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DEVELOPMENT AND CHARACTERIZATION OF A RAPID DECHLORINATING ENRICHMENT STIMULATED FROM AN AQUIFER CONTAMINATED WITH CHLORINATED ETHENES AND ETHANES

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By

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ABSTRACT

DEVELOPMENT AND CHARACTERIZATION OF A RAPID DECHLORINATING ENRICHMENT STIMULATED FROM AN AQUIFER CONTAMINATED WITH CHLORINATED ETHENES AND ETHANES

By

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Biostimulation has become an accepted potential remedial alternative for treating contaminated aquifers. This study evaluates a novel approach to enrich for rapid dechlorinating microbes by pulse feedings in a batch system over an extended period. It was demonstrated that by varying the electron donor to acceptor ratio during pulse feedings of chlorinated compounds, two different types of activity could be developed. Cometabolic dechlorination was dominant under high lactate conditions, and dehalorespiration activity was enriched under low lactate conditions. The high lactate/PCE enrichments exhibited slow and incomplete dechlorination of PCE with cDCE as the major degradation product. The low lactate/PCE enrichments completely dechlorinated PCE to ethene, and further reduction of ethene to ethane was observed. *Dehalococcoides* 16S rRNA gene targeted real –time PCR confirmed that two orders of magnitude higher amounts of *Dehalococcoides* DNA was present in low lactate enrichments compared to high lactate enrichments. This demonstrated that pulse feedings with low-level electron donor and a chlorinated ethenes (at a 1:10 electron acceptor/donor ratio) favors stimulation of

halorespirers. Using metabolic inhibitors revealed that the dechlorination observed in low lactate enrichments was not impacted by molybdate (inhibitor of sulfate-reducer), but was inhibited by high levels of sulfate, indicating sulfate reducers are not involved in the observed complete and rapid dechlorination. Low levels of BES (methanogen inhibitors) specifically inhibited methane productions, but the cultures retained dechlorination activity, indicating that the responsible microorganism(s) is (are) non-methanogenic. High levels of vancomycin (100 mg/L; acetogens inhibitors) successfully inhibited acetate production from lactate. The reduction in acetate production also inhibited dechlorination, further suggesting that acetate-utilizing dechlorinating microbes are responsible for the observed dechlorination.

The development of methodology for the stimulation of dehalorespiring microorganisms presented in this dissertation represents a potential strategy for the design of successful biostimulation systems for chlorinated solvents. It also has significant design implications for in-situ field systems.

DEDICATION

This dissertation is dedicated to my family and friends: my parents, NamYeun Chang and KiTae Kim; my sister and brother, SoAh Kim and ByungSoo Kim; and my biggest supporters, Y.A, T.L., and AJN. No matter where I go in life, you all are in my heart and prayers, now and for always. I could not have done this without you.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BES	2-Bromoethanesulfonic acid
CA	Chloroethane
c-DCE	cis-1, 2 Dichloroethene
t-DCE	trans-1,2 Dichloroethene
1,1-DCE	Dichloroethene
1,1,1-TCA	1,1,1-Trichloroethane
1,1-DCA	1,1-Dichloroethane
1,2-DCA	1,2-Dichloroethane
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
PCE	Perchloroethene
PCR	Polymerase chain reaction
rRNA	Ribosomal Ribonucleic acid
TCE	Trichloroethene
TOC	Total Organic Carbon
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
VC	Vinyl Chloride
VOC	Volatile Organic Compound

Chapter One: PROBLEM STATEMENT AND OBJECTIVES

Due to past agricultural practices, accidental chemical spills, and inappropriate disposal of industrial and commercial wastes, groundwater contamination is a significant national and international problem. In the United States, for example, groundwater contamination can be found in 1200 Superfund sites and an estimated 7000 Department of Defense sites (EPA, 1993). The most ubiquitous class of organic compounds polluting groundwater is the chlorinated solvents (EPA, 1998). Traditional approaches to water containing these contaminants have relied on groundwater extraction, followed by a physical/chemical process (e.g., air stripping and carbon adsorption). This approach has some major disadvantages such as high cost, inefficiencies in removing contaminants sorbed to the aquifer material, and requirement of pumping, treating, and ultimately disposal of large volumes of water. Accordingly, there has been a great deal of interest in in-situ alternative treatment strategies, many of which incorporate biological remediation of contaminants.

The three basic bioremediation approaches are natural attenuation, biostimulation, and bioaugmentation. Natural attenuation refers to unenhanced "natural" processes, which may occur at contaminated sites if sufficient nutrients and degrading microbes are present. This can include both abiotic and biotic mechanisms. For chlorinated organics, natural attenuation may be insufficient, and electron donor addition is often needed to accelerate reductive dechlorination or stimulate cometabolic activities. Biostimulation refers to enhancing the metabolic activity of indigenous microflora to transform target

compounds and usually requires amendments (electron donors and/or acceptors) to enhance the microbial populations already present.

In some cases, indigenous bacteria may be unable to mediate the desired transformation (Mayotte *et al.* 1996; Dybas *et al.* 1998; Ellis *et al.* 1999) and specific microorganisms with the desired traits need to be provided (bioaugmentation). Although bioaugmentation offers the promise of increased control over transformation of a specific compound, competition between indigenous microflora and the introduced organisms usually presents a challenge. Factors such as the nutritional requirements of dehalogenating microbes and electron donor competition between dechlorinating and non-dechlorinating microorganisms have presented challenges to implementation of this promising technology in the field. The research presented in this thesis provides insight into the issue of selective biostimulation: that is, how to enrich microbial populations that are capable of complete and rapid degradation of contaminant.

1.1. Hypothesis and objectives

The hypotheses of this research are:

1. Palmitate, a slow releasing carbon source, will enhance the rate of dechlorination compared to simple, readily available carbon sources.
2. Relative abundance of electron donor with respect to PCE will influence the level of dehalogenation populations such as *Dehalococcoides ethenogenes*, will impact rates, and will determine the extent of transformation. A lower abundance of electron donor with respect to PCE will produce a higher yield of

Dehalococcoides, which results in higher degradation rates and complete transformation.

The specific objectives were:

1. Evaluate the effect of various types of nutrient on stimulation of dechlorination.
2. Evaluate the effect of various chlorinated compounds and 2 different levels of lactate on stimulation of dechlorinating enrichments.
3. Characterize the mixed dechlorinating community by the inhibitors and hydrogen effects on dechlorination and growth.
4. Characterize the mixed dechlorinating community by using the molecular techniques such as RTm-PCR and T-RFLP.
5. Evaluate the abiotic degradation of chlorinated compounds.

The format of this thesis is as follows: theory supporting the aforementioned hypotheses are outlined in Chapter 2; Chapter 3 contains review of related researches; materials and methods are illustrated in Chapter 4; results from the screening of the various electron donors and byproduct analysis presented in Chapter 5; development strategies and complete dechlorination by enriched sediments are presented in Chapter 6; and effects of metabolic inhibitors on dechlorinating communities in Chapter 7; engineering applications in Chapter 8; conclusions and future research are summarized in Chapter 9.

1.2. Literature cited

U.S. Environmental Protection Agency. 1993. Cleaning Up the Nation's Waste Sites: Markets and Technology Trends. Office of Solid Waste and Emergency Response, EPA 542-R-92-012.

U.S. Environmental Protection Agency. 1998. National Water Quality. Inventory Report to Congress.

Mayotte, T., M. Dybas, and C. S. Criddle. 1996. Bench-scale evaluation of bioaugmentation to remediate carbon tetrachloride-contaminated aquifer materials. *Ground Water* 34 (2), 358-367.

Dybas, M. J., Barcelona, M., Bezborodnikov, S., Davies, S., Forney, L., Heuer, H., Kawka, O., Mayotte, T., Sepulveda-Torres, L., Smalla, K., Sneathen, M., Tiedje, J., Voice, T., Wiggert, D. C., Witt, M. E., and C. S. Criddle. 1998. Pilot-Scale Evaluation of Bioaugmentation for In-Situ Remediation of a Carbon Tetrachloride-Contaminated Aquifer. *Environ. Sci. Technol.* 32(22): 3598-3611.

Ellis, D.E., E.J. Lutz, R.J. Buchanan, M.D. Lee, C.L., Bartlett, M. Harkness, and K. DeWeerd. 1999. Bioaugmentation for accelerated in situ anaerobic bioremediation. In Platform Abstracts of the Fifth International Symposium on In-Situ and On-Site Bioremediation, Session D2, San Diego, California, April 19-22. Columbus, Ohio: Battelle

Chapter Two: BACKGROUND AND THEORY

2.1 Energetic Considerations

Microorganisms that are capable of utilizing the most energy rich electron acceptor/donor pair (the half reactions exhibiting the most positive electron E_H^o) will be dominant in a given environment. These organisms then in turn determine the redox conditions of the environment. The maximum free energy ($\Delta G^{o\prime}$) microorganisms may gain is commonly presented by reducing-oxidation reactions. Table 2.1 contains standard reducing potentials and free energies ($\Delta G^{o\prime}$) of redox couples and chlorinated compounds that are commonly found in contaminated groundwater at 25 °C. Thermodynamic consideration indicates that reductive dechlorination is exergonic and chlorinated compounds can be used as electron acceptors. Table 2.2 contains maximum free energies of selected organic acids and alcohols commonly used as electron donors in dechlorinating experiments.

McCarty (1969) proposed stoichiometric methodology to evaluate the energy requirement for cell synthesis from various carbon and nitrogen sources.

$$A = -\frac{\Delta G_p / k^m + \Delta G_c + \Delta G_n / k}{k\Delta G_r} \qquad (2.1)$$

where: A = electron equivalent of electron donor to energy/electron equivalent of cells synthesized : ΔG_p represents the energy required to reduce carbon source to pyruvate : k is cell efficiency of energy transfer : ΔG_n represents energy required to convert N source to NH_4^+ : ΔG_r presents energy available energy per electron equivalent of substrate

converted for energy : ΔG_c presents energy of conversion of one electron equivalent of intermediate to one electron equivalent of cells.

Table 2.1 Reduction Potentials of redox couples found in anaerobic dechlorinating environments.

Half-Reaction	$E_o'^{+}$ (V)	$\Delta G°'$ (kJ/mole)
$Fe^{3+} + e^- = Fe^{2+}$	0.77	-114
$SO_4^{2-} + 9H^+ + 8e^- = HS^- + 4\ H_2O$	-0.22	-146.7
$CO_{2\,(g)} + 8H^+ + 8e^- = CH_{4\,(g)} + 2\ H_2O$	-0.25	-131.2
$C_2Cl_4 + H^+ + 2e^- = C_2HCl_3 + Cl^-$	0.58	-191.0
$C_2HCl_3 + H^+ + 2e^- = C_2H_2Cl_2 + Cl^-$	0.54	-183.0
$C_2H_2Cl_2 + H^+ + 2e^- = C_2H_3Cl + Cl^-$	0.37	-151.1
$C_2H_3Cl + H^+ + 2e^- = C_2H_4 + Cl^-$	0.49	-173.7
$C_2H_4 + 2H^+ + 2e^- = C_2H_6$	0.1	-98.9
$C_2H_3Cl_3 + H^+ + 2e^- = C_2H_4Cl_2 + Cl^-$	0.57	-189.1
$C_2H_4Cl_2 + H^+ + 2e^- = C_2H_5Cl + Cl^-$	0.51	-177.5

[+] Redox-potentials were obtained from Criddle *et al.* (1991), Dolfing and Janssen (1994), and Vogel *et al.* (1987).

In heterotrophic reactions, the value of A is by definition *fe/fs* so that the fraction of organic substrate utilized for energy is

$$fe = \frac{A}{A+1} \qquad (2.2)$$

where: *fs* is fraction of electron donor synthesized and *fe* is fraction of electron donors converted to energy.

Table 2.2. Maximum free energies ($\Delta G°'$) for electron donors commonly used in dechlorination of chlorinated compounds[a]

Half-Reactions	$\Delta G°'$ kcal per mole electrons
$1/92 CH_3(CH_2)_{14}COO^- + 31/92\ H_2O \rightarrow 15/92\ CO_2 + 1/92\ HCO_3^- + H^+ + e^-$ Palmitate	-6.657
$1/12\ C_3H_5O_3^- + 1/3\ H_2O \rightarrow 1/6\ CO_2 + 1/12\ HCO_3^- + H^+ + e^-$ Lactate	-7.873
$1/14\ C_3H_5O_2^- + 5/14\ H_2O \rightarrow 1/7\ CO_2 + 1/14\ HCO_3^- + H^+ + e^-$ Propionate	-6.664
$1/6\ CH_4O + 1/6\ H_2O \rightarrow 1/6\ CO_2 + H^+ + e^-$ Methanol	-8.965

[a]Energetics and Bacterial Growth. Perry L. McCarty. Fifth Rudolf Research Conference, Rutgers. The State University, New Jersey, 1969. July 2.

2.2 Microbial Competition for Hydrogen

The ability of certain microorganisms to out compete others for the same growth nutrient is dependent upon both the kinetics and energetics of the competing reactions. When microbial strains compete for the same limiting nutrient, resource-based competition theory predicts that only one group of organisms will survive and all other will die out (Hansen *et al,* 1980). Experimental results have shown that two species were observed to coexist only if either (1) each was limited by a different resource and met the theoretical criteria for coexistence, or (2) the species were limited by the same resource and did not differ significantly in their resource requirements.

The rate of bacterial growth (r_g) and the rate of substrate consumption (r_{sc}) can be used to determine whether one organism has a kinetic advantage over another. The rate of bacterial growth is described mathematically by Monod's equations.

$$r_g = \frac{\mu_{max} XS}{K_s + S} \qquad (2.3)$$

Where; μ_{max} (hr^{-1}) is the organism's maximum specific growth rate; X represents the concentration of biomass (mg cells L^{-1}) ;S is the concentration of the growth-limiting substrate (mgL^{-1}) ; Ks is the half velocity constant which is the substrate concentration at half the maximum specific growth rate. The rate of substrate consumption, r_{sc} is related to the rate of bacterial growth by

$$r_{sc} = \frac{r_g}{Y} \qquad (2.4)$$

The organism's maximum yield coefficient (Y) represents the maximum mass of cells that can be produced per mass of substrate consumed. The Y and μ_{max} define the maximum specific substrate utilization rate k (mg substrate consumed mg $cells^{-1}$ h^{-1}) according to the relationship $k = \mu_{max}/Y$. Combining equation 2.3 into equation 2.4 yield the following expression for r_{sc}

$$r_{sc} = \frac{kXS}{K_s + S} \qquad (2.5)$$

Examination of the Monod's expression reveals that the rates of bacterial growth and substrate consumption are dependent upon values of Ks, Y, and k. Half-velocity rate constants represent the affinity of an organism for a substrate, and lower values of K_s are associated with higher affinities.

Many organisms like acetogens, methanogens, and sulfidogens use hydrogen produced from anaerobic metabolism of organic matters (Brock *et al.* 1999). Since hydrogen is also needed for the hydrogenotrophic organisms (such as hydrogen-utilizing PCE dechlorinators) to survive, the microbial competition for hydrogen can play an important role in the transformation of the chlorinated compounds. Values of half-velocity constants with respect to hydrogen, K_s (H_2), for hydrogenotrophic dechlorinators and methanogens and measurements of the maximum specific utilization rate reported are summarized are listed in Table 2.3. For hydrogenotrophic dechlorinators, half-velocity

Table 2.3. Values of half-velocity constants K_s and maximum specific utilization rate k with respect to hydrogen, for hydrogenotrophic PCE dechlorinators and methanogens.

	Ks (H_2)	k
Methanogens	2,500 –13,000 nM[a]	100–2,600μmol·mgprotein^{-1}hr^{-1} [c]
	960 ± 180 nM[b]	
Dechlorinators	100 ± 50 nM[b]	20 μmol·mgprotein^{-1}hr^{-1}[c]
	9-21 nM[c]	

[a] Robinson and Tiedje, 1984
[b] Smatlak *et al.* 1996
[c] Ballapragada *et al.* 1997

constants were found to be one-tenth that of the methanogenic organisms (Smatlak *et al.* 1996). The use of fermentable substrates that maintain low levels hydrogen was suggested to offer a competitive advantage for hydrogen utilizing dechlorinators.

The energetics of competing reactions can also be used to determine whether the competition will emerge between organisms utilizing the same electron donor. Cord-Ruwisch *et al.* (1988) observed that the hydrogen threshold is inversely correlated with changes in Gibbs free energy ($\Delta G°'$). As can be seen in Table 2.4, since dechlorination has lower free energy compared to other processes, dechlorinators are expected to grow at lower hydrogen partial pressure. Supporting observations had been made in many studies (Smatlak *et al.* 1996; Ballapragada *et al.* 1997).

Table 2.4. Threshold H_2 Concentrations and Energetics of H_2 Utilizing Reactions (adopted from Loffler *et al.* 1999).

Biological process	Equation	Threshold H_2 (nM)[a]	$\Delta G°'$ (KJ/mol H_2)
Dehalogenation	PCE + $4H_2 \rightarrow$ ethene + H^+ + Cl^-	< 0.3	-130 to -187
Methanogenesis	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	5 – 95	-33.9
Acetogenesis	$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	336- 3640	-26.1
Sulfate reduction	$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + H_2O$	1-15	-38.0

[a] In aqueous phase concentration

2.3 Design of Experiments

Significant factors

Many factors contribute to transformation of chemicals and it can be difficult to separate the contribution of any one factor to the overall degradation. Reported data on PCE and TCE transformation often does not include consideration of any role of abiotic mechanisms, assuming transformation occurred only biologically with microorganisms. To attempt to determine which factors are most significant, a statistical design approach (factorial design) is adopted which allows separation of significant factors from insignificant factors and thus the effects of interactions between each factor can be evaluated (Box *et al.* 1978).

Design Matrix

The main objective of factorial design is to see the effects of two or more independent variables (Wadsworth, 1990). It is usually more efficient to manipulate these variables in one experiment than run a separate experiment for each variable. Also it is possible to test for interactions among variables using factorial design. 2^k factorial designs need k parameters and helps in sorting out parameters of impact. The method uses a nonlinear regression model and assuming there are only 2 parameters, x_1, x_2, gives:

$$y=q_0+q_1*x_1+q_2*x_2+q_{12}*x_1*x_2 \qquad (2.4)$$

Here, y is the system performance metric; q_0 is the average performance; q_i is the effect on the performance of parameter x_i; q_{12} is the effect of the interaction of x_1 and x_2; The value of x_i is defined as:-1 if x_i assumes the minimum value in the experiment, otherwise, +1.

Experiment	x_1	x_2	x_1x_2	y
0	-1	-1	1	y_0
1	1	-1	-1	y_1
2	-1	1	-1	y_2
3	1	1	1	y_3

Table 2.5. Sign table for 2^k factorial designs

In this example, 2^2 experiments with 4 different combination of x_1,x_2 are needed. Then, by substituting x_i and y into the above performance equation, 4 equations with 4 unknowns q_0, q_1, q_2, q_{12} are obtained. Then, we can solve for q_0, q_1, q_2, q_{12} unknowns.

Analysis of Variance (ANOVA)

In order to further verify the degradation of chloroethenes from these preliminary results, collected data will be analyzed using (1) Analysis of variance (ANOVA), which tests the difference between 2 or more means by examing the ratio of variability between two conditions and variability within each condition; and (2) factorial design, which identifies positive and negative factors on degradation of chloroethenes. Values of the F

statistics and and p-value will be computed to determine the significance of main and interaction effects.

2.4. Literature cited

McCarty, Perry L. 1969. Energetics and Bacterial Growth. The Fifth Rudolf Research Conference, Rutgers, New Brunswick, New Jersey

Criddle, C.S. and P.L. McCarty. 1991. Electrolytic model system for reductive dehalogenation in aqueous environments. *Environ. Sci. Technol.* 25:973-978

Dolfing J. and D.B. Janssen. 1994. Estimates of Gibbs free energies of formation of chlorinated aliphatic compounds. *Biodegradation* 5: 21-28

Vogel, T.M., C.S. Criddle, and P.L. McCarty. 1987. Transformation of halogenated aliphatic compounds. *Environ. Sci. Technol.* 21: 722-736.

Hansen, S. R., and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. *Science.* 207:1491–1493.

Brock, T. D., Madigan, M. T., Martinko, J. M., and Parker, J. 1999. *Biology of Microorganisms*, Prentice-Hall, Inc., Englewood Cliffs.

Smatlak, C. R., Gossett, J. M., and Zinder, S. H. 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ. Sci. Technol.* 30 (9): 2850-2858.

Ballapragada, B. S., Stensel, H. D., Puhakka, J. A., and Ferguson, J. F. 1997. Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ. Sci. Technol*. 31(6): 1728-1734.

Robinson, J.A., and Tiedje, J.M. 1984. Competition between sulfate-reducing and methanogenic bacterial for H_2 under resting and growing conditions. *Archives of Microbiology*. 137: 26-32.

Cord-Ruwisch, R., H.-J. Seitz, and R. Conrad. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch. Microbiol.* 149:350-357.

Loffler F. E., James M. Tiedje, and Robert A. Sanford. 1999. *Appl. Environ. Microbiol.* 65(9): 4049-4056

Distefano, T. D., Gossett, J. M., and Zinder, S. H. 1992. Hydrogen as an electron-donor for dechlorination of Tetrachloroethene by an anaerobic mixed culture. *Appl. Environ. Microbiol.* 58 (11): 3622-3629.

Monod, J. 1949. "The growth of bacterial cultures." *Annual Review of Microbiology*.

Box, G.E. 1978. *Statistics for Experimenters:* An introduction to design, data analysis, and model building. John Wiley & Sons.

Harrison M. Wadswroth. 1990. Handbook of statistical methods for engineers and scientist. McCraw-Hill Publishing Company.

Chapter Three: LITERATURE REVIEW

This chapter provides previous research efforts on biotransformation of chlorinated ethenes under anaerobic conditions. The review begins with a general overview of chlorinated ethenes properties. The remaining review is divided into two sections. The first contains discussions on mechanisms and transformation kinetics of chlorinated solvents. A summary of previous research efforts on biotransformation of chlorinated ethenes follows shortly after.

3.1. Chlorinated Solvents

Due to the similarities in the electrical charges (or electronegativites) of oxygen and chlorine, chlorinated solvents are considered highly oxidized. Perchloroethylene (PCE), the most highly chlorinated ethylene molecule, is environmentally persistent partly because it only degrades anaerobically. PCE degradation proceeds through trichloroethylene (TCE), the TCE is then reduced to isomers of dichloroethylene. Although the cis-isomer (c-DCE) appears to be the predominantly produced dechlorination byproduct, trans-isomer (t-DCE) is also reported to be produced by *D. ethenogenes* 195 (Maymo-Gatell *et al.* 1999). A third isomer, 1, 1-dichloroethylene (1,1-DCE) is chemically possible, however, there is little evidence that it is biologically produced from TCE dechlorination. The next step of reductive dechlorination is the production of vinyl chloride (VC) from the dichloroethylene isomers. Removal of the final chlorine produces the innocuous ethylene molecule making this product the end point of the reductive dechlorination process, which satisfies the treatment purpose. Note

that because of the double bond in the ethylene molecule can be further reduced to ethane, which contains only a single bond between the carbons with three hydrogens on each carbon. All of the chlorinated ethylene are listed as priority pollutants under the Safe Drinking Water Act Amendments of 1986. Typical chemical and physical properties of these compounds are summarized in Table 3.1.

Table 3.1. Properties of Common Chlorinated Organic Compounds

Compound	Chemical Formula	Molecular Weight (g/mol)	Density (mg/m^3)[a]	Solubility in water (mM at 25 °C)[b]	Henry's Constant Hc[c] (dimensionless)
1,1,1-Trichloroethane	$CHCl_3$	133.41	1350	8.5	0.703
Tetrachloroethene	C_2Cl_4	165.83	1626	0.91	0.723
Trichloroethene	C_2HCl_3	131.39	1460	8.4	0.392
c-Dichloroethene	$C_2H_2Cl_2$	96.94	1284	36	0.167
Vinyl Chloride	C_2H_3Cl	62.50	911	43	1.137
Ethene	C_2H_4	28.04	2085	4.7	8.5[b]

[a] Data taken from Sawyer and McCarty (1994).
[b] Yaws, C. L. (1999)
[c] Gossett, J. M. (1987) at 24.8 °C

3.2. Biotransformation of Chlorinated Ethenes

Reductive Dechlorination

Reductive dechlorination or hydrogenolysis is a term to describe reactions where sequential electron and hydrogen additions replace chlorine atoms. Chlorinated ethenes are susceptible to biologically mediated reductive dechlorination reactions under anaerobic conditions. For example, PCE is sequentially reduced to TCE, c-DCE or t-DCE, VC, and then to ethene (Figure 3.1). The extent of reaction varies for different compounds and with differing environmental conditions. Generally more chlorinated compounds are more susceptible to rapid reductive dechlorination reactions while the more toxic intermediates such as DCE, VC are less readily degraded (Haston & McCarty, 1999; Vogel *et al.* 1987). These less chlorinated intermediates are susceptible to oxidation under aerobic and iron reducing conditions but PCE is only removed by anaerobically (Freedman and Gossett, 1989).

3.3. Mechanism of Chloroethenes Transformation: Halorespiration vs. Cometabolism

Most of these transformations were explained as cometabolism, which are fortuitous transformation of chemicals by enzymes or cofactors produced by organisms for other purposes. During the cometabolic process, microorganisms that are responsible for catalyzing such reactions do not obtain nutritional or energetic benefits. Researchers showed that the majority of chlorinated compounds could undergo cometabolic transformation to less chlorinated products and in some instances complete dechlorinations to benign products in environments. Reductive dechlorination has been reported to occur cometabolically under various redox environments including

denitrifying, sulfate reducing, fermentating, acetogenic, and methanogenic conditions (Fetzner & Lingens, 1994; Mohn & Tiedje, 1992).

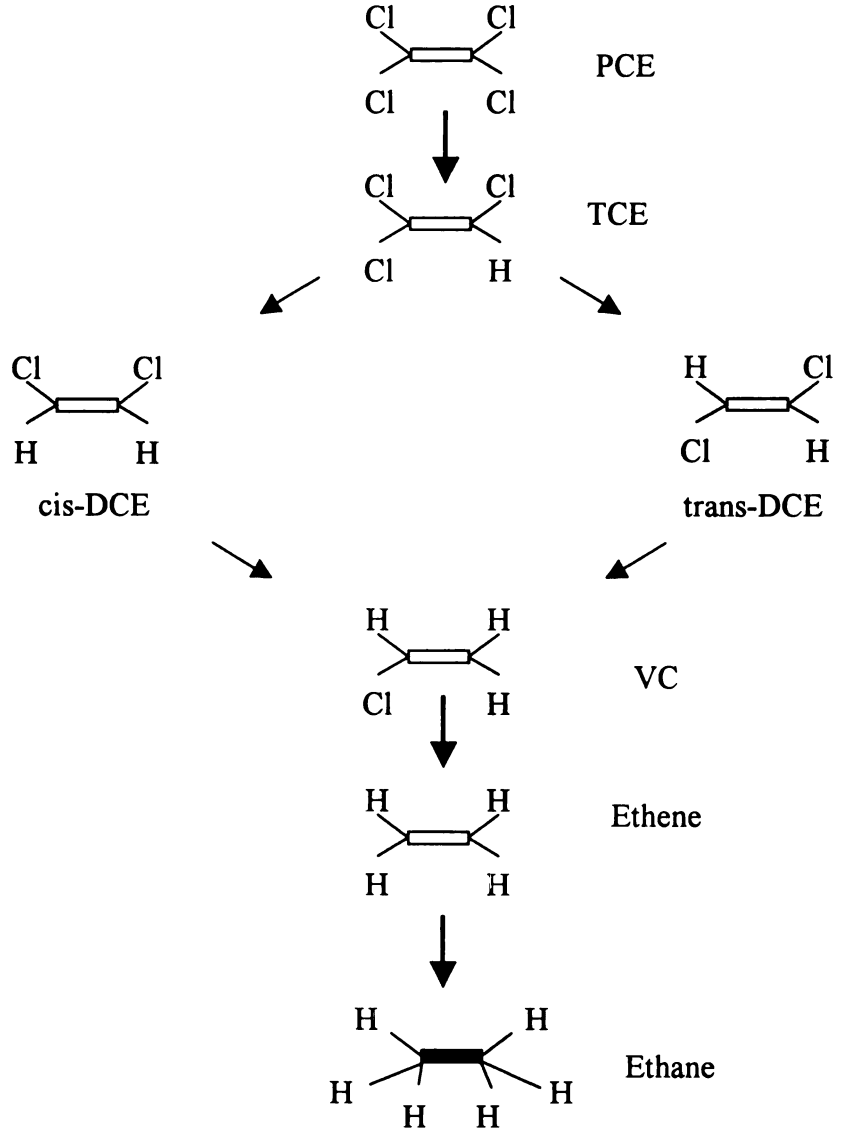


Figure 3.1 Pathway of microbial PCE transformation under anaerobic conditions.

However, recent research has indicated that in addition to cometabolic processes, anaerobic bacteria may degrade chlorinated ethenes by using them as an electron acceptor, which couples chlorinated ethenes directly to energy production (McCarty, 1997). In the absence of O_2, many anaerobes use electron acceptors such as nitrate, ferric iron, sulfate, protons, and CO_2. Reductive dechlorination has been shown to occur in all of the above mentioned alternative electron accepting environments, although it is more frequent in the environments with the lower redox potentials (Fetzner & Lingens, 1994; Mohn & Tiedje, 1992; Vogel *et al.* 1987). Due to similarity in redox potentials between chlorinated compounds to NO_3-/NO_2- redox couple, chlorinated compounds have the thermodynamic potential to serve as terminal electron acceptors in anaerobic microorganisms. Currently, only a few bacteria have been isolated that use chlorinated compounds as electron acceptors and conserve energy for growth.

Holliger *et al.* (1993) have described *Dehalobacter restrictus,* a highly purified enrichment culture that is able to grow by the reduction of PCE to c-DCE using hydrogen as the electron donor. This bacterium was able to grow on a limited number of electron donors, especially; hydrogen and formate, and could use only PCE and TCE as electron acceptors with carbon dioxide and yeast extract acting as carbon sources.

Five additional isolates, all capable of utilizing PCE as their terminal electron acceptors, have since been isolated: *Dehalospirillum multivorans*, strain TEA, *Desulfuromonas chloroethenica, Desulfitobacterium* sp. strain PCE1, and *Dehalococcoides ethenogens* strain 195. *Dehalosporillium multivorans* was able to grow on a range of electron donors including pyruvate, formate, hydrogen, lactate, ethanol and glycerol (Neumann *et al,* 1994). It was further demonstrated that only PCE and fumarate

could act as electron acceptors for this organism. Strain MS-1 has also been shown to dechlorinate PCE in pure culture, but PCE dependent growth has not been confirmed. Another organism capable of the incomplete dechlorination of PCE to c-DCE was the freshwater anaerobe TT4B, which used acetate or pyruvate as electron donor and only PCE or fumarate as an electron acceptor (Krumholz *et al.* 1996).

Of these isolates, only *Dehalococcodes ethenogenes* strain 195 catalyzes the complete dechlorination of PCE to ethene. *Dehalococcoides ethenogenes* was extracted from anaerobic digester supernatant and grown on hydrogen and PCE using carbon dioxide and yeast extract as carbon sources (Maymo-Gatell *et al.* 1997). It is reported that the growth of strain 195 was resistant to ampicillin and vancomycin and its cell wall did not react with a peptidoglycan –specific lectin and its ultra structure resembled S-layers of Archaea (Maymo-Gatell *et al.* 1997). Based on its 16S ribosomal DNA sequence, strain 195 clustered phylogenetically with the eubacteria but did not fall in any of the previously described eubacterial branches (Maymo-Gatell *et al.* 1997). The final step of VC transformation to ethene was found to occur only after PCE was completely degraded and cometabolic. No other known microorganisms capable of dechlorination beyond c-DCE have been reported so far.

3.4. Kinetics of Dechlorination

As reductive dechlorination proceeds, successive steps are increasingly slow and the conversion of VC to ethene has been observed to be a rate-limiting step in complete degradation (Freedman & Gossett, 1989). Studies of the kinetics of chlorinated ethene biodegradation have shown that DCE and VC have higher half velocity coefficients and

lower maximum transformation rates than their parent compounds so that their rate of degradation will be slower, especially at low concentrations (Haston and McCarty, 1999). The kinetics of dechlorination has been determined in both mixed and pure cultures, although inconsistencies in the manner the dechlorination rates were determined have made comparisons between studies difficult. Table 3.2 lists rate coefficients for various cultures and electron donors used to maintain the dechlorination activity.

Table 3.2. Rate coefficients for various mixed and pure cultures in dechlorination

Culture	Dechlorination	Electron Donor (s)	Rate of dechlorination	Reference
Methanosarcina sp.	PCE → TCE	Methanol and acetate	3.5 μmol·mgprotein^{-1}hr^{-1}	Fathepure *et al.* (1987)
Sulfate-reducing enrichment	TCE → c-DCE	Sodium lactate and acetate	213 μmol·L^{-1}d^{-1}	Pavlstathis & Zhuang (1991)
Enrichment culture	PCE → ethene	Methanol	275 μmol·L^{-1}d^{-1}	Distefano *et al.* (1991)
Fixed-bed column	PCE → ethane	Lactate	3.7 μmol·L^{-1}hr^{-1}	DeBruin *et al.* (1992)
Anaerobic sewage sludge – Batch	C-DCE → ethene, ethane	Glucose	4.2 μmolg^{-1} VSSd^{-1}	Komatsu *et al.* (1994)
Aquifer solids from polluted sediment – Batch	PCE → TCE TCE → c-DCE	Mixture of lactate, acetate and propionate	0.3 μMd^{-1}	Gibson *et al.* (1994)
Laboratory culture	PCE → VC	Methanol	4.6 ± 0.4 μmolg^{-1} VSSd^{-1}	Tandoi *et al.* (1994)
Anaerobic Reactor treating dechloromethane	TCE → DCE	Glucose	149 μMd^{-1}	Wild *et al.* (1995)

Dehalospirillum multivorans	PCE→ c-DCE	Pyruvate	50 nmol.min^{-1} (mg cell protein $^{-1}$)	Scholz *et al.* (1995)
Strain MS-1	PCE → c-DCE	Glucose, pyruvate, formate, lactate, acetate, yeast extract, amino acid	0.5 μmol·mgCell^{-1}hr^{-1}	Sharma & McCarty (1996)
Enriched contaminated soil sample	PCE → c-DCE	Citrate, pyruvate, succinate, formate, and acetate with hydrogen	0.4 μmol·mg VSS^{-1}hr^{-1}	Lee *et al.* (1997)
Enrichment from polluted sediment – Batch	c-DCE → ethene	H_2	16μMd^{-1}	Luijten *et al.* (1997)

3.5. Electron Donors for Reductive Dechlorination

A wide variety of fatty acids, alcohols, sugars and other compounds have been successfully demonstrated to support reductive dechlorination, and current researches focus on finding a particular electron donor that best support mixed dechlorinating anaerobes. Researchers reported that hydrogen is a key electron donor and organic compounds are mainly precursors to supply the needed hydrogen via fermentation (Distefano *et al.* 1992; Fennell *et al.* 1997). However, several studies have suggested the ability to enhance and sustain *in situ* dechlorination can be influenced by the selection of electron donor. Accordingly, many different types of electron donors were screened to stimulate and sustain dechlorination activity. Some researchers focused on hydrogen at

high partial pressures, where methanogens and other hydrogenotrophs may compete with dechlorinating microorganisms for hydrogen, resulting in incomplete dechlorination activity or its exclusion entirely (Ballapragada *et al.* 1997; Yang & McCarty, 1998). Meanwhile other researcher reported that the difference in the hydrogen partial pressure fail to represent long-term dechlorination activity (Fennell *et al.* 1997).

3.6. Methanogens vs. Sulfate Reducers

Anaerobic conditions encompass a range of redox potentials, depending on the terminal electron acceptors. Reductive dechlorination has been observed repeatedly in both natural and laboratory methanogenic environments (Fathepure *et al.* 1987; Fathepure & Boyd, 1988; DiStefano *et al.* 1992), under nitrate reducing conditions (Bouwer & McCarty, 1983; Jafvert & Wolfe, 1987), and in sulfate reducing conditions (Egli *et al.* 1987; Bagley & Gossett, 1990). Many reports on dechlorination in sulfate reducing conditions followed (Kohring *et al.* 1989; Stevens *et al.* 1988), but for most compounds, dechlorination occurred more readily when methanogenesis predominates (Bouwer & Wright, 1988; Gibson & Suflita, 1986). Therefore, early works on transformation of chloroethene were dominated in methanogenic mixed cultures or pure cultures of specific methanogens. However, many cases reported interactions/inhibitions in dechlorinations between microorganisms. Bacterial such as *Methanosarcina* sp. and *Methanosarcina mazei* could convert PCE to TCE with acetate as an electron donor and this conversion was found to be faster in mixed cultures and was inhibited when methanogensis was inhibited (Fathepure *et al.* 1987).

Freedman and Gossett (1989) reported a mixed culture capable of completely reducing PCE to ethene did experience inhibition when methanogenesis was inhibited, however, PCE degradation was only temporarily halted and did resume even with repeated additions of a methanogenesis inhibitor bromoethanesulfonic acid (BES). The same inhibitor was also found to prevent ethane production, but have no effect on other steps in reductive dechlorination of PCE to ethene in column studies (deBruin *et al.*1992). In other experiments with a methanol enrichment culture, high concentrations of PCE were reductively dechlorinated to ethene and methanogenic activity decreased as PCE loading and subsequent dechlorination increased (DiStefano *et al.* 1991).

