CHAPTER 1

BIOAUGMENTATION FOR GROUNDWATER REMEDIATION: AN OVERVIEW

Delina Y. Lyon and Timothy M. Vogel

Université de Lyon, Ecole Centrale de Lyon, 69134 Ecully Cedex, France

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

H.F. Stroo et al. (eds.), *Bioaugmentation for Groundwater Remediation*,
doi: 10.1007/978-1-4614-4115-1_1,

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

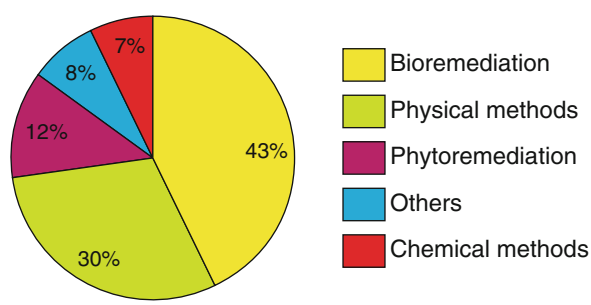

Figure 1.1. The use of common remediation methods by percentage as of 2009 (adapted from Pandey et al., 2009).

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

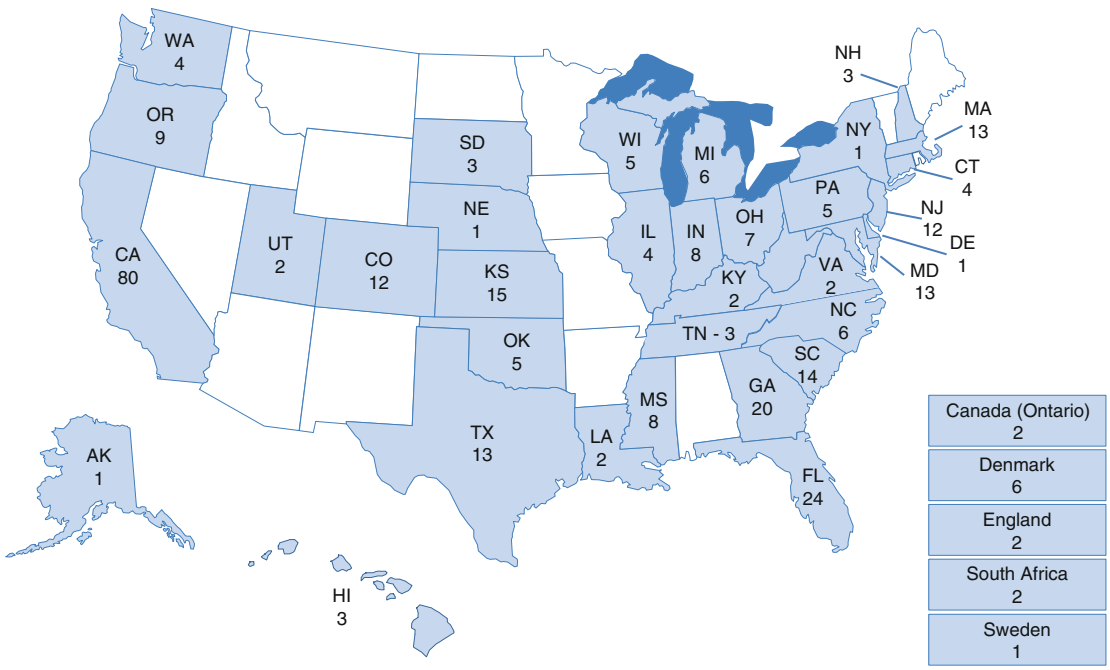

Figure 1.2. Recent census of bioaugmentation applications using *Dehalococcoides* spp. for site cleanup. Figure based on information provided in 2009 by R. J. Steffan (Shaw Environmental & Infrastructure, Inc., Lawrenceville, NJ), R. L. Raymond, Jr. (Terra Systems, Inc., Wilmington, DE) and P. C. Dennis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

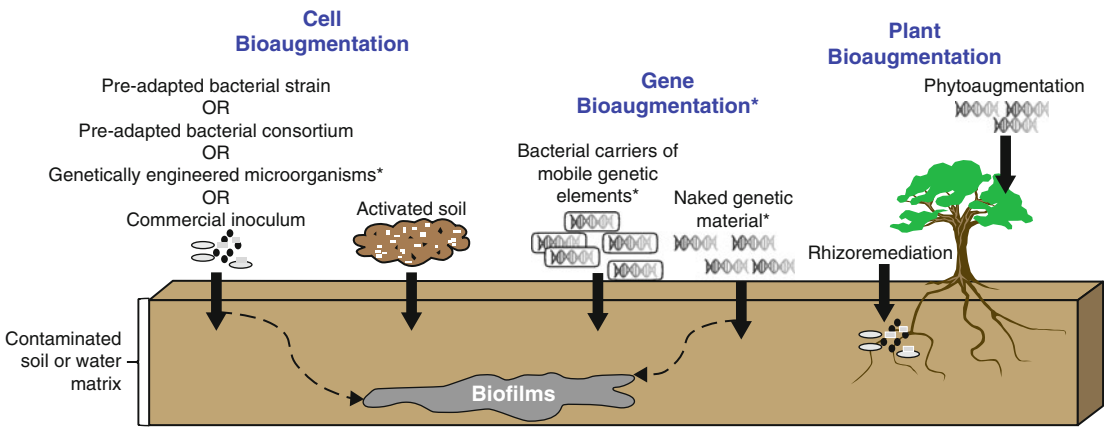

Figure 1.3. Summary of different bioaugmentation methods. * denotes methods that are yet to be commercially practiced.

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).

