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ASSESSMENT OF THE MICROBIAL COMMUNITIES
DERIVED FROM TWO TETRACHLOROETHENE
(PCE)-CONTAMINATED AQUIFERS

presented by

Rebekah R. Helton

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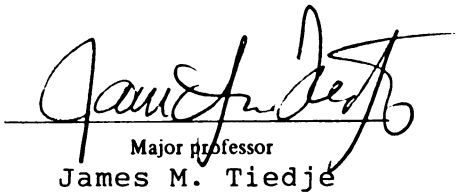
of the requirements for

M.S. degree in Crop & Soil Science



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ASSESSMENT OF THE MICROBIAL COMMUNITIES DERIVED FROM TWO TETRACHLOROETHENE (PCE)-CONTAMINATED AQUIFERS

By

Rebekah R. Helton

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ABSTRACT

ASSESSMENT OF THE MICROBIAL COMMUNITIES DERIVED FROM TWO TETRACHLOROETHENE (PCE)-CONTAMINATED AQUIFERS

By

Rebekah R. Helton

Tetrachloroethene (PCE) and trichloroethene (TCE) are among the US-EPA's listing of priority pollutants and are suspected human carcinogens. PCE and TCE have been used for many purposes including degreasing solvents and dry cleaning agents. However, poor handling, leaky disposal tanks, and intentional dumping has caused many groundwater systems to become contaminated.

The objective of this thesis was to examine the microbial communities associated with the anaerobic reductive dechlorination of PCE from two PCE-contaminated aquifers. The sites chosen for this study were Jacksonville, FL, and Oscoda, MI. Solvent Extraction Remediation Biotreatment (SERB) was used by at the Jacksonville, FL, site and bioaugmentation from a PCE-dechlorinating bioreactor was used at the Oscoda, MI, site. The Oscoda, MI, site showed no known dechlorinators in the microbial community of the PCE-to ethene (ETH) dechlorinating bioreactor inoculated from the Oscoda, MI, site sediments.

The indigenous microbial community structures were evaluated by PCR based molecular studies using 16S rDNA for terminal restriction fragment length polymorphism (T-RFLP). The Jacksonville, FL, site showed limited reductive dechlorination of PCE in anaerobic microcosms and a loss of microbial diversity from the ethanol treatment.

For Sampson, who will always remain my best friend

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Jamie Vanderwal, Leann Hopkins, Minna Laine, Olga Malstevea, Sveta Dedysh, Mike Aiello, and Joyce Wildenthal for their valuable discussions, patience, and suggestions.

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LIST OF ABBREVIATIONS

ARDRA.....amplified ribosomal DNA restriction analysis

ATSDR.....Agency for Toxic Substances and Disease Registry

cDCE.....*cis*-1,2-dichloroethene

CMEIAS.....Center for Microbial Ecology Image Analysis System

DNA.....deoxyribonucleic acid

GC-FID.....gas chromatography – flame ionization detection

LSM.....laser scanning confocal microscope

PCE.....tetrachloroethene

PCR.....polymerase chain reaction

SERB.....solvent extraction remediation biotreatment

TCE.....trichloroethene

tDCE.....*trans*-1,2-dichloroethene

TRFs.....terminal restriction fragments

T-RFLP.....terminal restriction fragment length polymorphism

US-EPA.....United States Environmental Protection Agency

VC.....vinyl chloride

CHAPTER I

INTRODUCTION AND HISTORY

OVERVIEW

Chlorinated aliphatic compounds such as tetrachloroethene (PCE) and trichloroethene (TCE) are among the United States Environmental Protection Agency's (US-EPA) listing of priority pollutants given that the compounds are suspected human carcinogens. PCE and TCE have been widely used for a multitude of purposes including military and industrial de-greasing solvents and dry cleaning agents. Unfortunately, the lack of adequate handling, unsecured disposal tanks, and the intentional dumping of chloroethenes has polluted the groundwater supply in some regions.

PCE can be reductively dechlorinated (Figure 1.1) by some microorganisms under anaerobic conditions by sequential dechlorination through intermediates TCE, *cis*-1,2-dichloroethene (cDCE) or *trans*-1,2-dichloroethene (tDCE), vinyl chloride (VC) finally to ethene (ETH) (de Bruin, *et al.*, 1992; Freedman and Gossett, 1989; Tandoi, *et al.*, 1994). PCE was observed to be completely reduced to ETH biotically under laboratory conditions (DiStefano, *et al.*, 1992; Magnuson, *et al.*, 1998; Wu, *et al.*, 1998) as well as completely detoxified under field conditions after biostimulation and bioaugmentation techniques (Beeman, *et al.*, 1994; MacNaughton, *et al.*, 1999; McCarty, 1993; Munakata-Marr, *et al.*, 1997; Spuij, *et al.*, 1997). Abiotic reductive dechlorination of PCE to lesser

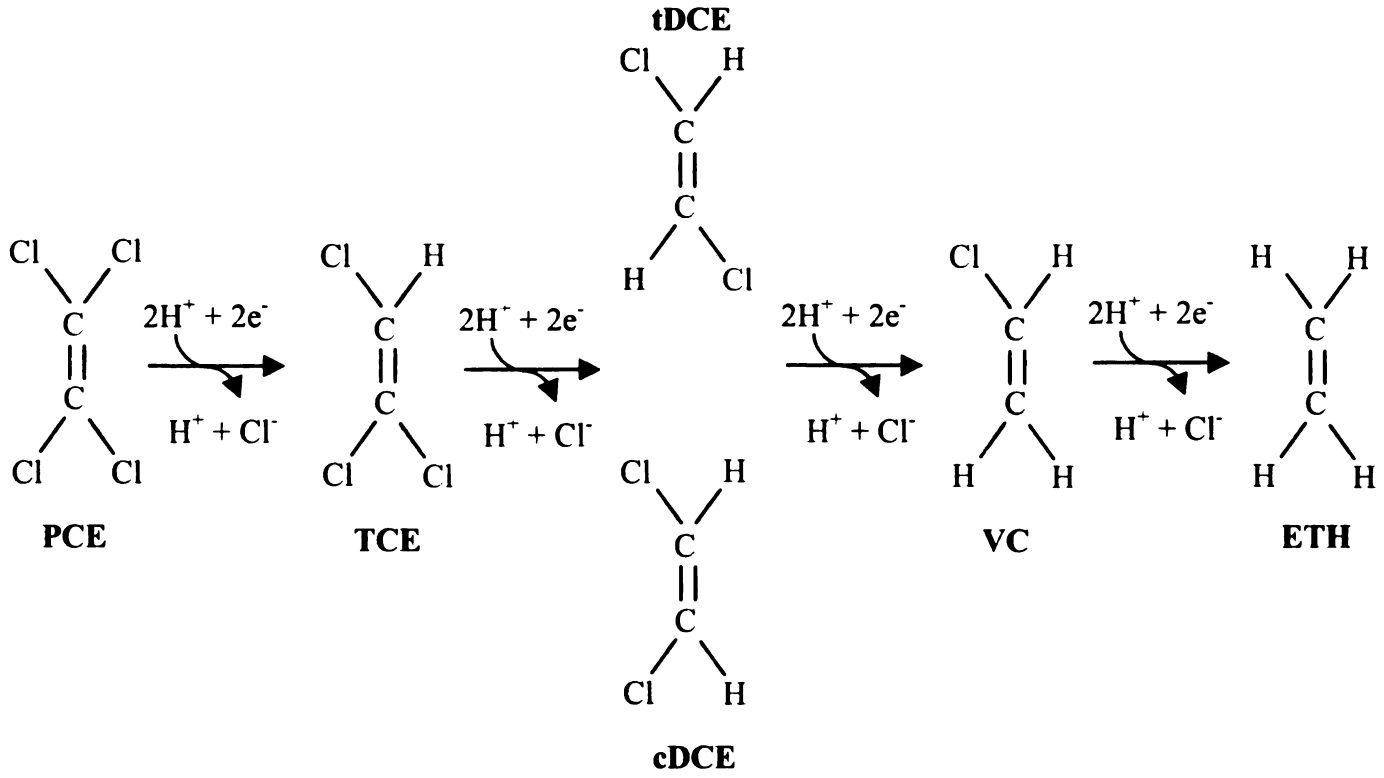


Figure 1.1. Sequential reductive dechlorination of tetrachloroethene (PCE) to ethene (ETH). Each reduction step requires an electron donor and a hydrogen to dislocate the attached chlorine.

chlorinated ethenes is also possible with reduced metal co-factors and some metals (Burris, *et al.*, 1996).

BACKGROUND

Occurrence of chloroethenes. All chloroethenes are toxic, although vinyl chloride is the only chloroethene proven to be a human carcinogen (ATSDR, 1997, 1997, 1997). Much research has focused on the biodegradation of xenobiotic chlorinated ethenes, but there has been little attention to their natural occurrence. Even though there are large anthropogenic sources, TCE and PCE have been found to naturally occur in sea water and volcanoes, possibly as biosynthetic intermediates and often in fairly high concentrations (Abrahamsson, *et al.*, 1995; Gribble, 1994, 1996). This natural production may be responsible for the evolution of microbial mediated catabolic activities currently used for bioremediation of xenobiotic sources.

Even though human production of these compounds has increased the concentrations in certain environments, natural sources account for approximately 2000 halogenated compounds (Gribble, 1994), including PCE and TCE. But this does not compare with the pollution caused by human production. Chlorinated ethenes have been manufactured since the 1940's for a multitude of purposes (Table 1.1). With accidental and intentional dumping, poor handling and waste drums that leak, these harmful compounds have caused serious concern after entering many groundwater systems in high concentrations (Table 1.2). The adverse health effects of these chemicals such as central nervous system disorders and leukemia are summarized in Table 1.3.

Table 1.1. Industrial and domestic sources of chloroethenes.

Dry cleaning agents	Typewriter correction fluid	Industrial metal de-greasers	Auto polish, cleaners and brake quieters	Water repellants
Spot removers and aerosol cleaners	Wood cleaners	Adhesives	Oven cleaners	Electrical transformer cooling agent
Refrigerants	Caffeine extractant for coffee, soda and teas	Suede and leather protectors	Printing inks	Silicone and brake lubricants
Paper, pulp and rubber manufacturing	Iron and steel production	Pesticides and fumigants for insects and rodents	disinfectant for surgical instruments	Cosmetic and pharmaceutical manufacturing
PVC production	Ignition wire driers	Electroplating of metals	Surgical anesthetic	Rug and upholstery cleaners
Paint and varnish removers	Textile finishes	Nonstructural caulking compounds and sealants	Laundry starch preparations	Solvents for fats, waxes and gums

Table 1.2. Total environmental release of PCE, TCE, and VC. States are ranked according to those with the most chloroethenes, as well Michigan and Florida, the two states sampled for this study. Amounts listed are in pounds.

	PCE	
1.	Kansas	1,162,486
2.	California	1,001,183
3.	Ohio	698,132
4.	Virginia	343,755
5.	Connecticut	338,219
22.	Michigan	61,070
24.	Florida	42,754
	TCE	
1.	Illinois	2,600,761
2.	Indiana	1,770,804
3.	Pennsylvania	1,623,785
4.	Wisconsin	843,630
5.	Virginia	834,043
7.	Michigan	802,492
19.	Florida	282,683
	VC	
1.	Texas	240,215
2.	Delaware	144,300
3.	Louisiana	122,625
4.	Pennsylvania	121,501
5.	Illinois	170,005
9.	Florida	15,380
10.	Michigan	10,095

Table 1.3. Exposure source and health effects associated with PCE, TCE and VC as described by the Agency for Toxic Substances and Disease Registry (ATSDR).

Tetrachloroethene (PCE)		**Trichloroethene (TCE)**		**Vinyl Chloride (VC)**	
Exposure	*Effects*	*Exposure*	*Effects*	*Exposure*	*Effects*
breathing contaminated air	respiratory tract irritation	breathing contaminated air	respiratory toxin	breathing contaminated air	liver and nerve damage
ingestion of contaminated water or food	liver and kidney cervical, skin, urogenital damage and cancer	ingestion of contaminated water or food	fetal mal-development in exposed mothers	ingestion of contaminated water	sperm and testes, lung and kidney damage
medicine to eliminate hookworms, intestinal flukes, nematodes in humans	central nervous system: dizziness, headache, disorientation, difficulty in speech or movement, unconsciousness, death	vapors from contaminated drinking, swimming and shower waters	impaired heart functions, coma, narcosis, death, hallucinations, convulsions, drowsiness, jaundice, vomiting, psychosis, depression	as a breakdown product of other xenobiotics	liver, brain and lung cancers
dermal contact to dry-cleaned clothes		an anesthetic in surgical procedures		tobacco smoke	leukemia
breast milk of exposed pregnant woman (possible concentration)	leukemia	breast milk of exposed pregnant woman	neurotoxin to liver, facial nerves, kidneys, reproductive toxin	vapors from contaminated drinking, swimming and shower waters	immune reactions
an anesthetic in surgical procedures	fetal mal-development in exposed mothers cancer		skin vesication, paralysis of the fingers, dermatitis		fetal defects in exposed mothers, and miscarriages
					central nervous system: dizziness, drowsiness, death, headache, disorientation

Reductive dechlorination. Reductive dechlorination through biological means has been well documented in anaerobic ecosystems (Bolesch, *et al.*, 1997; de Bruin, *et al.*, 1992; DiStefano, 1999; DiStefano, *et al.*, 1991; Fathepure, *et al.*, 1987; Freedman and Gossett, 1989). The reduction of a chloroethene to a chloride with hydrogen is exergonic with the resulting $\Delta G^{\circ\prime}$ of –376 kJ/mol of chloride produced. In the dechlorination pathway PCE is reduced sequentially by several dehalogenase enzymes (Figure 1.1).

PCE had previously been found to be degraded completely to ETH via a consortium of anaerobic bacteria (Cabirol, *et al.*, 1996; DiStefano, *et al.*, 1991; Wild, *et al.*, 1995; Zinder and Gossett, 1995). By supplying dechlorinating cultures with an electron donor, the rate and extent of dechlorination could be increased. Currently known organisms capable of reductive dechlorination of PCE include *Desulfitobacterium dehalogenans* strain PCE1, *Dehalobacter restrictus*, *Dehalospirillum multivorans*, *Desulfitobacterium chlororespirans* strain SF3 and *Dehalococcoides ethenogenes*.

Each of those mentioned microorganisms reduce PCE to cDCE with the exception of *D. ethenogenes*, which is capable of complete reductive dechlorination to VC and a co-metabolic reduction of VC to ETH. Although undefined dechlorinating cultures are capable of using various electron donors such as methanol, formate, hydrogen, acetate or glucose, *D. ethenogenes* requires hydrogen as the electron donor for the reduction of PCE (Maymo-Gatell, *et al.*, 1999). No other substrates have been identified which support growth of *D. ethenogenes*.

MICROBIAL COMMUNITIES

Microbial community composition. What makes up a microbial community? What resources does a community need to function and survive in the environment? These questions are slowly being addressed. Microbial communities in nature are complex, diverse and important. However, very little is known about their structure and their responses to environmental stresses. Microbes maintain global processes by cycling nutrients including those in wastes generated by humans. The diversity and complexity of their environment provides numerous ecological niches, which are dependent on factors such as location, pH, nutrient and water availability, and temperature. Changes to any of these factors, due to spatial heterogeneity, surface area, macro- and micro-aggregate formations, and human influence can alter or create new niches in the environment for these microorganisms (Cho and Kim, 2000; Kaufman, *et al.*, 1999; MacNaughton, *et al.*, 1999; Nüsslein and Tiedje, 1998; Ovreas, *et al.*, 1998; Snaidr, *et al.*, 1997). However, the entire microbial habitat and structure is still not fully understood for most environments.

16S rRNA use to define communities. The study of microbial community diversity after 100 years of pure culture study is vastly incomplete. The total number of microbial species in culture account for only a small fraction of all microorganisms thought to exist in nature, (*e.g.* Torsvik, *et al.*, 1990). Until the middle of the last decade, enumeration and identification of environmental microbes relied on phenotypic methods, which restricted knowledge to those microbes capable of being cultivated on media. Other less abundant or less responsive microorganisms were not detected, providing an incomplete community profile. "It is estimated that less than 10% of the organisms from

the natural environment can be isolated using traditional techniques," (Brock, 1987). Unfortunately, due to the dynamics of microbial communities and limitations of enrichment conditions, culture results are often not reproducible. Serious bias can be introduced through laboratory cultivation given that direct plating experiments are typically dependent on the media type and purification techniques used (Boivin-Jahns, *et al.*, 1995). Activities of microbes *ex situ* may also differ from those *in situ* due to the lack of interactions with the environment and other organisms.

Using molecular techniques based on phylogenetic analysis of 16S rDNA, a culture independent approach was developed to more comprehensively evaluate the structure of microbial communities. The comparison of 16S rDNA sequences has advanced microbial identification to an evolutionarily based paradigm (Olsen, *et al.*, 1986; Woese, 1987). All forms of life are now separated into three major domains based on an organism's 16S rRNA genes and some other key features; these are Bacteria, Archaea or Eukaraya (Woese, *et al.*, 1990). The analysis of 16S rDNA sequences is widely accepted as an investigative biomarker for numerous reasons. 16S rRNA is essential to protein synthesis, ubiquitous in all organisms, and structurally as well as functionally conserved. These genes are readily isolated and identified, and possess both variable and highly conserved regions in both the primary and secondary structures. The rRNA gene sequences slowly evolve without horizontal gene transfer. This molecule has been useful for ecological studies of the microbial communities in complex environments.

