5.4.2 Cost for Case 2: Shallow Barrier

The cost breakdown for Case 2 is presented in Table 11.5. A summary of the costs is presented in Table 11.6.

In the case of the biobarrier, the higher O&M costs are due to the need for amendment additions every 3 years to provide a continuing supply of food for the dechlorinating bacteria. Reamendment costs are evenly distributed between material costs (primarily EVO) and labor cost associated with the injection process. Monitoring costs still constitute about 45% of the total cost for this remedy reflecting the high cost of long-term monitoring. The cost to include bioaugmentation as part of the implementation adds 1.8% to the total cost of the remedy.

Table 11.5. EISB Cost for Case 2

Cost Element	EISB Cost ($)
Design	
Pre-design sampling and utility survey	15,000
Laboratory studies	20,000
Groundwater modeling	20,000
In-field hydraulic testing	2,000
Detailed design	10,000
Permitting and reports (including bioaugmentation related)	13,000
Total design	*80,000*
Capital	
Site preparation	2,000
Injection/monitoring well installation and development	23,500
Process equipment	9,000
Materials	12,000
Labor	13,000
Cost for bioaugmentation culture	4,000
Additional cost for injection of culture	4,000
Waste management and disposal	1,000
Reports	5,000
Total capital	*73,500*
Operation and Maintenance (per event every 3 years)	
Amendment re-addition	25,000
Reports	2,500
Average annual O&M	*9,000*
Annual Monitoring Costs (for first 5 years)	
Labor	5,000
Analytical, groundwater	7,000
Reports	10,000
Total monitoring (per year)	*22,000*
Annual Monitoring Costs (6–30 years)	
Labor	2,500
Analytical, groundwater	3,500
Reports	5,000
Total monitoring (per year)	*11,000*

11.5.4.3 Cost Analysis for Case 3: Deep Barrier

The cost analyses for the three technologies considered in Case 3 are presented in Table 11.7. A summary of costs is presented in Table 11.8.

As with the biobarrier in Case 2, the higher O&M costs are due to the need for amendment additions every 3 years to provide a continuing supply of growth substrate for the dechlorinating

Table 11.6. EISB Cost Summary for Case 2

Cost Element	EISB Cost
Total design costs	$80,000
Total capital costs without bioaugmentation	$65,500
Design and capital cost for bioaugmentation	$11,000
NPV of total O&M costs (assumed for 30 years)	$176,000
NPV of total monitoring costs (assumed 30 years)	$266,000
NPV of total cost (30 year O&M and monitoring)	$596,000
Bioaugmentation costs as a percentage of NPV total cost	1.8%

Table 11.7. Cost Comparison for Case 3

Cost Element	EISB Cost ($)
Design	
Pre-design sampling and utility survey	15,000
Laboratory studies	20,000
Groundwater modeling	20,000
In-field hydraulic testing	2,000
Detailed design	10,000
Permitting and reports (including bioaugmentation related)	13,000
Total design	*80,000*
Capital	
Site preparation	2,000
Injection/monitoring well installation and development	61,000
Process equipment	9,000
Materials	35,000
Labor	29,000
Cost for bioaugmentation culture	8,000
Additional cost for injection of culture	7,000
Waste management and disposal	1,000
Reports	5,000
Total capital	*157,000*
Operation and Maintenance (per event every 3 years)	
Amendment re-addition (per event)	64,000
Reports	2,500
Average annual O&M	*22,000*
Annual Monitoring Costs (for first 5 years)	
Labor	5,000
Analytical, groundwater	10,000
Reports	10,000
Total monitoring (per year)	*25,000*

(*continued*)

Table 11.7. (continued)

Cost Element	EISB Cost ($)
Annual Monitoring Costs (6–30 years)	
Labor	*2,500*
Analytical, groundwater	*5,000*
Reports	*5,000*
Total monitoring (per year)	*12,500*

Table 11.8. EISB Cost Summary for Case 3

Cost Element	EISB Cost
Total design costs	$80,000
Total capital costs without bioaugmentation	$142,000
Design and capital cost for bioaugmentation	$18,000
NPV of total O&M costs (assumed for 5 years)	$431,000
NPV of total monitoring costs (assumed 10 years)	$302,000
NPV of total cost (5 year O&M and 10 year monitoring)	$970,000
Bioaugmentation costs as a percentage of NPV total cost	1.9%

bacteria. Monitoring costs constitute only about 30% of the total cost for this remedy (versus about 45% for Case 2) since the costs to implement and operate the remedy increase more than the extra cost to monitor a larger system. As with the biobarrier in Case 2, the cost to include bioaugmentation as part of the implementation is relatively small, about 1.9% of the total cost of the remedy.

11.6 SUMMARY

The key potential economic benefits of bioaugmentation are: (1) reduction in the time required to achieve complete dechlorination of chlorinated solvents (or complete degradation of other target compounds), thereby reducing the overall costs for injection of electron donor and reducing groundwater monitoring costs; (2) reduction in regulatory oversight by achieving treatment objectives sooner; (3) reduction in the time required to return the groundwater to beneficial use by achieving treatment goals in a shorter period of time; and (4) ability to apply EISB at sites where this approach would otherwise not be effective and where other more expensive approaches would be required. These benefits will be realized at sites where suitable microorganisms are not present or are present at low initial concentrations. If the remediation objectives for a site dictate that biodegradation needs to be initiated in a short timeframe, bioaugmentation can provide a significantly greater assurance that the objectives will be achieved.

A rough planning level cost estimate for bioaugmentation has been developed based on the information presented in this chapter. Assuming that 1 L of culture is used to bioaugment 35,000 L of groundwater, the culture costs $200/L to purchase and $100/L to inject, and the porosity is 30%, the cost to bioaugment this site would be $2.60/$m^3$ of aquifer. These costs are

typically less than 3% of total lifecycle costs for an EISB remedy and 6–8% of the initial injection costs.

The key factors affecting the incremental cost of bioaugmentation include:

- Whether a separate mobilization is required to bioaugment, or whether bioaugmentation can be done at the same time as electron donor additions;
- Whether complete degradation must be occurring throughout the target treatment volume immediately after bioaugmentation, or whether there is time available for the introduced microbes to grow, disperse and colonize the subsurface;
- Whether EISB is used for source treatment or as a reactive barrier;
- Site conditions that affect organism growth and survival (e.g., pH, ORP or presence of cocontaminants); and
- Any site conditions that impact the ability to deliver the inoculum (e.g., depth, heterogeneity or permeability).

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CHAPTER 12

RESEARCH NEEDS FOR BIOAUGMENTATION

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12.1 INTRODUCTION

Successful bioaugmentation depends on an ability to identify and monitor the organisms, proteins and genes needed for biodegradation, as well as the biomarkers of other organisms upon which they rely or with whom they compete. Innovations in biotechnology are leading to a better understanding of microbial communities and their environments. New technologies such as metagenome sequencing, transcript profiling, shotgun proteomic analysis, metabolic modeling and synthetic biology are allowing researchers to study the important functional and regulatory elements in complex microbial systems. These technologies will enable more accurate predictions of bioremediation outcomes. Such basic scientific understanding will become critical for developing successful bioaugmentation inocula, assessing the need for bioaugmentation at specific sites and obtaining regulatory approval for bioaugmentation in many jurisdictions.

Buoyed by the stunning example of a successful merger of basic science and practical application represented by the *Dehalococcoides* (*Dhc*) story for chlorinated ethene bioaugmentation, researchers are pursuing additional discoveries and developments that will have practical applications for bioremediation. Examining specifically why bioaugmentation can work so well for chlorinated ethenes, but yet has offered little added benefit for petroleum hydrocarbon remediation, will hopefully provide clear direction for future approaches. This chapter describes some of these promising research directions and identifies some of the current research needs in basic science at various scales. Some specific examples illustrating research needs directly targeted to extending the application of bioaugmentation to broader environments and new contaminants are described.

12.2 RESEARCH NEEDS IN BASIC SCIENCE

As the previous chapters have shown, several different bioaugmentation approaches can be successful. One lesson from these approaches is that the better the understanding of the microorganisms at the heart of the process, the more effective the implementation will be. There are four scales at which basic science is critical to a better understanding of bioaugmentation: (1) at the molecular scale, particularly at the levels of gene regulation and the enzymatic mechanism for dechlorination; (2) at the organismal scale, such as in the detailed understanding of an isolated culture; (3) at the community scale, understanding microbial community dynamics and interspecies connections; and (4) at the environment, ecosystem or field scale, where community dynamics interact with the physical and chemical environment and issues of partitioning and transport come into play.

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12.2.1 Molecular Scale

On a molecular scale, metabolites and metabolic pathways, enzymes, and their activity and regulation can be stitched together to create a model of an organism's life cycle. From this model, some predictions of how to improve or change the organism's activity can be made. For specific reactions, researchers attempt to identify proteins of interest from deoxyribonucleic acid (DNA) information and subsequently work to characterize the proteins' activities. In the field of bioremediation, particular attention has focused on the biodegradative enzymes responsible for contaminant transformation. These are typically classified as dehalogenases or oxygenases: families of proteins responsible for the degradation of chlorinated compounds.

In the case of anaerobic reductive dechlorination, current understanding of the biochemical mechanism at the enzyme level is severely behind what is known for other catalytic enzymes, such as the aerobic hydrolytic dehalogenases (Chan et al., 2010) or the oxygenases (Arora et al., 2009; Nebe et al., 2009). Many gene sequences of predicted reductive dehalogenases have been identified, but this has not led to an understanding of how these enzymes break the carbon-halogen bond. Considerable effort has been directed at characterizing these enzymes (Maillard et al., 2003; Van De Pas et al., 1999), but of the over 280 putative reductive dehalogenase genes known, only 12 have been biochemically characterized or been identified though molecular biology techniques (Adrian et al., 2007; Bisaillon et al., 2010; Cheng and He, 2009; Grostern and Edwards, 2009; Krajmalnik-Brown et al., 2004; Krasotkina et al., 2001; Magnuson et al., 1998; Maillard et al., 2003; Marzorati et al., 2007; Miller et al., 1998; Muller et al., 2004; Nakamura et al., 2006; Tsukagoshi et al., 2006).

Reductive dehalogenases are a challenge to purify from cultures containing dechlorinating organisms. These strictly anaerobic organisms do not grow to high cell density, and the reductive dehalogenases are membrane-associated and oxygen-sensitive, so yields of purified protein are very low or of an inactivated form, and are thus not adequate for many subsequent biochemical analyses. Despite these limitations, enzyme assays on crude extracts and partially purified proteins can yield valuable insights into the kinetics (Adrian et al., 2007; Grostern et al., 2009) or the activities of individual enzymes (Adrian et al., 2007).

An alternative to *de novo* purification is heterologous expression – i.e., placing the gene for the enzyme in another "tamed" organism (such as *E. coli*) and instructing this organism to make large amounts of the protein. To date, this approach has not led to expression of a functional reductive dehalogenase, but eventually this problem will be solved. Once this is achieved, researchers will be able to synthesize every predicted reductive dehalogenase enzyme, test substrate ranges and kinetics, specifically identify all steps in the dechlorination reaction, and optimize the enzymes for new halogenated contaminants by modifying key amino acids in the active site. Mutant studies with aerobic (hydrolytic) dehalogenases have enabled construction of dehalogenases with increased efficiency (Bosma et al., 2002) and led to the identification of defluorinases (Chan et al., 2010).

In the absence of a purified protein product, a variety of both traditional biochemical and larger-scale transcriptomic/proteomic studies can be used to identify important proteins in contaminant-degrading organisms, particularly those whose genomes have been sequenced. For example, transcriptomic and proteomic studies that examine the complete profile of gene or protein expression at the organismal level have been of some help in identifying substrate ranges for some of the reductive dehalogenases (Johnson et al., 2008; Morris et al., 2007; Rahm and Richardson, 2008). Most recently, substrates for dehalogenases have been identified using non-denaturing polyacrylamide gel electrophoresis to first partially purify enzymes from crude cell extracts from mixed or pure cultures. Partially-purified enzymes in gel slices are assayed for dehalogenating activity, and associated proteins identified using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) (Adrian et al., 2007). This approach is being applied

to a variety of cultures and will assist in assigning function to many dehalogenases only known from a gene sequence.

Efforts also are currently directed at elucidating all proteins in the electron transport chain (e.g., hydrogenases, cytochromes, ferredoxin, or other electron carriers), the mechanism of reductive dehalogenation, its regulation and the specific conditions under which the different dehalogenases are recruited. Similar interests exist for understanding the oxygenases and other enzymes involved in aerobic mechanisms of dechlorination. The ever-increasing availability of genomic data provides a mine for identifying genes encoding undiscovered biocatalysts for biotransformation reactions. However, much work remains to fully characterize many of the poorly annotated genes and proteins whose activities are correlated with biotransformation.

Research Needs: Basic Science – Molecular Scale

Mechanistic understanding of the reactions that break down pollutants in groundwater.

- Identification of substrates and molecular mechanisms of enzyme-catalyzed contaminant transformation reactions in biodegrading microbial communities.

12.2.2 Organismal Scale

A variety of aerobic and anaerobic bacteria capable of contaminant transformation have been isolated in pure culture. Pure cultures derived from a single cell (i.e., clones) provide a clean system in which to test hypotheses and understand the specific growth requirements of an organism. Unfortunately, cultivated strains drastically under-represent the microbial and functional diversity in the environment, in microcosms, enrichment cultures, and bioaugmentation cultures. Novel cultivation techniques are needed not only to increase the number of isolated strains, but also to study defined microbial communities in action. Certainly genome and metagenome sequences and genome-wide assays (microarrays and shotgun proteomics) have been very useful in helping to identify gene and protein responses to specific stresses and growth conditions (N'guessan et al., 2010; Nicolau et al., 2009; Selesi et al., 2010; Zhou, 2003).

Better understanding of an organism's nutrient requirements through identification of up-regulated transport and synthesis systems may provide clues to better cultivate these organisms, with the potential for increased yields and contaminant degradation rates. In particular, genome-scale mathematical models of microbial metabolism and regulatory networks, which provide a framework onto which to anchor disparate "omic" data, are a particularly powerful tool for extracting useful information from studies of uncultivated organisms (Lee et al., 2006; Zhao et al., 2010).

Despite the advantages of the novel "omic" technologies that can examine a mixed bioaugmentation culture as a whole, highly enriched or purified strains continue to be the basic unit for building the tree of life, and understanding cellular evolution. A specific example of the benefits of prospecting for and isolating new organisms is provided by the search for *Chloroflexi* (Section 2.2.1). Genome-enabled models of microorganisms and their impact are described in Section 2.2.2. Both of these approaches have the potential to impact bioremediation and bioaugmentation strategies.