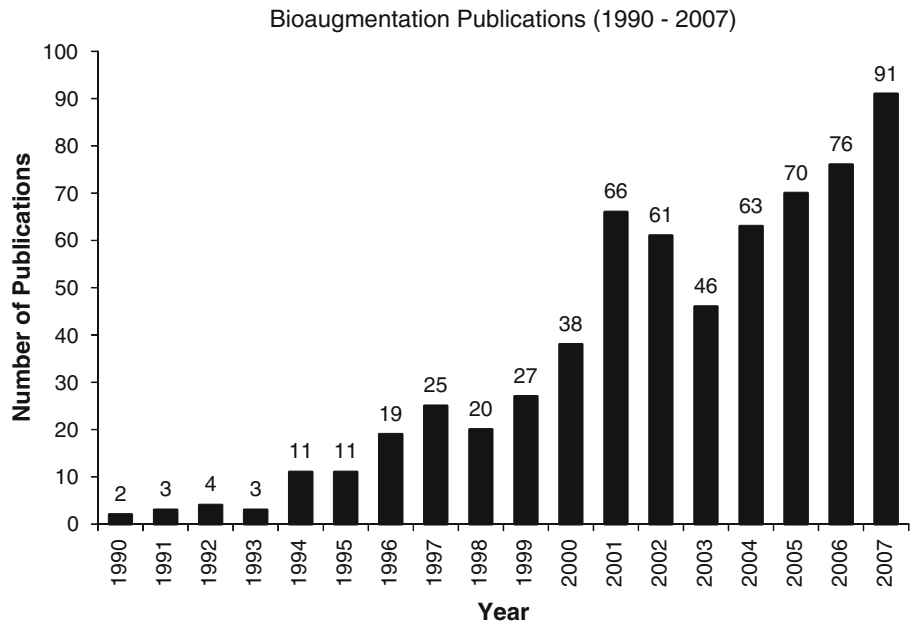

Figure 1.4. Number of international bioaugmentation publications (1991–2007) (adapted from the Science Citation Index).

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,

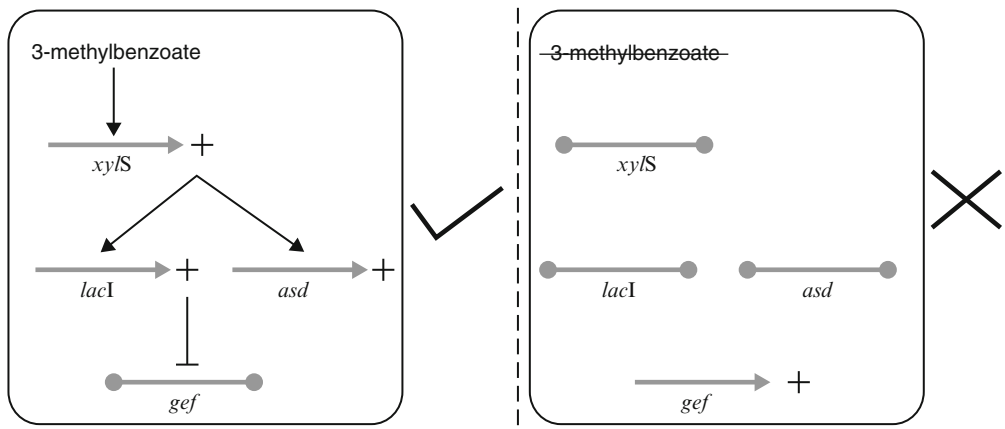

Figure 1.5. Example of a control strategy for GEMs (adapted from Davison, 2005). When the pollutant of interest, 3-methylbenzoate, is present, it activates *xylS*, which then positively activates the transcription of the *asd* gene (for the essential diaminopimellic acid) and *lacI* gene. *LacI* represses 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.