While culture methods suggest a less than 10% recovery in natural communities (Boivin-Jahns, *et al.*, 1995; Torsvik, *et al.*, 1990; Ward, *et al.*, 1990), molecular

approaches can examine a higher level of diversity (Tiedje, *et al.*, 1999; Tiedje, *et al.*, 1997; Ward, *et al.*, 1990). To date, over 16,000 aligned prokaryotic sequences have been made available via the Ribosomal Database Project, release 8.0 (Maidak, *et al.*, 2000). The development and application of PCR has made rRNA sequences easily obtainable, and other studies in molecular ecology from various environments have showed this technique to be appropriate (Borneman, *et al.*, 1996; Buckley, *et al.*, 1998; Giovannoni, *et al.*, 1990; Lee, *et al.*, 1996; Moyer, *et al.*, 1995; Schmidt, *et al.*, 1991). Microbial community diversity can be examined and evaluated by sequencing 16S clone libraries to reveal extensive diversity of rRNA genes and novel sequences that are only distantly related to those known from cultivated species (Zhou, *et al.*, 1997).

Yet, 16S rDNA-based studies include potential biases by not reflecting the total microbial community. A clone library of PCR products may not be representative of the most successful community members due to patchy distribution or aggregation, unequal lysis of cells, lost DNA during purification procedures, humic inhibition of PCR amplification, or preferential amplification of certain organism's rDNA. Also, the PCR amplification can produce mixed genomes leading to the formation of chimeras , or the amplification of DNA included in the *Taq* polymerase (Amann, *et al.*, 1995; Liesack and Stackebrandt, 1992; Schmidt, *et al.*, 1991; Wang and Wang, 1997).

Because reductive dechlorinators are difficult to impossible to culture, I used a 16S rDNA based methodology and used well characterized eubacterial primers thought to recover most of the known bacterial domains in the RDP. For both the Florida and Michigan sites studied, 16S rDNA was amplified from aquifer communities and subsequently analyzed by terminal restriction length polymorphism (T-RFLP). For the

Michigan site, the amplified ribosomal DNA restriction analysis (ARDRA) was also used.

Terminal restriction fragment length polymorphism of 16S rRNA genes can demonstrate the presence of phylogenetically distinct bacteria within a complex microbial community. T-RFLP is an easy, rapid procedure requiring no cloning steps. It reveals differences between community profiles. However, it does lack detailed resolution and information about microbial abundance can only be qualitatively estimated by peak area or height. The T-RFLP technique is the best method to monitor the appearance and disappearance of TRFs (terminal restriction fragments) as well as detection or no detection of TRFs in combination with other molecular methods (Avaniss-Aghajani, *et al.*, 1996; Horz, *et al.*, 2000; Liu, *et al.*, 1997; Marsh, 1999; Moeseneder, *et al.*, 1999).

BIOREMEDIATION

Major research efforts are being focused toward developing feasible processes that will clean up contaminated sites and reduce the risk of human exposure to toxic and carcinogenic compounds. Bioremediation techniques hold promise as effective, low cost means to detoxify or eliminate some pollutants (Alexander, 1994; Baker, 1994; Hazen, 1997; McCarty, 1993; Semprini, 1995; Tiedje, 1993). It can only be successful if the contaminant can be degraded, the microbes able to degrade it are present and if any environmental limitations are present, so that there are feasible remedies to implement. In an ideal world, the native microorganisms would completely metabolize the contaminant into harmless end-products of CO_2, Cl^-, and H_2O, releasing energy from

catabolic reactions (Bolesch, *et al.*, 1997; de Bruin, *et al.*, 1992; DiStefano, *et al.*, 1991; Talley and Sleeper, 1997).

Several types of remedial alternatives are available, including natural attenuation, air sparging, pump and treat, biostimulation, and bioaugmentation. Natural attenuation relies on natural, unaided processes to degrade the pollutants to non-toxic levels without human manipulation of the environment (Lorah, *et al.*, 1997; Parker and Mohr, 1996; Pope and Jones, 1999). However, to be considered a successful means of remediation, natural attenuation must accomplish the desired effects within a reasonable period. Often times, this is not the case and more active processes must be implemented in order to complete the remediation process. The biological reductive dechlorination of PCE can also lead to end products that are more hazardous, such as the known human carcinogen, VC. Without enhanced remediation techniques, the resulting chloroethene product would prove to be a more dangerous problem than the initial contamination problem.

High concentrations PCE and TCE also tend to localize in dense nonaqueous-phase liquids (DNAPLs) in aquifers (Nielsen and Keasling, 1999). Due to the fact that the DNAPLs density is much greater than that of the groundwater, these pools of PCE and TCE are recalcitrant to degradation and require more effective remediation techniques for removal (Nielsen and Keasling, 1999; Rothmel, *et al.*, 1998). Although pump and treat methods have been typical remediation techniques, as well as air sparging, they are not cost effective because of the excessively long pumping times to remove the DNAPLs (Rothmel, *et al.*, 1998; Semprini, 1995). Methods of *in-situ* remediation processes are important to develop in order to speed remediation and

remediation costs. The bioremediation techniques used at the PCE-contaminated aquifer sites evaluated in this thesis were biostimulation and bioaugmentation.

In biostimulation, the goal is to promote the activity of indigenous microbes with nutrients and other substrates that will enable them to more rapidly degrade the contaminant of interest. Factors affecting degradation of PCE include: bioavailability, moisture content, pH, and temperature. Microbial communities require both an electron donor and an acceptor in order to capture energy and grow. Previous studies have shown that supplying an electron donor, such as ethanol, stimulates the reductive dechlorination of PCE (Carr and Hughes, 1998; Gibson and Sewell, 1992; Middeldorp, *et al.*, 1998). High concentrations of PCE inhibit methane producing organisms. Methanogens have been shown to be partly responsible for the reduction of chloroethenes (Anderson and McCarty, 1997; Fogel, *et al.*, 1986; Freedman and Gossett, 1989; Semprini, *et al.*, 1990; Sullivan, *et al.*, 1998; Vogel and McCarty, 1985). However, PCE is now known to be more readily dechlorinated by organisms other than methanogens (DiStefano, *et al.*, 1991; Maymo-Gatell, *et al.*, 1995). Over-stimulation is possible as well, with excessive microbial growth clogging the aquifer pores and limiting the biostimulation efforts. This can be overcome by inoculating in pulses to permit migration and substrate adaptation. However, this is more often a problem with aerobic processes due to low cell yield. However, once biostimulation is better understood and evaluated, it could quickly and efficiently assist the bioremediation attempts from other PCE-contaminated sites by providing the necessary requirements.

Bioaugmentation is the process of amending a site with external microorganisms along with their supporting nutrients, to reduce or eliminate the environmental

contamination. It is necessary to determine if the existing microbial community is inadequate for sufficient contaminant removal. This method requires having in hand microorganisms that are able to perform the task and a method of producing enough for field inoculation. In order to prove the effectiveness of the inoculation, a suitable method for tracking the movement of the bacteria in the aquifers is needed (Harvey, 1993). Unfortunately there is no set method to determine what nutrients are optimally required for bioaugmentation of all contaminated sites. Hence, preliminary studies and experiments are required to establish an efficient level of nutrients able to support the desired bioremediation effects, which then are only site specific. By understanding the complexities of other sites, one may be able to identify a more time efficient means of establishing the correct remediation efforts for new contaminated sites.

The goal for PCE bioremediation is to reduce the chloroethene concentrations to below the regulatory standards, and hence to non-hazardous levels. Reductive dechlorinating PCE to ETH cannot now be reliably achieved in the field, probably because different indigenous communities yield different outcomes. Hence, site specific research is attempted to identify which remediation strategy to use. Knowledge in microbial communities may also help in choosing the best remediation strategy.

RESEARCH PURPOSE

The primary objective of this thesis is to characterize the microbial communities associated with the anaerobic reductive dechlorination of PCE from PCE-contaminated aquifer sediments at two sites. The sites chosen for this study were located in

Jacksonville, Florida, and Oscoda, Michigan. Each of the afore mentioned sites was contaminated with PCE originating from former dry cleaning establishments.

Jacksonville, Florida, site. The goals for evaluating the microbial community at the Jacksonville site were to (i) evaluate the microbial population response to a large ethanol addition (SERB process), (ii) determine which electron donor(s) support anaerobic reductive dechlorination of PCE, and (iii) determine the effect of residual ethanol on the reductive dechlorination process.

The former Sages Dry-cleaning establishment had contaminated the aquifer with PCE. The Solvent Extraction Remediation Biotreatment (SERB) technology was employed to flush the highly contaminated source area with a high concentration of ethanol (95%) to remove the DNAPL. However, while a majority of the solubilized chlorinated ethenes and injected ethanol was recovered, a residual mixture of ethanol and chloroethenes remained. I attempted to evaluate any shift in the microbial community due to the large ethanol flush. Understanding the effects of the SERB treatment on the microbial community is necessary in order to determine whether the microbial reductive dechlorination process can be employed as a polishing step to detoxify residual chloroethenes. The indigenous microbial community structures were evaluated from aquifer sediment samples which were collected prior to the SERB in June, 1998, and post-SERB in July, 1999.

Reductive dechlorination of PCE was assessed under chlororespiring conditions in anaerobic microcosms constructed from site materials amended with PCE. These microcosms were supplied with several electron donor conditions including acetate, lactate, hydrogen, ethanol, succinate, whey, molasses, starch, and no electron donor as a

control. PCE dechlorination to TCE and cDCE, with a transient appearance of tDCE, was detected in all treatments, and in microcosms that were not amended with additional substrates.

This study was conducted under the State of Florida Dry-cleaning Solvent Cleanup Program, together with the owners of the contaminated site. The program is managed through the Florida Department of Environmental Protection (FDEP). Funding for the project came from the US-EPA – TIO, the State of Florida, the Strategic Environmental Research and Development Program (SERDP) and the Federal Integrated Biotreatment Research Consortium (FIBRC). Groups involved with work at the site included Michigan State University, the University of Florida, Levine Fricke Recon (LFR), and the US-EPA, Ada, Okalahoma Laboratories.

Oscoda, Michigan, site. The aim of the study at the Bachman Road site in Oscoda, was to evaluate the microbial community of a bioreactor to be used for the bioaugmentation of the site. Bachman Road aquifer sediments were previously evaluated for dechlorinating activity in microcosms with acetate, glycerol, glucose, formate, lactate, molasses, and whey as electron donors. Following several months of incubation, complete dechlorination of PCE was observed with lactate, fumarate, glucose and whey. A chlororespiring bacterium, BRS-1, was isolated from the site and identified as a *Desulfuromonas* species (Löffler, *et al.*, 2000). An enrichment was also established from the site that readily converted cDCE to ETH with H2 as the electron donor (Loffler, *et al.*, 2000). Initial experiments evaluated the kinetics of PCE or cDCE dechlorination using BRS-1 or the cDCE dechlorinating enrichment to evaluate the feasibility of a field bioaugmentation study (Löffler, *et al.*, 2000). Strain BRS-1 and the cDCE-dechlorinating

mixed culture were used to inoculate a fed-batch bioreactor to grow sufficient inoculum for a field experiment. The reactor, under non-sterile conditions, successfully operated for over 12 months and completely dechlorinated PCE to ETH. Sufficient biomass was produced for a field bioaugmentation experiment in autumn 2000.

Reactor samples were taken at various time points to examine microbial community dynamics and the overall stability of the reactor. For my study, a reactor sample taken on August 14, 2000, was used to determine the composition of the PCE-dechlorinating community. The bioaugmentation procedure was performed by transferring the bioreactor inoculum to the previously lactate amended field site in October, 2000.

Feasibility studies for the field demonstration of this technology were funded by the State of Michigan Division of Environmental Quality. The bioreactor was maintained by EFX Systems, Inc., located in Lansing, Michigan. The study, Remediation of Chlorinated Solvents at the Bachman Road Site Using Innovative Technologies: Microbial Halorespiration and Surfactant-Enhanced Aquifer Remediation, was performed in collaboration with the University of Michigan and Georgia Institute of Technology through the Great Lakes and Mid-Atlantic US-EPA Hazardous Substance Research Center (GLMA-HSRC).

CHAPTER II

MICROBIAL COMMUNITY ANALYSIS OF A TETRACHLOROETHENE (PCE)-CONTAMINATED AQUIFER AFTER SOLVENT EXTRACTION REMEDIATION BIOTREATMENT (SERB).

INTRODUCTION

Tetrachloroethene (PCE) and its reduced daughter products, trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), and vinyl chloride (VC), are a class of ubiquitous environmental pollutants which have members classified as probable or actual human carcinogens (ATSDR, 1997, 1997, 1997; ECO-USA, 1990, 1995, 1995). Several instances demonstrated that PCE and TCE contamination is a serious public health concern. For instance, in Woburn, Massachusetts, several children died from leukemia after prolonged consumption of TCE-contaminated groundwater. Many Woburn adult residents suffered from symptoms associated with solvent poisoning, *e.g.* nausea, abdominal cramps, sore throats, rashes, diarrhea, skin eruptions, and death (Bair and Wood, 1999; Harr, 1996). While the cause of these effects was never clearly established, the correlation was remarkable.

Chlorinated ethenes have been included on the U. S. Environmental Protection Agency's (US-EPA) listing of priority pollutants (US-EPA, 2000). PCE and TCE have been widely used as industrial solvents and dry cleaning agents. Unfortunately, due to poor handling, leaky disposal tanks and direct dumping, these hazardous materials have accumulated in aquifers (Holliger, 1995).

Obviously, the most effective means of dealing with environmental contamination is to prevent the release of these toxic compounds. Unfortunately, post-handling and proper disposal methods have been less than adequate. Effective management of hazardous waste handling and proper disposal is crucial to protect humans and nature. Because this has not consistently occurred, major research efforts are focused on determining feasible processes that enhance remediation of currently contaminated sites and on reducing the risk of human exposure to toxic and carcinogenic compounds while returning the contaminated sites as close as possible to their original composition.

PCE was shown to be reductively dechlorinated under anaerobic conditions by sequential dechlorination through the intermediates TCE, cDCE, VC, and finally to ethene (ETH) (de Bruin, *et al.*, 1992; Freedman and Gossett, 1989; Magnuson, *et al.*, 1998; Tandoi, *et al.*, 1994). Complete dechlorination of chloroethenes has been observed under laboratory conditions, and a few field studies have demonstrated that complete detoxification is possible through biostimulation and bioaugmentation (Beeman, *et al.*, 1994; MacNaughton, *et al.*, 1999; McCarty, 1993; Munakata-Marr, *et al.*, 1997; Spuij, *et al.*, 1997). Ideally, the dechlorinating microorganisms completely transform the contaminants into non-toxic, environmentally benign end-products of CO_2, Cl^-, and H_2O, while releasing energy for growth thus generating microbial biomass. Microbial remediation processes have attracted considerable attention over the last decade due to the fact that a bioremediation process is often less costly than traditional pump and treat technology.

The objective of this study was to evaluate the microbial community in an aquifer contaminated with PCE from the Sages Dry Cleaning establishment in Jacksonville,

Florida. The PCE-DNAPL (dense non-aqueous phase liquids) source area was flushed and biostimulated by using a solvent extraction remediation biotreatment (SERB). The US-EPA installed injection wells upstream of the source area and injected 9,000 gallons of 95% ethanol/ 5% water. The mixture was hydraulically removed from downstream extraction wells. More than 90% of the PCE (40 L free phase) and 99% of the injected ethanol were recovered. The ethanol that remained in the aquifer was expected to stimulate reductive dechlorination of the residual chloroethenes. The site was monitored for 13 months post-SERB for the PCE concentration changes in the plume (Figure 2.1), and the appearance of daughter chloroethene products (Figures 2.2 and 2.3). The plume concentration data is extrapolated based on concentrations seen at the available monitoring wells. The US-EPA estimated that the complete removal of chloroethenes from the site will take from 3-30 years following SERB.

Aquifer sediment was collected near the injection wells and monitoring wells downgradient of the source area pre- and post-SERB. The shifts in the indigenous microbial community and its ability to reductively dechlorinate the residual contaminants were monitored. The extent of PCE dechlorination was assessed in anaerobic microcosms, initiated by S.J. Flynn. The microbial community was also examined using fluorescence microscopy and image analysis. In addition, a 16S rRNA gene based molecular approach was used to monitor the community dynamics due to SERB.

