The Feasibility of Bioaugmentation for the Remediation of Chlorinated Solvents: A Microcosm Study

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reductive dechlorination, bioremediation and natural attenuation
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ABSTRACT

Chlorinated solvents are among the most prevalent contaminants at Superfund sites. Perchloroethylene (PCE) and its degradative byproducts pose a particular problem because of their persistence in the subsurface and their threat to ecological health. In this study, microcosms were used to test the viability of bioaugmentation as a possible remediation strategy at a PCE contaminated site at the Naval Amphibious Base at Little Creek located in Virginia Beach, Virginia. All microcosms were created in duplicate using spatially diverse soils and the bioaugmented series innoculated with a mixed microbial culture provided by the Dr. Frank Loffler. This culture has been found to be capable of completely degrading PCE to ethene. The aqueous ethene concentration was monitored over time. It is clear from the results that bioaugmentation successfully increased the degree of reductive dechlorination over their static counterpart. Without innoculation, shallow static microcosms showed an accumulation of cis-DCE, while deep soils never showed conversion beyond TCE. Shallow bioaugmented microcosms showed the production and loss of vinyl chloride indicated probable complete conversion of PCE to ethene while deep soils showed the production of *cis*-DCE. These differences in dechlorination between shallow and deep soils indicate a possible disparity in reduction capacity. At day 78, microcosms were spiked with higher concentrations of PCE resulting in a reduction in dechlorination activity. Static microcosms exhibited similar degradative trends but bioaugmented batches experienced dramatic reductions in dechlorination activity indicating possible inhibition effects of native organisms due to concentration or potential toxic shock. It appears that bioaugmentation is a remediation alternative worthy of further study including possible delivery methods, toxicity or inhibition effects of concentration, and fate/transport studies.

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This thesis is dedicated to my family, Charlton, Carolyn and Elizabeth Ellis, without which none of this would have been possible. Their continued support, both financially and emotionally, has allowed me to focus on this task at hand and continue my pursuit of happiness.

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CHAPTER 1:
INTRODUCTION

Tetrachloroethene (PCE) and trichloroethene (TCE) are nonflammable, non-corrosive commercial solvents that have been used over the past 75 years predominantly as chemical intermediates in production processes and to lesser extents as a dry cleaning solvent and metal degreaser (5). The potential negative environmental and health related effects of chlorinated ethenes and their degradative byproducts have been of concern for the last 25 years. Following a peak in usage in the late 1970's, increased recycling practices and a transition to more environmentally benign alternatives have led to a steady decline in the production of PCE. As of 1995, estimated production of PCE was at 490 million pounds (102). Unfortunately, poor handling and disposal practices have led to extensive loss of this compound into the environment. It is estimated that nearly 80 percent of the PCE commercially used in industry ends up in the atmosphere (110). This is of particular concern because PCE and each of its degradation byproducts is a possible carcinogen (23). Acute exposure to PCE and it derivatives often leads to nervous system damage and irritation to skin and nasal passages. Long term exposure causes liver and kidney damage as well as cancer (121). Vinyl Chloride (VC) is the most toxic byproduct of PCE degradation, known by the Department of Health and Human Services to cause liver cancer (6). The chemical and physical properties of chlorinated ethenes are given in Table 1.

Table 1. Chemical and Physical Properties of Chlorinated Ethenes

Characteristic/Property	***PCE***	***TCE***	***cis-DCE***	***VC***
Molecular Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	C_2H_3Cl
CAS Registry No.	127-18-4	79-01-2	156-59-2	75-01-4
Approx. Annual World Production[a] (metric tons)	1,100,000	600,000	NA	NA
MCLG[b] (µg/L)	0	0	70	0
MCL[b] (µg/L)	5	5	70	2
Molecular Weight (g/mol)	165.83	131.39	96.94	62.50
Human Carcinogenity[c]	Probable	Probable	Probable	Known
Density[d] (g/cm3)	1.626	1.46	1.28	0.91
Water Solubility[d] (mg/L at 25°C)	150	1,100	800	1.1
Vapor Pressure[e] (mm at 25°C)	14	60	200	2,660
Henrys' Law Constant[f] (dimensionless at 24.8°C)	0.723	0.392	0.167	1.137
Octanol/Water Partition Coefficient[d], log K_{OW}	2.88	2.29	1.86	1.38
Sorption Partition Coefficient[d], log K_{OC}	2.56	2.03	1.69	1.76
Sorption Partition Coefficient[d], log K_{OM}	2.32	2.78	NA	NA

NA - Data not available.
a Source: Schwarzenbach et al. (1993).
b MCLG- Maximum Contaminant Level Goal, MCL- Maximum Contaminant Level; U.S. EPA National Primary Drinking Water Contaminant Standards. Source: American Water Works Association (1999).
c Source: American Water Works Association (1999).
d Source: Mackay, D., et al. (1993).
e Source: Fetter, C.W. (1999).
f Source: Gossett, J.M. (1987).

PCE readily evaporates when exposed to air and exists as a non-aqueous phase liquid (NAPL) in water. It is also relatively insoluble in water and does not tend to bind to soil. These

characteristics lead to high mobility in the subsurface and difficulty in remediation. The net negative environmental impact is further worsened by the widespread use of PCE as a dry cleaning agent, which has led to its release in numerous locations. Both PCE and TCE are the most common groundwater pollutants at hazardous waste sites (82). After entering into the environment, PCE can be anaerobically degraded via reductive dechlorination and forms the daughter products of TCE, three isomers of dichloroethene, vinyl chloride, and eventually ethene. The reductive dehalogenation pathway for PCE and its intermediates is given in Figure 1.

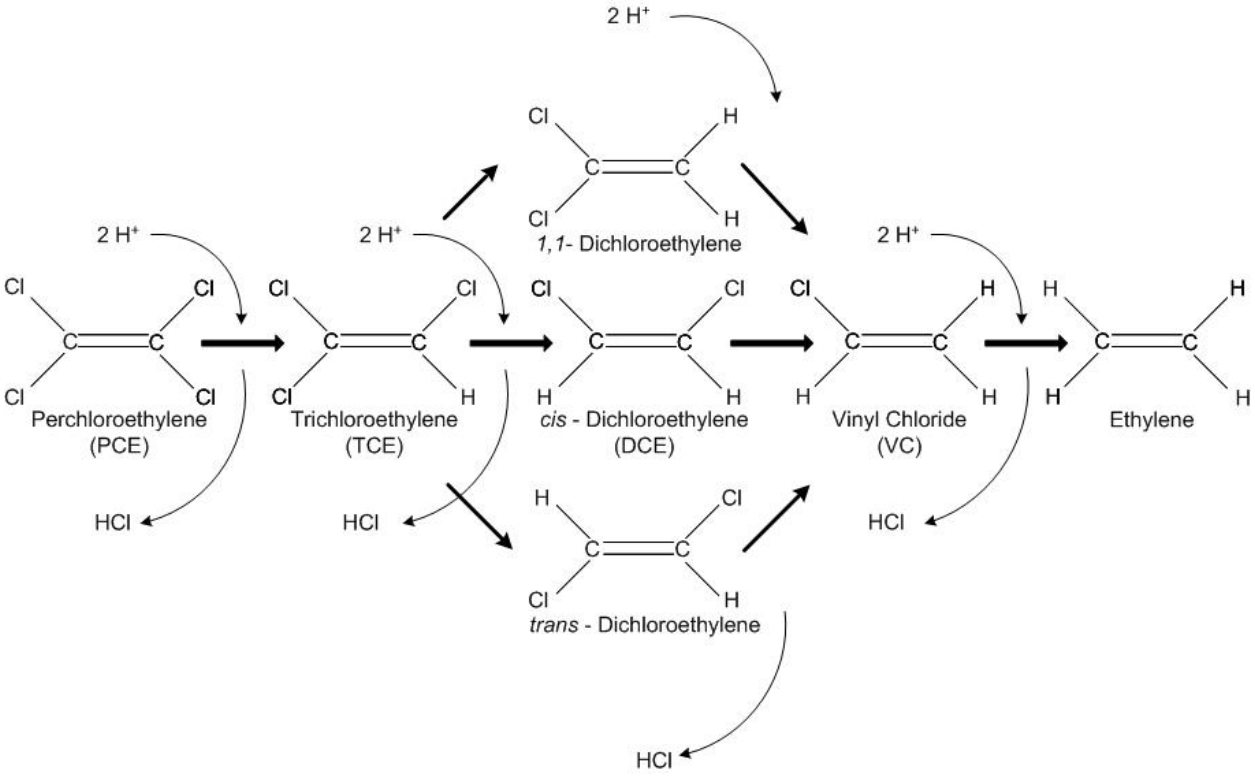


Figure 1. Reductive Dehalogenation Pathway

Ultimately, the goal of remediation is to reduce contaminant levels below the government issued maximum contaminant level (MCL). The MCL for PCE, TCE, DCE, and VC is 5, 5, 7, and 2 parts per billion respectively (115). There have been several remediation technologies proposed to clean up affected sites. Pump and treat has been most widely used. In 1989, the EPA conducted a study of 19 contaminated sites in which pump and treat was used as the primary mode of clean-up (111). The EPA study concluded that the most significant drops in contamination were experienced during the days immediately following groundwater extraction. Contaminant level quickly leveled off and some sites even experienced a "rebound", most likely due to the flushing effect of the pumping process. It was determined that the presence of NAPLs were not considered in the design of these systems and that cleanup goals could not be met in a timely manner using pump and treat systems (112).

Other systems studied by the EPA included air-sparging coupled with enhanced in situ biodegradation. Beyond the expense of these systems, the mass flux of oxygen that can be reliably imparted to the subsurface is relatively small compared to the mass of organics present. Research is currently being conducted to test the viability of adding oxygen and hydrogen release compounds as well as special electron acceptors (119) (70).

With only limited field success of other remediation technologies, many site managers are resorting to monitored natural attenuation as a primary contaminant mitigation strategy. Natural attenuation refers to all physical, chemical, and biological mechanisms responsible for

lowering the contaminant concentrations, mass, mobility, volume, and toxicity at a given site. One of the most important means is through biotic transformation to less toxic byproducts. The effectiveness of this process, of course, relies on the viability of the microorganisms present. This literature review focuses on the processes affecting monitored natural attenuation (particularly intrinsic bioremediation) and current research being conducted to test the practicability of adding known dechlorinating cultures to contaminated sites to stimulate reduction.

CHAPTER 2: LITERATURE REVIEW

MONITORED NATURAL ATTENUATION

The increased use of synthetic chemical compounds in manufacturing and industry coupled with negligent disposal procedures has led to their extensive release into the environment. Many of these chemicals pose serious threats to both human health and the well-being of the surrounding ecosystem. Fortunately, nature has an intrinsic capability to degrade or disperse the majority of xenobiotic substance that we introduce. When the goal of a remediation effort is to simply remove a contaminant, methods like incineration have such a drastic effect on soils that we are left with nothing more than a useless pile of waste, stripped of its capacity as a natural resource. Other methods become technically infeasible based on specific site conditions. With more emphasis being place on reuse, many government regulators are turning to remediation methods that maintain inherent characteristics of natural systems while reducing contaminant concentrations. One of these techniques is monitored natural attenuation (MNA).

Monitored natural attenuation refers to the observed reduction in contaminant concentrations spatially outward from a contaminant source. The decline is due to a number of fate and transport mechanisms including advection, dispersion, diffusion, sorption, recharge, volatilization, biodegradation, abiotic degradation, and phase partitioning (122). It is quickly becoming the primary remediation strategy at sites where other methods have been deemed ineffective or cost prohibitive. The biggest hurdle in gaining widespread public support is the pervasive belief that MNA is a passive approach and that responsible parties are simply doing nothing. It has been shown that earth's natural remediation capacity often deems an engineered remediation system unnecessary (10) (92). As is the case with any technique, MNA has its advantages and limitations. A summary of these is given as Table 2.

Table 2. Advantages and Disadvantages of Monitored Natural Attenuation

Advantages	***Limitations***
During intrinsic bioremediation, contaminants can ultimately be transformed to inoccuous by-products (e.g. carbon dioxide, ethene, chloride, and water) not just transformed to another phase or location within the environment	Natural attenuation is subject to natural and anthropogenic changes in local hydrogeologic conditions, including changes in groundwater flow direction or velocity, electron acceptor and donor concentrations, and potential future releases.
Natural attenuation is nonintrusive and allows continuing use of infrastructure during remediation	Responsibility must be assumed for long-term monitoring and its associated cost, and the implementation of institutional controls
Natural attenuation does not involve generation or transfer of wastes	Time frames for complete remediation can be long
Natural attenuation is often less costly than other currently available remediation technologies	The hydrologic and geochemical conditions amenable to natural attenuation are likely to change over time and could result in renewed mobility of previously stabilized contaminants and may adversely affect remedial effectiveness.
Natural attenuation can be used in conjuction with, or as a follow-up to, other intrusive remedial measures	Aquifer heterogeneity may compicate site characterization, as it will with any remedial approach
Natural attenuation is not subject to limitations imposed by the use of mechanized remediation equipment (e.g. no equipment downtime)	Intermediate products of biodegradation [e.g vinyl chloride (VC)] can be more toxic than the original [e.g. trichloroethene (TCE)]

Weidemeier et al (1999)

As the public has gained a higher level of environmental consciousness, new concerns have been voiced, often calling MNA a "do nothing" approach to contaminant cleanup. According to Diane Heminway of the Citizens' Environmental Coalition in Medina, New York, many feel that, "in an effort to reduce the number of sites on the Superfund list, governmental regulators are simply cleaning up these sites with an eraser" (63). In an effort to increase the perceived legitimacy of MNA in light of limited scientific knowledge, the OSWER Directive 9200.4-17 (113), released in 1997 offered a more specific framework when dealing with chlorinated aliphatics asserting that evidence must include

1) Historical ground water and/or soil chemistry data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. In the case of a ground water plume, decreasing concentrations should not be solely the result of plume migration. In the case of inorganic contaminants, the primary attenuating mechanism should also be understood.
2) Hydrogeologic and geochemical data that can be used to demonstrate indirectly the type(s) of natural attenuation processes active at the site, and the rate at which such processes will reduce contaminant concentrations to required levels. For example, characterization data may be used to quantify the rates of contaminant sorption, dilution, or volatilization, or to demonstrate and quantify the rates of biological degradation processes occurring at the site.

3) Data from field or microcosm studies (conducted in or with actual contaminated site media) which directly demonstrate the occurrence of a particular natural attenuation process at the site and its ability to degrade the contaminants of concern (typically used to demonstrate biological degradation processes only).

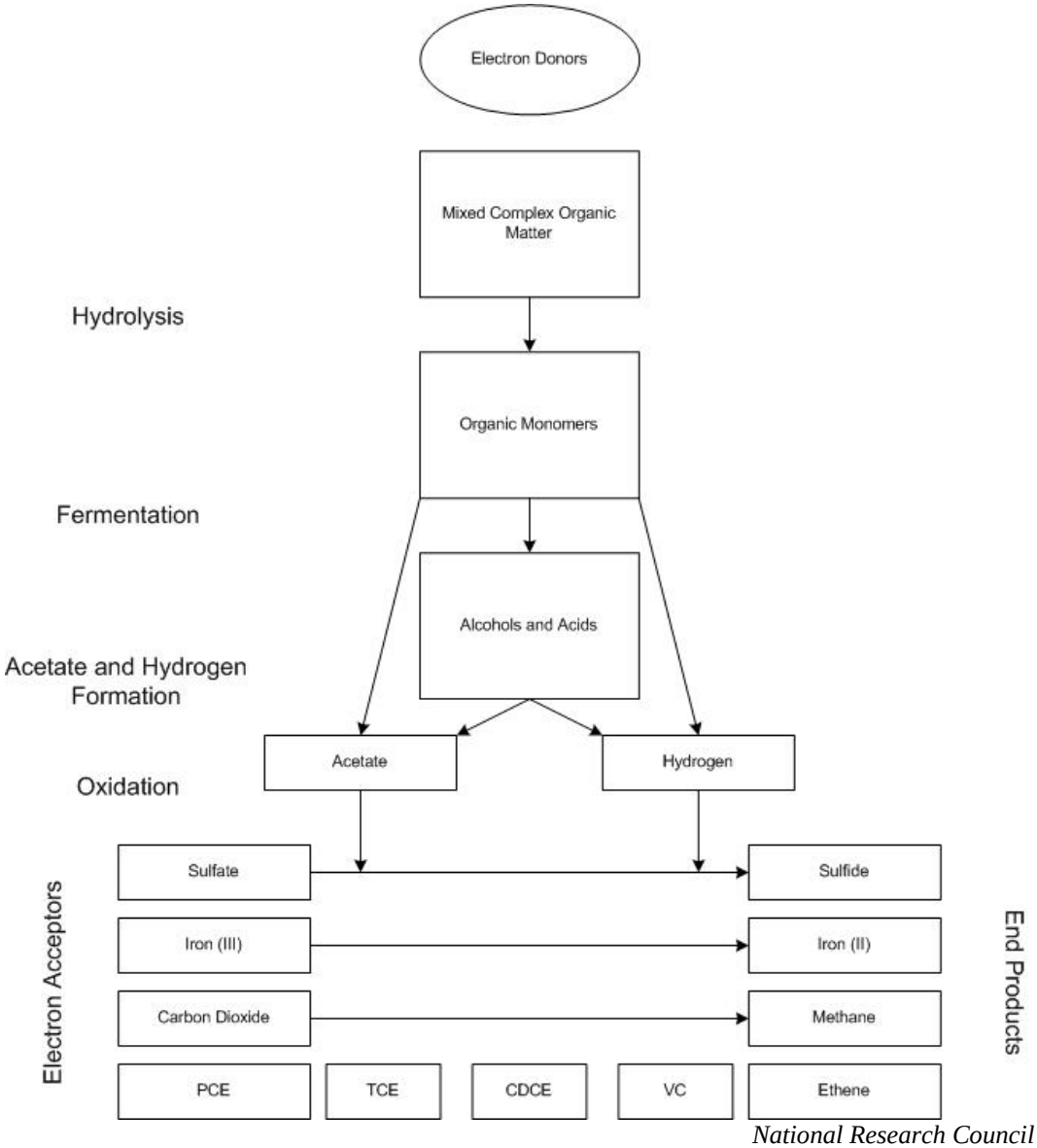
A recent EPA publication entitled *Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Groundwater* offers the following guidance in interpreting the aforementioned evidence stating (114)

> *Unless EPA or the implementing state agency determines that historical data (Number 1 above) are of sufficient quality and duration to support a decision to use monitored natural attenuation, EPA expects that data characterizing the nature and rates of natural attenuation processes at the site (Number 2 above) should be provided. Where the latter are also inadequate or inconclusive, data from microcosm studies (Number 3 above) may also be necessary. In general, more supporting information may be required to demonstrate the efficacy of monitored natural attenuation at those sites with contaminants which do not readily degrade through biological processes (e.g., most non-petroleum compounds, inorganics), at sites with contaminants that transform into more toxic and/or mobile forms than the parent contaminant, or at sites where monitoring has been performed for a relatively short period of time. The amount and type of information needed for such a demonstration will depend upon a number of site-specific factors, such as the size and nature of the contamination problem, the proximity of receptors and the potential risk to those receptors, and other physical characteristics of the environmental setting (e.g., hydrogeology, ground cover, or climatic conditions).*

At most contaminated sites, the bulk of the contaminant is located in the "source zone". These sources may include ruptured transport pipes, leaking underground storage tanks and drums, landfills, etc. It would, of course, seem reasonable that the removal of these persistent sources would expedite MNA remediation efforts. The goals of source removal would be to remove as much contaminant mass as practical, in the hope of reducing the longevity and perhaps concentration of the contaminant plume, and thereby avoid any changes that would reduce the effectiveness of natural attenuation (88). The DNAPL character of chlorinated solvents makes source removal problematic because detection of DNAPL and characterization of mass is costly and technically difficult. PCE and TCE tend to pool at the crest of confining units and thus can even travel counter to hydraulic gradients.