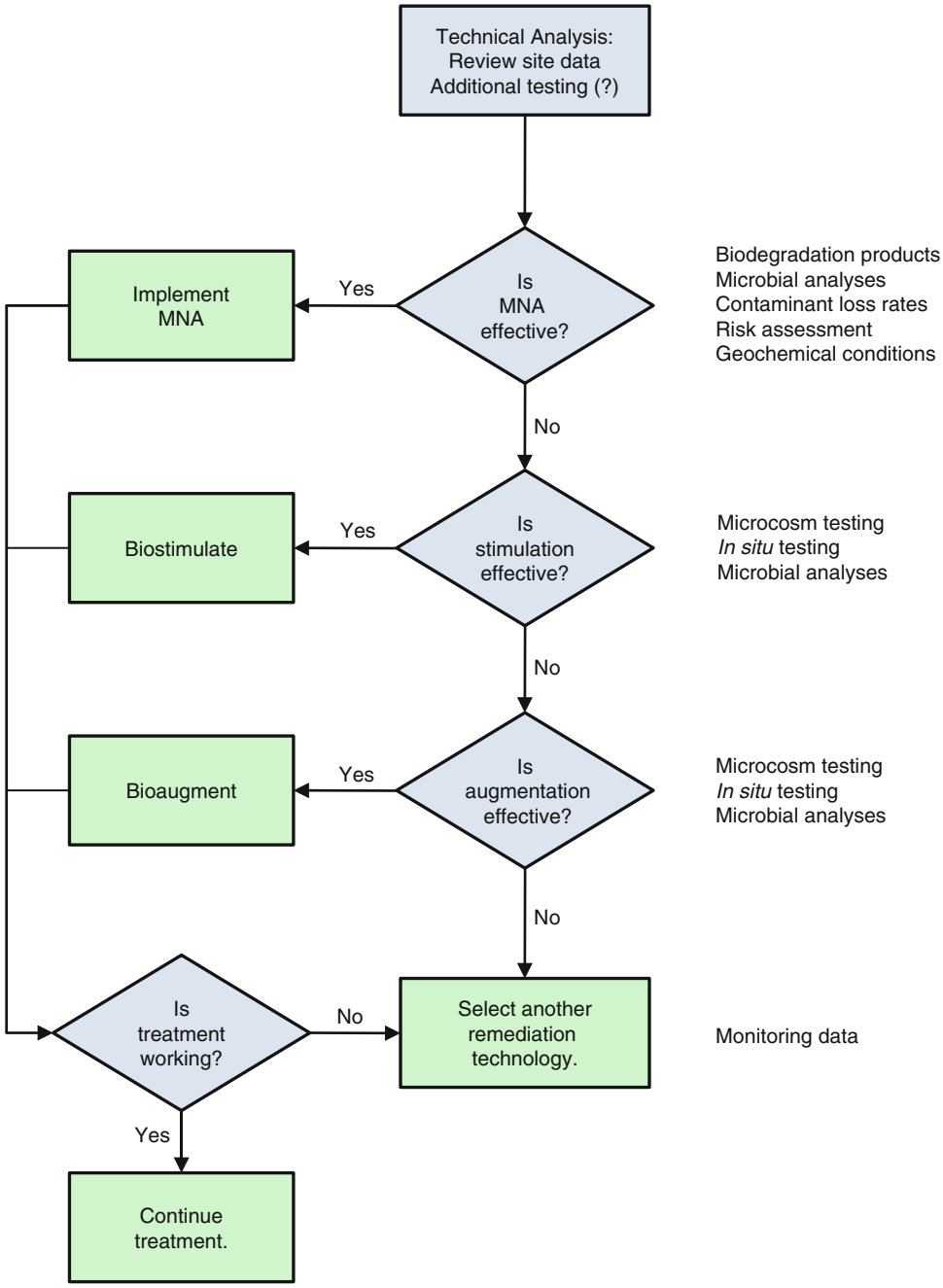

Figure 1.6. Overview of the bioaugmentation selection process. Information needs are listed along the right side of the decision flowpath.

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).

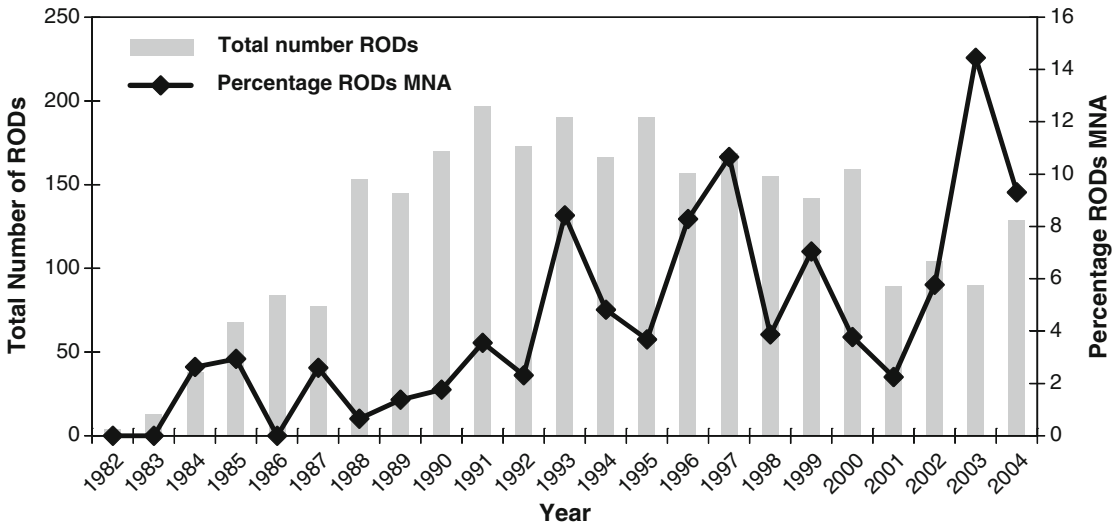

Figure 1.7. RODs for MNA at National Priorities List (NPL) sites (adapted from USEPA, 2007). The bars represent the number of RODs per year, and the line shows the percentage of those RODs that were for MNA for that year.

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

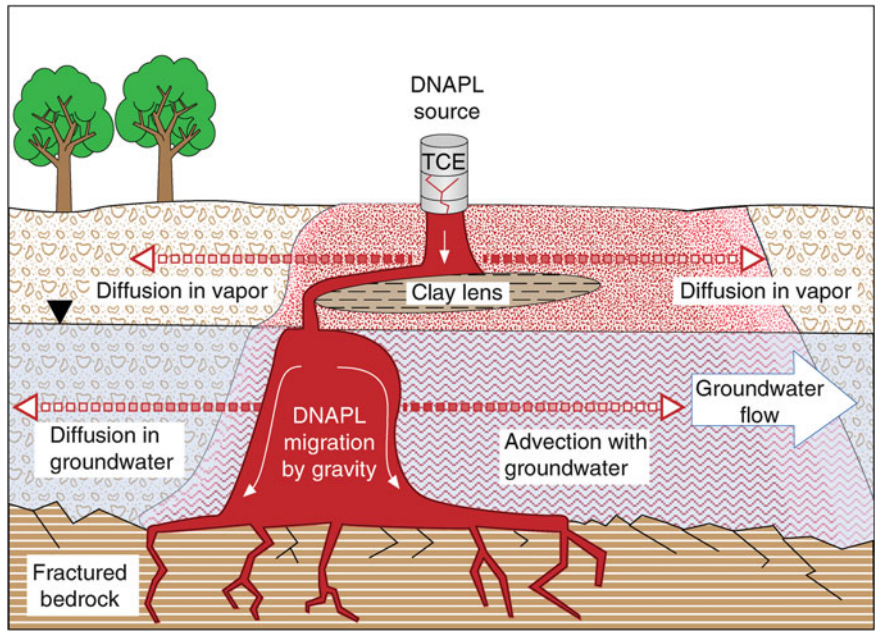

Figure 1.8. Conceptual diagram of a DNAPL-contaminated site (USEPA, 2007).

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.

REFERENCES

Abhilash PC, Jamil S, Singh N. 2009. Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. Biotechnol Adv 27:474–488.

Adamson DT, McDade JM, Hughes JB. 2003. Inoculation of a DNAPL source zone to initiate reductive dechlorination of PCE. Environ Sci Technol 37:2525–2533.

Amos BK, Suchomel EJ, Pennell KD, Löffler FE. 2008. Microbial activity and distribution during enhanced contaminant dissolution from a NAPL source zone. Water Res 42:2963–2974.

Atagana HI, Haynes RJ, Wallis FM. 2003. Optimization of soil physical and chemical conditions for the bioremediation of creosote-contaminated soil. Biodegradation 14:297–307.

Atlas R. 1993. Bacteria and bioremediation of oil spills. Oceanus 36:71–73.

Atlas RM, Bartha R. 1972. Degradation and mineralization of petroleum in sea water: Limitation by nitrogen and phosphorous. Biotechnol Bioeng 14:309–318.