Total community DNA was isolated, and bacterial 16S rRNA genes were PCR-amplified using universal primers 8F and 1392R. Amplified 16S rDNA was analyzed using terminal restriction fragment length polymorphism (T-RFLP). Specific primers for detecting PCE-dechlorinating *Desulfuromonas* species and *Dehalococcoides* species

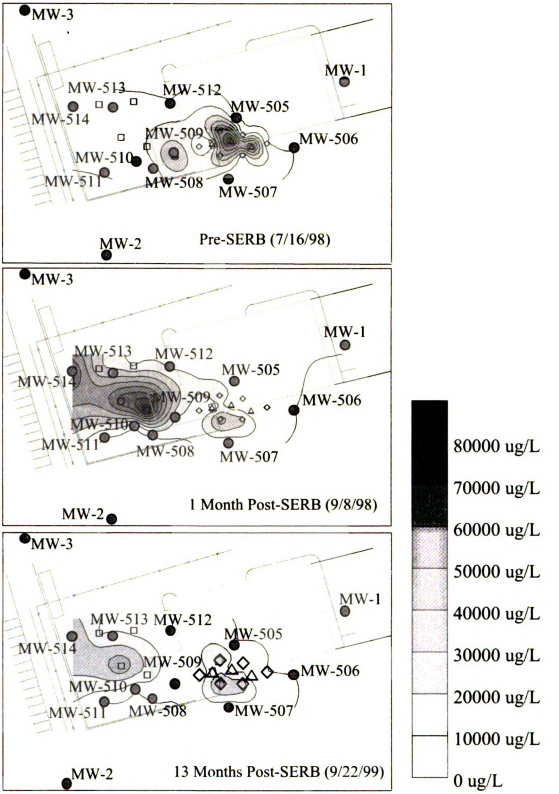


Figure 2.1. PCE plume maps of the site indicating the changes in PCE concentrations over the time period of 13 months. Figures are kindly supplied by G.W. Sewell.

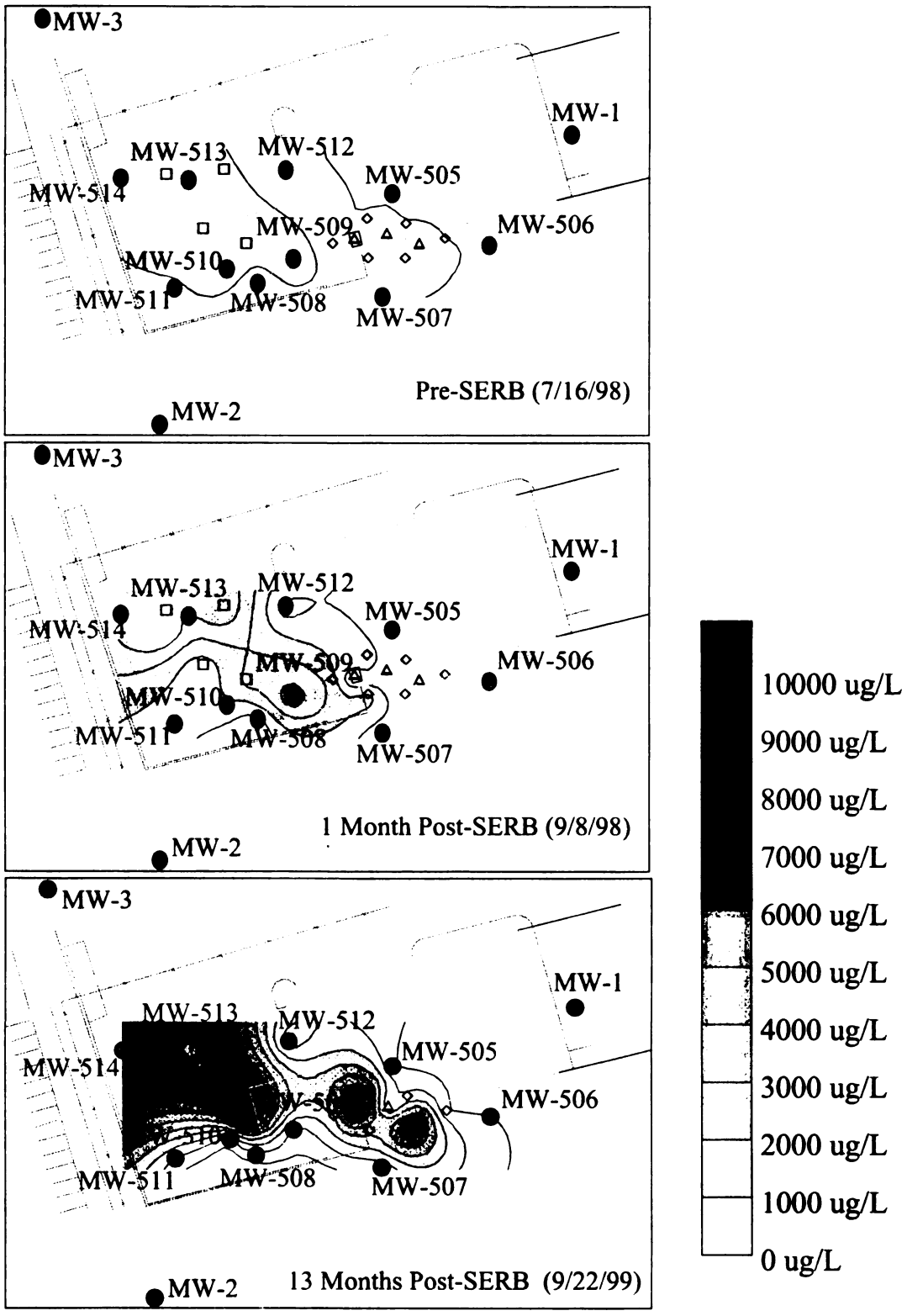


Figure 2.2. TCE plume maps of the site indicating the changes in TCE concentrations over the time period of 13 months. Figures are kindly supplied by G.W. Sewell.

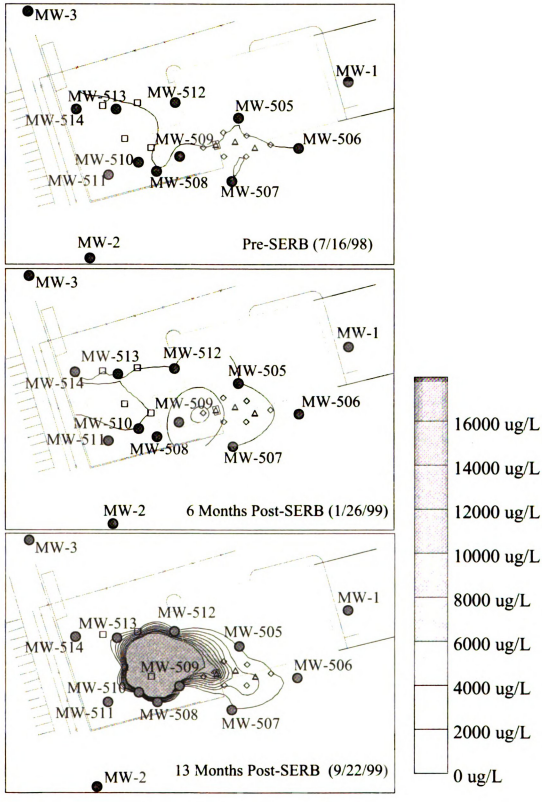


Figure 2.3. cDCE plume maps of the site indicating the changes in concentrations over the time period of 13 months. Figures are kindly supplied by G.W. Sewell.

were used to determine the presence of such populations (Löffler, *et al.*, 2000). *Dehalococcoides* species are the only known group of bacteria that completely dechlorinates PCE to ETH (Maymo-Gatell, *et al.*, 1999).

MATERIALS AND METHODS

Chemicals. PCE, TCE, and cDCE were obtained from Aldrich Chemical Co. (Milwaukee, WI). Gaseous VC was obtained from Fluka Chemical Corp. (Ronkonkoma, NY) and ethene from AGA Gas, Inc., (Cleveland, OH). Standards were prepared as described by Gossett (1987).

Aquifer sample collection. Aquifer core samples were collected downgradient from the former Sages Dry Cleaning establishment in Jacksonville, FL, now covered by a cement cap. The sampling site was located directly beside a canal that flows into the St. Johns River which flows into the Atlantic Ocean. The site has an average subsurface temperature of 24.3°C and a pH range of 3.28 – 6.76. Cores from the source area, dissolved plume and unaffected zones were taken at average depths of 30 ft below ground surface (Figure 2.4). Post-SERB sediments were collected after the ethanol front had passed the monitoring wells. Samples were immediately shipped to the Center for Microbial Ecology (CME) in ice-packed coolers and promptly stored at 4°C upon arrival. Soil physical and chemical analyses were done at the Soil Analysis Laboratory at Michigan State University, and by Levine-Fricke-Recon, site engineers.

Microcosms. Microcosms were established in sterile 20 ml serum vials assembled within an anaerobic chamber with a nitrogen-hydrogen content of 97/3

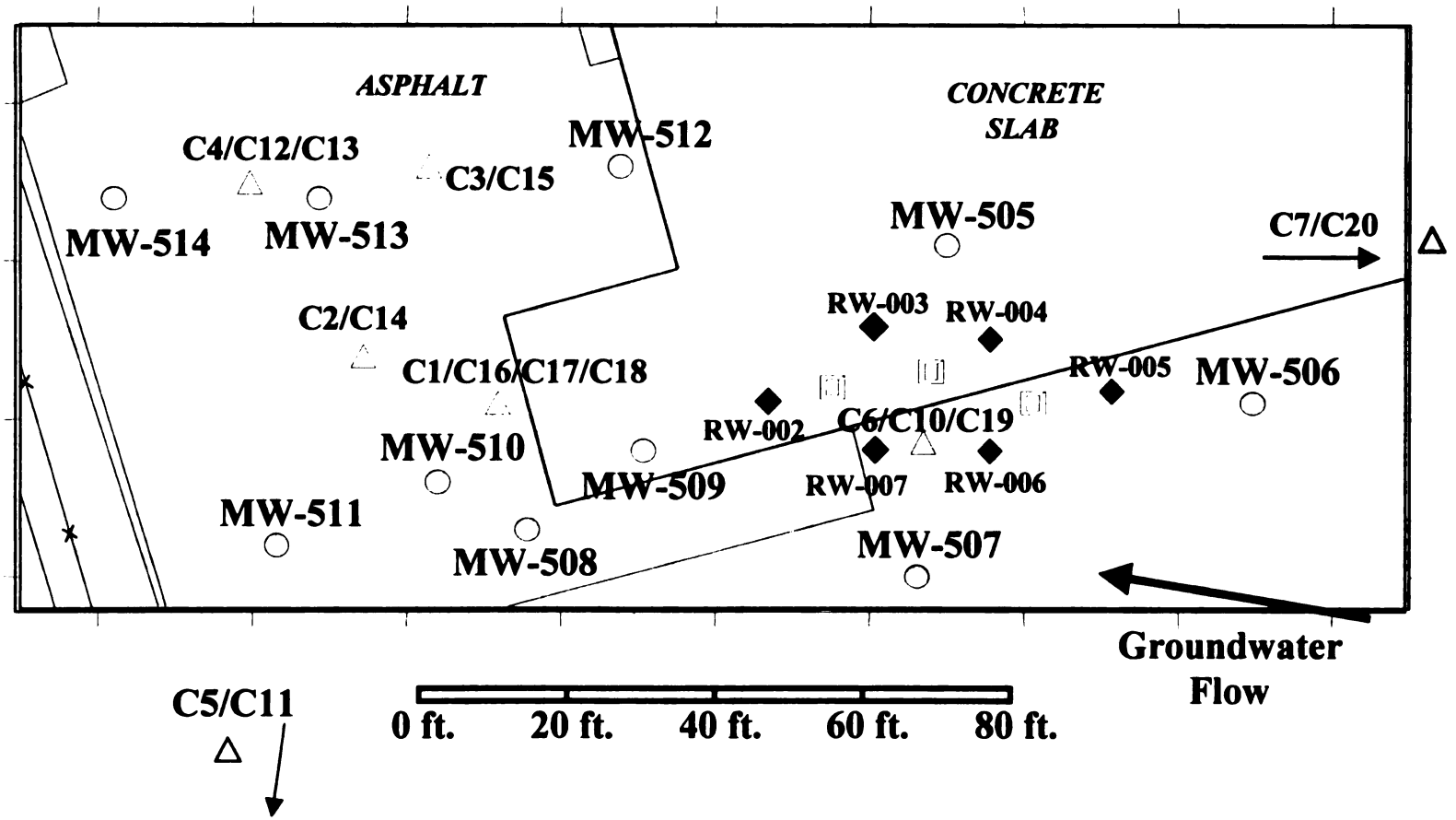


Figure 2.4. Site map of the former Sages Dry Cleaning establishment in Jacksonville Florida. Symbols on the map indicate the following: C =cores, MW = monitoring well, RW = recovery well, and the three squares represent the injection wells. Figure kindly supplied by G.W. Sewell and altered for this thesis by R.R. Helton.

[vol/vol]. Pre-SERB aquifer sediments were thoroughly mixed and approximately 2 g (wet weight) was added to the vials containing 10 ml of degassed phosphate buffered saline solution. Microcosms were then flushed with sterile hydrogen-free dinitrogen to remove residual hydrogen. In order to evaluate potential electron donors for reductive dechlorination, each microcosm received 9 ppm PCE and one of the following electron donor additions: acetate, hydrogen, lactate, succinate, glycerol, ethanol (2 mM each), starch, molasses, whey (0.5 mg/ml each), or no donor. Each treatment was established in triplicate, and one additional microcosms served as an autoclaved (killed) control. All serum vials were sealed using Teflon-lined rubber stoppers with aluminum crimp caps, and incubated in inverted position at 30°C in the dark.

Detection of chlorinated ethenes. Quantification and identification of the chlorinated ethenes (CEs) in the microcosms was discerned by headspace analysis via gas chromatography (GC). All CEs were measured in headspace samples at 25°C. For each microcosm, 0.2 ml headspace samples were analyzed on a Varian GC (model 3700) equipped with a Megabore model DB-624 column (45 m length x 0.543 mm diameter) and a flame ionization detector. Helium was used as the carrier gas. The temperature was held at 50°C for 4 min followed by an increase of 50°C/min to 200°C. Gas-tight 250-µl glass syringes (Hamilton, Reno, NV) with gas-tight Teflon valves and Luer Lock adapters were used for all headspace measurements.

Nucleic acid isolation and 16S rDNA amplification. Total community DNA was isolated and purified from aquifer sediments (1.0 g [wet weight]) using the UltraClean Soil DNA Kit from Mo Bio Laboratories, Inc.(Solana Beach, CA) according to the manufacturer's recommendations, except for an altered freeze thaw cycle between

–80°C and 65°C. Purified DNA was dissolved in ultra-pure water (Sigma, St. Louis, MO), and immediately PCR amplified. PCR amplifications were performed in 50 µl reaction volumes. Each reaction was performed using the pair of universal bacterial primers, 8F [5' AGAGTTTGATCCTGGCTCAG 3'], labeled with hexachlorofluorescene (Hex) at the 5' end for eventual use in the T-RFLP procedure, and 1392R [5' ACGGGCGGTGTGT 3'], to give near-complete 16S rRNA gene sequences. PCR mixtures included *Taq* DNA Polymerase (Gibco BRL, Gaithersburg, MD) and were done according to manufacturer's recommendations with the addition of 0.2 mg/ml of bovine serum albumin (Sigma Chemical Co., St. Louis, MO). PCR amplification was conducted in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT).

The protocol consisted of an initial denaturation step at 94°C for 3 min followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 57°C for 45 sec, elongation at 72°C for 1 min 30 sec and one extension cycle at 72°C for 7 min to finalize the PCR. Negative controls included tubes that received no template DNA, as well as positive controls containing pure culture genomic DNA. Aliquots (10 µl) of the PCR products were separated by electrophoresis in a 1.5% agarose gel using 1 X TAE buffer. The gel was stained with ethidium bromide (500 ng/ µl) and visualized by ultraviolet excitation.

Terminal restriction length polymorphism (T-RFLP). T-RFLP analysis was performed as previously done (Bruce, 1997; Brunk, *et al.*, 1996), with modifications (Ayala del Rio, 1999; Braker, *et al.*). Amplified PCR products were separately digested with the restriction endonucleases *Hha*I, *Hpa*II, and *Rsa*I overnight at 37°C following the manufacturer's recommendations (Gibco). Resulting fragments were resolved on an

ABI 373A sequencer in a 6% urea-containing polyacrylamide gel, *PE*Applied Biosystems Sequencer, (Foster City, CA), running in gene scan mode. The resulting electropherograms were analyzed for similarities using GeneScan software version 2.1 (*PE*Applied Biosystems).

Microscopy and image analysis. Microscopic observations were done using a Zeiss 10 Laser Scanning Confocal Microscope (LSM). Sediment samples were serially diluted in phosphate buffered saline, stained with acridine orange, and filtered through a 0.2 μm pore-size black polycarbonate filter (Bloem, 1995). The filters were then mounted on ethanol washed slides with non-fluorescent immersion oil and sealed with nail varnish to prevent cover slip movement. Each slide was observed under a 65X oil immersion objective using the 488 nm laser line in confocal fluorescence. A BP 520 – 560 barrier filter was used instead of a LP 520 in order to reduce the fluorescence from the humic materials. A total of 99 fields of view were examined. Images were taken digitally using the LSM and then transferred to a computer according to the LSM course manual (Whallon, 1993). Digital images collected with the LSM were then prepared and enhanced with Adobe Photoshop as required and analyzed using the Center for Microbial Ecology Image Analysis System (CMEIAS) (Liu, *et al.*, 1998).

Direct counts for post-SERB. Direct colony counts were performed using the colony plate count method as described by Koch (1994).