12.2.2.1 Expanding the Dechlorinators: Novel Chloroflexi and Beyond

The discovery of *Dehalococcoides* strain 195 as a dechlorinating organism (Freedman and Gossett, 1989; Maymó-Gatell et al., 1997) marked the beginning of enquiry into this group as potential bioremediation tools. Members of the *Dehalococcoides* group have been

identified at many geographically-diverse contaminated and uncontaminated sites, and have been successfully enriched from soil microcosms to liquid culture in several laboratories. Several isolates of *Dehalococcoides* spp. have been obtained, and complete genome sequences are currently available for five strains (strains 195, CBDB1, BAV1, VS, and GT) (Kube et al., 2005; McMurdie et al., 2009; Seshadri et al., 2005; Frank Löffler 2009, University of Tennessee, personal communication). Metagenome sequences of *Dehalococcoides*-containing cultures are available for three cultures: DonnaII, KB-1©, and ANAS (publicly available through the Joint Genomes Institute) (Duhamel et al., 2004; Fennell et al., 1997; West et al., 2008).

The depth of information available for the *Dehalococcoides*, while still incomplete, represents an abnormally high level of characterization compared to the rest of the bacterial phylum Chloroflexi, of which *Dehalococcoides* represents a relatively deep-branching group (Figure 12.1). The 16S ribosomal ribonucleic acid (rRNA) gene diversity within the *Dehalococcoides* ranges from 98% to 100% nucleotide identity, indicating that all currently isolated strains are extremely closely related evolutionarily despite their relatively diverse substrate profiles. The ubiquity of the *Dehalococcoides* as dechlorinating organisms identified at contaminated sites and the close genomic conservation seen within the group may be evidence of an extremely restricted group filling a specific niche, or may be an artifact of shared culture and isolation techniques

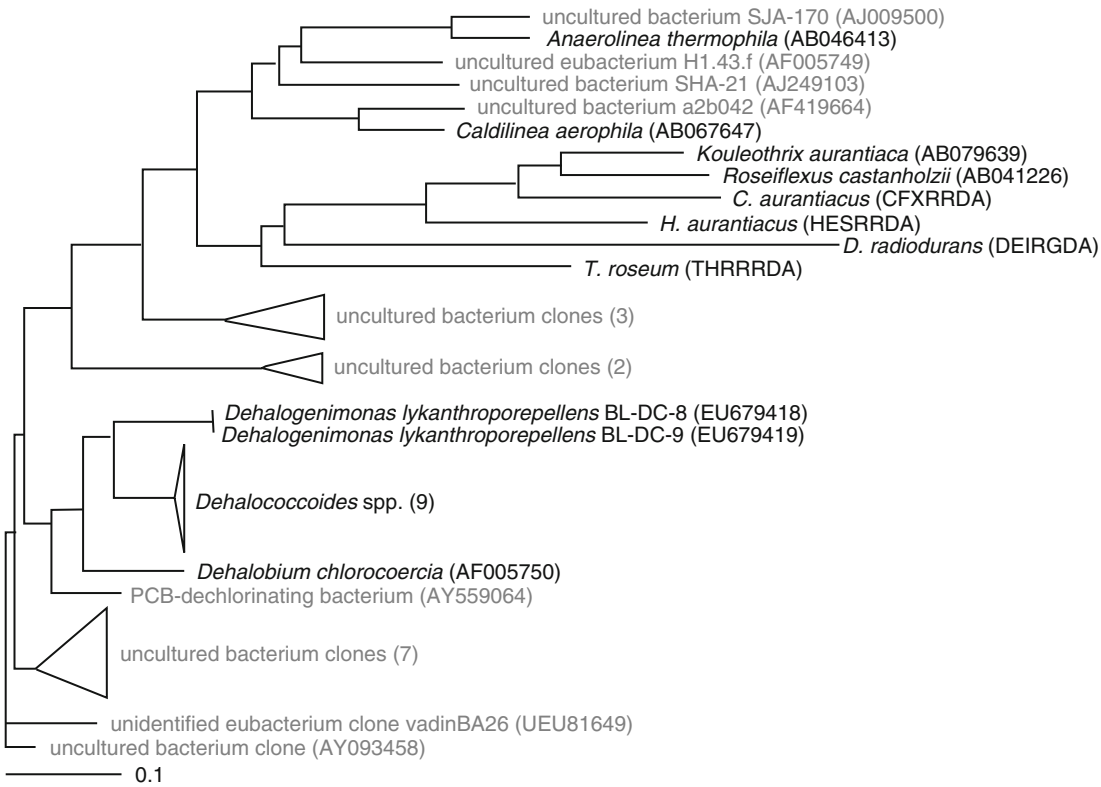

Figure 12.1. Maximum likelihood phylogeny of the known 16S rRNA diversity within the phylum Chloroflexi, including representative environmental clones. Cultured strains are labeled in black while environmental clones are in grey. Species sharing high sequence similarity (>95%) have been condensed into wedges, where the depth of the wedge represents the average length of the condensed branches. Numbers after the wedge labels indicate the number of branches condensed.

between laboratories working with bioaugmentation. It is unclear if the low diversity within the *Dehalococcoides* is indicative of the level of diversity to be expected within the Chloroflexi.

Recently, the bioremediation field has been actively searching for other, non-*Dehalococcoides* organisms with the potential to contribute to bioaugmentation efforts. Much of this effort has been focused on expanding current knowledge of the Chloroflexi, including searching for near-neighbors to the *Dehalococcoides*. In 2008, a pair of Chloroflexi bacteria capable of degrading 1,2,3-trichloropropane was isolated from a contaminated site in Louisiana (Moe et al., 2009; Yan et al., 2009). The 16S ribosomal DNA (rDNA) genes for these isolates share only 90% nucleotide identity with *Dehalococcoides*, indicating that they are a new lineage within the Chloroflexi. This lineage has been named *Dehalogenimonas lykanthroporepellens* (Moe et al., 2009), and represents the first discovery of a non-*Dehalococcoides* Chloroflexi capable of dechlorination.

The identification of closely related Chloroflexi to the *Dehalococcoides* group is exciting, as it indicates that further expansion of the known Chloroflexi is likely to yield industrially relevant organisms with the potential to address recalcitrant contaminants. This has already been borne out, as a novel Dehalogenimonas was recently identified that dechlorinates trans-DCE (Manchester et al., 2012). Additionally, the added diversity that the *Dehalogenimonas* provide will allow for deeper examination of *Dehalococcoides* genome evolution, including examination of reductive dehalogenase movement and inheritance within and between genomes. This could shed light on the mechanisms for acquisition of reductive dechlorination in these bacteria, a current mystery.

A recently announced research effort in Germany seeks to discover and characterize novel Chloroflexi from marine systems (Adrian, 2009). While it is difficult to predict the outcome of a search for unknown organisms from uncharacterized environments utilizing novel metabolic pathways, this style of large-scale environmental screening has the potential to yield numerous interesting and novel organisms. Efforts of this nature will need to utilize many different culture conditions in order to successfully enrich for unknown organisms. The future of bioaugmentation lies in the discovery of novel biodegradation pathways, which will only be found through this style of broad-scale search with varied culture conditions.

Outside of the Chloroflexi, several organisms capable of dehalogenation of chlorinated substrates are known. *Dehalobacter* (*Dhb*) species are members of the Clostridia (Holliger et al., 1998), while *Geobacter* and *Shewanella* belong to the delta- and gamma-Proteobacteria, respectively (Lovley et al., 1993; Macdonell and Colwell, 1985). While these phyla are significantly better characterized than the Chloroflexi, there still exists the potential for expansion of the known species within these groups, including identification of organisms relevant to bioaugmentation. The advantage to discovering novel organisms within better-characterized phyla lies in the potential for generating a genetic system for examination of dehalogenation and other processes more directly. Moreover, genes with high similarity to known dehalogenases have been identified in the genomes of organisms not known for this activity, including *Mesotoga* and even in the Archeaon *Ferroglobus* (Dr. C. Nesbo, personal communication, 2010; Hafenbradl et al., 1996). While the substrates for these putative dehalogenases have not been identified, these findings suggest that dehalogenating activity is more widely distributed than previously thought.

12.2.2.2 Genome Scale Reconstructions and Mathematical Models of Microbial Metabolism

With the growing number of completely sequenced microbial genomes, interest has grown in developing computational tools that use this genome-scale information to reconstruct metabolic pathways and develop models of microbial growth and activity. Constraint-based models

describe a biological system by a set of constraints that can be described mathematically, and the set of possible solutions define the range of valid states and behaviors of the biological system (Lee et al., 2006). These constraints can be physicochemical (e.g., physical laws of conservation of mass and energy), topological (e.g., spatial restrictions on metabolites within cellular compartments), and environmental (e.g., nutrient availability and pH).

Some models attempt to predict fluxes between different metabolic pathways, both to understand the microbial physiology and to predict growth yields and metabolite usage within the cell. Flux balance analysis is specifically concerned with determining a set of steady-state fluxes that optimize a specific parameter (e.g., maximizing biomass production). Once the system is defined, optimization techniques may be applied to evaluate the performance of the biological system to perturbation(s) using computer simulations. The resulting sets of simulated flux data can be compared with experimental data and ultimately the collection of possible fluxes can be used to predict the response of large-scale biochemical networks exposed to different conditions (Lee et al., 2006).

This constraint-based modeling approach can accurately predict microbial growth under a variety of environmental conditions (Edwards et al., 2001; Feist et al., 2009; Ibarra et al., 2002; Mahadevan et al., 2006; Oh et al., 2007). This approach may be particularly well-suited to modeling microorganisms in heterogeneous environments because it does not assume constant yield coefficients and has been shown to account for the changes in the metabolic network in response to nutrient limitations (Schuetz et al., 2007; Varma et al., 1993).

The potential of metabolic modeling for understanding and predicting microbial physiology is evident by the rapidly expanding database of models (~40) for a diversity of microorganisms. However, apart from a few notable exceptions, most of the research efforts have focused on well-studied model organisms, motivating the need for expanding these modeling approaches to non-model organisms and microbial communities. For example, the Department of Energy (DOE) has recently initiated such a modeling effort, focused on microbial processes relevant to the missions in bioenergy generation (Rittmann et al., 2008), carbon cycling and bioremediation (Ahsanul Islam et al., 2010; Scheibe et al., 2009; Yu et al., 2010). Finally, most of the models are based on metabolic networks reconstructed from pure cultures, further highlighting the need for network reconstruction and modeling methods from metagenome sequences.

Research Needs: Basic Science – Organismal Scale

Enrich for and cultivate new organisms and newly-defined communities of organisms, on different contaminants and under different conditions.

- Refer to Section 12.3.4 on The Enrichment Paradox.

Develop new strategies for cultivating microbes from the subsurface.

- Focus on both isolated organisms and defined communities.

Better understand the evolution of these organisms and the origins of the activity (e.g., dechlorination).

- How were these traits acquired and why?
- What are the natural substrates and niches for these organisms?

Apply genome scale models of metabolism and regulation to enhance microbial growth.

- Take advantage of the large available datasets of genomic information.
- Models should be applied to real systems, not model organisms.

12.2.3 Community Scale

A better understanding of the factors that affect growth and activity of microorganisms in their environment requires insight into the relationships between different microbes in a community. This insight is critical for defining input parameters for organism- or genome-scale modeling as described earlier. It also is required to understand the contribution of ancillary microbes to the primary contaminant degraders, such as the contribution of non-dechlorinators to promoting dechlorination rates.

It is commonly observed that mixed cultures and consortia exhibit faster dechlorination than pure cultures of dechlorinating organisms. This difference is likely due to the ability of other microbes to provide nutrients (e.g., vitamin B12, acetate) and secondary metabolites to the dechlorinators (Heimann et al., 2006; Johnson et al., 2009; West et al., 2008). On a physical environment level, these associated organisms help control the hydrogen partial pressure, which provides the reducing environment required for dechlorination reactions to be energetically favored. Associated organisms can potentially contribute a competitive advantage to bioaugmentation inocula by controlling the concentrations of metabolites and other required factors.

Understanding the complete metabolic potential of a bacterial community also can inform the choice of electron donor at a site. For example, the donor provided during bioaugmentation may be converted to a less tractable but higher efficiency donor through interspecies nutrient transfer, which will influence community structure and dechlorination.

Understanding the interactions of microorganisms within a community is critical for predicting the competitive advantage of an introduced community over indigenous populations. With increased knowledge of the interactions within a bioaugmentation culture, researchers can apply metabolic modeling on a community scale to describe the potential effects of different site conditions. However, gathering the raw data to inform a community-scale model is a large undertaking (Yu et al., 2010), and represents a current bottleneck in this area.

Research Needs: Basic Science – Community Scale

Develop clever tools to identify interspecies metabolites and signals in communities of microbes.

Apply more systematic analyses of defined consortia.

Understand the ecological basis of competition.

- Refer to the Enrichment Paradox below.

Apply genome scale models of metabolism to communities of organisms.

12.2.4 Environmental/Ecosystem and Earth Scale

Finally, interaction between microbial communities and their environment at a larger scale is a critical consideration for successful bioaugmentation. Communities not only need to contain the appropriate catabolic capabilities, but they also need to have the ability to adapt

to the water activity, pH, temperature, cocontaminants and other physicochemical conditions of the environment to which they will be introduced (Cameron et al., 2010; Kristensen et al., 2010; Schaefer et al., 2010; Schuetz et al., 2007). Microbes also disburse across environments, and the longer-range transport of microbes and their catabolic activity, including their genes, has implications for the long-term fate of contaminants and the impact and regulation of introduced bioaugmentation cultures. The ability of many microbial communities to form aggregates on particles and in biofilms has implications for their ability to utilize sorbed contaminants (as compared to the limitations of free-living microbes) and also is a critical consideration for transport (short- and long-range) and survival of the introduced microbes, and by extension the fate of the target contaminants.

Microbial interactions with solid surfaces are an influencing factor in interspecies nutrient transfer and microbial survival. From a contaminant perspective, both abiotic chemical transformations and biotic metabolic and cometabolic transformations need to be included when developing a mechanistic understanding of the ultimate fate of contaminants. Combined remedies that take optimal advantage of physical, chemical and biological attenuation mechanisms for a given contaminant suite are very much needed.

A major challenge to this work is the inherent heterogeneity in natural environments. This is a very difficult challenge, as each point in space will offer a different environment with different concentrations of donors, acceptors, other nutrients and inhibitors. A microbial consortium must be robust to the full range of conditions present within a site. Therefore, perhaps the most important determinant of success is the degree to which the site under remediation is hydrogeologically and chemically characterized.

