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Characterization of Tetrachloroethene-Dechlorinating Bacteria and Investigation into Their Ability to Enhance Removal Rates of Tetrachloroethene-Containing Nonaqueous Phase Liquids

by

Cynthia Schmidt Carr

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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ABSTRACT

Characterization of Tetrachloroethene-Dechlorinating Bacteria and Investigation into Their Ability to Enhance Removal Rates of Tetrachloroethene-Containing Nonaqueous Phase Liquids

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Cynthia Schmidt Carr

The influence of electron donors on the ability to sustain tetrachloroethene (PCE) dechlorination was investigated in recycle columns containing a PCE-dechlorinating mixed culture. Over a period exceeding one year, it was demonstrated that equivalent amounts of lactate, methanol, and hydrogen could sustain rate and extent of dechlorination equally. Dechlorination was not impacted by competition for electron donor at high hydrogen partial pressures, despite the presence of an actively methanogenic community. Results from these experiments imply that cost and method of delivery may dictate electron donor selection for stimulation of anaerobic in situ dechlorination.

Efforts to isolate PCE-dechlorinating organisms from the mixed culture resulted in the development of a highly purified co-culture that dechlorinated PCE to cis-dichloroethene and was dominated by curved, motile rods and cocci. Preliminary molecular biology techniques were employed to characterize the ecology of the purified culture. It was discovered that multiple microorganisms, one of which shared 98% 16S rDNA sequence identity with the fermentative coccus, Lactosphaera pasteurii, were
present. Experiments characterizing the nutritional requirements of the purified co-culture determined dechlorination sustainment required the addition of filter-sterilized cell extract prepared from the parent mixed culture. It was determined that hydrogen, pyruvate, glucose, ethanol, and yeast extract could sustain dechlorination, while formate, acetate, acetaldehyde, lactate, propionate, butyrate, and methanol could not.

Experiments to determine the effect of dechlorination on the longevity and composition of PCE-containing NAPLs were conducted in continuous-flow stirred-tank reactors (CFSTRs). Comparisons between biotic and abiotic CFSTRs demonstrated that dechlorination resulted in a factor of 14 increase in PCE removal rates from the NAPL. Dechlorination daughter products partitioned between the aqueous and NAPL phases, resulting in temporal changes in NAPL composition. The combined effects of dissolution and dechlorination on the removal of chlorinated ethenes from the NAPL were described using a mathematical model that approximated dechlorination as a pseudo first-order process. It was determined that total chlorinated ethenes removal from the NAPL would be achieved in 13 days in the biotic CFSTRs, as compared to 77 days in the abiotic CFSTRs – corresponding to an 83% reduction in the longevity of the chlorinated ethenes component of the NAPL.
ACKNOWLEDGMENTS

This research was funded in part by the Gulf Coast Hazardous Substance Research Center, the Energy and Environmental Systems Institute (Rice University), and the Eleanor and Mills Bennett Fellowship in Hydrology.

Many people contributed to the research presented herein and deserve special recognition for their efforts. I sincerely appreciate the guidance of my dissertation adviser, Joe Hughes, who possesses the amazing ability to turn any negative into a positive. I will always appreciate the opportunities he afforded me and the investment he made in my education. I would also like to thank my committee members, Phil Bedient, Jordan Konisky, and Bill Rixey, for serving on my dissertation committee. Their insights and valuable suggestions contributed greatly to the preparation of this work.

A very special thank you is extended to the post-doctoral associates with whom I collaborated on several projects. Sanjay Garg wrote the dissolution-dechlorination model presented in Chapter 6, and Betsy McLaughlin-West, Dandan Zheng, and John Spear (University of Colorado, Boulder) served as my source of information on molecular biology techniques.

The generosity of the labs of George Bennett and Frederick Rudolph (Biochemistry and Cell Biology, Rice University) were very much appreciated. Use of their equipment, and the assistance of Mary Harrison and Sandra Clark, made much of the molecular work
possible. Richard Gomer (Biochemistry and Cell Biology, Rice University) assisted in obtaining the photomicrographs presented in Chapter 5.