While these studies suggested that methanogens are still involved in partial PCE or TCE reductive dechlorination and may be necessary members of bacterial populations of complete reductive dechlorination, other works found that PCE and TCE could be degraded in the complete absence of methanogenesis. Bagley and Gossett (1990), reported that PCE degradation to TCE and c-DCE was enhanced in sulfate reducing cultures when methanogenesis was inhibited. There are still no published reports of complete degradation of PCE to ethene under sulfate reducing conditions in mixed cultures. But pure cultures of *Desulfovibrio desulfuricans* are reported to degrade PCE without any other productions of dichloroethene (Fathepure *et al.* 1987). In addition, sulfidogenic bacterium DCB-1 (Shelton & Tiedje, 1984; Stevens *et al.* 1988) has been shown to dechlorinate PCE to TCE, and *Desulfobacterium autotrophicum* reduces PCE quantitatively to TCE and DCE (Egli *et al.* 1987), indicating the existence of reductive dechlorination capabilities in sufidogenic and sulfate-reducing bacteria. The research efforts on biotransformation of chlorinated ethenes are summarized in Table 3.3.

3.7. Abiotic degradation of chlorinated compounds

The biological reductive dechlorination of PCE, TCE to ethene and ethane has been demonstrated at a number of sites. However, biological activity often leads to incomplete or slow degradation of a specific compound. This is generally due to a deficiency in an electron donor or acceptor, other nutrients required for the dechlorinating microbial population, and/or absence of appropriate dechlorinating microbial populations.

FeS has been identified as a soil precipitate in sulfate-reducing environments where it is formed through the biologically mediated reduction of sulfate to sulfide and subsequent reaction of sulfide with available iron species (Rickard 1969; Freney 1979). Several forms of ferrous sulfide can be produced; amorphous iron sulfide, makinawite ($Fe_{0.995\text{-}1.023}S$), greigite (Fe_3S_4), pyrrohotitie ($FeS_{1.1}$), and pyrite (FeS_2). These soil minerals are reported to transform halogenated organic compounds. (Magnetite: Sivavec and Horney 1997; McCormick *et al.* 1998), pyrite (Kriegman-King & Reinhard, 1991 and 1994). Butler and Hayes (1999) have demonstrated that ferric sulfides and ferric disulfides such as mackinwite and pyrite can promote the abiotic dechlorination of chloroethenes. The major byproduct of was acetylene after 120 days. Lee and Batchelor (2002) also reported that pyrite degraded TCE to 3.3% c-DCE, 43% acetylene, 2.2% ethene, and 50 % residual TCE after 32 days. The research efforts on abiotic transformation of chlorinated ethenes are also summarized in Table 3.3.

Table 3.3. Summary of Research Efforts regarding the degradation of chlorinated ethenes and ethanes in biotic and abiotic conditions.

(a) Electron donors and source of inoculums in methanogenic mixed and pure cultures.

Source of Inoculum	Electron Donor (s) and Dechlorination	Summary	Reference
Methanogenic mixed culture from laboratory digester	None, except methanol used in PCE and TCE solutions PCE → DCE TCE → DCE	Degradation of PCE and TCE in anaerobic and aerobic cultures. Both compounds were recalcitrant under aerobic conditions and appreciable degradation under anaerobic conditions was not observed within 16 weeks.	Bouwer and McCarty (1981)
Same as above	Acetate PCE → TCE	Under methanogenic conditions, PCE (100 < μg/L) was reductively dechlorinated to TCE. Addition of BES causes a reduction in acetate utilization, but did not affect the extent of PCE dechlorination. The role of acetoclasts in PCE dechlorination remained uncertain.	Bouwer and McCarty (1983)
Soil from a TCE contaminated site	Soybean meal PCE → DCE	With radiolabeled TCE, authors proved that it reduced to 1, 2-DCE isomers in soil microcosms.	Kleopfer *et al.* (1985)
Laboratory culture	Acetate PCE → DCE	PCE dechlorinate to TCE, DCEs, and VC in fixed film methanogenic columns. 24 % of the initial PCE added was reported to be mineralized to CO_2	Vogel and McCarty (1985)
Pure cultures of acetoclastic methanogens	Methanol and acetate PCE → TCE	Pure cultures of anaerobic bacteria capable of dechlorinating PCE were identified. DCB-1, a chlorobenzoate fed anaerobe, and two strains of Methanosarcina dechlorinated PCE. DCB-1 stoichiometrically converted PCE to TCE. When DCB-1 was mixed with the two Methanosarcina strains, higher rates of PCE dechlorination were achieved and TCE degraded.	Fathepure, Nengu, and Boyd (1987)

Sewer sludge and acetoclastic methanogens in pure culture	Methanol and acetate PCE → TCE	BES significantly inhibited PCE dechlorination and methanogensis. Acetoclastic methanogens, Methanosarcina sp. and Ms. mazei were shown to dechlorinate PCE in pure cultures. Relationships may exist between PCE dechlorination and methanogesis.	Fathepure and Boyd (1988)
Pure culture of Methanosarcina sp. Strain DCM	Methanol, acetate, methylamine, and trimethylamine PCE → TCE	Methanosarcina sp. Strain DCM as shown to dechlorinate PCE to TCE while growing on methanol, acetate, methylamine and trimethylamine. PCE dechlorination was observed only during mathanogenesis, and was found to be contingent upon methanol utilization. Authors suggest that a reduced electron carrier diverted electrons generated during methane synthesis to PCE, and that stimulating methanogenesis could enhance PCE dechlorination.	Fathepure and Boyd (1988)
Laboratory anaerobic digester seeded with digested sludge from NY wastewater treatment plant	Methanol, hydrogen, formate, acetate, and glucose PCE → ethene	First publication of the complete dechlorination of PCE to ethene in mixed cultures. Under methanogenic conditions, the reduction of VC to ethene was found to be the rate-limiting step in the dechlorination process. Hydrogen, formate, acetate, and glucose were found to sustain dechlorination, although methanol was the most effective. TCE dechlorination and methanogenesis were inhibited in cultures amended with BES. BES did not immediately inhibit PCE dechlorination, but resulted in the accumulation of TCE and 1,2-DCEs. Authors concluded that methanogens may have played a key role in PCE dechlorination, yet emphasized that complete dechlorination was only occurring in mixed cultures.	Freedman and Gossett (1989)
Aquifer solids	Toluene PCE → DCE	Metabolism of toluene was found to create an initial source of reducing equivalents for the dechlorination of PCE. Either benzoate or acetate, both toluene metabolites, served as the final electron donor.	Sewell and Gibson (1991)
Laboratory culture used by Freedman and Gossett	Methanol PCE → DCE	PCE (55mg/L) was degraded to ethene (<1% remaining as VC) within 4 days by a methanol-fed anaerobic enrichment culture. Thirty-one percent of the methanol added was used for dechlorination, and the remainder was utilized for acetate production. Dechlorination occurred in the	Distefano, Gossett, and Zinder. (1991)

		absence of methanogenesis. This observation was inconsistent with previous work, which indicated that methanogens participated in PCE dechlorination. Authors concluded that other organisms, potentially acetogens, played a role in PCE dechlorination.	
Aquifer Solids from Traverse City, MI.	Simple organics and alcohols PCE → DCE	Lactate- and ethanol-fed cultures dechlorinated PCE (5 mg/L) with smaller lag times than did cultures fed butyrate, crotonate, or propionate. Dechlorination activity was not enriched using acetate, isopropanol, or methanol. Unlike the other substrates tested, methanol and acetate usually do not produce large amounts of hydrogen during their anaerobic metabolism. Therefore, the authors concluded that hydrogen, generated through alcohol and fatty acid metabolism, was the final electron donor in PCE dechlorination.	Gibson and Sewell (1992)
Laboratory culture used by Freedman and Gossett (1989)	Methanol and Hydrogen PCE → DCE	Hydrogen has been proposed as the direct electron donor, derived from the fermentation or breakdown of other more complex compounds. Hydrogen was found to sustain dechlorination in an anaerobic enrichment culture for a period of 14 to 40 days. Dechlorination beyond 40 days could not be sustained without the addition if nutritional factors from a culture containing the same inoculum but fed methanol. Acetogenesis was inhibited in both the methanol- and hydrogen-fed cultures by vancomycin, and eubacterial inhibitor of cell wall synthesis. In the presence of vancomycin, dechlorination was inhibited in only the methanol-fed cultures. Authors concluded that dechlorination in the methanol-fed cultures was sustained by hydrogen produced during acetogenesis of methanol. BES was found to inhibit dechlorination in cultures fed methanol and hydrogen, suggesting that hydrogen-utilizing methanogens, not acetogens, were possibly the dechlorination microorganism.	DiStefano, Gossett, and Zinder (1992)
Rhine River sediment and anaerobic granular sludge	Lactate PCE → Ethane	PCE (1.5 ppm) was dechlorinate to ethene and ethane in a fixed-bed column. When BES inhibited methanogenesis, ethene was not reduced to ethane. Both river sediment and sludge were need for complete dechlorination	DeBruin *et al.* (1992)

PER-K23 (Dehalobacter restrictus)	Hydrogen and formate PCE → Ethane (?)	A gram-negative bacterium (PER-K23) was insolated from the inoculum from DeBruin et al (1992). First isolate to couple the dechlorination of PCE to c-DCE to growth. Growth on PCE or TCE could only be sustained using hydrogen or formate. Biomass and dechlorination were found account for all of the electrons generated from hydrogen and formate utilization.	Hollinger, Schraa, Stams, and Zehnder (1993)
Aquifer solids from Traverse City, MI	Mixtures of lactate, acetate, and propionate PCE → DCE	A mixture of fatty acids, at three different concentrations, was fed in conjunction with 5 mg/L, PCE to microcosms containing aquifer solids. The amount of PE dechlorinated was similar regardless of fatty acid concentration, although higher substrate concentrations led to shorter lag periods. A zero order rate constant of 0.3 $\mu M \cdot day^{-1}$ was calculated for PCE and TCE dechlorination. TCE and the DCE isomers were the reduced end products formed. The authors concluded that butyrate oxidation supported dechlorination activity since it preceded the onset of CE reduction to TCE.	Gibson, Robertson, Russell, and Sewell (1994)
Enrichment culture seeded with *D. tiedjei* DCB-1	3-chlorobenzoate PCE → DCE	Biofilm reactor packed with an enrichment culture containing Desulfomonile tiedjei DCB-1 was shown to dechlorinate PCE to cis- and trans-DCE. Acetate, methanol, glucose, and benzoate could not replace 3-chlorobenzoate (3-CB) as electron donor. Dependence of the culture on 3-CB indicated that strain DCB-1 of a similar microorganism was involved in dechlorination. The maximum PCE dechlorination rte was 10.3 $\mu mol \cdot L^{-1} \cdot hr^{-1}$.	Fathepure and Tiedje (1994)
Laboratory culture used by Freedman and Gossett	Methanol PCE → VC	Anaerobic enrichment culture degraded 53 mg/L PCE to VC within 20 hours. The PCE degradation rate was calculated to be 4.6 ± 0.4 μmol PCE$\cdot mg\ VSS^{-1} \cdot day^{-1}$. VC dechlorination was inhibited by the presence of PCE. Zero order kinetics were used to describe PCE, TCE, cis-DCE and 1,1-DCE reductions to VC. Trans-DCE conversion to VC was modeled using first order kinetics.	Tandoi *et al.* (1994)
Anaerobic digested sewage sludge	Glucose, yeast extract, propionate, hydrogen, methanol and acetate	Cis-DCE dechlorination in anaerobic culture was supported by glucose, yeast extract, propionate, and to a lesser extent by hydrogen. The rate of cis- dechlorination was slower in methanol-fed cultures than in	Komatsu *et al.* (1994)

	c-DCE→ ethene, ethane	cultures in which no electron donor was added. This implied that methanol might have had an inhibitory effect on dechlorination.	
Dehalospirillum multivorans	Pyruvate, hydrogen, and formate PCE → c-DCE	*Dehalospirillum multivorans*, an anaerobe capable of utilizing PCE as its terminal electron acceptor, dechlorinated PCE to cis-DCE. Pyruvate, formate, and hydrogen could serve as electron donors. Fumarate was found to be an alternative terminal electron acceptor. The enzyme PCE dehalogenase was recovered in cell free extracts.	Neumann *et al.* (1994)
D. multivorans	Pyruvate and PCE PCE → DCE TCE → DCE	*D. multivorans* grew in defined medium with PCE and H_2 as sole energy sources and acetate as carbon source. Alternatively to PCE, fumarate and nitrate could serve as electron acceptors: sulfate could not. The organism utilized a variety of electron donors for dechlorination (pyruvate, lactate, ethanol, formate, glycerol, H_2).	Scholz-Muramatsu-Heidrun *et al.* (1995)
Laboratory culture used by Freedman and Gossett	Hydrogen PCE → VC, ethene	Using the anaerobic enrichment culture developed by (Freedman and Gossett), a hydrogen-utilizing culture capable of growing on PCE was isolated. The hydrogen-PCE culture was capable of dechlorinating PCE to VC and ethene, but required supplements of vitamin B_{12}, supernatant from an anaerobic digester sludge, and acetate as a source of carbon. Methanol and acetate could not replace hydrogen as an electron donor, and the culture did not produce methane or acetate. This suggests that the hydrogen-PCE culture did not contain methanogens or acetogens.	Maymo-Gatell, Tandoi, Gossett, and Zinder (1995)
Butyrate enrichment culture seeded from Distefano *et al.* (1991)	Hydrogen and formate PCE → VC	Dechlorinating organisms are able to utilize lower concentrations of hydrogen than competing bacteria such as methanogens.	Smatlak, Gossett, and Zinder (1996)
Strain MS-1	Glucose, pyruvate, formate, lactate, acetate, yeast extract, amino acids	A facultative aerobe capable of dechlorinating PCE at 0.5 μmol PCE•hr^{-1}•mg (dry weight) cell^{-1} was isolated from a site in Victoria, TX, contaminated with PCE. PCE was dechlorinated to cis-DCE.	Sharma and McCarty (1996)

	PCE → c-DCE	The presence of oxygen and nitrate inhibited dechlorination, suggesting that strain MS-1 would only dechlorinate when more thermodynamically favorable terminal electron acceptors were absent. Numerous electron donors could sustain dechlorination, although high concentrations of fermentable compounds were inhibitory. Characteristics of strain MS-1 were found to be very similar to the Enterobacteriaceae family. Enterobacter agglomerans was also shown to dechlorinate PCE to c-DCE.	
Strain TT4B	Acetate or pyruvate PCE → c-DCE	Strain TT4B was isolated from stream sediments contaminated with TCE and toluene. The isolate dechlorinated PCE to c-DCE, and was found to use other terminal electron acceptors (TCE, fumarate, and ferric nitroacetate).	Krumholz, sharp, and Fishbain (1996)
Laboratory methanogenic consortium	Lactate, acetate, hydrogen, and propionate PCE → TCE VC, ethene	Higher dechlorination rates were observed to correspond to higher hydrogen partial pressures. Dechlorinators were found to have an advantage in competing with methanogens for hydrogen (K values for hydrogen uptake by dechlorinators were reported to be 12-28 ppm).	Ballapragada *et al.* (1997)
Laboratory culture	Butyric acid, ethanol, lactic acid, propionic acid PCE → DCE	Four electron donors were compared for their ability to stimulate and sustain dechlorination in short and long-term studies. It was found that for butyrate and propionate, which are fermented at low hydrogen partial pressures of 10-3.5 and 10-4.4 at respectively and ethanol and lactic acid fermented at partial pressures orders of magnitude higher, long term differences were negligible. In all cases, increased electron donor levels resulted in greater ethene production	Fennell *et al.* (1997)
Contaminated soil sample	Citrate, pyruvate, succinate, formate, acetate, and acetate with hydrogen PCE → DCE	Demonstrated that dechlorinating bacteria are relatively abundant in nature by showing that cultures from both contaminated and uncontaminated sites could dechlorinate PCE (nominal concentration of 10 mg/L) within two weeks. PCE dechlorinating activity increased with increasing PCE concentration up to 150 mg/L nominal concentration (58 mg/L in aqueous phase).	Lee *at al.* (1997)

		The optimum values for pH and temperature were 7.0 and 30°C, respectively. Yeast extract was required to maintain activity, and all electron donors tested supported dechlorination. Dechlorinating rate was determined to be 0.4 μmol PCE•mg VSS^{-1}•hr^{-1}	
Sediments taken from several contaminated sites	Methanol, lactate, acetate, and sucrose PCE → TCE	Tested the ability of several electron donors to enrich PCE dechlorination in contaminated sediments. Long lag-times were observed before PCE dechlorination commenced in all systems. Lactate was the only electron donor that was able to support dechlorination in more than one of the sediments tested. Lactate also supported greater extents of dechlorination.	Gao, Skeen, Hooker, and Quesenberry (1997)
Dehalococcoides ethenogenes strain 195	Hydrogen PCE → ethene	An anaerobic bacterium was isolated that had the capability to dechlorinate PCE to ethene. Strain 195 coupled the reduction of PCE with growth on hydrogen, and required the addition of anaerobic digester sludge supernatant for growth. This is the first report of an isolate with the capability of reducing PCE completely to ethene.	Maymo-Gatell *et al.* (1997)
Laboratory enrichment culture seeded from an anaerobic upflow sludge blanket; sediments from a contaminated site in Texas	Methanol, lactate, and hydrogen PCE → VC, ethene	A high-rate PCE dechlorinating culture was enriched from a culture with no previous exposure to chlorinated ethenes. The ability to enrich and sustain dechlorination activity with various electron donors was tested with this culture either alone or with a (1:1) mixture containing a second culture derived from contaminated aquifer sediments. Over extended periods of time (approx. 430 days), it was demonstrated that similar rates and extents of PCE dechlorination could be achieved regardless of electron donor fed or inoculum used. Later studies demonstrated that PCE dechlorination could be sustained at high hydrogen partial pressures despite the presence of an actively methanogenic community.	Carr and Hughes (1998)
Laboratory culture	Butyric acid, ethanol, lactic acid, and propionic acid PCE → c-DCE, VC	Using data obtained from a laboratory, a model was developed to predict the formation of hydrogen from the fermentation of various substrates coupled with the competition for hydrogen among methanogens and dechlorinating bacteria. Model simulations suggested that	Fennell and Gossett (1998)

		compensating for competition of electron donor by adding excess donor eventually led to failure of dechlorination and the development of a predominantly methanogenic population.	
Aquifer material from a PCE-contaminated groundwater site in Victoria, TX	Benzoate and propionate c-DCE → ETH	Demonstrated that in batch studies containing benzoate and c-DCE, dechlorinators maintained hydrogen concentrations at levels below that which support methanogenesis. Cultures fed propionate, which is fermented to hydrogen at slower rates than benzoate, had 100% conversion to ethene rather than 73% in benzoate-fed cultures. Dechlorinators had higher hydrogen utilization efficiencies in continuous flow, completely mixed systems than in batch reactors, indicating that different approaches may be used to favor dechlorination over other competing microbial processes.	Yang and McCarty (1998)
Aquifer material from a PCE-contaminated groundwater site in Victoria, TX	Benzoate and propionate	The maximum degradation rates and half-velocity coefficients for PCE, TCE, c-DCE, and VC dechlorination were determined in batch cultures. Degradation rates were highest with PCE (77 μM/day), and degradation rates for c-DCE and VC were similar (14 μM/day, 13 μM/day, respectively). Half-velocity coefficients for PCE, TCE, c-DCE, and VC were 0.11, 1.4, 3.3, and 2.6 μM, respectively. Authors concluded that the common observation of slow or incomplete dechlorination to the level of c-DCE and/or VC could be partially explained by kinetics.	Haston and McCarty (1999)
TCE-contaminated aquifer	Glucose PCE → VC	Rates of PCE and TCE dechlorination were shown to increase with increasing PCE and TCE concentrations, including concentrations near the solubility limit. Under saturating conditions (i.e., DNAPL present), PCE and TCE dechlorination did not result in the buildup of VC, potentially due to less competition for electron an electron donor. PCE dechlorination was shown to follow Monod kinetics, and TCE and VC dechlorination were first-order.	Nielsen and Keasing (1999)

(b) Electron Donors and source of inoculums in sulfate-reducing mixed and pure cultures.

Source of Inoculum	Electron Donor (s)	Summary	Reference
Soil sediments	Acetate	Trace halogenated aliphatics were transformed under denitrifying, sulfate-reducing, and methanogenic conditions.	Bouwer and Wright (1988)
Laboratory culture	Lactate PCE → DCE TCE → DCE	PCE dechlorinated to TCE and c-DCE under sulfate-reducing conditions. Extent and rate of PCE dechlorination was less than that observed in mixed methanogenic cultures. Neither lactate, acetate, methanol, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid, succinic acid, nor hydrogen appeared directly to support tetrachloroethene dechlorination, although lactate-fed inocula demonstrated longer-term dechlorinating capability.	Bagley and Gossett (1990)
Enrichments from contaminated subsurface soil	Sodium lactate and acetate TCE → c-DCE	TCE reductively dechlorinated to c-DCE by sulfate-reducing. The highest observed transformation rate of TCE was 213 micromoles l^{-1} per day at 35°C. No further dechlorination of c-DCE. A decrease in the rate and extent of TCE transformation was observed with an increase in the concentration of bromoethanesulfonate up to 50 mM.	Pavlostathis & Zhuang (1991)
Desulfitobacterium sp. Strain PCE1	Lactate, pyruvate, butyrate, formate, succinate, and ethanol PCE → DCE	Anaerobic bacterium was isolated from a PCE dechlorinating enrichment culture containing sulfate reducers and acetogens. *Desulfitobacterium* could utilize PCE, 2-chlorophenol, 2, 4, 6-trichlorophenol, fumarate, sulfite, thiosulfate, and 3-chloro-4-hydroxy-phenylactetate as terminal electron acceptors. PCE was dechlorinated to TCE, and c-DCE and t-DCE. Hydrogen was found to inhibit dechlorination.	Gerritse *et al.* (1996)
Laboratory Enrichment	Acetate (1 mM) 1,1,1-TCA → DCA and DCA → unknown products	The rate of transformation and the by-products depended on the concentrations of TCA, acetate and sulfate. Both packed-bed reactor studies and batch experiments with BEA and molybdate demonstrated the involvement of methanogens and sulfate-reducing bacteria in transformation of TCA.	DeBest *et al.* (1997)

Subsurface soils	Methanol, lactate, acetate and sucrose PCE → TCE	Various levels of sulfate-reducing, acetogenic, fermentative, and methanogenic activity were observed in all sediments. PCE dechlorination was detected in all microcosms, but the amount of dehalogenation varied by several orders of magnitude. Lactate-amended microcosms showed large amounts of dehalogenation but elevated levels of dehalogenation were not consistently associated with any observable anaerobic metabolisms.	Gao-Jianwei *et al.* (1997)
PCE-enrichment	Yeast extract, sodium lactate PCE → TCE	A methanogenic and sulfate-reducing consortium showed that dehalogenation was due to the direct activity of methanogens. In the presence of sulfate, methanogenesis and dechlorination decreased because of interspecific competition, probably between the H_2-oxidizing methanogenic and sulfate-reducing bacteria in batch conditions.	Cabirol *et al.* (1998)
PCE contaminated soil	PCE → c-DCE, TCE	PCE was transformed to c-DCE and TCE under sulfate-reducing conditions using acetate, lactate, and methanol as electron donors. Biotransformation of PCE was dependent upon the type of electron donor used.	Ndon *et al.* (2000)

(c) Summary of dechlorination by iron-reducing or iron related abiotic mechanism.

Source	Dechlorination	Summary	Reference
Anaerobic aquifer sediments	Vinyl chloride added as substrate VC → CO_2	First demonstration of vinyl chloride can be oxidized to CO_2 under iron (III)-reducing conditions. Transformation was dependent upon the bioavailability of Fe (III), which was added as Fe-EDTA.	Bradley and Chapelle (1996)
Creek bed sediments obtained near discharge of contaminated groundwater	c-DCE and VC added as substrates C-DCE, VC→ CO_2	c-DCE and VC mineralization were observed under both methanogenic and Fe (III)-reducing conditions. In the methanogenic microcosms, 5% to 44% of ^{14}VC and 4% to 14% of ^{14}DCE were recovered as $^{14}CO_2$. Under iron reducing conditions, the recovery of $^{14}CO_2$ from labeled VC was twice that of the methanogenic microcosms, and similar for labeled c-DCE. The kinetics of DCE and VC mineralization varied between the two compounds: DCE was modeled using first order kinetics, and VC was modeled with Michaelis-Menten kinetics.	Bradley and Chapelle (1997)
1.1 M Na_2S added to 0.57 M $FeCl_2$ in anaerobic glove bag	PCE → acetylene TCE → acetylene	TCE and PCE were transformed by 10 g/L FeS in aqueous solution at pH 8.3. Acetylene was the major reaction product for both TCE and PCE. 1,1-DCE was persistent over 120 days.	Butler and Hayes (1999)
Zero-valent iron		Reductive β-elimination accounts for 87 % of PCE, 97% of TCE, 94 % of c-DCE, and 99 % of t-DCE. Reaction of 1,1-DCE gives rise to ethylene, consistent with a reductive α-elimination pathway.	William and Roberts (2000)
Pyrite and magnetite		Abiotic reductive dechlorination of chlorinated ethenes by pyrite and magnetite was monitored in batch system. Target organics were mainly transformed to acetylene and small amount of chlorinated intermediates, which suggests that β-elimination, was the main dechlorination pathway.	Lee and Batchelor (2002)

3.8. Literature cited

Maymo-Gatell, X.,Anguish, T. and S. H. Zinder. 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by *Dehalococcoides ethenogens* 195. *Appl. Environ. Microbiol.* 65(7): 3108-3113.

Sawyer, C. N., McCarty, P.L., and G. F. Parkin. 1994. Chemistry for environmental engineering, McGraw-Hill, Inc., New York

Yaws, C. L. 1999. Chemical properties handbook: physical, thermodynamic, environmental, transport, safety, and health related properties for organic and inorganic chemicals, McGraw-Hill, Inc., New York.

Gossett, J. M. 1987. Measurement of Henry's Law Constants for C1 and C2 chlorinated hydrocarbons. *Environ. Sci. Technol.* 21(2): 202-208.

Haston, Z.C., and P.L. McCarty, 1999. Chlorinated ethene half-velocity coefficients (Ks) for reductive dechlorination. *Environ. Sci. Technol.* 33(2): 223-226.

Vogel, T.M., C.S. Criddle, and P.L. McCarty. 1987. Transformation of halogenated aliphatic compounds, *Environ. Sci. Technol.* 21; 722-736.

Freedman, D. L. and J. M. Gossett, 1989. Biological reductive dechlorination of Tetrachloroethylene and Trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Fetzner, S., and F. Lingens. 1994. Bacterial dehalogenation; Biochemistry, genetics, and biotechnological applications. *Microbiol. Rev.* 58: 641-685

Mohn, W.W., and J.M. Tiedje. 1992. Microbial reductive dehalogenation. *Microbiol. Rev.* 56: 482-507

McCarty, Perry. 1997. Breathing with Chlorinated Solvents. *Science.* 6(276) 1521-1522

Holliger, C., G. Schraa, Stams Alfons, J. M., Zehnder J. B. Alexander. 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl. Environ. Microbiol* 59(9): 2991-2997.

Neumann, Anke., Scholz, Muramatsu Heidrun., Gabriele Diekert. 1994. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Archives of Microbiology*, 162(4): 295-301.

Krumholz, Lee R, Sharp, Richard, Fishbain, S. Susan. 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation, *Appl. Environ. Microbiol.* 62(11): 4108-4113

Maymo-Gatell, X., Chien, Y. T., Gossett, J. M., and S. H. Zinder, 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276(5318): 1568-1571.

Distefano, T. D., Gossett, J. M., and S. H. Zinder, 1992. Hydrogen as an electron-donor for dechlorination of Tetrachloroethene by an anaerobic mixed culture. *Appl. Environ. Microbiol* 58(11): 3622-3629.

Fennell, D. E., Gossett, J. M., and S. H. Zinder, 1997. Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.* 31(3): 918-926.

Ballapragada, B. S., Stensel, H. D., Puhakka, J. A., and J. F. Ferguson, 1997. Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ. Sci. Technol*. 31(6): 1728-1734.

Yang, Y. R. and P. L. McCarty, 1998. Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environ. Sci. Technol*. 32(22): 3591-3597.

Fatherpure. B. Z., Nengu, J. P., S. A. Boyd. 1987. Anaerobic bacteria that dechlorinate perchloroethene. *Appl. Environ. Microbiol.* Washington: American Society for Microbiology. Nov 1987. v. 53 (11): 2671-2674.

Fathepure, B. Z. and S. A. Boyd, 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters* 49(2): 149-156.

Bouwer, E.J. and P.L. McCarty. 1983. Transformations of halogenated organic compounds under denitirifying conditions. *Appl. Environ. Microbiol.* 45(4): 1295-1299

Jafvert, C.T. and N. L. Wolfe. 1987. Degradation of selected halogenated ethanes in anoxic sediment-water systems. *Environ. Toxicol. Chem.* 6;827-83

Egli, C., R.Scholtz, A.M. Cook and T. Leisinger. 1987. Anaerobic dechlorination of tetrachloromethane and 1, 2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. *FEMS Microbiol. Lett.* 43: 257 –261.

Bagley, D. M. and J. M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and cis-1,2-dichloroethene by sulfate-reducing enrichment cultures. *Appl. Environ. Microbiol.* 56(8): 2511-2516.

Kohring, G., X. Zhang and J. Wiegel. 1989. Anaerobic dechlorination of 2,4-dichlrophenol in freshwater sediments in the presence of sulfate. *Appl. Environ. Microbiol.* 55(10): 2735-2737

Stevens, T.S., T.G. Linkfield and J. M. Tiedje. 1988. Physiological characterization of strain DCB-1, a unique dehalogenating sulfidogenic bacterium. *Appl. Environ. Microbiol.* 54; 2938-2943.

Bouwer, E.J. and J. P. Wright. 1988. Transformations of trace halogenated aliphatics in anoxic biofilm columns. *J. Contam. Hydrol.* 2; 155-169.

Gibson, S.A. and J. M. Suflita. 1986. Extrapolation of biodegradation results to groundwater aquifers; reductive dehalogenation of aromatic compounds. *Appl. Environ. Microbiol.* 52(4): 681-688

Debruin, W. P., Kotterman, M. J. J., Posthumus, M. A., Schraa, G., and Zehnder, A. J. B. 1992. Complete biological reductive transformation tetrachloroethene to ethane. *Appl. Environ. Microbiol.* 58(6): 1996-2000.

DiStefano, T. D., J. M. Gossett and S. H. Zinder. 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl. Environ. Microbiol.* 57: 2287-2292.

Pavlostathis, S.G. and P. Zhuang. 1991. Transformation of trichloroethylene by sulfate-reducing cultures enriched from a contaminated subsurface soil. *Appl. Microbiol. Biotech.* 36 (3): 416-420.

Komatsu, T, Momonoi, K., Matsuo, T., K. Hanaki. 1994. Biotransformation of c-DCE to ethylene and ethane under anaerobic conditions. *Water Science and Technology.* Kidlington, Oxford, UK; Elsevier Science Ltd. v. 30 (7): 75-84.

Gibson, S.A., Roberson, D.S., Russell, H.H., and G.W. Sewell. 1994. Effects of three concentrations of mixed fatty acids on dechlorination of tetrachloroethene in aquifer microcosms. *Environmental Toxicology and Chemistry*, 13 (3): 453-460.

Tandoi, V., Distefano, T. D., Bowser, P. A., Gossett, J. M., and S. H. Zinder. 1994. Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. *Environ. Sci. Technol.* 28(5): 973-979.

Wild, A. P., Winkelbauer, W., and T. Leisinger. 1995. Anaerobic dechlorination of trichloroethene, tetrachloroethene and 1,2-dichloroethane by an acetogenic mixed culture in a fixed-bed reactor. *Biodegradation* 6(4): 309-318.

Scholz. Muramatsu Heidrun, Neumann. Anke, Messmer. Michael, Moore. Edward, and Diekert. Gabriele. 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Archives of Microbiology*; 163 (1): 48-56.

Sharma. P. K. and P.L. McCarty. 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1,2-dichloroethene. *Appl. Environ. Microbiol.* 62 (3): 761-765.

Lee, T. H., Yoshimi, M., Ike, M., and M. Fujita. 1997. Characterization of an anaerobic soil enrichment capable of dechlorinating high concentrations of tetrachloroethylene. *Water Science and Technology.* 36(6/7): 117-124.

Luijten. M.L.G.C., S.W.M. Kengen, G. Schraa, and A.J.M. Stams. 1997. Complete reductive dehloagnation of chlorinated ethenes. In: H.Verachtert and W.Verstraete (Ed.), Intern. Symposium. Environ. Biotechnol., pp. 24-25.

Shelton, D.R., Boyd, S.A. and J.M. Tiedje. 1984. Anaerobic biodegradation of phthalic acid esters in sludge. *Environ. Sci. Technol.* 18 (2) p. 93-97.

Rickard, D. T. 1969. The microbiological formation of iron sulphides, *Stockholm Contr, Geol.* (26): 49-6

Freney, J. R. 1979. Sulfur Transformations, in R. W. Fairbridge, and C. W. Finkl, Jr., Eds, *The Encyclopedia of Soil Science Part 1, in Encyclopedia of Earth Science* Volume XII, Dowden, Hutchison and Ross, Inc., Stroudsburg, PA, pp. 536-544.

Sivavec, T.M. and D. P. Horney. 1997. Reduction of chlorinated solvents by Fe(II) minerals. Preprints of papers presented at the 213 th ACS National Meeting, April 13-17, 1997, San Francisco, CA, Vol 37 (1), pp. 115-117

McCormick, M.L., Kim, H.S., Bouwer, E.J., and P. Adriaens. 1998. Abiotic transformation of chlorinated solvents as a consequence of microbial iron reduction: An investigation of the roles of biogenic magnetite in mediating reductive dechlorination, Proceedings of the 13 th Mid-Atlantic Industrial and Hazardous Waste Conference, Suri, R.P.S. and Christensen, G.L., eds., Villanova University, Philadelphia, PA, July 12-15, 1998, pp. 339-348.

Kriegman-King, M. R. and M. Reinhard. 1991. Reduction of hexachloroethane and carbon tetrachloride at surfaces of Biotite, Vermiculite, Pyrite, and Marcasite, in R. Baker, Ed., Organic Substances and Sediments in Water, Vol. 2., Processes and Analytical, Lewis Publishers, Inc. Chelsea, Michigan, pp. 349-364.

Kriegman-King, M. R. and M. Reinhard. 1994. Transformation of carbon tetrachloride by Pyrite in aqueous solution, *Environ. Sci. Technol* (28), 692-700.

Butler, E., and Kim F. Hayes. 1999. Kinetics of the transformation of Trichloroethylene and Tetrachloroethylene by iron sulfide. *Environ. Sci. Technol.* 33(12): 2021-2027

Lee, W., and B. Batchelor. 2002. Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 1. Pyrite and Magnetite, *Environ. Sci. Technol*, 36(23): 5147-5154

Bouwer, E. J., B. E. Rittmann, and P. L. McCarty 1981. Anaerobic degradation of halogenated 1- and 2-carbon organic compounds. *Environ. Sci. Technol.* 15: 40-49.

Kleopfer, R.D., Easley. D. M. , Haas, B.B., Deihl, T.G., Jackson, D.E., and C.J. Wurrey. (1985). Anaerobic degradation of trichloroethylene in soil. *Environ.Sci. Technol,* 19(3): 277-280.

Vogel T. M. and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.*, 49(5): 1080-1083.

Fathepure, B. Z. and S. A. Boyd. 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* Sp. Strain Dcm. *Appl. Environ. Microbiol.* 54(12): 2976-2980.

Sewell, G. W., and S. A. Gibson. 1991. Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. *Environ. Sci. Technol.*, 25(5): 982-984

Gibson, S.A., and G.W. Sewell. 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol*, 58 (4): 1392-1393.

Fathepure, B.Z. and J.M. Tiedje. 1994. Reductive dechlorination of tetrachloroethylene by a chlorobenzoate enriched biofilm reactor. *Environ. Sci. Technology*. 28:746-752.

Maymo-Gatell, X., Tandoi, V., Gossett, J. M., and S. H. Zinder. 1995. Characterization of an H_2-utilizing enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis. *Appl. Environ. Microbiol.* 61(11): 3928-3933.

Smatlak, C. R., Gossett, J. M., and S. H. Zinder. 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ. Sci. Technol.* 30(9): 2850-2858.

Gao, J. W., Skeen, R.S., Hooker, B. S., and R. D. Quesenberry. 1997. Effects of several electron donors on tetrachloroethylene dechlorination in anaerobic soil microcosms. *Water Research.* 31(10): 2479-2486.

Carr, C. S. and J. B. Hughes. 1998. Enrichment of high rate PCE dechlorination and comparative study of lactate, methanol, and hydrogen as electron dopers to sustain

activity. *Environ. Sci. Technol.* 32(12): 1817-1824.

Nielsen, R. B. and J. D. Keasing 1999. Reductive dechlorination of chlorinated ethene DNAPLs by a culture enriched from contaminated groundwater. *Biotechnol. Bioeng.* 62 (2): 160-165.

Gerritse, J., Renard, V., Gomes, T. M. P., Lawson, P. A., Collins, M. D., and J. C. Gottschal. 1996. *Desulfitobacterium sp.* strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. *Archives of Microbiology* 165(2): 132-140.

DeBest.J. H., Jongema. H, Weijling. A, Doddema. H.J., Janssen. D. B., and W. Harder. 1997. Transformation of 1,1,1-trichloroethane in an anaerobic packed-bed reactor at various concentrations of 1,1,1-trichloroethane, acetate and sulfate. *Applied Microbiology and Biotechnology.* 48 (3): 417-423.

Gao, J. W., Skeen, R.S., Hooker, B. S., and R. D. Quesenberry. 1997. Effects of several electron donors on tetrachloroethylene dechlorination in anaerobic soil microcosms. *Water Research.* 31(10): 2479-2486.

Cabirol. Nathalie, Jacob. Francois, Perrier. Joseph, Fouillet. Bruno, and P. Chambon. 1998. Interaction between methanogenic and sulfate-reducing microorganisms during dechlorination of a high concentration of tetrachloroethylene. *Journal of General and Applied Microbiology.* 44 (4): 297-301.