RESULTS

PCE dechlorinating microcosms. The potential and extent of biological PCE reductive dechlorination was assessed in anaerobic microcosms amended with PCE as the electron acceptor under a variety of different electron donor treatments. The sequential reduction of PCE to cDCE, with a transient appearance of TCE, was detected under all electron donor conditions tested (Table 2.1). PCE to cDCE dechlorination was also observed in the microcosms that were not amended with additional electron donors indicating that sufficient suitable electron donor was present in the aquifer materials. However, the predominant dechlorination end-product observed was cDCE in all microcosms.

The fastest dechlorination rates were observed in the microcosms amended with whey and molasses (Figure 2.6), averaging a 50% decrease in PCE concentration in less than 20 days (Figure 2.5). Acetate and hydrogen amended microcosms showed similar end products of cDCE (Figure 2.7). Ethanol-amended microcosms dechlorinated PCE to cDCE as well, however, one vial (E2) showed no further reduction past TCE (Figure 2.8.A). Slow rates of reductive dechlorination were also seen in the microcosms amended with only PCE and no electron donor (Figure 2.8.B). Starch proved to be the least effective electron donor; complete reductive dechlorination to ETH was not achieved in any of the vials. No dechlorination was observed in the autoclaved (killed) controls.

Table 2.1. Summary of PCE dechlorinating microcosms which showed reductive dechlorination. *The ethanol amendments had one microcosm (E2) that did not dechlorinate beyond TCE. No VC or ETH was detected in any of the microcosms by GC-FID analysis.

Electron donor condition	Concentration amount	Dechlorination product	
		TCE	cDCE
Whey	0.5 mg/ml	+	+
Molasses	0.5 mg/ml	+	+
No donor	-	+	+
Succinate	2 mM	+	+
Hydrogen	2 mM	+	+
Acetate	2 mM	+	+
Ethanol	2 mM	+	+*
Glycerol	2 mM	+	+
Lactate	2 mM	+	+
Starch	5 mg/ml	+	+

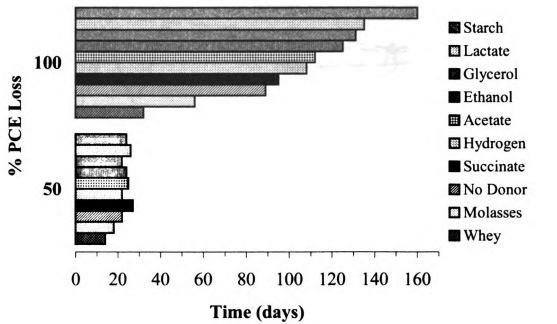
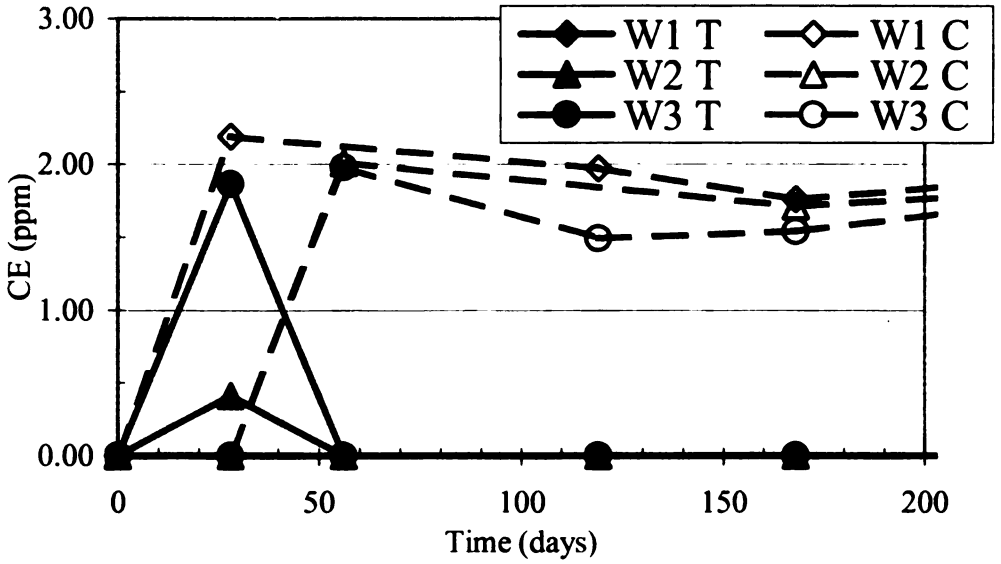
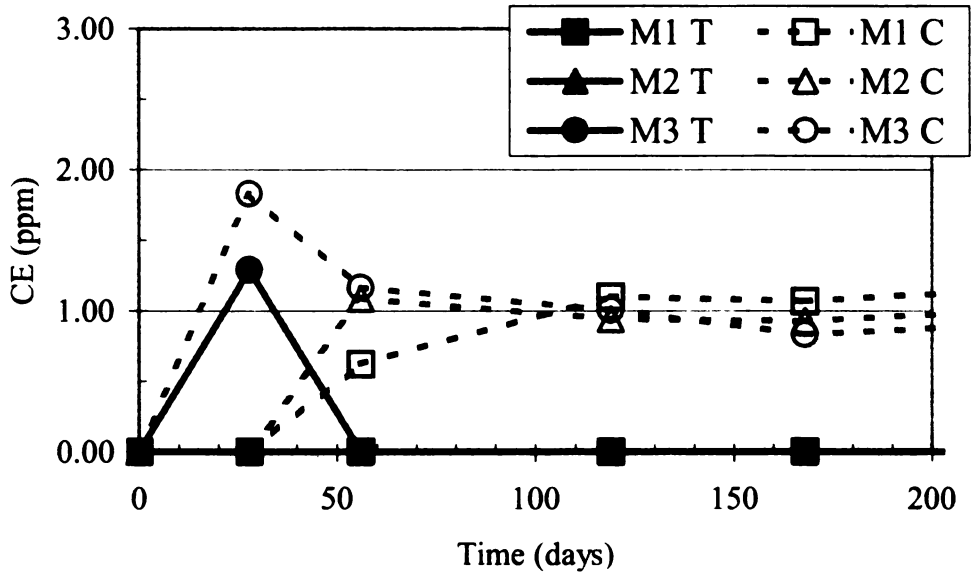


Figure 2.5. Time required for 50% and 100% loss of PCE as detected by GC-FID analysis in PCE amended microcosms with the respective electron donor condition. The most rapid average losses occurred with whey and molasses as electron donors. Each set of bars indicates the percent loss. There is no significant difference between all of the treatments using a 0.50 level (50% loss P value = 0.39; 100% loss P value = 0.001)

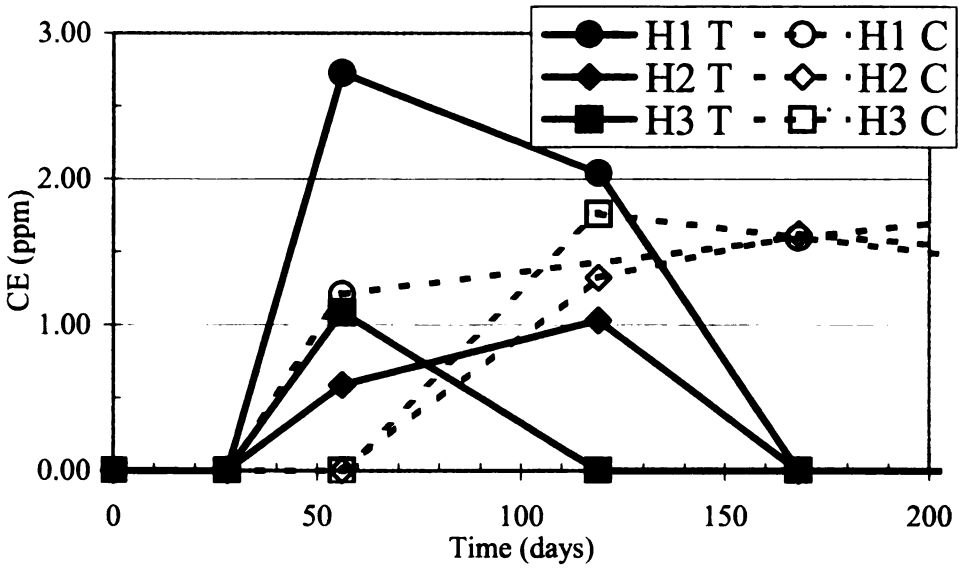


A.

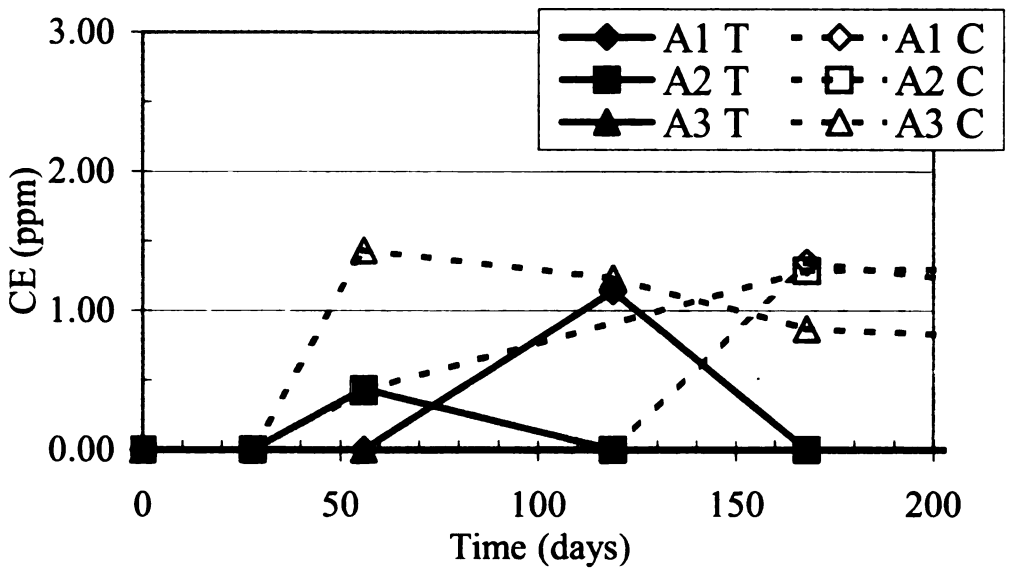


B.

Figure 2.6. GC-FID analysis of the appearance and disappearance of the PCE reduction products from the three replicate amended microcosms fed whey (A), and molasses (B). The chloroethenes are abbreviated as T = TCE and C = cDCE for each of the replicates.

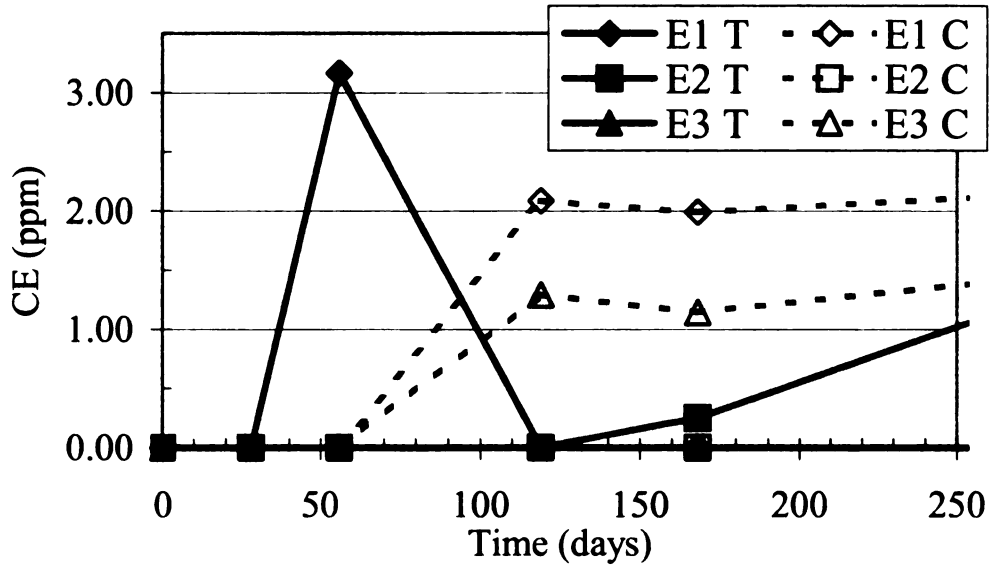


A.

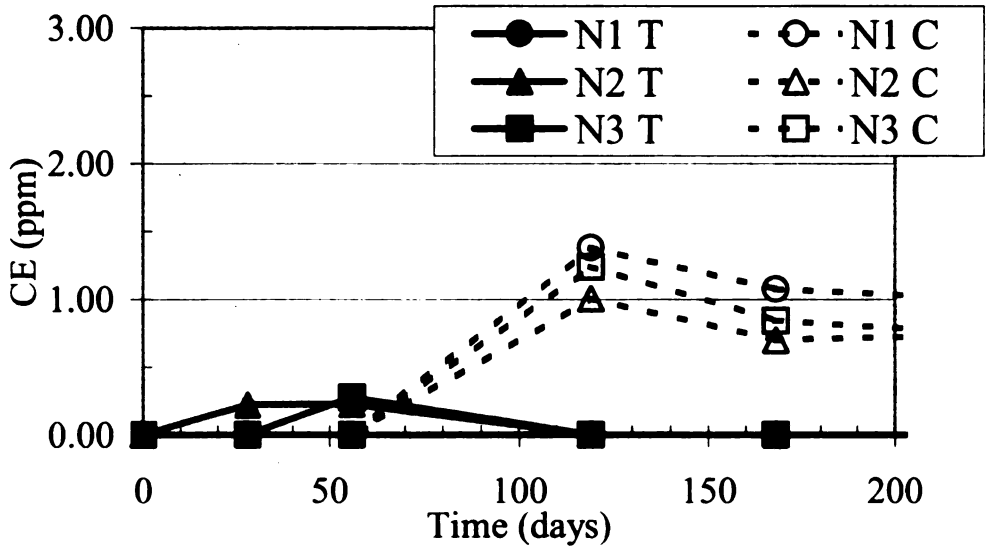


B.

Figure 2.7. GC-FID analysis of the appearance and disappearance of the PCE reduction products from the three replicate amended microcosms fed hydrogen (A), and acetate (B). The chloroethenes are abbreviated as T = TCE and C = cDCE for each of the replicates.



A.



B.

Figure 2.8. GC-FID analysis of the appearance and disappearance of the PCE reduction products from the three replicate amended microcosms fed ethanol (A), and no donor (B). The chloroethenes are abbreviated as T = TCE and C = cDCE for each of the replicates.

DNA extraction. Total community DNA was extracted from aquifer material collected at the same location, pre- and post-SERB. DNA extraction from the sediments was extremely difficult due to the simultaneous co-extraction of humic materials. The aquifer solids contained significant amounts of humic material coupled with low biomass concentrations. The aquifer pH also ranged from 3.28 – 6.76 with an average temperature of 24.3°C. Within humic materials, fulvic acids are commonly soluble under these conditions and also may contribute to these difficulties. The samples used for this study showed a pH of 5.5 and a total organic carbon of 0.46%. A sample taken outside of the PCE contaminated plume area showed a pH of 4.5 and a total organic carbon of 1.15%. A variety of different DNA extraction procedures were repeatedly tested to obtain PCR-amplifiable DNA (Appendix). None of the different DNA extraction or clean-up methods resulted in DNA that could be reproducibly PCR amplified.

Modifications to the DNA extraction protocols such as PVPP, EDTA, etc., to relieve the inhibitory effect of humics gave no positive result. Final eluates of DNA showed straw-colored liquid rather than the typical clear colored liquid obtained from DNA purification procedures. Furthermore, attempts to separate the humics from the DNA also did not yield PCR amplifiable template DNA. For instance, electrophoresis on agarose gels failed to separate the humic materials from the DNA. The resulting gels showed no visible bands under UV excitation. In addition, the bacterial biomass in the post-SERB aquifer was very low, estimated at 10^4 per gram of aquifer material by direct plate counts. The expected DNA yields for the pre-SERB sediments were 1-8 μg/g [dry wt] of sediment from the injection well area and 2-9 μg/g [dry wt] of sediment from the monitoring well area. No DNA was recovered from the injection well area sediments and

30 ng/g DNA was recovered from the monitoring well area sediments. The post-SERB sediments from the monitoring well area had an expected DNA yield of 0.5-2 μg/g [dry wt] of sediments yet again only 30 ng/g DNA was recovered. All calculations were performed using the methods as described by Zhou, *et al.* (1996).

Amplification did, however, occur in a second PCR using internal universal primers indicating that sufficient template DNA was present in the pre-SERB aquifer material. Hence, a nested PCR approach with specific primers targeting the PCE-dechlorinating *Desulfuromonas* group and the *Dehalococcoides* group was used to test for the presence of these populations in the Jacksonville aquifer. A positive signal was obtained with the *Dehalococcoides*-targeted primers, indicating the presence of one or more *Dehalococcoides*-type populations in the Jacksonville aquifer (Löffler, *et al.*, 2000). These results were obtained with the Mo Bio DNA extraction kit, included a freeze-thaw cycle. We failed to reproduce this result when other DNA extraction methods were used. No evidence for the presence of acetate-oxidizing, PCE-dechlorinating *Desulfuromonas* species was obtained with the nested PCR approach. These results demonstrated that bacteria related to *Dehalococcoides ethenogenes* were present in the aquifer (Löffler, *et al.*, 2000).