As stated before, there are many processes, both physical and abiotic, that tend to immobilize or concentrate contaminants. From an environmental health standpoint, it would be most effective to physically transform hazardous chemicals into more innocuous products. Some naturally-occurring chemical reactions achieve this but the primary method is through biological conversion. Many microorganisms are capable of utilizing contaminants as primary growth substrates or are able to cometabolize contaminants, thus reducing toxicity. A more in depth review of specific metabolic pathways follows this section. Intrinsic bioremediation is the strict reliance on native populations and geochemistry to carry out this process. More commonly, systems must be emplaced which provide deficient nutrients or the population themselves. This is known as engineered bioremediation. The basic processes involved in bioremediation are shown in Figure 2 (82). Bioremediation can contribute significantly to any MNA approach but

cannot be relied upon solely therefore it is important to understand its advantages and pitfalls to determine the efficacy of this technology on a site specific basis.



National Research Council (2000)

Figure 2. Basic Steps in the Bioremediation of Chlorinated Solvents

IN SITU BIOREMEDIATION

The greatest enigma facing the effectiveness of in situ bioremediation in the field is the great deal of uncertainty with natural systems. Due to site complexities, the site best suited for this type of technology is one which mimics most closely the lab scale setting in which initial tests are performed, one which can be easily predicted and monitored. Of course, spatial heterogeneity and lack of reliable data often prevent a clear understanding of system dynamics. To further complicate matters, contaminants and the microorganisms capable of degrading them occur under limited site specific conditions therefore there is no one set of site conditions amenable to in situ bioremediation.

When predicting the success of intrinsic bioremediation, the most important factor beyond the available microorganisms and contaminant itself is the nature of tainted soil. If the goal of a remediation project is to not only remove harmful substances from the subsurface but to restore the soil to its preexisting condition, one must possess a clear understanding of the complexities of the medium and it many functions (3). Soil properties vary on a wide scale based on its material composition and grain size, climatic setting, and age of contaminant discharge. These factors can be classified as physical and structural, chemical, and biological (Table 3).

Table 3. Physical, Chemical and Biological Properties of Soil

Physical/Structural Properties	***Chemical Properties***	***Biological***
Hydraulic conductivity	Contaminant concentration	Vegetation present
Permeability	pH	Animals present
Pore size	Anion/cation exchange	Microorganisms present
Temperature	capacity	Root structures
Moisture	Presence of nutrients, salts,	Leaf litter
Presence of organic matter	minerals, organic matter	
Degree of homogeneity		

Cheng et al (2000)

In determining how resident and inoculated populations function as part of a remediation system, it is imperative to understand what conditions are necessary to initiate and sustain biological activity. A thorough overview of specific metabolic processes is given in subsequent sections. Microorganisms require certain nutrients for survival. Assimilatory reactions garner C, N, S, and P for biomass production while dissimilatory reactions use other micronutrients for assimilatory and maintenance processes. In the event that the required nutrients are not present in a system, microorganisms will be left incapable of performing basic functions and thus lose the ability to degrade contaminants. Microorganisms are dependent on carbon leached from surface materials or materials delivered from adjacent aquifers and the capillary rise of groundwater (3) as well as hydrogen derived from naturally occurring substrates. Up to this point, there has only been one culture proven capable of transforming PCE completely to ethene (80). This culture uses hydrogen as its direct electron acceptor in a process called dehalorespiration. Scientists have concluded that dehalorespiration follows the preceding general form

$$H_2 + C\cdots Cl \Rightarrow H\cdots Cl + H^+ + C$$

This pathway indicates the importance of maintaining adequate hydrogen concentrations in contaminant fields. Only recently has work been done focusing on the importance of direct addition of hydrogen as an electron donor (56), (101), (8). Due to the difficulty of direct hydrogen introduction in the field it is often necessary to rely on other methods. Much of the prior work has been focused on the addition of secondary hydrogen sources such as organic substrates which, through a fermentation reaction, yield hydrogen (118),(51),(54). Some research, based on microcosm and co-culture experiments, has also considered the manner in which hydrogen is introduced in the system and how this affects both the degradation endpoint and kinetics (70), (134). Jianzhong et al (70) concluded that the addition of H_2 or a fermentable substrate (e.g. acetate) may be sufficient to support complete reductive dechlorination. Yang et al. (134) found that addition of fermentable substrates such as olive oil and pentanol could increase DNAPL dissolution three fold while mediating methanogenic substrate utilization (134).

The presence of hydrogen in a system is necessary for biological transformation to occur but its sheer existence does not imply that reductive dechlorination will occur. Recent work has shown that dechlorinating population can utilize hydrogen at lower concentrations than that of methanogens (47), (133), (70). In an environment containing excess hydrogen, viable dechlorinators may be outcompeted, consequently slowing degradation kinetics and reducing populations. An engineered approach to this problem has resulted in the development of hydrogen reducing compounds (HRCs) which are able to release hydrogen in amounts sufficient to maintain dechlorinating species but able to starve competing methanogens (91).

The success of any bioremediation strategy is also governed by the oxidation-reduction potential (ORP) of the site. Because microorganisms use contaminants for energy, it is thermodynamically favorable to utilize reactions which yield the greatest energy. The energy produced in these reactions is governed by the Gibb's free energy of the reaction or the maximum useful energy change for a chemical process at constant temperature and pressure. Common electron acceptors found in aquifer are, in decreasing order of reductive capacity, oxygen, nitrate, manganese, iron, sulfate and carbon dioxide. Electron acceptors tend to be depleted consecutively in order of decreasing reductive power yielding a contaminant plume with the characteristic redox conditions shown in Figure 3. The distance from the source within a plume controls which electron acceptor will be present and in what quantities. Figure 4 illustrates the region of the electron tower in which reductive dechlorination is the primary method of biologic transformation. Because of the apparent recalcitrance of PCE and TCE under aerobic conditions (117), much work has been in the area of alternative electron acceptors. A thorough but not exhaustive list of this work is given as Table 4.

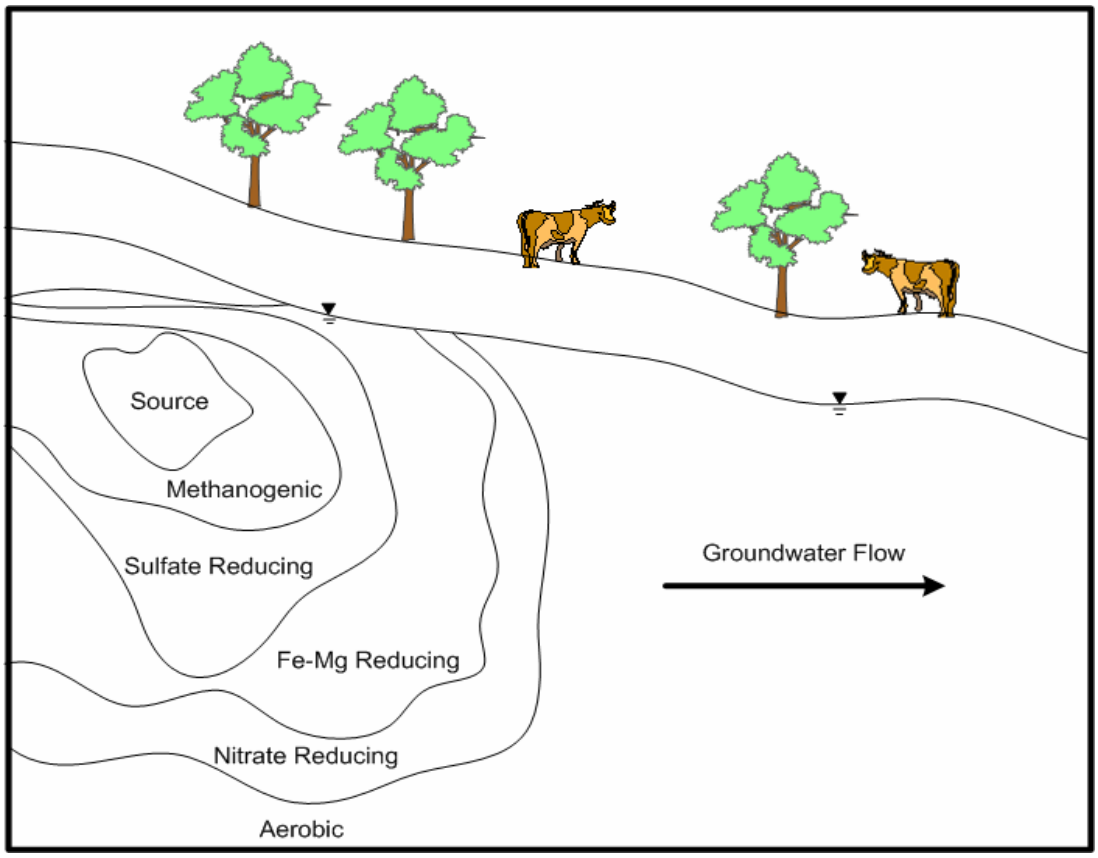
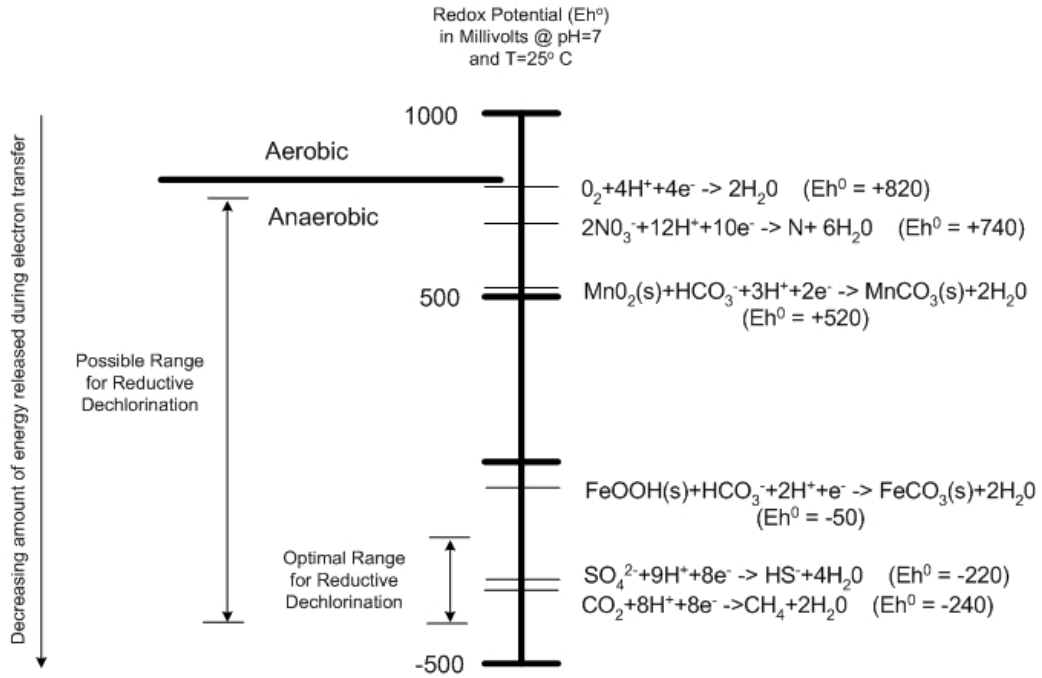


Figure 3. Characteristic Plume Profile and Redox State



Wiedemeyer (1999)

Figure 4. Possible Redox Range for Reduction Dechlorination

Table 4. Research Regarding Reductive Dechlorination with Alternate Electron Acceptors

Compound	***Electron Acceptor***	***Products***	***Reference***
Tetrachloroethene (PCE)	SO_4	TCE, cDCE	(7)
	CO_2	TCE, cDCE,VC, ethene, ethane, carbon dioxide	(118),(31)
	O_2	Not identified	(73)
	O_2 + CoM	TCE-epoxide, CO, glyoxylic acid, trichloroethanol, trichloroacetyldyhyde, trichloroacetate, dichloroacetate, glyoxylate, formic acid, CO_2	(98),(35)
Tricloroethene (TCE)	NO_3	Not identified	(73)
	SO_4	cDCE	(7)
	CO_2	cDCE, tDCE, VC, CO_2	(26)
	CO	cDCE, tDCE, 1-1-DCE, VC, ethene	(69)
	O_2	CO_2	(19),(71)
	O_2 + CoM	cDCE-epoxide, tDCE-epoxide	(67)
cis-1,2-Dichloroethene (cDCE)	Fe^{3+}	CO_2	(18)
	CO_2	VC, ethene, ethane	(72)
	Humic Acid	CO_2	(20)
trans-1,2-Dichloroethene (cDCE)	O_2	CO_2	(68)
	O_2 + CoM	tDCE-epoxide	(71)
1,1-Dichloroethene (1DCE)	O_2	Not identified	(128)
	O_2 + CoM	Not identified	(34)
	CO_2	VC	(128)
Vinyl Chloride (VC)	O_2	CO_2	(19;30)
	O_2 + CoM	Not identified	(34)
	Fe^{3+}	CO_2	(17)
	CO_2	Ethene, ethane, CO_2, CH_4, chloromethane	(13),(31),(18)
	Humic Acid	CO_2	(20)

Lee et al (2000)

It is well understood that, within the scope of MNA, successful bioremediation is one of the most promising methods to transform chlorinated solvents into more innocuous byproducts but with mass loss arising from other abiotic processes (sorption, advection, volatilization, etc…), it is often difficult to be certain that biological degradation, particularly reductive

dechlorination is occurring. The National Research Council (NRC) offered the first set of protocols for evaluating the efficacy of intrinsic bioremediation. These guidelines, proposed, by top experts in the field (88), require three lines of evidence that biodegradation is occurring including:

1) documented loss of contaminants from the site
2) laboratory assays or technical literature showing that microorganisms from the site samples have the potential to transform the contaminant under the expected site conditions; and
3) One or more pieces of information showing that the biodegradation potential is actually realized in the field.

Assessing the success of in situ bioremediation in the field can be a difficult task. Limitations of analytical methods and the inability to obtain representative samples often produce cloudy data at best but there are certain key indicators that denote successful biodegradation. The following rules of thumb signify that reductive dechlorination is occurring (122)

1. Ethene is being produced (even low concentrations are indicative of biodegradation)
2. Daughter products are being produced (such as *cis*-DCE or vinyl chloride)
3. Chloride concentrations are elevated
4. Methane is being produced
5. Fe(II) is being produced
6. Hydrogen concentrations are greater than 1 nM
7. Dissolved oxygen concentrations are low

Once it has been determined that bioremediation is a viable remediation alternative at a site, the time of remediation must be estimated. The complexity of relevant dechlorinating processes and site specific characteristics make accurate estimation of rate constants is difficult. However, much work has been done to estimate time dependent variables in site cleanups. Generally, biodegradation rate coefficients for chlorinated ethenes were found to be 0.0001 to 0.01 (half lives of about 20 years to 0.2 year) without significant variance between the compound (122) (Table 5). It is widely accepted that anaerobic biodegradation is most likely not a first order decay process or an instantaneous reaction but the mathematical simplicity and relatively good data fit of first order rates has resulted in their use in models. The determination of the constants has relied primarily on one of two methods of determination. Other methods that assume steady state equilibrium, which couples the regression of contaminant concentration versus downgradient distance with an analytical solution of the 1-D advection/dispersion equation (22) or conservation of mass methods (93). Some work, however has used carefully controlled laboratory experiments to estimate kinetic coefficients using the Monod model (33),(106). The changeability in this data suggests a strong spatial dependence on rate constant within the plume which could depend on many factors including reducing conditions, microbial population, and relevant abiotic sinks.