The single most difficult problem to overcome at full scale is mixing. Thus, a major research need is developing clever and cost effective ways to bring the microbial community, contaminant and electron donor together. Part of this effort requires an improved mechanistic understanding and better models of microbial transport, growth and dechlorination, particularly models that incorporate the complexity of electron donor fermentation and dechlorination in competition with methanogenesis and other terminal electron accepting processes (TEAPs) in the context of a flowing multiphase system. In addition, such models should consider combined and integrated remedies involving chemical or physical treatment, as well as biological treatment.

To continue improving bioremediation, fully instrumented field sites, such as those at Bemidji (Minnesota, USA), Moffett Federal Airfield (California, USA), Canadian Forces Base (CFB) Borden (Canada), and others have been and will continue to be essential to developing practical tools that capture the important rate-determining processes at real sites. Only by working at a field scale, while integrating knowledge from all scales (from molecular to ecosystem), can practical solutions be discovered. How complex subsurface ecosystems behave was a major focus of a DOE Office of Biological and Environmental Research (BER) workshop in August, 2009 (DOE/SC-0123, March 2010; www.science.gov/ober/BER_workshops.html). The workshop report identified three major research gaps: (1) research approaches must embrace a hybrid of bottom-up reductionism with top-down complexity; (2) such hybrid research efforts are needed on relevant field sites with iterative experimental and modeling activities; and (3) novel complex system science approaches are needed.

Research Needs: Basic Science – Ecosystem Scale

Understand the distribution of these organisms around the globe.

- Where did they come from?
- How do microbes and their catalytic activities spread to contaminated sites?

Need to develop practical combined remedies.

- Chemical/Biological
- Thermal/Biological

Need fully-instrumented field sites.

- Similar to CFB Borden, Moffett Federal Airfield, and other sites.
- Allow testing of hypotheses at field scale.

12.3 KEY CONCEPTS FOR BIOAUGMENTATION RESEARCH

This section will consider some fundamental concepts for future bioaugmentation research and development. Three concepts are discussed: the ecological niche, the value of microcosms and the enrichment paradox. The thesis is that an improved understanding of these concepts will foster innovation and lead to breakthroughs in the application of bioaugmentation.

12.3.1 The Niche Concept and Its Importance for Bioaugmentation

The word "niche" is derived from the Middle French word *nicher*, meaning *to nest*. The full range of environmental conditions (biological and physical) under which any organism can exist describes its fundamental niche. Ecologists further refine this definition considering the effects of resource availability, competition and predation to define a realized niche that is narrower than an organism's fundamental niche. Natural selection drives competing species into different patterns of resource use, in other words into different niches. This process allows two species to partition certain resources so that one species does not outcompete the other. The result of prolonged competition and natural selection has created very narrowly defined niches for some organisms. *Dehalococcoides* is a case in point. The following section explores what this concept means for bioaugmentation.

12.3.1.1 Anaerobic Dehalogenation and *Dehalococcoides*' Niche

Physiological and genomic analyses have confirmed that the *Dehalococcoides* niche is similar to that of methanogenic Archaea from an electron donor and oxidation-reduction potential (redox) perspective. Specifically, like most methanogens, *Dehalococcoides* have an obligate requirement for highly reduced conditions, and require hydrogen as an electron donor. Hydrogen is provided in nature by fermentation of a broad range of substrates, and therefore is not a significant limitation in the ecological sense. However, from the electron acceptor perspective, *Dehalococcoides* are highly adapted to a very specific niche, as they can only use halogenated organic substrates as terminal electron acceptors. This degree of specialization is remarkable, and was quite unexpected. Now it seems probable that other dechlorinating organisms, including *Dehalobacter* and *Dehalogenimonas,* may have similarly restricted metabolisms.

"Why" this is so is a question for fundamental research, but the result of this specialization has profound implications for bioaugmentation. Most obvious is that these organisms grow specifically on the substrates that are contaminants of interest for bioaugmentation. Moreover, because organisms that dechlorinate typically cannot do anything else to gain metabolic energy, they have few competitors and their presence is strongly indicative of dehalogenation. This linkage between presence and activity is highly useful for tracking bioaugmentation inocula, and for assessing sites for potential dechlorination, because detectable levels of *Dehalococcoides* DNA can be considered reliable evidence that dechlorination is occurring. The only competition of concern within a dechlorination system is the potential for competition between dehalogenators for substrates. It is thus important to select for strains of organisms known to catalyze the dechlorination reaction to a nontoxic end product. Dechlorinating organisms do have to compete for electron donor (hydrogen) and other nutrients, but easily have the advantage when there is ample halogenated organic present.

It is clear now that one of the major limitations to many early bioaugmentation schemes was the inability of introduced organisms to survive and compete, and the challenge of promoting the expression of contaminant-degrading activity under *in situ* conditions. This is particularly true for most organisms that degrade petroleum hydrocarbons. Such organisms can typically metabolize a wide variety of carbon-containing substrates and will only degrade contaminants as a last resort. Thus when introduced into a contaminated site, the desired degradation of the contaminants often is not seen. The petroleum hydrocarbon situation is further complicated by the fact that there are multiple compounds with different functionalities (alkanes, alkenes, arenes and combinations thereof), and it is difficult to generate an enrichment culture capable of complete breakdown of the multitude of compounds present at a petroleum-contaminated site. These observations have led to a proposed optimal scenario for bioaugmentation (below).

Optimal Scenario for Successful Bioaugmentation

An optimal scenario for efficient and successful bioaugmentation is to create an environment that can be populated with organisms (native or introduced) whose existence in that niche *solely* depends on the transformation of the contaminants(s) of interest.

The obligate dehalogenating lifestyle of *Dehalococcoides* is the most clear-cut example of a system that meets this requirement. Moreover, each strain of *Dehalococcoides* harbors a selective suite of enzymes that distinguish them and further narrow their niche. Successful bioaugmentation relies on creating an environment where reducing conditions (i.e., hydrogen), the contaminant of interest, and organism(s) who are obligate degraders of the contaminant of interest are co-located. Let us investigate if this optimal scenario holds for other examples of successful bioaugmentation.

12.3.1.2 *Pseudomonas stuzeri* KC and Cometabolic Transformations

In the case of *Pseudomonas stuzeri* strain KC (see Chapter 9) (Dybas et al., 1995; Essen et al., 2007; Zawadzka et al., 2006), the environment has to be changed to create a niche for the introduced organism. In a high-pH environment, most organisms become severely iron-limited. In strain KC, a higher pH triggers the production of an iron-scavenging siderophore that fortuitously dechlorinates carbon tetrachloride (CT) rapidly. At high pH, and in combination with a specific set of donor and acceptor (acetate/nitrate), strain KC's existence is solely dependent on the production of the CT-degrading siderophore.

Thus, bioaugmentation with strain KC at high pH and acetate/nitrate conditions would appear to be a case that fits the above optimal scenario. However, this bioaugmentation strategy fails when other acetate/nitrate-consuming organisms with a different siderophore for scavenging iron at high pH displace strain KC. Thus, while the existence of strain KC in this niche solely depends on the production of the siderophore, its ability to dechlorinate is not part of its selective advantage in this case. Since the contaminant-transforming organism may be outcompeted, even within its selective niche, bioaugmentation with strain KC is more difficult and less predictable than bioaugmentation with *Dehalococcoides*.

In general, cometabolic transformations of all kinds are less efficient precisely because of the lack of feedback between the contaminant transformation process and microbial growth, and the competitive advantage that this feedback would provide to the bioaugmented organism(s). Thus, while cometabolic bioaugmentation schemes can be effective, it is a short-term effect, until the ecosystem adapts to the change in state brought about by the addition of donors and acceptors that are required to create the niche for the desired organism. An optimal strategy therefore would be to select for organisms that can gain a selective advantage when the contaminant is transformed, even in a cometabolic situation.

This is not to say that cometabolic transformations should be discounted. These transformations have a very important role to play in cases where the contaminant concentrations are too low to support the growth of organisms (Nalinakumari et al., 2010; Sharp et al., 2005), or when there are diverse mixtures of contaminants and the transformation of some can be cometabolic. Nonetheless, a bioaugmentation strategy should rely on finding that combination of niche and organism(s) that provides the greatest selective advantage to the desired process.

In the case of metabolic transformations, there is the opportunity for long-term inoculation of an ecosystem with sustainable contaminant transformation ability, provided the niche still exists. It is clear that biological processes often continue at sites long after the active remedial stage, and that they play an important role in dampening rebound after more aggressive remedial efforts (chemical and thermal treatments, for example) (McGuire et al., 2006). This extended treatment reflects the ability to establish a metabolic transformation activity for long periods of time in an ecosystem where it was not previously found.

12.3.2 Hydrocarbons and Other Reduced Contaminants

Many bioremediation efforts have focused on petroleum hydrocarbons, and with just cause – these are ubiquitous pollutants and many microbes can degrade them, under a wide variety of conditions. This fact would suggest that bioaugmentation should be easy. But these same features make bioaugmentation – specifically to accelerate remediation or to target a particular constituent of petroleum – a difficult challenge.

For example, benzene is a major toxic constituent of petroleum, and a frequent driver at contaminated sites. Can one imagine an environment that can be created in the subsurface and populated with organisms whose existence in that niche ***solely*** depends on benzene transformation? The answer, with the current state of knowledge, is no. All known benzene-degrading organisms, regardless of whether they are aerobic or anaerobic respirers, also use other substrates – derivatives of natural organic matter (sugars, amino acids, fatty acids) and other petroleum hydrocarbons – and do not solely rely on benzene for growth. Moreover, the niche for hydrocarbon degraders overlaps with so many other organisms that metabolic diversity is their key strategy for survival.

Due to this metabolic flexibility, creating sufficient selective pressure to result in specifically enhanced biodegradation of a particular petroleum contaminant has proven to be very difficult. Instead, the approach has been to stimulate all microbial activity, in the hopes of also accelerating benzene degradation. This approach works, though it is highly non-specific.

In this context, it is clear why bioaugmentation has not worked, as there is little selective advantage for the introduced organism. Significant breakthroughs in increasing the specificity of hydrocarbon degradation will arise from identifying novel organisms with obligate hydrocarbon-degrading activity and novel niches for such activity, or in engineering a strain to have a competitive advantage in a niche that can be created *in situ*.

Recently, the genome sequence of a hydrocarbon-degrading marine bacterium, *Alcanivorax borkumensis*, has revealed that this organism is highly adapted to utilizing *only* oil hydrocarbons (alkanes), and does not possess the genes for using sugars or amino acids as carbon substrates (Schneiker et al., 2006). The obligate-hydrocarbon consuming nature of the organism, along with a variety of other niche-specific genes (including genes conferring the ability to scavenge for nitrogen, to form biofilms at the oil–water interface and to produce biosurfactants), gives this strain a competitive edge in oil-polluted environments and points to design strategies for oil spill remediation. This particular organism and the associated niche (marine oil spills) appear to provide the optimal scenario for bioaugmentation. Not surprisingly, *Alcanivorax borkumensis* is found enriched in marine oil spills around the globe (Schneiker et al., 2006), much as *Dehalococcoides* is found enriched in chlorinated solvent sites around the globe. Similarly, specific hydrocarbon-degrading microbes, related to psychrophilic *Oleispira* have been found enriched in the Deepwater Horizon blowout in the Gulf of Mexico (Hazen et al., 2010).

Are biostimulation and bioaugmentation approaches likely to be more successful when the contaminant is the electron acceptor, rather than the electron donor in an organism's metabolism? There is a notion that the diversity of electron acceptors (electron poor compounds) used by microbes may be lower than the diversity of electron-donating (electron rich) substrates. For example, in considering microbial ecosystems, it is common to describe various redox zones on the basis of the predominant TEAP, ranging from oxygen, through nitrate, nitrite, iron, manganese, sulfate and finally to carbon dioxide (CO_2). Certainly other electron acceptors are known, including common contaminants such as halogenated organics, perchlorate and uranium. However, given the widely different energetic requirements associated with the reduction of these electron acceptors, each particular organism typically has evolved to use a relatively limited set of acceptors. Conversely, electron donors, at least the most common carbon-based donors, all tend to feed rapidly into central metabolism, and thus, a given organism can easily harbor the potential to degrade a myriad of different organic compounds, with relatively little energetic or evolutionary penalty.

Based on this apparently greater microbial diversity in anaerobic environments, researchers seeking obligate contaminant-degrading organisms to create conditions for optimum bioaugmentation may have more luck when the contaminant is an electron acceptor. There may be fewer alternatives for the microorganisms, and thus it may be easier to create an environment where the desired electron acceptor (the contaminant) is a specific organism's only option.

Niche Concept and Necessity for Successful Bioaugmentation

The goal is to create conditions that are selective for the desired contaminant-degrading activity *in situ*. Experience suggests that this goal is easier to achieve if:

- The contaminant degradation is linked to microbial growth (i.e., metabolic)
- The microbes are obligate contaminant-degraders
- The contaminants are electron acceptors (because there are fewer alternatives, compared to electron donors)
- The site is contaminated only by a narrow range of pollutants.

12.3.3 The Much-Maligned Microcosm and the Need for Activity-Based Tests

A microcosm is a small, representative system serving as an analogy to a larger system in constitution, configuration or development. The word is derived originally from Greek, meaning "little world" (*micros kosmos*).

Microcosms are frequently used to represent or mimic the subsurface environment and the interplay of physical, chemical and microbial activity within. Their design ranges from very simple batch microcosms, consisting of subsurface material and groundwater in a sealed bottle, to more complex flowing columns and increasingly larger tank systems (Da Silva et al., 2006; Reeves et al., 1999). Microcosms can be designed to mimic as closely as possible the *in situ* environmental conditions or can be used to explore the effects of defined perturbations, such as the addition of specific nutrients, donors or acceptors, or the modification of parameters such as pH, redox or temperature.

The conditions imposed on the microcosm will define the niche and therefore which microbes grow, and careful planning of the imposed enrichment conditions is critical. Microcosm studies have been the source of practically all contaminant-degrading enrichment cultures and isolates. From the perspective of subsurface remediation, microcosms prepared with material from long time, historically-impacted environments have been the most successful. In such environments, the native microbes have been chronically exposed to the contaminants, and have had a much longer time to adapt to prevailing contaminants. Microcosms and the subsequent enrichment cultures derived from them continue to be a wonderful resource for discovering novel microbial activities.

The microcosm study, particularly the relatively rapid and easily parallelizable batch microcosm study, has long been a powerful diagnostic and decision tool in bioremediation. But recently, with the advent of molecular biological tools (MBTs) (MBTs – DNA based detection) to detect biomarkers correlated with activity, the use of the microcosm has been sidelined. The attraction to MBTs is clear. The analyses are easily done on extracted groundwater, as with other chemical analyses, and turnaround times can be less than a few weeks. Costs are comparable to chemical analyses too.