I would also like to thank my lab mates and friends at Rice, all of whom have made my time here memorable and fun.

My parents and family have been a constant source of support and enthusiasm no matter what I have attempted to accomplish, and for this I am most grateful. Thank you for the sacrifices you made, none of which went unnoticed or unappreciated. And to Christian - who has survived and sustained me through my darkest moments of self-doubt and frustration - thank you for everything. Being next to you is the best place I have ever known.

CSC
in memoriam

Mary Etta Sego
(1917-1997)

my paragon
of tolerance and selflessness
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NOTATION AND ABBREVIATIONS USED IN TEXT

Chemical Compounds
BES 2-bromoethanesulfonic acid
cis-DCE cis-dichloroethene
Lact lactate
MeOH methanol
PCE tetrachloroethene
TCE trichloroethene
VC vinyl chloride

Biological and Engineering Notation
\( \alpha_c \) cell yield coefficient (eq. cells formed/eq. H\(_2\) consumed\(^{-1}\))
bp base pairs
BTEX benzene, toluene, ethylbenzene, and xylenes
\( C''\) NAPL concentration (g/cm\(^3\) NAPL\(^{-1}\))
\( C'\) aqueous concentration (g/cm\(^3\) water\(^{-1}\))
CFSTR continuous-flow stirred-tank reactor
DNA deoxyribonucleic acid
DNAPL dense nonaqueous phase liquid
ED electron donor
eq. equiv. equivalent
FSCE filter-sterilized cell extract
FYE prefermented yeast extract
\( \Delta G^\circ_r \) Gibbs free energy of formation
\( \Delta G^\circ' \) Gibbs standard free energy change for a reaction (1 M concentrations, 1 atm pressure, pH 7)
GC gas chromatograph
\( H_c \) dimensionless Henry’s constant
HRT hydraulic retention time (h\(^{-1}\))
$k$ maximum specific substrate utilization rate (mg substrate consumed·mg cells$^{-1}$·h$^{-1}$)

$k_i$ pseudo-first order dechlorination rate coefficient (h$^{-1}$)

$K_a$ lumped mass transfer rate constant (s$^{-1}$)

$K_{"NAPL/water"}$ NAPL/water partition coefficient (cm$^3$ water·cm$^3$ NAPL$^{-1}$)

$K_r$ half-velocity constant (mg·L$^{-1}$)

NAPL nonaqueous phase liquid

PCE$_{aq}$ PCE saturated aqueous solution

PCR polymerase chain reaction

$Q$ flow rate (L·h$^{-1}$)

$r_g$ rate of bacterial growth (mg cells·L$^{-1}$·h$^{-1}$)

$r_i$ homogeneous reaction in aqueous phase removing species $i$

$r_{sc}$ rate of substrate consumption (mg substrate consumed·L$^{-1}$·h$^{-1}$)

$R_i$ dimensionless retardation coefficient

rDNA ribonucleic DNA

RDP Ribosomal Database Project

RFLP restriction fragment length polymorphism

$S$ concentration of growth-limiting substrate (mg·L$^{-1}$)

$t_{1/2}$ half-life (h)

TE tris-EDTA buffer

$v_i$ interstitial velocity (cm·s$^{-1}$)

$V$ volume (L)

VSS volatile suspended solids

$x$ distance within the source zone (cm)

$X$ concentration of biomass (mg cells·L$^{-1}$)

$Y$ maximum yield coefficient (mg cells produced·mg substrate consumed$^{-1}$)
Greek Terms

\( \theta_w \)  saturation of water \((\text{cm}^3 \text{ water} \cdot \text{cm}^3 \text{ pores}^{-1})\)

\( \theta_n \)  saturation of NAPL \((\text{cm}^3 \text{ NAPL} \cdot \text{cm}^3 \text{ pores}^{-1})\)

\( \mu_{\text{max}} \)  maximum specific growth rate \((\text{h}^{-1})\)

\( \tau \)  hydraulic retention time \((\text{h}^{-1})\)
Chapter One: PROBLEM STATEMENT AND OBJECTIVES

Tetrachloroethene (PCE) and trichloroethene (TCE) are two of the most prevalent and persistent groundwater contaminants in the United States (1,2). These compounds have been used historically as industrial solvents, degreasers, paint removers, and dry cleaning agents. Through improper storage and disposal techniques, large volumes of these contaminants have been released into the subsurface compromising both the quality of the environment and the health of potential receptors. Once in the subsurface, PCE and TCE pose a formidable challenge to current remediation technologies, particularly due to their tendency to form nonaqueous phase liquids (NAPLs).