Ndon, U. J., Randall, A. A., and T. Z. Khouri. 2000. Reductive dehalogenation of tetrachloroethylene by soil sulfate reducing microbes under various electron donor conditions. *Journal of Environmental Monitoring and Assessment*, 60: 329-336.

Bradley, P. M., and F. H. Chapelle. 1996. Anaerobic mineralization of vinyl chloride in Fe (III)-reducing, aquifer sediments. *Environ. Sci. Technol.* 30 (6): 2084 –2086

Bradley, P.M., and Chapelle, F.H. 1997. Kinetics of DCE and VC mineralization under methanogenic and Fe(III)-reducing conditions. *Environ. Sci. Technol.* 31:2692-2696.

Arnold, W. A.and A. L. Roberts. 2000. Pathways and kinetics of chlorinated ethylene and chlorinated acetylene reaction with Fe(0) particles. *Environ. Sci. Technol*, 34(9): 1794-1805.

Chapter Four: MATERIALS AND METHODS

4.1. Chemicals and Reagents.

All chemicals used in this thesis were ACS reagent grade or higher. The chlorinated compounds used as substrates in microcosms studies were obtained in neat liquid forms from Sigma-Aldrich (Milwaukee, WI): PCE (HPLC grade, 99.9+%); TCE (certified ACS grade); c-DCE (certified ACS grade); 1,1,1,-TCA, trans-1,2-dichloroethene (t-DCE), 1,1-dichloroethene (1,1-DCE), 1,1-dichloroethane (1,1-DCA). Analytical standards such as PCE, TCE, c-DCE, t-DEC, 1,1-DCE, 1,1,1-TCA, 1,1-DCA, 1,2-DCA, VC, and CA (chloroethane) were purchased from Supelco (Bellefonete, PA) in methanol-dissolved forms.

Gas standards including vinyl chloride (1000 ppm), methane (100%), hydrogen (100%), ethene (100 ppm), acetylene (1 %), carbon dioxide (100 %), and ethane (100%) were purchased from Scott Specialty Gases.

4.2. Description of Site and Sediments.

The study site is an aquifer located approximately 16 km south of Kalamazoo, MI (Figure 4.1). This aquifer (designated by Michigan Department of Environmental Quality as Plume G) has been contaminated with mixed volatile organic compounds (VOCs) and metal compounds due to PCE use and improper disposal of wastes by a plastic and rubber manufacturer, which impacts $1.7x10^7$ cubic yards of sediments (Mayotte, 1994). Immediately down gradient of the Plume G source, groundwater and sediments are contaminated with chromium and arsenic from a lumber preserving

operation (Plume F). Migration of these two plumes to the southeast has resulted in a zone of significant hexavalent chromium/chlorinated organic contamination. Data collected from 1988 on has revealed evidence of intrinsic reductive dechlorination that the daughter products (i.e.TCE and c-DCE), not the parent compound (PCE) prevail. The background chlorinated compounds and their levels in plume G site are summarized in Table 4.1.

Table 4.1. Typical Contaminants found from The Study Area[a]

Contaminants	Concentration (μg/L)
TCE	1015
1,1,1-TCA	232
c-DCE	1518
1,1-DCA	249
VC	19
Chromium	70
Arsenic	152[b]

[a]VOC concentrations from location MPS-5. No PCE was detected in MPS-5 vicinity.
[b]Maximum concentration detected in MDEQ monitoring wells. No significant arsenic is present in MPS-5 vicinity

The Plume G aquifer is comprised of relatively homogeneous glacial outwash sands. This glacial outwash consisted of mostly coarse to medium sand with two gravel and cobble layers located just above the confining clay layer (depth 80-83 ft). Sulfate, nitrate, dissolved iron concentrations and total organic carbon analysis indicated that the

site groundwater was predominantly anaerobic and low in electron donors (Table 4.2). The mean annual temperature of this plume is about 12°C. The sediment used in this study was collected from the mixed waste impacted section of the plume G site, depth between 50 ft and 75 ft, which were mainly composed of fine to medium sand. Batch experiments were designed to imitate field environment (temperature, electron acceptor levels, and chloroethene concentrations) as much as possible.

Table 4.2. Typical Chemical Composition of G plume site[a]

Parameter	Value	Parameter	Value
Dissolved iron (aqueous)	12-20 mg/L	Nitrate	45 mg/L
Total iron	2.5-8.2 mg/g	Sulfate	38 mg/L
pH	7.2	Total Organic Carbon	< 0.04 %[b]

[a]Anion concentrations from groundwater (MW-MSU1). Iron concentrations from core sediment samples.
[b]Provided by Xianda Zhao.

4.3. Microcosms

All experiments were conducted either in 250–ml serum bottles containing 200 ml of groundwater or 40-ml vials containing 35 ml of anaerobically collected groundwater from the same aquifer location (MW-MSU1) at 65 ft below groundwater surface (bgs). Bottles were sealed with Mini-inert® (teflon) valves or sealed with screw cap containing 22-mm teflon-lined butyl rubber septa. Groundwater was supplemented to create a sulfate reducing media as described in Atlas (2). The detailed description of the amended media is shown in Table 4.3. The groundwater medium was kept under O_2 free N_2-CO_2 (80:20) prior to addition of $NaHCO_3$ and vitamin solutions. The vitamin solutions were filter

sterilized. The pH of the medium was adjusted to 7.3 to 7.5 with a sterile HCl or Na_2CO_3 solution. Approximately 20g of soil with large particles removed by sieving with 3.35 mm screen were added to 250-ml bottle. Abiotic (pasteurized) controls were included in all experiments. For abiotic controls, the medium and sediments were pasteurized for 8 hours in 70°C water bath and cooled to room temperature under O_2 free N_2-CO_2 (80:20) prior to addition of $NaHCO_3$ and vitamin solutions. Sorption controls were added to account for potential partitioning associated with large-chain hydrophobic carbon sources such as CMB and palmitate.

The pH of cultures was routinely checked with a pH meter and probe (Orion). A sub-sample of the culture was removed and pH was determined both before and after adjustment additions. The pH probe was calibrated before each use (Fisher Scientific).

Table 4.3. Basal Medium for Microcosm[a]

Component		Trace metal solution		Vitamin solution	
Na_2SO_4	48 mg	$FeCl_2 \cdot 4H_2O$	1.5 g	Thiamin	0.01 g
NH_4Cl	0.16 g	$CoCl_2 \cdot 6H_2O$	0.12 g	p-Aminobenzoic acid	5 mg
KH_2PO_4	28 mg	$MnCl_2 \cdot 4H_2O$	0.1 g		
Resazurin	10 mg	$ZnCl_2$	0.07 g	Vitamin B_{12}	5 mg
$NaHCO_3$	5.0 g	H_3BO_3	0.06 g	Biotin	1 mg
Trace metal solution	1 mL	$Na_2MoO_4 \cdot 2H_2O$	0.025 g	Groundwater	100 mL
Vitamin solution	1 mL	$NiCl_2 \cdot 6H_2O$	0.025 g		
Groundwater	1000 mL	$CuCl_2 \cdot 2H_2O$	0.015 g		
		HCl (25 %)	6.5 mL		
		Groundwater	1000 mL		

[a] Modified from Atlas (1993)

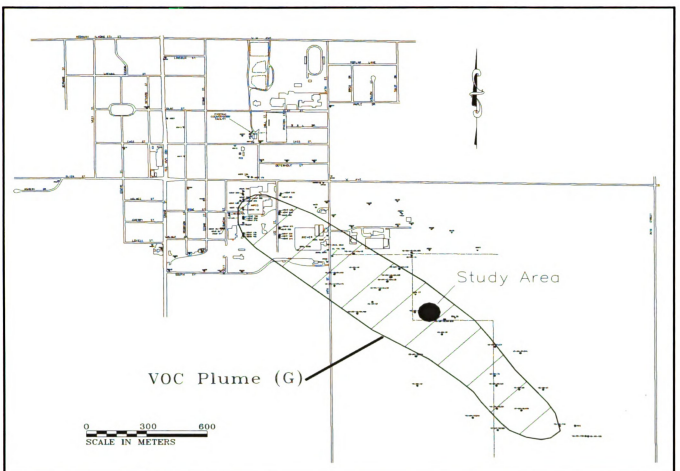


Figure 4.1. Location of study area. (obtained from Graulau-Santiago. 2004)

4.4. Analysis of Volatile Organics

4.4.1. Acetylene, Methane, Ethane, Ethene, and CO_2

Concentrations of acetylene, ethane, ethene, and methane were routinely determined by gas chromatographic (GC) analysis utilizing PerkinElmer (Autosystem) equipped with a flame-ionization detector and a 1 % SP-1000 on 60/80 Carbopack-B (3.2 mm x 2.44 mm) column from Supelco. Standards and identification were accomplished by matching the retention times of certified gas standards with peaks detected in headspace samples. The oven program was as follows: 35 °C, hold 2 min, 35 °C/min to 180 °C, no hold, flow rate was at 20 ml/min. The detector and injection port temperatures were 200°C. Standards were prepared in serum bottles closed with aluminum crimp cats and teflon lined butyl rubber stoppers. The detection limit of ethene was 65 nmol/L.

In case of high concentrations of methane and CO_2, these were quantified by direct injection of headspace samples (500 μL) into GC equipped with a thermal conductivity detector (TCD) and a molecular sieve 5A column (15 ft x 1/8 in. stainless steel 60/80 Carboxen-1000: Supelco) using helium (30 ml/min) as carrier gas. The GC oven was operated isothermally at 200°C. Standards were prepared by volumetric dilutions of certified gas standards.

4.4.2. Chlorinated compound

Liquid samples (0.5 mL) were collected with a gas-tight syringe and immediately dispensed to 40 mL EPA/VOA vials (0.125" TFE/Silicone Liner: I-Chem), which contains 4.5 mL of 2 % sodium bisulfate as a preservative. Vials were sealed immediately with

Teflon lined septa and kept in the refrigerator until final analysis. All samples were analyzed within a 14-day holding period.

Identification and quantification of PCE, TCE, c-DCE, t-DCE, 1,1-DCE, and VC was made by gas chromatography/mass spectrometry (Agilent 5973 Mass Selective Detector) combined with a purge-and-trap system (Tekmar/Dohrmann Precept II and Tekmar/Dohrmann 3100 Sample Concentrator). GC calibration was performed by adding known masses of PCE, TCE, c-DCE, trans-DCE, 1,1-DCE, and VC directly to a 40-mL EPA/VOA vial containing 5-ml chilled distilled deionized water. Quantification of chlorinated ethenes and ethene was corrected for partitioning between the aqueous and gas phases. Dimensionless Henry's Gas Law constants were obtained from Gossett (1987) except for gases. These values are as follows: TCE, 0.392; c-DCE, 0.154; VC, 1.077; ethane, 20.4 (Schwarzenbach *et al.* 1993); ethylene, 8.5 (Yaws, 1999); methane, 33.1 (Freedman & Gossett, 1989), and acetylene, 0.887 (Butler & Kim, 1999). Throughout this thesis, all reported aqueous and gas-phase concentrations have been corrected for partitioning between aqueous and gas phase.

4.5. Analysis of Volatile Fatty Acids

Liquid samples were dispensed into Eppendolfs and clarified by centrifuge at 18, 000 rpm for 5 minutes. The supernatant was the acidified with 50 µl of 1.25 N H_2SO_4, and dispensed into a 1.5 ml glass vial (Sun Microsystems). The samples were kept frozen until analysis. Acetic, propionic, and lactic acids were measured by high performance liquid chromatography using a Supelco Discovery C8® column (ultraviolet absorbance at 210 nm) and the flow rate was 0.6 mL/min. Acetonitrile was used to regenerate the column between

samples and 3.4 g/L of KH_2PO_4 was used as an eluent. Chromatograms were recorded and data integrated using Turbochrom R 4 software (Perkin Elmer Corp.).

4.6. Anion Analysis

Anions were assayed by ion chromatography with suppressed conductivity detection on a Dionex model 2000i/SP ion chromatography equipped with a Dionex AS4A IonPac column and utilizing a 1.8 mM biocarbonate-1.7 mM carbonate mobile phase (3mL/min). Chromatograms were recorded and data integrated using Turbochrom R 4 software (Perkin Elmer Corp.). Five–point calibration curves were prepared by diluting primary anion standards into secondary deionized water standards. 1 mL liquid samples were taken from bottles and filtered through a 0.22 μm nylon filter (Scientific Resources Inc.). 120 microliter of filtered samples were diluted with 480 μl of deionized water, and dispensed into a polypropylene sample vial (Alcott Chromatography). Samples were stored at -20 °C until analysis was performed. Each sample was analyzed for acetate, bromide, chloride, nitrate, nitrite, phosphate, and sulfate.

4.7. Hydrogen Analysis

Hydrogen levels were monitored by injecting 0.5 mL of headspace gas into a mercury vapor reduction gas analyzer (RGA2 Reduction Gas Analyzer;Trace Analytical, Menlo Park, CA). Data was collected with Turbochrom R4 software. Hydrogen partial pressures were measured in the headspaces of bottles at room temperature. The detection limit for hydrogen in headspace was 10 ppb (0.4 nM).

Standards were prepared in serum bottles purged with N_2 and capped with butyl septa prior to adding H_2. Hydrogen values are expressed in parts per million by volume (1 ppmv = 0.1 pa = 10^{-6} atm) according to the convention adapted by Conrad (1996). Since most of the values published in other studies are expressed as hydrogen dissolved in aqueous, dissolved H_2 concentrations were calculated by using the following equations:

$$H_s(dissolved) = \frac{LP}{RT} \qquad (4.1)$$

Where, H_2 (dissolved) is the aqueous concentration in moles per liter; L is the Ostwald coefficient for H_2 solubility, which is 0.01913 at 25°C (Wilhelm *et al.* 1977); R is the universal gas constant (0.0821 liter atm $K^{-1}mol^{-1}$); P is the absolute pressure (in atmospheres); and T is the Kelvin temperature (Lovley *et al.* 1994). Therefore, 1 ppmv = ca. 0.78 nM at 25 °C.

4.8. Hydrogen Sulfide Analysis

Hydrogen sulfide was colormetrically measured using sulfide kit (Chemtrics, Inc.) based on Methylene blue method.

4.9. Data Reduction

Transformation of chlorinated compounds was decided using first-order kinetics. First-order rate constant k can be obtained by plotting natural logarithm of measured aqueous concentrations of chlorinated compound as a function of time. Evidence for fit of

the data to such a rate law includes the good agreement between experimental data and rate laws. First order reaction rate (r) in the liquid phase:

$$r = (dC_{aq} / dt) = -k_{obs} C_{aq} \qquad (4.2)$$

Due to the existence of headspace in batch systems, a fraction of the chlorinated ethenes partitioned to the headspace and was effectively withdrawn from the reaction phase (aqueous phase). As a result, the measured rates of transformation can be impeded. The inherent rates of transformation occurring in aqueous phase can be determined from measured rates using the volumes of the gaseous and aqueous phases, assuming equilibrium partitioning occurring between these phases at all times. The inherent rate is the product of the measured rate, corrected by factor *f* (Burris *et al.* 1996)

$$f = 1 + H \frac{V_g}{V_{aq}} \qquad (4.3)$$

Where H is the dimensionless Henry's law constant ($molL^{-1}_{gas}$ / $mol\ L^{-1}_{aqueous}$), and V_g and V_{aq} are the gas and aqueous volume respectively. Values of k were corrected to account for the effects of partitioning of the reactant between the aqueous and gas phases ($k_{obs} = f * k$).

4.10. DNA Analysis

4.10.1. DNA Extraction and RTm-PCR

Microcosm DNA samples were extracted from vigorously mixed suspended sediment slurries (3 ml). After collection, sediment slurries were centrifuged (10,000 rpm) for 6 minutes to remove supernatant and DNA was extracted from sediment pellets using DNA extraction kit (MO BIO Laboratories. Inc. Solana Beach, CA.) following manufacturer's guidelines.

Total DNA quantification was accomplished by detecting and quantifying double-stranded DNA (dsDNA) in sediment using the PicoGreen® method (Molecular Probes co. Eugene, OR). PicoGreen® quantification reagent is an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solutions. Quantification assays were performed using 1-10 % dilutions of sample extracts resuspended in 50 µL of 10 mM TRIS-HCl [pH 7.5]. The samples were excited at 485 nm and the fluoresecence emission intensity was measured at 538 nm using a spectroflurometer. Fluoresecence emission intensity was then plotted versus DNA concentrations. Sample quantitations were accomplished by using MFX Microliter Plate Flourometer and integration software.

Real-time PCR (TaqMan-PCR) was used to quantify *Dehalococcoides* populations and conducted by following the outlines by Gruntzig, et al. (Gruntzvig *et al.* 2001). PCR amplification was performed in 50 µl reaction volumes. The reaction mixture for real-time PCR consisted of 1X TagMan Universal Master Mix (containing AmpliTaq Gold DNA polymerase, AmpErase Uracil-N-glycosylase, which degrades PCR carryover products from previous reactions, deoxynucleoside triphosphates with

dUTP, a passive reference [6 carboxy-X-rhodamine], and optimized buffer components) (PE Applied Biosystems), 50 nM forward primer and 50 nM reverse primer. MicroAmp optical caps and tubes were used for the final reactions. The 16S rRNA gene forward and reverse primers were 5' CTGGAGCTAATCCCCAAAGCT 3'; 5' CAACTTCATGCAGGCGGG 3'(He *et al.* 2002)'. PCR conditions were as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, then 40 cycles of 15 seconds at 95 °C and 1 minutes at 60 °C. Negative controls with no template DNA or no probe were run in each reaction.

The increase in fluorescence emission, due to the degradation of the probe by the DNA polymerase in each elongation step, was monitored during PCR amplification using the 7700 Sequence Detector (PE Applied Biosystems). The fluorescence signal was normalized by dividing the emission of the reported dye (6-carboxyflourscene) by the emission of the passive reference dye, 6-carboxy-X-rhodamine. The parameter threshold cycle (T_C) is the fractional cycle number at which the fluorescence emission crosses an arbitrarily defined threshold within the logarithmic increase phase (0.1 in our reactions). The higher the amount of initial template DNA, the earlier the fluorescence will cross the threshold and the smaller will be T_C. The TC values obtained for each sample were compared with a standard curve to determine the initial copy number of the target gene.

4.10.2. T-RFLP analysis

T-RFLP analysis was performed as previously described by Liu, *et al.* (1997). The amplification was performed using the pair of universal bacterial primers, 8F [5' AGAGTTTGATCCTGGCTCAG 3'] labeled with hexachlorofluorescene (Hex) at the 5'

end (synthesized by Operon Technologies, Inc., Alameda, CA). PCR mixtures included Taq DNA Polymerase (Gibco BRL, Gaithersburg, MD) and were done according to manufacturer's recommendations with the addition of 0.2 mg/ml of bovine serum albumin (Sigma Chemical Co., St. Louis, MO). PCR amplification was conducted in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The protocol consisted of an initial denaturnation step at 94 °C for 30 sec, annulling at 57 °C for 45 sec, elongation at 72 °C for 1 min 30 sec and one extension cycle at 72 °C for 7 min to finalize the PCR. Negative controls included tubes that received no template DNA, as well as positive controls containing pure culture genomic DNA. Aliquots (10 µl) of the PCR products were separated by electrophoresis in a 1.5 % agarose gel using 1 X TAE buffer. The gel was stained with ethidium bromide (500 ng/ µl) and visualized by ultraviolet excitation (Helton, 2000).

Amplified PCR products were purified using the Wizard PCR purification kit (Promega, Madison, WI) and separately digested with the restriction endonucleases *HhaI* overnight at 37 °C. Resulting fragments were resolved on an ABI 373A sequencer in a 6 % urea-containing polyacrylamide gel (PE Applied Biosystems Sequencer), running in gene scan mode. The resulting electropherograms were analyzed for similarities using GeneScan software version 3.1 (PE Applied Biosystems).

4.11. Literature cited

Mayotte, Timothy. J. 1994. Field Trips Guidebook. The geological society of America. North Central Section.

Ronald, M. Atlas. 1993. Handbook of Microbiological Media. CRC Press, Florida

Jaime A. Graulau-Santiago. 2003. Development and Application of a Methodology to Evaluate Natural Attenuation for Chlorinated Solvents using Conceptual and Numerical Models. A Doctoral Thesis. Civil and Environmental Engineering. Michigan State University.

Gossett, J. M. 1987. Measurement of Henrys Law Constants For C1 And C2 Chlorinated Hydrocarbons. *Environ. Sci. Technol.* 21(2): 202-208.

Schwarzenbach R.P., P.M. Gschwend, and D.M. Imboden. 1993. Environmental Organic Chemistry. Wiley and Sons, Inc.

Yaws, C.L. 1999. Chemical Properties Handbook. McGraw-Hill.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. . *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Butler, E., and Kim F. Hayes. 1999. Kinetics of the transformation of trichloroethene and tetrachloroethene by Iron Sulfide. *Environ. Sci. Technol.* 33: 2021-2027

Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO_2, CH_4, OCS, N_2O, and NO). *Microbiol. Rev.* 60: 609-640

Wilhelm, E., R. Battino, and R.J. Wilcock. 1977. Low pressure solubility of gases in liquid water. *Chem. Rev.* 77:219-262

Lovley, D.R., F.H.Chapelle, and J.C. Woodward. 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anaerobic groundwater. *Environ. Sci. Technol.* 28: 1205-1210

Burris, D.R., C.A. Delcomyn, M.H. Smith, and A.L. Roberts. 1996. Reductive dechlorination of tetrachloroethylene and trichloroethylene catalyzed by vitamin B-12 in homogeneous and heterogeneous systems. *Environ. Sci. Technol.* 30:3047-3052.

Gruntzvig, V., Nold, S.C., Zhou, J., and J.M. Tiedje. 2001. *Pseudomonas stutzeri* Nitrite Reductase gene abundance in environmental samples measured by RT-PCR. *Appl. Environ. Microbiol.* 67, 760-768.

He, J., Sung, Y., Dollhopf, M.E., Fathepure, B.Z., Tiedje, J. M., and F.E. Loffler. 2002. Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environ. Sci. Technol.* 36, 3945-3952.

Liu, W., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of microbial diversity by determining Terminal Restriction Fragment Length Polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 63:4516-4522

Helton, Rebekah R. 2000. Assessment of the microbial communities derived from two tetrachloroethene contaminated aquifers. M.S. Thesis. Michigan State University.

Chapter Five: EVALUATION OF ELECTRON DONOR EFFECTS ON THE TRANSFORMATION OF TCE AND c-DCE USING ANAEROBIC SEDIMENTS

5.1. Abstract

Anaerobic microcosms prepared with sediment and groundwater from an aquifer contaminated with mixed chlorinated compounds (perchloroethene (PCE), 1,1,1-trichloethane (1,1,1-TCA), and daughter products (c-DCE and vinyl chloride)), and chromium were used to investigate the effects of electron donors on dechlorination. Screened substrates were ethanol, methanol, lactate, palmitate, propionate, and commercial food grade palmitate (Crisco®) delivered via Microporous Beads (CMB). Substrates were added at levels calculated to stoichiometrically reduce all available anions in the system. Hydrogen levels were ranged from 0.3 to 1.1 mM in biotic bottles. But no clear relationship was observed between extent of dechlorination and hydrogen levels. In microcosms spiked with TCE, the greatest transformation was observed with CMB (44 %) or lactate (36 %) as carbon sources. For all electron donors tested, the predominant biotransformation daughter product was c-DCE, with little further dechlorination beyond c-DCE over a 240-day period. Productions of acetylene have indicated that abiotic degradation by iron sulfide also participated in dechlorination of TCE.

5.2. Introduction

Chlorinated volatile organic compounds (VOCs) are the most frequently occurring type of contaminant in groundwater at Superfund sites in US (EPA, 1994). It has been estimated by EPA that cleanup of these sites will cost more than $45 billion (EPA, 2000). Biological remediation of these compounds offers the potential for economical and destructive removal of these hazardous compounds from contaminated aquifers. Biological treatments may be passive (natural attenuation) or engineered. Naturally occurring biological activity often leads to incomplete or slow attenuation or incomplete degradation of contaminants. This is generally due to a deficiency in an electron donor or acceptor, other nutrients required for the dechlorinating microbial population, and/or absence of appropriate dechlorinating microbial populations. In these cases, biostimulation or bioaugmentation may be successful. Biostimulation can be a less complicated and costly alternative compared to bioaugmentation, if the appropriate population(s) are present.

Many substrates have been shown to stimulate dechlorination of chlorinated solvents (Gibson *et al.* 1994; Gibson and Sewell, 1992; Gao *et al.* 1997). These are generally organic acids and alcohols. Four fatty acids (lactate, palmitate, propionate, and CMB) and two alcohols (methanol and ethanol) were used as electron donors to support dechlorination in this study. Palmitate and CMB were evaluated as potential slow degrading/persistent electron donors for dechlorination. Propionate is a common fermentation intermediate that could be produced during anaerobic metabolism. Alcohols such as ethanol and methanol were used as easily degradable substrates, and

methanol has been often used to support methanogens (Fennell *et al.* 1997; Carr and Hughes, 1998; Fathepure and Boyd, 1988 (a)(b)). Furthermore, fermentation of these substrates is known to produce different levels of hydrogen (Fennell *et al.* 1997; Carr and Hughes, 1998). Ethanol and lactate are known to generate relatively high hydrogen levels, whereas propionate is known to generate lower hydrogen levels. Since differences in dechlorination might be due to the possible competition between dechlorinators and other hydrogen-utilizing microorganisms for hydrogen, hydrogen production was monitored.

Much of the work on biodegradation of VOCs has evaluated degradation under methanogenic conditions (Fathepure and Boyd, 1988 (a)(b); Freedman and Gossett, 1989). Fewer studies have been conducted on the transformation of chlorinated compounds under sulfate reducing conditions (Pavlostathis and Zhuang, 1991) in spite of the dechlorinating potential of sulfate-reducing bacteria and the relative abundance of sulfate in subsurface environments.

FeS has been identified as a soil precipitate in anaerobic environments where it is formed through the biologically mediated reduction of sulfate to sulfide and subsequent reaction of sulfide with available iron species (Richard, 1969; Freney, 1979). Several forms of ferrous sulfide can be produced; amorphous iron sulfide, mackinawite ($Fe_{0.995-1.023}S$), greigite (Fe_3S_4), and pyrrohotitie ($FeS_{1.1}$). Formation of amorphous iron sulfide predominates at low pH, while pyrite (FeS_2) can be developed by further reaction with elemental sulfur.

Butler and Hayes (1999) have demonstrated that ferric sulfides and ferric disulfides such as mackinwite and pyrite can promote the abiotic dechlorination of

chloroethenes. Trichloroethene was transformed to 65% acetylene, 6% c-DCE, and 9% residual TCE (at pH 8.3) after 120 days. Lee and Batchelor (2002) reported that pyrite degraded TCE to 3.3% c-DCE, 43% acetylene, 2.2% ethene, and 50% residual TCE after 32 days. However, reports by Hassan *et al.* (1995) and Doong and Wu (1992) indicated that FeS is unreactive in the transformation of chlorinated compounds. Discrepancies in experimental conditions among these different studies may be responsible for these disparate results.

The purpose of the studies reported here were to screen for the biostimulation potential of the native site microbes when augmented with different electron donors. I sought to demonstrate that by adjusting the levels and types of substrates, I could poise the systems reducing potential above methanogenesis and thus selectively influence which indigenous populations were enriched. I also attempted to evaluate the production and reactivity of FeS since it is naturally occurring in sulfate-reducing environments and known to be involved in dechlorination of chlorinated compounds (Butler and Hayes, 1999; Lee and Batchelor, 2002; Hassan *et al.* 1995; Doong and Wu, 1992). Here I report on comparative studies in TCE/c-DCE degradation by both biostimulation and biogenic iron sulfide.

5.3. Materials and Methods

All experiments were conducted in 250–ml serum bottles containing 200 ml of groundwater anaerobically collected from the same aquifer location (MW-MSU1) at 65 ft below groundwater. The bottles were sealed with mini-inert® teflon valves, and incubated in the dark, at aquifer temperature (15°C) and inverted to minimize volatile loss. The bottles were shaken periodically (twice a week). Reagent-grade chemicals were used in nutrient medium preparation. Groundwater was supplemented to create a sulfate reducing media as described in previously in Chapter 4. In order to study the effect of reductant (Na_2S) on dechlorination of TCE and c-DCE, some bottles were spiked with 0.15 or 1.5 mM of sodium sulfide. Not only abiotic (pasteurized) controls, but also sorption controls were added to account for potential partitioning associated with hydrophobic carbon sources in all experiments. To prevent the formation of siderite ($FeCO_3$), and mimic in-situ conditions, the pH was maintained between 7.0-7.5 by addition of NaOH.

The concentrations of TCE and c-DCE in the bottles were 15 μM and 20 μM, respectively. To study the effect of different substrates in dechlorination, soluble nutrients including ethanol, 2 mM; lactate, 2 mM; methanol, 4.25 mM; palmitate, 0.25 mM; propionate, 1.5 mM; and slow release nutrients (CMB, 400 mg/L each) were added, respectively. CMB carbon beads were prepared by loading molten Crisco® into clay hydrophobic plant growth media. Substrate concentrations were calculated using McCarty's stoichiometric approach at standard conditions to poise the reducing potential to sulfate reducing environments to prevent methanogensis due to excess carbon (See Appendix A). The equations for the heterotrophic sulfate reducing of various electron

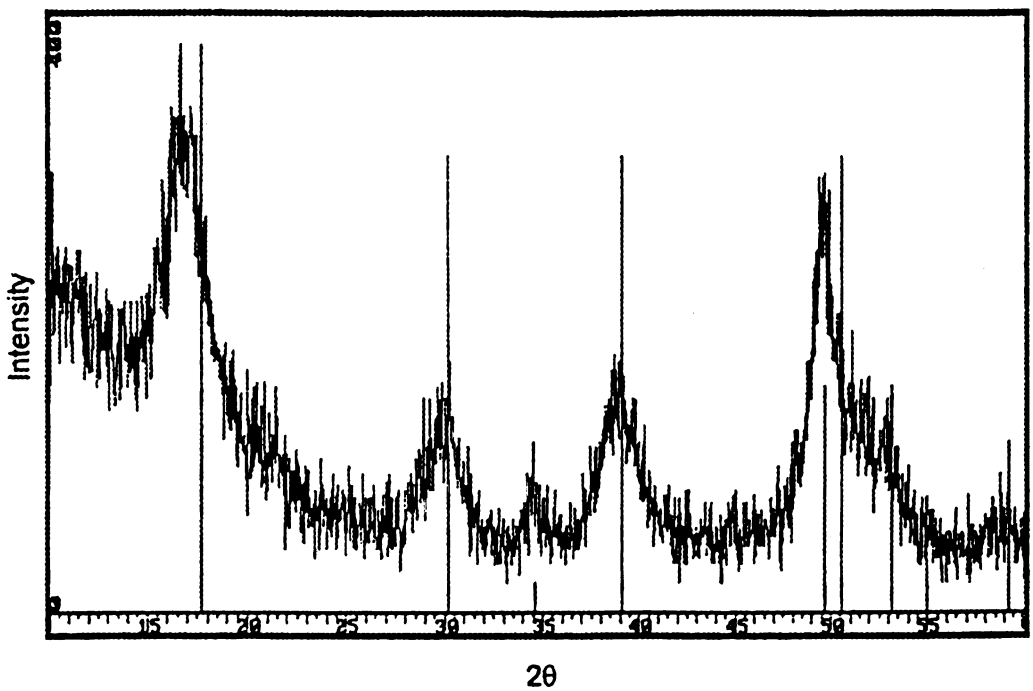
donors were calculated using cell efficiency of energy transfer as 0.6 and ammonia as N-source (McCarty, 1969).

The identification of iron sulfide's crystalline structure produced in the microcosms was made by powder x-ray diffraction (XRD) analysis (McCormick *et al.* 2002). Figure 5.1 shows a X-ray diffraction pattern for the experimentally-prepared FeS match with the mackinawite (The mackinawite power diffraction pattern is from the Powder Diffraction File, International Center for Diffraction Data, Swarthmore, PA). Poorly-crystalline mackinawite and amorphose FeS are found in natural systems (Butler, 1998). X-ray diffraction analyses were conducted using a Rigaku Rotaflex rotating anode XRD apparatus (Cu Kα radiation, 40 kV, 100 mA). XRD samples were prepared by freeze-drying and back-filling under nitrogen to potential oxidation. Samples were mounted on glass slides in the anaerobic glovebox and then sealed under tape to prevent sample oxidation during analysis.

Headspace samples (acetylene, ethane, ethene, and methane) were determined by gas chromatographic (GC) analysis (PerkinElmer) as described in Chapter 4.

Figure 5.1. Powder X-ray diffraction pattern by experimentally prepared FeS.

(The mackinawite power diffraction pattern is from the Powder Diffraction File, International Center for Diffraction Data, Swarthmore, PA).



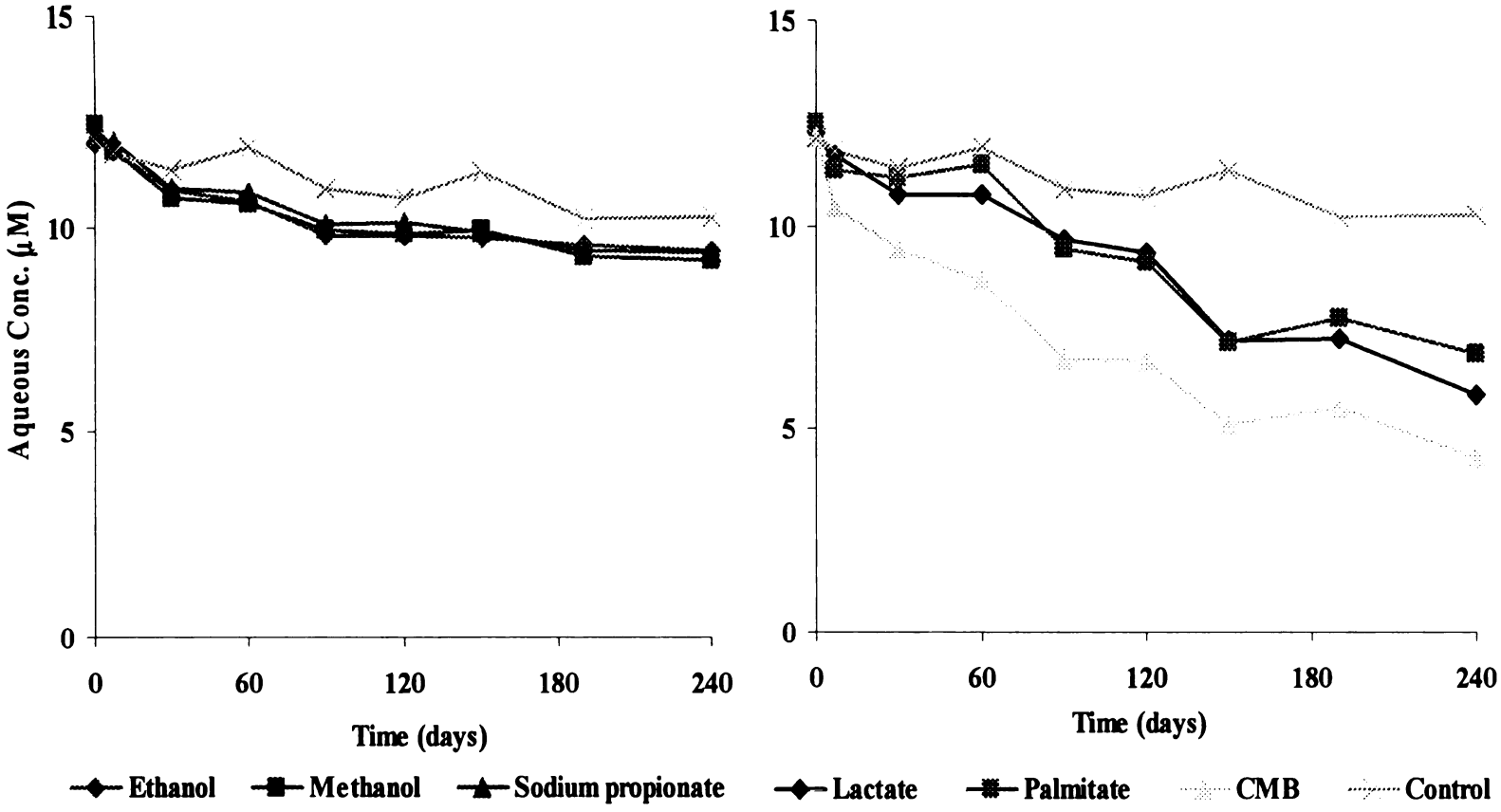
5.4. Results

5.4.1. Effect of carbon sources on transformation of c-DCE and TCE

1. TCE transformation

In order to determine which substrates support enhanced dechlorination activity, multiple carbon sources were screened, including both alcohols and fatty acids. Long-term incubation cultures were provided with carbon sources including methanol, ethanol, lactate, palmitate, CMB, and propionate at the beginning of incubation only. Within a week of nutrient addition, nitrate presented in the site groundwater (average concentration of 35 ppm) was consumed. All biotic bottles blackened within two weeks and the presence of sulfide was confirmed by Chemetrics kit (methylene blue method). Anion levels, turbidity, and color remained unchanged in pasteurized abiotic controls throughout the 240-day period.

All bioactive (non-control) microcosms exhibited some dechlorination of TCE over a 240-day period. The activity generally initiated after approximately 60 days of incubation, however the onset of degradation and 240 day removal amounts varied among replicates. Lactate, CMB, and palmitate were effective in stimulating TCE degradation (Figure 5.2). Propionate, methanol, and ethanol stimulated slight dechlorination of TCE. The dominant byproduct of TCE degradation was c-DCE, but small amount of acetylene was detected in most replicates. The percent removal of TCE by fatty acids and their sorption controls are shown in Figure 5.3. Sorption controls are included for large molecular weight (C-16) carbon sources to accounts for partitions. After 240 days of incubation, CMB and lactate showed the most significant removal (about 44 % and 36 % removal respectively, corrected for partition). An approximately 15 % decrease in TCE



(a) Slow

(b) Rapid

Figure 5.2. Differences in (a) slow vs. (b) rapid TCE transformation due to electron donors during 240 days.

level was observed in the CMB specific abiotic control bottles, most likely due to partitioning to the hydrophobic Crisco® in the CMB beads (Figure 5.2). This indicated the removal in the CMB treated microcosms were not due to partitioning. However, without additional amendments, c-DCE appeared to resist further dechlorination under the conditions of this study.