However, MBTs are only as good as what you know, as they target only known organisms and genes. Moreover, DNA-based assays, which are by far the most common owing to their ease, do not provide quantitative data on rates. RNA-based methods are problematic as well, as it is difficult to correlate transcript levels with expression and rate. Comparatively, activity-based assays, such as the microcosm, or derivatives thereof, provide valuable kinetic information without the need for any *a priori* knowledge of the system.

The lack of a requirement for such *a priori* information is one of several advantages microcosm studies have over the newer MBT techniques. Specifically, microcosms provide valuable information for determining:

- Which contaminants at a site can be degraded at all
- The products of degradation
- Relative rates – or rates normalized to some total biomass estimation
- Matrix effects of the subsurface on degradation
- Cocontaminant effects
- If bioaugmentation is needed
- If inhibitors are present
- Which electron donors, nutrients, and electron acceptors are most effective
- What conditions can be changed to enhance activity (e.g., pH, temperature, and redox).

To date, few MBTs can address these confounding factors directly. In fact, a combination of MBTs and microcosms is the most powerful diagnostic choice, and is an ongoing area of research to further validate cheaper molecular (i.e., MBT) analyses. A related research need and a worthwhile goal is to develop a mechanism to carry out a rapid activity-based assay for a given process, e.g., dechlorination of vinyl chloride (VC). A simple system, where one could take a groundwater sample, add a reagent and measure color formation with time, or a similarly straightforward assay, would allow determination of VC dechlorination rate normalized to a total biomass or protein measurement.

An assay connecting dechlorination rates directly to biomass would be a powerful tool. Such measurements are difficult given the typically low biomass concentrations in groundwater, but should be possible with sensitive detection chemistry. Such assays are similar to common measurement techniques used in marine and freshwater sediment ecosystems, where assays to measure methane production rates, sulfate-reduction rates and CO_2 fixation rates are commonplace. Such rates also would be highly useful input for groundwater and transport models, increasing the informative nature of this hypothetical assay.

Microcosms and the Need for Activity-Based Tests

Microcosm studies and other direct measurements of microbial activity are the best and most definitive way to understand complex processes at a given site.

- More time-consuming but more informative than simple measurements of chemical biological markers.

Activity-driven assays do not rely on pre-existing knowledge of microbial identity.

Microcosms are essential to enriching novel microbes.

Microcosms can more readily detect combined abiotic and biotic processes, cometabolism, presence of inhibitors, and substrate interactions and interferences.

12.3.4 The Enrichment Paradox

When setting up microcosms and subsequent enrichment cultures to explore the biodegradation of a certain contaminant of interest, a researcher is guided by the reductionist principle, to simplify the system to its essential components. Multiple transfers of enrichment culture into defined medium are designed to weed out non-essential organisms while maintaining targeted activity, often while striving for an "isolate" of the organism of interest. However, such selection techniques, while leading to decreased culture complexity, often also lead to decreased degradative capacity and decreased culture robustness. The latter are critical components of effective bioaugmentation cultures, and thus bioaugmentation and basic research needs (i.e., enrichment) can be at odds in this regard.

As an example, the dechlorination of 1,1,2-trichloroethane (TCA) requires at minimum two distinct dechlorinating organisms, a *Dehalobacter* that dihaloeliminates 1,1,2-TCA to VC, and a *Dehalococcoides* that dechlorinates VC to ethene. Depending on the enrichment conditions, one or the other, or both of these organisms can be maintained in an enrichment culture (Grostern and Edwards, 2006) (see Table 12.1). Enrichment on 1,1,2-TCA supported both organisms. Enrichment cultures fed both 1,1,2-TCA and 1,2-dichloroethane (1,2-DCA) selected for *Dehalobacter* at the expense of *Dehalococcoides*. Enrichment on 1,2-DCA and a complex donor interestingly also supported both *Dehalococcoides* and *Dehalobacter*, as each of these

Table 12.1. Effects of Enrichment Conditions on Activity of Culture "WL"

Culture and Electron Donor/Acceptor Pairs				
Culture	**Parent**	**Subculture**	**Subculture**	**Subculture**
"WL"	TCA/EtOH	TCA/DCA/EtOH	DCA/EtOH	DCA/H_2
1,1,2-TCA	+	+	+	–
1,2-DCA	+	+	+	+
PCE	+	–	+	–
TCE	+	+	+	–
cis-DCE	+	–	+	–
VC	+	–	+	–
Organisms present	*Dhb* and *Dhc*	*Dhb* only (multiple strains)	*Dhb* and *Dhc*	*Dhb* only (fewer strains)

Note: *cis*-DCE–*cis*-dichloroethene; EtOH–ethanol; PCE–perchloroethene

organisms is capable of using this substrate. Enrichment on 1,2-DCA and hydrogen, a more stringent condition, resulted in a pure population of *Dehalobacter* that could no longer dechlorinate TCA or trichloroethene (TCE). The original enrichment thus contained at least two distinct *Dehalobacter* strains with different catabolic activities, as well as at least one strain of *Dehalococcoides*. These strains were competing for electron acceptors, and hence different enrichments could lead to pure strains, but at the expense of the dechlorination substrate range of the culture.

Many sites contain multiple contaminants and other reactive molecules and this mixed contamination and chemistry can lead to complex inhibition dynamics. This inhibition can be acting on the native bacterial communities as well as on the bioaugmented organisms. A bioremediation system therefore must be robust to the presence of inhibitory cocontaminants, or, ideally, capable of degrading the full spectrum of contaminants present at a site.

For example, the reductive dechlorination of TCE to ethene by *Dehalococcoides* is slowed in the presence of elevated concentrations of 1,1,1-TCA. Similarly, the reductive dechlorination of 1,1,1-TCA to chloroethane by *Dehalobacter* is slowed by the presence of chlorinated ethenes, and neither *Dehalococcoides* nor *Dehalobacter* can dechlorinate all of the substrates present in such a system. Mixed together, the combined enrichment culture not only has a broader substrate range, it also accelerates the rate of total dechlorination by alleviating cross inhibition between the chlorinated ethenes and ethanes (see Table 12.2) (Grostern et al., 2009). This is an example of a general concept that can be applied for bioaugmentation – mixing of enrichment cultures maintained on defined substrates can allow a bioaugmentation effort to target a larger range of contaminants.

The examples above illustrate the importance of two different concepts: (1) functional diversity and (2) functional redundancy. Diversity is needed to tackle real sites with complex mixtures, so that the bioaugmentation inocula contain the metabolic capacity to degrade all (or most) of the contaminants present at a site. Redundancy is needed to provide robustness. Functional redundancy can be selected for within the biodegrading population (e.g., multiple *Dehalobacter* or *Dehalococcoides* strains with overlapping substrates). Additionally, the functional redundancy of the supporting organisms (methanogens and acetogens) in higher-complexity consortia allows rapid adjustment to perturbations in the system, including

Table 12.2. Combined Cultures have Broader Substrate Range and Alleviate Inhibition

Substrate	Culture and Electron Acceptor		
	Dhb-TCA 1,1,1-TCA	KB-1© TCE	*Dhb* + KB-1© 1,1,1-TCA and TCE
1,1,1-TCA	+	–	+
1,1-DCA	+	–	+
TCE	–	+	+
cis-DCE	–	+	+
VC	–	+	+
Organisms	Multiple *Dhb* strains	Multiple *Dhc* strains	*Dhb* and *Dhc*

utilization of a wider variety of electron donors. This redundancy increases the flexibility of the consortia and can lower the effective cost of a bioremediation effort.

Even though researchers understand the value of diversity and redundancy in the field, they are often trapped by the enrichment paradox – in order for an enrichment consortium to be understood and fully characterized, it needs to be simplified. Classic microbiological techniques involve enrichment and isolation of target organisms, which is the easiest way to generate a defined culture, which is then easier to describe for environmental certification processes (e.g., by U.S. Environmental Protection Agency (USEPA) or Environment Canada). The simpler the system becomes through an enrichment process, the stronger the questions that can be asked using it, and the clearer the answers from those experiments. For research purposes, especially for determining processes taking place, identifying enzymes integral to those processes, and determining organismal roles, having a clean and simplified system is crucial.

However, the enrichment required for characterization of cultures generally also results in lowered functional redundancy, slower growth and lowered degradative capacity, none of which are ideal for use of the culture in an industrial setting or in research-based experiments. For example, the bioaugmentation culture KB-1© is typically maintained on TCE, with methanol and ethanol as electron donors. It is a stable and robust culture containing multiple dechlorinators (at least two *Dehalococcoides* and a *Geobacter*) as well as many functionally redundant supporting acetogens and methanogens. In an enrichment study, KB-1© was maintained on VC alone, with hydrogen as an electron donor. After several transfers, the KB-1© culture had been enriched to essentially a co-culture of one *Dehalococcoides* strain and an acetogen of the genus *Sporomusa*. While this system presented an excellent system for examining dechlorination and was easily defined in terms of organismal diversity, the co-culture had lost the ability to degrade PCE, and the rate of dechlorination had significantly slowed. Together with the example from the WL enrichment process (Table 12.1), it is clear that enrichment often leads to a decrease in degradative capability within the cultures, even though it generates a simplified microbial system that is preferable for characterization and research.

In a deliberate attempt to maintain multiple species in an enrichment culture, the WBC-2 culture (Jones et al., 2006) is maintained on multiple chlorinated substrates, including 1,1,2,2-tetrachloroethane, c*is*-1,2-dichloroethene (*cis*-DCE) and 1,1,2-TCA, and two generic electron donors, lactate and ethanol. This strategy does seem to hold advantages for maintaining functional diversity and redundancy, yet makes dechlorination activity and microbial population dynamics difficult to predict, particularly when any stress occurs. Indeed, the WBC-2 culture is not very robust when diluted more than 10-fold, presumably as a result of

disruption of the delicate balance between numerous dechlorinating populations and their respective substrates.

At heart, the ideal of simplicity and deconvolution that is central to basic research is directly opposed to the research needs for developing a field application. The past reliance on classical microbiological procedures has led to significant research into single organism isolates removed from their native bacterial network. But this approach provides an incomplete picture of the more complex consortia needed for bioremediation efforts. With the increased need for systems robust to inhibition from cocontamination comes an interest in and a need for novel avenues for examining bacterial systems that are independent of isolate strains. These novel mechanisms must allow researchers to perturb and examine the enrichment consortia as a system, while still providing the ability to ask specific questions within this framework.

The Enrichment Paradox

Higher culture complexity = greater degradation capability.

Higher culture complexity increases difficulty in pursuing specific research questions.

Bioremediation in the field and research in the laboratory are at odds when it comes to culture enrichment.

Can examine complex systems as a whole, using novel systems biology approaches.

12.4 APPLIED RESEARCH NEEDS

The discussions in the prior section regarding niches, microcosms and the enrichment paradox, are central to wisely approaching other practical research needs that relate to bioaugmentation. Examples of such other practical research needs include:

- Better monitoring tools
- Commercial-scale production, storage and shipping of bioaugmentation culture
- Delivery and mixing of bioaugmentation culture into a field site
- Selection of appropriate electron donor
- Obtaining data for regulatory approval
- Modeling of sites, dechlorination and biological activity.

Each of these research needs will be described briefly below, as they represent the areas in which significant optimization of existing bioaugmentation systems can occur, and are simultaneously the variables that will need to be examined for any novel organisms or enrichment cultures to be commercialized as bioaugmentation tools.

12.4.1 Monitoring Tools

There has been a significant effort to develop tools and techniques to delineate geology, chemistry, water flow, contaminant distribution and microbial distribution at field sites. Certainly, there is always a need to improve on these tools, and molecular biological tools are no exception. Molecular tools, that use the genetic signatures of organisms as biomarkers for a particular activity, are already highly effective at measuring the relative abundance of specific populations from one location to the next, or from one sampling time to the next at a given site.

These techniques also have contributed significantly to site characterization and modeling efforts. With the discovery of new microbes and new genes, these tools will increase in scope and usefulness.

Certainly the ability to monitor genes and their products will continue to improve. The ever-growing genomic database allows the design of highly specific primers for functional genes with unprecedented specificity. It is already possible to rapidly assay the abundance of hundreds or thousands of genes in a sample, but what is less clear is how to integrate that information in a highly efficient and useful way into existing site models.

In many instances, the monitoring tools are not the bottleneck for better bioaugmentation performance, but rather it is the paucity of measurements and the extreme degree of extrapolation that is the problem. A few discrete measurements cannot be extrapolated reliably to a whole site. Therefore, the driving force for optimizing or designing monitoring tools has to be cost and ease of use, or there never will be sufficient measurements taken to adequately represent a site.

12.4.2 Production, Storage and Shipping

Each individual bioaugmentation culture will require optimization of production on a large scale. For example, the commercial culture KB-1© is grown in 100-liter (L) anaerobic tanks under specific headspace-to-liquid volume ratios at pH 7. While KB-1© is relatively robust to different conditions, many cultures will require more specific optimization steps in order to grow adequate volumes of high cell density cultures for bioaugmentation applications. Likewise, the conditions used for storage and shipping of an inoculum will be culture-dependent, with nutrient requirements, rate of gas generation (and thus pressure buildup in transport containers), oxygen tolerance and community stability as factors that need to be considered.

12.4.3 Delivery and Mixing

Microbial introduction into the subsurface can proceed via direct injection of free-living bacteria or by introduction of encapsulated bacteria, with or without amendment supplementation. Direct injection is most commonly used currently, and is generally effective for introducing an inoculum into the environment. However, dispersal of the inoculum and subsequent spread of the dechlorination ability within the subsurface can be inefficient depending on the subsurface composition and groundwater flow.

For encapsulation methods, a variety of different compounds can be used as the carrier material, including alginate and gellan gum. Encapsulation can serve a protective role as well as control the availability of the contaminant to bacteria. For microbeads, the size of the capsules can influence the degree of exposure and can be modified according to pore size considerations for different environments. In some cases, electron donors may be included as part of the encapsulation construct. Incorporating the donor can have beneficial influences on the degradative activity, although it may be detrimental in environments with multiple non-target electron acceptors.

12.4.4 Electron Donor Choice

As mentioned above, the choice of electron donor for a system can have significant impact on the bacterial community that flourishes and hence the dechlorination processes and substrates that are seen. A more restrictive electron donor (e.g., hydrogen in the case of KB-1©)

may specifically target the organism of interest for dechlorination, while a more widely used electron donor (methanol, lactate, ethanol, vegetable oil or some combination) may stimulate the growth of non-target organisms, leading to increased biomass but not necessarily increased dechlorination capacity or rates. In choosing an electron donor, the more industry-based factors of transport, delivery and cost must be balanced against creating the correct niche at the site for the organism of interest.