Anaerobic in situ bioremediation may offer a unique ability to restore aquifers contaminated with chlorinated ethenes. Research conducted over the last two decades has revealed that certain anaerobic bacteria possess the ability to transform chlorinated solvents to less chlorinated (3-6) and non-toxic species (7-9), a process generally referred to as dechlorination or reductive dechlorination. More recent research has revealed the existence of several microorganisms, known as halorespirers, which are capable of coupling rapid rates of PCE dechlorination to growth (10-18). The discovery of this unique metabolic pathway has significantly heightened interest in the application of anaerobic bioremediation to chlorinated ethenes contaminated sites.

Implementation of this technology in the field has been somewhat limited, however, due to the lack of knowledge regarding the extent of dechlorination that can be achieved, the potential for such problems as electron donor competition among
dechlorinating and nondechlorinating microorganisms, and the nutritional requirements of dechlorinating bacteria. Several studies have indicated that the selection of electron donor may impact the ability to sustain dechlorination in situ. Although many electron donors have been observed to sustain dechlorination in mixed cultures, hydrogen has been proposed to serve as the final electron donor for rapid dechlorination (19,20). More recent investigations have indicated that at high hydrogen partial pressures, methanogens and other hydrogenotrophs may compete with dechlorinating microorganisms for hydrogen, resulting in incomplete dechlorination activity or its exclusion entirely (21-23). These studies suggest that the use of fermentable substrates that maintain low levels of hydrogen would be necessary to offer dechlorinators a competitive advantage in situ.

Beyond electron donor requirements, very little information is known regarding other nutritional requirements of dechlorinating bacteria (e.g., terminal electron acceptors, carbon source, vitamins, etc.). Although these requirements may be best understood when studied in pure culture, of the PCE-halorespiring bacteria isolated to date, no nutritional homogeneities have been observed among all six other than the ability to utilize PCE as terminal electron acceptor. Several halorespiring isolates have well characterized growth requirements, while others can not survive in pure culture without the addition of an undefined growth factor such as filter-sterilized spent medium. Only through investigations with highly purified cultures and isolates can an understanding of the growth requirements and interspecies dependencies of these organisms be gained. This knowledge can then be used to better characterize how these microorganisms might
behave in a given environment, and whether issues such as electron donor competition are relevant.

Another aspect of in situ anaerobic bioremediation that warrants investigation is the application of this technology within the source zone where NAPLs are present. To date, most bioremediation processes are employed downstream of the source zone within the contaminant plume. Because source zones contain the greatest mass of contaminant and act as continuous sources of contamination, they are the foremost environmental concern. The discovery of PCE halorespiration represents a paradigm shift in the understanding of the ability to rapidly biodegrade chlorinated ethenes, and the potential for applying bioremediation processes in NAPL source zones. Because halorespiring bacteria require PCE for growth, it may be possible to stimulate dechlorination within the source zone, thereby increasing the rate of chlorinated ethenes removal as compared to dissolution alone.

The purpose of this research was to further the understanding and application of PCE reductive dechlorination by investigating electron donor requirements and the role of hydrogen in mixed, methanogenic cultures where dechlorination was present; by characterizing the ecology and nutritional requirements of a highly purified dechlorinating culture; and by investigating the influence of dechlorinating microorganisms on rates of PCE and chlorinated ethenes removal from nonaqueous phase liquids. Specifically, the objectives were as follows:
1. Evaluate the impact of electron donor selection on the long-term sustainability of dechlorination, measured as the rate of PCE disappearance and the distribution of dechlorination end products.

2. Determine the impact of high hydrogen partial pressures on dechlorination when present in an actively methanogenic community.