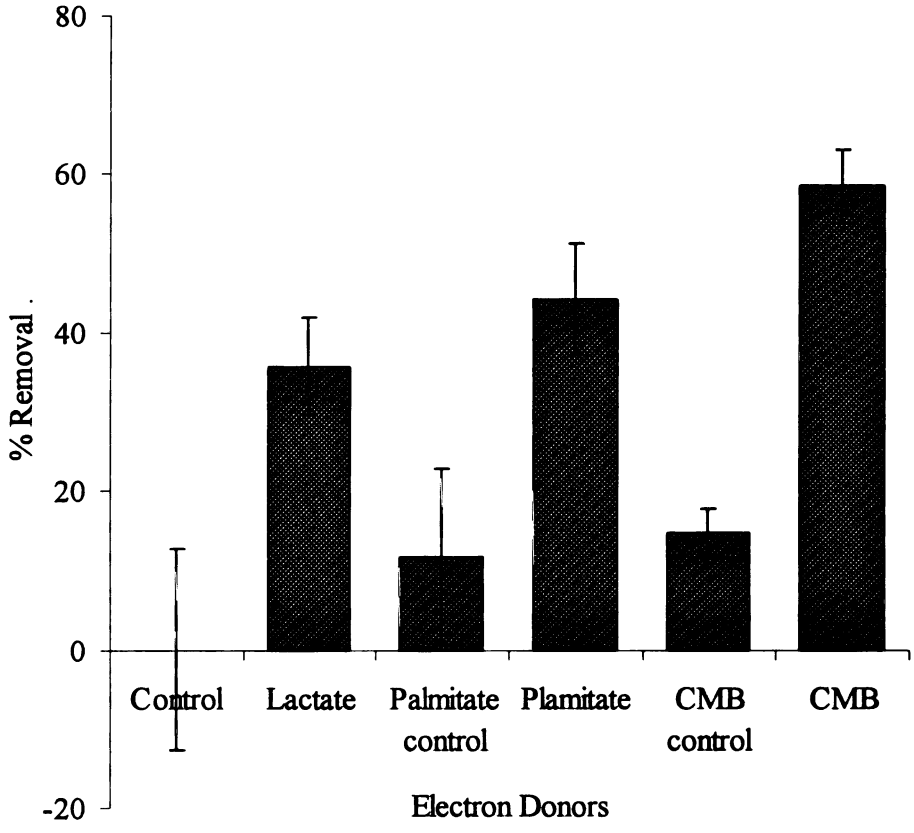


Figure 5.3. The differences in dechlorination of TCE after 240 days expressed as percentage removal of abiotic soil control. This experiment was conducted in the presence of Na_2S (1.5 mM). Sorption controls are included for large molecular weight (C-16) carbon sources. Values are averages of 4 replicates and the error bars represent the standard deviation.

2. Cis-DCE transformation

Unlike TCE, only slight degradation of c-DCE was observed over a 240-day period. Palmitate and CMB amended microcosms demonstrated some degradation of c-DCE (Figure 5.4). For c-DCE-amended microcosms, minimal degradation daughter products were detected. As can be seen on Table 5.2, about 25 % and 12 % degradation of the initial c-DCE were observed in the CMB and CMB specific abiotic control bottles, respectively, after 240 days of incubation. This again indicated partitioning did not account for c-DCE removal in the CMB fed microcosms.

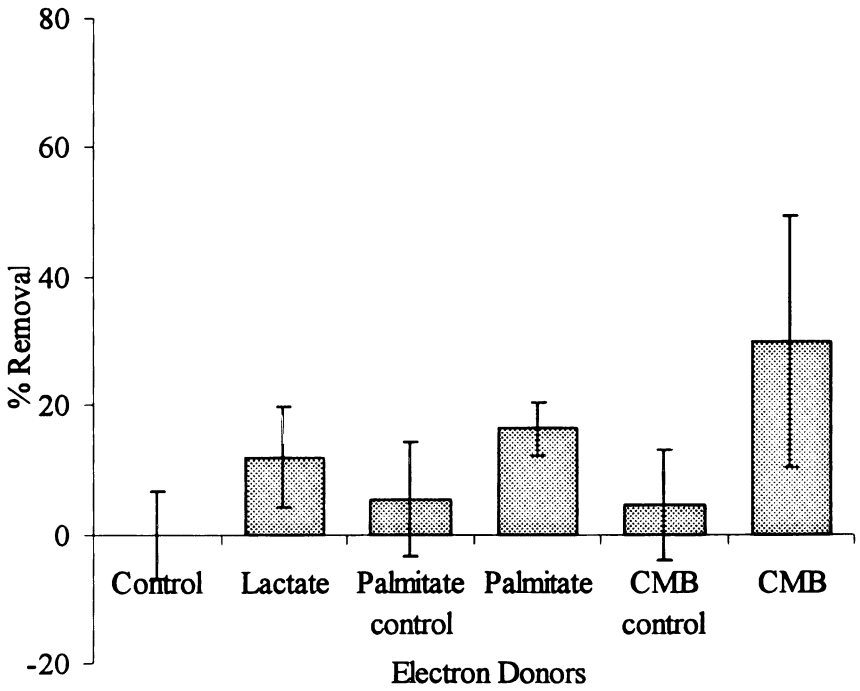


Figure 5.4. The differences in dechlorination of c-DCE after 240 days expressed as percent removal of abiotic soil control. This experiment was conducted in the presence of Na_2S (1.5 mM). Sorption controls are included for large molecular weight (C-16) carbon sources. Values are averages of 4 replicates and the error bars represent the standard deviation.

5.4.2. Oxidation-Reducing Potential and pH

The pH of the bottles dropped slowly during the course of incubation and remained stable around 6.8, while the oxidation-reduction potential (ORP) changed rapidly at first (from an initial value of -102 mV to about -230 mV on day 30), then remained relatively constant. The transformation of chloroethenes, pH/ORP final values after 240 days of incubation, and hydrogen production after 25 days of incubation at 15°C was summarized in Table 5.1.

Table 5.1. Effect of substrates on transformation of chloroethenes[a] after 240 days of incubation in sulfate-reducing conditions[b].

Reactant	Electron donors	pH/ORP	Hydrogen (mM)	Chlorinate ethene Reduction (as %)[c]
TCE	Controls	7.2/-138	0	8.6 ± 2.6
	No nutrient	7.2/-189	0	12.8 ± 2.6
	CMB	6.8/-206	0.4 ± 0.03	43.7 ± 4.7
	Ethanol	7.2/-168	0.4 ± 0.12	14.2 ± 3.3
	Lactate	6.9/-192	0.3 ± 0.07	35.7 ± 4.1
	Methanol	7.1/-211	1.1 ± 0.18	17.0 ± 3.0
	Palmitate	6.9/-182	0.3 ± 0.09	32.5 ± 2.9
	Propionate	7.0/-202	0.5 ± 0.03	14.1 ± 3.6
c-DCE	Controls	7.1/-123	0	12.1 ± 2.7
	No nutrients	7.2/-147	0	11.6 ± 2.7
	CMB	6.8/-248	0.5 ± 0.08	25.3 ± 5.6
	Ethanol	7.1/-180	0.6 ± 0.12	9.1 ± 2.8
	Lactate	6.9/-217	0.4 ± 0.09	11.8 ± 3.8
	Methanol	7.0/-199	1.0 ± 0.28	13.0 ± 4.4
	Palmitate	7.0/-192	0.4 ± 0.09	10.9 ± 3.2
	Propionate	7.0/-185	0.5 ± 0.01	13.2 ± 3.5

[a] Values represent average of triplicate. ± indicates standard deviation
[b] All experiments are done in the presence of reductant Na_2S of 1.5 mM; Methane levels were below 0.1 μM at the end of the incubation; At end of incubation H_2 levels were below 0.01 mM.
[c] Based on initial moles of chloroethenes individually.

5.4.3. Anion Utilization

Within a week of carbon addition, complete removal of available nitrate/nitrite was observed. When Na_2S was not added as a reductant, all sulfates present in the groundwater (65 to 92 ppm) were removed within 12 days (Figure 5.5). In order to see the effect of sulfate on TCE transformation, a similar amount of sulfate was respiked on 110-th day for CMB fed microcosms (Figure 5.6). Even though complete removal of sulfate occurred again, no significant enhancement on TCE transformation was observed during this period.

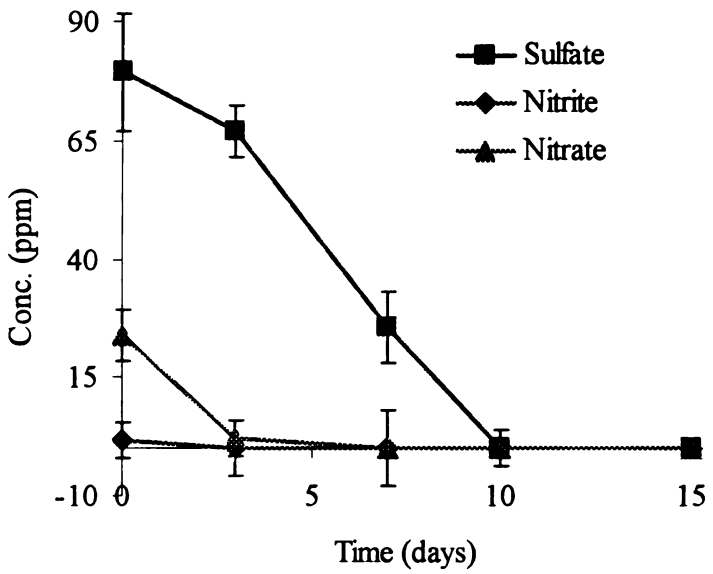


Figure 5.5. Anion degradation in CMB fed microcosm. Values represent average of triplicate. The error bars indicate standard deviation

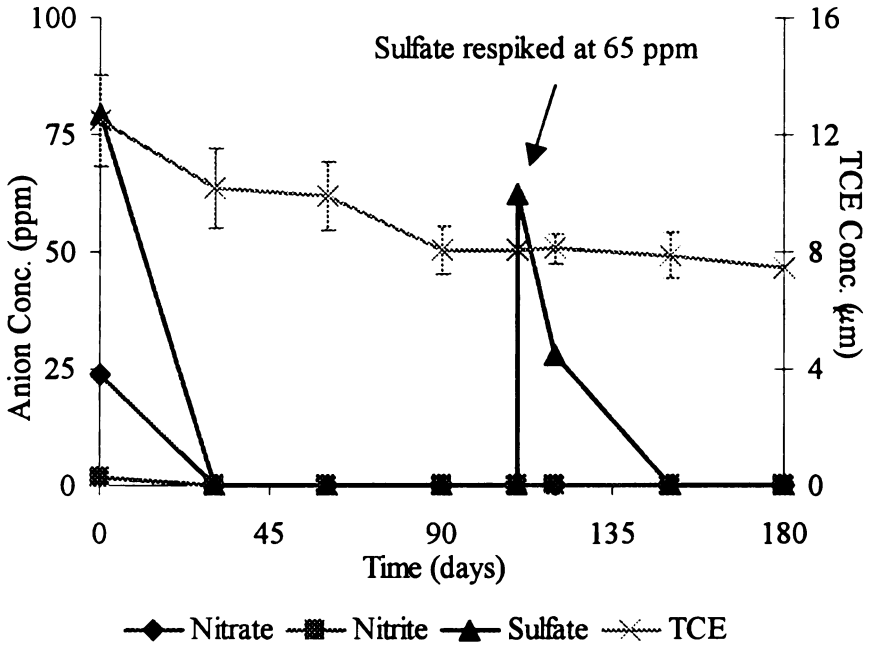


Figure 5.6. Effect of sulfate on transformation of TCE in CMB fed microcosm.

5.4.4. Hydrogen production

High levels of hydrogen were produced by fermentation of substrates at first (Table 5.2). Ethanol fed microcosms exhibited the highest levels of hydrogen immediately following the electron donor addition. But no clear relationship was observed between extent of dechlorination and hydrogen levels. Methane production was minimum in all biotic bottles. All these observations indicate that amount of added substrate was enough to sustain sulfate reducers but not for methanogens.

5.4.5. Effect of sodium sulfide on TCE transformation

To determine the effect of reductant on transformation of TCE, additional microcosms were prepared with 2 different levels of sodium sulfide (0.15 and 1.5 mM). Approximately 32 % of TCE were transformed with and without reductant, respectively (lactate as substrate). Addition of reductant did not lead to a significant increased in TCE transformation (Table 5.2). Sterile controls amended with 1.5 mM of sodium sulfide showed no significant degradation of TCE (i.e. < 7 %), and no c-DCE or other daughter products were detected. Microcosms with and without added Na_2S, demonstrated similar ORP values at the same substrate levels (ranging from -182 to -248). Thus, not only microcosms *with reductant,* but also those *without reductant* sustained desirable reducing conditions, indicating the biological processes, as well as added reductants were capable of generating the drop in reducing potential. Therefore, carbon loading (not use of reductant) may be sufficient to control ORP in the field. Table 5.2 shows transformation of TCE in the presence and absence of reductant using CMB and lactate as substrates.

Table 5.2. Reduction of TCE depending on presence of reductant after 180 days of incubation at 15°C.

Substrate	Reductants (mM)	Reduction (as %)[a]	ORP
CMB	0	37.8 ± 5.8	-187 ± 7.3
	0.15	40.0 ± 4.4	-209 ± 5.9
	1.5	41.4 ± 7.2	-211 ± 4.5
Lactate	0	32.4 ± 1.9	-201 ± 5.7
	0.15	31.4 ± 2.1	-196 ± 4.5
	1.5	32.7 ± 3.7	-213 ± 3.1

[a] Values represent average of triplicate. ± indicates standard deviation.

5.4.6. Byproduct Analysis

Table 5.3 shows the byproduct investigation after the dechlorination of TCE after 180-day period. Productions of trace amount of ethene and acetylene initiated approximately 90 days after incubation, however the onset of degradation and 180-day removal amounts varied among replicates. The principal reaction byproduct for TCE transformation using CMB as substrate was c-DCE and acetylene; c-DCE accounted 65 % of the transformed TCE, while acetylene accounted for 27 %. Even though trace amounts of ethene were detected in some bottles, neither t-DCE nor 1,1-DCE was observed above the method detection limit.

Unlike TCE, c-DCE treated microcosms exhibited little degradation of c-DCE. However, trace amount of acetylene was observed above the method detection limit (acetylene: 50 nM) using CMB as substrate. No 1,1-DCE or VC was observed above the method detection limit. Mass balance of c-DCE shown in Table 5.3 shows more than 17 percent discrepancy, which may have resulted from VOCs loss from handling and sampling.

In order to see the effect of sulfate on TCE transformation, a similar amount of sulfate was respiked on 110-th day for CMB fed microcosms. Even though complete removal of sulfate was observed again, no significant enhancement in acetylene production was observed during this period.

The crystalline structure of microcosms is illustrated in Figure 5.7. The figures show wide peaks and relatively small intensities. These diffraction patterns by the microcosm sediment shows poor degree of crystallinity and thus, indicating that the iron sulfide produced was amorphous. A crystal structure of microcosm treated with added

sulfate can be seen in Appendix B (no difference in crystal structure was observed due to added sulfate).

Table 5.3. The byproducts and mass recoveries for transformation of c-DCE and TCE after 180 days[a].

Reactant	Amendment	Byproduct	Mass recovery (%)[b]
TCE	CMB	Acetylene	7.3 ± 4.4
		Ethene	2.2 ± 3.7
		c-DCE	17.5 ± 4.9
		TCE remaining	62.2 ± 5.8
		Total	89.2 ± 6.8
	Ethanol	Acetylene	5.3 ± 2.0
		c-DCE	1.8 ± 0.5
		TCE remaining	87.7 ± 7.3
		Total	94.8 ± 8.3
	Lactate	Acetylene	8.2 ± 3.7
		Ethene	2.1 ± 1.3
		c-DCE	15.2 ± 2.4
		TCE remaining	67.6 ± 1.9
		Total	93.1 ± 8.9
	Methanol	Acetylene	4.7 ± 1.2
		c-DCE	1.5 ± 0.9
		TCE remaining	84.9 ± 5.8
		Total	91.1 ± 6.7
	Palmitate	Acetylene	6.3 ± 2.1
		Ethene	2.2 ± 0.7
		c-DCE	7.5 ± 3.9
		TCE remaining	68.2 ± 9.3
		Total	84.2 ± 12.8
	Propionate	Acetylene	1.9 ± 1.4
		c-DCE	2.0 ± 1.1
		TCE remaining	88.2 ± 5.8
		Total	92.2 ± 6.8
c-DCE	CMB	Acetylene	7.3 ± 1.0
		c-DCE remaining	75.5 ± 4.9
		Total	82.8 ± 5.8

[a]The pH of microcosms was maintained and nearly constant between 7-7.5. Reductant (Na_2S) was not added. Only trace amounts of VC were detected. Values represent average of triplicates. ± indicates standard deviation
[b]Based on initial moles of chloroethenes individually.

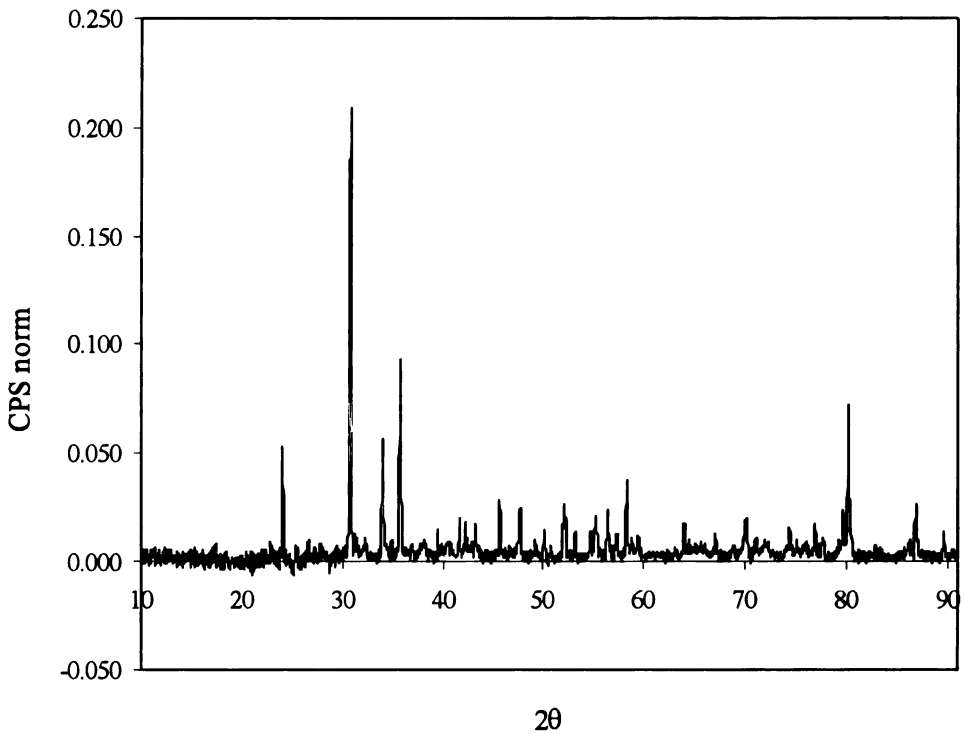


Figure 5.7. Powder x-ray diffraction pattern by iron sulfide produced in microcosm.

5.4.7. Field Estimation

The extrapolation of laboratory result to field-scale processes is an uncertain endeavor, particularly in environmental microbiology (Madsen, 1996). However, studies of several sites contaminated with chlorinated ethenes suggest that rates of reductive dechlorination estimated from laboratory microcosms are similar to those estimated from field studies (Wilson *et al.* 1996). Half-life prediction of chloroethene in the groundwater was made using first order rate equation to obtain treatment estimation. As can be seen in Table 5.4, remediation of TCE and especially c-DCE in groundwater will results in prolonged operation time and high cost without electron donor addition. Natural attenuation occurring in the aquifer seems not only incomplete, but also insufficient. The effects of sulfate, reductant (Na_2S) and nutrient and their interactions on the dechlorinations are summarized on Appendix C.

Table 5.4. Estimated transformation rate and half-time of TCE and c-DCE biodegradation in Plume G

Chloroethene	Electron donor	k (1/month)[a]	Half-time (month)[b]
TCE	CMB	0.13	5.6
	Ethanol	0.09	8.0
	Lactate	0.11	6.5
	Methanol	0.08	8.3
	Palmitate	0.08	9.0
	Propionate	0.1	6.6
	No nutrient	0.006	108
c-DCE	CMB	0.03	20.3
	Ethanol	0.01	62.4
	Lactate	0.03	24.5
	Methanol	0.02	37.5
	Palmitate	0.01	47.1
	Propionate	0.01	53.7
	No nutrient	0.003	256

[a] Based on first-order rate equation $C=C_oe^{-kt}$.
[b] $t_{1/2} = \ln 2/k$.

5.5. Conclusion and Discussion

Several researchers have demonstrated the stimulation of reductive dechlorination of chlorinated ethene by addition of organic supplements or hydrogen (Gibson *et al.* 1994; Fathepure and Boyd, 1988(a); Freedman and Gossett, 1989). These studies were conducted in soils that were rich in organic material (Gibson *et al.* 1994; Freedman and Gossett, 1989) or a pure culture derived from anaerobic sludge (Fathepure and Boyd, 1988(a)). In contrast, our study used anaerobic materials from an oligotrophic aquifer (maximum total organic carbon < 0.04 %) impacted with mixed chlorinated compounds (PCE, 1,1,1-TCA, and daughter products), and chromium to investigate the effects of nutrients on dechlorination. TCE degradation was achieved by selectively stimulating indigenous microflora using fatty acids such as CMB and lactate. The predominant transformation daughter product was c-DCE and acetylene.

Previous studies on the transformation of chloroethenes under sulfate-reducing conditions have also resulted in the production and accumulation of c-DCE (Pavlostathis and Zhuang, 1991; Bagley and Gossett, 1990). Even though TCE degradation continued after depletion of sulfate, it occurred at a much lower rate in our study. This suggests these conditions led to enrichment of sulfate reducers that the degradation observed might have been linked to sulfate reducing populations such as *Desulfomonile tiedjei* or *Desulfovibrio fructosivorans* (Drzyzga and Gottschal, 2002; Mohn and Tiedje, 1991).

Hydrogen is an important intermediate in the anaerobic degradation of organic matter (Conrad, 1999). Microbial competition for H_2 plays an important role in the natural attenuation of chloroethenes, and recent studies have focused on the role of H_2 as a key electron donor for the reductive transformation of these compounds. In our study, we

designed a sulfate-reducing environment where sulfate is depleted with minimal methane production. Even though high H_2 production was observed in some cases, no clear relationship with transformation rate was observed.

Even though the predominant transformation byproduct was c-DCE, small amounts of acetylene, known as an abiotic dechlorination indicator (Butler and Hayes, 1999; Lee and Batchelor, 2002), were also detected. This observation suggested that the abiotic degradation by iron sulfide have participated in dechlorination of TCE in microcosm.

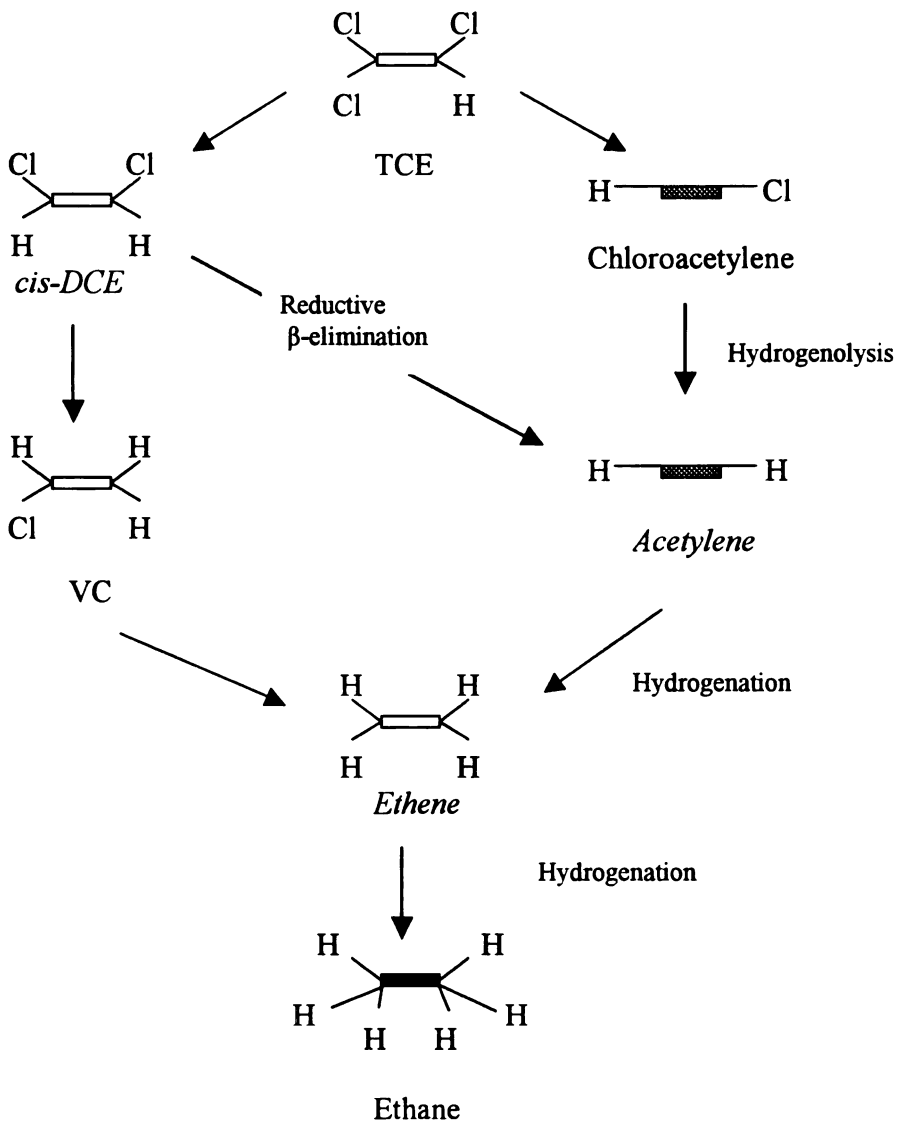
A plausible scheme for the reduction of the TCE to acetylene by abiotic degradation (hydrogenolysis (replacement of a halogen by hydrogen), reductive elimination (dihaloelimination), and hydrogenation (reduction of multiple bonds)) has been proposed by Lee *et al* (2002). Because acetylene was one of the main transformation products, a reductive elimination pathway would appear occur (Figure 5.8). The removal of TCE without observing stoichiometric production of a chlorinated intermediate suggests that the acetylene formed during the TCE dechlorination is produced via an unstable intermediate (chloroacetylene) that quickly decays.

The diffraction pattern by XRD indicated poor-crystalline (amorphous) formation of FeS in microcosm. According to Berner (1964), the poorly-crystalline mackinawite and amorphous FeS are often found in natural systems and their crystallinity is similar to the materials precipitated from sodium sulfide and ferrous sulfate.

Additional sulfate, which would have provided added source of the reductive power in microcosm, did not coincide with rapid enhanced dechlorination. No immediate increase on the TCE degradation by the added sulfate indicated that the dechlorinations

by indigenous sulfate reducers and iron sulfide are very slow. This suggests that the pulse feeding strategy is required to achieve sufficient dechlorinating activity.

Figure 5.8. Possible pathways for the reductive dechlorination of chlorinated ethenes by iron sulfide (modified from Lee *et al.* (2002)). Chemical compounds in italic characters were detected in this experiment



5.6. Literature cited

EPA. 1994. "Common Chemicals found at Superfund Sites", Office of Emergency and Remedial Response, Washington, D.C.

EPA. 2000. Engineered Approaches to In Situ Bioremediation of Chlorinated Solvents: Fundamentals and Field Applications.

Gibson, S.A., Roberson, D.S., Russell, H.H., and G.W. Sewell. 1994. Effects of three concentrations of mixed fatty acids on dechlorination of tetrachloroethene in aquifer microcosms. *Environmental Toxicology and Chemistry*, 13 (3): 453-460.

Gibson, S.A., and G.W. Sewell. 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol.* 58 (4): 1392-1393.

Gao, J. W., Skeen, R.S., Hooker, B. S., and R. D. Quesenberry. 1997. Effects of several electron donors on tetrachloroethylene dechlorination in anaerobic soil microcosms. *Water Research*, 31(10): 2479-2486.

Fennell, D. E., Gossett, J. M., and S. H.Zinder. 1997. Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.*, 31(3): 918-926.

Carr, C. S. and J. B. Hughes. 1998. Enrichment of high rate PCE dechlorination and comparative study of lactate, methanol, and hydrogen as electron donors to sustain activity. *Environ. Sci. Technol.*, 32(12): 1817-1824.

(a) Fathepure, B. Z. and S. A. Boyd. 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. Strain Dcm.. *Appl. Environ. Microbiol.* 54(12): 2976-2980.

(b) Fathepure, B. Z. and S. A. Boyd. 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters* 49(2): 149-156.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Pavlostathis, S. G. and Ping Zhuang. 1991. Transformation of trichloroethylene by sufate-reducing cultures enriched from a contaminated subsurface soil. *Appl. Microbiol. Biotech*, 36(3): 416-420.

Rickard, D. T. 1969. The microbiological formation of iron sulphides, *Stockholm Contr, Geol.* (26): 49-6

Freney, J. R. 1979. Sulfur Transformations, in R. W. Fairbridge, and C. W. Finkl, Jr., Eds, *The Encyclopedia of Soil Science Part 1, in Encyclopedia of Earth Science* Volume XII, Dowden, Hutchison and Ross, Inc., Stroudsburg, PA, pp. 536-544.

Butler, E., and Kim F. Hayes. 1999. Kinetics of the Transformation of Trichloroethene and Tetrachloroethene by Iron Sulfide. *Environ. Sci. Technol.* 33: 2021-2027

Lee, W., and B. Batchelor. 2002. Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 1. Pyrite and Magnetite, *Environ. Sci. Technol*, 36(23): 5147-5154

Hassan, S. M., Wolfe, N.L., and M.G. Cippolone. 1995. Reaction mechanisms involved in the reduction of halogenated hydrocarbons with sulfated iron, "*209th ACS National Meeting*, April 2-7, Anaheim, CA, Vol. 35 (1), pp.73-737.

Doong, R. A. and S. C. Wu. 1992. Reductive dechlorination of chlorinated hydrocarbons in aqueous solutions containing ferrous and sulfide ions, *Chemosphere* 24, 1063-1075.

McCarty, P. L.1969. Energetics and Bacterial Growth. *5th Rudolf Research Conf.* Rutgers.

McCormick, M.L. E.J. Bouwer, and P. Adriaens. 2002. Carbon tetrachloride transformation in a model iron-reducing culture: relative kinetics of biotic and abiotic reactions. *Environ. Sci. Technol.* 36(3); 403-410

Butler, Elizabeth Constance. 1998. Transformation of haolgenated organic compounds by iron sulfide. A Ph.D. Thesis. pp. 28. Univeristy of Michigan.

Madsen, E.L. In Soil Biochemistry. Vol.9; Stotzky, G., Bollag, J., M. Eds.; Marcel Dekker: New York, 1996. pp.287-370

Wilson, J.T.; Kampbell, D.H. and J.W. Weaver. In U.S. Environmental Protection Agency Symposium in natural attenuation of chlorinated organics in ground waters. Sept. 11-13, 1996; Dallas, TX; EPA/540/R-96/509; 1996; pp.124-127

Bagley D. M. and J. M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and cis-1,2- dichloroethene by sulfate-reducing enrichment cultures. *Appl. Environ. Microl.* 56(8): 2511-2516.

Oliver Drzyzga and Jan C. Gottschal. 2002. Tetrachloroethene dehalorespiration and growth of *Desulfitobacterium frappieri* TCE1 in strict dependence on the activity of *Desulfovibrio fructosivorans*. *Appl. Environ. Microbiol.*, p. 642-649, Vol. 68, No. 2

Mohn, W.W., and J.M. Tiedje. 1991. Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. *Arch. Microbiol.* 157:1-6.

Conrad, R. 1999. Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiol. Ecol.* 28, 193-202.

Berner, R. A. 1964. Iron sulfides formed from aqueous solution at low temperatures and atmospheric pressure. *J. Geol.* 72, 293-306.

Chapter Six: STIMULATION OF HIGH-RATE COMPLETE DECHLORINATION ACTIVITY BY PULSE FEEDINGS OF CHLOROETHENES

6.1. Abstract

Once-fed microcosms that demonstrated initial positive dechlorinating activity were further characterized for chloroethene/ethane transformation potential by pulse-feedings of VOCs, lactate, and native levels of sulfate using plume G groundwater. Experiments were performed in batch microcosms receiving two levels of lactate; low (0.5 mM) or high (12.5 mM). PCE was fed approximately 15 µmol/ 2 month for the first year followed by 15-µmol/ month in the second year. High lactate enriched bottles exhibited limited transformation of PCE with TCE or c-DCE as byproduct (0.036/day). Low lactate enrichments demonstrated high rate of degradation; PCE, TCE, c-DCE, t-DCE, 1,1-DCE, and VC to ethenes (rates ranged from 0.19 to 0.82/day). Further reduction to ethane was observed in many cases. Highest rate of PCE transformation was observed at 25 °C in low lactate/PCE enrichments. 16S rRNA gene-based RTm-PCR identified that the pre-enrichments contain no quantifiable amounts of *Dehalococcoides* populations. Significant levels of *Dehalococcoides* were detected in all of the post-enrichment samples. The low lactate/PCE microcosms contained *Dehalococcoides* at least two orders of magnitude higher than high lactate/PCE enriched microcosms. This demonstrated that pulse feedings of chlorinated ethenes, and low levels of electron donor effectively stimulated *Dehalococcoides* populations.

6.2. Introduction

Methanogens are known to dechlorinate PCE to TCE and c-DCE (Fathepure and Boyd, 1988(a)(b); Freedman and Gossett, 1989). However, the dechlorination by methanogens is known to be incomplete and proceed at low rates. So far only *Dehalococcoides ethenogenes* is known to fully dechlorinate PCE to ethene. It uses H_2 as a sole electron donor, grows slowly in pure culture, and is fastidious in its growth conditions (Maymo-Gatell *et al.* 1997). In pure culture, *Dehalococcoides ethenogenes* requires the extract of an anaerobic sludge community for sustained growth, suggesting that it relies on biochemical collaboration with other microorganisms and that it benefits significantly by growing in a mixed community.

Several molecular approaches are currently available as means of exploring microbial communities including T-RFLP and RTm-PCR (Hendrickson *et al.* 2002; Liu *et al.* 1997). This research discusses the characterization of highly enriched mixed cultures that reductively dechlorinates PCE to ethene/ethane with lactate as an electron donor using two molecular techniques. Effects on the community structure and activity on the original community by prolonged exposure to high versus low lactate/PCE feedings were studied. Terminal restriction length polymorphism (T-RFLP) analyses were employed to identify community structure in the mixed cultures and to track population dynamics after enrichment by PCE. Additionally, specific primers for detecting *Dehalococcoides* species were used to determine the presence and enumeration of such *Dehalococcoides* populations.

Studies presented here focus on the enrichment of a high-rate PCE dechlorinating culture through pulse feedings of PCE, lactate, and native levels of groundwater sulfate over 2-year period. Microcosms which demonstrated initial positive dechlorinating activities, were further enriched using 2 different ratios of lactate and pulse feedings of chlorinated ethenes to evaluate the effect of excess electron donor on stimulating dechlorinating activity. I have evaluated the effect of lowering the ratio between substrate to PCE, along with pulse feedings on the substantial enrichment of *Dehalococcoides*. Characterization of these enrichments and enumeration of *Dehalococcoides* are presented later in this chapter. Finally, I conducted daughter product analysis to evaluate the completeness of PCE transformation by rapidly dechlorinating enrichments.

6.3. Materials and Methods

Enrichment Procedure

Anaerobic microcosms assembled from plume G sediments initially exhibited neither rapid nor complete transformation of parent compounds (TCE and c-DCE). In order to enhance dechlorinating activity, enrichments that demonstrated the highest dechlorination activity were subcultured and incubated with three different concentrations of lactate (0, 0.5 mM, and 12.5 mM) and equal amount of PCE (15 μmol/bottle). Table 6.1 shows the additions of chlorinated compounds and lactate corresponding to the appropriate experiment. Chlorinated compounds were added neat using 5μL microsyringe except for VC, which was added as a gas using airtight syringe. The Roman numeral corresponds to the chronological order in which the experiments were performed. Experiment I indicates two conditions that favor one or the other process based on electron donor/acceptor ratio.

Experiment II was designed to screen for the substrate specificity of PCE over TCE, and c-DCE. Experiment II was set up using c-DCE long-term microcosms from chapter 5. Only low levels of lactate (see Table 6.1 for exact concentration) were fed once every 2 months for 6 months and once a month thereafter for c-DCE and TCE. TCA microcosms were spiked once every 2 months, as the TCA microcosms never exhibited high rates of dechlorination. Therefore, all chloroethene enrichments consumed similar amounts (12 cycles of 15 μmol) before the rate experiments except TCA. TCA enrichments had only 6 cycles of 15 μmol over 360 days of enrichment. The additions and consumptions of chlorinated compounds for experiment II are depicted in

Appendix D. The overview of enrichments and experiments performed according to their time sequence is described in Figure 6.1.