Baldi F, Leonardi V, D'Annibale A, Piccolo A, Zecchini F, Petruccioli M. 2007. Integrated approach of metal removal and bioprecipitation followed by fungal degradation of organic pollutants from contaminated soils. Eur J Soil Biol 43:380–387.

Bamforth S, Singleton I. 2005. Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. J Chem Technol Biotechnol 80:723–736.

Barbeau C, Deschenes L, Karamanev D, Comeau Y, Samson R. 1997. Bioremediation of pentachlorophenol-contaminated soil by bioaugmentation using activated soil. Appl Microbiol Biotechnol 48:745–752.

Bathe S, Schwarzenbeck N, Hausner M. 2005. Plasmid-mediated bioaugmentation of activated sludge bacteria in a sequencing batch moving bed reactor using pNB2. Lett Appl Microbiol 41:242–247.

Beck EC. 1979. The Love Canal Tragedy. EPA Journal. January. http://www.epa.gov/history/topics/lovecanal/01.html. Accessed March 15, 2012.

Bej AK, Perlin M, Atlas RM. 1991. Effect of introducing genetically engineered microorganisms on soil microbial community diversity. FEMS Microbiol Ecol 86:169–176.

Bento FM, Camargo FA, Okeke BC, Frankenberger WT. 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour Technol 96:1049–1055.

Boon N, Goris J, De Vos P, Verstraete W, Top EM. 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. Appl Environ Microbiol 66:2906–2913.

Bott TL, Kaplan LA. 2002. Autecological properties of 3-chlorobenzoate-degrading bacteria and their population dynamics when introduced into sediments. Microb Ecol 43:199–216.

Bouwer EJ, McCarty PL. 1983. Transformations of halogenated organic compounds under denitrification conditions. Appl Environ Microbiol 45:1295–1299.

Brooksbank AM, Latchford JW, Mudge SM. 2007. Degradation and modification of fats, oils and grease by commercial microbial supplements. World J Microbiol Biotechnol 23:977–985.

Bumpus JA. 1989. Biodegradation of polycyclic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 55:154–158.

Button DK, Robertson BR, McIntosh D, Juttner F. 1992. Interactions between marine bacteria and dissolved-phase and beached hydrocarbons after the Exxon Valdez oil spill. Appl Environ Microbiol 58:243–251.

Cases I, de Lorenzo V. 2005. Genetically modified organisms for the environment: Stories of success and failure and what we have learned from them. Int Microbiol 8:213–222.

Cerniglia C. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368.

Cherian S, Oliveira MM. 2005. Transgenic plants in phytoremediation: Recent advances and new possibilities. Environ Sci Technol 39:9377–9390.

Cohen Y. 2002. Bioremediation of oil by marine microbial mats. Int Microbiol 5:189–193.

Contreras A, Molin S, Ramos JL. 1991. Conditional-suicide containment system for bacteria which mineralize aromatics. Appl Environ Microbiol 57:1504–1508.

Coppotelli BM, Ibarrolaza A, Del Panno MT, Morelli IS. 2008. Effects of the inoculant strain *Sphingomonas paucimobilis* 20006FA on soil bacterial community and biodegradation in phenanthrene-contaminated soil. Microb Ecol 55:173–183.

Cupples AM, Spormann AM, McCarty PL. 2003. Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. Appl Environ Microbiol 69:953–959.

Damborsky J. 1999. Tetrachloroethene-dehalogenating bacteria. Folia Microbiol 44:247–262.

Davison J. 2005. Risk mitigation of genetically modified bacteria and plants designed for bioremediation. J Ind Microbiol Biotechnol 32:639–650.

Deeb RA, Scow KM, Alvarez-Cohen L. 2000. Aerobic MTBE biodegradation: An examination of past studies, current challenges and future research directions. Biodegradation 11:171–186.

Desaint S, Arrault S, Siblot S, Fournier JC. 2003. Genetic transfer of the mcd gene in soil. J Appl Microbiol 95:102–108.

DiStefano TD, Gossett JM, Zinder SH. 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. Appl Environ Microbiol 57:2287–2292.

Duhamel M, Wehr SD, Yu L, Rizvi H, Seepersad D, Dworatzek S, Cox EE, Edwards EA. 2002. Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride. Water Res 36:4193–4202.

Dybas MJ, Barcelona M, Bezborodnikov S, Davies S, Forney L, Heuer H, Kawka O, Mayotte T, Sepúlveda-Torres L, Smalla K, Sneathen M, Tiedje J, Voice T, Wiggert DC, Witt ME, Criddle CS. 1998. Pilot-scale evaluation of bioaugmentation for in-situ remediation of a carbon tetrachloride-contaminated aquifer. Environ Sci Technol 32:3598–3611.

Dybas MJ, Hyndman DW, Heine R, Tiedje J, Linning K, Wiggert D, Voice T, Zhao X, Dybas L, Criddle CS. 2002. Development, operation, and long-term performance of a full-scale biocurtain utilizing bioaugmentation. Environ Sci Technol 36:3635–3644.

El Fantroussi S, Agathos SN. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr Opin Microbiol 8:268–275.

Ellis DE, Lutz EJ, Odom JM, Buchanan Jr RL, Bartlett CL, Lee MD, Harkness MR, Deweerd KA. 2000. Bioaugmentation for accelerated in situ anaerobic bioremediation. Environ Sci Technol 34:2254–2260.

ESTCP (Environmental Security Technology Certification Program). 2005. Bioaugmentation for Remediation of Chlorinated Solvents: Technology Development, Status, and Research Needs. ESTCP, Arlington, VA, USA. Available at: http://www.serdp-estcp.org/Tools-and-Training/Environmental-Restoration/Groundwater-Plume-Treatment/Bioaugmentation-for-Remediation-of-Chlorinated-Solvents-Technology-Development-Status-and-Research-Needs. Accessed March 15, 2012.

EU (European Union). 2001. Directive 2001/18/EC of the European Parliament and of the Council; March 12, 2001; On the Deliberate Release into the Environment of Genetically Modified Organisms and Repealing Council Directive 90/220/EEC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2001L0018:20031107:EN:PDF. Accessed June 18, 2012.