The attempts to extract DNA from the post-SERB samples did not result in sufficient DNA to visualize on agarose gels after staining with ethidium bromide. Even after PCR amplification with universal primers, no visible bands were detected in agarose gels after ethidium bromide staining and UV excitation. However, positive signals of PCR amplified 16S rDNA resulted from using the Mo Bio kits following the vortex and heat method altered with a freeze thaw cycle at -80°C to 65°C, yet this was not

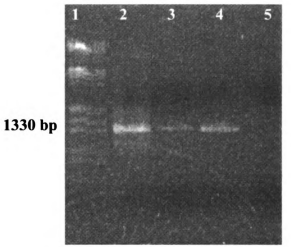


Figure 2.9. PCR amplification of 16S rRNA gene using the primer set 8F and 1392R. Lane 1, Lambda *Eco*RI *Hind*III molecular weight marker; lane 2, positive *E. coli* control; lane 3, Jacksonville aquifer sediment sample; lane 4, Bachman Road aquifer sample; lane 5, negative control containing no included DNA to PCR reaction. The Bachman Road aquifer sample was used as comparison of extractable DNA from another PCE contaminated aquifer, without humic interference.

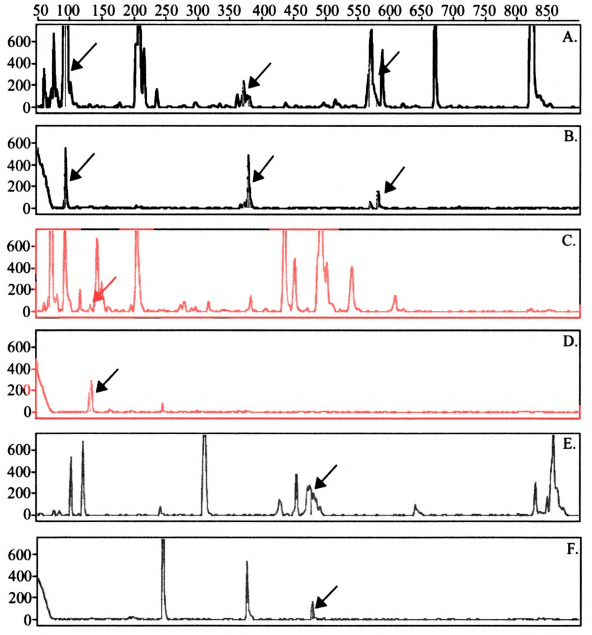


Figure 2.10. T-RFLP patterns of pre- and post-SERB sediments. Electropherograms are grouped by enzymatic restriction digests. Set A. and B. are *Hha*I digests, C. and D. are *Hpa*II digests, and E. and F. are *Rsa*I digests. The sequence of each set is pre treatment profile above post treatment profile. Approximately 30 ng of DNA initially used for each T-RFLP analysis. Arrows denote persistence of peaks, suggesting the presence of related bacteria. Fragment sizes are indicated along the x-axis and the peak heights may be indicative of the abundance of organisms possessing that specific terminal fragment length. A definite community shift is indicated between the pre- and post-SERB sediments. Images in this thesis are presented in color.

reproducible (Figure 2.9). Unfortunately, using nested PCR with specific primers targeting the PCE-dechlorinating *Desulfuromonas* group and the *Dehalococcoides* group for the post-SERB material, did not result in the same positive signals as it had for the pre-SERB aquifer materials.

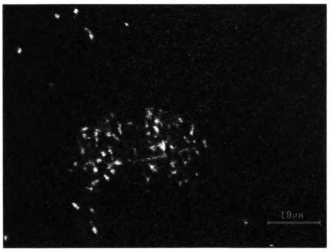
T-RFLP analysis of total community DNA. Sufficient amounts of DNA were extracted and PCR amplified from the pre- and post-SERB aquifer materials for T-RFLP analysis using one of the extraction procedures. T-RFLP was used to determine if the microbial community at the site had changed as a result of the SERB treatment. Community shifts were indicated by changing peak patterns, *i.e.* the terminal restriction fragments (TRFs), on the electropherograms. The aquifer microbial community from the same core location, showed extensive shifts from pre-SERB to one year post-SERB (Figure 2.10). The patterns show that the numbers of terminal restriction fragments (TRFs) detected in the post-SERB aquifer samples have significantly decreased from those in the pre-SERB aquifer samples. In the *Hpa*II and *Rsa*I enzymatic digests, only one TRF remained in common between the samples (Figure 2.10, C-F). Three TRFs of identical size remained after the *Hha*I enzymatic digest of the pre- and post-SERB samples (Figure 2.10 A-B). *Escherichia coli* DNA was used as a positive control in the PCR amplifications with bacterial universal primers, enzymatic digestion, and in T-RFLP analysis as a positive control.

Microscopic evaluations. A qualitative and systematic examination of the aquifer sediments was performed using laser scanning confocal microscopy and image analysis. The morphology and total number of indigenous bacteria in pre- and post-SERB aquifer samples was examined.

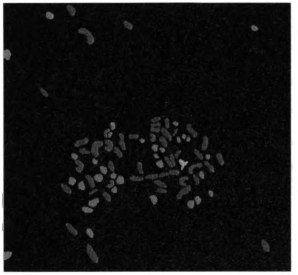
The pre-SERB monitoring well sediments had an observed cell count of 1.3×10^9 cells/g with an organic carbon content of 20 μgC/g of sediment and the pre-SERB injection well sediments had a cell count of 1.0×10^8 cells/g of sediment with an organic carbon content of 2 μgC/g. Pre- and post-SERB aquifer samples contained a high degree of humic material, which caused major interferences in the microscopic examination. Efforts to analyze the cell count of the post-SERB sediments by microscopic examination were not feasible. Thus, cell counts were estimated using the plate count techniques; 3.8×10^4 CFU/g of sediment were found. Unfortunately, this method detects only viable cells, whereas microscopic methods detect viable and dead cells.

The humic material emitted fluorescent light at the same wavelength as the acridine orange stained cells requiring the use of a band-pass (BP) filter to eliminate wavelengths below 520nm and above 560nm. Using this experimental setup, humic material appeared to provide a tight colonization area for the bacteria (Figure 2.11). When no humic material was within the field of view, bacteria were fewer in number or not detected within the field of view (Figure 2.12). Counting cells proved to be impossible due to this problem. Most frequently, the field of view showed few to no visible cells and infrequently showed the humic material with bacteria.

Each of the microscopic images was processed through the Center for Microbial Ecology Image Analysis System (CMEIAS). By comparing the images with the humic materials to the images lacking the humic materials, a percent abundance of each morphotype was calculated (Table 2.2). In addition, the total organic carbon present in microbial biomass was computed from the available images by CMEIAS to be 13 μg C/g

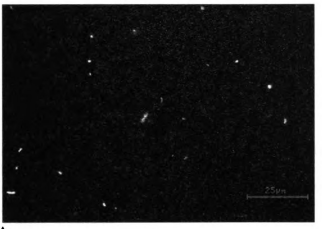


A.

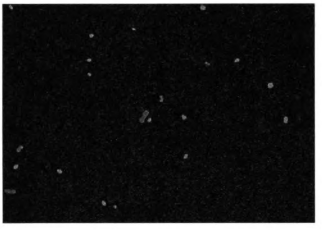


B.

Figure 2.11. Digital LSM image (A) and computed image analysis (B). The images were taken at 488 nm, 65X objective lens with a 60X zoom, BP 520-560 barrier filter to reduce humic fluorescence. The bacteria were stained with acridine orange. The cluster of bacteria is positioned in humic material, the empty black space around the cluster contained no visible humic material. The different colors reflect the morphotype class assigned by CMEIAS. Images in this thesis are presented in color.



A.



B.

Figure 2.12. Digital LSM image (A) and computed image analysis (B). The images were taken at 488 nm, 65X objective lens with a 20X zoom, BP 520-560 barrier filter to reduce humic fluorescence. The bacteria were stained with acridine orange. No cluster of bacteria was found within the field of view with little humic material present. The different colors reflect the morphotype class assigned by CMEIAS. Images in this thesis are presented in color.

Table 2.2. Percent abundance of morphotypes as examined by CMEIAS computer image analysis of the LSM digital images showing bacteria in clusters or no clusters. The presence of humic material altered the composition of morphotypes. The values represent the percent abundance per field of view using a 65X objective lens. A total of 99 fields of view were examined.

Morphotype	Cluster	No Cluster	Sample Variance
Coccus	40.3	78.2	718.2
Curved Rod	10.9	4.3	21.8
U-Shaped Rod	1.2	0	0.7
Regular Rod	45.3	13	521.6
Club-Shaped	1.2	4.3	4.8
Rudimentary Branch	1.2	0	0.7

of sediment with humic material and 1.6 μg C/g of sediment without humic materials present in the two fields of view (Figures 2.11 and 2.12).

DISCUSSION

Microcosm and field studies performed on the pre- and post-SERB sediments of the PCE-contaminated aquifer in Jacksonville, FL, demonstrated the presence of chloroethene-dechlorinating bacteria. The electron donor which supported the most rapid reductive dechlorination of PCE to cDCE was whey rather than the ethanol which was used for PCE extraction at the field site. The primary reduced chloroethene produced from PCE was cDCE. The results from the microcosm studies suggest that the rates of PCE dechlorination to TCE and cDCE depended on the type of electron donor present.

Even though 16S rDNA primers revealed the presence of PCE-dechlorinating *Dehalococcoides* species (Löffler, *et al.*, 2000) at this site, none of the laboratory microcosms completely reduced PCE to ETH. Possible reasons for incomplete dechlorination could be to uneven spatial distribution of the organisms within the aquifer, or to the conditions in the microcosms that were not conducive for complete dechlorination. In the autoclave (killed) microcosms, PCE reduction was never detected, thus indicating that the observed dechlorination of PCE was not due to abiotic processes. The microcosms were sealed with Teflon-lined butyl rubber stoppers to avoid extensive sorption of chloroethenes to the stoppers. However, repeated piercings of the stoppers for analyses resulted in some leakage of chloroethenes preventing the closure of mass balances. Instead, the conditions were based on daughter products formed.

T-RFLP proved to be a quick, reliable and simple procedure for comparison between microbial communities. The method, however, does not resolve closely related organisms or organisms that happen to share the same restriction sites and hence yielding the same TRF. Therefore, a single peak in the electropherogram may represent more than one organism.

T-RFLP was used to discern the microbial community's response to the SERB ethanol treatment. Distinctive patterns of the complex microbial communities on the electropherograms are rapidly generated by T-RFLP (Avaniss-Aghajani, *et al.*, 1996; Brunk, *et al.*, 1996). Although there are drawbacks to T-RFLP, these can be rectified by using multiple enzymatic digestions (Horz, 2000; Moeseneder, *et al.*, 1999). The high humic content of these sediments hindered much of the molecular and microscopic work. The results showed that the SERB process reduced the microbe population diversity since the number of TRFs was reduced approximately 78%. This was likely due to lysis and death of the native bacteria by the 9000 gallons 95% pure ethanol injected into the aquifer and the selection of only a few surviving populations that would grow rapidly in the ethanol.

Based on the sediments obtained from the aquifer, T-RFLP is an appropriate method to assess the microbial community dynamics resulting from engineered projects such as SERB. The microscopic examination is in agreement with the molecular studies showing low bacterial numbers and reduced diversity following SERB. Both sets of samples contained the same high amount of unusual humic materials, however, it was much more difficult to extract DNA and to obtain a suitable fluorescent microscopic image in post-SERB sediments. Assuming that the humic materials contained the same

density of bacteria in both samples, the fact remains that the microbial community suffered a drastic loss following SERB treatment.

Whether SERB is a generally appropriate technology for environmental cleanup of PCE-contaminated source areas remains uncertain. The data presented by the US-EPA shows the loss of PCE and its dechlorination products following the use of SERB, but continued removal to acceptable levels of chloroethenes could still take 3-30 years. The studies reported here suggest that the SRB treatment has a more negative effect on the indigenous microbial populations than previously suspected. This impact on level of time for clean-up and overall site restoration is unexplained. This information is probably needed before the performance of processes can be reliably predicted at other sites.

This project was managed through the Florida Department of Environmental Protection (FDEP). Funding was generously supplied from the US-EPA TIO, the State of Florida and SERDP (FIBRC-WES). This project was considerately arranged as an add-on examination of the microbial ecology associated with SERB by Guy W. Sewell, Ph.D. at the US-EPA. We also would like to acknowledge others from US-EPA associated with this project including, Susan C. Mrakik, M.S., project liaison, and Frank Beck, Ken Jewell, and Tony Lee, site engineers. We also would like to thank the State of Florida for this study opportunity as well as the field engineering consulting firm, Levine-Fricke Recon.

CHAPTER III

EXAMINATION OF THE MICROBIAL COMMUNITY STRUCTURE OF A PCE-TO-ETHENE-DECHLORINATING BIOREACTOR

INTRODUCTION

Aquifers contain the essential groundwater resources for human consumption and use as well as for many industrial purposes. Yet chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE), have become ubiquitous environmental pollutants by accumulating in these valuable aquifers. PCE and TCE were extensively used as dry cleaning agents and industrial de-greasing solvents, but were not always disposed of or contained properly. As a consequence, chlorinated ethenes have become widespread environmental pollutants of groundwater. The U. S. Environmental Protection Agency has ranked PCE, TCE, and vinyl chloride (VC) as 38th, 43rd and 44th, respectively, among its list of priority pollutants (US-EPA, 2000). PCE and TCE are suspected human carcinogens and VC, the most hazardous of the chloroethenes, has been proven to be carcinogenic to humans (ATSDR, 1997, 1997, 1997; ECO-USA, 1990, 1995, 1995).

Considerable effort thus has been employed to treat and contain chloroethene pollution. A promising and cost effective approach to detoxify contaminated aquifers is the stimulation of microbial populations that reductively dechlorinate chloroethenes. Complete reductive dechlorination of PCE and TCE to lesser chlorinated ethenes, and

even to ethene, has been observed under anaerobic conditions in the laboratory (de Bruin, *et al.*, 1992; DiStefano, *et al.*, 1992; Freedman and Gossett, 1989; Magnuson, *et al.*, 1998; Tandoi, *et al.*, 1994; Wu, 1998) and in the field through *in-situ* biostimulation and bioaugmentation (Beeman, *et al.*, 1994; Bradley, *et al.*, 1998; Hopkins, *et al.*, 1993; MacNaughton, *et al.*, 1999; McCarty, 1993; Munakata-Marr, *et al.*, 1997; Roberts, *et al.*, 1990; Semprini, 1995; Spuij, *et al.*, 1997; Steffan, *et al.*, 1999; Yager, *et al.*, 1997).

Dehalococcoides ethenogenes is currently the only known organism, which can completely dechlorinate PCE to ethene (Maymo-Gatell, *et al.*, 1999), however, it specifically requires hydrogen as an electron donor and only cometabolizes VC to ETH. All other currently known PCE-dechlorinating isolates including *Desulfuromonas* and *Desulfitobacterium* species cannot dechlorinate PCE beyond TCE or cDCE (Bolesch, *et al.*, 1997; de Bruin, *et al.*, 1992; el Fantroussi, *et al.*, 1998; Flynn, *et al.*, 1999; Gerritse, *et al.*, 1999; Krumholz, *et al.*, 1996; Löffler, *et al.*, 2000; Miller, *et al.*, 1998; Mohn and Tiedje, 1992; Neumann, *et al.*, 1998; Nielsen and Keasling, 1999; Tandoi, *et al.*, 1994; Vogel and McCarty, 1985; Wild, *et al.*, 1996).

Since the dechlorinating populations are often not present in high enough numbers to result in high-rate dechlorination, or are not evenly distributed throughout the contaminated aquifer, bioaugmentation is a viable approach to overcome these limitations. The desired remediation effects can be achieved by inoculating the contaminated aquifer with organisms possessing the metabolic capabilities to reductively dechlorinate PCE and its reduced daughter products.

The aim of this study was to evaluate the microbial community structure of a PCE-to-ETH-dechlorinating bioreactor used for a field trial of bioaugmentation in a PCE-

contaminated aquifer in Oscoda, MI. The source of the contamination stems from a now defunct dry cleaning establishment. The dissolved contaminants have migrated in a small confined plume towards Lake Huron shore. The site, known as Bachman Road site, has been monitored since 1986. Groundwater measurements over that time indicated that some reductive dechlorination occurred. The rates, however, were slow and dechlorination stalled at cDCE (Löffler, *et al.*, 2000).

Microcosm studies with aquifer materials collected inside the plume demonstrated that complete dechlorination to ethene is possible with the indigenous microflora. Sequential transfers and enrichment with PCE or cDCE as the only available electron acceptors yielded a pure culture of a PCE-to-cDCE-dechlorinating organism (Löffler, *et al.*, 2000). Complete 16S rRNA gene sequencing identified the isolate as a *Desulfuromonas* species, and the organism was designated strain BRS-1. Enrichment with cDCE as the electron acceptor yielded a highly enriched mixed culture that dechlorinated cDCE to ethene. Strain BRS-1 and the cDCE-dechlorinating mixed culture were used to inoculate the fed-batch reactor to produce enough biomass for a field inoculation. The reactor was maintained by EFX Systems, Inc. A dechlorination pilot test area was designed for passive plume control via bioaugmentation procedures downgradient of the source area (Figure 3.1).