Table 5. Compiled Kinetic Data for Chlorinated Ethenes

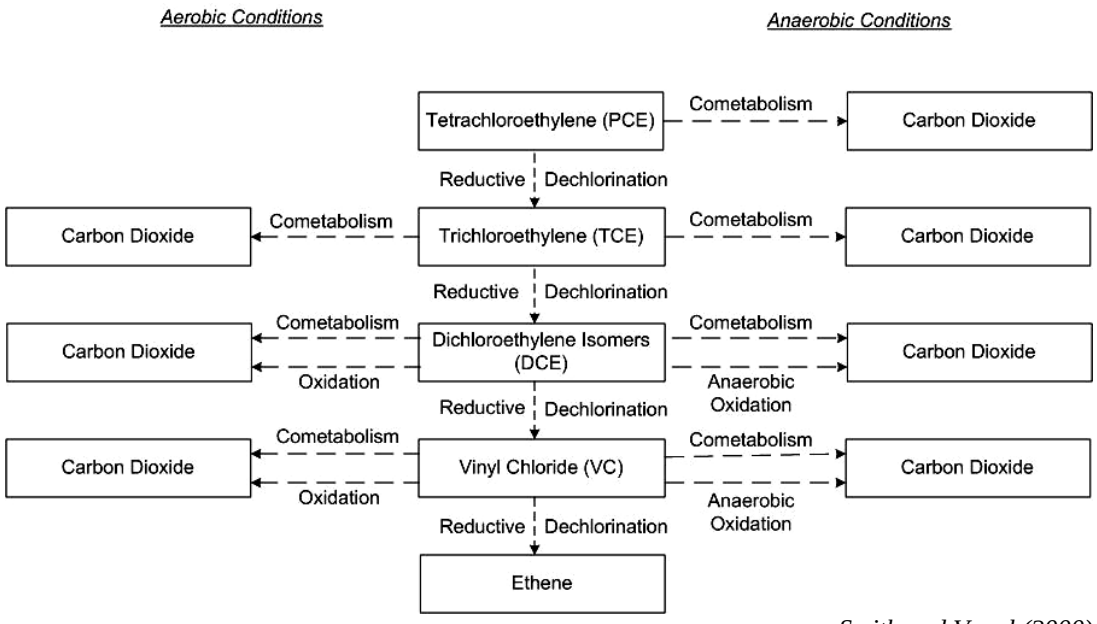
Compound	*First-Order Biodegradation Half-Life (days)*	*Microcosm*	*Field Scale*	*Reference*
PCE	13			(15)
	35	X		(90)
	877-1019		X	(40)
	2	X		(49)
	89	X		(127)
	33-90	X		(12)
	141	X		(129)
	141-210	X		(62)
	231-6930	X		(126)
TCE	116-347		X	(55)
	147-845		X	(120)
	182-210		X	(39)
	198-1100		X	(124)
	231		X	(27)
	365		X	(75)
	788		X	(14)
	877-1540		X	(40)
	990-1155		X	(37)
	1386		X	(105)
DCE	116	X		(18)
	277-4331		X	(124)
	495-1019		X	(40)
	2	X		(49)
	77	X		(130)
	27-82	X		(126)
	77-976		X	(105)
	158-495		X	(39)
	289		X	(120)
	1386		X	(27)
	89-347			(9)
	139	X		(131)
	77			(107)
	58	X		(30)
	23,100	X		(11)
VC	82		X	(27)
	98-1690		X	(120)
	533-1824		X	(124)
	693-806		X	(40)

Wiedemeyer et al (1999)

DEGRADATIVE METABOLIC PATHWAYS

In an effort to clean-up chlorinated solvents, in particular, it is important to know the metabolic pathways by which certain microorganisms can degrade these contaminants. The biological transformation of chlorinated ethenes has been a heavily researched topic for its promise as a remediation technology. The processes by which this biodegradation occurs fall into three categories 1) direct oxidation 2) cometabolism, in which contaminants are concurrently degraded along with primary substrates but no energy gained and 3) reductive dechlorination, by which the contaminant is used as the primary growth substrate and energy source. These processes can occur either or without the presence of oxygen. A summary of these processes is given as Figure 5. It is probable that no one process is responsible for

complete transformation of contaminants to harmless byproducts but rather, spatial sequencing within the plume utilizes appropriate redox conditions and microbial consortiums to fully convert wastes.



Smith and Vogel (2000)

Figure 5. Metabolic Pathways Pertinent to Chlorinated Solvents

DIRECT OXIDATION

When oxygen is used unequivocally as the electron acceptor and the contaminant as the electron donor, the chlorinated ethane can be systematically to more innocuous byproducts. To date, however, the higher chlorinated PCE and TCE have not demonstrated the capability of undergoing this process. The isomers of DCE have not been directly proven to degrade via this pathway but new data suggest that certain isomers of DCE could serve as a possible electron donor (19)

The most convincing work pertinent to chlorinated ethane removal is the work with vinyl chloride aerobically oxidizing directly to carbon dioxide and water (60),(59). Chapelle and Bradley have shown that VC (16) and possibly DCE (18) could be used as electron donors under iron reducing conditions.

Although direct aerobic oxidation in thought to only occur to partially chlorinated aliphatics, it is quite possible that it plays an integral part during the sequential process of biodegradation whereby the end products of methanogenic and sulfate reducing processes are further degraded downgradient where aerobic and iron reducing conditions are likely to be found.

COMETABOLISM

Cometabolism is a process by which microorganism produce certain enzymes in an effort to gain energy from a source other than the contaminant of interest. In the process of metabolizing this substrate, other compounds are transformed. Each of the chlorinated ethenes, less PCE, has been shown to degrade via aerobic cometabolism (87),(83),(116). For cometabolism to proceed, an oxygenase-inducing electron donor such as phenol, methane, or toluene must be present in

concentrations high enough to sustain suitable populations and must be added. These constituents do not often coincide with chlorinated solvents such as PCE or TCE. When they are present, the ratios of electron donor to the contaminant are not adequate to maintain the reaction (4), (96). Work by Vogel and coworkers have suggested that the degree of oxidation increases as the degree of chlorination decreases (116). The cometabolic pathway proceeds through a series of intermediates such as chlorinated oxides, aldehydes, ethanols and epoxides coupled with increased concentrations of chlorides, carbon mono/dioxides and numerous organics acids (84), (83).

Although anaerobic oxidative cometabolism has been shown to occur in the lab, it is widely held that alternate substrates are not present in natural systems in sufficient quantity to sustain this process (132). Reductive dechlorination can also occur cometabolically but is typically so "slow and incomplete that a successful natural attenuation strategy typically cannot completely rely upon it" (56).

The chlorinated aliphatic that holds the most promise of being remediated using the cometabolic pathway is TCE. Wilson and Wilson presented the first lines of evidence that TCE was aerobically degraded via methanotrophic bacteria in a soil enriched with CH_4 and oxygen (O_2) (132). Further studies further elucidated that the enzyme *methane monooxygenase* (MMO) was responsible for catalyzing the oxidation of TCE (2), (64). Cometabolism tends to be an unsustainable process under stagnant conditions because of substrate competition and enzyme inhibition and inactivation (42). Competition occurs between the natural substrates, such as CH_4, NH_3, or toluene, and chlorinated solvents for binding on the active site of the nonspecific oxygenase enzyme (97). The nature of most contaminated groundwater systems is generally anaerobic and would most likely require engineering systems capable of introducing oxygen into the system.

REDUCTIVE DECHLORINATION

Reductive dechlorination (RD) (more specifically, dehalorespiration) is a process in which contaminants are used as the primary growth substrate and electron acceptor. In this process, one chlorine ion is released as the molecule accepts two electrons from an electron carrier (Figure 1). Reductive dechlorination has been the metabolic pathway of primary focus in natural systems due to its prevalence under anaerobic conditions. It was first thought that this process occurred strictly cometabolically but in 1994, Hollinger et al discovered a microorganism capable of carrying out this transformation biologically (66). Freedman and Gosset were the first to show complete reduction of PCE to ethene under methanogenic conditions (51).

Reductive dechlorination has also been shown to occur cometabolically under nitrate reducing, sulfate reducing, iron reducing, and methanogenic conditions (85), (48), (44). Biologically significant redox carrier molecules such as B_{12}, hemes, and F_{430} have been cited as catalysts in cometabolic reductive dechlorination (52). Although reaction rates of this form of RD might be slower than traditional dehalorespiration, this process could prove significant in wetlands, landfills or other environments where high organic fractions and methanogenic respirations are likely to be found (74).

Hydrogen, the direct electron donor, is fundamental in this process and must be derived from naturally occurring fermentable substrates such as alcohols and organic acids making hydrogen levels significant in measuring MNA capabilities. The reductive chlorination process often vies for available electrons with other electron accepting processes (101), (133). When RD unsuccessfully competes for free electrons, it requires the oxidation of excessive amounts of hydrogen producing substrates coupled with only limited reduction of chlorinated compounds. Rarely is there a sufficient concentration of electron donor in natural systems and dechlorination

is often incomplete, ceasing at cDCE or VC (88), (82). However, reaction kinetics and field data analyses have shown that reductive dechlorination probably account for the majority of biotic loss at sites where biotransformation is significant in the attenuation of the plume

KNOWN DECHLORINATING CULTURES

Several anaerobic organisms have been isolated in pure culture, adept at partial metabolic dehalogenation of PCE. One such strain, *Dehalobacter restrictus*, uses molecular hydrogen as an electron donor but ceases dehalogenation at *cis*-DCE (65), (125). The bacterium *Desilfitobacterium sp. Strain PCE1* rapidly dechlorinates PCE to TCE in the presence of formate. The process is slower in the presence of propionate but leads to the production of vinyl chloride and ethene (53). Yet another homoacetogenic bacteria, *Sporomusa ovata*, was found to dechlorinate PCE to TCE following a pseudo first order kinetic (108). Neumann et al used an activated sludge sample to isolate *Dehalospirillum multivorans*, capable of the dechlorination of tetrachloroethylene to dichloroethene with H_2, pyruvate, ethanol or glycerol as electron donor (89), (95). Scientists have also isolated two cultures, *Burkholderia cepacia* G4 and *Methylosinus trichosporium*, capable of aerobic cometabolic degradation of TCE in the presence of toluene (77), (104). At present there is only one isolate, *Dehalococcoides ethenogenes*, proven able to dechlorinate PCE completely to ethene (80), (79), (81). More recently, Edwards et al claims to have isolated yet another species (KB-1) capable of full dechlorination but further verification is required (38).

Until recently, PCE was thought to be recalcitrant under aerobic conditions (117) but recent work using, *Pseudomonas Stutzeri* OX1, containing the aerobic toluene-o-xylene enzyme suggests that perhaps PCE could be metabolized in oxygen rich environments (99).

With regard to mixed cultures, metabolic RD of PCE to VC or ethene has been obtained with various microbial methanol-consuming consortia selected after long acclimatization (DiStefano et al 1992). The dechlorinating microbial consortia show PCE disappearance rates intermediate between those obtained with metabolic and cometabolic processes in pure cultures.

BIOAUGMENTATION

Bioremediation has been a viable remediation technology since the late 70's. Optimal bioremediation requires the presence of a substrate, appropriate electron acceptors, sufficient peripheral nutrient concentrations, and suitable microbial populations. Public concern over the introduction of non-indigenous microbial species into the environment had led most researchers and remediation engineers to focus on biostimulation, the addition of the first three key components into the system in order to make conditions ideal for contaminant removal. Recently, a clearer understanding of microbial dynamics has suggested that perhaps partial or incomplete contaminant removal is due to incomplete microbial consortiums capable of degrading the waste. Bioaugmentation (BIO) refers to the engineered addition of microbes into the subsurface adept at promoting degradation, most often the transformation of contaminants into harmless byproducts. There is, however, a contention among some scientists, those of the "ubiquity principle", who believe that all bacteria are available at all sites of any contamination and contend bioaugmentation is never necessary (50).

The first large scale clean-up using bioaugmentation was in 1970's on British beaches contaminated by the *Torrey Canyon* spill. Since that time, BIO has been gaining increasing support from U.S. environmental agencies and culminated in its resounding success after the Exxon Valdez oil spill in 1989 (50). Bioaugmentation may broaden the list of sites suitable for bioremediation and Forsyth et al (50) proposed its use under the following conditions:

- Sites with low or nondetectable concentrations of contaminant-degrading microbes
- Sites contaminated with compounds requiring long acclimation or adaptation times
- Sites containing compounds requiring multi-process remediation, including processes detrimental or toxic to microbes
- Sites requiring cleanup under time constraints that may not be met using biostimulation alone
- Small-scale sites on which costs for extensive testing exceed costs for bioaugmentation

After determining the likelihood of a site for successful bioaugmentation, a suitable culture must be chosen. At this point, microbes may be purchased commercially and incubated in a manner which optimizes their dechlorinating capacity. Cultures used in bioaugmentation may be genetically engineered to improve regulatory control over the expression of certain biodegradative pathways. Due to concern over the release of these mutant strains into municipal settings, the use of these organisms has been limited to laboratory studies. A novel alternative to traditional genetic engineering has been the breeding of naturally occurring microbial populations under selective environmental pressures (21). The growth of microorganisms under restricted conditions leads to increased expression of beneficial phenotypic characteristics which can increase degradation rates. Both methods enhance the breadth of organisms capable of degrading target pollutants and thus increase the number of sites amenable to bioremediation. Some favorable characteristics that may be used for selection of a culture include (Adapted from Forsyth et al 1995):

- The ability to grow at low or high temperatures
- The ability to withstand high or low concentrations of contaminants
- Heavy metal tolerance
- The ability to survive in a wide range of conditions and/or media
- The ability to produce surfactants to make contaminants more accessible

Bioaugmentation is most successful when cultures are chosen that have already been adapted to the target contaminant and site conditions. Many commercially available products are actually a collection of microbial strains able to degrade contaminants likely to be found in concert such as the BTEX compounds or the chlorinated ethene suite. It is an economically sound practice to perform lab and pilot scale studies prior to field scale implementation to increase the probability of success. To date, much work has been done on all scales to prove the feasibility of BIO as a remediation strategy when dealing with chlorinated ethenes.

LAB SCALE STUDIES

Due to the complex character of the subsurface and the implementation expense of full scale tests, much of the bioaugmentation research has been done in the laboratory. The use of microcosm and column studies has given researchers insight into system dynamics under controlled conditions. Much of the early work with lab-scale bioaugmentation studies is covered by Daughton et al (29). More recent studies have focused on the cometabolism under semi-aerobic conditions. Munakata-Marr et al utilized a genetically engineered, phenol-fed mutant of *Burkholderia (Pseudomonas) cepacia* G4 in a microcosm study and found that biological addition led to a two-fold reduction in TCE concentration versus the non-augmented batch (86). Microcosm experiments using the still unsubstantiated KB-1 strain at 5 sites of varying

concentrations show promise of bioaugmentation in the treatment of PCE/TCE source areas and highly fractured environments (38)

Carr et al set up continuous-flow stirred tank reactors to estimate the influence of dechlorinating microorganisms in NAPL source zones (24). Culture addition resulted in a 14x greater reduction in NAPL concentrations versus the unamended microcosms. Similar results were demonstrated in the innoculated columns of Cope et al (25). This data confirms the previous hypothesis that the effectiveness of MNA is governed by biotic sinks (88).

Column studies from a well studied bioaugmentation test site at the Dover Air Force Base, Area 6, were used to test the disparity between biostimulation and bioaugmentation. In this study, soil amendments such as organics acids and other key nutrients were added without any apparent change in the cDCE endpoint. After a culture, derived from the DOE Pinellas site (109), was added, full dechlorination to ethane was achieved (58). The culture was also successful in degrading TCE at both high and low concentrations suggesting a resiliency to toxic shock.

PILOT/FIELD SCALE STUDIES

The ultimate goal of any laboratory experiment is to gain knowledge that will lead to successful practice in the field. This coupled with the inability to mimic realistic hydrologic variables and time scales in the lab has driven many scientists to set up experiments on larger scale.

The first of its kind, Fantroussi et al used a 500 L soil bioreactor to ascertain the long-term viability of inoculated pure cultures in soil and discover if long term monitoring was practical for systems of this scale (43). In this study, both sterile and non-sterile soil were innoculated with a known dechlorinating culture and fed a continuous stream of chlorobenzoate. The use of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis confirmed similar reports (45) that spatial population heterogeneities were directly correlated with degradation capacities .

The column work performed by Harkness et at (58)was implemented in the field at Dover Air Force base in 2000 by Ellis et al (41). A 3000 m^3 situ volume was amended with nutrients and substrate and monitored for nearly nine months at which time dechlorination by indigenous species ceased at cDCE, confirming laboratory experiments. The volume was then inoculated with the *Pinellas* culture (109) and complete transformation occurred within 8 months. Unfortunately, the aquifer used in this study was not studied in its natural state. The aquifer was naturally aerobic thus requiring the addition of lactate as a reducing agent. This, no doubt, created niches for other well-adapted peripheral microbial consortiums (41). Steffan et al (103) were able to use a 900 m^3 plot of an aquifer in its in situ state combined with the *Burkholderia cepacia* ENV435 strain (32) to achieve similar results (103). It appears that success can be achieved regardless of the method of culture introduction. In the Ellis analysis, a dehydrated culture was added in relatively low quantities ("35 g on a dry weight basis, based on optical measurements" (41)) while Steffan inundated 550 L of liquid culture ($1*10^{11}$ cfu/ml) into the test plot through upgradient wells or pumped through downgradient ones.

In 2000, another successful field investigation of bioaugmentation was performed at the Aerojet Superfund site in California (28). Once again, biostimulation failed to drive dechlorination beyond the cDCE endpoint. Within 1 week VC and ETH were observed. Seventy-five days later, microbial characterization using sequence specific primers confirmed that the *Dehalococcoides* KB-1 innoculum had been transported at least 50 ft from the original injection point and survived. Similar results using the same culture were obtained at the Kelly Air Force Base in South Central Texas under forced reducing conditions (76).