3. Characterize the ecology of a highly purified PCE-dechlorinating culture through molecular biology techniques, and determine the electron donors and growth factors required to sustain dechlorination.

4. Determine whether dechlorinating bacteria can reduce the longevity of PCE in a PCE-containing NAPL, and to evaluate the transient effect of dechlorinating bacteria on the chlorinated ethenes distribution between the NAPL and aqueous phase.

The format of the thesis is as follows: theory supporting the aforementioned objectives is outlined in Chapter Two; Chapter Three contains a review of related research efforts; results from the electron donor and hydrogen studies are presented in Chapter Four; the ecology and nutritional requirements of the highly purified enrichment culture are detailed in Chapter Five; Chapter Six contains the results from the NAPL studies; general conclusions are summarized in Chapter Seven; and engineering significance and topics of further research are covered in Chapters Eight and Nine, respectively.

References


Chapter Two: BACKGROUND AND THEORY

This chapter provides the reader with a review of background information and theoretical considerations central to the research presented in latter chapters. In the engineering literature, electron donor competition among organisms is commonly presented mathematically using Monod’s limiting-substrate model and the change in free energy of competing reactions. Because these concepts are discussed in Chapters 3 and 4 with regard to hydrogen competition among dechlorinators and other hydrogenotrophs, a review of the formation of hydrogen via organic substrate degradation and the kinetics and energetics of electron donor competition are presented. Formation and dissolution of NAPLs are discussed in the last two sections of this chapter as a review for information presented in Chapter 6.

*Thermodynamic Considerations for the Metabolism of Organic Electron Donors to Hydrogen*

Hydrogen and acetate are the frequent products of anaerobic microbial metabolism of organic electron donors (1). Table 2.1 contains reactions involving the metabolism of select organic acids and alcohols to acetate and hydrogen (note that these are simplified models and not all organic substrate transformations result in acetate and hydrogen production). The energetics of these reactions, represented by the change in free energy ($\Delta G^\circ$), are listed in Table 2.2. As shown in Table 2.2, several reactions are thermodynamically unfavorable (i.e., $\Delta G^\circ$ is positive) under standard conditions (i.e., 25°C; 1 M concentrations of reactants and products; 1 atm $H_2$; pH 7 for reactions resulting in $H^+$ formation). In the presence of 1 atm $H_2$, propionate, butyrate, ethanol, and...
Table 2.1  Simple Models of Organic Electron Donor Metabolism to Acetate and Hydrogen

<table>
<thead>
<tr>
<th>electron donor</th>
<th>overall reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>(2CH_4O \rightarrow C_2H_3O_2^- + H^+ + 2H_2)</td>
</tr>
<tr>
<td>pyruvate</td>
<td>(C_3H_6O_3^- + H_2O \rightarrow C_2H_3O_2^- + CO_2 + H_2)</td>
</tr>
<tr>
<td>lactate</td>
<td>(C_3H_4O_3^- + H_2O \rightarrow C_2H_3O_2^- + CO_2 + 2H_2)</td>
</tr>
<tr>
<td>ethanol</td>
<td>(C_2H_6O + H_2O \rightarrow C_2H_3O_2^- + H^+ + 2H_2)</td>
</tr>
<tr>
<td>butyrate</td>
<td>(C_4H_8O_4^- + 2H_2O \rightarrow 2C_2H_3O_2^- + H^+ + 2H_2)</td>
</tr>
<tr>
<td>propionate</td>
<td>(C_3H_4O_3^- + 2H_2O \rightarrow C_2H_3O_2^- + CO_2 + 3H_2)</td>
</tr>
<tr>
<td>benzoate</td>
<td>(C_7H_8O_7^- + 6H_2O \rightarrow 3C_2H_3O_2^- + CO_2 + 2H^+ + 3H_2)</td>
</tr>
</tbody>
</table>

Table 2.2  Change in Free Energy (\(\Delta G^o\)) Values as a Function of Hydrogen Partial Pressure for Organic Electron Donors Metabolized to Acetate and Hydrogen