Due to the limitations of culture-dependent methods, a culture independent approach was chosen for the evaluation of the microbial community structure of the dechlorinating bioreactor. Total community DNA was extracted and examined by terminal restriction fragment length polymorphism (T-RFLP) and by amplified rDNA restriction analysis (ARDRA) followed by sequencing of unique clones. The analysis

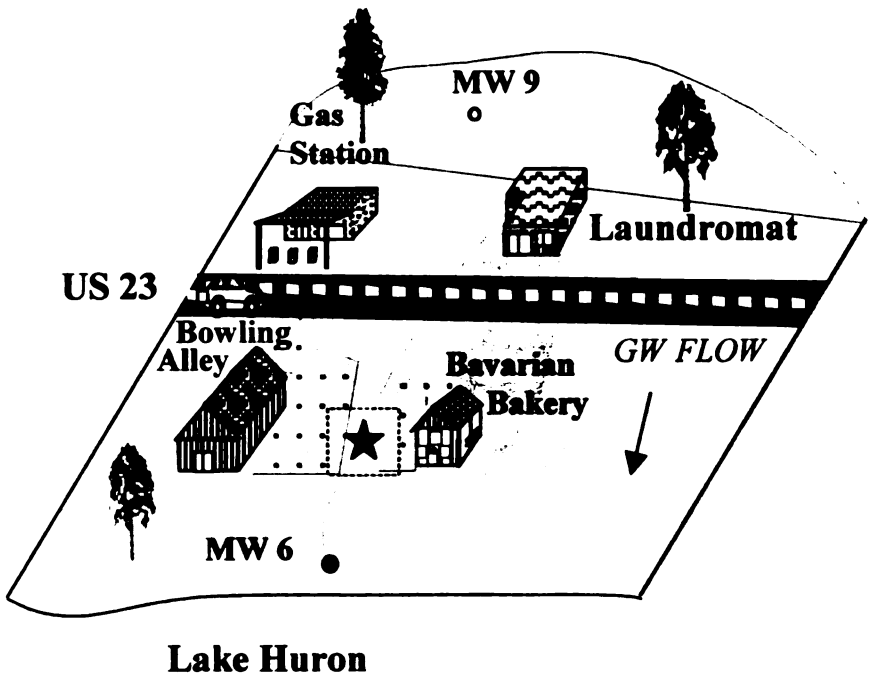


Figure 3.1. Site and plume map of the Bachman Road aquifer in Oscoda, Michigan. The star indicates the area designated as the dechlorination pilot test area. Figure kindly supplied by M.E. Dollhopf and altered by R.R. Helton.

revealed a direct correlation between the two methods and gave a clearer picture of the community matrix established in the dechlorinating bioreactor. This information provides the initial methods for tracking the inoculated population in the field and gives a preliminary indication of the diversity, composition and physiologies of the PCE to ETH chlororespiring reactor community.

MATERIALS AND METHODS

Sample collection. Reactor samples were collected by Ms. Jing Shi, M.S., from the dechlorinating reactor set up at EFX Systems, Inc, Lansing, MI. The samples were taken via a valve port on the bioreactor and transferred to 160 ml serum bottles. Each bottle was filled nearly completely from the base up and immediately sealed with butyl rubber stoppers and aluminum crimp caps to limit exposure to air.

Nucleic acid isolation and 16S rDNA amplification. *All nucleic acid isolation procedures and PCR reactions for this section were completed by M.E. Dollhopf.* Total community genomic DNA was isolated from reactor samples using the Ultra-Clean Soil DNA Kit from Mo Bio Laboratories, Inc. (Solana Beach, CA). A total of 4.5 ml of reactor culture fluid was concentrated in pellet form by centrifugation and resuspended in 500 µl sterile water, separated into two 250 µl aliquots and added to the kit supplied tubes. DNA was isolated via manufacturer's recommendations. The DNA extract was concentrated using the ethanol precipitation method described in the kit protocol and the

product from two kit extractions was combined and resuspended in 20 µl of DNA suspension buffer supplied with the Mo Bio kit.

16S rRNA genes were PCR amplified in duplicate 50 µl reaction volume sets. For use in the T-RFLP procedure, one set used the pair of universal bacterial primers, 8F [5' AGAGTTTGATCCTGGCTCAG 3'], labeled with hexachlorofluorescene (Hex) at the 5' end, and 1392R [5' ACGGGCGGTGTGT 3'], to give near-complete 16S rRNA genes. The second set used the same primer sequences but without a Hex label on the 8F primer in order to be used for cloning and sequencing of the amplified products. PCR mixtures included 2.5 µl of 10x reaction buffer containing $MgCl_2$ (Roch, Mannheim, Germany), 1.25 mM deoxynucleoside triphosphates (dNTPs) (Perkin-Elmer, Norwalk, CT), 1U *Taq* DNA Polymerase (Roche), 0.2 mg/ml of bovine serum albumin, (Roche) and were conducted according to manufacturer's recommendations.

PCR amplification was performed in a GeneAmp PCR system 9600 Thermal cycler (PE Biosystems, Norwalk, CT). The PCR program consisted of an initial denaturation step of 94°C for 3 min followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and one extension cycle at 72°C for 7 min to finalize the PCR. Negative controls containing no added DNA, as well as positive controls containing pure culture genomic DNA, were included alongside reactions. Aliquots (10 µl) of PCR products were separated via electrophoresis in a 1.5% (wt/vol) agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide (0.5 µg/ml) and visualized by ultraviolet excitation. The PCR products were immediately used for either T-RFLP or cloning procedures.

Terminal Restriction Length Polymorphism (T-RFLP). *All T-RFLP procedures were completed by M.E. Dollhopf.* T-RFLP procedures were performed as in chapter II, with the same modifications by H. Ayala del Rio, for samples taken from the initial set-up, August 14, 2000, and August 28, 2000. The PCR products were purified using Microcon 100 spin tubes and the protocol supplied (Amicon). Hex-labeled PCR amplified products were digested separately with the restriction endonucleases *Hha*I, *Msp*I, and *Rsa*I (Gibco BRL, Gaithersburg, MD) for 3.5 h at 37°C followed by enzyme inactivation at 80°C for 10 min. The restriction fragments were resolved with an ABI 373A sequencer (*PE*Applied Biosystems, Foster City, CA) on a 6% urea-containing polyacrylamide gel running in gene scan mode, performed at the Michigan State University Sequencing Facility. The resulting electropherograms were analyzed for similarities using GeneScan Version 2.1 (*PE*Applied Biosystems).

16S rRNA gene cloning. The non-Hex labeled PCR products were quantified by comparing the band intensity in 10 µl volumes to the known concentrations of the standard marker ΦX-174 DNA-*Hae*III (Finnzymes Oy, Espoo, Finland). The PCR products were ligated directly into the pCR 2.1 vector with T4-ligase using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA), as per manufacturer's recommendations. Briefly, a total volume of 2 µl was transformed into INVαF' competent *E. coli* cells (Invitrogen) following the manufacturer's recommendations for heat shock at 42°C. Transformed cells were incubated at 37°C with aeration for 1 h in SOC medium (Invitrogen). Aliquots of 50 µl were spread on Luria-Bertani (LB) (Difco Laboratories, Detroit, MI) agar plates containing 40 mg/ml X-Gal (Sigma, St. Lousi, MO) and 50 µg/ml Ampicillin (Sigma) and incubated inverted at 37°C overnight.

A selection of 100 white colonies, indicating the presence of a PCR insert , were randomly selected and plated onto LB Amp_{50} X-Gal plates with sterile toothpicks. From this, 60 clones were selected for direct PCR amplification in 25 µl reaction volumes containing 2.5 µl of 10x reaction buffer with $MgCl_2$ (Roch), 1.25 mM deoxynucleoside triphosphates(dNTPs) (Perkin-Elmer), 1U *Taq* DNA Polymerase (Roche), 0.2 mg/ml of bovine serum albumin, (Roche) and the M13 F and M13 R primers (*PE*Applied Biosystems) suggested by the Original TA Cloning Kit (Invitrogen). PCR amplification was conducted in a GeneAmp PCR system 9600 Thermal cycler (PE Biosystems). Thermal cycler conditions consisted of 95°C for 5 min followed by 30 cycles consisting of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and one extension cycle at 72°C for 10 min to finalize the PCR. The amplified PCR products were visualized in 1.5% agarose (wt/vol) gels and 46 were selected for further analysis.

ARDRA analysis. A restriction analysis of the 60 selected clones was performed with two tetrameric restriction endonucleases. Digests were performed in a total volume of 10 µl containing 0.3 U of each enzyme *Eco*RI and *Msp*I (Gibco) simultaneously. The digests were incubated at 37°C overnight inactivated by heating at 65°C for 15 min followed by freezing at –20°C overnight to ensure inactivation. The digested PCR products were separated in 3.5% (wt/vol) MetaPhor agarose gels (FMC, Indianapolis, IN) in fresh 1 X Tris-borate-EDTA (TBE) buffer at 120 V/cm in a 4°C cold room for 4 h. Gels were stained with ethidium bromide (0.5 µg/ml) for 15 min and the bands visualized by ultraviolet excitation. The RFLP patterns were compared by eye and 10 unique

patterns were identified. The unique clones were further analyzed by sequence analysis and labeled as BRDR 1 through BRDR 10 (Bachman Road Dechlorinating Reactor).

16S rRNA sequencing and phylogenetic analysis. PCR products from the 10 selected clones with unique RFLP patterns were purified with the Qiagen PCR Purification Kit (Qiagen, Chatsworth, CA), dissolved in a final volume of 30 µl of TE buffer and quantified on a 2% agarose gel. A total of 30 ng PCR product was combined separately with primers 8F, 357F and 787R, and sequenced at the Michigan State University DNA Sequencing Facility.

Sequences were assembled using SeqEd, Version 1.0.3 (Applied Biosystems). Each of the 5' ends of the sequences were aligned to the 16S rRNA sequence of *Escherichia coli* (GenBank accession J01695) using the GDE sequence editor (Genetic Data Environment, Version 2.3), and examined with the CHECK-CHIMERA tool, both obtained from the Ribosomal Database Project, Version 8.0 (Maidak, *et al.*, 2000). The number of bases between the 8F T-RFLP primer and the 5' start of the sequences was estimated from this alignment. Restriction maps were generated electronically using Sequencer, Version 3.0 (Gene Codes Corp.) and the resulting 5' terminal restriction fragments (TRFs) compared to the T-RFLP electropherograms. Phylogenetically related sequences and a preliminary alignment were derived from the RDP programs SUBALIGNMENT and ALIGN SEQUENCE.

The alignment was completed using GDE and a neighbor-joining tree was constructed with the programs DNADIST and NEIGHBOR from the PHYLIP package (Felsenstein, 1993). A weighting mask was used to include only unambiguously aligned positions and all other program options remained at their default settings. Sequences

included are (GenBank accession in parenthesis): *Sporomusa silvacetica* (Y11332), *Anaerovibrio burkinabensis* (AJ010961), *Clostridium propionicum* (X77841), *Desulfitobacterium dehalogenans* (L28946), environmental clone WCHB1-80 (AF050563), *Comamonas testosteroni* (M11224), *Ideonella dechloratans* (X72724), *Acinetobacter johnsonii* (X81663), *Desulfovibrio desulfuricans* (M34113), *Desulfomonile tiedjei* (M26635), *Dehalococcoides ethenogenes* (AF004928), *Anaeromusa acidaminophila* (AF071415), *Clostridium pasteurianum* (M23930), clone SJA-65 (AJ009473), *Desulfuromonas chloroethenica* (U49748), *Desulfovibrio dechloracetivorans* SF-3 (AF230530).

Reactor and field site. The all glass and stainless steel, 75 L fed-batch reactor was amended with an initial concentration of 50 mg/L lactate and 5 mg/L PCE by EFX Systems staff. The reactor was able to reductively dechlorinate PCE completely to ETH anaerobically after inoculation. The PCE-dechlorinating reactor had been successfully operating over a time period of 12 months and continued to completely dechlorinate PCE to ETH. The reactor received periodic additions of PCE and lactate when no PCE or lactate was detected in the effluent. The amendments occur approximately 8-10 times prior to the biomass being harvested. An automated pH control system maintained a constant pH between 6.6 and 6.8, which was found optimal for the reactor dechlorinating populations.

The bioaugmentation procedure of the Bachman Road site will be completed by anaerobically transferring the reactor inoculum to the site. The field site had been amended with lactate approximately 3 weeks prior to inoculation to ensure reducing conditions. The test area consists of two separate hydraulically controlled plots, one is

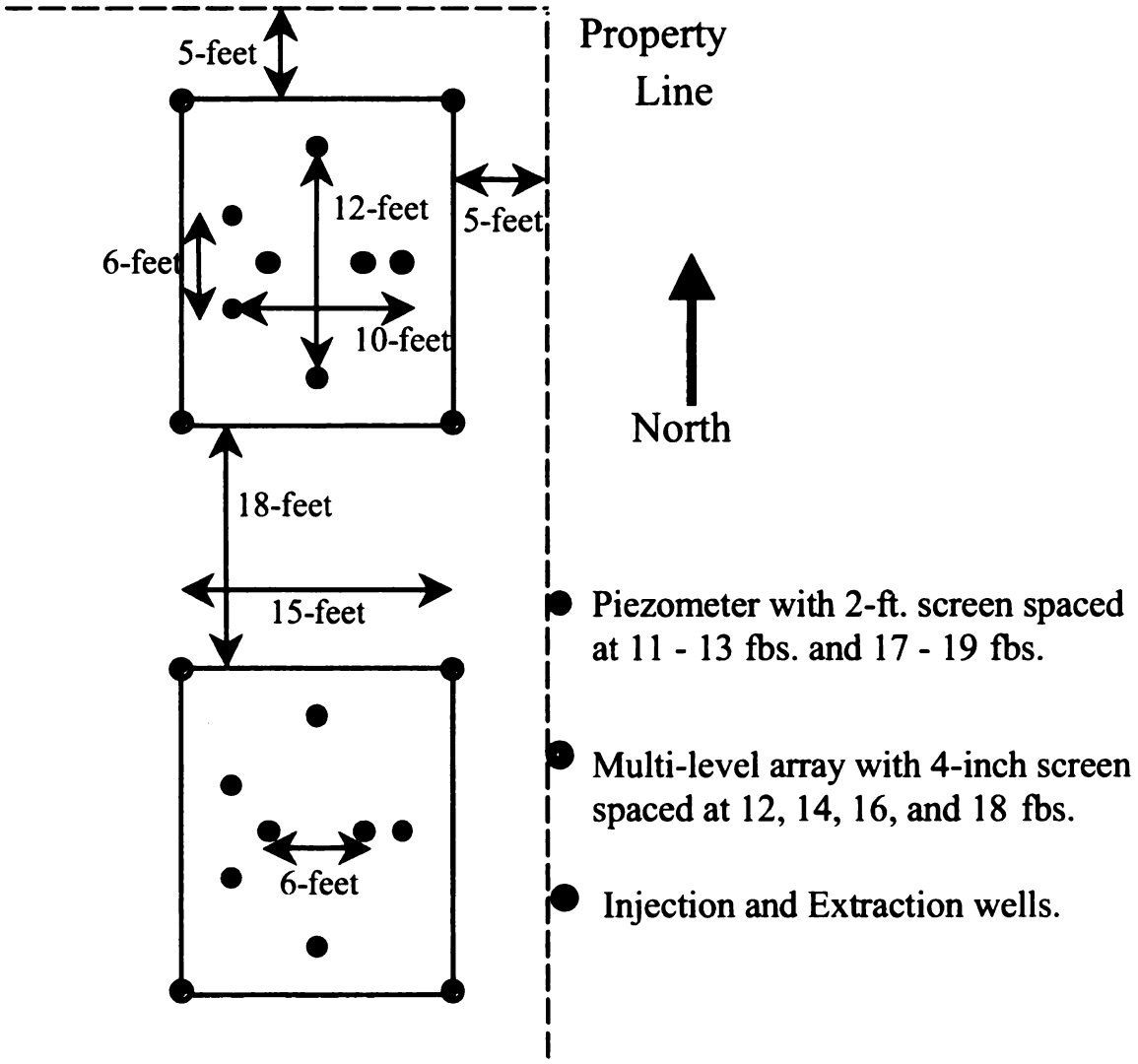


Figure 3.2. Configuration of the dechlorination pilot study test grids for Plume A at the Bachman Road site aquifer. Figure kindly supplied by M.E. Dollhopf.

for bioaugmentation and the other is an un-inoculated control plot experiencing all the same conditions and treatments as the bioaugmented plot (Figure 3.2). The inoculum will be introduced into the aquifer through an injection well in the bioaugmentation test plot and repeated as necessary based on field evaluations of cell breakthrough at the extraction well. Reactor samples were taken at various time points to evaluate the microbial community dynamics and the overall stability of the reactor. For this study, a reactor sample taken on August 14, 2000, was used to evaluate the composition of the dechlorinating community. The reactor performance was stable and continued to completely reduce PCE to ethene. Later samples were taken to establish similarity and stability.

RESULTS

ARDRA analysis. Total community DNA was extracted from 4.5 ml of culture fluid obtained from the PCE-dechlorinating bioreactor sample taken two months before field inoculation. Restriction patterns from a total of 46 clones were compared. The RFLP pattern distribution was analyzed by eye, ten different RFLP patterns were discerned from the 46 clones (Figure 3.3). The most dominant clone pattern present corresponded to clone 2. A cumulative abundance curve, indicating the frequency of clone diversity in the reactor as detected by RFLP analysis, is shown in Figure 3.4. Identical patterns were grouped together and the frequency distribution analyzed (Figure 3.5). Clones representing the ten selected RFLP types were chosen for further analysis.