Fennell DE, Nijenhuis I, Wilson SF, Zinder SH, Haggblom MM. 2004. *Dehalococcoides ethenogenes* strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ Sci Technol 38:2075–2081.

Field J, de Jong E, Costa G, de Bont J. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl Environ Microbiol 58:2219–2226.

Finneran KT, Lovley DR. 2001. Anaerobic degradation of methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA). Environ Sci Technol 35:1785–1790.

Fogel MM, Taddeo AR, Fogel S. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl Environ Microbiol 51:720–724.

Freedman DL, Gossett JM. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 55:2144–2151.

Garbisu C, Alkorta I. 1999. Utilization of genetically engineered microorganisms (GEMs) for bioremediation. J Chem Technol Biotechnol 74:599–606.

Gentry TJ, Rensing C, Pepper IL. 2004. New approaches for bioaugmentation as a remediation technology. Crit Rev Environ Sci Technol 34:447–494.

Glaser J. 1993. Engineering approaches using bioremediation to treat crude oil-contaminated shoreline following the Exxon Valdez accident in Alaska. In Flathman P, Jerger DE, Exner JH, eds, Bioremediation Field Experience. Lewis Publisher, Boca Raton, FL, USA, Chapter 5.

Goldstein RM, Mallory LM, Alexander M. 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl Environ Microbiol 50:977–983.

Goulding C, Gillen CJ, Bolton E. 1988. Biodegradation of substituted benzenes. J Appl Bacteriol 65:1–5.

Gribble G. 1998. Naturally occurring organohalogen compounds. Acc Chem Res 31:141–152.

Häggblom MM, Youngster LK, Somsamak P, Richnow, HH. 2007. Anaerobic biodegradation of methyl tert-butyl ether (MTBE) and related fuel oxygenates. Adv Appl Microbiol 62:1–20.

Hazen T, Tabak H. 2005. Developments in bioremediation of soils and sediments polluted with metals and radionuclides: 2. Field research on bioremediation of metals and radionuclides. Rev Environ Sci Biotechnol 4:157–183.

He J, Ritalahti K, Yang K, Koenigsberg S, Löffler F. 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nat 424:62–65.

He Q, Sanford R. 2002. Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. Biodegradation 13:307–316.

Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC. 2002. Molecular analysis of *Dehalococcoides* 16 S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Appl Environ Microbiol 68:485–495.

Hesselsoe M, Bjerring ML, Henriksen K, Loll P, Nielsen JL. 2008. Method for measuring substrate preferences by individual members of microbial consortia proposed for bioaugmentation. Biodegradation 19:621–633.

Holliger C, Wohlfarth G, Diekert G. 1999. Reductive dechlorination in the energy metabolism of an anaerobic bacteria. FEMS Microbiol Rev 22:383–398.

Hood ED, Major DW, Quinn JW, Yoon WS, Gavaskar A, Edwards EA. 2008. Demonstration of enhanced bioremediation in a TCE source area at Launch Complex 34, Cape Canaveral Air Force Station. Ground Water Monit Remediat 28:98–107.

Hristova KR, Lutenegger CM, Scow KM. 2001. Detection and quantification of methyl tert-butyl ether-degrading strain PM1 by real-time TaqMan PCR. Appl Environ Microbiol 67:5154–5160.

Hunter WJ, Shaner DL. 2010. Biological remediation of groundwater containing both nitrate and atrazine. Curr Microbiol 60:42–46.

ITRC (Interstate Technology & Regulatory Council). 2008. *In Situ* Bioremediation of Chlorinated Ethene: DNAPL Source Zones. BioDNAPL-3. Prepared by the Bioremediation of DNAPLs Team.

Jacques RJ, Okeke BC, Bento FM, Teixeira AS, Peralba MC, Camargo FA. 2008. Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. Bioresour Technol 99:2637–2643.

Jansson JK, Bjorklof K, Elvang AM, Jorgensen KS. 2000. Biomarkers for monitoring efficacy of bioremediation by microbial inoculants. Environ Pollut 107:217–223.

Johnson DR, Brodie EL, Hubbard AE, Andersen GL, Zinder SH, Alvarez-Cohen L. 2008. Temporal transcriptomic microarray analysis of *Dehalococcoides ethenogenes* strain 195 during the transition into stationary phase. Appl Environ Microbiol 74:2864–2872.

Juhanson J, Truu J, Heinaru E, Heinaru A. 2007. Temporal dynamics of microbial community in soil during phytoremediation field experiment. J Environ Eng Landsc Manag 15:213–220.

Kane A, Vidumsky J, Major DW, Bauer NB. 2005. In-situ bioremediation of a chlorinated solvent residual source in unconsolidated sediments and bedrock using bioaugmentation. In Calabrese EJ, Kostecki PT, Dragun J, eds, Contaminated Soils, Sediments and Water: Science in the Real World 9:45–55.

Kappeli O, Auberson L. 1997. The science and intricacy of environmental safety evaluations. Trends Biotechnol 15:342–349.

Keppler F, Borchers R, Pracht J, Rheinberger S, Scholer H. 2002. Natural formation of vinyl chloride in the terrestrial environment. Environ Sci Technol 36:2479–2483.

Khomenkov VG, Shevelev AB, Zhukov VG, Zagustina NA, Bezborodov AM, Popov VO. 2008. Organization of metabolic pathways and molecular-genetic mechanisms of xenobiotic biodegradation in microorganisms: A review. Prikl Biokhim Mikrobiol 44:133–152.

Kiesel B, Muller RH, Kleinsteuber R. 2007. Adaptative potential of alkaliphilic bacteria towards chloroaromatic substrates assessed by a gfp-tagged 2,4-D degradation plasmid. Eng Life Sci 7:361–372.

Kramer U. 2005. Phytoremediation: Novel approaches to cleaning up polluted soils. Curr Opin Biotechnol 16:133–141.

Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJ. 2004. Rhizoremediation: A beneficial plant-microbe interaction. Mol Plant Microbe Interact 17:6–15.

Lebeau T, Braud A, Jezequel K. 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environ Pollut 153:497–522.