CAVEATS TO BIOAUGMENTATION

Bioaugmentation has been proven successful in the laboratory and holds promise for full scale implementation but still faces opposition from proponents of traditional remediation. It appears that researchers have found strains of microorganisms capable of degrading chlorinated ethenes in the lab but a number of issues that must be dealt with before bioaugmentation enters the mainstream.

The biggest logistical constraint is how to disperse these cultures in the subsurface. A thorough review of subsurface microbial transport is given by Harvey et al (61). Initial data suggests that the state of the culture (liquid or dry) makes little difference (57). The likelihood of the culture to disseminate into the groundwater system depends on many variables. Naturally, the most readily available mode of transport of microbes is concurrent with the movement of groundwater through the site. The same mechanism causing plume migration may also be a tool for biological transmittance. Soils with greater conductivities (sands, gravels) would have greater potential for microbial transport. As pore sizes decrease, however, natural filtering may occur. The greatest contributor to the retardation of microbial distribution is adhesion to organic matter. The degree of adhesion is governed by soil composition as well as electrolyte concentration (100). Some researchers have looks into the use of surfactants and foams to deal with the problem (94) or the use of strains resistant to adhesion (36).

The question of culture viability over the course of the project still remains. Many researchers have studied the competitive behavior of native microbial consortia in the presence of an environmental perturbation (78),(1). Newly introduced microbial populations may have a metabolic advantage over indigenous creatures in the presence of the target contaminant but must still compete for macronutrients such as H, N, P, etc. Some studies suggest that the fierce competition for hydrogen may govern the entire dechlorinating process (46), (70). Growth rate, too, may have an effect on the ability of foreign strains to survive. Some data shows doubling time on the order of hours and days (58), (41),(81). Faster growth rates not only lead to faster proliferation of species but resistance to predation by protozoa.

The activity of a microbial syndicate is also of concern within aquifer systems. Although lab results suggest wide concentration ranges in which dechlorinating strains can survive (58), it is still unclear whether particular dechlorinators could be susceptible to toxic shock or perhaps “starvation”. Other environmental factors such as temperature, pH, and salinity may have significant impacts on the wellbeing of introduced communities.

CONCLUSION

The extent of chlorinated ethene contamination and the push for reclamation within the urban landscape has led scientists to search for new ways to expedite the cleanup of contaminated lands. The understanding of natures own capability to remove xenobiotic substances (particularly chlorinated solvents) through bioremediation is in its infancy but is being guided by the discovery of new microbial species capable of metabolizing these components through novel degradative pathways. The effect of environmental factors also plays an important role in the ability of these microorganisms to remove contamination. From initial studies on varied scales, bioaugmentation shows promise to expand the reach of bioremediation to sites previously deemed discordant. Further work must be done to full reconcile the responsible mechanisms and limitations of this technology so it can be used reliably in the future.

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CHAPTER 3: MATERIALS AND METHODS

SITE DESCRIPTION

The Naval Amphibious Base Little Creek (NABLC) (Virginia Beach, VA.) is an operating military facility that provides logistic and support services for amphibious warfare operations. NABLC was commissioned in 1945 and spans over 2,000 acres of beachfront adjacent to the Chesapeake Bay (Figure 6).

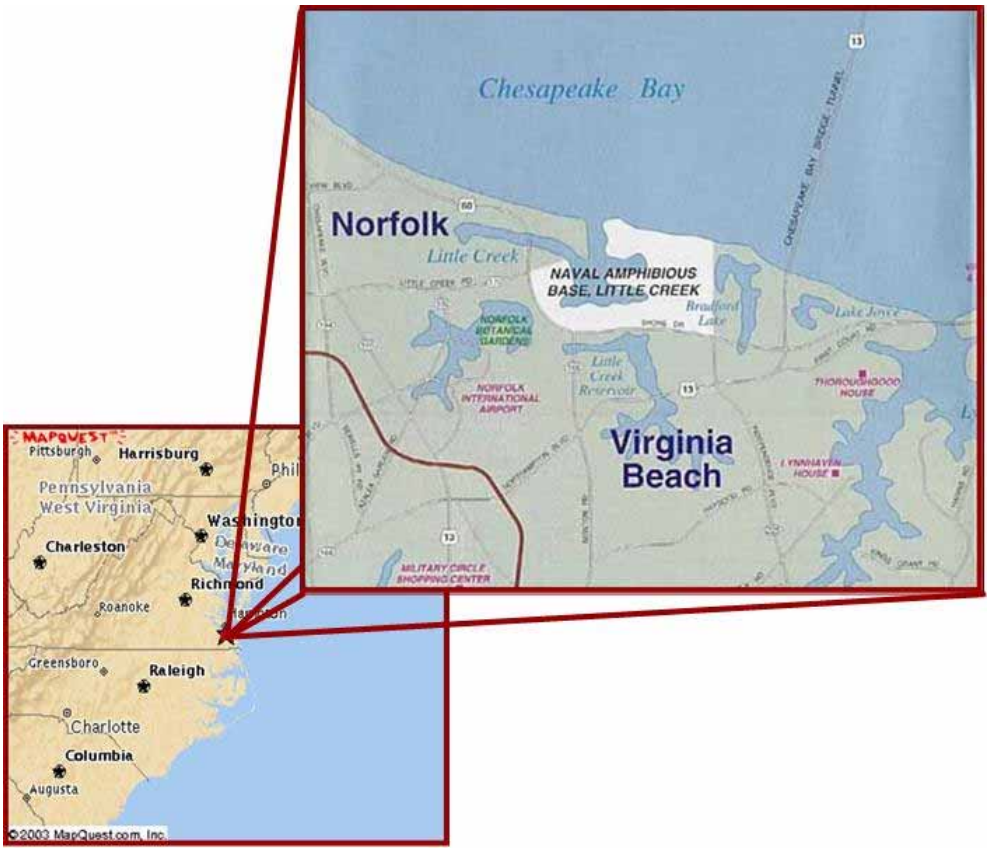


Figure 6. Naval Amphibious Base Little Creek (NABLC) Map

The PCE impacted area of concern for this study is a former laundry and dry-cleaning facility located on NABLC where an estimated 200 gallons of PCE were dumped into a leaking on-site storm sewer from 1973 to 1978 (9). The impacted zone is in an unconfined aquifer of the Columbia Group, composed of fine to medium sand with intermittent fine clay and silt layers. Groundwater depth is approximately 2 m below ground surface (bgs). A 9- to 12-meter confining clay aquitard separates the impacted aquifer from the Yorktown Aquifer, a regional drinking water source. Testing of groundwater samples collected from the underlying confined aquifer have not detected contaminants above drinking water standards, confirming that contamination is limited to the upper aquifer.

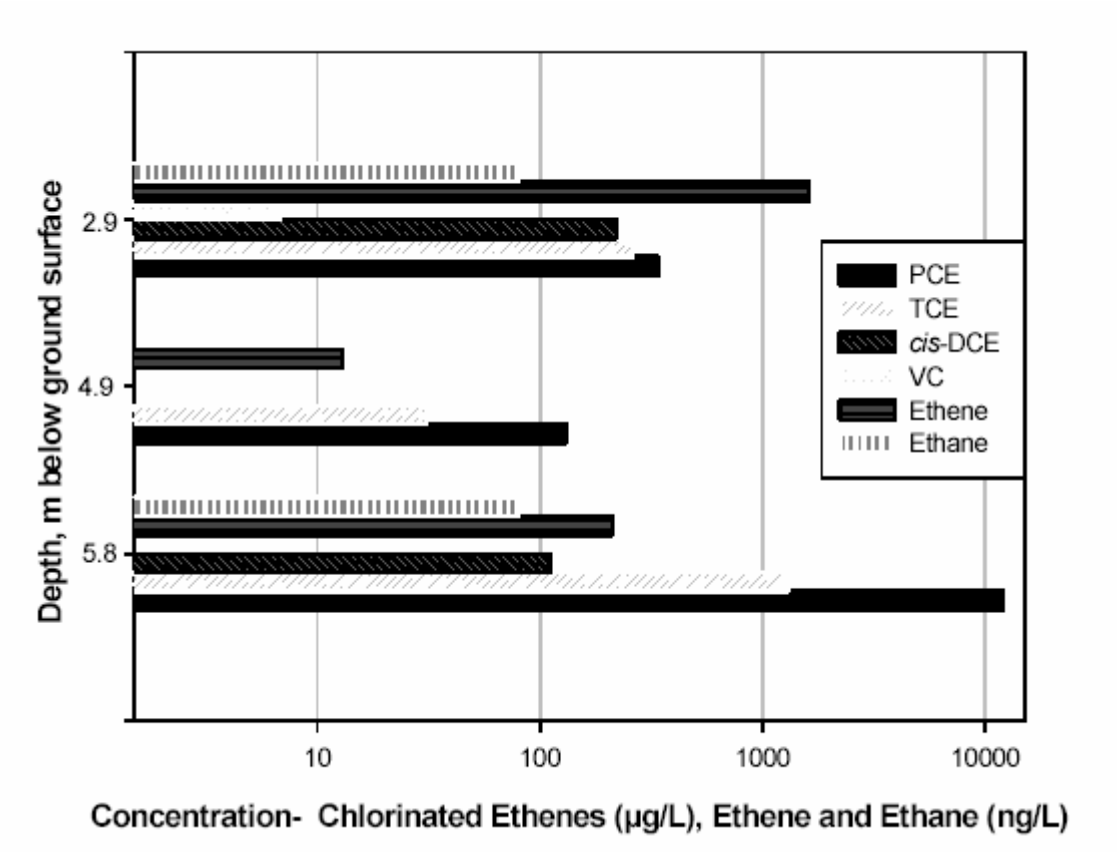
An array of traditional and multi-level sampler (MLS) monitoring wells were installed at the site to elucidate contamination profiles. Site groundwater samples were analyzed by a certified commercial laboratory in June 1999. The presence of PCE degradation byproducts not native to the source, and favorable environmental conditions (Table 6 and Figure 7) establish that reductive dechlorination by indigenous microorganisms is occurring at the site (1).

Table 6. Natural Attenuation Indicator Parameters from Selected Monitoring Wells at NABLC

Analyte	Well Location (distance along transect)				
	MLS-15 (Background)	MLS-11 (0 m)	MLS-12 (43 m)	MLS-22 (70 m)	MLS-20 (96 m)
PCE (µg/L)	ND	2290	2907	4217	914
TCE (µg/L)	ND	7.57	362	830	179
cis-DCE (µg/L)	ND	ND	107	38.5	45.3
VC (µg/L)	ND	ND	10.6	ND	ND
Ethene (µg/L)	ND	0.016	3.45	1.66	0.077
Ethane (µg/L)	ND	0.013	0.055	0.652	0.027
Methane (µg/L)	32.5	10.32	44.4	37.1	843
Dissolved Hydrogen (nM)	NA	NA	2.88	NA	2.56
Carbon Dioxide (mg/L)	144.6	137.8	105.4	75.4	54.2
Chloride (mg/L)	21.4	27.6	36.5	29.3	29.6
Sulfate (mg/L)	40.2	42.7	74.9	41.8	14.7
Sulfide (mg/L)	0.14	0.11	0.11	0.1	0.14

a Depth averaged concentrations for individual multi-level sampler monitoring wells.
ND - Not detected above method detection limits.
NA - Not analyzed.

Wang (2000)



Wang (2000)

Figure 7. Reductive Dechlorination Products in Well MLS-12 (Source Area) at NABLC

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The high proportion of PCE to the other degradation intermediates in the source area indicates that reductive dehalogenation processes may be slow or incomplete. However, since ethene is present, this indicates organisms capable of complete dehalogenation are present. Analysis of the electron acceptor conditions in groundwater samples collected in June 1999 indicate that sulfate reduction is the chief terminal electron-accepting process (11) and is sufficient for dehalorespirator growth (26). However, background and source zone sulfate/sulfide concentrations make an assessment of sulfate reduction difficult to verify. Chapelle et al. (11) note that geologic sources commonly produce sulfate, and that the presence of metals can precipitate sulfides from solution, therefore an accumulation of sulfides is not necessarily as an indicator of active sulfate reduction.

Prior studies conducted at this site by Berry et al. (1) using laboratory microcosm experiments under simulated natural (unamended) conditions demonstrated that indigenous dehalorespirators were capable of only partial dechlorination of PCE to *cis*-DCE. The purpose of this study was to investigate the feasibility of bioaugmentation as a possible source reduction measure in support of an MNA approach at NABLC. This study attempts to achieve complete reductive dechlorination by adding a microbial consortium to static microcosms and test the hypothesis that incorrect or incomplete PCE degrading communities exist at the site. All dechlorinating culture-amended microcosms were inoculated with a culture capable of transforming PCE to ethene.

AQUIFER SAMPLING

Due to monetary and logistical constraints, all aquifer sediments used in microcosm construction were obtained from microcosms previously established to test the viability of intrinsic bioremediation at the site (23). After lengthy discussion, it was determined that the proper storage of these soils had kept them viable for further study.

Microcosms were established using existing soil and tested for dechlorinating activity by spiking with 4 µmol PCE and monitoring solvent concentrations for 16 weeks (data not shown). Observed daughter product formation was consistent with prior Virginia Tech research using the same soil. This activity serves as the scientific basis for using preexisting soil for current work.

GROUNDWATER SAMPLES

Groundwater samples were collected by CH2MHill on January 8th, 2003. Two one-liter amber bottles were collected from both the blue and yellow ports of a multi-level sampler located at MLS-12 (4-6 ft and 10-12 ft depth respectively). Samples were packed on ice and shipped overnight to the Virginia Tech campus on January 9th, 2003. Once received, groundwater samples were immediately placed in the 4º C room for long term storage. These groundwater samples were used for microcosm construction.

CHEMICALS AND SOLUTIONS

Liquid PCE and TCE (Fisher Scientific, Pittsburgh, PA) were used for preparing stock feed solutions and analytical standards. *Cis*-DCE, 100 µg /mL in methanol and VC, 100 µg/mL in methanol (Fisher Scientific, Pittsburgh, PA), were used as analytical standards. Sodium acetate and lactic acid (Fisher Scientific) was used as electron donors and supplemental nutrient/carbon sources.

A mineral salts solution was created as a growth media for the bioaugmentation culture. It was adapted from Frank Loffler (see Appendix A)

MICROCOSM PREPARATION

Laboratory microcosm experiments were prepared in an anaerobic chamber under an N_2 atmosphere using modified Hungate techniques (18) and conducted in 160 mL serum bottles (125 mL nominal volume) (Wheaton, Millville, NJ) containing 50 grams of saturated aquifer sediment and approximately 100 mL of ground water (Figure 8).

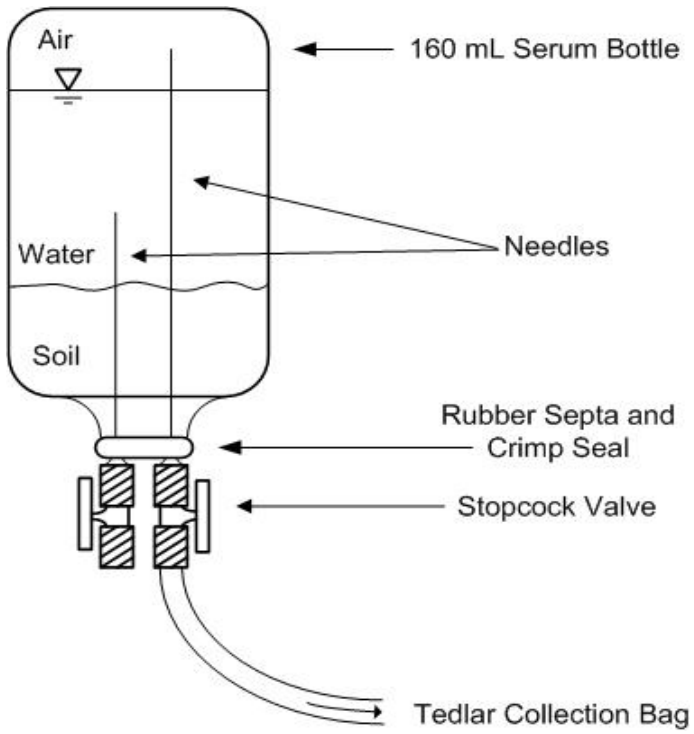


Figure 8. Microcosm Diagram

Duplicate microcosms were prepared representing the field conditions shown in Table 7. Prior to microcosm establishment, aquifer sediments were blended to reduce the effects of spatial heterogeneities.

Table 7. Microcosm Test Matrix

Well	***Depth***	***Added Bioaugmented Culture***
MLS-12	Shallow	Yes
		No
MLS-22	Shallow	Yes
		No
	Deep	Yes
		No
Abiotic Controls	Shallow	No
	Deep	No
Culture Control	No Soil	3X

Mixed samples for both shallow and deep depths were used for abiotic controls. Control soil was autoclaved a minimum of 20 times over the course of 2 weeks to allow for killing of germinated spores. Five mL of a 0.1 M sodium azide solution was also added to controls to further inhibit microbial activity.

All microcosms maintained a solids to water ratio of 67.7% (wt./wt.) to simulate aquifer conditions, and headspace volume of approximately 10 mL to allow for gas production. Anaerobic conditions were monitored through the use of a .1 M resazurin solution and achieved through the subsequent addition of a .065 M sodium sulfide solution. All active microcosms were fed .4 mL of a 10,000 ppm acetate solution. This mass was carefully calculated in an effort to keep hydrogen levels low and based on the molar conversion of PCE through to ethene. Calculated molar quantities were increased 4 times to account for the fact that hydrogen production and utilization is not 100% efficient. This follows current research suggesting that dechlorinators are able to out compete methanogens at lower hydrogen concentrations (19, 20, 30).