<table>
<thead>
<tr>
<th>electron donor</th>
<th>(\Delta G^o) (kJ) [1 atm H_2]</th>
<th>(\Delta G^o) (kJ) [10^2 atm H_2]</th>
<th>(\Delta G^o) (kJ) [10^4 atm H_2]</th>
<th>(\Delta G^o) (kJ) [10^6 atm H_2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>-58.50</td>
<td>-81.25</td>
<td>-104.01</td>
<td>-126.76</td>
</tr>
<tr>
<td>pyruvate</td>
<td>-52.01</td>
<td>-63.39</td>
<td>-74.76</td>
<td>-86.14</td>
</tr>
<tr>
<td>lactate</td>
<td>-8.83</td>
<td>-31.58</td>
<td>-54.34</td>
<td>-77.09</td>
</tr>
<tr>
<td>ethanol</td>
<td>9.64</td>
<td>-13.11</td>
<td>-35.87</td>
<td>-58.62</td>
</tr>
<tr>
<td>butyrate</td>
<td>48.28</td>
<td>25.53</td>
<td>2.77</td>
<td>-19.98</td>
</tr>
<tr>
<td>propionate</td>
<td>71.61</td>
<td>37.48</td>
<td>3.35</td>
<td>-30.78</td>
</tr>
<tr>
<td>benzoate</td>
<td>86.25</td>
<td>52.12</td>
<td>17.99</td>
<td>-16.14</td>
</tr>
</tbody>
</table>

For all reactions, pH 7 was assumed. Free energies of formation (\(\Delta G^o\)) values were taken from (1).
benzoate transformations do not result in the release of free energy. However, all the reactions become thermodynamically favorable at hydrogen concentrations less than $10^{-4}$ atm (see Table 2.2).

In mixed cultures, the hydrogen produced via organic electron donor degradation is used to support the growth of hydrogenotrophic organisms, such as hydrogen-utilizing PCE dechlorinators (i.e., hydrogen-based dechlorination). Metabolism of these organic substrates is dependent upon the existence of hydrogen-consuming organisms capable of maintaining the concentration of hydrogen at a level that makes electron donor degradation thermodynamically favorable. This syntrophic phenomenon between the hydrogen-producing and hydrogen-consuming microorganisms is referred to as interspecies hydrogen transfer. Because organisms growing on hydrogen do so at very specific hydrogen concentrations (the minimum concentration supporting growth is called the hydrogen threshold), the selection of an appropriate organic electron donor can be of the utmost importance in selecting for certain organisms in engineered biological systems.

**Role of Kinetics and Energetics in Determining Microbial Competition for Hydrogen**

The ability of certain microorganisms to out compete others for the same growth substrate is dependent upon both the kinetics and energetics of the competing reactions. The rate of bacterial growth ($r_x$) 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 the Monod expression (2)

$$r_x = \frac{\mu_{\text{max}} X S}{K_s + S}$$  \hspace{1cm} (2.1)
where: $\mu_{\text{max}}$ (h$^{-1}$) 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 (mg·L$^{-1}$); and the half-velocity constant, $K_s$ (mg·L$^{-1}$), represents the substrate concentration at half the maximum specific growth rate. The rate of substrate consumption, $r_{sc}$, ($r_{sc}$ is defined as the absolute value of the conventional term "rate of substrate utilization". $r_{su}$) is related to the rate of bacterial growth by the following relationship:

$$r_{sc} = \frac{r_e}{Y}$$  \hspace{1cm} (2.2)

The organism's maximum yield coefficient ($Y$) represents the maximum mass of cells that can be produced per mass of substrate consumed. The coefficients $Y$ and $\mu_{\text{max}}$ define the maximum specific substrate utilization rate ($k$: mg substrate consumed·mg cells$^{-1}$·h$^{-1}$) according to the relationship $k = \mu_{\text{max}}Y$. Substituting equation 2.1 into equation 2.2 yields the following expression for $r_{sc}$:

$$r_{sc} = \frac{kYS}{K_s + S}$$  \hspace{1cm} (2.3)