12.4.5 Regulatory Considerations

Among the considerations when generating a bacterial culture for use as an industrial bioremediation agent are the regulatory requirements for applying the culture at a site. Typically these regulations stem from environmental protection agencies connected to state and federal governments, and are geared towards preventing significant perturbations to the environment and native flora at a contaminated site, as well as assessing the potential for pathogenicity within the bioremediation culture. In order to address these requirements, a proper characterization of the enrichment cultures must be undertaken, including identification of the organisms present, as well as a detailed description of the active processes involved in the remediation itself. Both of these prerequisites require a significant amount of research prior to the culture being marketed as a commercial remediation tool.

12.4.6 Modeling of Sites, Dechlorination and Biological Activity

To be truly useful, the information gleaned from research into biological processes like inhibition and dechlorination rates, as well as the physical characteristics known about the site, need to be integrated into organism, microbial community, and flow and transport models to provide truly predictive and diagnostic tools.

Here, a clear research need is to coordinate field measurements and information from disparate measurement techniques into an effective site model, in order to get more value from a limited set of monitoring data. Better communication between modelers and researchers developing the physical, chemical and biological monitoring tools and approaches would aid both sides in generating useful tools. Modelers need to focus on identifying measurable parameters that can guide experimentalists, and conversely, experimentalists need to consider how their data could be used in a model. As mentioned above, activity-based assays may actually be more informative for flow and transport models than data from gene abundance and gene expression measurements, and hence researchers could specifically focus on the set of parameters that will aid modelers in defining a site's characteristics.

The ultimate goal in site modeling is to be able to accurately predict microbial and contaminant fate at a given site, under a given set of conditions. Validated field scale models, built on sound basic science and informed with experimental data that integrate processes operating at all scales, are one way to account for parallel processes occurring at differing time and length scales. These are complicated but powerful studies, and more of this kind of research needs to occur.

12.5 FUTURE PERSPECTIVES

This section will examine some potential areas for expanding the capabilities of bioremediation in the coming decades and centuries. While some techniques discussed below may be "out there" in terms of their current feasibility, they represent potential avenues for novel tools, targeted

approaches and paradigm-shifting techniques that might alter the current definition of bioaugmentation permanently.

12.5.1 Biosensors as MBTs

An underexplored area of research that may have a profound impact on tracking of bioaugmentation inocula or determining native flora dechlorination capabilities at a site is the development of biosensors. Biosensors are capable of detecting or "sensing" a chemical based on molecular recognition, by a biological component linked to some detector that converts the sensed signal to digital information. Detection of the biologically-sensed signal is based on electrochemical, optical, gravimetric or thermal effects, which are subsequently changed to a digital signal (Wei et al., 2009).

The main requirement for a biosensor is a system that will sense the condition of interest (e.g., degradation, cell growth) whose output can be connected to a detector (e.g., a heat sensor or an electric potentiometer). The main challenge in developing a biosensor is thus to create a system whose output can be measured in a meaningful way. Biosensors have the potential to address many current issues in site remediation trials, including providing a fast assay for coupling dechlorination rates to biomass at a site, the need for which was discussed above.

The goal of biosensor studies is to quantify chemicals, and in some cases microbes, rapidly, cheaply and accurately. In the last couple of years, biosensor research has turned to approaches that integrate electronics and biology to build biosensors on the nanoscale level. Nanoscale detection allows measurements along local concentration gradients, in single cells and even for specific protein interactions. Researchers therefore can make precise measurements across heterogeneous samples, even on a tiny scale. One example of a successful biosensor that exists today is a peroxidase enzyme immobilized onto chitosan microspheres, which can detect chlorophenols at very low levels (El Ichi et al., 2009). Another group showed that attaching bacteria to polyaniline nanofibrils modified the electrical conductivity of the system in a linear fashion once above a threshold number of cells. This system could be used as a nanobiodetector for cell growth in suspension (Langer et al., 2009).

These integrated approaches that promote interfacing between biological sensors and the environment open new scopes and opportunities in environmental monitoring and analytics. Development of detection systems linked to outputs meaningful in bioremediation studies will not be trivial, but the potential gain from such monitoring tools is easy to envision.

12.5.2 Designer Microbes and Synthetic Biology

Much discussion and debate has centered around the introduction of genetically modified organisms into the environment (De Lorenzo, 2009). Many jurisdictions are reticent or simply refuse any release of genetically modified organisms. Nevertheless, in the case of bioremediation systems, significant research has examined the potential for introducing degradative genes into heterologous hosts – i.e., bacteria that previously did not contain these genes, but are capable of synthesizing and utilizing them. These research efforts have thus far been unsuccessful in creating an effective genetically modified bioaugmentation inoculum. However, it is highly probable that novel heterologous expression systems in tractable and environmentally robust organisms will be developed (for instance, an oxygen-tolerant anaerobic organism, or a dechlorinator with a broader electron donor substrate range). Such advances could reduce or remove some of the limitations to bioaugmentation.

The considerations in developing such so-called designer microbes are twofold: (1) to introduce novel genetic capabilities into the subsurface in hopes of promoting degradative

activities that may not be present, or may be present at very low levels in the natural environment, and (2) to select hardy, environmentally adapted microbes for genetic engineering and introduction, thereby increasing the likelihood of microbial establishment and survival. This latter consideration is critical in difficult to remediate sites where mixed chemical pollution or harsh conditions exist. In some cases, the introduced organism may provide an activity (i.e., one or more enzymatic steps) that bridges the degradative pathways of the indigenous communities or allows activity under the prevailing site conditions (e.g., anaerobic vs. aerobic conditions). As the understanding of transformative pathways (such as reductive dehalogenation or dissimilatory iron reduction) improves, it will be possible to construct strains expressing more efficient enzymes and to identify gaps in degradative pathways that can be filled with designed microbes.

Other advanced genetic alterations may be at the level of regulation. Altering regulatory controls can be used to overcome limitations on bioremediation performance, such as those associated with inhibition from mixed contaminants or particular environmental conditions (e.g., pH or redox levels). In some cases genetic modifications may involve introduction of novel transporters allowing uptake of contaminants not otherwise accessible to the cell (Saleem et al., 2008). In addition to genetic modification to obtain strains of interest, improved culturing methods have been developed which promote adaptive evolution (i.e., mutations producing desired strain alterations) (Suenaga et al., 2009). Strains of interest produced by this method are highly desirable from a bioaugmentation standpoint as they bypass some of the policy concerns associated with introduction of organisms into the environment. Furthermore, this natural adaptation, as opposed to expression of a heterologous activity, may have a lower energy cost, making these organisms better able to survive and thrive after introduction into the subsurface environment.

Another application of designer strains has been as biosensors for detecting and measuring biological activities, contaminant levels, biological oxygen demand or other features of the environment. Applications on this front have involved introductions of chromosomally associated detection genes or genes which promote surface-based modifications. In the first case, fluorescence genes such as luciferase (*lux*) or green fluorescent protein (*gfp*) are fused downstream of target genes, with activation by appropriate substrates resulting in cell fluorescence (Dawson et al., 2008). Though not quantitative, this approach can be useful as an indicator of contaminant presence and/or conversion depending on the choice of physiological gene. Alternatively, designer microbes that provide surface expressed biological detectors (such as single chain variable fragments) can be constructed (Dhillon et al., 1999). In these cases, the cell surface functions as a detector of a particular contaminant or transformation product. Developments in nanoscale biosensors (comments above) may have a profound influence on both the need and the modifications applied to bacterial strains for application in the environment.

The ability to synthesize, manipulate and clone large fragments of DNA has led to the successful cloning of a complete synthetic bacterial genome (Gibson et al., 2010). It now seems possible to eventually generate a whole organism with a specific suite of genes. However, it is still unknown how well such a designer organism will fare when introduced into a variable and resource competition driven environment, or which genes to include to ensure survival at a contaminated site. These are still a long way off.

12.5.3 Bioaugmenting with Genes

The capacity for DNA transfer among bacteria presents the possibility of introducing new genetic capabilities into environments without the need to specifically engineer strains or

introduce non-native organisms (Cortez et al., 2009). In this approach, DNA encoding the genes required for the reaction of interest (e.g., degradation) is delivered to the contaminated site. The delivery of this DNA can be accomplished by bioaugmentation with strains harboring donor DNA, or by injecting DNA encapsulated in delivery materials such as alginate. Through this approach, new functions could be stably introduced into indigenous populations already adapted to the environment at hand. Key considerations for application of this approach are gene degradation rates and the survival times of delivery strains. In some cases, the donor strains themselves can survive in the introduced environments, promoting continued gene transfer and presenting an additional active group capable of target degradative ability. In these cases, bioaugmentation is occurring both through DNA transfer as well as the more classical introduction of a bacterial population. It is actually highly likely, given that we now realize that some reductive dehalogenase genes are on mobile genetic elements (Maillard et al., 2005; McMurdie et al., 2011), that we may be unintentionally transferring reductive dehalogenase genes to native competent species during bioaugmentation with, for example, *Dehalococcoides*-containing cultures. Such DNA transfer, if it is occurring, is beneficial to remediation efforts, as it would accelerate the propagation of desired traits.

The less classical approach, injection to the subsurface of encapsulated DNA, requires that the DNA survive injection, that a cell within the environment take in the released DNA, and that the DNA is successfully transcribed into an active protein product. This approach may sound implausible, but the mechanisms for plasmid DNA uptake and regulation of gene expression within bacteria are becoming better understood, and it is not impossible to foresee this system becoming industrially applicable. The main advantage of this option is enhanced delivery, because the encapsulated DNA may be transported in the environment without the additional need of donor cell survival. DNA encapsulation in synthetic materials allows extended delivery possibilities in harsh environments where establishment of nonindigenous populations would be limited.

12.5.4 Bioaugmenting with Viruses

Though not extensively studied, the possibility of using lysogenic bacteriophages to deliver novel genetic traits has been proposed. In this system, bacteriophages, possibly encased in biodegradative polyester microspheres, would be delivered to the subsurface. This approach may present advantages over plasmid DNA delivery (described above in Section 12.5.3), because the bacteriophage DNA is inserted on the chromosome, which provides more stable maintenance of genetic elements than plasmid insertions.

Considerations with this approach relate to phage specificity and the ability of phages to attack microbial strains of interest in the subsurface environment. Currently, it is uncertain how conditions in the environment may modulate prophage switching between lysogenic and lytic phases, so it is difficult to control or target bacteriophage infections. But the ability of phages to replicate in the cell and then infect novel hosts offers a promising approach to promoting greater transfer of desired genetic elements within the subsurface (Sobecky and Coombs, 2009).

Development of a viral bioaugmentation assay requires a gene whose function is clearly defined in terms of activity and substrate range. A fully characterized gene would allow introduction of a targeted activity to a site, and hence tracking of the success of the bioaugmentation. While this seems like a reasonable requirement, the reductive dehalogenases, as a putative family of interest for this technique, have proven difficult to characterize, and for many, the substrate range is entirely unknown. This technique also would require an infected bacterium to properly synthesize the novel gene. This is most likely to be successful if the gene is introduced to an organism capable of synthesizing similar proteins.

Early trials of this technique, if it is developed, will likely rely on the basic science research that has characterized much of the biology in the well developed bioaugmentation systems. A hypothetical case study, discussing the steps to successfully introducing a vinyl chloride reductase gene (*vcrA*) into a contaminated environment using this technique, is described below.

12.5.4.1 Viral Bioaugmentation of *vcrA*: A Thought Experiment

The reductive dehalogenase *vcrA* is one of the few biochemically characterized reductive dehalogenases (RDases). It is active in the crucial dechlorination step of VC to ethene (Muller et al., 2004), and is thus of significant interest for both bioaugmentation of sites containing chlorinated ethenes and as a marker gene for determining a site's native dechlorination potential. With *vcrA*'s known substrate specificity, it might be possible to target VC dechlorination by transferring this RDase to natively occurring *Dehalococcoides* or other dechlorinating organisms. This transfer could potentially be accomplished by introducing the *vcrA* gene on a virus, bacteriophage or mobile element into the environment in high copy numbers. Subsequent phage infection or other mode of DNA intake could lead to integration of the *vcrA* gene into a native dechlorinating organism. Ultimately this could lead to an introduction of VC reductase capability at a site that was previously stalled at the VC to ethene dechlorination step.

Interestingly, the *vcrA* gene in *Dhc* strain VS has a codon bias indicative of a recent acquisition of this gene within the genome (McMurdie et al., 2007). A similar unusual codon bias is present in the VC reductase *bvcA* within *Dhc* strain BAV1 (Krajmalnik-Brown et al., 2004; McMurdie et al., 2007). Both of these key genes are present in high plasticity regions of the *Dhc* genome structure, where the majority of genetic integration events have taken place, and are hypothesized to be present on "genomic islands" – large mobile elements within the genomes (McMurdie et al., 2009).

The evidence for *vcrA* and *bvcA* being recent acquisitions within *Dhc* genomes supports the potential for their use as independent bioaugmentation tools, as there is clear evidence that *Dhc* strains are capable of integrating and subsequently transcribing a novel reductive dehalogenase gene, leading to an increased substrate range. There has been little to no research on the mechanisms of DNA uptake and gene transfer in *Dehalococcoides*, and so it is unclear whether this strategy could be effective on an industrial scale. However, if a system of *vcrA* delivery, genomic incorporation and enzyme translation could be developed, it would be a powerful new tool for bioaugmenting sites that exhibit partial dechlorination profiles.

Future Perspectives

Advances in bioremediation and bioaugmentation will come both from:

- Discovery of new organisms with new degradative capabilities.
- Development of entirely novel approaches, likely combining biotic and abiotic technologies.

Biosensors are potentially a powerful tool for monitoring site remediation.

Designer microbes and directed lateral gene transfer are useful genetic modification techniques for developing targeted, site-specific bioremediation strategies.

Viruses could be used to inoculate a specific site with specific gene or function, without the need for bacterial cultures.

12.6 CONCLUSIONS

The story of chlorinated ethene bioremediation and bioaugmentation, encompassing both the discovery of a novel group of microbes and the development of a truly successful bioremediation approach, has reenergized the scientific and engineering communities to look more carefully for other opportunities to improve bioremediation. Research in this field has been fueled by a curiosity-driven quest to discover new life, by rapidly evolving technologies to study microbes and processes that occur at the molecular scale and below, and by the need to find cost-effective approaches for cleaning up the environment. It is absolutely certain that many more microbes with unusual modes of life will be discovered. Such organisms, and the techniques developed to study them, also will provide important strategies and tools needed for greener approaches to remediation and site management.