Bioaugmented batches were inoculated with 2 mL (2% by volume, ca 10^8 cells (based on Lofflers estimates) of a known dechlorinating mixed consortia provided by Dr. Frank Loffler of the Georgia Tech Institute of Technology. This culture has been shown to fully dechlorinate PCE to ethene and is composed predominantly *dehalococcoides* and *dehalobacter* species.

Each microcosm was spiked with approximately 6 µM PCE via direct injection. Some of the microcosms had residual PCE remaining from the prior experience therefore PCE levels may have been higher than that attributed to the spike. The bottles were then sealed with Teflon-lined rubber butyl septa and aluminum crimp caps, inverted, and statically incubated at room temperature in the dark. All microcosms were stored in a fabricated stand which allowed for simple transport and sampling events (Figure 9).

Figure 9. Microcosm Incubation Stand

On day 78 of testing, all microcosms were respiked with an additional 15 µM PCE, .7 ml (.122 M) lactic acid solution, and .6 ml of a .065 M Na_2S solution via direct injection. Once again, the amount of added lactic acid was determined to maintain hydrogen concentrations below the threshold of methanogenic communities. The choice to use lactic acid in place of sodium acetate was base on personal correspondence from the research group at Georgia Tech responsible for providing the culture. It should be noted that lactic acid is the primary component of hydrogen release compound (HRC), a commercially available product used for these purposes. This additional contaminant spike was carried out with the belief that previously injected microbial consortia undergo an acclimation period subsequent to initial exposure to contaminant introduction.

On day 91, after an observed reduction in both degradation rate and daughter product formation within the soil microcosms, three soil free microcosms were established containing a solution of 100 mL growth media, 26 µM PCE, and 5 mL of the dechlorinating culture (ca 10^8 cells (estimated by Loffler)). These microcosms would serve as an indicator of matrix effects as well as give an indication of the significance of increased concentrations on the culture. These three microcosms will be referred to as the Culture Control Microcosms.

ANALYTICAL TECHNIQUES

Chlorinated ethenes (PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC) were analyzed in the aqueous phase using a Model 3000 (Tekmar Co., Cincinnati, OH) purge-and-trap concentrator followed by a Model 9001 (Tremetrics Inc., Austin, TX) gas chromatograph (GC) equipped with a Model 1000 Hall detector (Tracor Instruments, Austin, TX) and capillary column (30 m . 0.53 mm, 2.0 µm film thickness, Rtx-Volatiles; Restek Corp., Bellefonte, PA). The oven temperature was held isothermal for 5 min. at 35°C, followed by temperature ramps of 6°C/min. up to 95°C and 26°C/min. up to 200°C. Liquid samples were extracted from microcosms using a gas-tight, locking, glass syringe (Hamilton Co., Reno, NV). Due to its proximity to the Atlantic Ocean, NABLC groundwater contained high levels of native chloride therefore increased chloride levels could not be measured and subsequently used as a line of evidence that biological PCE degradation was occurring.

MICROBIAL CULTIVATION AND ENUMERATION

After a literature review of known complete PCE-degrading cultures, the decision was made to use an isolate from the research team at Georgia Tech led by Frank Loffler (16, 27). After receiving approximately 300 ml (ca 10^8 cells), the decision was made to begin cultivation of additional quantities for use in subsequent research. A 1:1 ratio of glycerol (cryoprotective agent) to culture solution was created using three 5 ml aliquots and put in a 4°C freezer for preservation.

Remaining culture was developed using a 3% (vol/vol) addition into the aforementioned mineral salts medium (see Appendix A) within sterile 100 ml Pyrex media containers (Wheaton, Millville, NJ). Cultures were fed a molar-balanced combination of PCE (e^- acceptor) and sodium acetate (e^- donor/carbon source). Electron donors/acceptors were periodically added to sustain growth. Cultures were eventually transferred to autoclaved, rubber septa sealed 160 ml serum bottles for long term storage.

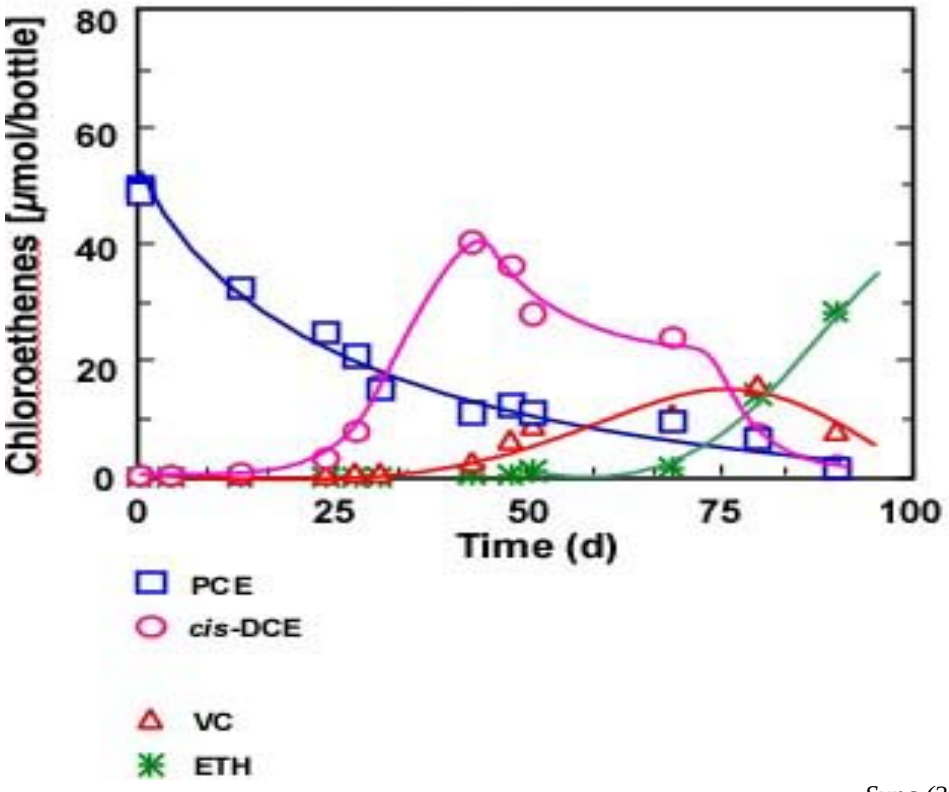
CHAPTER 4: RESULTS

INITIAL CULTURE VIABILITY DATA

A microbial culture capable of degrading PCE to ethene was provided by Dr. Frank Loffler of Georgia Tech. Independent lab data demonstrating that this particular culture was capable of completely dehalogenating PCE to ethane was included with the culture. Data for the culture is given in Figure 10 (27). Preliminary microbial identification performed at Georgia Tech denotes *dehalococcoides* and *dehalobacter* species as the dominant species in the mixed culture.

The degradation pattern shown in Figure 10 contains several notable features. One is the absence of TCE. Conversations with Mr. Sung (Georgia Tech) confirmed that TCE was never measured above 1 µmol/bottle. He also agreed that it is highly unlikely that the biological transformation is not proceeding through the TCE intermediate, but rather, that the conversion of TCE to DCE is rapid relative to the conversion of PCE to TCE, so TCE does not appear in solution.

For the data shown in Figure 10, a very high concentration of PCE was used, on the order of 80 ppm. This is well above the range of the source zone concentration at NABLC (400-1000 ppb). *Cis*-DCE was the only DCE isomer detected through the course of Loffler's enrichment culture experiments, which agrees with many field studies where *cis*-DCE is the primary isomer that is biologically produced (23). The significant production of *cis*-DCE in the culture microcosms suggest that the conversion of PCE to ethene is primarily biologically mediated.



Sung (2002)

Figure 10. Microcosm Results from the Georgia Tech Study Using a Mixed Culture Spiked with PCE

MICROCOSM EXPERIMENTS

A series of soil microcosms were created to determine the effect of the addition of Dr. Loffler's dechlorinating culture on the degradation of PCE using aquifer sediment from NABLC. Within the first 3 days of initial PCE injection, all microcosms (including killed controls) showed a decline in PCE in the liquid phase on the order of 60-85 %. This loss, considered to be abiotic, is attributed in part to adsorption onto aquifer sediments but is most likely an artifact of the sample not being well mixed. Other, less significant abiotic losses of PCE arise from partitioning into the gaseous phase. Calculations using Henry's Law suggest that losses due to aqueous-gas phase segregation account for only about 1% of the total contaminant decline. Therefore, the day one data was discounted and the value for the initial PCE concentration was taken as the value measured on day three.

Although the microcosm setup was designed to capture volatile gases, including ethene, measurable quantities of VC or ethene were not detected in any of the microcosms at any time. It is possible that there was some release of volatiles through leaks in the system but it is likely that the amount of ethene produced fell below the detection limit of the analytical equipment. At no point in the experiment were the *trans* or 1-1 DCE isomers found. Similarly, no biodegradation byproducts were detected in any of the killed controls.

Controls showed loss of PCE over the course of the experiment (Figure 11) on the order of 51 % and 60 % for the shallow and deep controls, respectively. These losses are similar to the results by Rectanus (23) using identical samples. Her experiments resulted in a 50 % loss of PCE through 245 days in the killed controls. At no point were any degradation end products seen in the killed controls. Because the headspace was vented to a tedlar bag, partial pressures were assumed to be atmospheric. Although PCE is less volatile that the other degradation byproducts (Henry's Constant of .015 atm m^3/mole)there was some loss to volatilization. PCE loss rates of 0.04 and 0.05 d^{-1} were observed in the shallow and deep controls, respectively. These rates are similar to control rates reported by Brauner et al. in a study of the degradation of aromatic compounds in soils (8). Brauner et al. attributed these losses to adsorption

Adsorption is probably responsible for the abiotic PCE losses from the aqueous phase. Abiotic reductive dechlorination can not be entirely ruled out because microcosm head space gas was not tested for short lived intermediates such as acetylene which result from the beta elimination pathway (25). Prior work by Rectanus (23) using identical soil samples attempted to quantify reductive capacities, including ferrous iron and sulfate, suspected catalysts for abiotic dechlorination (23). Groundwater analyses indicate "partial reductive dechlorination to *cis*-DCE and possible anaerobic oxidation to of *cis*-DCE to VC and ethene under sulfate-reducing and methanogenic conditions" (25).

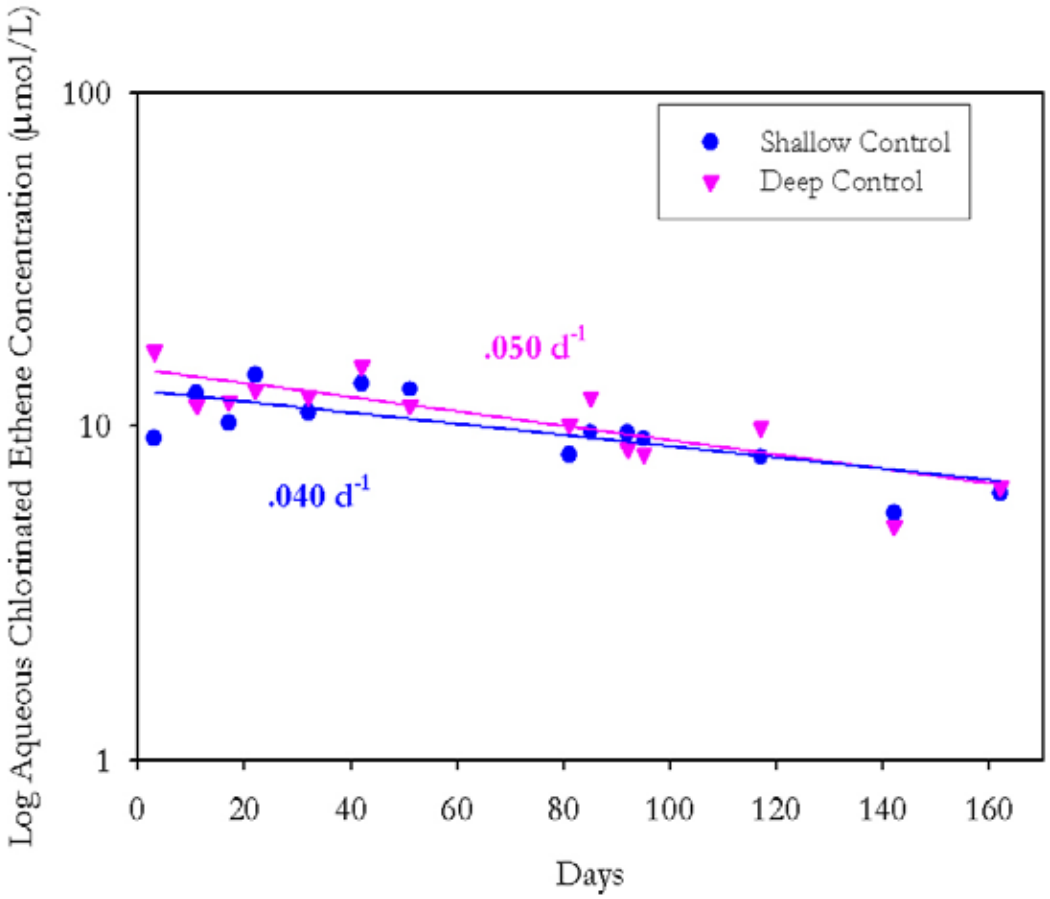


Figure 11. Deep and Shallow Killed Control Results for Total Chlorinated Ethenes

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The change in electron donor in the microcosms from sodium acetate to lactate on day 36 was made based on correspondence with members of the Georgia Tech research team responsible for the development of the dechlorinating culture. All cultures were developed at Georgia Tech using lactate as the principal hydrogen donor. It is known that dechlorinating cultures exhibit some electron donor preference, principally when the culture is developed in the lab (19, 27). It appears that dechlorination trends were unaffected by the change and no evidence suggests any effect of changing organic electron donor from acetate to lactate as indicated in Figures 2.7 and 2.8.

NON-AUGMENTED MICROCOSMS

Soil microcosms were constructed to test the ability of site soil to degrade PCE without the addition of a PCE degrading culture. At no point was vinyl chloride observed in any of the static microcosms that did not receive the addition of the PCE degrading culture (Figures 12, 13, 14). At the onset of the experiment, there was some concern about the viability of the aged soil and the effect that storage might have on resident microbial populations. However, the data are similar to microcosm responses for this soil when it was fresh (23). Rectanus calculated zero order PCE degradation rates of .07 and .11 µmol/day for shallow and deep soils respectively (23). Prior findings indicated that *cis*-DCE was the predominant dechlorination end product for this soil barring the addition of a dehalogenating culture capable of producing ethene (23).

It is evident from the data that the reductive capacities for different shallow soils from the Navy site differed from location to the other. The shallow soil samples from MLS-12 experienced only a 12% loss in PCE over the first 78 days while soil from MLS-22 Shallow had complete conversion of PCE and TCE to *cis*-DCE. After the addition of 15 µM PCE at day 78 of the experiment, there was a 72% and 70% reduction in aqueous PCE concentration in MLS-12 Shallow and MLS-22 Shallow respectively. The increase in degradation within the shallow soils could be attributed to the activation of dormant dechlorinating populations within the MLS-12 Shallow soil at higher concentrations or perhaps the initiation of pathways capable of degrading PCE within existing consortia. It may also be a result of population growth among dechlorinating microorganisms. It appears that the data for the respiking would best represent the response in these soils in the presence of contamination by PCE.