Sampling and Analysis

Over the initial period of enrichment, complete transformation of PCE was not observed; however, periodic respiking with PCE was conducted. During enrichment, one-quarter of the bottle (liquid volume, 200-mL), contents were replaced with fresh groundwater media (typically once/2 month), to replenish vitamin, phosphate, and lactate (0.5 or 12.5 mM). Groundwater media was made by adding phosphate (KH_2PO_4, 0.2g/L), sodium bicarbonate ($NaHCO_3$, 5g/L), and vitamin supplements (see Table 4.3) to plume G groundwater, which contained native amounts of sulfate (range between 35 to 60 ppm) and nitrate (range between 25 to 40 ppm). After lactate addition, PCE was added at 15 μmol/bottle. Throughout these studies, bottles were purged thoroughly after sampling but prior to each PCE addition to remove VC and ethene, and thus to prevent potential toxicity (Schink, 1985). The pH was monitored and maintained between 6.8-7.5 by addition of small amounts of 1 M $NaHCO_3$ solution. Chlorinated compounds, headspace, anion, and fatty acid analyses were done as described previously in Chapter 4. Terminal restriction length polymorphism (T-RFLP) and Real time PCR (RTm-PCR) analyses were performed as described in Chapter 4.

Effect of Temperature

All bottles were kept in a constant room temperature during 2-year enrichment period. Nine low lactate/PCE enriched bottles were incubated at three different

temperatures (15, 25, and 35 °C) for 3 weeks to evaluate temperature effects on transformation of PCE.

Table 6.1. Experiment arrangements for respective conditions. Microcosms were exposed to pulse feedings of PCE over 1.5 years for Experiment I and pulse feedings of TCE, c-DCE, and TCA over 1 year for Experiment II[a].

Conditions	Chloroethene added (μmol)	Lactate/PCE (equiv/equiv)	μequiv of lactate	μmol of lactate
Experiment I: Cometabolic reductive dechlorination favoring versus chlororespiration favoring conditions (using TCE exposed bottles from Chapter 5)				
PCE 1	15	0/0	0	0
PCE 2	15	**10**/1	1200	100
PCE 3	15	**250**/1	30000	2500
Experiment II: Chlororespiration favoring conditions (using c-DCE exposed bottles from Chapter 5)				
TCE	15	**10/1**	900	75
c-DCE	15	**10/1**	600	50
TCA	15	**10/1**	600	50

[a]The electron donor ratio was calculated assuming: PCE 8 equiv/mol; TCE 6 equiv/mol; c-DCE 4 equiv/mol; and TCA 4 equiv/mol. Lactate was assumed to provide 12 equiv/mol (on CO_2 basis).

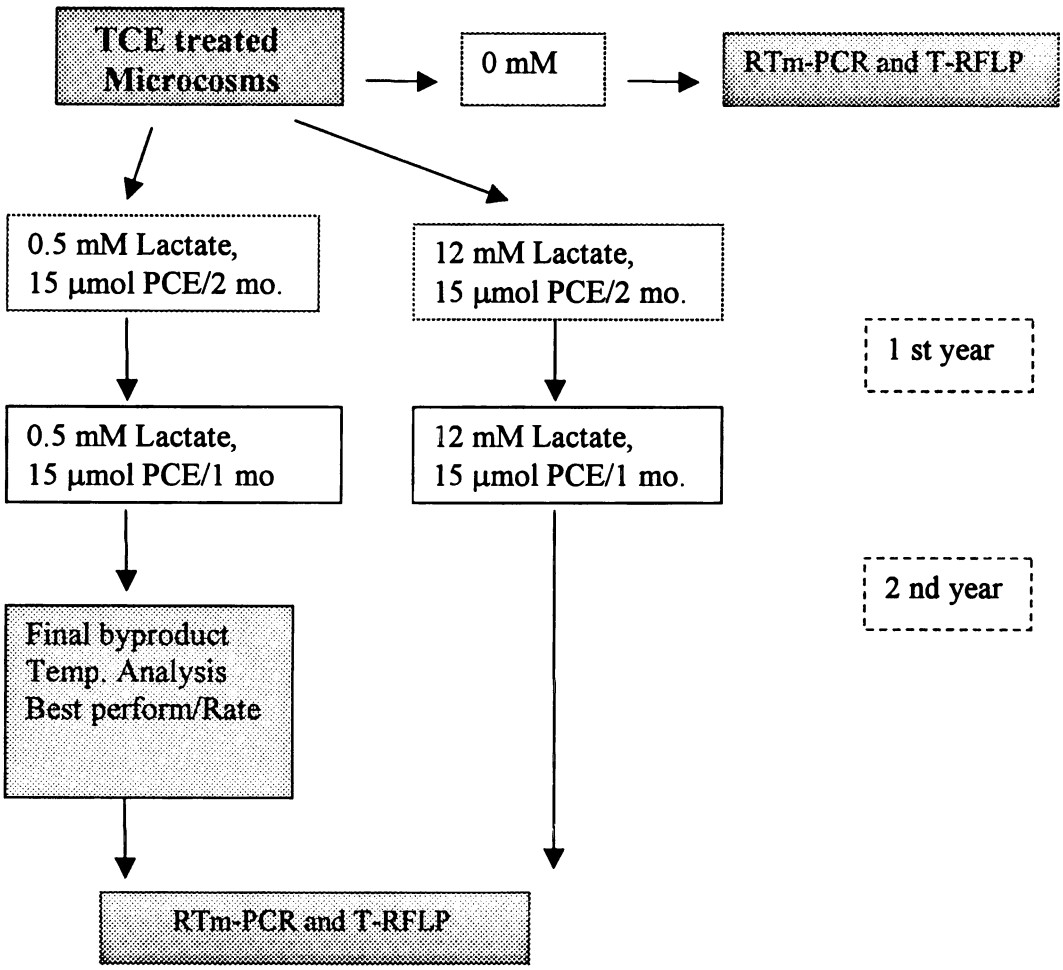


Figure 6.1. The overview and sequence of the experiments performed during enrichments.

6.4. Results

6.4.1. High lactate conditions

6.4.1.1. Transformation of PCE

After one year of repeated feedings, transformation rates became faster (once a month feeding of PCE) and c-DCE was produced as byproduct for some bottles. Figure 6.2. depicts repeated additions and consumptions of PCE at the early stages of enrichment period. However, transformation beyond TCE was limited, with only slight c-DCE degradation during 2 years. Figure 6.3 illustrates PCE transformation by high lactate/PCE enrichments and the first order rate achieved by high and low lactate/PCE enrichments.

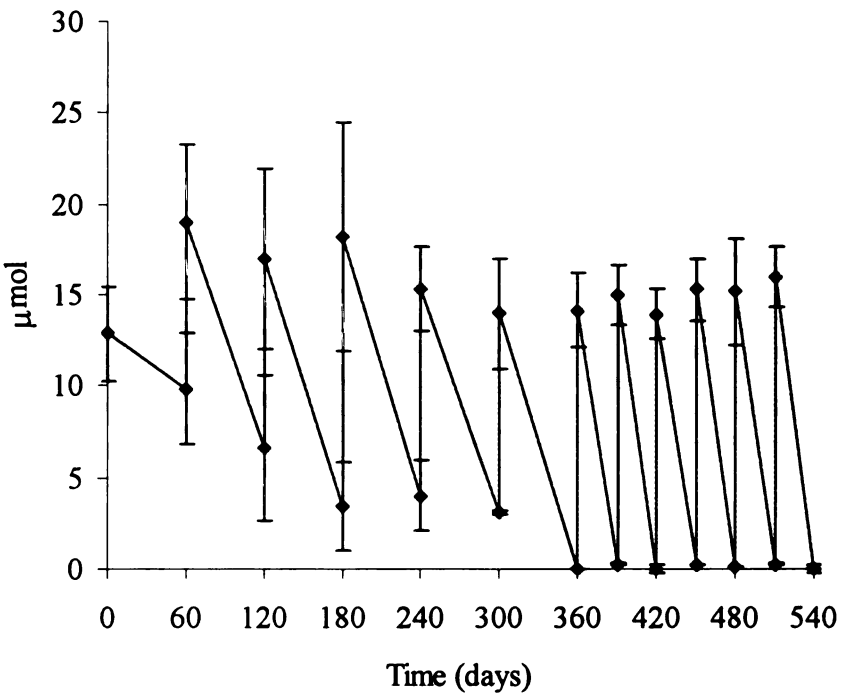


Figure 6.2. PCE additions and consumption by high lactate/PCE treatment over 1.5 years. 12 cycles of PCE was added.

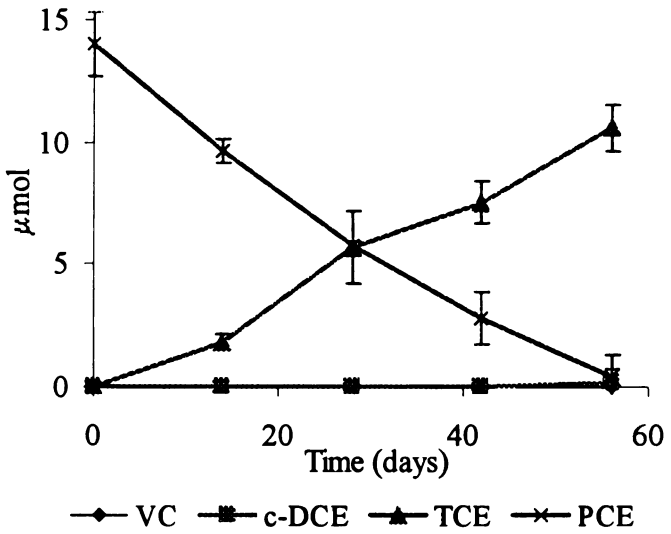


Figure 6.3. Effects of prolonged exposure of high lactate (12.5 mM) on enhancing transformation activity (after 12 month of incubation).

Table 6.2. Effects of prolonged exposure of high versus low lactate on enhancing transformation activity (after 12 month of incubation).

	Transformation rate (1/day)[a]	Half life (day)[b]	Final products
Low lactate/PCE	0.71	0.98	ethene and ethane
High lactate/PCE	0.04	19.4	c-DCE and TCE

[a] Based on first-order rate equation $C=C_oe^{-kt}$.
[b] $t_{1/2} = \ln 2/k$.

6.4.1.2. Fermentation balance

An electron balance was determined after 10 days of fermentation. To accomplish this, fatty acid transformation, methane, and hydrogen were monitored 10 days after the spiking of lactate and PCE. Using lactate and PCE as starting compounds, microequivalents of lactate consumed were balanced with the sum of the microequivalents of reduced products formed. The electron balance for one of the six replicate bottles is shown in Table 6.3. It is apparent that nearly 76.5 % of the reducing equivalents from lactate were used to produce acetate and propionate and the other 10 % being used in methane production. Dechlorination and hydrogen production were insignificant sinks (< 1.5 %) for electrons on 10^{th} day electron balance.

The other five replicate bottles exhibited similar results. The fraction of lactate equivalents used in dechlorination varied from 0.7-1.5 % and averaged 1.2 % among 5 bottles. The electron equivalents for dehalogenation were calculated assuming complete dechlorination from PCE to ethene requires 8 equiv/mol and lactate breaks down to propionate and acetate. Biomass increment was ignored. However, CO_2 change during this 10 day was not negligible. The missing 8 % of reducing equivalents produced from lactate consumption may have used in nitrate or sulfate reduction. Negative and positive values represent consumption and production during 10 days.

Table 6.3. Electron balance for a single, PCE degrading microcosm (15 μmol PCE /bottle, 12.5 mM (2500 μmol) lactate; data obtained at day 10)

Compound	Amount present (μmol)	Reducing Equivalents (eq/mol)	Electron equiv. consumed (μeq)	Electron equiv. formed (μeq)	Percent[a] (%)
Lactate	-476	4	-1904		
Propionate	52	6		312	16.4
Acetate	129	8		1032	54.2
CH_4	23	8		184	9.7
H_2	0.27	2		0.54	0.03
PCE	-13	0			
TCE	13	2		26	1.4
DCE isomers	0	4			
VC	0	6			
Ethene	0	8			

[a]Percent calculated by dividing reducing equivalents produced over total reducing equivalents consumed.

6.4.2. Low lactate conditions

6.4.2.1. Enrichment of high rate activity

Low lactate/PCE bottles were respiked with PCE and replenished with fresh groundwater media just like high lactate/PCE bottles. However, lactate was supplied 25 times less than high lactate/PCE bottles (100 μmol). Reducing potential for low lactate/PCE reached approximately -200 and pH was monitored and maintained between 6.8-7.5. After 4 cycles of repeated feedings, transformation rates became faster (once a month feeding of PCE) and more complete. For example, primarily VC was produced as byproduct. By 9 cycles of repeated feedings (12 month), bottles treated with low lactate ones exhibited complete transformation of PCE to ethene and 20 times faster than high lactate treated ones (Figure 6.4).

Since complete transformation of PCE was only observed under the low lactate conditions, the ranges of VOCs, which could be degraded under these conditions, were determined. As parent products, TCE, c-DCE, and TCA were evaluated since these compounds already present in plume G. TCE and c-DCE were completely transformed to ethene after 6 month of repeated feedings. However, limited number of TCA fed microcosms (5 out of 12) transformed to chloroethane (CA). Ethane production from CA was not observed over 60 days.

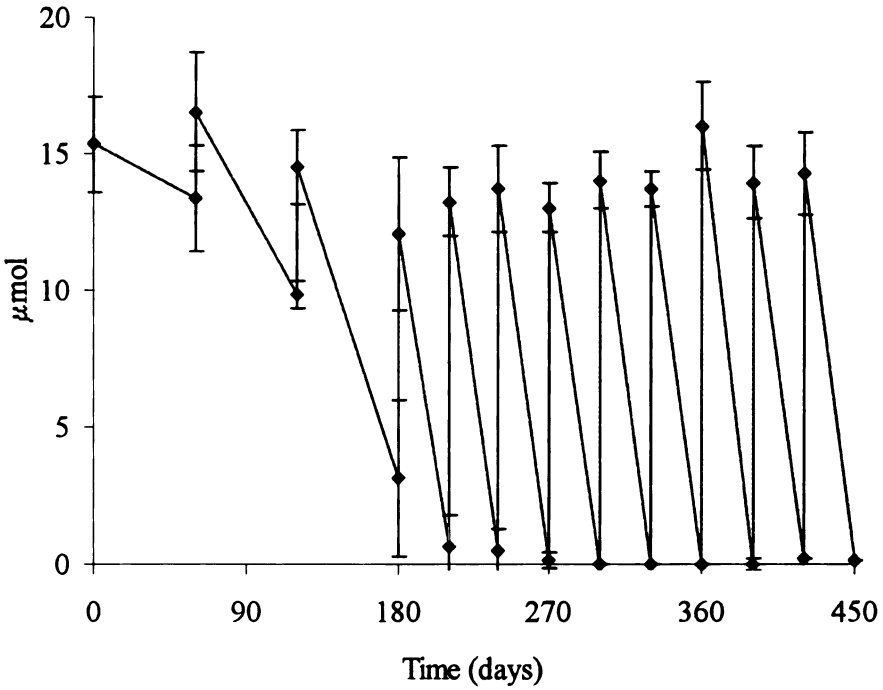


Figure 6.4. PCE additions and consumption by low lactate/PCE treatment during 450 days (12 cycles).

6.4.2.2. Fermentation balance

An electron balance was calculated after 10 days of fermentation for low lactate treated microcosms. To accomplish this, fatty acid transformation, methane, and hydrogen were monitored 10 days after the spiking of lactate and PCE. Using lactate and PCE as starting compounds, microequivalents of lactate consumed were balanced with the sum of the microequivalents of reduced products formed. The electron balance for one of the six replicate bottles is shown in Table 6.4. It is apparent that about nearly half of the reducing equivalents from lactate were used to produce acetate (43%) and the other 20 percent being used in reductive dechlorination. Methanogensis and hydrogen production were insignificant sinks (4 %) for electrons on 10^{th} day electron balance.

The other five replicate bottles exhibited similar results. The fraction of lactate equivalents used in dechlorination varied from 12-28 % and averaged 24 % among 5 bottles. The electron equivalents for dehalogenation were calculated assuming complete dechlorination from PCE to ethene requires 8 equiv/mol and lactate breaks down to propionate and acetate. Biomass increment was ignored and CO_2 change during this 10 day was negligible. Negative and positive values represent consumption and production during 10 days.

Table 6.4. Electron balance for a single, PCE degrading microcosm (15.2 μmol PCE /bottle, 0.5 mM, lactate; data obtained at day 10)

Compound	Amount present (μmol)	Reducing Equivalents (eq/mol)	Electron equiv. consumed (μeq)	Electron equiv. formed (μeq)	Percent[a] (%)
Lactate	-102	4	-408		
Propionate	13	6		78	19
Acetate	22	8		176	43
CH_4	0.2	8		1.6	0.4
H_2	< 0.01	2		0.02	< 0.005
PCE	-14	0			
TCE	0	2			
DCE isomers	3.2	4		12.8	3
VC	11.5	6		69	17
Ethene	0	8			

[a]Percent calculated by dividing reducing equivalents produced over total reducing equivalents consumed.

6.4.2.3 . Utilization of Chlorinated Ethenes

Low lactate/PCE enrichments which showed complete degradation of PCE were employed to determine the ability to degrade other chlorinated compounds. In addition, degradation rates were compared with bottles enriched with TCE and c-DCE. The transformation rates observed in these experiments are summarized in Table 6.4. The enrichments usually required 5-15 days to completely degrade PCE depending on the bottles and concentration of parent products. There was stoichiometric conversion of PCE to VC, and VC dechlorination occurred only when all other chlorinated intermediates were nearly consumed. Figure 6.5 shows the sequence and duration of intermediates produced by high concentration of PCE dechlorination. The productions of TCE and c-DCE were observed as PCE degraded. Cis-dichloroethene was completely dechlorinated to VC in approximately 20 days.

When high concentrations of PCE or TCE were received, some bottles produced t-DCE as intermediate, which persisted longer than c-DCE. When t-DCE alone was added in the bottles, its rate of degradation was again slower than c-DCE. VC production from t-DCE was slow and transformation of VC was substantially slower than any other chloroethenes tested. Utilization of 1,1-DCE by the culture was similar to c-DCE in that conversion from VC to ethene only occurred after depletion of 1,1-DCE (Figure 6.6). When VC was added alone, it was converted to ethene with no lag period.

All these tests were done in the bottles that were previously enriched with repeated PCE feedings over a 2-year period. However, the disappearance of TCE and c-DCE from TCE, c-DCE incubated bottles did not show any increase in rate of transformation compared to PCE enriched bottles. For example, the rates of

transformation of c-DCE in PCE enriched bottles were not significantly different from that of c-DCE enriched bottles (Table 6.5).

Figure 6.5. Transformation of PCE and intermediate productions from lactate/PCE enrichments received high dosage of PCE (80 μmol).

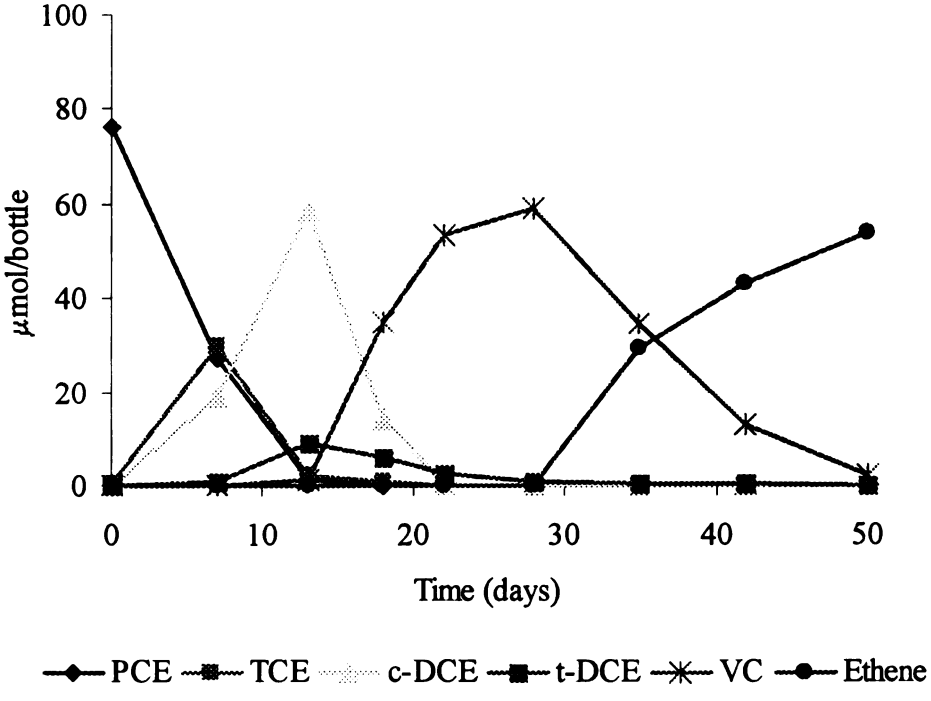
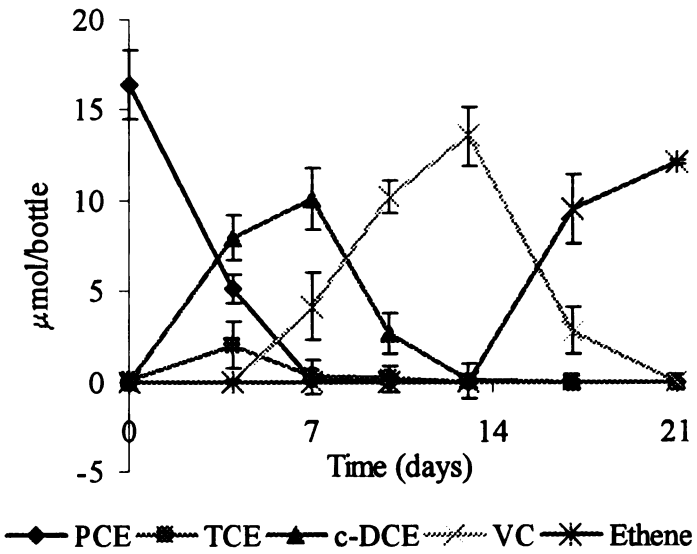
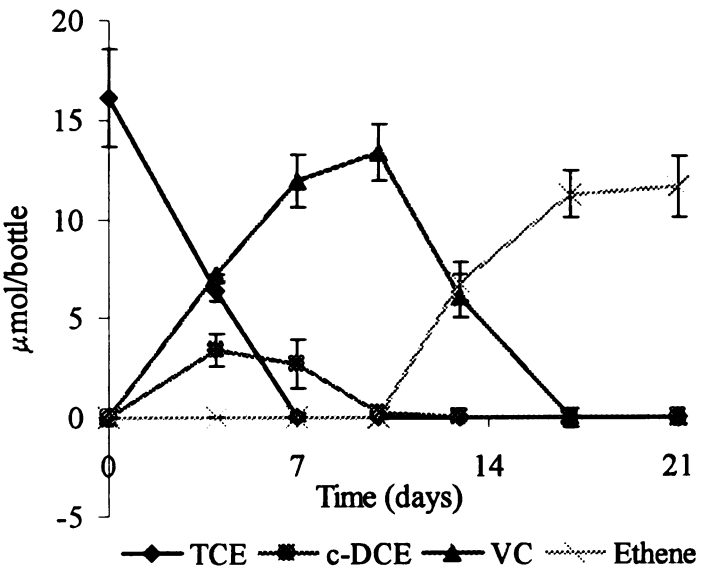


Figure 6.6. Conversion of chlorinated ethenes to ethene by low lactate/PCE enrichment. This experiment was done with the microcosms showing highest activity. Approximately 15 µmol of chlorinated ethenes and 0.5 mM of lactate were used: (a) PCE; (b) TCE; (c) c-DCE; (d) t-DCE; (e) 1,1-DCE; and (f) VC. Error bars represent the standard deviation of triplicates.

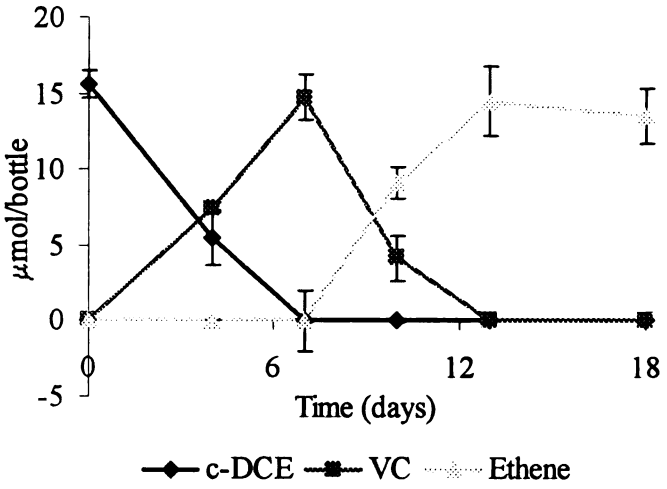
(a) PCE



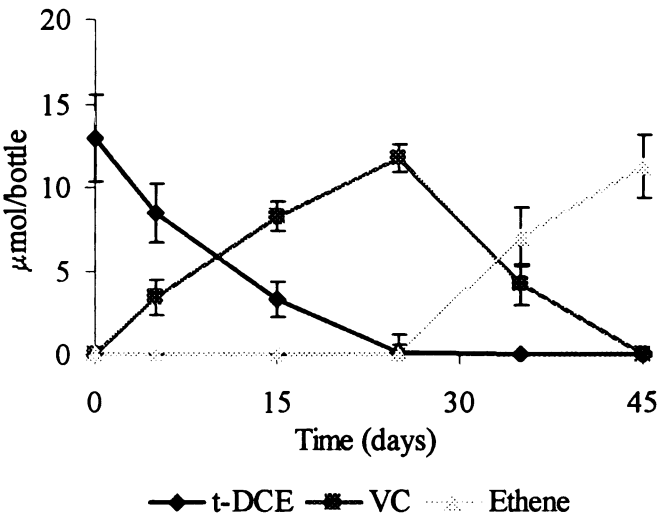
(b) TCE



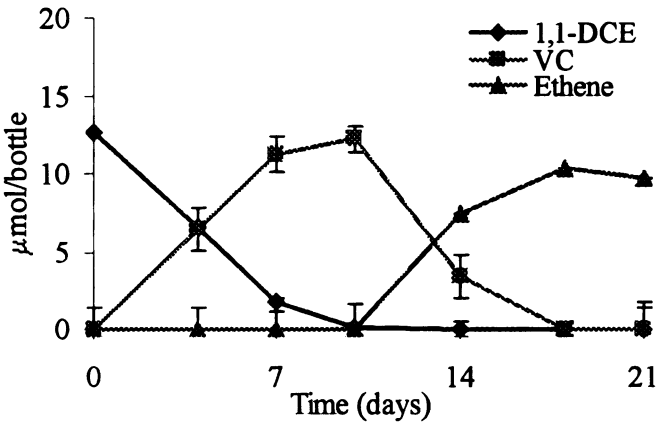
(c) c-DCE


20
15
10
5
0
-5
µmol/bottle
0
6
12
18
Time (days)
c-DCE
VC
Ethene

(d) t-DCE


20
15
10
5
0
µmol/bottle
0
15
30
45
Time (days)
t-DCE
VC
Ethene

(e) 1,1-DCE


20
15
10
5
0
µmol/bottle
0
7
14
21
Time (days)
1,1-DCE
VC
Ethene

(f) VC

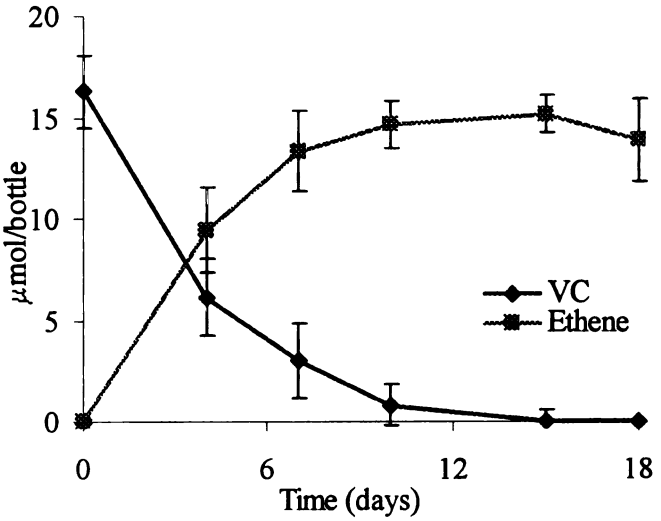

20
15
10
5
0
µmol/bottle
0
6
12
18
Time (days)
VC
Ethene

Table 6.5. Summary of dechlorination rate observed by low lactate/PCE, low lactate/TCE, and low lactate/c-DCE enrichments (/day)[a,b]

Compound	PCE Enriched	TCE Enriched	c-DCE Enriched
PCE	0.82 ± 0.07	N/A[c]	N/A[c]
TCE	0.69 ± 0.08	0.76 ± 0.02	N/A[c]
c-DCE	0.66 ± 0.04	0.70 ± 0.04[d]	0.67 ± 0.03
t-DCE	0.19 ± 0.06	N/P[e]	N/A[c]
1,1-DCE	0.45 ± 0.04	N/P[e]	N/A[c]
VC	0.29 ± 0.03	0.29 ± 0.03[d]	0.31 ± 0.04[d]

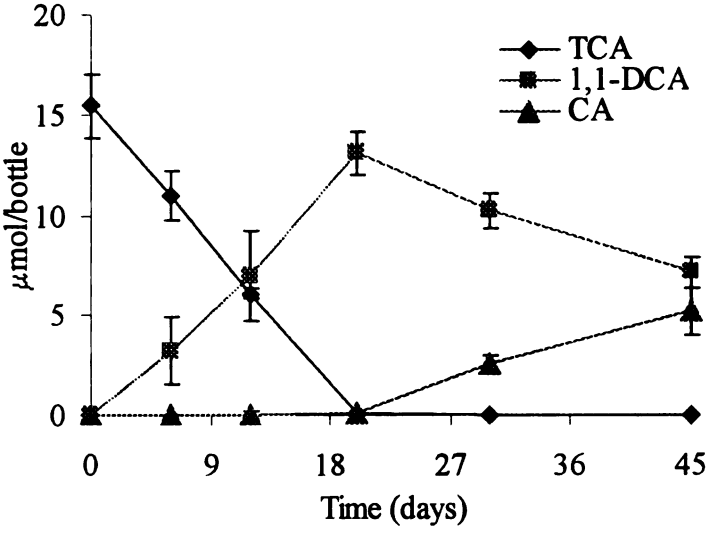
[a] Based on first-order rate equation $C=C_oe^{-kt}$. Microcosms had 15 cycles of respking.
[b] Experiments were conducted in triplicates (± indicates standard deviation from triplicates).
[c] N/A = Not Available.
[d] Some compounds like c-DCE and VC were produced as daughter products.
[e] Some compounds are NP (Not Present or Not Produced).

6.4.2.4. Metabolism of chlorinated ethanes.

The enrichments (pulse fed TCA for 12 month) also transformed 1,1-DCA and TCA in a rather different way. Some of the bottles received TCA did not show any signs of degradation. Some bottles showed rapid transformation after a 1-month lag phase. Only bottles that showed TCA transformation degraded 1,1-DCA. 1,1-DCA had been found to be the main product of TCA biotransformation. Chloroethane was the final product and no ethane was observed over 2-month period (Table 6.6).

Figure 6.7. Conversion of chlorinated ethanes by low lactate/TCA enrichment. Individual serum bottles have received 15 μmol of chlorinated ethanes and lactate of 0.5 mM: (a) TCA and (b) 1,1-DCA. Error bars represent the standard deviation of triplicates.

(a) TCA



(b) 1,1-DCA

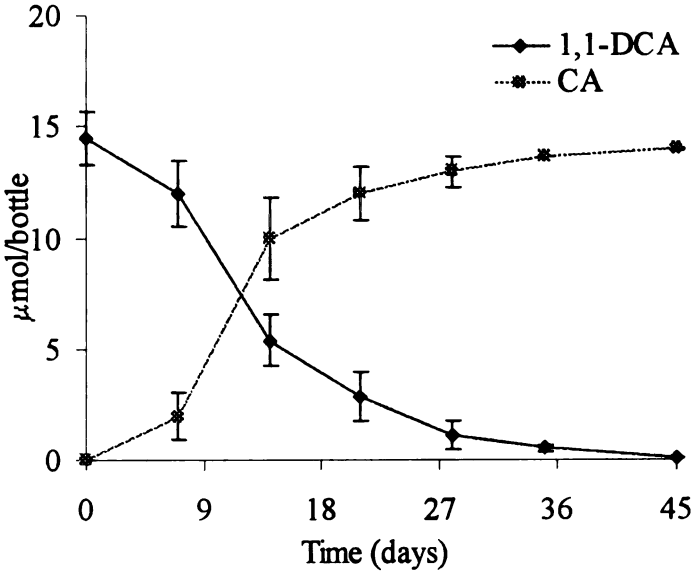


Table 6.6. Effects of prolonged exposure of TCA on stimulation of transformation activity (after 12 month of incubation).

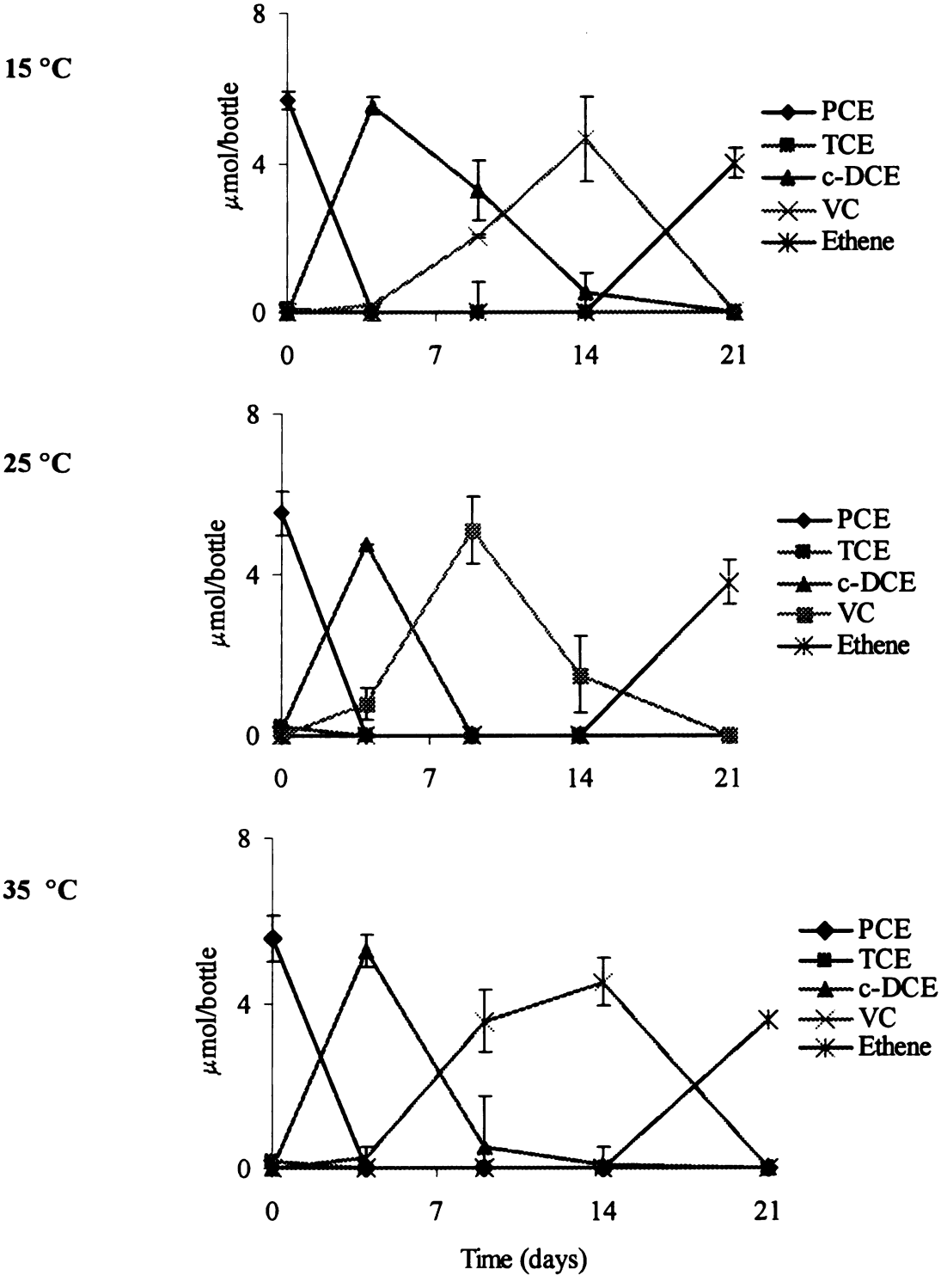
	Transformation rate (1/day)[ab]	Half life (day)[c]	Final product
TCA	0.23 ± 0.03	3.0	1,1-DCA and CA
1,1-DCA	0.12 ± 0.02	3.7	CA

[a] Based on first-order rate equation $C=C_oe^{-kt}$.
[b] Experiments were conducted in quadruplicates (± indicates standard deviation from quadruplicates).
[c] $t_{1/2} = \ln 2/k$.

6.4.2.5. Effect of temperature

The microcosms spiked with 6 μmol/bottle of PCE were kept in stationary and dark place at different temperatures (15 °C, 25 °C, and 35 °C). The effects of temperature on PCE transformation were monitored over 3 -week periods. As can be seen from Figure 6.8, all microcosms exhibited similar rates of PCE transformation. However, the byproduct disappearance was difference among treatments. The ethene production from 25 °C started at day 10, when others started at day 14. In additions, cis-DCE persisted longer at low temperature (15 °C) than others.

Figure 6.8. Temperature effects on PCE transformation on low lactate/PCE enrichment. Individual serum bottles have received 6 μmol of PCE and lactate of 0.5 mM. Error bars represent standard deviation of triplicates.