The research needs identified span a wide range of scales, from that of individual molecules to that of entire ecosystems. Some of the most important and potentially rewarding research needs for enhanced application of bioaugmentation have been reviewed and succinctly summarized in text boxes within this chapter. One overarching need is to consider sustainability in the development of new compounds and materials. Bioaugmentation researchers must work to ensure that the collective knowledge gained from the study of the capabilities of microbes and their environments is used by the chemical industry and regulatory agencies to design, develop and permit new compounds that look and behave more like those that exist already in nature, and to which organisms have become adapted, so that they are more likely to be degraded when released into the environment.

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APPENDIX A
LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

°C	Degrees Celsius
µg	Microgram(s)
µg/L	Microgram(s) per liter
µL	Microliter(s)
µm	Micrometer
μm^3	Cubic micrometer(s)
µM	Micromolar
µmol	Micromole(s)
1-D	One-dimensional
2-D	Two-dimensional
3-D	Three-dimensional
1,1-DCA	1,1-dichloroethane
1,1,1-TCA	1,1,1-trichloroethane
1,1,2-TCA	1,1,2-trichloroethane
1,1,2,2-TeCA	1,1,2,2-tetrachloroethane
1,2-D	1,2-dichloropropane
1,2-DCA	1,2-dichloroethane
1,2,3-TCP	1,2,3-trichloropropane
2,4-D	2,4-dichlorophenoxyacetic acid
ACAD	AcylCoA dehydrogenase
AFB	Air Force Base
AFCEE	Air Force Center for Environmental Excellence (renamed Air Force Center for Engineering and the Environment)
AIHA	American Industrial Hygiene Association
ASU	Arizona State University
atm	Atmosphere
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BDI	Bio-Dechlor INOCULUM
BER	Biological and Environmental Research
bgs	Below ground surface
BMO	Butane monooxygenase enzyme
BTEX	Benzene, toluene, ethylbenzene and total xylenes
C_T	Cycle threshold
ca.	Approximately
CAH	Chlorinated aliphatic hydrocarbon
CAS	Chemical Abstract Services
CF	Chloroform
CFB	Canadian Forces Base
CFC-11	Trichlorofluoromethane
CFC-113	1,1,2-trichloro-1,2,2-trifluoroethane
CFC-22	Monochlorodifluoromethane
cfm	Cubic feet per minute
CFU	Colony forming unit(s)
***cis*-DCE**	*cis*-1,2-dichloroethene
cm	Centimeter(s)
CMO	Cyclohexanone monooxygenase
COD	Chemical oxygen demand
CSIA	Compound specific isotope analysis
CT	Carbon tetrachloride
cy	Cubic yard
d	Day
Da	Dalton(s)
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis

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Dhb	*Dehalobacter*
Dhc	*Dehalococcoides*
DI	Deionized
d-MTBE	Deuterated MTBE
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAPL	Dense nonaqueous phase liquid
DO	Dissolved oxygen
DoD	Department of Defense
DOE	Department of Energy
DPA	Dipicolinic acid
DWT	Dry weight
EISB	Enhanced *in situ* bioremediation
eq/L	Equivalent weight per liter
ESTCP	Environmental Security Technology Certification Program
ETBE	Ethyl *tertiary* butyl ether
EU	European Union
EVO	Emulsified vegetable oil
FAD	Flavin adenine dinucleotide
FISH	Fluorescence *in situ* hybridization
FLiPS	Free-living, pleomorphic spirochetes
ft	Foot/feet
ft^2	Square feet
ft^3	Cubic feet
g	Gram
g/cm^3	Gram(s) per cubic centimeter
g/L	Gram(s) per liter
g/mL	Gram(s) per milliliter
g/mol	Gram(s) per mole
GABA	Gamma-aminobutyric acid
gal	Gallon(s)
GC	Gas chromatography
GEM	Genetically engineered microorganism
gfp	Green fluorescent protein
GMO	Genetically modified organism
gpm	Gallons per minute
ha	Hectare
HAD	Haloacid dehalogenase
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HFCS	High-fructose corn syrup
HGT	Horizontal gene transfer
HIBA	2-hydroxyisobutyrate
HPR	High plasticity regions
hr	Hour
HRC®	Hydrogen Release Compound
IARC	International Agency for Research on Cancer
in	Inch(es)
IR	Iron-reducing
ISCO	*In situ* chemical oxidation
ITRC	Interstate Technology & Regulatory Council
K_h	Henry's Law Constant
Kcal	Kilocalorie(s)
kDa	Kilodalton(s)
kg	Kilogram
kJ	Kilojoule(s)
km	Kilometer(s)
kPa	Kilopascal(s)
L	Liter
lb	Pounds
LBG	Luria-Bertani
LC-MS/MS	Liquid chromatography tandem mass spectrometry
lux	Luciferase
m	Meter(s)
M	Molar
m^2	Square meter(s)
m^3	Cubic meters
Mb	Mega base(s)
MBT	Molecular biological tool
MC	Methanogenic conditions
MCA	Metabolic control analysis
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
MFS	Major facilitator superfamily
mg	Milligram(s)
mg/L	Milligram(s) per liter
MGE	Mobile genetic element
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
MMO	Methane monooxygenase enzyme
mmol	Millimole
MNA	Monitored natural attenuation
mol	Mole
MPN	Most probable number
mRNA	Messenger ribonucleic acid
MS	Mass spectrometer
mS/cm	Millisiemens per centimeter

MSM	Minimal salts medium
MTBE	Methyl tert-butyl ether
mV	Millivolt(s)
MW	Molecular weight
MX	Mixed electron acceptor conditions
NADH	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAD(P)H	Reduced form of Nicotinamide adenine dinucleotide phosphate
NAPL	Nonaqueous phase liquid
NAS	Naval Air Station
NAVFAC ESC	Naval Facilities Engineering Service Center
NBVC	Naval Base Ventura County
NFESC	Naval Facilities Engineering Service Center
nm	Nanometers
nM	Nanomolar
NPL	National priorities list
NPV	Net present value
NR	Nitrate-reducing
NSF	National Science Foundation
O&M	Operation and maintenance
OD	Optical density
OMB	Office of Management and Budget
ORF	Open reading frames
ori	Origin of replication
ORP	Oxidation reduction potential
OSHA	Occupational Safety and Health Administration
***o*TOM**	*o*toluene monooxygenase
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDDs	Polychlorinated-dibenzo-*p*-dioxins
PCE	Perchloroethene (also termed perchloroethylene or tetrachloroethylene)
PCR	Polymerase chain reaction
PDTC	Pyrrolidine dithiocarbamate
PdtF	Cysteine desulfurylase
PeCB	Pentachlorobenzene
PGPR	Plant growth promoting rhizobacteria
PHB	Poly-β-hydroxybutyrate
PHS	U.S. Public Health Service
PLC	Programmable logic controller
pMMO	Particulate methane monooxygenase enzyme
ppb	Part(s) per billion
ppm	Part(s) per million
psi	Pounds per square inch
PVC	Polyvinyl chloride
QA	Quality assurance
QC	Quality control
qPCR	Quantitative PCR
RABBITT	Reductive anaerobic biological *in situ* treatment technology
RAMM	Revised anaerobic mineral medium
RDase(s)	Reductive dehalogenase
rDNA	Ribosomal DNA
RMD	Retrievable media devices
RNA	Ribonucleic acid
ROD	Record of decision
ROI	Radius of influence
rpm	Revolution(s) per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Reverse transcriptase quantitative PCR
s	Second(s)
SERDP	Strategic Environmental Research and Development Program
SJCA	St. Julien's Creek Annex
sMMO	Soluble methane monooxygenase enzyme
SR	Sulfate-reducing
SRS	Savannah River Site
SU	Standard units
TAME	*Tertiary* amyl methyl ether
Tat	Twin arginine translocation
TBA	Tert-butyl alcohol
TBF	*Tertiary*-butyl formate
TCA	Trichloroethane
TCE	Trichloroethene
TDO	Toluene dioxygenase
TDS	Total dissolved solids
TEAPs	Terminal electron accepting process
TGGE	Temperature gel gradient electrophoresis

TMO	Toluene monooxygenase
TOC	Total organic carbon
***trans*-DCE**	*trans*-1,2-dichloroethene
T-RFLP	Terminal restriction fragment length polymorphism
TRF	Terminal restriction fragment
TSCA	Toxic Substances Control Act
TSS	Total suspended solids
USEPA	U.S. Environmental Protection Agency
USGS	United States Geological Survey
VC	Vinyl chloride
vcrA	Vinyl chloride reductase gene
VFA	Volatile fatty acid
VOC	Volatile organic compound
vss	Volatile suspended solid
v/v	Volume per volume
w/v	Weight/Mass per volume
w/w	Weight per weight
YE	Yeast extract
yr	Year(s)

APPENDIX B
UNIT CONVERSION TABLE

Multiply	By	To Obtain
Acres	0.405	Hectares
Acres	1.56 E-3	Square miles (statute)
Centimeters	0.394	Inches
Cubic feet	0.028	Cubic meters
Cubic feet	7.48	Gallons (U.S. liquid)
Cubic feet	28.3	Liters
Cubic meters	35.3	Cubic feet
Cubic yards	0.76	Cubic meters
Feet	0.305	Meters
Feet per year	9.66 E-7	Centimeters per second
Gallons (U.S. liquid)	3.79	Liters
Hectares	2.47	Acres
Inches	2.54	Centimeters
Kilograms	2.20	Pounds (avoir)
Kilograms	35.3	Ounces (avoir)
Kilometers	0.62	Miles (statue)
Liters	0.035	Cubic feet
Liters	0.26	Gallons (U.S. liquid)
Meters	3.28	Feet
Miles (statue)	1.61	Kilometers
Ounces (avoir)	0.028	Kilograms
Ounces (fluid)	29.6	Milliliters
Pounds (avoir)	0.45	Kilograms
Square feet	0.093	Square meters
Square miles	640	Acres

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APPENDIX C
GLOSSARY

Abiotic - Occurring without the direct involvement of organisms.

Acclimation - Time required for physiological adjustment by an organism to environmental change.

Acetogen - A bacterium that generates acetate as a product of anaerobic respiration.

Actinomycetes - A group of Gram-positive bacteria commonly found in soil, more recently classified as Actinobacteria.

Activated carbon - A highly adsorbent form of carbon used to remove odors and/or toxic substances from liquid or gaseous emissions.

Active treatment - *In situ* bioremediation approach in which water soluble amendments are added to the subsurface intermittently, frequently, or even continuously, by pumping liquid solutions into injection wells. Extraction may also be used to recover water prior to amendment addition and/or to recirculate amendments through the target treatment zone.

Aerobic - Environmental conditions where oxygen is present.

Air sparging - Technology in which air or oxygen is injected into an aquifer to volatize or biodegrade contaminants.

Aliphatic compounds - Any chemical compound belonging to the organic class in which the atoms are not linked together to form a ring.

Alkane - A hydrocarbon consisting of single carbon-carbon bonds without any cyclic structures.

Alluvial - Relating to or involving sand deposited by flowing water.

Amplicon - A piece of deoxyribonucleic acid (DNA) formed as the product of natural or artificial amplification events.

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Anaerobic - "Without air." Generally refers to occurring or living without oxygen present. Thus, in an anaerobic groundwater system, the chemistry is characterized by reductive conditions. Sometimes anaerobic is used (e.g., in wastewater treatment) to indicate a lack of any electron acceptors (including nitrate and sulfate). In groundwater, a dissolved oxygen concentration below 1.0 mg per liter (mg/L) is generally considered anaerobic.

Anneal - The pairing of complementary DNA or ribonucleic acid (RNA) sequences via hydrogen bonding to form a double-stranded polynucleotide.

Anoxic - "Without oxygen." For example, anoxic groundwater is groundwater that contains no dissolved oxygen.

Aquifer - An underground geological formation that stores groundwater. A confined aquifer lies beneath a confining unit of lower hydraulic conductivity. An unconfined aquifer does not have a confining unit and is defined by the water table.

Aquitard - An underground geological formation of low permeability that does not readily transmit groundwater.

Attenuation - Reduction of contaminant concentrations over space or time. Includes both destructive (e.g., biodegradation, hydrolysis) and non-destructive (e.g., volatilization, sorption) removal processes.

Bacteriophage - Any one of a number of viruses that infect bacteria, often shortened to simply "phage." Bacteriophages are extremely abundant and widespread. In nature, phages often transfer DNA among bacteria and have been used as designed carriers of DNA to spread desirable genes into bacteria.

Bedrock - The solid or fractured rock underlying surface solids and other unconsolidated material or overburden.

Bioaugmentation - Addition of microbes to the subsurface to improve the biodegradation of target contaminants. Microbes may be "seeded" from populations already present at a site or from specially cultivated strains of bacteria.

Bioavailability - The degree or ability to be absorbed and ready to interact in an organism.

Biobarrier - A remediation technology designed to intercept and biologically treat a contaminant plume as it passes through a permeable subsurface barrier. Biobarriers are created by installing wells or trenches across the width of a plume to deliver substrate to the microorganisms in the aquifer as groundwater flows through the barrier.

Biodegradation - Biologically mediated conversion of one compound to another.

Biofouling - Impairment of the functioning of wells or other equipment as a result of the growth or activity of microorganisms.

Biomarker - A biochemical within an organism that has a particular molecular feature that makes it useful for identifying a specific biological activity.

Biomass - Total mass of microorganisms present in a given amount of water or soil.

Bioremediation - Use of microorganisms to control and destroy contaminants.

Biostimulation - The modification of the subsurface to stimulate existing bacteria capable of bioremediation.

Biotransformation - Biologically catalyzed transformation of a chemical to some other product.

Capture zone - The three-dimensional region that contributes the groundwater extracted by one or more wells or drains.

Catalyst - A substance that promotes a chemical reaction but does not itself enter into the reaction.

Chlorinated solvent - A hydrocarbon in which chlorine atoms substitute for one or more hydrogen atoms in the compound's structure. Chlorinated solvents commonly are used for grease removal in manufacturing, dry cleaning, and other operations. Examples include trichloroethene (TCE), perchloroethene (PCE), and trichloroethane (TCA).

Coenzyme - A substance that enhances the action of an enzyme.

Cometabolism - The simultaneous metabolism of two compounds in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate). For example, in the process of degrading methane, some bacteria can degrade hazardous chlorinated solvents that they would otherwise be unable to attack.

Compound specific isotope analysis (CSIA) - Isotopic characterization of individual compounds that can be used to assess degradation processes.

Conjugation - The direct transfer of genetic material from one cell to another.

Daughter products - Generally refers to compounds produced during the degradation of other "parent" compounds. In the case of chlorinated ethenes, PCE and TCE are generally the parent compounds, and dichloroethene (DCE) and vinyl chloride (VC) are the daughter products.

Dechlorination - A type of dehalogenation reaction involving replacement of one or more chlorine atoms with hydrogen.