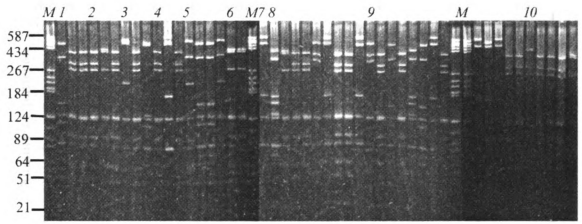


Figure 3.3. ARDRA restriction patterns of amplified 16S rDNA clones from the BRDR reactor sample. The clones were restriction digested with *Eco*RI and *Msp*I. The bands found at 124 and 89 are from the vector pCR2.1, which contains two *Eco*RI sites. Lanes marked *M* are molecular weight marker V; the lane pattern numbers correspond to the clone numbers selected for further analysis in sequencing.

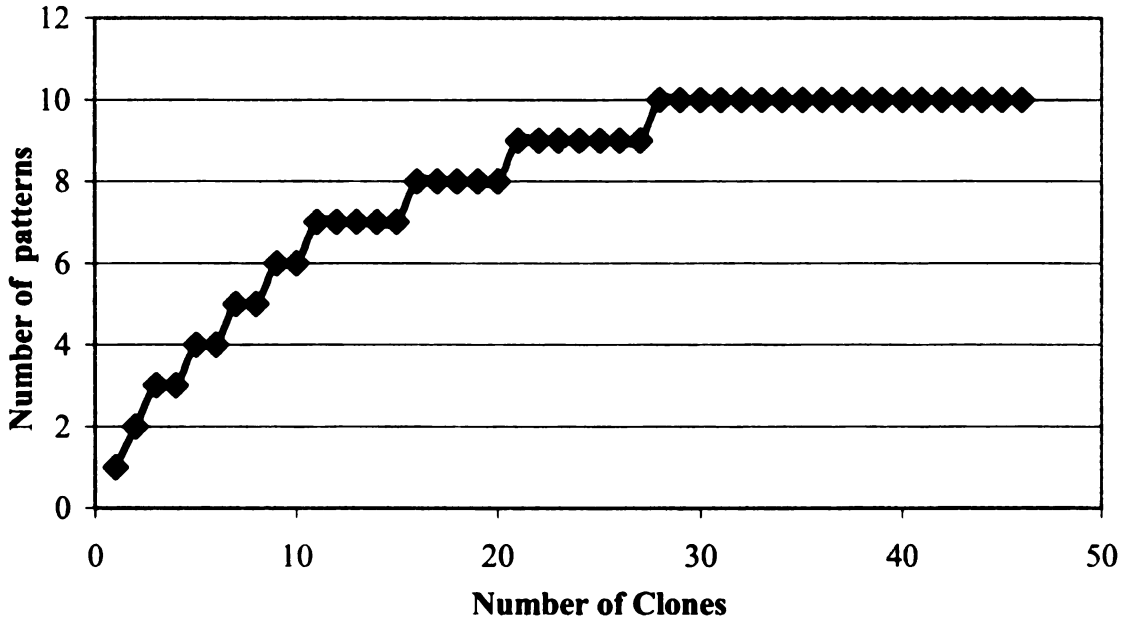


Figure 3.4. Cumulative abundance curve indicating the diversity of clones detected by RFLP analysis in the Bachman Road dechlorinating reactor sample.

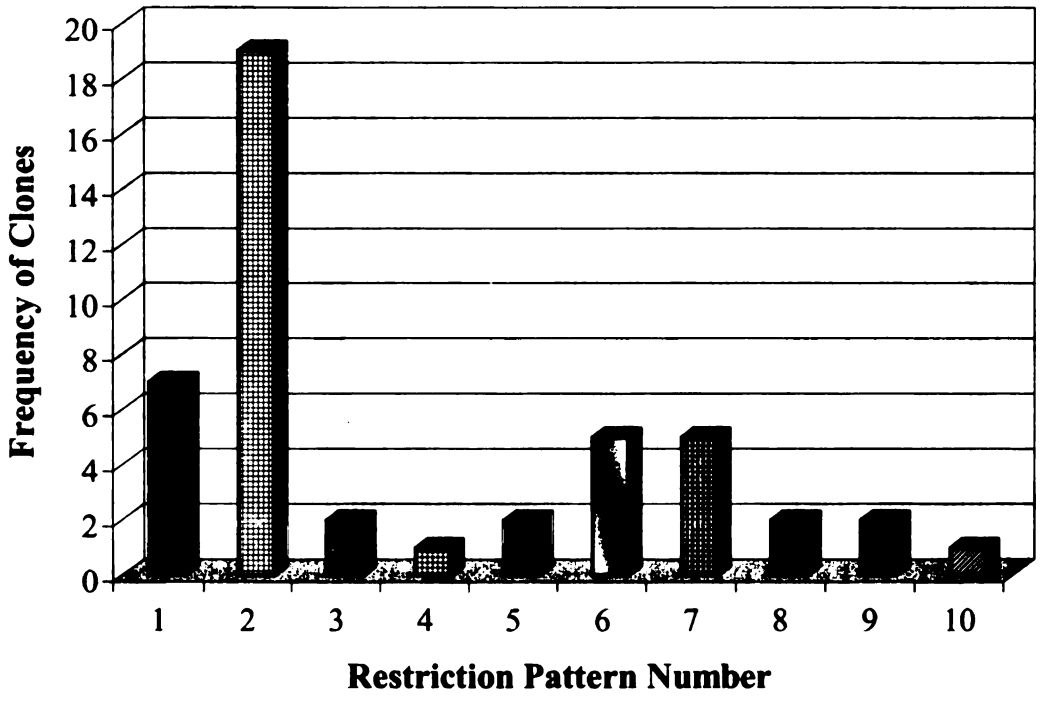


Figure 3.5. Distribution of redundant RFLP patterns from Bachman Road dechlorinating reactor sample taken on August 14, 2000.

16S rDNA sequence analysis. Each of the clones representing the 10 unique RFLP patterns was sequenced. An average sequence length of 743 bp was obtained for each and aligned to *Escherichia coli* positions 61 to 828. Tables 3.1 and 3.2 summarize the similarities of the sequenced clones as analyzed using the Ribosomal Database Project (RDP). All sequences showed a greater than 96.6% similarity to the rDNA of sequences listed in the RDP. Of the sequenced clones, four were placed in Proteobacteria with two *Beta*, one *Gamma* and one *Delta* subdivision members. Five others fell into the Gram Positive Bacteria with three Sporumusa, one Eubacterium and one Clostridium subdivision members. Only one clustered with Environmental Clone WCHB subdivision of Bacteria. BRDR 6 possessed an unusual insert at *Eschericia coli* positions 69 to 99, consisting of 122 bases. No chimeric sequences were identified.

Phylogenetic relationships. The phylogenetic relatedness was determined by construction of a neighbor joining tree for 10 selected clones (Figure 3.6). The relationships were based on a matrix of pair wise similarity values of 701 positions aligned to *E. coli* positions 61 to 828, except for clone 2, which ended at *E. coli* position 750 and BRDR 1, which ended at *E. coli* position 751. Clones BRDR 2 and BRDR 10 are identical over available sequence, however, BRDR 2 had less available sequence for comparisons. The most frequently encountered pattern, BRDR 2, as well as BRDRs 6, and 10, were closely related to both *Anaeromusa acidaminophila* and *Anaerovibrio burkinabensis*. Due to the limited resolving power of 16S rDNA and near identity of these sequences, the exact relationship cannot be determined between BRDRs 2, 6, 10 and *A. acidaminophila* and *A.burkinabensis*. BRDR 1 was related to *Ideonella dechloratans*, BRDR 3 was phylogeneticaly located near the Clostridium clone, Clone

Table 3.1. Similarity indices for clones BRDR 1, 3, 5, 7, and 9. All clones showed a greater than 96.6% similarity to the rDNA sequences listed in the Ribosomal Database Project.

	Ideonella dechloratans	Clone SJA-65	env WCHB 80	*Acinetobacter johnsonii*	*Clostridium propionicum*	BRDR 1	BRDR 3	BRDR 5	BRDR 7	BRDR 9
Ideonella dechloratans	100.0									
Clone SJA-65	78.7	100.0								
env WCHB 80	74.0	75.4	100.0							
Acinetobacter johnsonii	82.8	79.6	75.3	100.0						
Clostridium propionicum	77.9	84.2	73.7	77.5	100.0					
BRDR 1	99.2	77.2	73.8	82.2	76.2	100.0				
BRDR 3	78.2	97.6	73.2	79.8	84.2	76.6	100.0			
BRDR 5	73.9	75.7	96.6	75.6	73.4	73.9	75.6	100.0		
BRDR 7	81.6	79.3	74.3	98.6	76.9	81.4	79.4	74.4	100.0	
BRDR 9	77.9	84.2	73.8	77.5	99.9	80.8	84.2	73.5	76.9	100.0

Table 3.2. Similarity indices for clones BRDR 2, 4, 6, 8, and 10. All clones showed a greater than 96.6% similarity to the rDNA sequences listed in the Ribosomal Database Project. BRDR 2, 6 and 10 were the most similar to each other.

	Anaeromusa acidaminophila	*Anaerovibrio burkinabensis*	*Desulfovibrio desulfuricans*	*Comamonas testosteroni*	BRDR 2	BRDR 4	BRDR 6	BRDR 8	BRDR 10
Anaeromusa acidaminophila	100.0								
Anaerovibrio burkinabensis	99.8	100.0							
Desulfovibrio desulfuricans	77.6	77.8	100.0						
Comamonas testosteroni	75.4	75.4	77.1	100.0					
BRDR 2	99.4	99.7	76.8	74.7	100.0				
BRDR 4	75.1	75.4	77.1	100.0	74.7	100.0			
BRDR 6	99.6	99.9	77.8	75.4	99.9	75.4	100.0		
BRDR 8	77.1	77.3	99.0	77.0	76.5	77.0	77.3	100.0	
BRDR 10	99.5	99.8	77.8	75.4	100.0	75.4	99.9	77.3	100.0

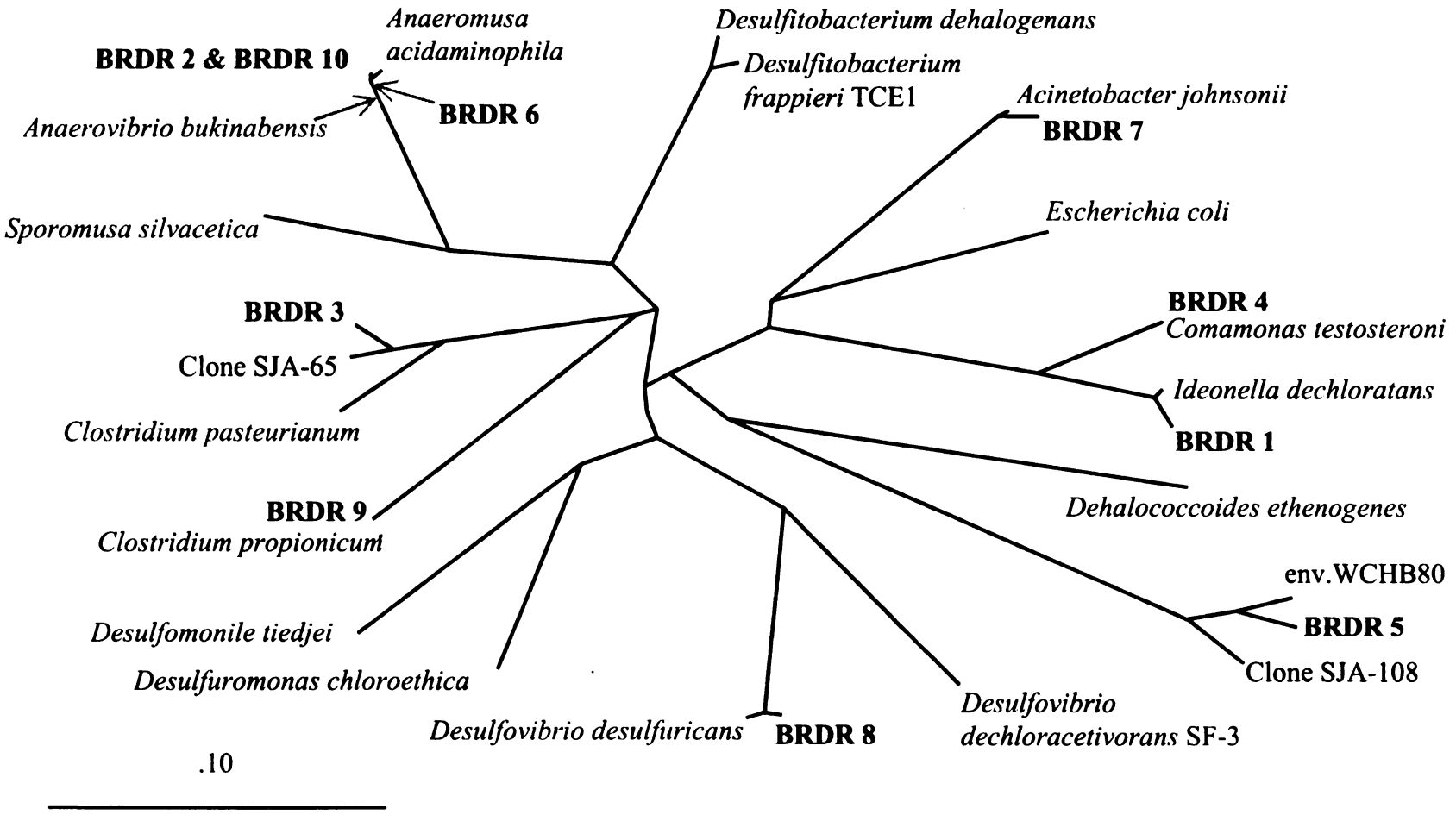


Figure 3.6. Phylogenetic neighbor-joining tree showing the relationship of the clones from the Bachman Road PCE-dechlorinating reactor. The tree was constructed using the programs DNADIST and NEIGHBOR from the PHYLIP package. A weighting mask was used to include only unambiguously aligned positions. The scale bar represents 10 substitutions per 100 bases.

SJA-65, and BRDR 7 had *Acinetobacter johnsonii* as its closest neighbor. BRDR 4 was 100% identical to the 16S rRNA sequence of the type strain of *Comamonas testosteroni*, and BRDR 9 was 99.9% similar to *Clostidium propionicum*. The other clones were placed with BRDR 5 neighboring the environmental clone WCHB80 and BRDR 8 fell closely to *Desulfovibrio desulfuricans*.

T-RFLP comparisons to ARDRA RFLPs. In order to determine if all of the clones examined were the dominant representatives of the microbial bioreactor community, as reflected by T-RFLP, T-RFLP fragments were compared to predicted restriction sites from the sequenced clones. Since it is possible for different organisms to share a common restriction site in the 16S genes, only a single peak on the electropherogram would be shown for that fragment. Therefore, three restriction enzyme digests were used to eliminate this ambiguity. Three independent restriction digests using the restriction enzymes *Hha*I, *Msp*I and *Rsa*I were used to obtain a T-RFLP community fingerprint from the extracted DNA of the reactor sample. Computer simulation of the same enzymatic restrictions used for the T-RFLP analysis was performed on the reactor clone sequences (Moyer, *et al.*, 1996). The resulting fragments were consistent with the peaks found for the T-RFLP electropherograms.