Lee JH, Dolan M, Field J, Istok J. 2010. Monitoring bioaugmentation with single-well push-pull tests in sediment systems contaminated with trichloroethene. Environ Sci Technol 44:1085–1092.

Lee K, Levy E. 1987. Enhanced biodegradation of a light crude oil in sandy beaches. Proceedings, 1987 International Oil Spill Conference, pp 411–416. American Petroleum Institute Publication Number 4452, Washington DC, USA.

Lee PK, Macbeth TW, Sorenson Jr KS, Deeb RA, Alvarez-Cohen L. 2008. Quantifying genes and transcripts to assess the in situ physiology of *Dehalococcoides* spp. in a trichloroethene-contaminated groundwater site. Appl Environ Microbiol 74:2728–2739.

Leisinger T. 1983. Microorganisms and xenobiotic compounds. Experientia 39:1183–1191.

Lendvay JM, Löffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, Gebhard M, Heine R, Helton R, Shi J, Krajmalnik-Brown R, Major CL, Barcelona MJ, Petrovskis E, Tiedje JM, Adriaens P. 2003. Bioreactive barriers: A comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. Environ Sci Technol 37:1422–1431.

Lenski RE. 1993. Evaluating the fate of genetically modified microorganisms in the environment: Are they inherently less fit? Experientia 49:201–209.

Little CD, Palumbo AV, Herbes SE, Lidstrom ME, Tyndall RL, Gilmer PJ. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl Environ Microbiol 54:951–956.

Lopes Ferreira N, Malandain C, Fayolle-Guichard F. 2006. Enzymes and genes involved in the aerobic biodegradation of methyl tert-butyl ether (MTBE). Appl Microbiol Biotechnol 72:252–262.

Maes A, van Raemdonck H, Smith K, Ossieur W, Lebbe L, Verstraete W. 2006. Transport and activity of *Desulfitobacterium dichloroeliminans* strain DCA1 during bioaugmentation of 1,2-DCA-contaminated groundwater. Environ Sci Technol 40:5544–5552.

Major D, Cox E. 1992. Survey of microbial inoculants for bioremediation and identification of information requirements suitable for the feasibility evaluation and validation of bioremediation. Prepared for the Hazardous Contaminants Branch, Ontario Ministry of the Environment. PIB 2152.

Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36:5106–5116.

Margesin R. 2007. Alpine microorganisms: Useful tools for low-temperature bioremediation. J Microbiol 45:281–285.

Mathew M, Tan LR, Su Q, Yang X, Baxter M, Senior E. 2006. Bioremediation of 6%[w/w] diesel-contaminated mainland soil in Singapore: Comparison of different biostimulation and bioaugmentation treatments. Eng Life Sci 6:63–67

May HD, Miller GS, Kjellerup BV, Sowers KR. 2008. Dehalorespiration with polychlorinated biphenyls by an anaerobic ultramicrobacterium. Appl Environ Microbiol 74:2089–2094.

McGechan M, Lewis D. 2002. Transport of particulate and colloid-sorbed contaminants through soil, part 1: General principles. Biosyst Eng 83:255–273.

Mehmannavaz R, Prasher SO, Ahmad D. 2002. Subsurface irrigation as a microbial delivery tool for bioaugmentation: Transport, distribution and survival in large packed soil columns. Environ Technol 23:707–717.

Mohn W, Tiedje JM. 1990. Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch Microbiol 153:267–271.

Morrill PL, Lacrampe-Couloume G, Slater GF, Sleep BE, Edwards EA, McMaster ML, Major DW, Sherwood Lollar B. 2005. Quantifying chlorinated ethene degradation during reductive dechlorination at Kelly AFB using stable carbon isotopes. J Contam Hydrol 76:279–293.

Morse JJ, Alleman BC, Gossett JM, Zinder SH, Fennell DE, Sewell GW, Vogel CM. 1998. Draft Technical Protocol: A Treatability Test for Evaluating the Potential Applicability of the Reductive Anaerobic Biological in situ Treatment Technology (RABITT) to Remediate Chloroethenes. ESTCP, Arlington, VA, USA. http://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/ER-199719/ER-199719. Accessed May 31, 2012.

Nancharaiah YV, Joshi HM, Hausner M, Venugopalan VP. 2008. Bioaugmentation of aerobic microbial granules with *Pseudomonas putida* carrying TOL plasmid. Chemosphere 71:30–35.

Newby DT, Josephson KL, Pepper IL. 2000. Detection and characterization of plasmid pJP4 transfer to indigenous soil bacteria. Appl Environ Microbiol 66:290–296.

Newcombe DA, Crowley DE. 1999. Bioremediation of atrazine-contaminated soil by repeated applications of atrazine-degrading bacteria. Appl Microbiol Biotechnol 51:877–882.

Nojiri H, Shintani M, Omori T. 2004. Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. Appl Microbiol Biotechnol 64:154–174.

Oldenhuis R, Vink RL, Janssen DB, Witholt B. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl Environ Microbiol 55:2819–2826.

Otte M-P, Gagnon J, Comeau Y, Matte N, Greer CW, Samson R. 1994. Activation of an indigenous microbial consortium for bioaugmentation of pentachlorophenol/creosote contaminated soils. Appl Microbiol Biotechnol 40:926–932.

Padmavathiamma PK, Li LY. 2007. Phytoremediation technology: Hyper-accumulation metals in plants. Water Air Soil Pollut 184:105–126.

Pandey J, Chauhan A, Jain RK. 2009. Integrative approaches for assessing the ecological sustainability of in situ bioremediation. FEMS Microbiol Rev 33:324–375.

Park D, Lee DS, Kim YM, Park JM. 2008. Bioaugmentation of cyanide-degrading microorganisms in a full-scale cokes wastewater treatment facility. Bioresour Technol 99:2092–2096.

Pepper IL, Gentry TJ, Newby DT, Roane TM, Josephson KL. 2002. The role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soils. Environ Health Perspect 110:943–946.

Pfaender WF, Maggard SP, Gander LK, Watrud LS. 1997. Comparison of three bioremediation agents for mineralization and transformation of pentachlorophenol in soil. Bull Environ Contam Toxicol 59:230–237.