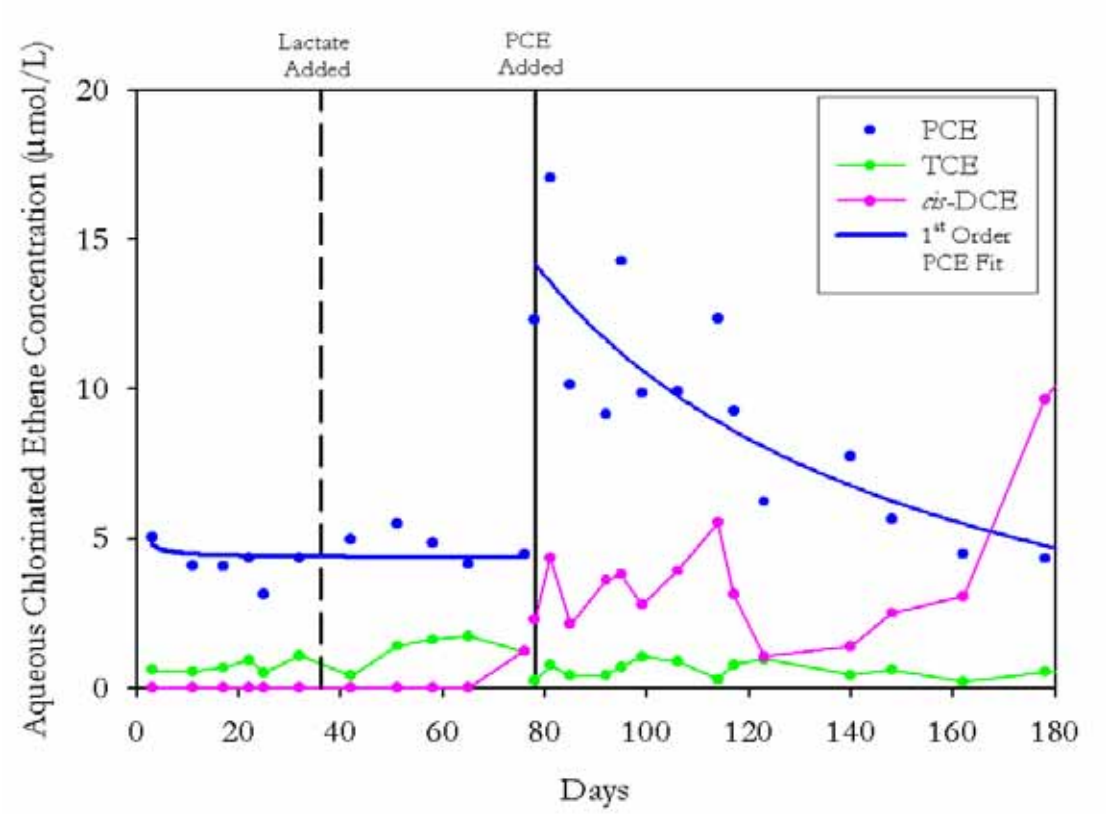

Figure 12. Measured Concentration of Chlorinated Ethenes in Unamended MLS-12 Shallow Microcosm Spiked with PCE

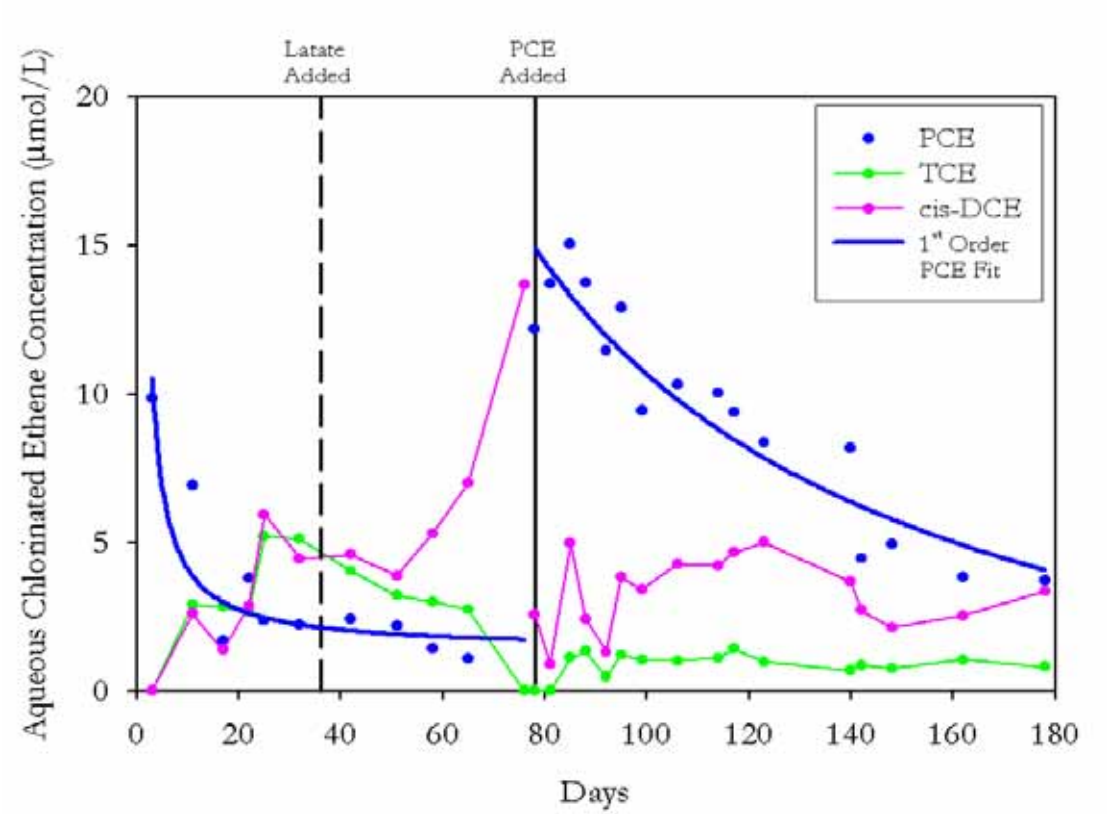

Figure 13. Measured Concentration of Chlorinated Ethenes in Unamended MLS-22 Shallow Microcosm Spiked with PCE

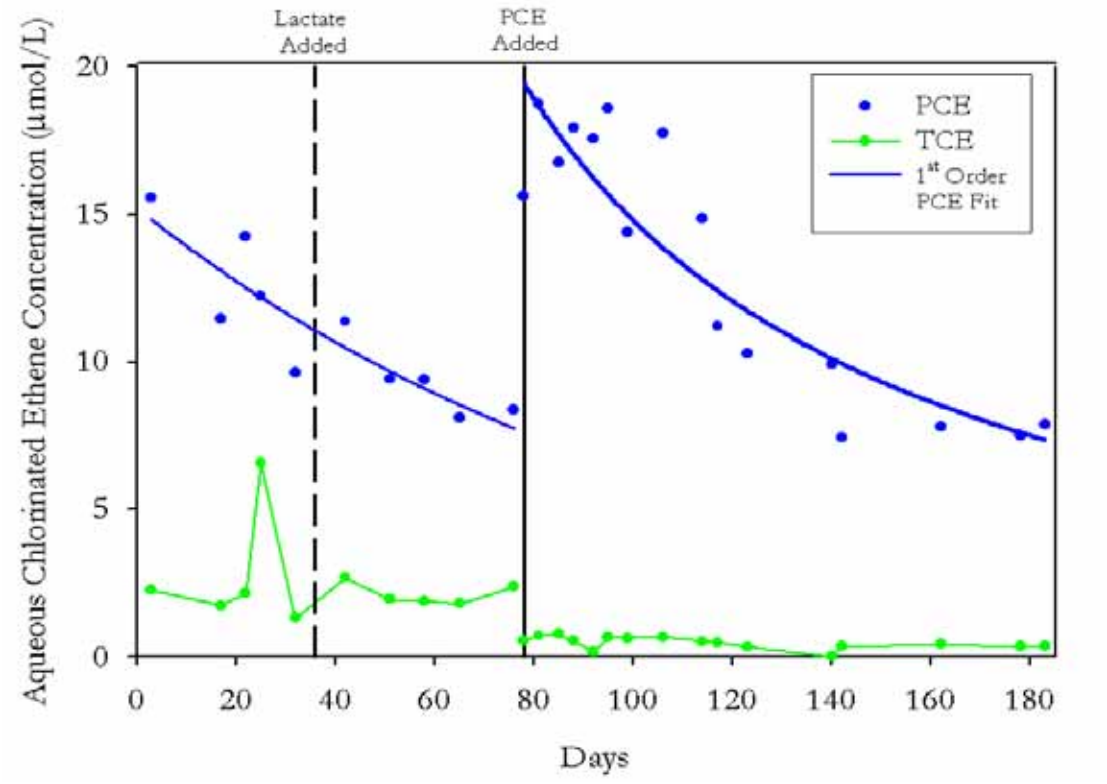

Figure 14. Measured Concentration of Chlorinated Ethenes in Unamended MLS-22 Deep Microcosm Spiked with PCE

For the deep soil at MLS-22, dehalogenation was less prolific (Figure 14). At no point was *cis*-DCE or VC produced. Small amounts of TCE were detected. However, the amounts changed little over time. During the first 78 days of the experiment, PCE levels were reduced by 47% while the additional mass added at day 78 was reduced by 58% over the remaining 105 days of the experiment. Calculated first order degradation rates are given at Table 8. Average PCE loss rates for the unamended microcosms (.09 d^{-1}) were greater than the control (.05 d^{-1}) suggesting that some biological activity was taking place. For deep soils, there appeared to be greater loss of PCE over controls without measurable production of TCE or *cis*-DCE. This could be the result of the direct conversion of TCE to ethane (14). It is possible that the deep soils from the Navy site do not possess the reductive capacity, whether biologically or geochemically, to drive dechlorination beyond PCE.

Table 8. Calculated First Order PCE Degradation Rate Constants (d^{-1})

Microcosm		*Post 6 µM injection*		*Post 15 µM injection*		*Rate Increase due to Concentration*
Unamended	*MLS 12S*	0.00_a	K_{avg} = 0.06	0.09	K_{avg} = 0.11	45%
	MLS 22S	0.09		0.11		
	MLS 22D	0.09		0.12		
Bioaugmented	*MLS 12S*	0.04	K_{avg}^{c} = 0.17	0.22	K_{avg} = 0.20	15%
	MLS 22S	0.73_b		0.19		
	MLS 22D	0.10		0.19		
Rate Increase due to Bioaugmentation		65%		45%		
Enrichment Culture [d]	*500 µM*	$4.17 * 10^{-9}$				
	25 µM	$8.00 * 10^{-9}$				
Controls	*Deep*	0.04				
	Shallow	0.05				

Notes:
[a] = *Slope was actualy positive due to data scatter*
[b] = *only used two data points to determine slope due to rapid loss of PCE*
[c] = *used half of .73 (.34) for average calculation*
[d] = *calculated on a per cell basis*

BIOAUGMENTED MICROCOSMS

All bioaugmented microcosms resulted in an increased production of degradation byproducts over their unamended counterparts (Figures 15, 16, 17). Both shallow samples at MLS-12 Shallow and 22 Shallow appeared to be more active than MLS-12 Deep, as confirmed by the production of VC. Although it could not be confirmed by head space samples, it is likely that the decline in aqueous VC coincided with production of ethene. Although attempts were made to avoid volatile release in the design of the microcosm and in the sampling protocol, one could assume that, due to its high volatility, some gaseous VC was lost from the system. It can also be seen that neither of the shallow soil microcosms generated levels of TCE above background; a very distinct characteristic of the aqueous ethene profile produced by the additive consortia (Figure 6). A distinct characteristic is that VC was produced concurrent with the first spike of PCE but not following the respike of PCE. It appears that an anaerobic oxidizing culture in the native soils was activated and dominated over the bioaugmented cultures after the second spike.

It is suspected that a probable sink for VC and cis-DCE may be possible diffusion of these byproducts through the plastic tubing. The concurrent loss of PCE and lack of degradation end products support this proposal. The direct oxidation and mineralization of VC to CO_2 has been well documented for VC in the presence of humic substances (HS) (3) and under methanogenic conditions (5, 6). Direct oxidation has also been documented for cis-DCE and TCE under anaerobic conditions (7, 10). This process has been proven under manganese reducing conditions. Other studies suggest that microorganisms capable of HS cometabolic oxidation of chlorinated ethenes are phylogenetically diverse and ubiquitous (12). At this point is unclear to what extent anaerobic direct oxidation played in the removal of VC and *cis*-DCE.

Data for MLS-22 Shallow best elucidated the benefits of bioaugmentation, showing complete conversion of PCE to zero background levels over the first 78 days of the experiment. The aqueous PCE concentration in MLS-12 and 22 dropped by 64% and 62% respectively.

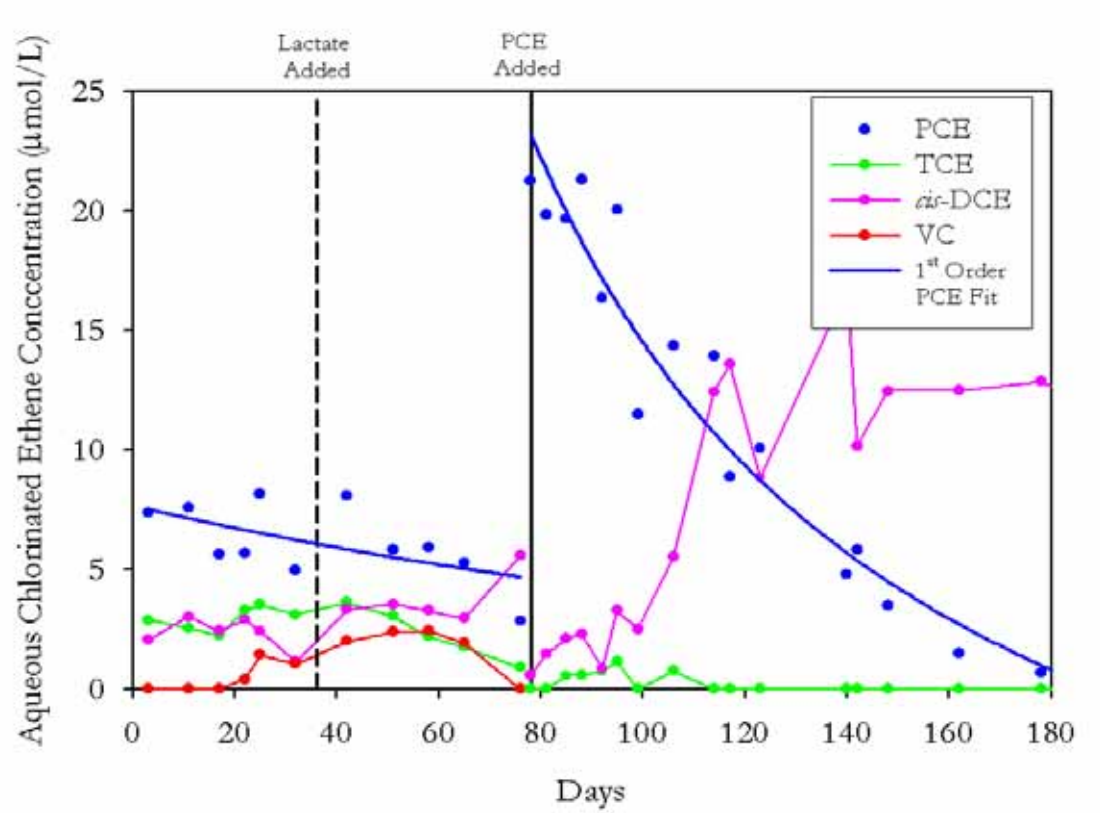

Figure 15. Measured Concentration of Chlorinated Ethenes in Bioaugmented MLS-12 Shallow Microcosm Spiked with PCE

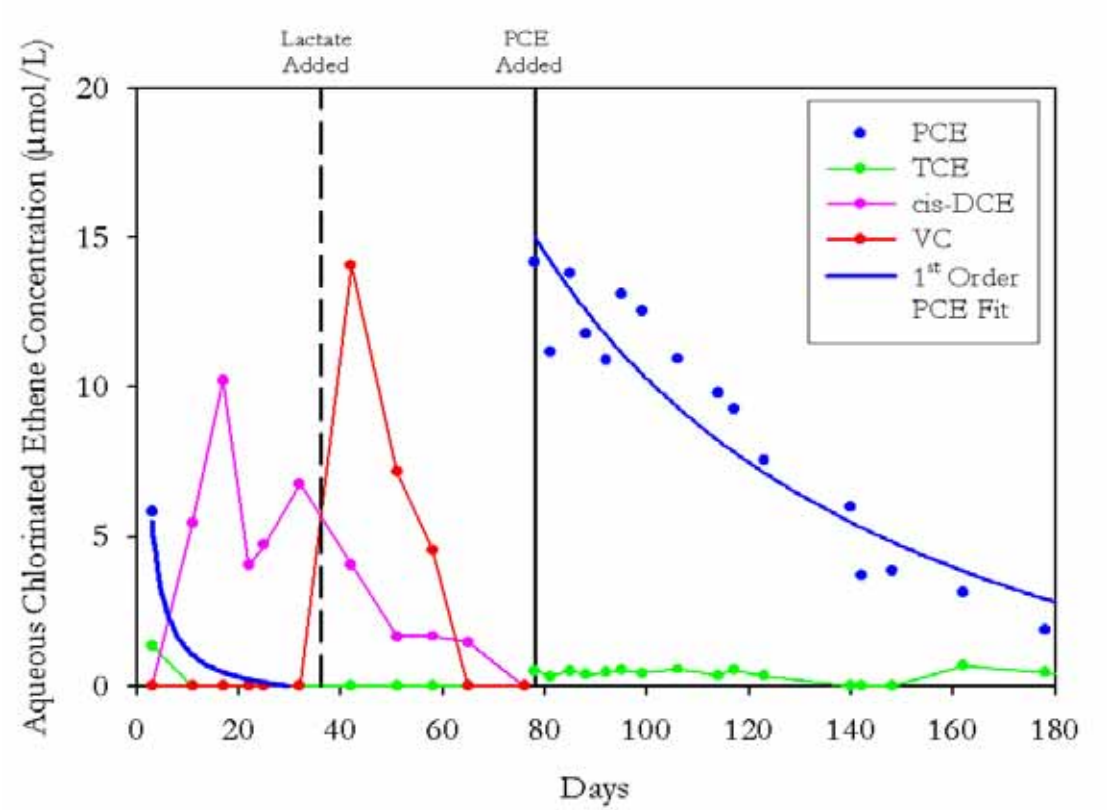

Figure 16. Measured Concentration of Chlorinated Ethenes in Bioaugmented MLS-22 Shallow Microcosm Spiked with PCE

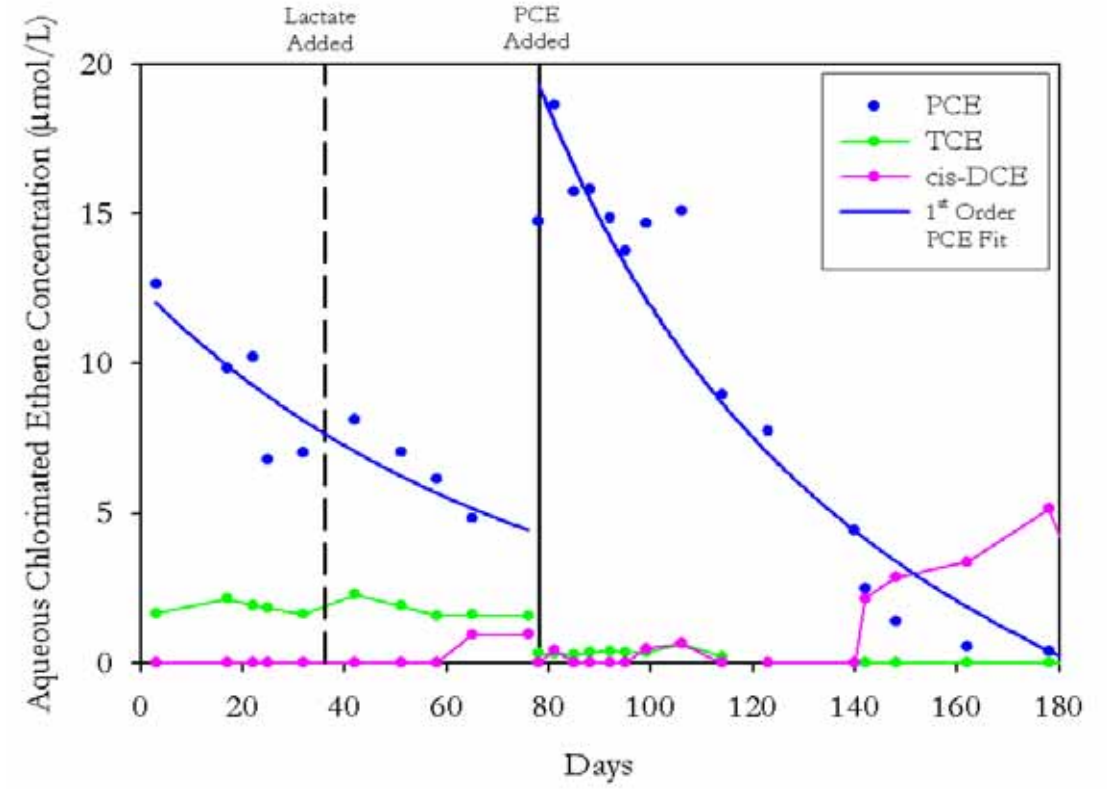

Figure 17. Measured Concentration of Chlorinated Ethenes in Bioaugmented MLS-22 Deep Microcosm Spiked with PCE

When the bioaugmented microcosms were respiked with 15 μM PCE at day 78, PCE degradation rates increased at MLS-12 Shallow, 22 Shallow, and 12 Deep to .22, .19, and .19 d^{-1}, respectively, an increase of 2.4 times (MLS-12 Shallow), 1.72 times (MLS-22 Shallow), and 58 times (MLS-12 Deep) over their non-augmented counterpart (Table 7). The similarity of these rates suggest the added culture was controlling the degradation of PCE. At no point was VC detected in any of the bioaugmented microcosms after the respike. It is possible that the lack of VC production was due to volatilization and diffusion through the plastic tubing and to a limited extent direct anaerobic oxidation of *cis*-DCE and VC to CO_2 by an indigenous anaerobic oxidizing species whose metabolic pathways were stimulated after the initial spike (2). Both MLS-12 Shallow and MLS-12 Deep microcosms were characterized by an accumulation of *cis*-DCE while MLS-22 Shallow showed a decline in PCE concentrations of 83%, but with little production of dechlorination intermediates.