6.4.2.6. Did it go all the way to ethane?

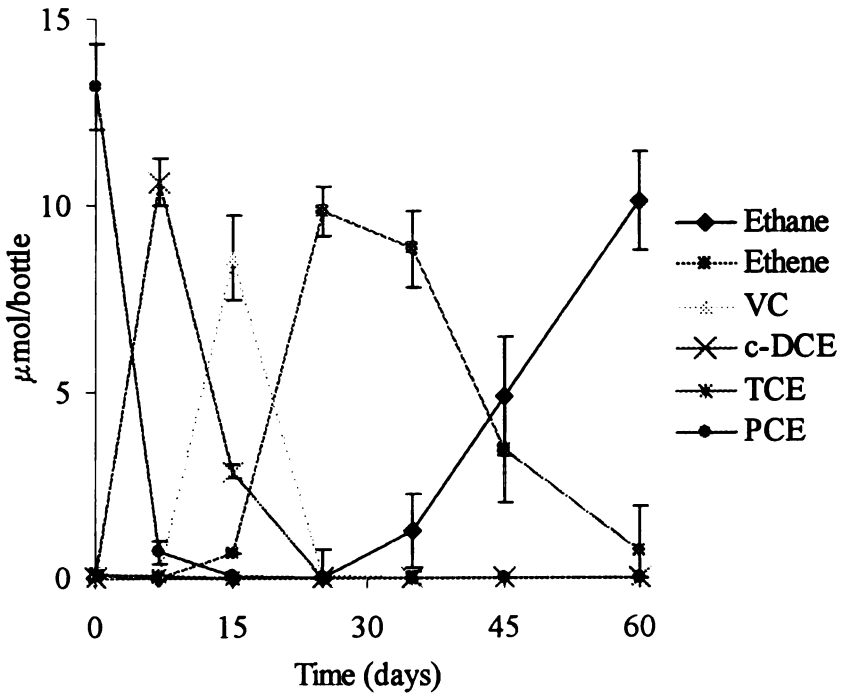
Reduction of ethene to ethane has been previously demonstrated under methanogenic condition by enrichment (deBruin *et al.* 1992). In order to examine whether the enriched mixed community can produce ethane from ethene, 8 bottles were injected with PCE and sampled every 10 days. Ethane production was observed from ethene after 60 days. Utilization of ethene by the culture was similar to the rest of the chloroethenes, in that the conversion from ethene to ethane only occurred after depletion of all chlorinated parent and intermediate products (Figure 6.9). However, some bottles exhibited very slow reduction of ethene to ethane. As can be seen from high standard deviation of total ethane produced, the degree of reduction from ethene to ethane varied between microcosms. Mass balance of PCE to ethane is shown in Table 6.7.

Table 6.7. Mass balance determined for PCE transformation to ethane after 60 days

Total PCE added (μmol ± SD)[a]	Total Ethene produced (μmol ± SD)	Total Ethane produced (μmol ± SD)	Conversion (%)
13.15 ± 1.15	0.69 ± 1.24	10.10 ± 1.36	82%

[a] SD – standard deviation from 6 out of 8 microcosms tested. The rest 2 bottles had very slow ethane production that they were not included in the calculations.

Figure 6.9. Final PCE transformation byproducts by low lactate/PCE enrichments. 6 out of 8 microcosms tested. The rest 2 bottles had very slow ethane production that they were not included in this figure. Individual serum bottles have received 15 μmol of PCE and lactate of 0.5 mM.



6.4.3. Microbial Community Analysis

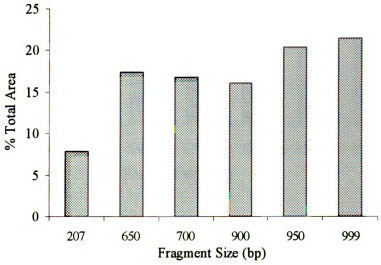
After extracting DNA from 3-ml samples of sediment groundwater slurry of pre-enriched, low, and high lactate enrichment, PCR is amplified for T-RFLP analysis and Real time PCR (RTm-PCR) for the quantification of dechlorinating populations. T-RFLP was used to determine if the microbial community had been changed over prolonged exposure to chlorinated compound loadings and lactate. Community shifts were indicated by changing peak patterns, i.e. the terminal restriction fragments (TRFs), on the electropherograms. Since it is possible for different organisms to share a common restriction site in the 16S genes, only a single dominant peak on the electropherogram was considered for that fragment.

Using digestions enzyme *HhaI*, pre-enrichment and enrichments showed differences in fragments numbers. The patterns show that the numbers of terminal restriction fragments (TRFs) detected in the enriched ones have significantly increased from those in the pre-enrichment samples. All low lactate chloroethene enrichment (PCE, TCE, and c-DCE) had similar fragments and overall bacterial community structure, indicating that the dominant indigenous populations were similar to each other (Figure 6.10). The TCA and high lactate enrichments had different patterns from low chloroethene enrichments in terms of terminal restriction fragments (TRFs) sizes indicating change in overall bacterial community structure.

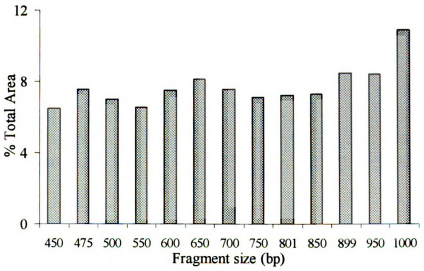
T-RFLP profile fail to show a single fragment that could represent as *Dehalococcoides* specific fragment (List of the T-RFLP fragments and peak areas using *HhaI* are shown on Appendix D).

Figure 6.10. T-RFLP profiles generated using pre-enrichment, low lactate/PCE, and high lactate/PCE enrichments using *HhaI*. Each fragment size indicates percent fragment area: (a) pre-enrichment; (b) high lactate/PCE; (c) low lactate/PCE; (d) low lactate/c-DCE; (e) low lactate/TCE; and (f) low lactate/TCA

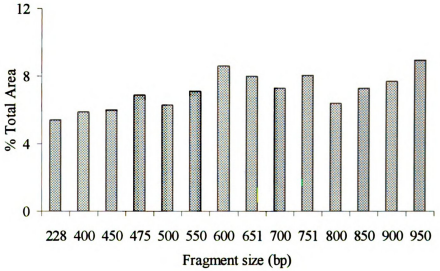
(a) Pre-enrichment



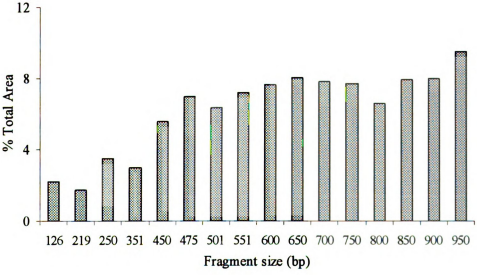
(b) High Lactate/PCE Enrichment



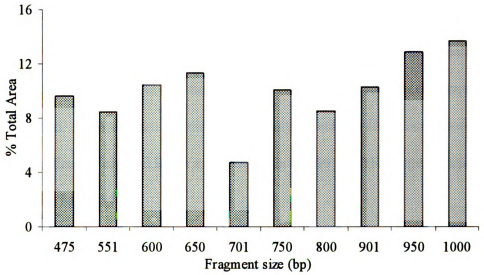
(c) Low Lactate/PCE Enrichment



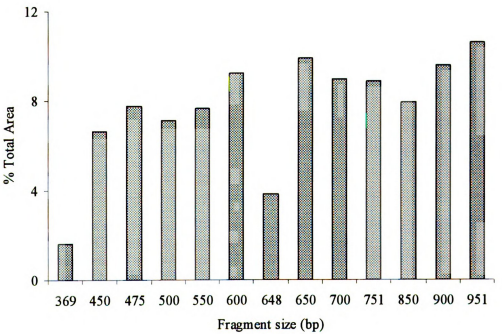
(d) Low Lactate/TCE Enrichment



(e) Low Lactate/c-DCE Enrichment



(f) Low Lactate/TCA Enrichment



Compared to pre-enrichment bottles which exhibited non-quantifiable amounts of *Dehalococcoides*, greater numbers of *Dehalococcoides* were observed in enriched bottles. As described in Table 6.7, the enrichment by low lactate/PCE treatment contained at least 160 times higher numbers of *Dehalococcoides* populations than the high lactate/PCE enrichments. The differences in total DNA for low chlorinated compounds were small. However, the differences in *Dehalococcoides* concentration were substantial. TCA enrichment did not have any detectable *Dehalococcoides*. The quantitative estimates on total DNA and *Dehalococcoides* 16S rRNA gene copies are summarized in Table 6.8.

Table 6.8. Quantitative Estimation on Total DNA and *Dehalococcoides* populations in pre-enrichment, Low/PCE, High/PCE, c-DCE, TCE, and TCA enriched bottles using PicoGreen and RTm-PCR[a]

	Total DNA (ng/ml)	*Dehalococcoides* (pg/ml)	16S rRNA gene (copies/ml)[b]
Pre-enrichment	85.33 ± 16.42	NQ[c]	NR[d]
High lactate/PCE	186.28 ± 64.25	1.7[e]	[1.0 ± 0.78] x 10^3
Low lactate/PCE	129.60 ± 77.41	256.8	[1.6 ± 0.11] x 10^5
Low lactate/c-DCE	130.57 ± 47.33	195.3	[1.2 ± 0.57] x 10^5
Low lactate/TCE	78.76 ± 4.25	118.1	[7.2 ± 0.40] x 10^4
Low lactate/TCA	90.59 ± 15.70	ND[f]	NR[d]

[a]Triplicate samples of slurry extracted DNA were used except for the high lactate/PCE enrichment. Three PCR reactions were run for each sample point.
[b]16S rRNA gene copies/ml = DNA (μg/ml)x6.023x10^{23}/(1.5x10^6x660x10^6) from He et al. (16)
[c]NQ = detectable but Not Quantifiable by RTm-PCR.
[d]NR = Not Reported due to the absence of data
[e]4 out of 8 results were used in calculated due to the high variation of DNA between samples.
[f]ND = Not Detectable by RTm-PCR.

6.5. Conclusion and Discussion

After 2 years of pulse feedings, once-fed microcosms (microcosms from chapter 5), which initially exhibited slow degradation, showed much higher rates of PCE dechlorination. The high lactate/PCE treatments exhibited improved rates of PCE dechlorination and produced TCE and c-DCE as final byproducts. The low lactate/PCE treatments were capable of sequentially dechlorinating chlorinated ethenes to ethene, and then ethane as final byproduct. These results provide evidence that the ratio between electron acceptor and donor is important. Even though 16S rRNA gene targeted primers revealed the presence of PCE-dechlorinating *Dehalococcoides* in high lactate enrichments, none of the laboratory microcosms completely reduced PCE to ethene. Possible reasons for incomplete dechlorination could be that the high nutrient environment resulted in elevated H_2 levels, which stimulated methanogenic populations, and resulted in limited growth of halorespirating populations.

In this study, 100-µmol lactate (electron donor) and 15 µmol of PCE were used. This is 10 times the minimum reducing equivalents necessary for a complete reduction of the 15 µmol PCE provided to ethene, and is similar to the amounts supplied in other studies (Freedman and Gossett, 1989; Bagley and Gossett, 1990; Vogel and McCarty, 1985). It is not clear from our study whether a smaller amount of lactate or other electron donors would have been a better candidate for even faster and complete dechlorination.

The highest rate of transformation was 0.82/day using PCE as parent product. VC, 1,1-DCE, and even t-DCE were dechlorinated by the PCE enrichment, but degradation rates were slow compared to PCE. Transformation of chlorinated ethenes

did not show specificity over what compound it was enrich with. TCE dechlorination by TCE enrichment have similar rate as TCE dechlorination by PCE enrichments.

Lowering the ratio between substrate to PCE, along with pulse feedings has resulted in substantial enrichment of *Dehalococcoides*. *Dehalococcoides* populations were detected in all of the enriched samples by RTm-PCR. On the contrary, the once fed long-term microcosms (from chapter 5) and TCA enrichments did not show detectable amounts of *Dehalococcoides*. Distinct differences in *Dehalococcoides* DNA levels were observed in low lactate and high lactate enrichments. The low lactate/PCE contained at least 160 times higher *Dehalococcoides* numbers than high lactate/PCE bottles, supporting the hypothesis that the *Dehalococcoides* cells compete better in a low rather than high nutrient environment.

T-RFLP was used to generate profiles of the bacterial communities in chlorinated compound enrichments and pre-enrichment. The number of fragments was consistently higher after enrichment. The detection of greater number of terminal fragments in chlorinated enrichment is an indication that a relatively higher bacterial diversity exists after enrichment on lactate/PCE.

The profiles generated by T-RFLP can vary in two ways. First, there can be variation in the number and size (in basepairs) of terminal restriction fragments (T-RFs) present in profile. Secondly, variation can be found in the height (and consequently the area) of any particular peak (Osborn *et al.* 2000). However, T-RFLP does not resolve closely related organisms or organisms that happen to share the same restriction sites and hence yielding the same TRF. Therefore, a single peak in the electropherogram may represent more than one organism.

A single common fragment present in all of the enriched microcosms (not found in pre-enrichment) by T-RFLP was not found. This may have resulted since these populations are a small percentage of the total community and only small sample amount (3 ml) was used to extract the DNA that it is not sufficient to represent whole microbial system in the bottle.

Even though CO_2 and methane have also been observed as dechlorination products under methanogenic conditions (Vogel and McCarty, 1985; Bradley and Chapelle, 1999), most biotransformation results in the accumulation of partially dechlorinated ethenes such as c-DCE or VC. Complete dechlorinations of chloroethene are known to produce ethene as a final transformation product (Maymo-Gatell, 1995). Despite the reports of ethene persistence in the subsurface (Oremland, 1981; Schink, 1985), ethene reduction was occurred predominant in low lactate enriched conditions. DeBruin (1992) also reported that ethane could be the final product of dechlorination in mixed community using lactate as an electron donor. These often-observed diverse transformations byproducts emphasize the need for better understanding of the underlying microbiological process involved.

Low temperature (15 °C) had no significant effects on transformation of PCE. Transformation of PCE was highest at room temperature instead of 35 °C. This might have resulted since the bottles have been enriched in room temperature over 2 years. The high and complete transformation of chlorinated compound, and the absence of chlorinated end products like VC, makes bioremediation an attractive method for the removal of chlorinated compounds at the plume G site.

It is not known which organisms are responsible for the reduction of ethene to ethane in our study. It is also not clear whether that same organisms are also responsible for TCA transformation since ethane production from CA was not observed. Since they were pulse fed with a different compound over an extended period, we suspect both communities are very different. More research will be required in the future to backup this assumption.

6.6. Literature cited

(a) Fathepure, B. Z. and Boyd, S. A. 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* Sp. Strain Dcm. *Appl. Environ. Microbiol.* 54(12): 2976-2980.

(b) Fathepure, B. Z. and Boyd, S. A. 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters.* 49(2): 149-156.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Maymo-Gatell, X., Y-T, Chien, J.Gossett, and S.H. Zinder. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276;1568-1571.

Hendrickson, E.R. *et al.* 2002. Molecular analysis of *Dehalococcoides* 16S Ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. *Appl. Environ. Microbiol.* 68 (2) 485-495.

Liu, Wen-Tso, Marsh T.L., Hans Cheng, and Larry J. Forney. 1997. Characterization of microbial diversity by determining Terminal Restriction Fragment Length Polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 63 (11) 4516-4522.

Schink, B. 1985. Inhibition of methanogensis by ethylene and other unsaturated hydrocarbons. *FEMS Microbiol. Ecol.* 31:63-69

Debruin, W. P., Kotterman, M. J. J., Posthumus, M. A., Schraa, G., and A. J. B. Zehnder, 1992. Complete biological reductive transformation tetrachloroethene to ethane. *Appl. Environ. Microbiol.* 58(6): 1996-2000.

Bagley, D. M. and J. M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and cis-1,2- dichloroethene by sulfate-reducing enrichment cultures. *Appl. Environ. Microl.* 56(8): 2511-2516.

Vogel T. M. and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.* 49(5): 1080-1083.

Osborn, A. M., E. R. B. Moore and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environm. Microbiol.* 2: 39-50.

Bradley, P. M. and F. H. Chapelle. 1999. Methane as a product of chloroethene biodegradation under methanogenic conditions. *Environ. Sci. Technol.* 33(4): 653-656

Maymo-Gatell, X., Tandoi, V., Gossett, J. M., and, S. H. Zinder. 1995. Characterization of an H_2 utilizing enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis. *Appl. Environ. Microbiol.* 61(11): 3928-3933.

Oremland, R.S. 1981. Microbial formation of ethane in anoxic estuarine sediments. *Appl. Environ. Microbiol.* 42: 122-129.

He, Jianzhong, Kirsti M. Ritalahti, Michael R. Aiello, and Frank Loffler. 2003. Complete detoxicification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species, *Appl. Environ. Microbiol.* 69(2): 996-1003.

Chapter Seven: CHARACTERIZATION OF DECHLORINATING COMMUNITY BY METABOLIC INHIBITORS

7.1. Abstract

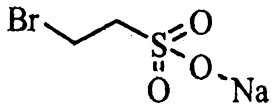
Metabolic inhibitors were used to characterize the anaerobic enrichment, which dechlorinates chloroethene to ethene/ethane using lactate as an electron donor. The effects of BES, molybdate, vancomycin, and sulfate on these enrichments were evaluated over 18 days. While the inhibitory effects depended on experimental concentrations, dechlorination was not hindered by molybdate (inhibitor of sulfate-reducer), but was inhibited by high levels of sulfate. High levels of sulfate stimulated PCE to c-DCE reduction, but not the complete and rapid dechlorination. Therefore, sulfate reducers appear to be not involved in the observed rapid and complete dechlorination. Low levels of BES (5 mM; methanogen inhibitor) specifically inhibited methane production, but the cultures retained dechlorination activity, indicating that the responsible microorganism(s) is (are) non-methanogenic. High concentration of BES (50 mM) inhibited the on-set of dechlorination and dechlorination stopped at c-DCE. The decrease in *Dehalococcoides* DNA in the presence of high levels of molybdate and BES suggests non-specific toxic effects. High levels of vancomycin (100 mg/L; acetogens inhibitors) successfully inhibited acetate production from lactate. The reduction in acetate production also inhibited dechlorination, further suggesting that acetate-utilizing dechlorinating microbes are responsible for the observed dechlorination.

7.2. Introduction

One of the main topics under investigation with regard to the process of reductive dechlorination is the interaction between microorganisms (methanogens, sulfate reducers, and acetogens, etc.) inhabiting contaminated groundwater and aquifer solids. To clarify these interactions and the roles of specific microorganisms in dechlorination, specific inhibitors were used to block the activity of potentially degradative bacteria.

Molybdate is often used as an inhibitor of sulfate reduction (Smith and Klug, 1981) and vancomycin is used to inhibit acetogenic bacteria since it is an inhibitor of cell wall synthesis in gram-positive eubacteria (Distefano *et al.* 1992). The 2-bromoethanesulfonic acid (BES) is frequently used to inhibit of methanogenesis (Distefano *et al.* 1992). Unlike vancomycin, molybdate, and BES, sulfate is not a specific inhibitor of any microbial physiological group. Sulfate addition usually stimulates growth of sulfate-reducing bacteria and thus inhibits metabolic activity of methanogenic bacteria.

The effects of molybdate, BES, vancomycin, and sulfate on PCE dechlorinating community enriched from mixed chlorinated solvents contaminated sediments was investigated. The main objective of this study was to characterize the anaerobic dechlorination activity observed in low lactate/PCE enrichment stimulated by pulse feedings of chlorinated compounds and lactate over 2 years. High rates of dechlorination indicated that the transformation observed in the lactate/PCE enrichment was occurring via halorespiration.

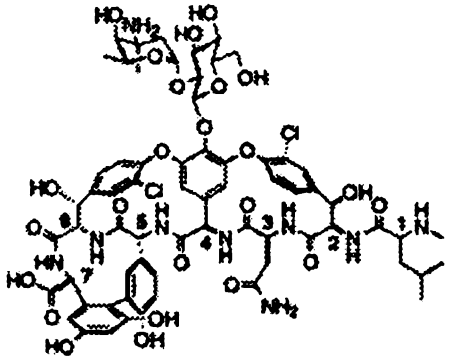


(a) BES

Mo

(b) Molybdate

SO_4^{2-}



(c) Sulfate

(d) Vancomycin

Figure 7.1. Chemical Structures of inhibitors used in this study. Vancomycin structure **obtained** from Doug *et al.* (2002).

Two molecular studies were conducted to characterize the effect of inhibitors on **enrichment**. Terminal restriction length polymorphism (T-RFLP) analyses were **employed** to identify community shifts due to the addition of inhibitors. Specific primers for **detecting** *Dehalococcoides* species were used to determine the effects of inhibitors on such **populations**.

7.3. Materials and Methods

Chemicals

Bromoethanesfulfonic acid (sodium salt, 98 %), vancomycine hydrochloride (4 % water content), and molybdate (sodium salt, 99+%) were purchased from Sigma-Aldrich (Milwaukee, WI).

Inhibitors Studies

Inhibitors were applied to low lactate/PCE vials to investigate the roles of acetogens, methanogens, and sulfate-reducers on the onset of dechlorination. The inhibitors were fed only once at the beginning of the experiment and their effects on transformation were monitored for 18 days. Approximately 4 g (wet weight) of sediments were anaerobically transferred to 40-ml serum vials each and sealed with screw cap containing 22-mm teflon-lined butyl rubber septa. Only low lactate/PCE microcosms were used in this study. The 30-mL of growth medium was amended with lactate (200 μmol), vitamin solutions, phosphate, and groundwater, as described previously (Chapter 4).

After transferred to their new 40-ml vials, vials were spiked with 3 μmol of PCE/month for 3 month. Thirty-three vials out of 45, which showed similar transformations rates to each other, were selected for this experiment in order to reduce the variation of PCE transformation between vials. A total of 33 vials were employed: 3 were prepared with no inhibitor, 9 had 3 different levels of BES (0.5, 5, 50 mM), and 6 had two different levels of vancomycin (25 mg and 100 mg), 9 had 3 different levels of

molybdate (0.5, 2, 6 mM), and 6 had 2 levels of sulfate (0.6 mM, 2 mM). The inhibitor concentrations were similar to other reported studies using dechlorinating enrichments for comparisons (Distefano *et al.* 1992; Pavlostathis and Zhuang, 1991;,DeBest *et al.* 1997; Bagely and Gossett, 1990; deBrun *et al.* 1992). Table 7.1 depicts the list of inhibitors and their concentrations used in this chapter.

Prior to each electron donor and PCE addition, the vials were thoroughly purged with 80% N_2- 20% CO_2. The intent of purging was to prevent accumulations of VC and/or ethene, which is a known inhibitor of methanogenesis (Schink. 1985). All vials had 200 µmol (5.72 mM) lactate as electron donor before 3 µmol (85 µM) PCE addition. Vancomycin was added directly after purging the vials. The rest of inhibitors (BES, molybdate, and sulfate) were bubbled with N_2, autoclaved, and then used for inhibition experiments. Vials were kept in a constant room temperature throughout the course of this experiment.

Table 7.1. The list of inhibitors and their concentrations used in this chapter

Samples	Inhibitor Concentrations (mM)[a]	Number of bottles
Control[b]	0	3
BES	0.5	3
	5	3
	50	3
Molybdate	0.5	3
	2	3
	8	3
Sulfate	0.6	3
	2	3
Vancomycin	25 mg/L	3
	100 mg/L	3

[a]Concentrations were in mM otherwise noted.
[b]Control had no inhibitor addition.

7.4. Results

7.4.1. Effects of BES

The effects of three different levels of BES on the dechlorination of PCE by low lactate/PCE enrichments was examined. Earlier microcosm results (Chapter 6) indicated that methane production was low, suggesting that methanogens do not play significant role in dechlorination. However, this observation does not completely rule out a role for methanogens in PCE dechlorination.

The effects of three levels of BES on PCE transformation during 18 days are depicted in Figure 7.3. Low levels of BES (0.5 mM) amendment showed no immediate effects on dechlorination, but 5 mM amendments exhibited delayed and partially inhibited dechlorination that DCEs and VC accumulated. Even though, 50 mM BES inhibited PCE dechlorination significantly, it did not stop PCE degradation completely. PCE transformation was delayed by 50 mM BES and did cause the production and accumulation of c-DCEs. This indicates that repeated dose of BES may be need to completely stop PCE dechlorination.

Ethene production was observed only by 0.5 mM BES treated vials during this 18-day period and both 0.5 mM BES treated vials and control vials reduced PCE in 6 days that VC and ethene were final byproduct after 18 days (Figure 7.2 and 7.3). This result suggests that small dose of BES had no effect on dechlorination.

Methane formation was inhibited by all doses of BES (Table 7.3). Methane productions from 0.5 mM and 5 mM BES were only 43 and 18 percent of the methane produced by control microcosms. This result shows that the residual PCE were probably not sufficient to suppress methanogenesis and BES was the one, which inhibited

methanogenesis. Hydrogen sulfide, lactate, acetate, and propionate concentrations were measured for all vials, where possible (Table 7.3). Even though BES inhibited PCE transformation, no apparent negative or positive effects on acetogenesis was observed (Table 7.3). Methane production in the BES 50 mM was less than 1 % of total equivalents were measured as methane, however non-specific effects were observed at this concentration. Sulfide production was not measured in the BES inhibited microcosms due to interference by BES.

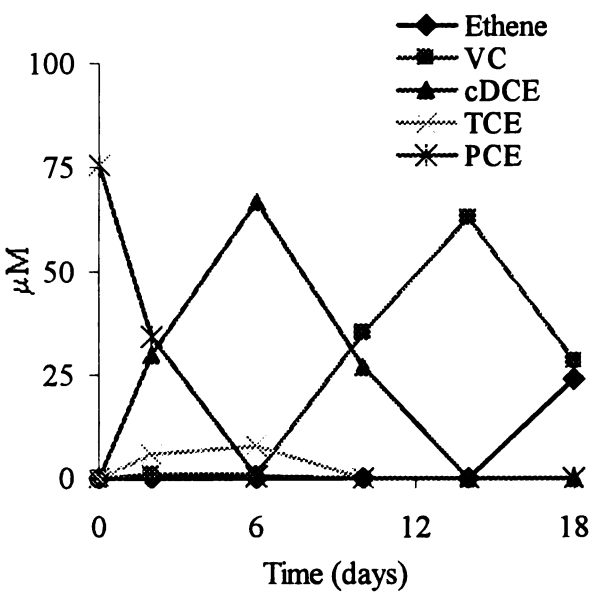


Figure 7.2. The PCE transformation by control.

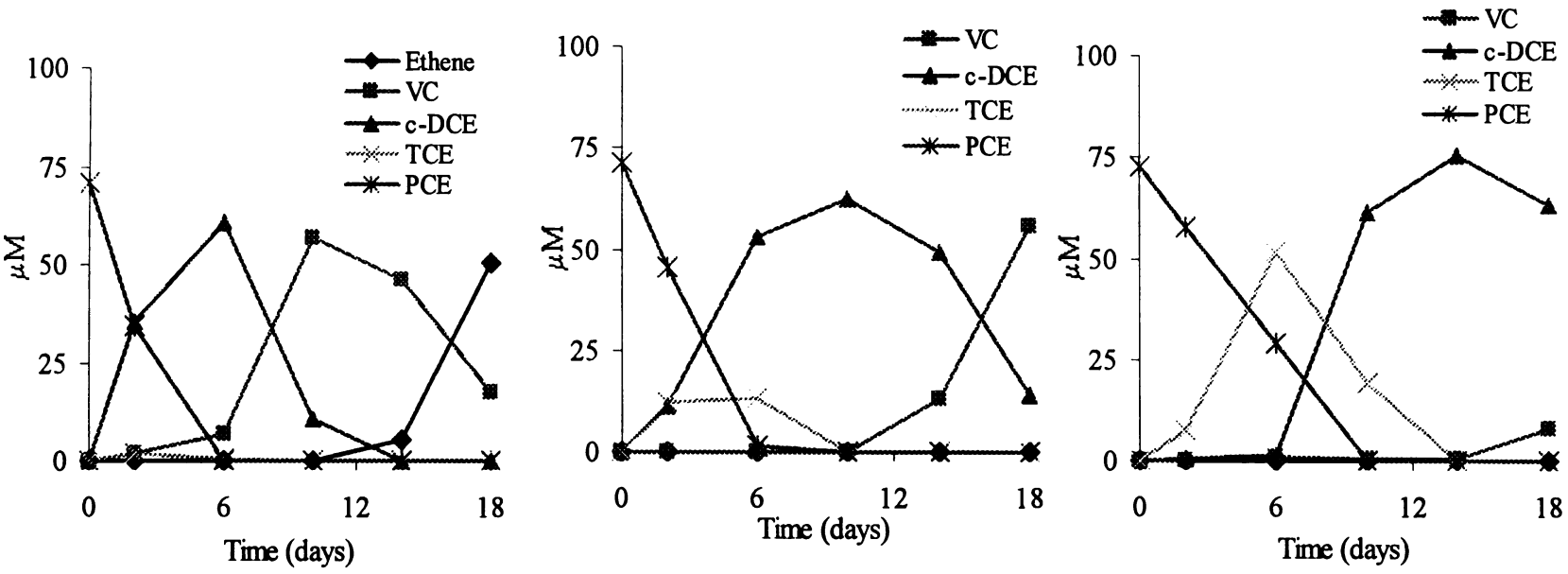


Figure 7.3. The effects of PCE transformation by 0.5 mM 5 mM, and 50 mM of BES. Microcosms were amended at 0.5 mM, 5 mM, and 50 mM of BES at zero time only.

7.4.2. Effects of Molybdate and Sulfate

Two levels of sulfate and three level of molybdate were added to observe their potential effects on PCE transformation. The effects of molybdate and sulfate on PCE dechlorination during 18-day period are shown on Figure 7.4 and Figure 7.5.

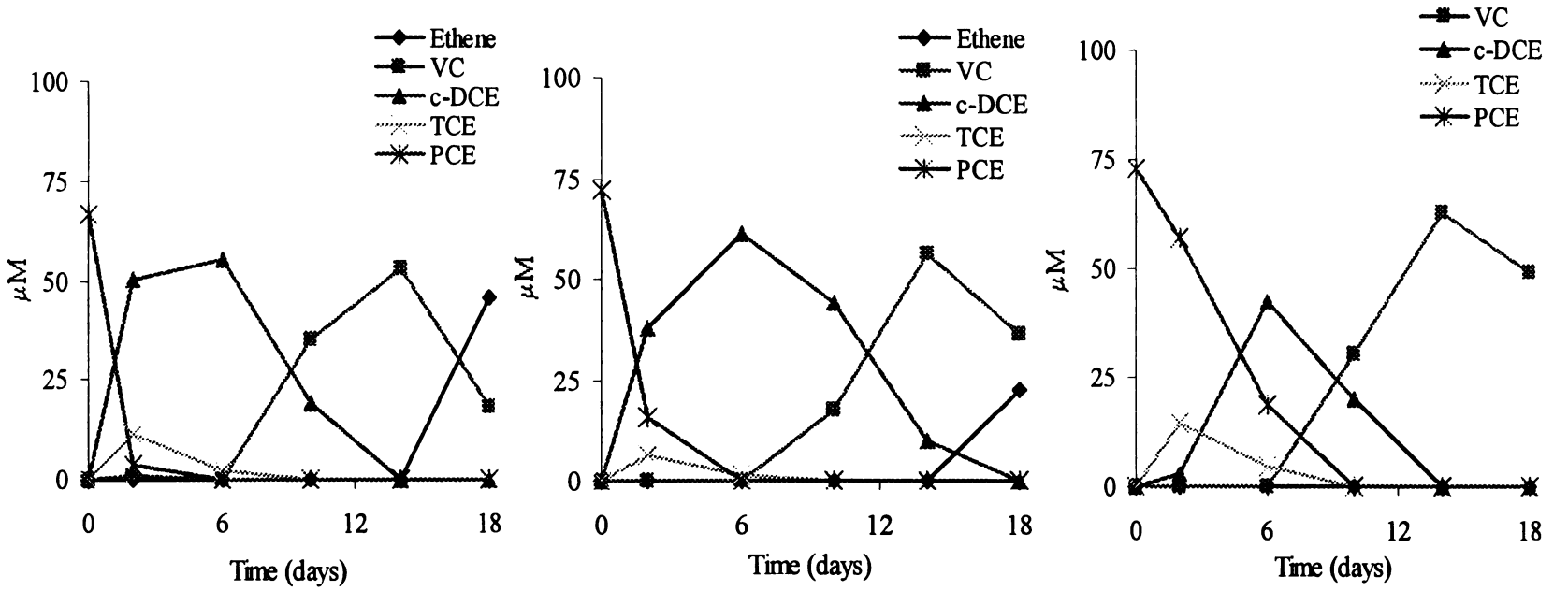
Dechlorination was neither inhibited nor improved by low levels of molybdate additions (0.5 and 2 mM). Both molybdate treated vials and control vials reduced PCE in 3 – 6 days and VC and ethene were final byproducts after 18 days (Table 7.2). High levels of molybdate (6 mM) amendments showed delayed and partially inhibited dechlorination that DCEs and VC accumulated. This suggests non-specific toxic effect of molydate on dechlorination.

The PCE dechlorination was neither delayed nor inhibited by 0.6 mM sulfate, but high levels of sulfate (2 mM) significantly reduced dechlorination of PCE that c-DCE were major byproduct after 18 days.

Molybdate did inhibit sulfate reduction (Figure 7.6). After 18 days of incubation, the residual sulfate from groundwater persisted in the microcosms amended with molydate. In sulfate-amended vials, low levels of sulfate (0.6 mM) were all reduced within 7 days, but high levels of sulfate (2 mM) persisted over 18 days.

Hydrogen sulfide, lactate, acetate, and propionate concentrations were measured for all vials, where possible (Table 7.3). Essentially stoichiometric sulfide production was observed in the 0.6 mM and 2 mM sulfate treated vials. Methane production was very low, accounting for only 1 % of the electron equivalents measured.

Both high sulfate and high molybdate inhibited methane production. Lesser amount of methane was observed by sulfate (0.6 mM and 2 mM) amendments compare to the molybdates and controls. However, high levels of molybdate (6 mM) did exhibit inhibitory effect on methanogenesis. As shown in Table 7.3, low levels of molybdate (0.5 and 2 mM) did not inhibit methane production while high level inhibited methanogensis up to 40 percent.

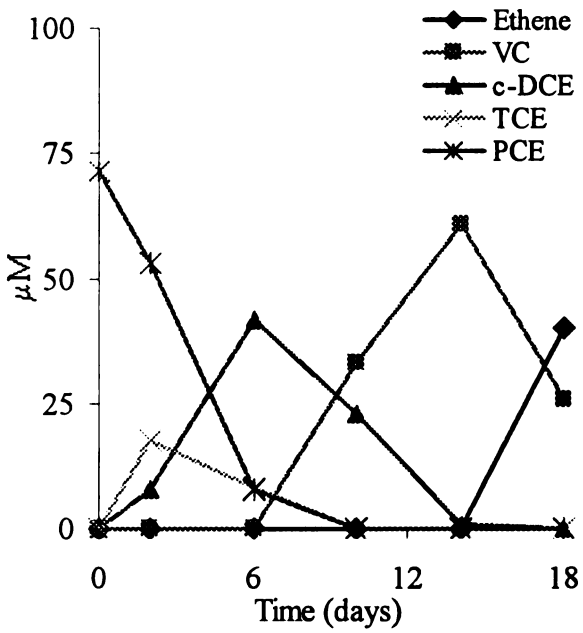


(a) Molybdate 0.5 mM (b) Molybdate 2 mM (c) Molybdate 6 mM

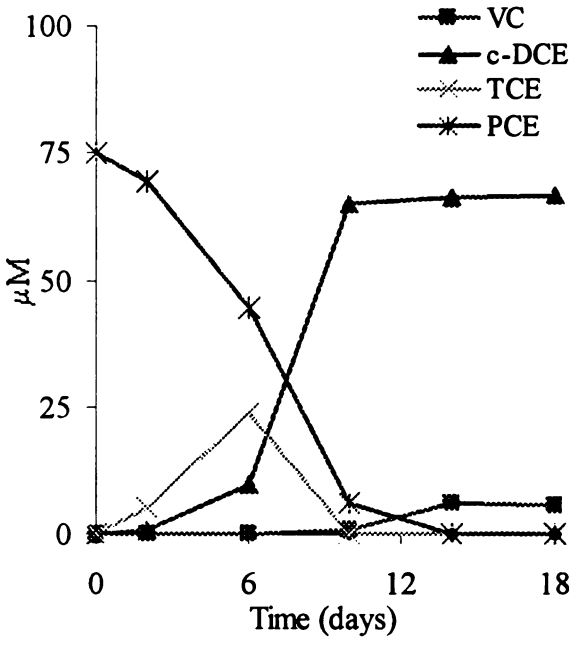
Figure 7.4. The effects of PCE transformation by 0.5 mM 2 mM, and 6 mM of molybdate. Microcosms were amended at 0.5 mM, 2 mM, and 6 mM of molybdate at zero time only.

Figure 7.5. The effects of PCE transformation by 0.6 mM and 2 mM of sulfate. Microcosms were amended with sulfate at time zero only.