Pointing SB. 2001. Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33.

Prince RC. 1993. Petroleum spill bioremediation in marine environments. Crit Rev Microbiol 19:217–242.

Pritchard PH. 1992. Use of inoculation in bioremediation. Curr Opin Biotechnol 3:232–243.

Rahm BG, Chauhan S, Holmes VF, Macbeth TW, Sorenson Jr KS, Alvarez-Cohen L. 2006. Molecular characterization of microbial populations at two sites with differing reductive dechlorination abilities. Biodegradation 17:523–534.

Raina V, Suar M, Singh A, Prakash O, Dadhwal M, Gupta SK, Dogra C, Lawlor K, Lal S, van der Meer JR, Holliger C, Lal R. 2008. Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with *Sphingobium indicum* B90A. Biodegradation 19:27–40.

Raymond RL. 1976. Beneficial stimulation of bacterial activity in groundwater containing petroleum hydrocarbons. American Institute of Chemical Engineers (AIChE) Symposium Series 73:390–404.

Richard J-Y, Vogel TM. 1999. Characterization of a soil bacterial consortium capable of degrading diesel fuel. Int Biodeterior Biodegrad 44:93–100.

Ripp S, Nivens DE, Werner C, Sayler GS. 2000. Bioluminescent most-probable-number monitoring of a genetically engineered bacterium during a long-term contained field release. Appl Microbiol Biotechnol 53:736–741.

Roane TM, Josephson KL, Pepper IL. 2001. Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. Appl Environ Microbiol 67:3208–3215.

Rothmel RK, Peters RW, Martin ES, DeFlaun MF. 1998. Surfactant foam/bioaugmentation technology for in situ treatment of TCE-DNAPLs. Environ Sci Technol 32:1667–1675.

Salanitro J, Johnson P, Spinnler GE, Maner PM, Wisniewski HL, Bruce CL. 2000. Field-scale demonstration of enhanced MTBE bioremediation through aquifer bioaugmentation and oxygenation. Environ Sci Technol 34:4152–4162.

Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM, Trevors JT. 2005. Microbial gene expression in soil: methods, applications and challenges. J Microbiol Methods 63:1–19.

Samanta SK, Singh OV, Jain RK. 2002. Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. Trends Biotechnol 20:243–248.

Satoh H, Okabe S, Yamaguchi Y, Watanabe Y. 2003. Evaluation of the impact of bioaugmentation and biostimulation by in situ hybridization and microelectrode. Water Res 37:2206–2216.

Sayler GS, Ripp S. 2000. Field applications of genetically engineered microorganisms for bioremediation processes. Curr Opin Biotechnol 11:286–289.

Sayre P, Seidler RJ. 2005. Application of GMOs in the U.S.: EPA research & regulatory considerations related to soil systems. Plant Soil 275:77–91.

Scheutz C, Durant ND, Dennis P, Hansen MH, Jorgensen T, Jakobsen R, Cox EE, Bjerg PL. 2008. Concurrent ethene generation and growth of *Dehalococcoides* containing vinyl chloride reductive dehalogenase genes during an enhanced reductive dechlorination field demonstration. Environ Sci Technol 42:9302–9309.

Scow KM, Hicks KA. 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Curr Opin Biotechnol 16:246–253.

Semprini L, Dolan ME, Mathias MA, Hopkins GD, McCarty PL. 2007. Laboratory, field, and modeling studies of bioaugmentation of butane-utilizing microorganisms for the in situ cometabolic treatment of 1,1-dichloroethene, 1,1-dichloroethane, and 1,1,1-trichloroethane. Adv Water Resour 30:1528–1546.

Shintani M, Yoshida T, Habe H, Omori T, Nojiri H. 2005. Large plasmid pCAR2 and class II transposon Tn4676 are functional mobile genetic elements to distribute the carbazole/dioxin-degradative car gene cluster in different bacteria. Appl Microbiol Biotechnol 67:370–382.

Short KA, Doyle JD, King RJ, Seidler RJ, Stotzky G, Olsen RH. 1991. Effects of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. Appl Environ Microbiol 57:412–418.

Simon MA, Bonner JS, Page CA, Townsend RT, Mueller DC, Fuller CB, Autenrieth RL. 2004. Evaluation of two commercial bioaugmentation products for enhanced removal of petroleum from a wetland. Ecol Eng 22:263–277.

Singer AC, van der Gast CJ, Thompson IP. 2005. Perspectives and vision for strain selection in bioaugmentation. Trends Biotechnol 23:74–77.

Singh R, Paul D, Jain RK. 2006. Biofilms: Implications in bioremediation. Trends Microbiol 14:389–397.

Smets BF, Pritchard PH. 2003. Elucidating the microbial component of natural attenuation. Curr Opin Biotechnol 14:283–288.

Smith AE, Hristova K, Wood I, Mackay DM, Lory E, Lorenzana D, Scow KM. 2005. Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl tertiary butyl ether (MTBE). Environ Health Perspect 113:317–322.

Springael D, Top EM. 2004. Horizontal gene transfer and microbial adaptation to xenobiotics: New types of mobile genetic elements and lessons from ecological studies. Trends Microbiol 12:53–58.

Stocking AJ, Deeb RA, Flores AE, Stringfellow W, Talley J, Brownell R, Kavanaugh MC. 2000. Bioremediation of MTBE: A review from a practical perspective. Biodegradation 11:187–201.

Strong L, McTavish H, Sadowsky M, Wackett L. 2000. Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. Environ Microbiol 2:91–98.

Stroo HF, Major DW, Gossett JM. 2010. Bioaugmentation for Anaerobic Bioremediation of Chlorinated Solvents. In Stroo HF, Ward CH, eds, *In Situ* Remediation of Chlorinated

Solvent Plumes. SERDP/ESTCP Remediation Technology Monograph Series (C.H. Ward, ed). Springer, New York, NY, USA, pp 425–454.

Suflita JM, Liang LN, Saxena A. 1989. The anaerobic biodegradation of *o*-, *m*-, and *p*-cresol by sulfate-reducing bacteria enrichment cultures obtained from a shallow anoxic aquifer. J Ind Microbiol 4:255–266.