ENRICHMENT CULTURE EXPERIMENT

Soil free microcosms were constructed in triplicate in order to remove matrix effects and test the effect of increased concentration of PCE on the bioaugmentation culture. A mixture was created using a 2% inoculation by volume to a mineral salts medium (Appendix A). Cell densities for the inoculation culture were estimated by Loffler to be on the order of 10^8 cells however it is difficult to estimate exactly what the cell densities were at the time of inoculation. The mixture was fed PCE at concentrations corresponding to those in the soil microcosms (26 μM) and aqueous ethene concentrations were measured over time (Figure 63).

Although, there was a marked decrease in the PCE concentration, degradation byproducts were not detected at any point during the 60 days of the experiment. After calculating the degradation rates on a per cell basis, the rates are very similar, 4.17 $*10^{-9}$ d^{-1} and 8.00 $*10^{-9}$ d^{-1} for Lofflers results and the current experiment respectively. These finding are significant considering PCE concentrations in Loffler's experiments were 500 μM (well above concentrations at the NABLC) compared to only 26 μM during the current experiment. This drastic reduction in kinetics can be attributed directly to the 2% dilution, leading to significantly lower dechlorinating populations.

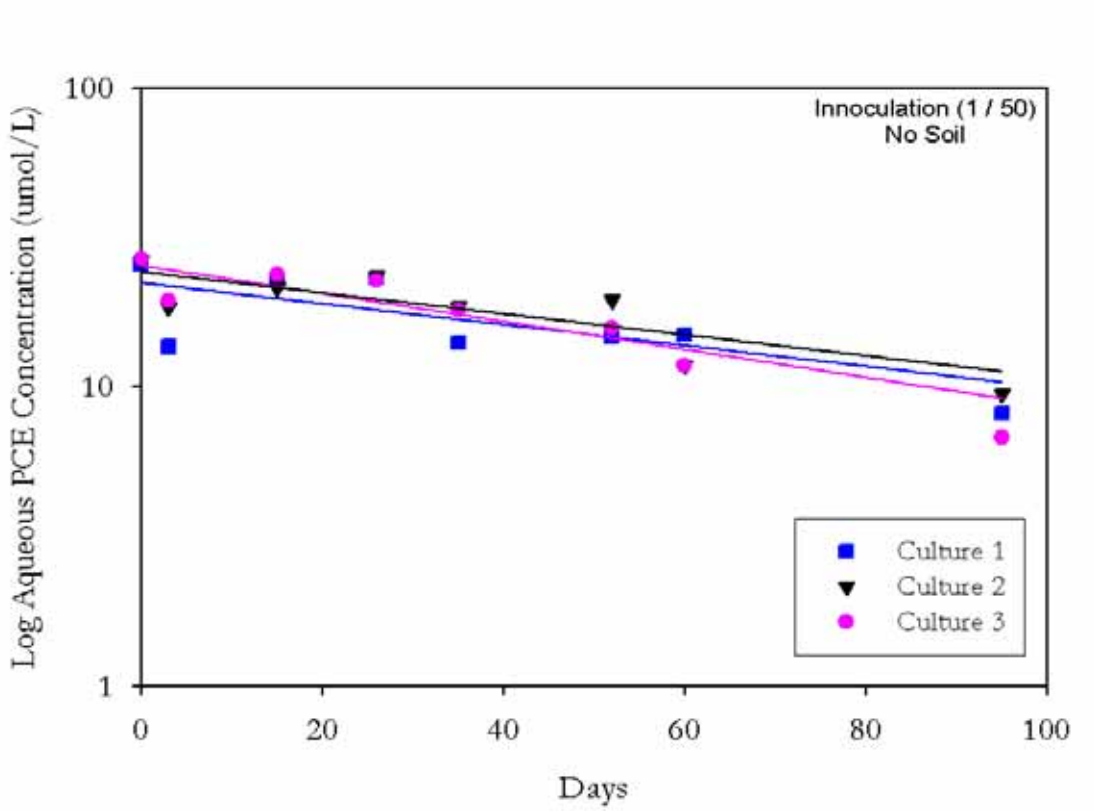

Figure 18. Enrichment Culture Experiment (26 µL PCE, No Soil)

EFFECT OF CULTURE ADDITION

In each of the microcosms, an increase in the concentration of PCE resulted in an increase in the rate of PCE degradation. The degradation rates in the bioaugmented microcosms increased from 90% to 550% when PCE concentrations were increased. For example, data in Figure 61 shows this lack of *cis*-DCE and VC production within a bioaugmented soil microcosm (MLS-22 Shallow) when the concentration is increased by a factor of 2.5. At no point after the respike was *cis*-DCE or VC detected. One possible explanation could be that higher concentrations of PCE may inhibit *cis*-DCE and VC production. PCE levels in bioaugmented microcosms quickly dropped below non-detect within the first ten days after spiking with the microcosms to 6 µM, however, after the spike to 15µM, PCE still remained at nearly 500 ppb after 80 days. Unamended microcosms exhibited a uniform degradation rate increase of 22.2% and 33.3% when the concentration of PCE was increased. Initial rate data for the non-augmented microcosm at MLS-12 Shallow could not be determined due to data scatter but conversion of PCE was minimal.

In order to further elucidate the relationship between the Loffler's soil-free culture results and the bioaugmented microcosm results, degradation profile data from Loffler's soil-free culture was normalized to degradation profile from MLS-22 Shallow (bioaugmented), given as Figure 16. Both data sets include data from the first 80 days. Note that the ethene concentration scale for the enrichment culture is 50 times higher than for the bioaugmented microcosm data. In this comparison, the three fold increase in PCE concentration in the soil microcosms appeared to stimulate reductive dechlorination. Rates of PCE conversion were 4.17 d^{-1} and 0.73 d^{-1} for the enrichment culture and MLS-22 Shallow bioaugmented microcosms respectively. Production of *cis*-DCE started almost immediately in the soil microcosm while there was nearly a ten day lag before *cis*-DCE was detected in the pure culture microcosm. VC production was also delayed

appearing around day 18 in the soil microcosm but not until day 23 in the enrichment culture. Significant amounts of TCE were not detected in either case. Although the kinetics are somewhat different, the resemblance in each profile lends credence to the cultures effectiveness.

The results of Loffler's soil-free culture indicate that VC is being transformed to ethene. The increased degradation rate coupled with the absence of dechlorination byproducts at higher concentrations in the bioaugmented microcosms indicate that either the mixed culture derived at Georgia Tech may contain microorganisms capable of direct anaerobic oxidation of *cis*-DCE and VC to CO_2 but that these microorganisms are being out-competed by native anaerobic oxidizing populations at higher concentrations. Alternatively, higher PCE concentrations may also initiate increased cometabolic enzyme production or the proliferation of new cometabolic pathways among indigenous populations. The increase in degradation rate and absence of dechlorination byproducts at higher concentrations may also be due to the population lag of both augmented and native species.

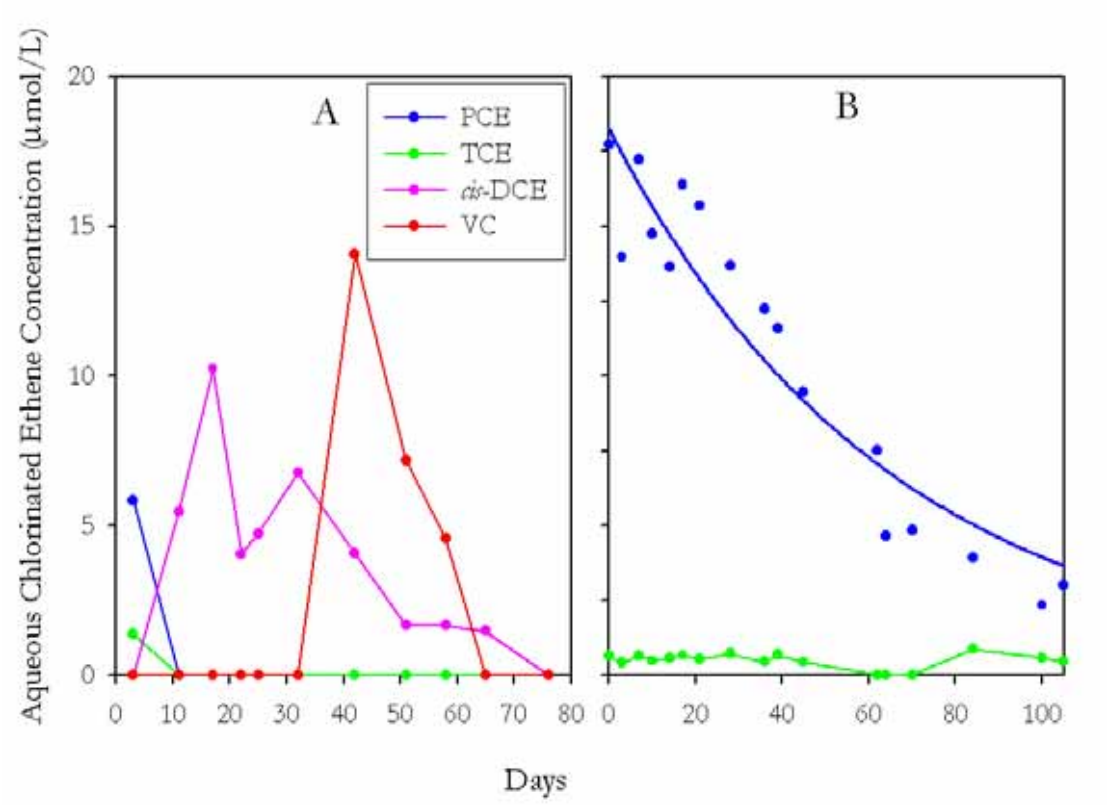

Figure 19. Concentration Effects on Dechlorinating Culture (Bioaugmented MLS-22 Shallow) A) Following 6 µm Injection and B) Following 15 µm Injection

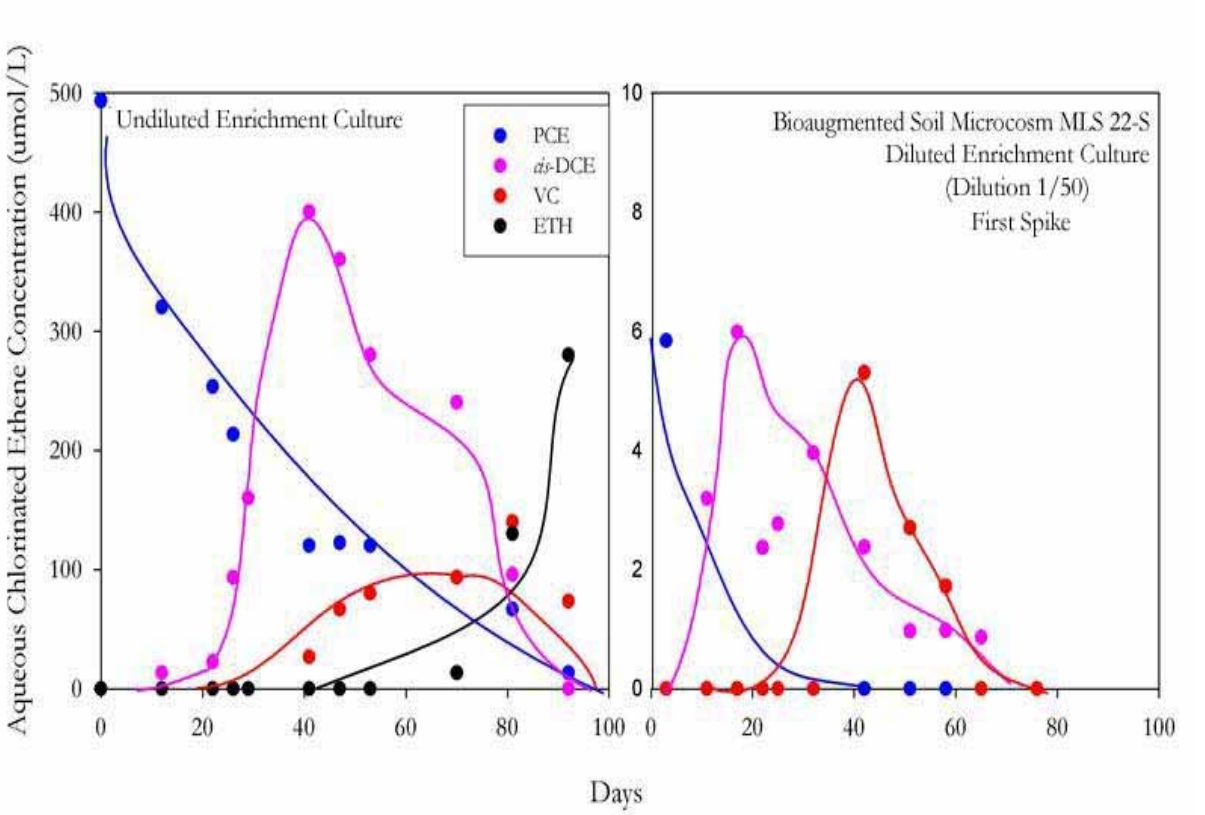

Figure 20. Measured Chlorinated Ethene Concentrations in Enrichment Culture (Left From Loffler) and Bioaugmented Soil Microcosm MLS-22 Shallow (Right)

It is likely that some features of the soil itself play a role in the determination of degradation rate and extent of dechlorination. Adsorption inhibits some degradation PCE by affecting its bioavailability. It is also doubtful that the bioaugmented culture is distributed uniformly throughout the soil, but rather, exhibits some preferential scattering based on physical and geochemical soil characteristics. Current work is being performed to better characterize the reductive capacity of soils at NABLC.

MASS BALANCE

Throughout the course of the experiment, carbon based mass balances were calculated based on aqueous ethene concentration for both non-augmented and bioaugmented microcosms (Figures 21 and 22, respectively). These values were calculated by simply converting each aqueous chlorinated ethene concentration to a molar basis and multiplying by the number of carbons contained within each ethene. This analysis was performed to track degradation within the system. Although it cannot be proven that the system is 100% gas tight, it is believed that the combination of microcosm inversion and the use of plumbers putty at gas collection points led to a relatively tight gas collection scheme. If this is true, then losses in total ethene based aqueous carbon can only be attributed to physical processes such as adsorption and partitioning as well as biologically mediated conversion.