(a) 0.6 mM Sulfate



(b) 2 mM Sulfate



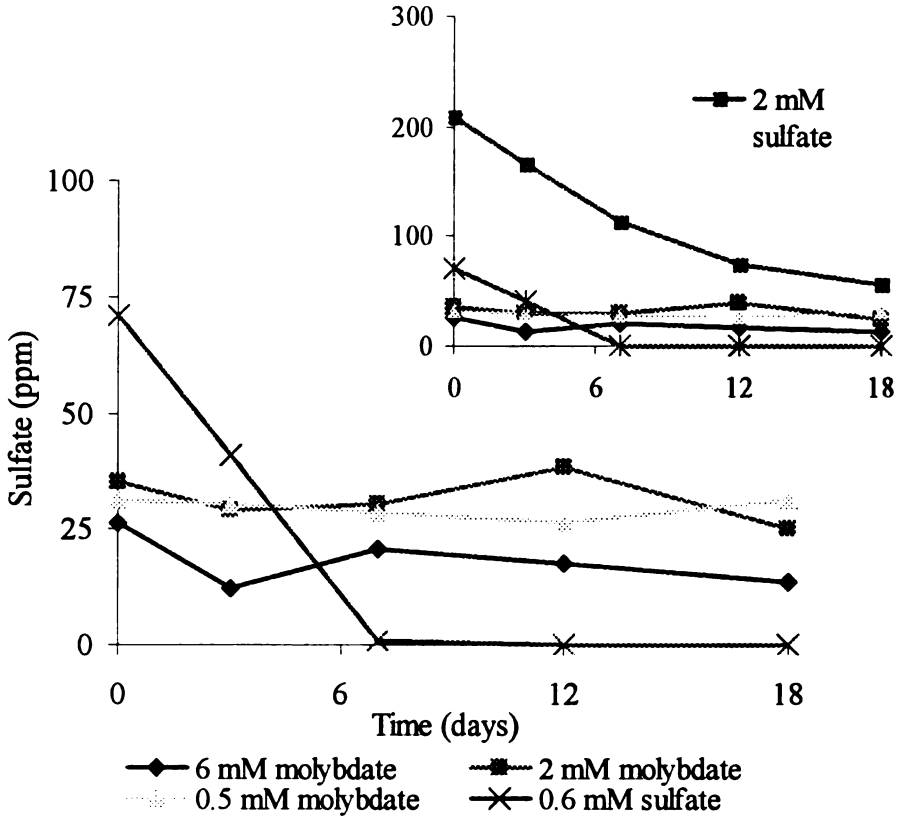


Figure 7.6. Sulfate reduction and effect of molybdate on residual sulfate in microcosms. The reduction of high sulfate (2 mM) is shown on the bigger scale.

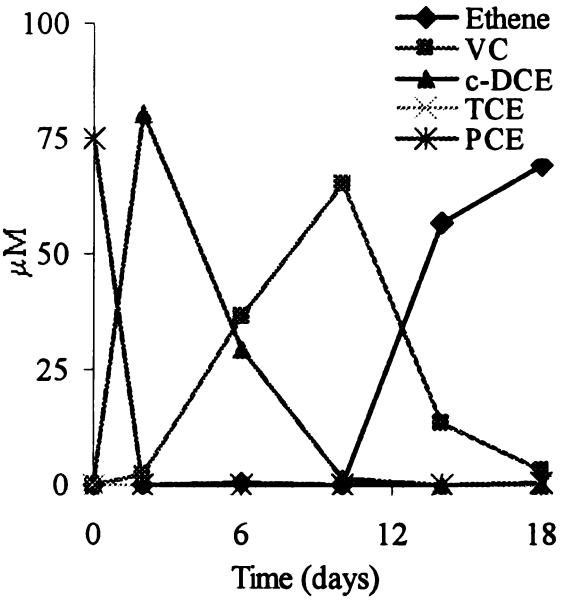
7.4.3. Effect of Vancomycin

Two levels of vancomycin were added to lactate/PCE enrichments to observe its potential effects on PCE transformation. On the basis of the electron balance from Chapter 6, acetogenesis from lactate was the predominant pathway, and the role of acetogens in the PCE dechlorination must be considered. Results for vancomycin (acetogenesis inhibitor)-amended bottles are shown in Figure 7.6. Figure 7.7 indicates that 25 mg/L of vancomycin had no significant effect on transformation while 100 mg/L of vancomycin delayed the transformation of PCE that VC and c-DCE was observed as final byproduct during 18 days.

Table 7.3. shows only low acetate production, demonstrating the inhibitory effect of vancomycin on acetogens. Methane productions from vancomycin treated vials were also smaller than controls.

The decline in ethene production and the detection of VC and DCEs by higher concentration of vancomycin indicated that acetogens are indirectly involved in PCE dechlorination. Lactate metabolism and the resultant products are shown in Table 7.3. Acetogenesis accounted for almost 50 percent of electron donor used for controls, and reduced products from PCE dechlorination represented nearly 10 percent.

(a) 25 mg/L



(b) 100 mg/L

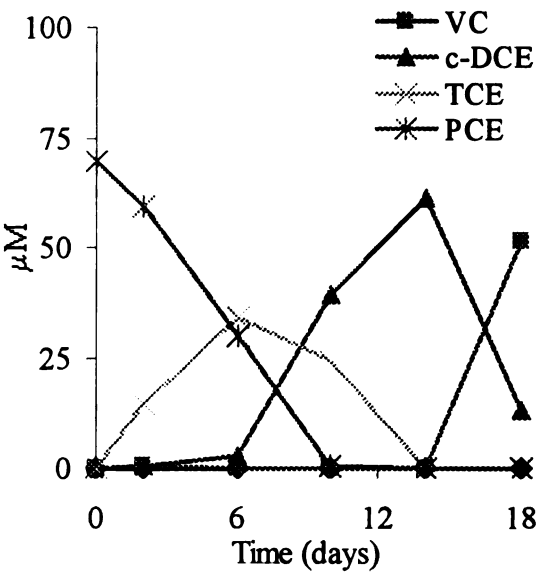


Figure 7.7. The effects of PCE transformation by 25 mg/L and 100 mg/L of vancomycin. Microcosms were amended with vancomycin at time zero only.

Table 7.2. Effect of inhibitors on reductive dechlorination of PCE after 18 days[a] (Initial PCE load was between 2.33 and 2.63 μmol/bottle).

Inhibitor	Amt of product formed (μmol)				
	PCE	TCE	c-DCE	VC	Ethene
BES					
0.5 mM	0	0	0	0.7	1.8
5 mM	0	0	0.5	1.9	0
50 mM	0	0	2.2	0.3	0
Sulfate					
0.6 mM	0	0	0	0.9	1.4
2 mM	0	0	2.3	0.2	0
Vancomycin					
25 mg/L	0	0	0	0.1	2.4
100 mg/L	0	0	0.5	1.8	0
Molybdate					
0.5 mM	0	0	0	0.6	1.6
2 mM	0	0	0	1.0	1.5
6 mM	0	0	0	0.5	2.0
No inhibitors	0	0	0	1.1	1.2

[a]Lactate (200 μmol) was supplied as electron donor. The data are means obtained for triplicates samples after 18 days. The standard deviation was $< \pm 0.2$.

Table 7.3. Influences of inhibitors on electron balance after 7 days[a]

Inhibitor	Produced μeqiv.				Lactate	Acetate/Lactate (%)	CH_4/Lactate (%)
	CH_4	HS^-	Acetate	Propionate			
BES							
0.5 mM	12	NM[b]	106	58	-242	44	7
5 mM	5		123	60	-273	45	3
50 mM	2		138	41	-267	52	1
Sulfate							
0.6 mM	5	167	70	42	-367	19	1
2 mM	5	275	73	30	-465	16	1
Vancomycin							
25 mg/L	18	33	27	9	-153	17	17
100 mg/L	11	33	15	7	-102	15	10
Molybdate[d]							
0.5 mM	33	0	88	21	-223	39	14
2 mM	34	0	101	28	-248	41	17
6 mM	20	0	110	17	-211	47	10
Control	28	33	135	36	-248	49	10

[a]Lactate (200 μmol) was supplied as electron donor. All units are μequivalent/vial, otherwise noted. Reducing equivalents were calculated assuming (μequiv/μmol): lactate, 4; propionate, 6; acetate, 8; CH_4, 8; and HS^-, 8.
[b]NM, not measurable due to BES interference with sulfide measurement.
[c]NR, not reported due to inability to measure sulfide concentration
[d]Measuring acetate from 2 mM and 6 mM molybdate was difficult due to interference by molybdate. Therefore, acetate concentrations reported here is obtained by IC, not HPLC.

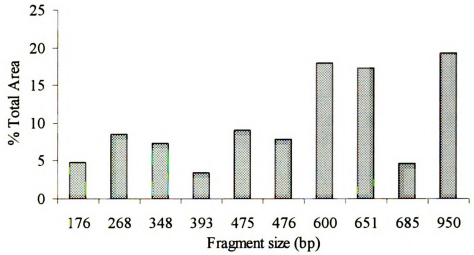
7.4.4. Microbial Community Analysis

After extracting DNA from 3-ml sediment slurry of microcosms treated with inhibitors for 18 days, PCR is amplified for T-RFLP analysis and Real time PCR was conducted for quantification of dechlorinating populations. T-RFLP was used to determine if the microbial community had been changed by the exposure to inhibitors. Changes in peak patterns and number of fragments can indicate community shifts. Since it is possible for different organisms to share a common restriction site in the 16S genes, only a single dominant peak on the electropherogram was considered for that fragment.

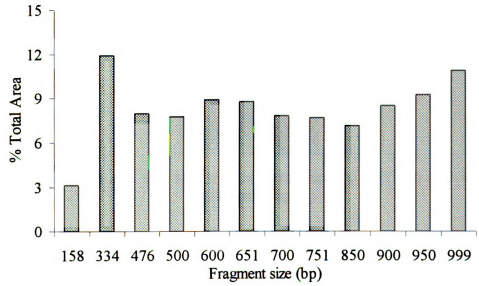
Not all T-RFLP produced usable patterns for inhibitor study. Many of them were over digested or not digested enough to represent the effect of inhibitors on microbial communities. Using digestions with the enzyme *HhaI*, inhibitors produced different patterns compare with controls. Each inhibit treated microcosms had different patterns from control in terms of terminal restriction fragments (TRFs) sizes indicating change in overall bacterial community structure (Figure 7.8). List of the T-RFLP fragments and peak areas using *HhaI* are shown on Appendix F.

Figure 7.8. T-RFLP profiles generated by using dechlorinating enrichments treated with inhibitors. Each fragment size indicates percent fragment area: (a) BES (0.5 mM); (b) molybdate (2 mM); (c) sulfate (0.6 mg/L); and (d) vancomycin (25 mg/L).

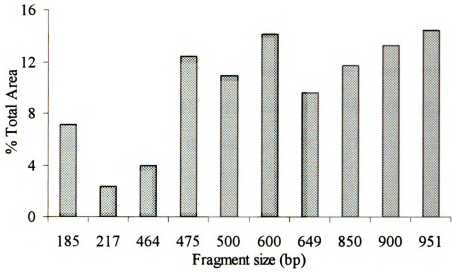
(a) BES



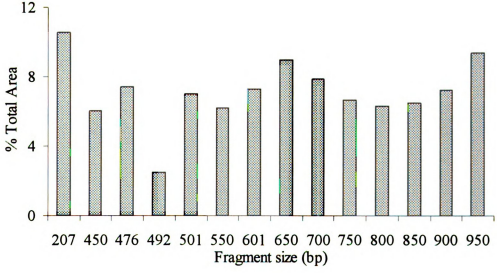
(b) Molybdate



(c) Sulfate



(d) Vancomycin



Microcosms amended with inhibitors such as BES showed significant decay in the total populations and *Dehalococcoides* populations at high levels (Table 7.4). These high levels (50 mM BES) exhibited non-specific toxic effect, which led to a 60 % decrease in total DNA. The 16S rRNA demonstrated a decrease in *Dehalococcoides* populations due to the high levels of BES. High levels of molybdate (6 mM) also exhibited non-specific toxic effects in that both total DNA and *Dehalococcoides* levels declined.

The low levels of sulfate and vancomycin did not significantly alter the total DNA levels and *Dehalococcoides* populations. While 25 mg/L vancomycin exhibited no effects on both DNA and *Dehalococcoides* yield, less total DNA and *Dehalococcoides* were observed at high levels (100 mg/L).

Table 7.4. Quantitative Estimation of Total DNA and *Dehalococcoides* populations in the BES, molybdate, sulfate, and vancomycin impacted microcosms using PicoGreen and RTm-PCR[a]

	Total DNA (ng/ml)		16S rRNA gene (copies/ml)[b]	
	before	after	before	after
BES				
0.5 mM	218 ± 39	430 ± 55	5.8×10^4	2.8×10^6
5 mM	295 ± 46	337 ± 38	4.3×10^5	8.3×10^6
50 mM	278 ± 22	103 ± 26	1.9×10^5	1.0×10^3
Sulfate				
0.6 mM	256 ± 19	477 ± 25	5.4×10^4	6.1×10^7
2 mM	219 ± 29	480 ± 27	4.2×10^3	7.9×10^5
Vancomycin				
25 mg/L	285 ± 55	323 ± 19	1.3×10^2	5.3×10^4
100 mg/L	247 ± 76	227 ± 76	5.7×10^4	8.8×10^4
Molybdate[d]				
0.5 mM	229 ± 54	450 ± 66	5.6×10^4	6.5×10^9
2 mM	198 ± 51	329 ± 64	0.7×10^7	5.3×10^{10}
6 mM	232 ± 17	287 ± 19	8.1×10^5	1.9×10^8
Control	185 ± 33	376 ± 46	5.0×10^6	8.7×10^{10}

[a]Triplicate samples of slurry extracted DNA were used. Three PCR reactions were run for each sample point.
[b]16S rRNA gene copies/ml = DNA (μg/ml)x6.023×10^{23}/($1.5 \times 10^6 \times 660 \times 10^6$) from He *et al.*(2003)
RTm-PCR data has shown as individual microcosms data (due to temporal variation between microcosm).
Gene copy numbers are reported for PCR runs in which dilution series, minimum signal and cycle parameters where met.

7.5. Conclusion and Discussion

Characterization of the dechlorination activities in an undefined microbial community is an important step in the identification of the responsible microbial populations. Information from these studies can provide a basis for further enrichment, and may facilitate the identification and isolation of microorganisms capable of dechlorination. The microbial group specific inhibitors can sometimes be used to distinguish which group is responsible for a specific activity (Oremland and Capone, 1988).

To investigate the role of methanogenic, acetogenic and sulfate-reducing microorganisms on the transformation of PCE, 4 inhibitors; BES, molybdate, sulfate, and vancomycin were used. Although methane production was low in lactate/PCE enrichments, this fact alone does not rule out a role for methanogens in PCE reduction. BES, considered a selective inhibitor of methyl coenzyme M reductase, the enzyme that catalyzes the final step in methanogensis (Gunsalus *et al.* 1978), was therefore used to inhibit methanogenesis in the enrichments. Adding BES eliminated methanogenic activity, which plays an important role in anaerobic microbial processes (Bhatnagar *et al.* 1991; Zeikus, 1977), but a partial dechlorination activity was still remained. Repeated additions of BES might have been required in order to completely stop dechlorination, possibly because microbes can degrade BES. Schink (1985) has noted this is a draw back to the use of BES.

Many puzzling (diverse) results have been published regarding the use of BES. Previous works have demonstrated that methanogens can dechlorinate PCE (Fathepure *et al.* 1987; Fathepure and Boyd, 1988). However, when methanogenesis stops, PCE

dechlorination by methanogens also stops. In previous research conducted by Freedman and Gossett (Freedman and Gossett, 1989), 5 mM BES was found to be sufficient for the complete cessation of methane production and TCE dechlorination by mixed methanogenic cultures.

In the case of mixed sulfate-reducing cultures spiked with 50 mM BES, methane was not produced and PCE dechlorination declined by approximately 25 % as compared to non-inhibited cultures (Bagley and Gossett, 1990). Loffler *et al.* (1997) showed that BES (2 mM) inhibited dechlorination in the absence of methanogens, indicating BES results should be interpreted with caution.

From our study, BES inhibited dechlorination beyond DCEs when added at 50 mM. Methane production in the presence of 50 mM BES was less than 1 % of the total measured electron equivalents, indicating very little methanogenic activity. However, PCE dechlorination was not completely stopped. These observations deviate substantially from the pattern of PCE dechlorination observed with methanogenic systems. Therefore, it appears probable that dechlorinators are responsible for the observed dechlorination.

Lactate/PCE enrichment also had high acetate production that a majority of reducing equivalents was accounted for acetate production with the remainder being accounted for by PCE reduction (refer to Chapter 6). Vancomycin effectively inhibited acetogenesis in lactate fed bottles in every concentration. Vancomycin is a eubacterial peptidoglycan synthesis inhibitor, (is bacteriocidal,) and is more effective against gram-positive than gram-negative eubacteria (Bock and Kandler, 1985; Joklik *et al.* 1984). It is unlikely that vancomycin directly inhibited acetogenesis in the microcosms but rather the

inhibition was probably due to the bacteriocidal effects of vancomycin on acetogens, most of which are gram positive (Ljungdahl, 1986). In contrast to 100 mg/L-amended bottles, 25 mg/L amended bottles continued to dechlorinate well after the inhibition of acetogenesis.

The inhibition of PCE dechlorination by the high levels of vancomycin (100 mg/L) suggests that PCE dechlorinator could not use lactate directly but rather required lactate metabolism, most likely acetate (or H_2) as the electron donor for reductive dechlorination. Figure 7.9 shows a hypothetical model of the lactate metabolism and PCE transformation by the enrichment, which includes the hypothesis that acetate and H_2 as the actual electron donor for PCE dechlorination.

Control (no inhibitors) performed similarly with respect to electron donor use; acetate production accounted for a majority of reducing equivalents, with the remainder being accounted for by PCE reduction and methane production.

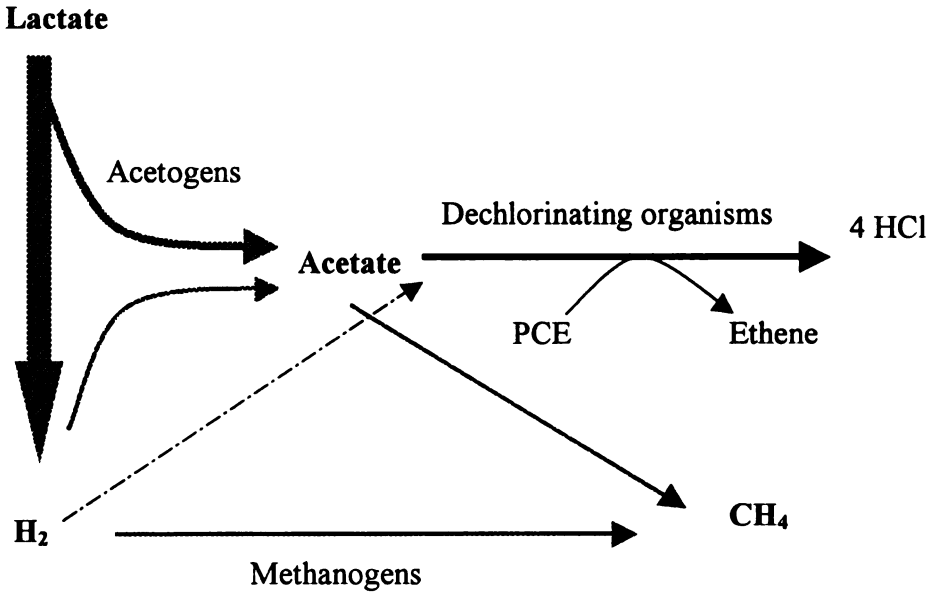
Molybdate is generally regarded as a specific inhibitor of sulfate-reducing bacteria (SRB) (Taylor and Oremland, 1979) and thus widely used in microbiology studies due to its specificity (Oremland and Capone, 1988). Molybdate is an analog that compete with sulfate for the active site of ATP sulfurylase, resulting in formation of an unstable analog-AMP complex, which readily hydrolyzes to AMP and the sulfate analog; the latter is then available to again react with ATP sulfurylase (Wilson and Bandurski, 1958). Repetition of these events depletes intracellular ATP, thereby halting growth of the bacteria and, as a result, inhibiting sulfate reduction.

Even though sulfate reduction was efficiently prevented by small amounts of molybdate (0.5 and 2 mM), no inhibition on PCE dechlorination was observed. The

inhibition of PCE dechlorination by high molybdate concentrations might have been resulted by a toxic effect since it can bind free sulfide ions to form a molybdosulfide complex (Tonsager and Averill, 1980), and this could influence microorganisms requiring sulfide ions (Oremland and Capone, 1988) e.g. methanogens (Bhatnagar *et al.* 1991; Zeikus, 1977). The methane production from 6 mM molybdate treated vials was less than 0.5 mM or 2 mM, which confirms the toxic effects on methanogens.

T-RFLP was used to discern the microbial communities's response to the inhibitor treatments. The restriction enzyme digest using the restriction enzymes *HhaI* was used to obtain a T-RFLP community fingerprint from the extracted DNA of the microcosm sample. Profiles of the bacterial communities showed extensive shifts due to inhibitors in terms of terminal restriction fragments (TRFs) numbers/sizes.

Figure 7.9. Hypothetical model based on DiStefano *et al.* (1992), for carbon and electron flow in a lactate-PCE enrichment



7.6. Literature cited

Smith, L.R. and M.J. Klug. 1981. Electron donors utilized by sulfate-reducing bacteria in eutrophic lake sediment. *Appl. Environ. Microbiol.* 42:116-121

Distefano, T. D., Gossett, J. M., and S. H. Zinder. 1992. Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl. Environ. Microbiol.* 58(11): 3622-3629.

Steven D. Dong, Markus Oberthür, Heather C. Losey, John W. Anderson, Ulrike S. Eggert, Mark W. Peczuh, Christopher T. Walsh, and Daniel Kahne. 2002. The structural basis for induction of vanB resistance. *Am. Chem. Soc.,* 124 (31), 9064 -9065

Pavlostathis, S.G.and P. Zhuang. 1991. Transformation of trichloroethylene by sulfate-reducing cultures enriched from a contaminated subsurface soil. *Appl. Microbiol. Biotech.* 36 (3): 416-420.

DeBest, J. H., Jongema, H.,Weijling. A., Doddema, H. J., Janssen.,D. B. and W. Harder. 1997. Transformation of 1,1,1-trichloroethane in an anaerobic packed-bed reactor at various concentrations of 1,1,1-trichloroethane, acetate and sulfate. *Applied Microbiology and Biotechnology.* 48 (3): 417-423.

Bagley, D. M. and J. M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and cis-1,2-dichloroethene by sulfate-reducing enrichment cultures. *Appl. Environ. Microbiol.* 56(8): 2511-2516.

DeBruin, W. P., Kotterman, M.J. J, Posthumus, M.A., Schraa, G., and A.J.B. Zehnder 1992. Complete biological reductive transformation of tetrachloroethene to ethane. *Appl. Environ. Microbiol.* 58(6) 1996-2000.

Schink, B. 1985. Inhibiton of methanogensis by ethylene and other unsaturated hydrocarbons. *FEMS Microbiol. Ecol.* 31:63-69

Ballapragada, B. S., Stensel, H. D., Puhakka, J. A., and J. F. Ferguson. 1997. Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ. Sci. Technol.* 31(6): 1728-1734.

Fennell, D. E., Gossett, J. M., and, S. H. Zinder. 1997. Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.* 31(3): 918-926.

He, Jianzhong, Kirsti M. Ritalahti, Michael R. Aiello, and Frank Loffler. 2003. Complete detoxicification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species, *Appl. Environ. Microbiol.* 69(2): 996-1003.

Oremland, R.S., and D.G. Capone. 1988. Use of specific inhibitors in biogeochemistry and microbial ecology. In K.C.Marshall (ed.), Advances in microbial ecology. Vol. 10. Plenum Publishing Co.

Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe. 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermotrophicum*. *Biochemistry* 17:2374-2376

Bhatnagar, L., M.K.Jain, and J.G.Zeikus. 1991. p. 251-270. Methanogenic bacteria. In variations in autotrophic life. Academic Press, Orlando, FL.

Zeikus, J.G. 1977. The biology of methanogenic bacteria. *Bacteriol. Rev.* 41:514-541

Fathepure, B., Nengu, J. P. and S.A. Boyd. 1987. Anaerobic bacteria that dechlorinate perchloroethene. *Appl. Environ. Microbiol.* 53(11): 2671-2674.

Fathepure, B. Z. and S. A. Boyd. 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters.* 49(2): 149-156.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Loffler, Frank E., K. M. Ritalahti.and James M. Tiedje. 1997. Dechlorination of chlorethenes is inhibited by 2 –Bromoethanesulfonate in the absence of methanogens. *Appl. Environ. Microbiol.* 63(12): 4982-4985

Bock, A. and O. Kandler. 1985. Antibiotic sensitivity of Archaebacteria, p. 525-544. In C. R. Woese and R.S. Wolfe (ed.), The bacteria: a treatise on structure and function, vol. VIII. Archaebacteria. Academic Press, Inc., New York.

Joklik, W.K., H.P. Willett, and D. B Amos. 1984. Zinsser microbiology. Appleton-Century-Crofts, East Norwalk, Conn.

Ljungdahl, L.G. 1986. The autotrophic pathwa of acetate synthesis in acetogenic bacteria. *Annu. Rev. Microbiol.* 40: 415-450.

Taylor, B. F., and R. S. Oremland. 1979. Depletion of adenosine triphosphate in Desulfovibrio by oxyanionsof group VI elements. *Curr. Microbiol.* 3:101-103

Wilson, L. G., and R. Bandurski. 1958. Enzymatic reactions involving sulfate, sulfite, selenate, and molybdate. *J. Biol. Chem.* 233:975-981

Tonsager, S.R., and B. A. Averill. 1980. Difficulties in the analysis of acid-labile sulfide in Mo-S and Mo-Fe-S systems, *Anal. Biochem.* 102:13-15.

Chapter Eight: ENGINEERING APPLICATIONS

8.1. Enrichment of Rapid and Complete Dechlorinating Activity

The research presented in this thesis provides insights into the practical application of selective biostimulation: how to enrich microbial populations that are capable of complete and rapid degradation of a contaminant. For oligotrophic aquifers such as Plume G, the native dechlorinating microflora are present at such small numbers that selective stimulation of the degradative indigenous soil microorganisms is required.

However, the success of stimulating dechlorinating activity may be severely limited by biological factors such as competition among dechlorinating and non-dechlorinating microorganisms, and the nutritional requirements of dechlorinating bacteria. Although the complete transformation of PCE to ethene have been observed (DiStefano *et al.* 1991; Freedman and Gossett, 1989), the PCE transformation often stopped at TCE (Fathepure and Boyd, 1988), or c-DCE (Sewell and Gibson, 1991) or VC (Vogel and McCarty, 1985). These incomplete reductive dechlorination reactions pose environmental risks because the intermediates c-DCE and VC are themselves hazardous environmental pollutants.

One of the most significant findings from this research has been the achievement of complete transformation of chlorinated ethenes by mixed cultures, which originally exhibited only limited and incomplete dechlorination. By pulse feedings of lactate and chlorinated compounds over extended periods, rapid and complete dechlorinating activity has been enriched. However, when enrichments were stimulated in high nutrient

conditions, slow and incomplete dechlorinating activity was expressed. The time sequences of the enrichments are summarized in Table 8.1. After 1 year of enrichment, 36 out of 36 bottles from low lactate/PCE enrichment showed complete activity compared to 0 out of 28 bottles from high lactate/PCE enrichments (Table 8.2). In addition, the transformation rate of PCE became 15 times faster in low lactate/PCE enrichments than high lactate/PCE enrichment after approximately same pulse feedings (12 cycles).

Table 8.1. Enhancement of first order rate k (1/day) on disappearance of parent products by pulse feedings over time.

Number of Pulse feedings	High Lactate/PCE	Low Lactate/PCE	Low Lactate/TCE	Low Lactate/c-DCE
3	0.008	0.012	0.01	0.01
6	0.04	0.38	0.42	0.33
12	0.06	0.85	0.72	0.62

Higher levels of *Dehalococcoides* populations were present in low lactate enrichments compare to high lactate enrichments. This demonstrated that pulse feedings strategy of chlorinated compounds and low electron donor could be a preferred method for the stimulation of halorespiring microbes. The mechanisms by which nutrient pulse feeding stimulates *Dehalococcoides* has two main components: (1) pulse feeding allows sustained H_2/acetate production and cofactors generation by fermentators, and (2) the relative low nutrient/high chlorinated ethene levels favor microbes that can exploit the energy ($\Delta G^{\circ\prime}$) present in the oxidized Cl-C bonds. Another examples of cofactors known to affect bioremediation are iron and copper for *Pseudomonas* KC (Tatara *et al.* 1993;

Dybas *et al.* 1995; Kim, 1998; Dybas *et al.* 1998). Schematics showing these relationships are included in Figure 8.1.

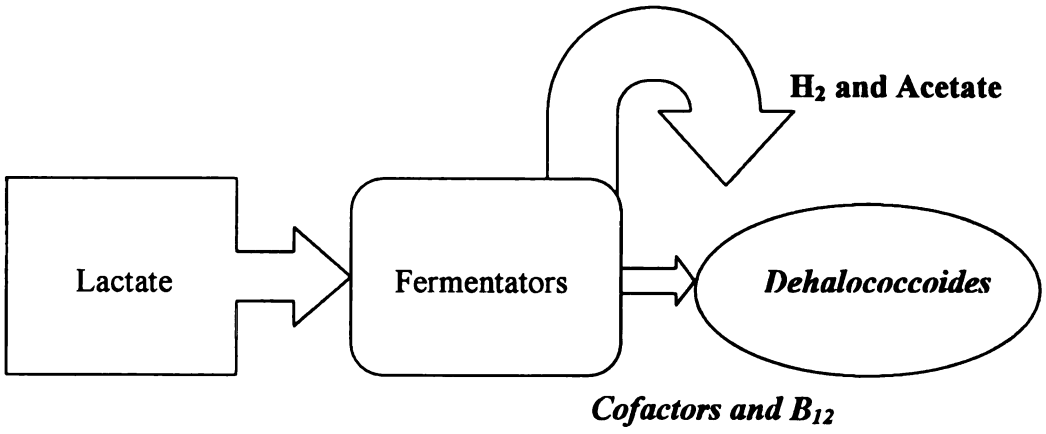


Figure 8.1. A schematic of the relationship between fermentators and *Dehalococcoides*.

Table 8.2. Comparisons of maximum rate between the pulse fed and once-fed enrichments in enhancing dechlorination activity observed from each condition. First order rates are obtained after the end of the enrichment experiment.

Method	Chloroethene	Electron donor	Maximum k (1/day)[a]	Success Rates[d]
Once-fed	TCE[b]	High Lactate	0.004	0/220
Pulse-fed	PCE[b]	High Lactate	0.08	0/28
Pulse-fed	PCE[b]	Low Lactate	0.82	36/36
Pulse-fed	TCE[b]	Low Lactate	0.73	36/36
Pulse-fed	cDCE[b]	Low Lactate	0.66	36/36
Pulse-fed	TCA[c]	Low Lactate	0.25	12/36

[a] Based on first-order rate equation $C=C_oe^{-kt}$.
[b] Final byproduct to ethene or ethane.
[c] Final byproduct to chloroethane.
[d] Occurrence of complete and rapid degradation.

8.2 Field Applications

The remediation of groundwater containing chlorinated compounds is challenging. Traditional approaches for groundwater remediation have relied on extraction, followed by a physical/chemical process (e.g., air stripping and carbon adsorption). This approach has some major disadvantages such as high cost, inefficiencies in removing contaminants absorbed to the aquifer material, and requirement of pumping, treating, and ultimately disposal of large volumes of water.

In situ bioremediation is an alternative approaches to these more traditional methods due to the discovery of bacterial populations that grow on chlorinated compounds, thus efficiently reducing and detoxifying these compounds. Major

advantages of employing such a method are potentially lower operation costs and minimum ecological disturbances. In addition, *in-situ* bioremedation does not generate waste solids for disposal.

Biostimulation refers to enhancing the metabolic activity of indigenous microflora to transform target compounds and usually requires amendments (electron donors and/or acceptors) to enhance the microbial populations already present. Factors such as the delicate nutritional requirements of dehalogenating microbes and electron donor competition between dechlorinating and non-dechlorinating microorganisms have presented challenges to implementation of this promising technology in the field.

Much of the work presented in this thesis has been directed toward feasibility of biostimulation in Schoolcraft Plume G site. For oligotrophic aquifers such as Plume G, natural attenuation may be insufficient, and electron donor addition is often needed to accelerate reductive dechlorination or stimulate cometabolic activities. Although bioaugmentation offers the promise of increased control over transformation of a specific compound, competition between indigenous microflora and the introduced organisms usually presents a challenge.

Laboratory and field investigations were used to develop cost effective methods of biologically generated reducing conditions to support reductive dehalogenation of PCE/TCE/TCA mixtures. We have evaluated the two levels of enhanced remediation in the Plume G: biostimulation and bioaugmentation: and compared their results over three-year period. Biostimulation could be part of a remediation strategy for a VOC plume (PCE and daughter products, 1,1,1-TCA and daughter products) co-mingled with a chromium and arsenic plume.

A series of periodic lactate additions were conducted in the field to test the hypothesis that pulse feedings of nutrient influence the stimulation of dechlorinating activity in the study region. Figure 8.1 depicts the delivery setups employed in the Schoolcraft Plume G. Groundwater was pumped from the flux control well to a mixing tank where lactate was added once a week using an in-tank recirculation system. Solution from the mixing tank was injected into the ground in delivery wells 1 and 2. The screened interval for these wells is between 18.9 and 25.0m bgs.

Biostimulation by weekly nutrient feeding resulted in TCE, c-DCE, and VC degradation (Figure 8.2). TCE and daughter products were degraded with a different motif than occurred under bioaugmentation conditions, with slower initial TCE removal, but more rapid c-DCE and VC removal. 1,1,1-TCA was more rapidly degraded in the biostimulation than the bioaugmentation test cell (data not shown).

Bioaugmentation with halorespiring microbes in a flow through system can be accomplished with a one-time inoculation of Bachman Road halorespiring consortium and weekly nutrient feeding. No pre-reducing of the aquifer was required. Degradation of TCE, c-DCE and VC has been observed, with aqueous phase reductions of up to 99% for TCE within 60 days. Degradation of TCE and daughter products has continued for over 15 months following inoculation. Both laboratory results and field evaluations have successfully demonstrated the importance of periodic feedings in stimulation of indigenous dechlorinating populations.

Observed enhanced dechlorination and enumeration of *Dehalococcoides* populations are summarized in the Table 8.3. Compared to a test grid inoculated with Bachman Consortium (0.2 %), biostimulation shows similar levels of *Dehalococcoides*.

Overall, *in situ* biostimulation using native flora to remove chlorinated compounds shows promise for cleaning up contaminated groundwater and sediments. The levels of *Dehalococcoides* stimulated in the biostimulation grid was comparable to the bioaugmentation system, suggesting that stimulation using indigenous flora shows promise for cleaning up chlorinated compounds contaminated aquifers in a cost-effective manner.

Table 8.3. Levels of *Dehalococcoides* DNA in the biostimuation and bioaugmentation test sites.