Degradation - The transformation of a compound through biological or abiotic reactions.

Deoxyribonucleic acid (DNA) - A nucleic acid that is the primary component of the chromosones of all organisms.

***Dehalococcoides* (*Dhc*)** - A genus of bacteria within class *Dehalococcoidetes* that obtain energy via the oxidation of hydrogen gas and subsequent reductive dehalogenation of halogenated organic compounds.

Dehalogenase - Enzyme catalyzing the loss of a halogen (chloride, fluoride, etc.).

Denature - A structural change in macromolecules (such as DNA) caused by extreme conditions such as high temperatures.

Denaturing gradient gel electrophoresis (DGGE) - A form of electrophoresis that uses a chemical gradient to denature the sample as it moves across an acrylamide gel. Commonly used to separate DNA or RNA into smaller fragments.

Dense nonaqueous phase liquid (DNAPL) - A liquid that is denser than water and does not dissolve or mix easily in water (it is immiscible). In the presence of water, it forms a separate phase from the water. Many chlorinated solvents, such as TCE, are DNAPLs.

Detection limit - The lowest concentration at which a chemical can reliably be detected.

Dichloroethene (DCE) - An organochloride solvent, also known as dichloroethylene, with the molecular formula $C_2H_2Cl_2$. DCE is found as three different isomers: 1,1-dichloroethene; *cis*-1,2-dichloroethene; and *trans*-1,2-dichloroethene.

Diffusion - Dispersive process resulting from the movement of molecules along a concentration gradient. Molecules move from areas of high concentration to areas of low concentration.

Dilution - The combined processes of advection and dispersion resulting in a net dilution of the molecules in the groundwater.

Dispersion - The spreading of molecules along and away from the expected groundwater flow path during advection as a result of mixing of groundwater in individual pores and channels.

Electron acceptor - Compound that receives electrons (and therefore is reduced) in the oxidation-reduction reactions that are essential for the growth of microorganisms and for bioremediation. Common electron acceptors in the subsurface are oxygen, nitrate, sulfate, iron and carbon dioxide. Chlorinated solvents (e.g., TCE) can serve as electron acceptors under anaerobic conditions.

Electron donor - Compound that donates electrons (and therefore is oxidized) in the oxidation-reduction reactions that are essential for the growth of microorganisms and bioremediation. Organic compounds (e.g., lactate) generally serve as an electron donor during anaerobic bioremediation. Less chlorinated solvents (e.g., VC) can also serve as electron donors. Hydrogen generated in fermentation reactions also can serve as an electron donor.

Enzyme - A protein created by living organisms to use in transforming a specific compound. The protein serves as a catalyst in the compound's biochemical transformation.

Ex situ - Latin term referring to the removal of a substance from its natural or original position, e.g., treatment of contaminated groundwater aboveground.

Expression - The manufacturing of gene products from genes. Generally, expression involves transcription from DNA to the complementary RNA sequence, and then translation from the RNA to individual amino acids that are the building blocks of proteins.

Fenton's Reagent - A solution consisting of hydrogen peroxide and an iron catalyst used to oxidize contaminants.

Fermentation - Oxidation of organic compounds occurring in the absence of an external electron acceptor.

Fluorescent *in situ* hybridization (FISH) - A molecular biological technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity, and fluorescence microscopy can then be used to identify cells containing the target DNA sequence and localize where the fluorescent probe binds to the chromosomes.

Fluorophore - A component of a molecule that causes a molecule to be fluorescent.

Fluxomics - The measurement of metabolic fluxes. A metabolic flux is the number of moles of a particular metabolite that are consumed or produced via a particular reaction per unit cell mass per unit time.

Ganglia - Small blobs of DNAPL trapped in individual pores or small groups of pores by capillary forces, as opposed to continuous accumulation of free-phase DNAPL (termed "pools").

Gene - Originally conceived as the fundamental "unit of heredity," the definition has become broader over time. A gene is a sequence of DNA that provides the basic instructions for manufacturing "gene products," notably proteins, but also RNA sequences that have regulatory functions.

Genome - The entire hereditary information of an organism. For example, the *Dehalococcoides* (*Dhc*) genome includes all of the genes, as well as the other non-coding DNA, that is contained in the *Dhc* chromosome. The human genome includes all DNA on our 23 chromosomes.

Genomics - The study of the genomes of organisms. The field includes efforts to sequence the entire DNA of given organisms, as well as to map the genes. For example, genomics has allowed sequencing of the entire DNA of strains of *Dehalococcoides*, and has revealed the locations of specific regions of its chromosome where dehalogenase genes are inserted.

Geochemical - Produced by or involving non-biochemical reactions of the subsurface.

Growth substrate - An organic compound upon which bacteria can grow, usually as a sole carbon and energy source.

Heterogeneity - A lack of uniformity in physical and/or chemical characteristics of an aquifer (as opposed to "homogeneity").

Heterologous - Literally means "derived from a different organism." Heterologous expression refers to the transfer of genetic material from one organism to another, so that the recipient cell manufactures ("expresses") a protein that it does not normally make.

Heterotrophic - Describes an organism that cannot synthesize its own food and is dependent on complex organic substances for nutrition (as opposed to autotrophic).

Hydrocarbon - Chemical compound consisting of hydrogen and carbon (although some or all of the hydrogen may be replaced by other atoms, such as chlorinated hydrocarbons).

Hydraulic conductivity - A measure of the rate at which water moves through a unit area of the subsurface under a unit hydraulic gradient.

Hydraulic fracturing - Method used to create fractures that extend from a borehole into rock and clay formations. Fractures are typically maintained by a proppant, a material such as grains of sand or other material that prevent the fractures from closing. Used to increase or restore the ability to transmit fluids.

Hydraulic gradient - Change in head (water pressure) per unit distance in a given direction, typically in the principal flow direction.

Hydraulic residence time - The average time water spends within a specified region of space, such as a reactor or a treatment zone within the subsurface.

Hydrocarbons - Chemical compounds that consist entirely of carbon and hydrogen.

Hydrogenolysis - Chemical reaction in which a carbon-carbon or carbon-heteroatom single bond is cleaved (or "lysed") by hydrogen. The heteroatom may vary, but it often is oxygen (O), nitrogen (N), or sulfur (S). Usually conducted catalytically using hydrogen gas.

Hydrolysis - The decomposition of organic compounds by interaction with water.

Hydrophobic - "Water-fearing." Hydrophobic compounds, such as oils and chlorinated solvents, have low solubilities in water and tend to form a separate nonaqueous phase.

Hydrogenotrophic - Using hydrogen as an electron donor.

In situ - Latin term meaning "in place"; in the natural or original position, e.g., treatment of groundwater in the subsurface.

***In situ* bioremediation** - The use of microorganisms to degrade contaminants in place with the goal of obtaining harmless chemicals as end products. Generally, *in situ* bioremediation is applied to the degradation of contaminants in saturated soils and groundwater, although bioremediation in the unsaturated zone can occur.

***In situ* chemical oxidation (ISCO)** - Technology that oxidizes contaminants in place by adding strong oxidants such as permanganate or peroxide, resulting in detoxification or immobilization of the contaminants.

***In situ* chemical reduction (ISCR)** - Technology that reduces contaminants in place by adding chemical reductants such as zero-valent iron, resulting in detoxification or immobilization of the contaminants.

***In situ* thermal treatment** - Treatment system that generates high temperatures to remove and destroy contaminants in place. In practice, three types of technologies have been used—steam injection, electrical resistance heating (generating heat by applying an electrical current) and thermal conductive heating (using electrical subsurface heaters to radiate heat outwards through the solid matrix).

Influent - Water, wastewater, or other liquid flowing into a reservoir, basin or *in situ* target treatment zone.

Inoculum - 1. Bacteria or fungi injected into compost to start biological action. 2. A medium containing organisms, usually bacteria or a virus, that is introduced into living organisms or environmental media.

Interfacial tension - The force at the interface between two immiscible liquids (such as a chlorinated solvent nonaqueous phase liquid [NAPL] and water) that results from the attractive forces between the molecules in the different fluids. Generally, the interfacial tension of a given liquid surface is measured by finding the force across any line on the surface divided by the length of the line segment (so that interfacial tension is expressed as force per unit length, equivalent to energy per unit surface area).

Intrinsic bioremediation - A type of *in situ* bioremediation that uses the innate capabilities of naturally occurring microbes to degrade contaminants without requiring engineering steps to enhance the process.

Intrinsic remediation - *In situ* remediation that uses naturally occurring processes to degrade or remove contaminants without using engineering steps to enhance the process.

Isotope - Any of two or more species of an element in the periodic table with the same number of protons. Isotopes have nearly identical chemical properties but different atomic masses and physical properties. For example, the isotopes chlorine 37 (^{37}Cl) and chlorine 35 (^{35}Cl) both have 17 protons, but ^{37}Cl has two extra neutrons and thus a greater mass.

Isotope fractionation - Selective degradation of one isotopic form of a compound over another isotopic form.

Kinetics - The rate at which a reaction occurs.

Lactate - A salt or ester of lactic acid.

Life cycle cost - The overall estimated cost for a particular remedial alternative over the time period corresponding to the life of the program, including direct and indirect initial costs plus any periodic or continuing costs of operation and maintenance.

Lipid - Amphiphilic molecule, possessing the ability to separate two different phases or layers (such as separating water and oil). Often refers to a cell's outer membrane. An amphiphile

possesses both a polar-charged region, which attracts water molecules, and a non-charged and non-polar area, which attracts non-polar oils and fats.

Long-term monitoring (LTM) - Monitoring conducted after a remedial measure achieves its objectives, to ensure continued protection and performance.

Lysogen - Refers to a lysogenic phage. Bacteriophages reproduce by two methods, referred to as the lytic and lysogenic cycles. In the lysogenic cycle, the phage can exist in a dormant state (a prophage) within its host bacterium, in which its DNA is integrated into the host's genome. Lysogenic phages, therefore, can be used to introduce foreign DNA into bacteria.

Mass balance - An accounting of the total inputs and outputs to a system. For dissolved plumes, it refers to a quantitative estimation of the mass loading to a dissolved plume and the mass attenuation capacity within the affected subsurface environment.

Mass discharge - The rate of mass flow across an entire plume at a given location. Also referred to as "total mass flux" or "integrated mass flux." Expressed in units of mass per time (e.g., grams per day [g/day]), mass discharge essentially integrates several individual mass flux measurements (expressed as mass/area/time, e.g., grams per square meter per day [g/m^2/day]).

Mass flux - The rate of mass flow across a unit area (typically measured in grams per square meter per day [g/m^2/day]). Typically calculated by integrating measured groundwater contaminant concentrations across a transect. Often used interchangeably with mass discharge or mass loading (expressed in grams per day [g/day]) to describe the mass emanating from a source zone or the mass passing a given transect across the plume.

Maximum contaminant level (MCL) - Standards set by the U.S. Environmental Protection Agency (USEPA) for drinking water quality that provides for a legal threshold limit on the amount of a hazardous substance that is allowed in drinking water under the Safe Drinking Water Act. The limit is usually expressed as a concentration in milligrams or micrograms per liter of water.

Messenger RNA (mRNA) - A molecule of RNA that serves as a template for protein synthesis. mRNA is transcribed from a gene and then translated by ribosomes in order to manufacture a protein. The sequence of a strand of mRNA is based on the sequence of a complementary strand of DNA.

Metabolite - The intermediates and products of metabolism.

Metabolomics - The measurement of metabolites and their levels. Used to understand metabolic pathways and the levels of different intermediates.

Metagenomics - The study of metagenomes (i.e., the genetic material that can be recovered directly from environmental samples). Also referred to as environmental genomics, ecogenomics, or community genomics, metagenomics allows research on organisms that are not easily cultured in a laboratory as well as studies of organisms in their natural environment.

Methane monooxygenase - Enzyme capable of oxidizing the C-H bond in methane, as well as other alkanes. Found in both soluble (sMMO) and particulate forms (pMMO) in methanotrophs (bacteria capable of growth on methane). Also capable of TCE degradation.

Methanogen (methanogenic bacteria) - A microorganism that exists in anaerobic environments and produces methane as the end product of its metabolism. Methanogens use carbon dioxide, or simple carbon compounds such as methanol, as an electron acceptor.

Methanogenesis - Process of producing methane gas during biological metabolism.

Methanotroph (methanotrophic bacteria) - A microorganism able to metabolize methane as its only source of carbon and energy. Methanotrophs can grow aerobically or anaerobically and require single-carbon compounds to survive.

Methyl tertiary butyl ether (MTBE) - Also known as methyl *tert*-butyl ether. MTBE is added to gasoline as an octane enhancer, originally developed to replace tetraethyl lead. It rapidly became a widespread groundwater contaminant because of its high solubility and recalcitrance to biodegradation.

Micelle - An aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution forms an aggregate with the hydrophilic "head" regions in contact with surrounding solvent, sequestering the hydrophobic single-tail regions in the micelle center.

Microautoradiography - An autoradiograph is an image produced by the radiation emitted from a specimen, such as a section of tissue, that has been treated with or has absorbed a radiolabeled isotope. In biology, this technique is often used to determine the location of a radioactive substance that has been introduced into a metabolic pathway or bound to a receptor or macromolecule. Microautoradiography refers to the application of these techniques to localize substances at the cellular level.

Microcosm - A laboratory vessel set up to resemble as closely as possible the conditions of a natural environment.

Mineralization - The complete degradation of an organic chemical or organism to carbon dioxide, water, and possibly other inorganic compounds or elements.

Molecular biological tool (MBT) - Laboratory test that can measure the presence and/or activity of microbes at a site. MBTs can be used to assess the potential for and performance of monitored natural attenuation and bioremediation strategies for remediation of environmental contaminants.

Monitored natural attenuation (MNA) - Refers to the reliance on natural attenuation processes (within the context of a carefully controlled and monitored site cleanup approach) to achieve site-specific remediation objectives within a timeframe that is reasonable compared to that offered by other more active methods.

Monod kinetics - Equation based on the Michaelis-Menten equation for enzyme kinetics that relates a bacterial culture's specific growth rate (μ) to the substrate concentration (s). Requires empirically derived parameters for the maximum growth rate (μ_{max}) with excess substrate available, and the half-maximal saturation constant (K_s), the substrate concentration at which the growth rate is half of μ_{max}. The fundamental equation is $\mu = \mu_{max} \frac{S}{K_s + S}$.

Nanoscale - Generally deals with structures of the size 100 nm or smaller. For example, reactive iron produced in this size range is referred to as nanoscale iron.

Niche - In ecology, a habitat that supplies the factors necessary for the existence of an organism or species, or the ecological role of that organism or species within the overall community of organisms. Derived from the meaning of niche as a place or role that is best suited for a particular person or thing.