At no point was ethene detected in the gaseous or aqueous phase. It is, however, evident that carbon is being removed from the aqueous phase

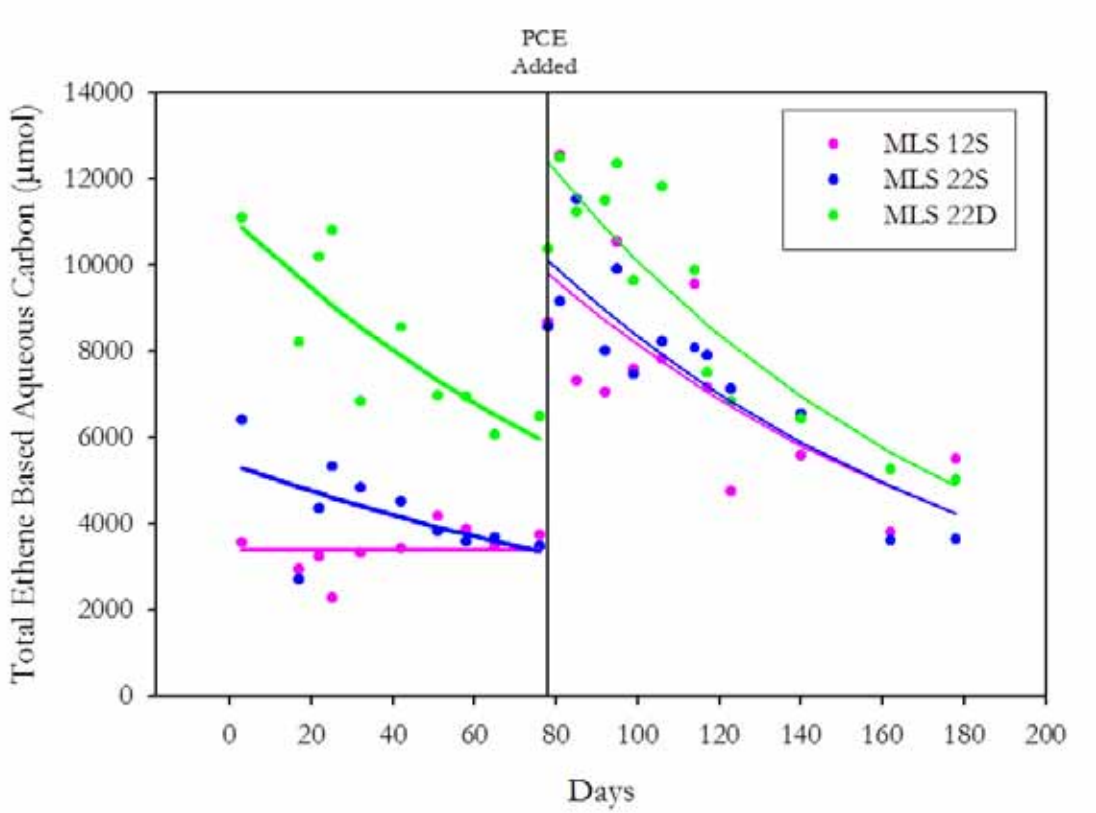

Figure 21. Unamended Carbon Balance Based on Aqueous Ethene Mass

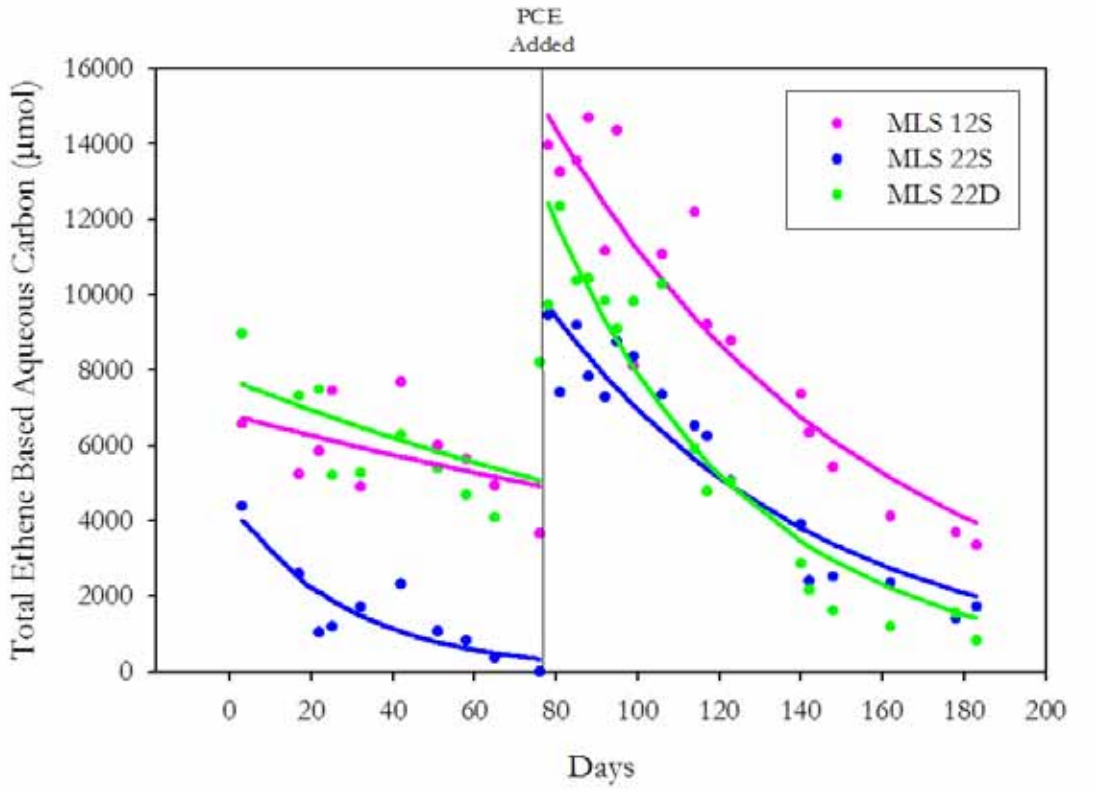

Figure 22. Bioaugmented Carbon Balance Based on Aqueous Ethene Mass

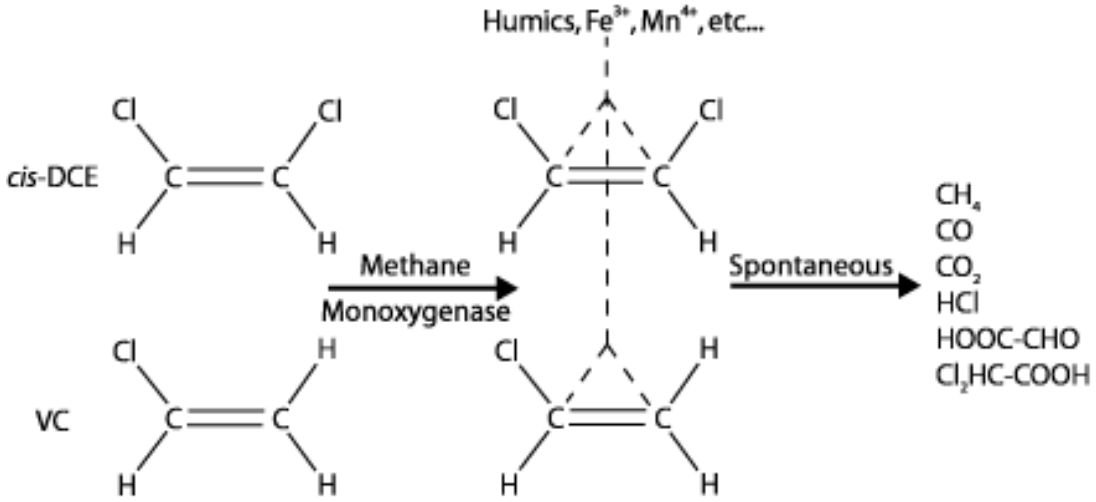

Figure 23. Proposed Direct Oxidation of cis-DCE and VC

CHAPTER 5: DISCUSSION

The data indicates that the addition of the mixed dechlorinating culture increases the PCE degradation rate in each of the bioaugmented microcosms over the unamended microcosms sharing the same soil. This trend is similar for the two PCE concentrations used in this test (both within the range for source concentrations at NABLC). It appears that PCE is degraded via several mechanisms. The addition of the dehalogenating culture appears to encourage biological dechlorination in shallow soils at lower concentrations (i.e. the formation of *cis*-DCE/VC) Although hydrogen concentrations were not measured during this experiment, hydrogen concentrations from Rectanus indicate that TEAP conditions are not conducive to significant losses due to anaerobic oxidation. It is more likely that VC was lost by diffusion through the tubing.

It is also apparent that the reductive capacities of shallow soils are different than that of deep soils. Further elucidation of terminal electron accepting processes (e.g. measurement of possible electron acceptors) would allow a better understanding of the contaminant conversion mechanisms. At no point was VC ever seen in any deep soil microcosms, yet degradation rates appear to be similar to those in shallow soils. It may be that the degradative capacity in shallow soils is amenable to reductive dechlorination and becomes more suitable for direct oxidation with depth. This reductive capacity is the cumulative effect of geochemical makeup, proximity to recharge/aeration zones, lithology, and native microorganisms.

Population densities have an impact on the PCE degrading ability of inoculated populations due to their ability to compete for biochemical reactants. Degradation rates per cell were very similar between Lofflers results and It is important to understand the growth rates of the cultures, whether pure or mixed, in order to determine what bioaugmentation volumes need to be used to produce viable populations in a timely manner.

Every attempt was made to maintain hydrogen concentrations below levels suitable for methanogens on order to allow the proliferation of reductive dechlorinators. There was no direct evidence (e.g. no evident impact on degradation trend with the shift from acetate to lactate as electron donor) that the electron donor type affected either the rate or extent of dechlorination but it is reasonable to assume that cultures developed using specific electron donors would be more successful if the donor remained the same. It is also important to realize that certain electron donors can impact native species. The use of lactate as an intermediate for hydrogen production has been well documented most notably by the commercially available Hydrogen Release Compound (HRC) (24). When mixed with water, the polylactate ester in HRC undergoes a fermentation reaction to produce H_2.

Based on the data obtained in this study, it appears that bioaugmentation is not a simple process and requires further research. Recent pilot-scale field tests of bioaugmentation have been reasonably successful (13, 21), but it is evident that bioaugmentation is not simply a matter of injecting a dehalogenating culture into the ground and expecting it to function as it did in the lab. Ultimately, the question that needs to be answered is whether or not bioaugmentation is a sustainable process in the field. There are a number of factors that may affect culture viability in the field.

To date, the *dehalococcoides* strain is the only pure culture proven capable of completely converting PCE to ethene. With our limited knowledge of bacterial capabilities, it is unlikely that this is the sole population capable of degrading these compounds. It is probable that other species or perhaps species in tandem have the ability to metabolize chlorinated ethenes with various levels of efficiency would be expected (28). It would be useful to run PCR analyses on the unamended soil samples to determine which of the known dechlorinating species exist at the site. If dechlorinating cultures are found, then there must be some other reason why dehalogenation in the soil systems differs from Loffler's enrichment culture. At this point, there seems to be no clear answer on the proper way to develop cultures intended for bioaugmentation. Bacterial cultivation is an expensive and time-consuming procedure. Therefore an understanding

of inoculation volumes is vital to estimating the cost of bioaugmentation. In these experiments, a 2% vol/vol inoculation was used and rates of PCE degradation were lower than for Loffler's culture data. When dealing with groundwater volumes typical of many Superfund sites, including NABLC, this volume could become excessively costly. In this experiment, a liquid culture was used but could the same result be achieved using dry cultures thus saving transportation costs?

Because microbes are developed under restricted conditions in the lab, there are likely growth environments that would yield population favorable to bioaugmentation. Concentration effects could be addressed by culturing populations at higher concentrations and thus, developing strains capable of withstanding higher contaminant levels.

The most important factor in determining the success of bioaugmentation might be hydrogen donor. Although there were no noticeable changes in dechlorinating trends coinciding with the change from acetate to lactate, there could be a dramatic influence on the bioaugmented culture if hydrogen conditions did not mimic the laboratory environment in which they were grown. Work is currently being done to try and answer these questions using this culture (19).

Probably the least understood mechanism in bioaugmentation is what happens to the culture after injection. Are they susceptible to flushing or perhaps small zones of influence? Work is currently being done to determine transport characteristics of bioaugmentation species (15, 17). The use flow through cells along with RFLP/PCR analysis could be used to test not only the ability of culture to disseminate itself spatially but also its ability to successfully compete with native populations.

It is obvious that remediation decisions must be made on a site specific basis but unfortunately these decisions are often at a scale that ignores micro-scale terminal electron accepter processes. It appears that these dissimilarities extend to microbial populations too as evidence by the difference in dechlorination activity between the deep and shallow soils from MLS-22. It is possible that wholly different microbial communities exist over small spatial scales. We must first understand why dechlorinating populations appear to be universal at some sites and wholly vacant from others. This biodynamic would become another criterion to determine sites amenable to bioaugmentation.

There is clearly a matrix effect on the performance of these dechlorinating cultures. Much of the novel activity demonstrated by the enrichment culture at high PCE concentrations was muted with the addition of soils and lower PCE concentrations similar to those at NABLC. This has far reaching implications for the feasibility of bioaugmentation as a source zone strategy at NABLC. The addition of the culture appears to expedite dechlorination in the lab scale, it is unclear whether significant reductions could be made in the time of remediation with given contaminant concentrations in the field.

Future work should include not only the exploration of more effective and less expensive electron donors but also a look into possible growth and delivery systems for both culture and bioenhancements. It may be necessary to obtain pure and mixed cultures from multiple sources proven to degrade PCE completely and compare concentration effects on biodegradation kinetics.

CHAPTER 6: CONCLUSIONS

The results of this study indicate that bioaugmentation is not a turnkey approach. Site-specific conditions appear to affect the efficacy of the inoculated dechlorinating cultures in the field.

First, the disparity in the degradation byproduct production between the soil-free and bioaugmented microcosms at different PCE concentrations suggest that there may be significant competition for macronutrients between native and non-native species and that this competition may be driven by concentration. Although sorption was not believed to be a source of abiotic PCE sequestration, soils may inhibit degradation by limiting the bioavailability of the contaminant to degrading populations.

There was not a noticeable change in degradation rate when using acetate versus lactate as the carbon and hydrogen source. It is possible that species native to the soil exhibit less of preference for donor type. Loffler's culture displays differences in both rate and extent of degradation when using acetate versus hydrogen as the primary electron donor (19). Competition between indigenous and bioaugmented species may have suppressed this trend. Bioaugmentation may need to be coupled with biostimulation using specific electron donors.

Higher inoculation volumes and concentration (as measured by culture density) increase the rate of degradation by providing a stable population capable of expanding as well as one apt to successfully compete with native populations. A 2% vol/vol inoculation resulted in reduced degradation rates by an order of magnitude (Figure 19). It is unknown what the threshold inoculation volumes yield populations capable of flourishing in situ.

Location in the contaminated zone also appears to affect the efficacy of bioaugmentation. The different geochemical makeup and associated the microbiological profile at each point can create very different extents of attenuation. The difference in degradation profiles between unamended shallow and deep wells (Figures 13 and 14, respectively) indicate that attenuation mechanisms are variably successful over even short distances within the plume.

Even at lab scale, these results suggest that bioaugmentation is not a direct correlation between enrichment culture and microcosm results. There are a number of questions that need to be answer to further elucidate its usefulness. These questions include culture development, the fate and transport of cultures in the subsurface and the effect of site heterogeneities on the introduced culture. The results in this study do, however, warrant further investigations into bioaugmentation as a tool in the remediation of chlorinated solvent sites.

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APPENDIX A: MINERAL SALTS RECIPE

Mineral Salts Medium ("DCB-1 medium")

Protocol

Salts	**1 x [g/L]**	**100 x stock [g/L]**
NaCl	1.0	100.0
$MgCl_2$ x 6 H_2O	0.5	50.0
KH_2PO_4	0.2	20.0
NH_4Cl	0.3	30.0
KCl	0.3	30.0
$CaCl_2$ x 2 H_2O	0.015	5

To prepare medium	**1 L**
100 x salts	10 ml
Trace element solution	1 ml
Se/Wo solution	1 ml
Resazurin (0.1% solution)	0.25 ml
Electron donor	optional
H_2O bidest.	add 1000 ml

Some chemicals (e.g. fumarate, pyruvate) should not be autoclaved

- boil, cool down to room temperature under flushing with N_2/CO_2 (80/20
- add 0.2 mM L-cysteine (MW 157.6) 0.031 g/L
- add 0.2 mM Na_2S x 9 H_2O 0.048 g/L [or: add 1 mM DL-dithiothreitol 0.154 g/L]
- add 30 mM $NaHCO_3$ 2.52 g/L
  adjust pH to 7.2 - 7.3 with CO_2 (to lower the pH with CO_2 is less time consuming)
- flush serum bottles with N_2/CO_2
- dispense medium (ca. 98 ml per bottle), close the bottles with black rubber stoppers (or Teflon-lined stoppers)
- autoclave after medium turned clear; autoclave bottles in a closed basket; do **NOT** remove bottles from autoclave before temperature of the medium has reached <70ºC

Electron donors (formate, acetate, lactate, propionate) and acceptors (PCE or *cis*-DCE) can be added prior to heat sterilization. Fumarate, pyruvate are heat-labile and should not be autoclaved.

Trace element solution

Per liter: HCl (25% solution, w/w), 10 ml; $FeCl_2$ x 4 H_2O, 5 g; $CoCl_2$ x 6 H_2O, 0.19 g; $MnCl_2$ x 4 H_2O, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; Na_2MoO_4 x 2 H_2O, 36 mg; $NiCl_2$ x 6 H_2O, 24 mg; $CuCl_2$ x 2 H_2O, 2 mg

Se/Wo solution

Per liter: 6 mg Na_2SeO_3 x 5 H_2O, 8 mg Na_2WO_4 x 2 H_2O and 0.5 g NaOH

Wolin Vitamins

Wolin, F. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:**2882-2886.

Vitamins	1000 x [mg/L]	Final conc.
biotin	20 mg/L	0.02 mg/L
folic acid	20 mg/L	0.02 mg/L
pyridoxine hydrochloride	100 mg/L	0.1 mg/L
riboflavin	50 mg/L	0.05 mg/L
thiamine	50 mg/L	0.05 mg/L
nicotinic acid	50 mg/L	0.05 mg/L
pantothenic acid	50 mg/L	0.05 mg/L
vitamin B_{12}	1 mg/L	0.001 mg/L
p-aminobenzoic acid	50 mg/L	0.05 mg/L
thioctic acid	50 mg/L	0.05 mg/L

adjust pH to ~7.5 with 10 M NaOH (takes some time)

aliquot in 20 ml portions, freeze, store in dark place (light sensitive)
prepare a 200 x working stock solution, filtersterilize