	Dehalococcoide DNA (%)	
	Days 36	Days 235
Bioaugmentation	2.08	0.024
Biostimulation	2.86	0.025

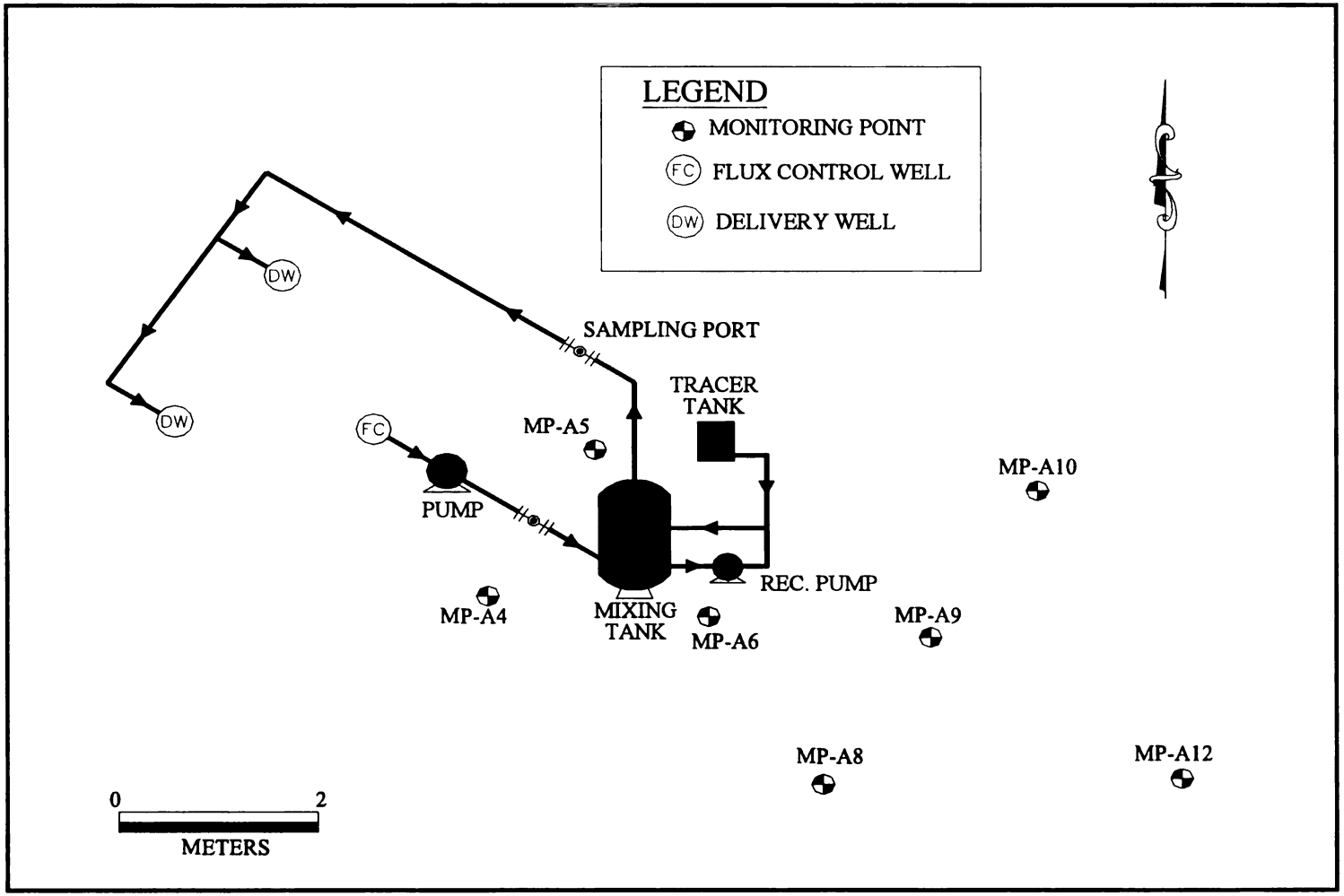
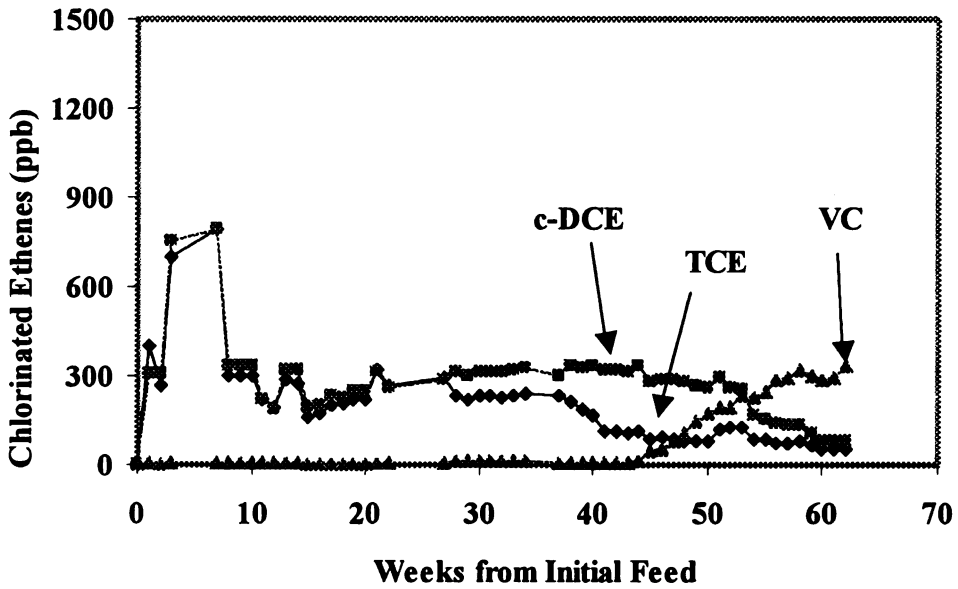


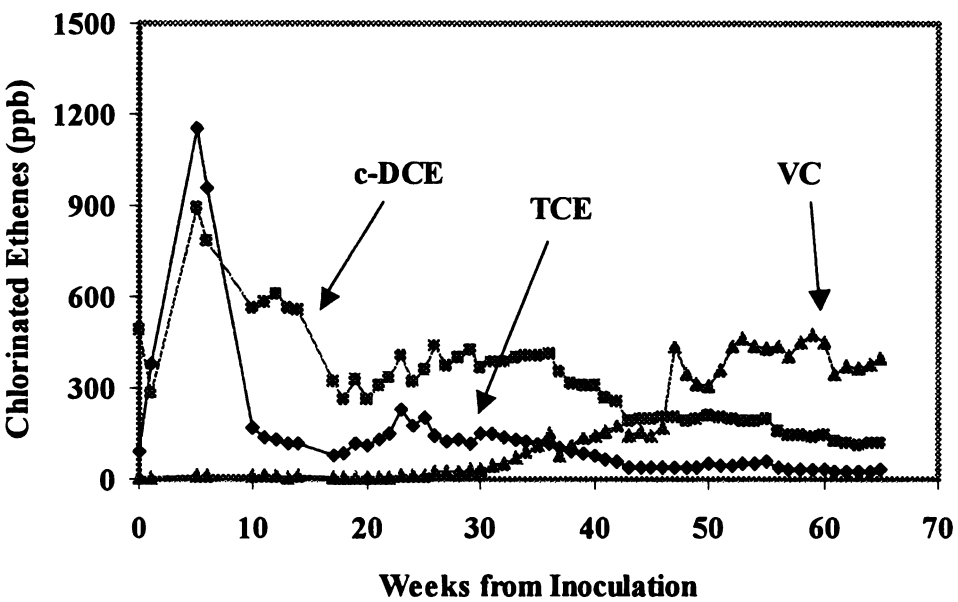
Figure .8.2 Layout of the tracer injection system (adopted from Jaime A. Graulau-Santiago)

Figure 8.3. Comparison on the Observed Reduction in Chlorinated Compounds in the biostimuation and bioaugmentation test sites.

(a) Biostimulation System



(b) Bioaugmentation System



8.3. Literature cited

DiStefano, T. D., J. M. Gossett and S. H. Zinder. 1991. Reductive dechlorination of high concentrations of Tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl. Environ. Microbiol.* 57: 2287-2292.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of Tetrachloroethylene and Trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55(9): 2144-2151.

Fathepure, B. Z. and S. A. Boyd. 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. *FEMS Microbiology Letters* 49(2): 149-156.

Sewell, G. W., and S. A. Gibson. 1991. Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. *Environ. Sci. Technol.*, 25(5): 982-984

Vogel T. M. and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.*, 49(5): 1080-1083.

Tatara, G. M., Dybas, M. J., and C. S. Criddle. 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. strain KC. *Appl. Environ. Microbiol.* 59(7), 2126-2131.

Dybas, M. J., Tatara, G. M., and C. S. Criddle. 1995. Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp. Strain KC. *Appl. Environ. Microbiol.* 61(2): 758-762.

Kim, HaeKyung. 1998. The role of trace copper in the transformation of carbon tetrachloride by *Pseudomonas stutzeri* KC. A MS Thesis, Department of Civil and Environmental Engineering, Michigans State University.

Dybas, M. J., Barcelona, M., Bezborodnikov, S., Davies, S., Forney, L., Heuer, H., Kawka, O., Mayotte, T., Sepulveda-Torres, L., Smalla, K., Sneathen, M., Tiedje, J., Voice, T., Wiggert, D. C., Witt, M. E., and C. S. Criddle. 1998. Pilot scale evaluation of Bioaugmentation for in-situ remediation of a carbon tetrachloride-contaminated aquifer. *Environ. Sci. Technol.* 32(22): 3598-3611.

Jaime A. Graulau-Santiago. 2003. Development and Application of a Methodology to Evaluate Natural Attenuation for Chlorinated Solvents using Conceptual and Numerical Models. A Doctoral Thesis. Civil and Environmental Engineering. Michigan State University.

Chapter Nine: CONCLUSIONS AND FUTURE STUDY

9.1. Conclusions

The following specific and general conclusions have been drawn regarding the development and characterization of dechlorinating microorganisms based on research performed using Plume G sediment and its enrichment:

- Incomplete and slow dechlorination is stimulated in Plume G by microcosms by addition of nutrients such as CMB, lactate, and palmitate.
- Cometabolic dechlorination was dominant in high lactate and PCE pulse fed conditions
- Halorespiration was dominant in low lactate and PCE pulse fed conditions
- Rates and extent of dechlorination increased with repeated feeding of chlorinated compounds
- High hydrogen concentration did not favor dechlorination over methanogenesis. Enhanced dechlorination activity under these conditions was not observed, which agrees with predictions by other researchers based on half-velocity constants with respect to hydrogen.
- *Dehalococcoides* 16S rRNA gene targeted real–time PCR confirmed that two order of magnitude higher amounts of *Dehalococcoides* DNA present in low lactate enrichments than high lactate enrichment.
- Metabolic inhibitors such as molybdate did not impact the low PCE/lactate enrichments, but high levels of sulfate inhibited dechlorination. This suggested that the sulfate-reducers are not involved in the observed dechlorination.

- Low levels of BES specifically inhibited methane production, but retained dechlorinating activity, indicating that the responsible microorganism(s) is (are) non-methanogenic.
- The reduction of acetate production was observed in vancomycin treated enrichment and inhibited dechlorination. This suggests that acetate-utilizing microbes are responsible for the observed dechlorination.

9.2. Future Study

Results from research presented in the previous chapters indicated that further research is required in specific areas. First, no attempts have been made to isolate PCE, TCE, and c-DCE dechlorinating microorganisms. The isolation of these dechlorinators from the enrichment appears to be necessary to better understand its nutritional dependencies on other organisms present in the culture. A better understanding of the growth requirements of TCA dechlorinating microorganisms can also be obtained through research using highly purified or pure cultures. Second, abiotic dechlorination observed in the microcosms has to be explored further due to the prevalence of high sulfate area (such as CA and CO) contaminated with chlorinated compounds. Third, the organism(s) responsible for the reduction of ethene to ethane has to be explored. This can be achieved again by purifying or isolating microorganisms from the enrichment. Fourth, the role of sulfate reducing dechlorinators such as *Desulfomonile tiedjei* in enrichment has to be addressed clearly. Enumeration of such microorganisms using specific primers would further dissect the enrichment community and their interactions with each other.

APPENDICIES

APPENDIX A. Electron Donor Calculations

(Adopted from McCarty McCarty, Perry L. 1969. Energetics and Bacterial Growth. The Fifth Rudolf Research Conference, Rutgers, New Brunswick, New Jersey)

Simple Models of Electron Donor Requirement Calculations. Microorganisms obtain energy for growth and maintenance by removing electrons from electron donors and transferring them via macromolecules to electron –deficient compounds, such as nitrate or sulfate, the terminal electron acceptors. A fraction of the electrons removed from the electron donor may also be used to reduce oxidized forms of carbon and nitrogen in the creation of new biomass. These two pathways for the consumption of reducing power are summarized in this equation where fs is the fraction of electrons diverted for synthesis and the fraction used for energy generation is termed fe.

$$R = Rd + feRa + fsRc$$

Where: Rd = half reaction for the oxidation of an electron donor normalized by the moles of electrons removed from the donor,

Ra = half reaction for the reduction of an electron acceptor used for energy normalized by moles of electrons added to the acceptor,

Rc = half reaction for the reduction of an electron accept used for synthesis normalized by the moles of electrons added to the acceptor, and fs + fe = 1

Electron donor	Half reaction	$\Delta G^{\circ\prime}$ (KJ/mol)
Methanol	0.167 methanol + 0.167 water => 0.167 $CO_2 + H^+ + e^-$	-37.53
Lactate	1/12 lactate + 1/3 water => 1/6 CO2 +1/12HCO_3^-+ $H^+ + e^-$	-32.96
Ethanol	1/12 ethanol + 3/12 water => 2/12 CO2 +1/12HCO_3^-+ $H^+ + e^-$	-31.13
Propionate	1/14 propionate + 5/14 H2O => 1/7 CO2 +1/14HCO_3^-+ $H^+ + e^-$	-27.9
Acetate	1/8acetate + 3/8 water => 1/8CO2 + 1/8HCO_3^-+ $H^+ + e^-$	-27.98
Palmitate	1/92 $C_{16}H_{31}O_2^-$ + 31/92 water => 15/92 CO_2 + 1/92HCO_3^-+ $H^+ + e^-$	-27.87

Electron acceptor	Half reaction	E°′ (V)	ΔG°′ (KJ/mol)
Nitrate reduction	$1/5\ NO_3^- + 6/5H^+ + e^- \Rightarrow 1/10N_2 + 2/5\ H_2O$	0.74	71.71
Sulfate reduction	$1/8\ SO_4^{2-} + 5/4H^+ + e^- \Rightarrow 1/8\ HS^- + ½\ H_2O$	-0.22	20.79
Cell synthesis	$¼\ CO_2 + 1/20\ NH_4^+ + H^+ + e^- \Rightarrow 1/20\ C_5H_7O_2N + 2/5\ H_2O$		

The appropriate value for fs depends upon the type of microorganism or enrichment involved, its electron donors and acceptors, and the amount of decay a culture experiences.

$$(fs)_{max} = \frac{-k\Delta Gr}{-k\Delta Gr + \frac{188 + \Delta Gn}{k} + \frac{\Delta Gp}{k^m}}$$

where: ΔGs = free energy per e- mole of the electron donor used for the creation of biomass = ΔGp + 18.8 + ΔGn

ΔGr = free energy per e- mole released by oxidation of the electron donor

ΔGp =free energy per e- mole of the electron donor– energy need for pyruvate

ΔGn = free energy need for N assimilation

k = the efficiency which energy is converted into new chemical

m = -1 if ΔGp < 0

m = 1 if ΔGp > 0

In our system:

*Resi*dual amounts of nitrate and sulfate in the groundwater; 65 ppm and 80 ppm each

*Assu*me ammonia is N-source

Kis 0.6

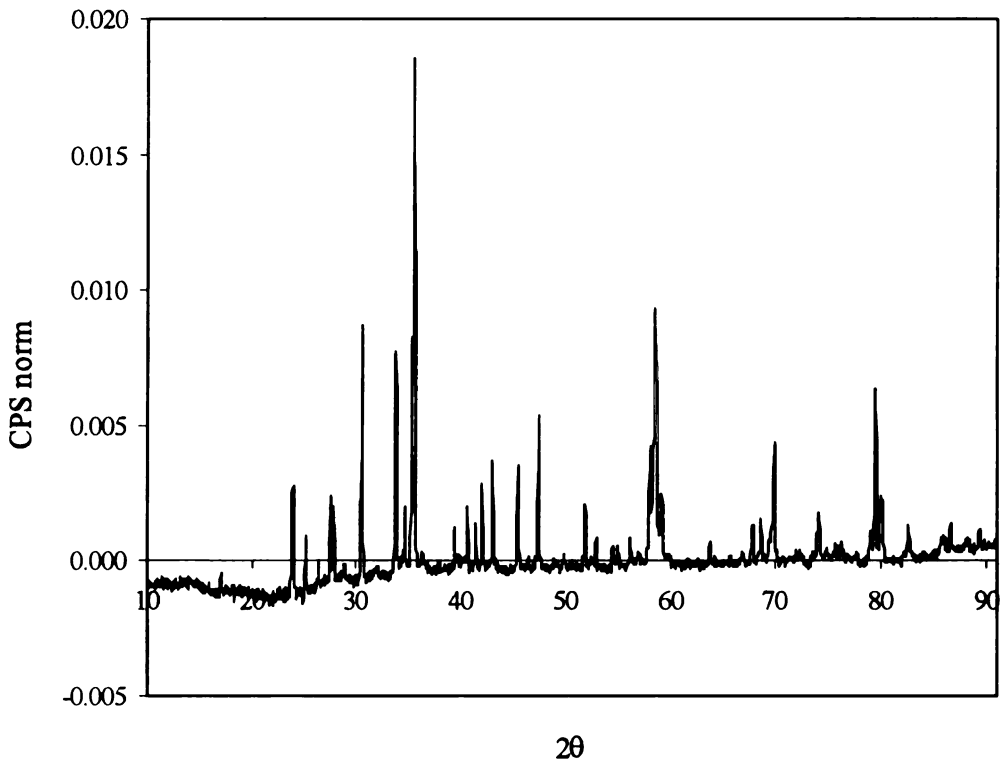
The amounts of electron donor required in our system.

	Nitrate	Sulfate	Total Electron Donor
Acetic acid	fs = 0.575, fe = 0.425 $\frac{0.125(59)}{0.2(0.425)62} \times 65 ppm = 90.96 pm$	fs = 0.09, fe = 0.91 $\frac{0.125(59)}{0.125(0.911)96} \times 80 ppm = 53.9 pm$	145 mg/L or 2.5 mM
Ethanol	fs = 0.6, fe = 0.39 $\frac{0.0833(46)}{0.2(0.39)62} \times 65 ppm = 51.52 pm$	fs = 0.137, fe = 0.863 $\frac{0.0833(46)}{0.125(0.863)96} \times 80 ppm = 2.6 pm$	81 mg/L or 2 mM
Lactic acid	fs = 0.636, fe = 0.364 $\frac{0.0833(89)}{0.2(0.364)62} \times 65 ppm = 106.7 pm$	fs = 0.17, fe = 0.83 $\frac{0.0833(89)}{0.125(0.83)96} \times 80 ppm = 59.4 pm$	166 mg/L or 2 mM
Methanol	fs = 0.684, fe = 0.316 $\frac{0.1667(32)}{0.2(0.316)62} \times 65 ppm = 88.5 pm$	fs = 0.25, fe = 0.75 $\frac{0.1667(32)}{0.125(0.75)96} \times 80 ppm = 47.4 pm$	136 mg/L or 4.25 mM

Palmitic acid	fs = 0.573, fe = 0.427 $\frac{0.0108(255)}{0.2(0.427)62} \times 65 ppm = 33.8 pm$	fs = 0.087, fe = 0.913 $\frac{0.0108(255)}{0.125(0.913)96} \times 80 ppm = 20 pm$	54 mg/L or 0.25 mM
Propionic acid	fs = 0.574, fe = 0.426 $\frac{0.125(73)}{0.2(0.426)62} \times 65 ppm = 64.13 pm$	fs = 0.088, fe = 0.912 $\frac{0.07(73)}{0.125(0.912)96} \times 80 ppm = 38.1 pm$	102 mg/L or 1.5 mM

APPENDIX B.

Effect of Added Sulfate on the Powder X-ray Diffraction Pattern by Iron Sulfide produced in Microcosm.



APPENDIX C. Design of Experiment

A statistical design of experiment was implemented to evaluate the effects of sulfate, reductant (Na_2S), and nutrient in the stimulation of dechlorination (Table 1). Replications were performed to gain a sufficient level of confidence in the estimated parameters. The main and interaction effects were analyzed by ANOVA and presented in Table 2. The two main effects such as sulfate and Na_2S exhibited as insignificant components on dechlorination. The nutrient alone had significant effect. According to the P-value, the two selected factors sulfate and reductant were insignificant at the 0.01 levels. The interactions between each factor were insignificant. In statistical analyses a *P* value of <0.01 was considered the criterion for significance.

Table 1. Experimental Design Matrix for Dechlorination

Run	Sulfate (A)	Reductant (B)	Nutrient (C)	Run label
1	-	-	-	(1)
2	+	-	-	a
3	-	+	-	b
4	+	+	-	ab
5	-	-	+	c
6	+	-	+	ac
7	-	+	+	bc
8	+	+	+	abc

Table 2. ANOVA by 2^3 Factor design

a	b	c	y
0	0	0	9.8
1	0	0	9.85
0	1	0	12.7
1	1	0	15.5
0	0	1	32.2
1	0	1	32.8
0	1	1	32
1	1	1	32.9
0	0	0	9.7
1	0	0	9.5
0	1	0	10.1
1	1	0	10.2
0	0	1	26.5
1	0	1	28.1
0	1	1	28.7
1	1	1	44.2
0	0	0	9.9
1	0	0	9.25
0	1	0	10.8
1	1	0	12.8
0	0	1	31.8
1	0	1	36.1
0	1	1	32.9
1	1	1	29.9

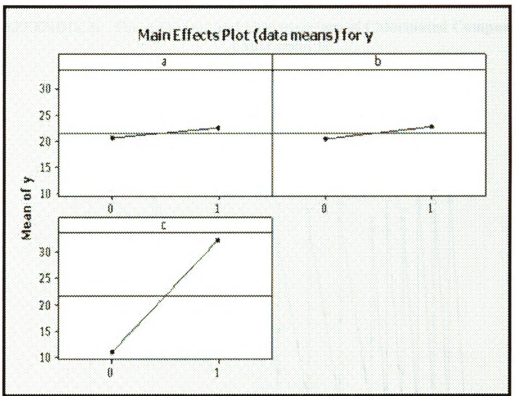
ANOVA: y versus a, b, c

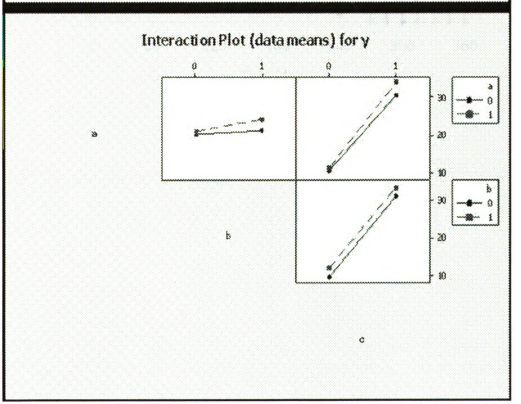
Factor	Type	Levels	Values
a	fixed	2	0, 1
b	fixed	2	0, 1
c	fixed	2	0, 1

Analysis of Variance for y

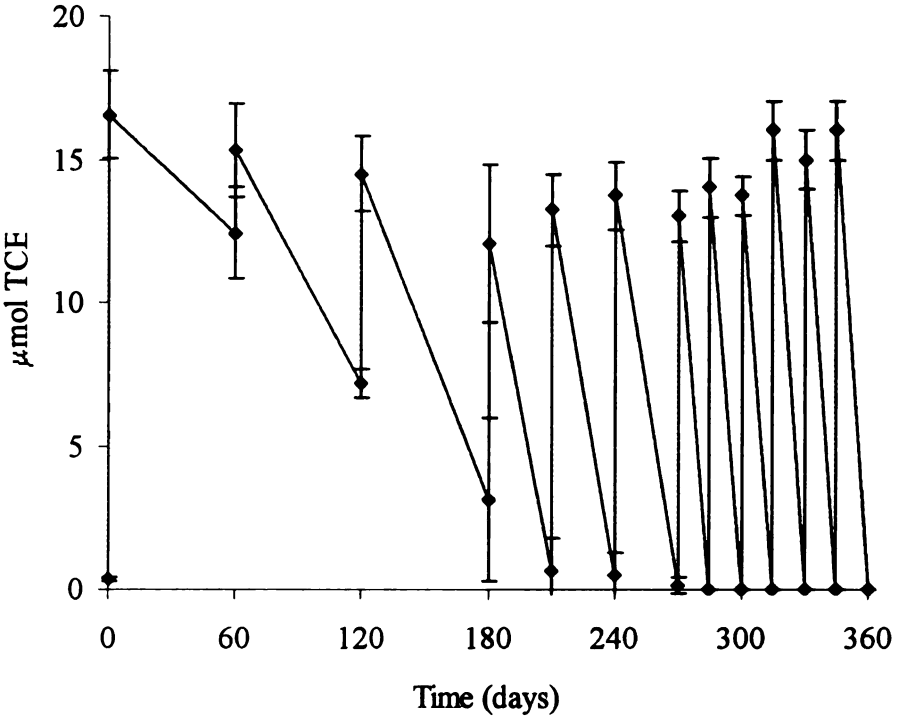
Source	DF	SS	MS	F	P
a	1	24.00	24.00	1.98	0.18
b	1	30.83	30.83	2.54	0.13
c	1	**2773.50**	**2773.50**	**228.80**	**0.00**
a*b	1	6.62	6.62	0.55	0.47
a*c	1	10.40	10.40	0.86	0.37
b*c	1	0.04	0.04	0.00	0.95
a*b*c	1	0.06	0.06	0.00	0.95
Error	16	193.95	12.12		
Total	23	3039.39			

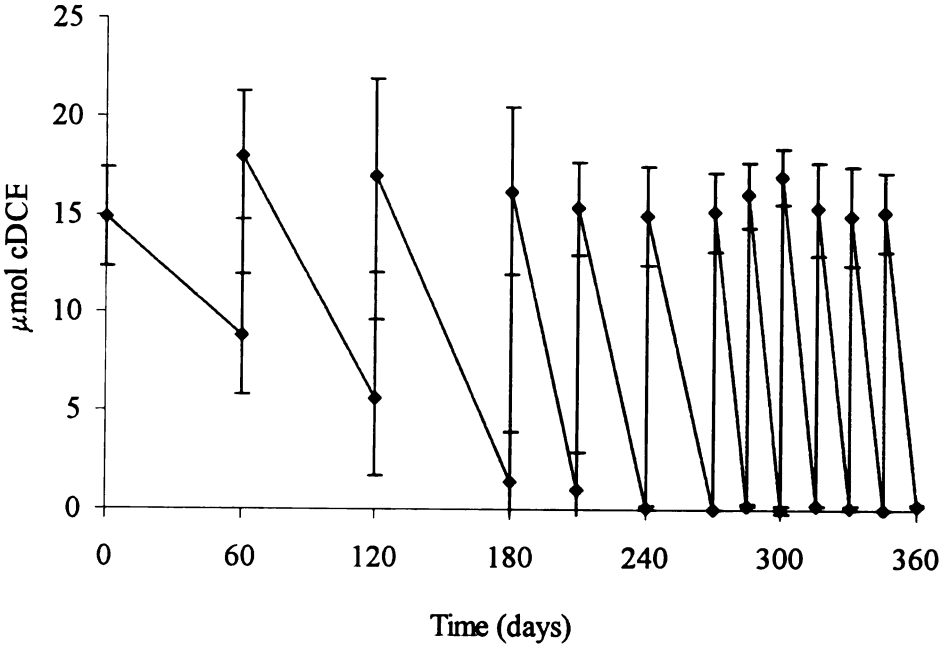
S = 3.48163 R-Sq = 93.62% R-Sq (adj) = 90.83%

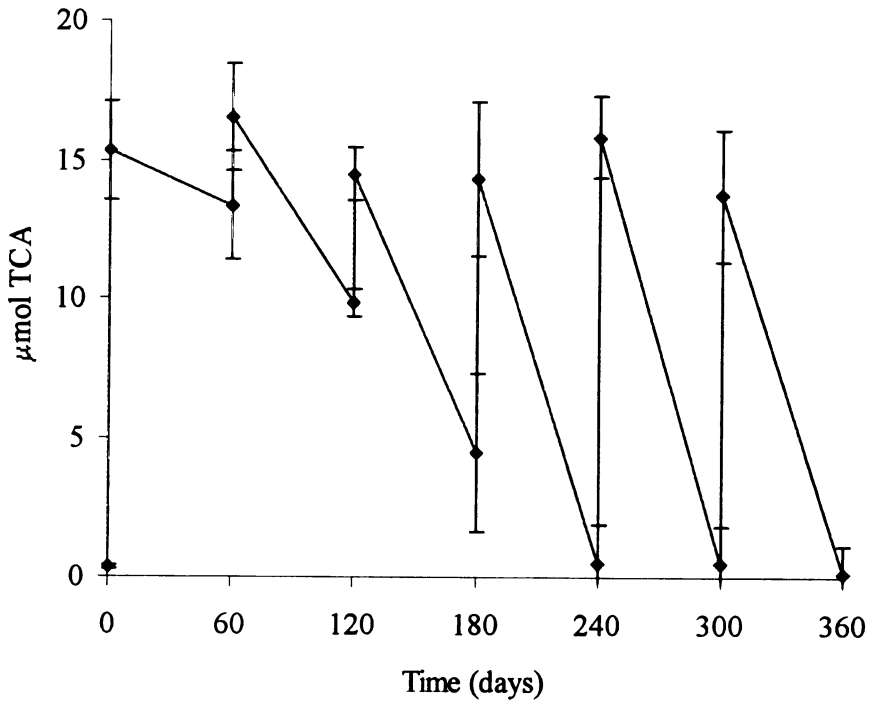

Main Effects Plot (data means) for y
a
b
c
Mean of y
30
25
20
15
10
0
1


Interaction Plot (data means) for y
a
b
c
0
1
30
20
10

APPENDIX D. The Additions and Consumptions of Chlorinated Compounds for Experiment II

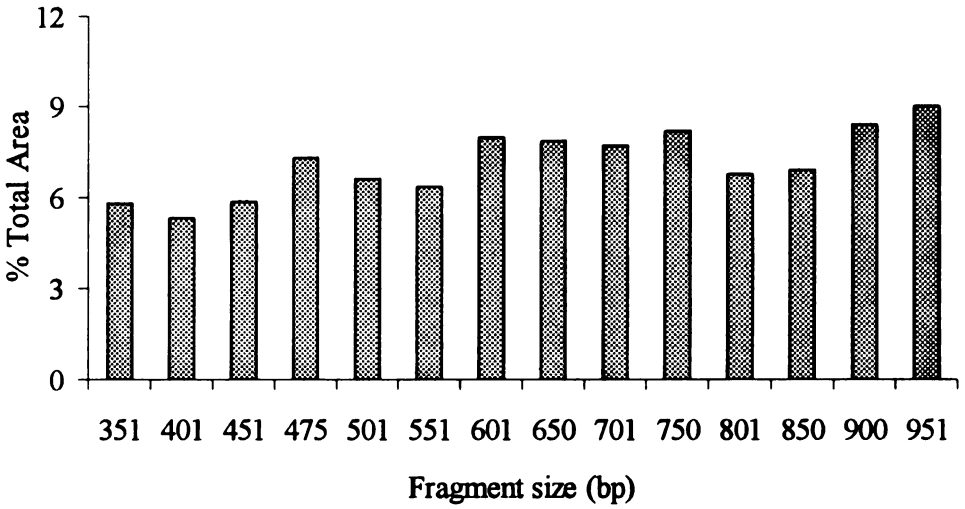



25
20
15
10
5
0
µmol cDCE
0
60
120
180
240
300
360
Time (days)


20
15
10
5
0
µmol TCA
0
60
120
180
240
300
360
Time (days)

APPENDIX E. T-RFLP Patterns from Chapter 6

1. EARLY microcosms after 1 yr of enrichment



Terminal fragment (bp)	Peak area
350.54	1335
400.64	1234
450.82	1355
475.3	1690
500.6	1532
550.63	1462
600.76	1845
650	1820
700.61	1784
750.26	1890
800.7	1558
850.15	1599
900	1932
950.7	2088

2. T-RFLP PATTERS SHOWN IN CHAPTER 6 (Figure 6.7).

(a) Pre-enrichment

Terminal fragment (bp)	Peak area
206.92	518
650.26	1144
700	1102
900.19	1055
949.8	1341
999.33	1416

(b) High Lactate/PCE

Terminal fragment (bp)	Peak area
449.68	1168
474.55	1352
499.58	1257
550.23	1170
600.12	1348
650.13	1455
699.86	1358
750.29	1272
800.62	1292
849.83	1308
899.46	1515
949.8	1503
999.56	1956

(c) Low Lactate/PCE

Terminal fragment (bp)	Peak area
228.09	1115
400.3	1211
450.34	1243
475.43	1427
500.44	1301
550	1473
600.48	1775
650.64	1654
700.4	1515
750.58	1664
800.31	1321
850.33	1512
899.82	1602
949.6	1853

(d) Low Lactate/TCE

Terminal fragment (bp)	Peak area
126.02	475
218.56	385
250.25	765
350.92	649
450.21	1223
475.2	1532
500.72	1388
550.53	1579
600.33	1670
650	1764
700.25	1713
750.4	1691
800	1446
850.45	1735
900.33	1752
950.36	2084

(e) Low lactate/cDCE

Terminal fragment (bp)	Peak area
475.29	1379
550.8	1208
600.31	1495
650.33	1625
700.92	683
750.49	1441
799.74	1221
900.6	1476
950.32	1851
1000	1970

(f) Low lactate/TCA

Terminal fragment (bp)	Peak area
368.61	252
450.11	1045
475.3	1227
500.41	1128
550	1207
600.44	1457
648.47	610
650	1561
699.87	1414
750.67	1403
850.15	1251
900	1517
950.72	1675

APPENDIX F. T-RFLP Patterns for Chapter 7

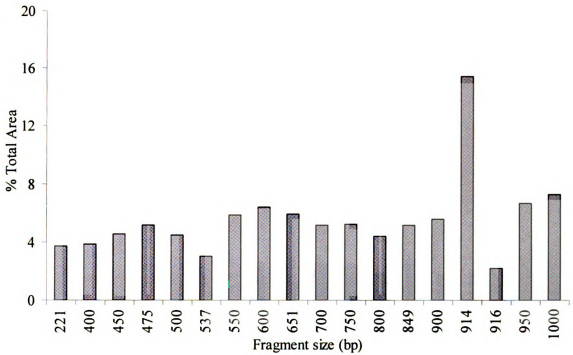
1. T-RFLP PATTERS SHOWN IN CHAPTER 7 (Figure 7.7).

(a) BESA (0.5 mM)		(b) Molybdate (2 mM)		(c) Sulfate (0.6 mM)		(d) Vancomycin (25 mg)	
Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area
175.58	311	157.76	465	185.47	732	206.81	1914
268.09	554	333.84	1782	217.39	240	450.22	1089
347.7	483	475.52	1185	463.73	404	475.53	1348
393.12	228	500.21	1159	475.1	1283	491.54	450
475.1	588	600.46	1333	500.31	1128	500.75	1266
475.51	512	650.5	1313	600.42	1452	550.11	1123
600.33	1174	700.26	1174	648.51	993	600.7	1323
650.84	1135	750.56	1152	850.15	1211	650.13	1628
685.01	304	850	1068	900.49	1371	700.26	1430
950.18	1259	900.18	1271	950.53	1485	749.58	1206
		950.39	1382			800.15	1143
		999.37	1624			849.67	1178
						899.64	1312
						950.1	1699

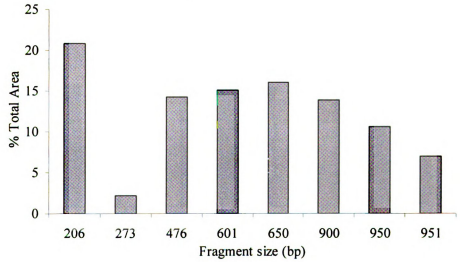
2. T-RFLP Patterns for different dosage

(a) BESA (5 mM)		(b) Molybdate (6 mM)		(c) Sulfate (2 mM)		(d) Vancomycin (100 mg)	
Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area	Terminal fragment (bp)	Peak area
220.94	1082	206.43	1831	74.93	3222	475.53	1171
400.1	1115	273.38	191	75	2411	550.34	580
450	1320	475.78	1255	163.61	454	600.47	1208
475.21	1504	600.54	1334	197.04	142	650.77	1382
500.11	1297	650.47	1419	279.81	265	750	1233
537.46	884	900.32	1226	350	610	850.17	1132
550	1706	949.65	931	475.39	1491	900.55	1361
600.35	1866	950.86	617	559.81	5742	950.4	1397
650.51	1713			600.21	1498	974.28	859
700.13	1507			650.58	1436	977.83	603
749.86	1514			700.36	1212	999.78	1629
800.31	1270			750.38	1533		
849.34	1504			800.82	1212		
899.82	1610			850.43	1241		
914.08	4504			900.16	1593		
916.49	646			951.36	1765		
950.2	1935			999.64	2082		
999.79	2110						

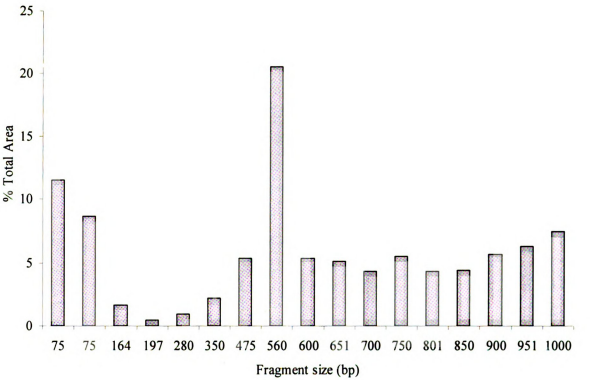
(a) BESA (5 mM)



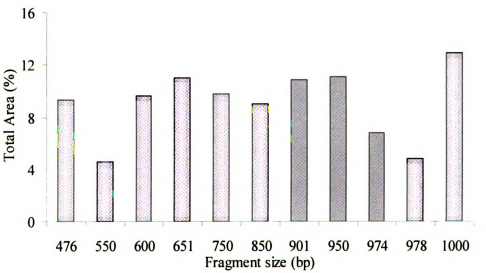
(b) Molybdate (6 mM)



(c) Sulfate (2 mM)



(d) Vancomycin (100 mg)



3. T-RFLP patterns before addition of inhibitors

(a) BESA before

Terminal fragment (bp)	Peak area
450.64	1170
475	1196
500.21	1164
550.43	1253
600.34	1530
650.24	1472
750.13	1348
795.24	377
800.14	1238
850.3	1375
899.67	1448
950.73	1682

(b) Molybdate before

Terminal fragment (bp)	Peak area
475.32	1506
500.53	1258
550.45	1356
600	1576
649.88	1657
699.87	1484
750	1477
800	1346
850	1369
899.64	1536
949.8	1888
999.79	2040

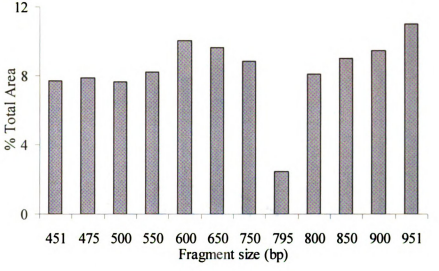
(c) Sulfate before

Terminal fragment (bp)	Peak area
147.91	1295
350.88	1203
400.7	1140
450	1251
475.55	1343
500.55	1278
549.89	1347
600.48	1438
650.13	1593
700.27	1414
749.57	1397
800.16	1333
850.68	1567
899.82	1409
950.2	1853

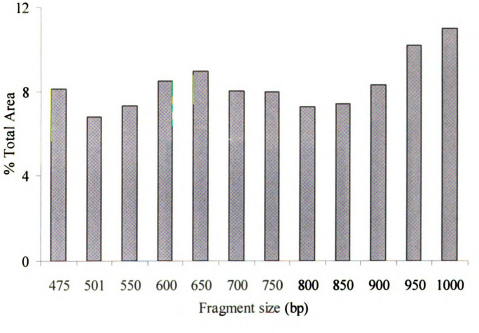
(d) Vancomycin before

Terminal fragment (bp)	Peak area
449.68	1168
474.55	1352
499.58	1257
550.23	1170
600.12	1348
650.13	1455
699.86	1358
750.29	1272
800.62	1292
849.83	1308
899.46	1515
949.8	1503
999.56	1956

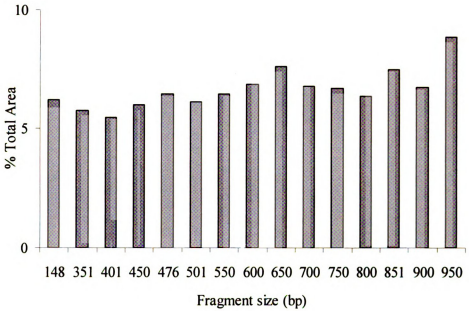
(a) BESA before



(b) Molybdate before



(c) Sulfate before



(d) Vancomycin before