Examination of the Monod expression reveals that the rates of bacterial growth and substrate consumption are dependent upon values of $K_s$, $Y$, 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. Values of half-velocity constants with respect to hydrogen, $K_s(H_2)$, for hydrogenotrophic dechlorinators and methanogens are listed in Table 2.3. Reported measurements of the maximum specific utilization rate are rare, but
Table 2.3 Values of Half-velocity Constants, $K_s(H_2)$, and Maximum Substrate Utilization Rates ($k$) Reported for Hydrogenotrophic Methanogens and PCE Dechlorinators

<table>
<thead>
<tr>
<th></th>
<th>$K_s(H_2)$</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanogens</td>
<td>2.500-13.000 nM$^a$</td>
<td>100-2,600 μmol·mg protein$^{-1}$·day$^{-1}$$^{(c)}$</td>
</tr>
<tr>
<td></td>
<td>960 ± 180 nM$^b$</td>
<td></td>
</tr>
<tr>
<td>dechlorinators</td>
<td>100 ± 50 nM$^b$</td>
<td>20 μmol·mg protein$^{-1}$·day$^{-1}$$^{(c)}$</td>
</tr>
<tr>
<td></td>
<td>9-21 nM$^c$</td>
<td></td>
</tr>
</tbody>
</table>

those that are available are listed in Table 2.3 as well. Calculated yield coefficients, which are used to normalize values for the maximum specific utilization rate, are listed in Table 2.4 for hydrogenotrophic methanogens and dechlorinators.

Half-velocity constants, yield coefficients, and maximum specific utilization rates must be taken into account to determine which organisms have greater rates of growth and substrate consumption and whether electron donor competition will be a factor among those organisms in co-culture. Theoretically, a competitive advantage will be held by organisms exhibiting the highest rates of growth and substrate consumption. Over extended periods of time, organisms with the highest rate of growth will dominate the mixed culture in terms of cell number, and these organisms will obtain the majority of hydrogen reducing equivalents present. One should note that these relationships are valid when there is only one growth-limiting substrate. Other factors affecting growth, such as availability of electron acceptor, are not considered.

The energetics of competing reactions can also be used to determine whether competition will emerge between two organisms utilizing the same electron donor. Specifically, Cord-Ruwisch, et al. (3) observed that hydrogen thresholds for hydrogenotrophic organisms are inversely related to the free energy of reaction. For example, the process of sulfate reduction, which has a higher free energy of reaction than methanogenesis ($\Delta G^\circ$ values are -38.9 and -33.9 kJ·mol$^{-1}$ H$_2$, respectively), is associated with organisms capable of growing at lower hydrogen partial pressures. Because hydrogen-based PCE dechlorination has a lower free energy of reaction ($\Delta G^\circ = -161.5$ kJ·mol$^{-1}$ H$_2$) as compared to methanogenesis and acetogenesis ($\Delta G^\circ = -23.7$ kJ·mol$^{-1}$ H$_2$),
Table 2.4 Calculated True Yield Coefficients for Hydrogenotrophic Methanogens and PCE Dechlorinators

<table>
<thead>
<tr>
<th>hydrogen consuming reaction</th>
<th>carbon source</th>
<th>$a_e$ (eq cells formed/eq H$_2$ consumed$^{-1}$)</th>
<th>$\gamma$ (mg cells formed/mg COD removed$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$</td>
<td>CO$_2$</td>
<td>0.042</td>
<td>0.030</td>
</tr>
<tr>
<td>$C_2Cl_4 + 4H_2 \rightarrow C_3H_4 + 4Cl^- + 4H^+$</td>
<td>CO$_2$</td>
<td>0.181</td>
<td>0.127</td>
</tr>
<tr>
<td>$C_2Cl_4 + H_2 \rightarrow C_2HCl_3 + Cl^- + H^+$</td>
<td>acetate</td>
<td>0.539</td>
<td>0.380</td>
</tr>
<tr>
<td>$C_2Cl_4 + 2H_2 \rightarrow C_3H_2Cl_2 + 2Cl^- + 2H^+$</td>
<td>acetate</td>
<td>0.536</td>
<td>0.377</td>
</tr>
<tr>
<td>$C_2Cl_4 + 3H_2 \rightarrow C_2H_3Cl + 3Cl^- + 3H^+$</td>
<td>acetate</td>
<td>0.525</td>
<td>0.369</td>
</tr>
<tr>
<td>$C_2Cl_4 + 4H_2 \rightarrow C_3H_4 + 4Cl^- + 4H^+$</td>
<td>acetate</td>
<td>0.521</td>
<td>0.367</td>
</tr>
</tbody>
</table>