Profiles of reactor samples taken on August 14, 2000, and on August 28, 2000, are shown in Figure 3.7. The terminal restriction fragments (TRFs) showed peaks consistent (+/- 3 bp) with the ten selected clones from the ARDRA analysis, as indicated by the highlighted peaks on the electropherograms (Figure 3.7). All of the selected clones compared to peaks for each of the enzymatic digestions for the August 14 sample except the *Hha*I digests for BRDRs 3 and 9, and *Msp*I digest for BRDR 4 (Table 3.3). A

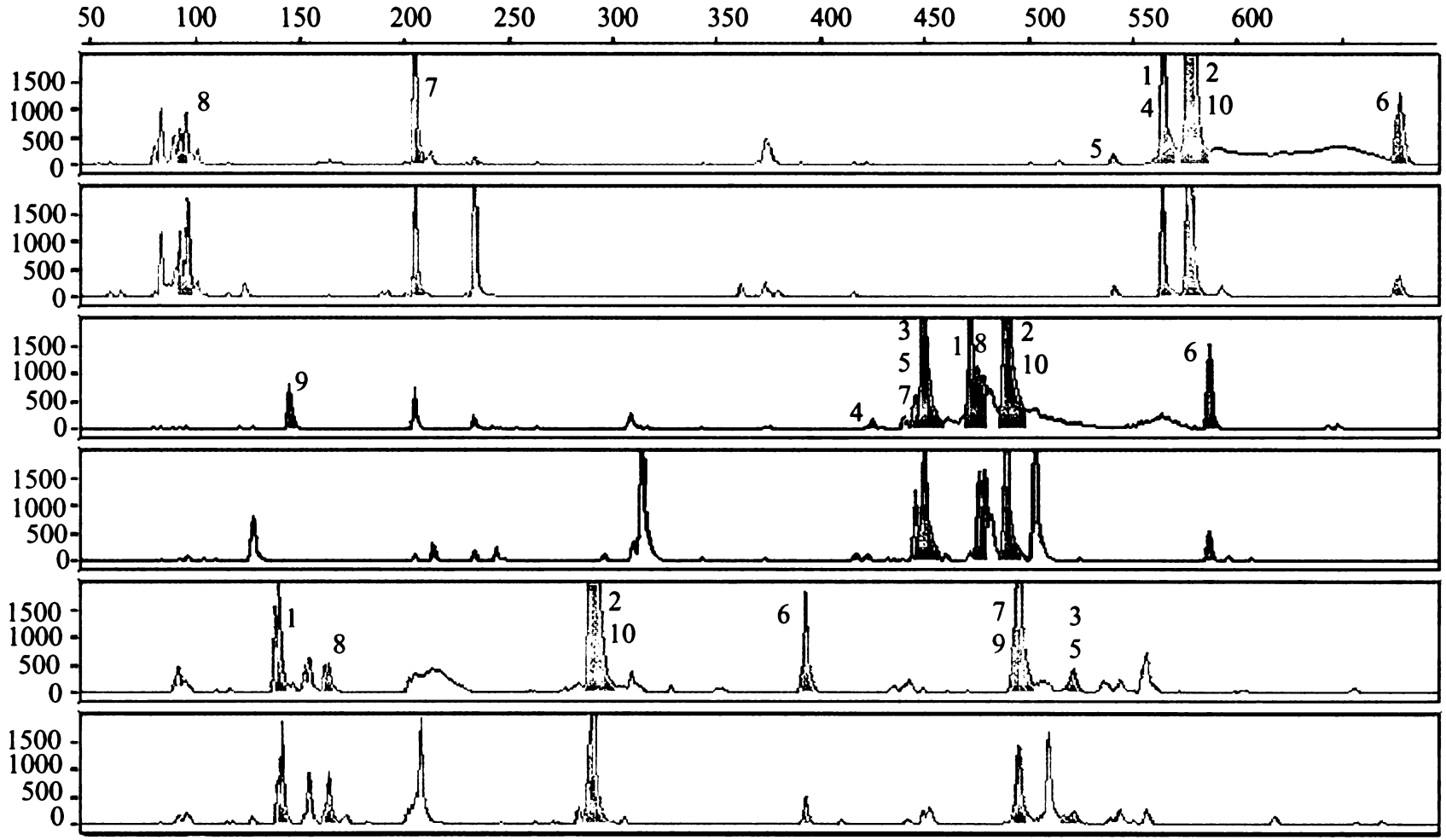


Figure 3.7. T-RFLP of Bachman Road PCE-dechlorinating reactor samples. In each set of electropherograms, the first one is from August 14, 2000 and the second is from August 28, 2000. The blue peaks are *Hha*I enzymatic digests, the black peaks are *Rsa*I digests, and the purple peaks are *Msp*I digests. Numbers beside highlighted peaks indicate corresponding ARDRA pattern clones. Figures in this thesis are presented in color.

Table 3.3. Detection of peaks on T-RFLP electropherograms from different samples from the Bachman Road PCE-dechlorinating reactor. A + indicates that a simulated fragment size for the 16S rRNA terminus of that clone had a corresponding peak in the T-RFLP electropherogram. A – indicates no such correspondence between the T-RFLP and the simulated fragment sizes.

Clone	Nearest relative	Mixed culture inoculum			Reactor sample 8-14-2000			Reactor sample 8-28-2000		
		***Hha*I**	***Rsa*I**	***Msp*I**	***Hha*I**	***Rsa*I**	***Msp*I**	***Hha*I**	***Rsa*I**	***Msp*I**
BRDR 1	*Ideonella dechloratans*	-	-	N/A	+	+	+	+	+	+
BRDR 2	*Anaeromusa acidaminophila* and *Anaerovibrio burkinabensis*	+	+	N/A	+	+	+	+	+	+
BRDR 3	Clone SJA-65 (Clostridium)	-	+	N/A	-	+	+	+	+	+
BRDR 4	*Comamonas testosteroni*	-	-	N/A	+	+		+	+	+
BRDR 5	Env. clone WCHB 80	+	+	N/A	+	+	+	+	+	+
BRDR 6	*Anaeromusa acidaminophila* and *Anaerovibrio burkinabensis*	-	+	N/A	+	+	+	+	+	+
BRDR 7	Clone T48 (Actinobacter)	-	-	N/A	+	+	+	+	+	+
BRDR 8	*Desulfovibrio desulfuricans*	+	-	N/A	+	+	+	+	+	+
BRDR 9	*Clostridium propionicum*	-	-	N/A	-	+	+	-	-	+
BRDR 10	*Anaeromusa acidaminophila* and *Anaerovibrio burkinabensis*	+	+	N/A	+	+	+	+	+	+

later reactor sample from August 28, used only for T-RFLP, showed peaks corresponding to all clones except the *Hha*I and *Rsa*I digests of BRDR 9.

DISCUSSION

This study was performed to investigate the microbial community of the PCE-dechlorinating bioreactor designed for bioaugmentation of a PCE contaminated aquifer in Oscoda, MI. The 75 L reactor was inoculated with site material from the PCE contaminated aquifer in hopes that organisms present, which are capable of PCE dechlorination, would be enhanced. Where other tests of bioaugmentation were used, exogenous organisms served as the inoculum with supplied oxygen (Steffan, *et al.*, 1999). The Bachman Road project was designed to use endogenous organisms that possess the desired metabolic capabilities in an anaerobic environment.

A combination of T-RFLP analysis and the construction of a 16S rDNA clone library were used to assess the microbial community of the Bachman Road PCE-dechlorinating reactor. Although there are drawbacks to T-RFLP in that a single peak could be representative of more than one organism which shares the same 16S rRNA terminus, this can be resolved by using multiple enzymatic digestions.

ARDRA analysis followed by sequencing provided a non-culture method to evaluate the microbial community composition. For the reactor sample from August 14, the selected clones correspond to T-RFLP patterns for the vast majority of the detected biomass. The T-RFLP patterns are also in very good agreement between the sample

taken on August 14, 2000, and the sample taken on August 28, 2000, indicating a stable microbial community over this time period. T-RFLP patterns from the original inoculum of more than a year earlier were very dissimilar to these, thus indicative of a community shift through enrichment process (data not shown).

Both of the methods used, T-RFLP and ARDRA, were beneficial in the evaluation of the complex microbial community composition of the PCE dechlorinating reactor. Knowledge of the microbial community matrix can help correlate which relevant organisms may be part of the reductive dechlorinating consortia that is reducing PCE to ETH. The phylogenetic tree based on the 16S rRNA sequences shows the diversity and relatedness of the groups of organisms detected in this dechlorinating community.

The reactor sample yielded ten different clones and they were not constrained to a single phylogenetic division. No clones were closely related to any of the known PCE degraders. Related types BRDR 2, 6, and 10 constituted 54% of the clones examined and correspond to most of the major peaks in the T-RFLP electropherograms (Figure 3.7). BRDR 2, 6, and 10 seemed to be nearest to two closely related organisms, *Anaerovibrio burkinabensis* and *Anaeromusa acidaminophila* (Figure 3.6). *A. burkinabensis* was isolated from a rice field in West Africa and is a strict anaerobe utilizing lactate as its sole carbon and energy source (Ouattara, *et al.*, 1992; Strompl, *et al.*, 1999). *A. acidaminophila* was previously isolated from an anaerobic digester and is limited to fermentation of glutamate, lactate, aspartate and pyruvate (Baena, *et al.*, 1999; Nanninga, *et al.*, 1987). These organisms may be the primary fermenters of the lactate in the bioreactor.

BRDR 6, which is phylogenetically similar to the former two organisms, has an unusual insert of 122 bases at *E. coli* position 69 to 99. This unusual insert is at exactly the same position as an insert found in the PCE dechlorinating bacterium *Desulfitobacterium frappieri* TCE1 (GenBank accession number X95972) (Gerritse, *et al.*, 1999). *D. frappieri* TCE1, isolated from a PCE-contaminated aquifer undergoing full-scale bioremediation (Gerritse, *et al.*, 1997), is capable of using lactate as an electron donor and uses PCE and TCE as electron acceptors to yield cDCE. Although this is not direct evidence of BRDR 6 being a PCE dechlorinating organism, it does seem to lend some interest to the possibility for dechlorination capabilities.

Another intriguing clone, BRDR 1, was placed closely to *Ideonella dechloratans* at 99.2% similarity. *I. dechloratans* has been described as an anaerobic microbe able to use chlorate as an electron acceptor (Coates, *et al.*, 1999; Malmqvist, *et al.*, 1994). Although it is unknown whether this organism participates in the reduction of PCE, it is known to grow by dissimilatory reduction of chlorate, which is also an environmental pollutant (Renner, 1998; US-EPA, 2000).

All clones had relatively closely related 16S sequences in the RDP database. The most unique were clones BRDR 3 and BRDR 5, with percent sequence similarity to the nearest neighbors of 97.6% and 96.6% (Table 3.1), respectively. Interestingly, the sequence obtained from clone BRDR 5 showed the highest sequence similarity to the environmental clone WCHB 80. This clone was from an aquifer contaminated with waste fuels and chlorinated solvents on the former Wurtsmith Air Force Base in Michigan, currently undergoing intrinsic bioremediation efforts (Dojka, *et al.*, 2000; Dojka, *et al.*, 1998). The presence of this clone, BRDR 5, may also suggest a possible

candidate for the reductive dechlorination of PCE. In addition to WCHB 80, BRDR 5 also placed near to several others from the same study and to several clones from an anaerobic trichlorobenzene metabolizing microbial consortium (von Wintzingerode, *et al.*, 1999). Although similar to these clones, BRDR 5 has as yet no identifiable cultured relatives (Figure 3.6). Although somewhat dissimilar in sequence, the nearest cultured member in this branch is *D. ethenogenes*, the organisms that dechlorinates PCE to ETH.

Other clones examined had closely related sequences in the RDP database as well. BRDR 4 was identical to the type strain of *Comamonas testosteroni*, BRDR 9 was 99.9% similar to *Clostridium propionicum*, and BRDR 8 was found to have 99.0% sequence similarity to the sulfate reducing bacterium, *Desulfovibrio desulfuricans*.

The use of molecular techniques has been quite useful in evaluating microbial communities composition of the PCE-dechlorinating bioreactor without the need for culture means or isolation methods. In this study, we were able to use a combination of T-RFLP and ARDRA techniques to examine the bacterial community of the PCE-dechlorinating bioreactor . The results are surprising in that no known PCE dechlorinators were detected by either method. Further evaluation of the organisms, the presence of dehalogenase genes, and the unusual insert in BRDR 6 are necessary in order to completely comprehend the PCE-dechlorinating reactor community. The results obtained here represent a good base to further investigation into the processes of anaerobic reductive dechlorination of PCE via bioaugmentation.

APPENDIX

APPENDIX

DNA EXTRACTION TECHNIQUES UTILIZED IN THE ATTEMPT TO ISOLATE MICROBIAL DNA FROM THE HIGH HUMIC SEDIMENTS OF A JACKSONVILLE, FLORIDA, AQUIFER

This appendix summarizes the DNA extraction procedures that were used in the attempt to obtain PCR amplifiable DNA from this aquifer. Each of the following methods was performed at least twice and several of the attempts were done as multiple samples with the final products pooled to concentrate the extracted material. All extractions were checked by gel electrophoresis and viewed under UV excitation and by UV spectrophotometer. Several of the extracted samples were PCR amplified using 16S rRNA primers even if no visible band was detected by UV excitation, and were amplified again in a nested PCR procedure with *Dehalococcoides* specific primers (FL-2). The only positive results came from the Mo Bio kits using the vortex and heat method altered with a freeze thaw cycle of -80°C to 65°C, however, this was not easily reproducible. All other attempts proved fruitless.

I believe the difficulties in DNA recovery and clean up stem from a combination of a relatively low biomass concentration and a high degree of humic material, the latter causing major interferences in the extraction of the DNA. The humic material seems to be rather different from other soils and sediments studied at the Center for Microbial Ecology. The attempts to clean out the Jacksonville type of humic material have not been successful by methods that worked on other sites high in humics. The humic content in the core sediments is very high as compared to the aquifer sediment sample from the Bachman Road site (Figure A.1).

The evidence for low biomass at the Jacksonville site comes from plate counts and direct microscopic counts. The aquifer pH also ranged from 3.28 – 6.76 with an average temperature of 24.3°C. Within humic materials, fulvic acids are commonly soluble under these conditions and also may contribute to these difficulties. The samples used for this study showed a pH of 5.5 and a total organic carbon of 0.46%. A sample taken outside of the PCE contaminated plume area showed a pH of 4.5 and a total organic carbon of 1.15%. Furthermore, the 95% ethanol flush of the site may have caused some biomass loss as well as altering organic matter in ways that may influence the extraction or clean up.

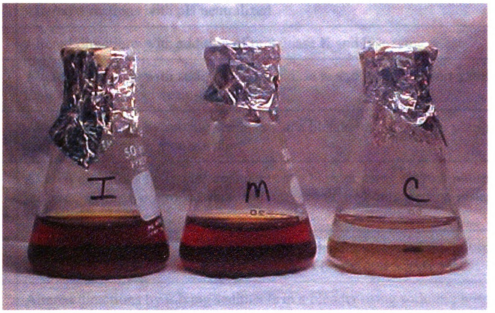


Figure A.1. Sediment slurrys showing the high humic material containing sediments from Jacksonville, Florida, (flasks I and M) and the typical aquifer sediments with little humic material from the Bachman Road site, Oscoda, Michigan, (flask C). Images in this thesis are presented in color.

Table A.1. Purchased kit protocols done in attempt to extract microbial DNA from the Jacksonville, Florida, SERB treated sediments, samples: 0.1, 0.5, 1.0, 5.0, and 15 grams.

A.	Mo Bio Ultra-Clean Soil DNA Kit
a.	Manufacturer's recommendations for vortex and heat at 70°C
b.	Manufacturer's recommendations for bead beating
c.	Manufacturer's recommendations for bead beating and heat at 70°C
d.	Altered directions with a freeze-thaw triple cycle between -80°C and 65°C
e.	Altered directions with a freeze-thaw triple cycle between liquid nitrogen and 65°C
f.	Altered directions with addition of polyvinylpolypyrrolidone (PVPP)
g.	Altered directions with addition of EDTA (1mM and 5mM)
h.	Altered directions with addition of SDS (10% and 20%)
i.	Altered directions with pH neutralized
j.	Altered directions with addition of proteinase K and lysozyme
k.	Altered directions with addition of proteinase K and lysozyme and a freez-thaw triple cycle between -80°C and 65°C
l.	Altered directions by mixing sediments in a blender using sodium phosphate buffered solution or groundwater
m.	Altered directions by mixing sediments in a blender using sodium phosphate buffered solution or groundwater and only using the liquid portion instead of the sediments
n.	Altered directions by mixing sediments in a blender using sodium phosphate buffered solution or groundwater with EDTA (1mM and 5mM)
o.	Altered directions by mixing sediments in a blender using sodium phosphate buffered solution or groundwater with EDTA (1mM and 5mM) and only using the liquid portion of the sediments
B.	Bio-101 Soil DNA Kit. Manufacturer's recommendations for vortex and heat.

Table A.2. Direct cell lysis protocols used to attempt DNA extraction from Jacksonville, Florida, SERB treated sediments, samples: 0.1, 0.5, 1.0, 5.0, and 15 grams.

a.	Zhou, J.Z., M.A. Bruns, J.M. Tiedje. 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62:316-322.
b.	Alteration of (a.) with proteinase K, freeze thaw triple cycle between -80°C and 65°C and a 2 hour stabilization after purification at 65°C (S.H. Haack).
c.	Alteration of (a.) with proteinase K and a 2 hour stabilization after purification at 65°C (E. Moss).
d.	Alteration of (a.) with proteinase K, freeze thaw triple cycle between -80°C and 65°C (M.S. Riley).
e.	Alteration of (a.) with proteinase K, freeze thaw triple cycle between -80°C and 65°C and pH neutralized.
f.	Alteration of (a.) with EDTA, proteinase K, freeze thaw triple cycle between -80°C and 65°C.
g.	Alteration of (a.) with of proteinase K, freeze thaw triple cycle between -80°C and 65°C and gel purification.
h.	Alteration of (a.) by blending sediments using sodium phosphate buffered solution or groundwater, EDTA, proteinase K and a freeze thaw triple cycle between -80°C and 65°C.
i.	Nüsslein, K., J.M. Tiedje. 1998. Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small subunit ribosomal DNA amplified from DNA fractionated on the basis of its Guanine and Cytosine composition. Appl. Environ. Microbiol. 64:1283-1289.
j.	van Elsas, J.D. and K. Smalla. 1995. Extraction of microbial community DNA from soils. In Akkermans, A.D.L., van Elsas, J.D., and de Bruijn, F.J. (ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, the Netherlands.
k.	Cullen, D.W. and P.R. Hirsh. 1998. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol. Biochem. 30: 983-993. (Both PVPP and Sephadex columns were used)
l.	Berthelet, M., L.G. Whyte, and C.W. Greer. 1996. Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. FEMS Microbiol. Ecol. 138:17-22.

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