Nonaqueous phase liquid (NAPL) - An organic liquid that is maintained as a separate phase from water.

Nucleotide - The fundamental building blocks of nucleic acids (RNA and DNA). Composed of a nucleobase (nitrogenous base), a five-carbon sugar (either ribose or 2′-deoxyribose), and one to three phosphate groups. The order of the nucleotides (A, T, C, or G for DNA) determines the structure of the genes and their products.

Oligonucleotide - A polynucleotide whose molecules contain a relatively small number of nucleotides.

Oligotrophic - 1. Any environment, ecosystem, or area that is low in nutrients and/or primary production. 2. Organisms adapted to low-carbon environments.

Organohalide respiration - Process by which organisms gain energy for maintenance or growth from reductive dechlorination reactions (i.e., the organisms benefit from contaminant transformation). Also called chlororespiration, dechlororespiration, halorespiration, dehalorespiration, chloridogenesis, catabolic reductive dechlorination, metabolic reductive dechlorination or respiratory reductive dechlorination.

Oxic - Containing oxygen or oxygenated. Often used to describe an environment, a condition, or a habitat in which oxygen is present.

Oxidation-reduction potential (ORP) - The tendency of a solution to either gain or lose electrons when it is subject to change by introduction of a new species. A solution with a higher (more positive) reduction potential than the new species will have a tendency to gain electrons from the new species (to be reduced by oxidizing the new species); a solution with a lower (more negative) reduction potential will have a tendency to lose electrons to the new species (to be oxidized by reducing the new species). A positive ORP indicates the solution is aerobic, while a negative ORP indicates reducing conditions are dominant.

Oxygenase - An enzyme that introduces oxygen into an organic molecule.

Passive treatment - *In situ* bioremediation approach in which amendments are added to the subsurface on a one-time, or infrequent basis. Passive treatment relies on the use of slow-release electron donors, which can be injected into the subsurface or placed in trenches or wells.

Pathogen - Microorganisms (e.g., bacteria, viruses, or parasites) that can cause disease in humans, animals, and plants.

Perchlorate - An anion consisting of one chlorine atom and four oxygen atoms, with the chlorine atom present at an oxidation state of +7. Perchlorate occurs naturally; because it is a potent oxidizer, it also has been manufactured and used for solid rocket propellants and explosives.

Perchloroethene (PCE, perchloroethylene, tetrachloroethene, tetrachloroethylene) - A colorless, nonflammable organic solvent, $CCl_2=CCl_2$, used in dry-cleaning solutions and as an industrial solvent.

Permeability - The rate at which liquids pass through soil or other materials in a specified direction.

Permeable reactive barrier (PRB) - A permeable zone containing or creating a reactive treatment area oriented to intercepting and remediating a contaminant plume.

pH - An expression of the intensity of the basic or acid condition of a liquid; may range from 0 to 14, where 0 is the most acid and 7 is neutral. Natural waters usually have a pH between 6.5 and 8.5.

Phytoaugmentation - The addition of bacterial genes into plants to confer degradation capacities.

Phytoremediation - The use of plants and, in some cases, the associated rhizosphere (root zone) microorganisms for *in situ* remediation of contaminants.

Planktonic - The organisms passively floating or drifting in a water body. Planktonic microbes in the subsurface are found in the aqueous phase, as opposed to those attached to solids.

Plasmid - A DNA molecule that is separate from, and can replicate independently of, the chromosomal DNA. Plasmids are double-stranded and, in many cases, circular. Plasmids usually occur naturally in bacteria, and can be used to introduce new genes into bacteria.

Plume - A zone of dissolved contaminants. A plume usually originates from a contaminant source zone and extends for some distance in the direction of groundwater flow.

Pneumatic fracturing - Injection of gas into the subsurface at pressures exceeding the natural *in situ* pressures and at flow volumes exceeding the natural permeability of the subsurface. Creates a network of artificial fractures in a geologic formation that can facilitate removal of contaminants out of the geologic formation; may be used to introduce remedial agents.

Polychlorinated biphenyls (PCBs) - A group of toxic, persistent chemicals used in electrical transformers and capacitors for insulating purposes, and in gas pipeline systems as lubricant. The sale and new use of these chemicals were banned by U.S. law in 1979.

Polymerase - An enzyme used to catalyze the polymerization of new DNA or RNA against an existing DNA or RNA template in the processes of replication and transcription.

Polymerase chain reaction (PCR) - Technique to amplify a single or a few copies of a specific DNA sequence by several orders of magnitude. Allows detection of a target gene or parts of a gene, even when present at low concentrations in soils or groundwaters, for example. PCR relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication.

Porosity - The fraction of the subsurface volume filled with pores or cavities through which water or air can move.

Precipitate - A substance separated from a solution or suspension by chemical or physical change.

Primary substrates - The electron donor and electron acceptor that are essential to ensure the growth of microorganisms. These compounds can be viewed as analogous to the food and oxygen that are required for human growth and reproduction.

Protein - Complex nitrogenous organic compounds of high molecular weight made of amino acids. Essential for growth and tissue repair. Many, but not all, proteins are enzymes.

Proteome - The entire complement of proteins in an organism or sample.

Proteomics - The large-scale study of proteins, particularly their structures and functions. Studying the proteome of an organism (i.e., all of its proteins) can reveal what enzymes are present at a given time and under given conditions, for example.

Protonophore - The ionophore carrying protons to facilitate crossing the lipid bilayer.

Pump-and-treat - Remediation strategy involving extraction of groundwater and aboveground treatment.

qPCR (quantitative polymerase chain reaction) - A laboratory technique used to amplify and simultaneously quantify a targeted DNA molecule. Also called real-time-PCR.

Radius of influence (ROI) - The radial distance from the center of an injection point or well to the point where there is no significant impact from the injected material.

Record of Decision - A public document that explains which cleanup alternative(s) will be used at National Priorities List sites where, under Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), trust funds pay for the cleanup.

Redox (redox potential) - Reduction/oxidation reactions in which atoms have their oxidation number changed. For example, carbon may be oxidized by oxygen to yield carbon dioxide or reduced by hydrogen to yield methane. The redox potential (ORP) reflects the tendency of a chemical species to acquire electrons and thereby be reduced. In a redox reaction, one chemical species—the reductant or reducing agent—loses electrons and is oxidized, and the other—the oxidant or oxidizing agent—gains electrons and is reduced.

Reducing - Environmental conditions that favor a decrease in the oxidation state of reactive chemical species (e.g., reduction of sulfates to sulfides).

Reductase - An enzyme catalyzing a reducing reaction.

Reduction - Transfer of electrons to a compound such as oxygen; occurs when another compound is oxidized.

Reductive dechlorination (hydrogenolysis) - Reaction involving removal of one or more chlorine atoms from an organic compound and their replacement with hydrogen atoms. A subset of reductive dehalogenation. Key reaction for anaerobic degradation of chlorinated solvents.

Reductive dehalogenation - The process by which a halogen atom (e.g., chlorine or bromine) is replaced on an organic compound with a hydrogen atom. The reactions result in the net addition of two electrons to the organic compound.

Remediation - Cleanup or other methods used to remove or contain contamination.

Rhizosphere - The soil on and near the roots of plants. This soil zone, directly influenced by root secretions, is teeming with microorganisms that feed on sloughed-off plant cells and the compounds released by roots.

Ribonucleic acid (RNA) - One of the three major macromolecules (along with DNA and proteins) essential for all known forms of life. Like DNA, RNA is made up of a long chain of nucleotides, but unlike DNA, RNA contains the sugar ribose (instead of deoxyribose) and contains the nucleobase uracil (instead of thymine). RNA molecules are used to catalyze biological reactions, control gene expression, and synthesize proteins. There are three main types of RNA: messenger RNA (which directs the assembly of proteins on ribosomes), transfer RNA (which delivers amino acids to the ribosome), and ribosomal (which links amino acids together to form proteins).

Ribosome - A component of cells that synthesizes protein chains. Ribosomes are the location for the process known as translation, in which the ribosome translates the genetic information from the messenger RNA (mRNA) by binding to an mRNA and using it as a template for determining the correct sequence of amino acids.

Saturated zone - Part of the subsurface that is beneath the water table and in which the pores are filled with water.

Seepage velocity - The average pore water velocity. Since groundwater flow actually occurs only through interconnected pores and not through the entire subsurface volume, as assumed in calculating the Darcy velocity (V), the seepage velocity (V_S) is equal to the Darcy velocity divided by the porosity (n), or $V_S = V/n$.

Semi-passive treatment - *In situ* bioremediation approach in which amendments are added to the subsurface intermittently (i.e., at intervals of a few weeks to a few months). Generally, water soluble compounds serve as the electron donor. The accumulation of biomass can also serve as a longer-term source of electron donors.

Sequencing - Generally refers to DNA sequencing, i.e., the process of determining the nucleotide order of a given DNA fragment.

Siderophore - A molecule that binds and transports iron in microorganisms.

Soil vapor extraction (SVE, soil venting) - An established technology for the *in situ* remediation of volatile organic compounds (VOCs) in soils. The process removes soil vapor contaminated with VOCs and enhances the mass transfer of VOCs from the soil pores to the vapor phase by applying a vacuum to extract soil contaminants and gasses.

Solubility - Ability of a substance to dissolve (or solubilize). The solubility of a specific solute is its maximum concentration in a given solvent at a reference temperature.

Sorption - Collection of a substance on the surface of a solid by physical or chemical attraction. Can refer to either absorption (in which one substance permeates another) or adsorption (surface retention of solid, liquid, or gas molecules, atoms, or ions).

Source zone - A subsurface zone that serves as a reservoir of contaminants to sustain a dissolved plume. The source includes the material that is or has been in contact with the separate phase (DNAPLs for chlorinated solvents); the source zone mass includes the sorbed and aqueous phase contaminants, as well as any residual NAPL.

Specific activity - A measure of the amount of target contaminant that can be degraded per unit of culture within a given time.

Stakeholder - A person other than regulators, owners or technical personnel, who has a legitimate interest in a contaminated site.

Steady-state - A condition of a physical system or device that does not change over time or in which any one change is continually balanced by another, such as the stable condition of a system in equilibrium.

Stoichiometry - The calculation of quantitative (measurable) relationships of the reactants and products in a balanced chemical reaction.

Substrate - A compound that microorganisms can use in the chemical reactions catalyzed by their enzymes.

Sulfate reducing bacteria (SRB, sulfate reducer) - Bacteria that convert sulfate to hydrogen sulfide. SRB often play important roles in the oxygen-limited subsurface.

Surfactant - A material that can greatly reduce the surface tension of water when used in very low concentrations. Primary ingredient of many soaps and detergents.

Synteny - The co-location of genes along the chromosome.

Syntrophic (syntrophism) - A biological relationship in which organisms of two different species or strains are mutually dependent upon one another for nutritional requirements.

Temperature gel gradient electrophoresis (TGGE) - A form of electrophoresis that uses a temperature gradient to denature a sample as it moves across a gel. Similar to DGGE, TGGE is commonly used to separate DNA or RNA into smaller fragments.

Terminal restriction fragment length polymorphism (T-RFLP) - A molecular biology technique used to profile microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The method is based on digesting a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer.

Thermodynamic - The study of the conversion of energy into work and heat and its relation to macroscopic variables, such as temperature and pressure.

Total dissolved solids (TDS) - Combined content of all inorganic and organic substances in a liquid that are present in a molecular, ionized, or micro-granular (colloidal sol) suspended form.

Total organic carbon (TOC) - Mass of carbon bound in organic compounds in soils, sediments, and water. Often used as a nonspecific indicator of water quality.

Toxicity - The degree to which a substance or mixture of substances can cause harm to organisms. Acute toxicity involves harmful effects in an organism through a single or short-term exposure. Chronic toxicity is the ability of a substance or mixture of substances to cause harmful effects over an extended period.

Transcription - The process of creating a complementary RNA copy (a transcript) of a DNA sequence. During transcription, a DNA sequence is read by an enzyme (RNA polymerase) that produces a complementary RNA strand. Transcription is thus the first step in gene expression, and is followed by translation of the RNA into proteins or other gene products.

Transcriptomics - The study of the transcriptome (all of the RNA molecules in a cell or population of cells). Transcriptomics thus allows the study of the genes that are being actively expressed at any given time, not just the genes that are present (i.e., genomics).

Transduction - Use of a bacteriophage (bacterial virus) to transfer genetic material between organisms.

Transformation - 1. DNA transfer through direct bacterial incorporation of extracellular DNA. 2. Abiotic or biotic catalyzed change of a chemical to some other product.

Translation - The decoding of messenger RNA to produce specific polypeptides. Occurs after transcription.

Transposon - A DNA sequence that can self-transpose (i.e., it can independently replicate itself and insert the copy into a new position within the same or another chromosome or plasmid). These so-called "jumping genes" (also known as transposable elements) are one form of "mobile genetic elements." Transposition has been useful to researchers as a method to alter the DNA within living organisms.

Trichloroethane (TCA) - An industrial solvent (CH_3CCl_3). Other names for it include methyl chloroform and chloroethane. Occurs in two isomers: 1,1,1-TCA and 1,1,2-TCA

Trichloroethene (TCE) - A stable, low-boiling-point, colorless liquid ($CHCl{=}CCl_2$). Used as a solvent or metal degreasing agent and in other industrial applications. Toxic if inhaled, and a suspected carcinogen. Also called trichloroethylene.

Vapor intrusion - Migration of volatile chemicals from the subsurface into overlying buildings.

Vapor pressure - A measure of a substance's propensity to evaporate. The force per unit area exerted by vapor in an equilibrium state with surroundings at a given pressure. It increases exponentially with an increase in temperature. A relative measure of chemical volatility, vapor pressure is used to calculate water partition coefficients and volatilization rate constants.

Vinyl chloride (VC) - A chemical compound (CH_2=CHCl) that is highly toxic and believed to be oncogenic. A colorless compound and an important industrial chemical chiefly used to produce the polymer polyvinyl chloride (PVC).

Viscosity - The molecular friction within a fluid that produces flow resistance.

Volatilization - Transfer of a chemical from the liquid to the gas phase (as in evaporation).

Volatile organic compound (VOC) - Any organic compound that has a high enough vapor pressure under normal conditions to significantly vaporize and enter the atmosphere.

Water solubility - The maximum possible concentration of a chemical compound dissolved in water. If a substance is water soluble, it can very readily disperse through the environment.

Water table - The top of an unconfined aquifer. Indicates the level below which subsurface solids and rock are saturated with water.

Wellhead - The assembly of fittings, valves, and controls located at the surface and connected to the flow lines, tubing, and casing of the well so as to control the flow from the reservoir.

Xenobiotic - A substance that is not normally found in the environment.

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