$^a$ Cell yield coefficients, $a_e$, were calculated as outlined in (21). Free energy of formation data were taken from references (1.21-24). For all reactions, pH 7 was assumed. $^b$ True yield coefficients were calculated from the cell yield coefficient assuming ammonium served as the nitrogen source for cell synthesis.
one would expect dechlorinators to be capable of growing at a lower hydrogen partial pressure. This observation had been made in two independent reports (4.5). In another case, dechlorinators were observed to maintain hydrogen levels at concentrations below that which would support methanogenesis and acetogenesis (6), thereby eliminating any potential competition for hydrogen.

In summary, to determine whether one organism can out compete another for hydrogen, both the kinetics and energetics of the two competing reactions must be evaluated. One should note that kinetic and thermodynamic comparisons between organisms may be an oversimplification of the complex interactions among members of an anaerobic consortium and may not be indicative of dechlorinator performance in a mixed culture. Environmental conditions, availability of growth factors other than electron donor, and nutritional dependencies of one organism on another may influence an organism's competitive advantage and should be considered in conjunction with the kinetic and thermodynamic analyses. Because these additional factors are unique to each culture, they can only be determined experimentally.

**Formation of Nonaqueous Phase Liquids**

Chlorinated solvents possess several physical and chemical properties that make them difficult to remediate once in the subsurface (physical and chemical properties used in this research are listed in Table 2.5). Characteristics of these compounds include low water solubilities, low interfacial tensions, low viscosities, and high densities. These properties govern the migration of the compounds through the subsurface as well as the contamination of the different subsurface phases.
### Table 2.5 Physical and Chemical Properties of Chlorinated Ethenes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g/mol)</th>
<th>Density (g/mL)(^a) 20 °C</th>
<th>Solubility in H₂O (mg/L) 20 °C</th>
<th>Henry's Constant(^d) 24.8 °C</th>
<th>(K_{wv}) 24°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetrachloroethene</td>
<td>C₂Cl₄</td>
<td>165.83</td>
<td>1.623</td>
<td>200(^b)</td>
<td>0.723</td>
<td>3060(^e)</td>
</tr>
<tr>
<td>trichloroethene</td>
<td>C₂HCl₃</td>
<td>131.39</td>
<td>1.464</td>
<td>1100(^b)</td>
<td>0.392</td>
<td>395(^e)</td>
</tr>
<tr>
<td>cis-dichloroethene</td>
<td>C₂H₂Cl₂</td>
<td>96.94</td>
<td>1.284</td>
<td>800 - 7.700(^c)</td>
<td>0.167</td>
<td>94(^e)</td>
</tr>
<tr>
<td>vinyl chloride</td>
<td>C₂H₃Cl</td>
<td>62.50</td>
<td>0.911</td>
<td>90 - 8.800(^c)</td>
<td>1.137</td>
<td>ND(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Data taken from (24). \(^b\) Data taken from (25). \(^c\) Range of reported solubilities as found in (26). \(^d\) Data taken from (27). \(^e\) Measured tridecane/water partition coefficients (see Chapter 6). \(^f\) Not determined.
Upon release, chlorinated solvents migrate through the vadose zone and below the water table where the compounds are dissolved forming a contaminated groundwater plume (see Figure 2.1). Low interfacial tensions allow the solvents to easily enter pore spaces forming residually trapped nonaqueous phases in the soil and aquifer matrices. In some instances, solvents may form pools on impermeable soil layers. Although low aqueous solubilities limit the dissolution of these immiscible, oily phases (7), groundwater concentrations are usually appreciable enough to exceed the U.S. Environmental Protection Agency (EPA) maximum contaminant level (MCL) (8). Even the smallest of NAPL residual saturations may represent a long-term, continuous source of groundwater contamination.