Suresh B, Ravishankar GA. 2004. Phytoremediation – A novel and promising approach for environmental clean-up. Crit Rev Biotechnol 24:97–124.

Swannell R, Head I. 1994. Bioremediation comes of age. Nat 368:396–397.

Sylvestre M, Macek T, Mackova M. 2009. Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs). Curr Opin Biotechnol 20:242–247.

Tagger S, Bianchi A, Juillard M, LePetit J, Roux B. 1983. Effect of microbial seeding of crude oil in seawater in a model system. Mar Biol 78:13–20.

Tam NF, Wong YS. 2008. Effectiveness of bacterial inoculum and mangrove plants on remediation of sediment contaminated with polycyclic aromatic hydrocarbons. Mar Pollut Bull 57:716–726.

Thompson IP, van der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: The challenge of strain selection. Environ Microbiol 7:909–915.

Timmis KN, Steffan RJ, Unterman R. 1994. Designing microorganisms for the treatment of toxic wastes. Annu Rev Microbiol 48:525–557.

Top EM, Springael D, Boon N. 2002. Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. FEMS Microbiol Ecol 42:199–208.

Top EM, Van Daele P, De Saeyer N, Forney LJ. 1998. Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. Antonie Leeuwenhoek 73:87–94.

Urgun-Demirtas M, Stark B, Pagilla K. 2006. Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. Crit Rev Biotechnol 26:145–164.

USEPA (U.S. Environmental Protection Agency). 1997. Microbial Products of Biotechnology: Final Rule. Federal Register 62:17910, April 11.

USEPA 2004. Cleaning up the Nation's Waste Sites: Markets and Technology Trends. EPA 542-R-04-015. USEPA, Washington, DC, USA. http://www.clu-in.org/market. Accessed March 15, 2012.

USEPA 2007. Treatment Technologies for Site Cleanup: Annual Status Report (Twelfth Edition). EPA-542-R-07-012. USEPA, Washington, DC, USA. http://www.epa.gov/tio/download/remed/asr/12/asr12_main_body.pdf.

Valdman E, Valdman B, Battaglini F, Leite SGF. 2004. On-line detection of low naphthalene concentrations with a bioluminescent sensor. Process Biochem 39:1217–1222.

Van Aken B. 2009. Transgenic plants for enhanced phytoremediation of toxic explosives. Curr Opin Biotechnol 20:231–236.

Van Aken B, Geiger S. 2011. Phytoremediation of Chlorinated Solvent Plumes. In Stroo HF, Ward CH, eds, *In Situ* Remediation of Chlorinated Solvent Plumes. SERDP/ESTCP Remediation Technology Monograph Series (Ward CH, ed). Springer, New York, NY, USA, pp 631–675.

Van Hamme JD, Singh A, Ward OP. 2003. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549.

Van Raemdonck H, Maes A, Ossieur W, Verthé K, Vercauteren T, Verstraete W, Boon N. 2006. Real time PCR quantification in groundwater of the dehalorespiring *Desulfitobacterium dichloroeliminans* strain DCA1. J Microbiol Methods 67:294–303.

van Veen JA, van Overbeek LS, van Elsas JD. 1997. Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61:121–135.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D, Mench M. 2009. Phytoremediation of contaminated soils and groundwater: Lessons from the field. Environ Sci Pollut Res 16:765–794.

Venosa A, Suidan M, Wrenn B, Strohmeier K, Haines J, Eberhart B, King D, Holder E. 1996. Bioremediation of an experimental oil spill on the shoreline of Delaware Bay. Environ Sci Technol 30:1764–1775.

Vogel TM. 1996. Bioaugmentation as a soil bioremediation approach. Curr Opin Biotechnol 7:311–316.

Vogel TM, Criddle CS, McCarty PL. 1987. Transformations of halogenated aliphatic compounds. Environ Sci Technol 21:722–736.

Vogel TM, Walter M. 2002. Bioaugmentation. In Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenbach LD, eds, Manual of Environmental Microbiology. ASM Press, Washington, DC, USA, pp 952–959

Vrionis HA, Anderson RT, Ortiz-Bernad I, O'Neill KR, Resch CT, Peacock AD, Dayvault R, White DC, Long PE, Lovley DR. 2005. Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl Environ Microbiol 71:6308–6318.

West KA, Johnson DR, Hu P, DeSantis TZ, Brodie EL, Lee PKH, Feil H, Andersen GL, Zinder SH, Alvarez-Cohen L. 2008. Comparative genomics of *Dehalococcoides ethenogenes* 195 and an enrichment culture containing unsequenced *Dehalococcoides* strains. Appl Environ Microbiol 74:3533–3540.

Weyens N, Van Der Lelie D, Artois T, Smeets K, Taghavi S, Newman L, Carleer R, Vangronsveld J. 2009. Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. Environ Sci Technol 43:9413–9418.

Wilson SC, Jones KC. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. Environ Pollut 81:229–249.

Winchell LJ, Novak PJ. 2008. Enhancing polychlorinated biphenyl dechlorination in fresh water sediment with biostimulation and bioaugmentation. Chemosphere 71:176–182.

Wu W-M, Carley J, Fienen M, Mehlhorn T, Lowe K, Nyman J, Luo J, Gentile ME, Rajan R, Wagner D, Hickey RF, Gu B, Watson D, Cirpka OA, Kitanidis PK, Jardine PM, Criddle CS. 2006. Pilot-scale in situ bioremediation of uranium in a highly contaminated aquifer. 1. Conditioning of a treatment zone. Environ Sci Technol 40:3978–3985.

Yang Y, Zeyer J. 2003. Specific detection of *Dehalococcoides* species by fluorescence in situ hybridization with 16 S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 69:2879–2883.

Zaidi S, Usmani S, Singh BR, Musarrat J. 2006. Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64:991–997.

Zanardini E, Pisoni C, Ranalli G, Zucchi M, Sorlini C. 2002. Methyl tert-butyl ether (MTBE) bioremediation studies. Ann Microbiol 52:207–221.

Zhuang X, Chen J, Shim H, Bai Z. 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environ Int 33:406–413.