**Dissolution of Nonaqueous Phase Liquids**

The dissolution of nonaqueous phase liquids is governed by several factors including the effective solubilities and aqueous phase diffusivities of NAPL components, the contact area between the NAPL and groundwater, and the groundwater velocity (7). The transfer of contaminants from the NAPL into the aqueous phase occurs through diffusion, and is limited by the contaminant's aqueous solubility. For single component NAPLs, the concentration of the contaminant in the aqueous phase may be equivalent to its aqueous solubility. Contaminants present in multicomponent NAPLs, however, will have lower effective solubilities as determined by the mole fraction of the contaminant in the NAPL mixture and its aqueous solubility. For multicomponent NAPLs, the most soluble contaminants are preferentially removed first.
Figure 2.1 Schematic of NAPL formation in the subsurface. The release of chlorinated solvents from a waste pit results in the formation of residually trapped NAPL (see insert). Excess solvent becomes trapped on a confining layer (bedrock) where it resides as a free phase. Groundwater passing through the contaminant source zone dissolves portions of the NAPL, creating a dissolved groundwater plume.
Groundwater velocity and the distribution of NAPL ganglia within the aquifer matrix determine whether dissolution is mass-transfer limited. If the groundwater velocity is too fast and/or can not flow through regions containing NAPL, equilibrium dissolution may not be achieved. Laboratory and theoretical data have suggested that at typical groundwater velocities (<1 m/day) and typical NAPL residual saturations, the mass transfer between the NAPL and aqueous phase is sufficient to result in aqueous concentrations equal to the chemical's saturation concentration (9.10). At high groundwater velocities (>1 m/day) or when the interfacial area between the NAPL and aqueous phase is reduced, dissolution may become mass-transfer limited (10-13). Because the presence of NAPLs in the subsurface limits the relative permeability of the medium, groundwater flow through an area of high NAPL residual saturation may be considerably decreased. This is often observed when NAPLs are present as pools.

Dissolution can be described mathematically using a mass balance equation for the aqueous and NAPL phases (partitioning to the soil is neglected in this case). The mass balance equation below is written for one dimension, and is based on the simplifying assumptions that dispersion within the source zone is minimal and that the saturations of water and NAPL, as well as porosity, do not change with time:

\[
\frac{\partial C_{i}^{*}}{\partial t} + \frac{\theta_{s}}{\theta_{a}} \frac{\partial C_{i}^{*}}{\partial t} = -v_{r} \frac{\partial C_{i}^{*}}{\partial x} - r_{i}^{*},
\]

(2.4)

where

\( C_{i}^{*} \) = aqueous concentration (g·cm\(^{-3}\) water\(^{-1}\)).

\( C_{i}^{*} \) = NAPL concentration (g·cm\(^{-3}\) NAPL\(^{-1}\)).
\[ \theta_w = \text{saturation of water (cm}^3 \text{ water-cm}^3 \text{ pores}^{-1}). \]

\[ \theta_n = \text{saturation of NAPL (cm}^3 \text{ NAPL-cm}^3 \text{ pores}^{-1}). \]

\[ v_i = \text{interstitial velocity (cm-s}^{-1}). \]

\[ x = \text{distance within the source zone (cm)}. \]

\[ r_i^{\prime} = \text{homogeneous reaction in aqueous phase removing species } i. \]

If linear partitioning is assumed, when mass-transfer effects are insignificant the concentrations of species \( i \) in the NAPL and aqueous phases can be related by the equilibrium expression.

\[ C_i^{\prime} = K_i^{\prime \rightarrow \ast} C_i^{\ast}. \quad (2.5) \]

where \( K_i^{\prime \rightarrow \ast} \) (cm\(^3\) water-cm\(^3\) NAPL\(^{-1}\)) represents the NAPL/water partition coefficient for species \( i \). Under mass-transfer limited conditions, the relationship between \( C_i^{\prime} \) and \( C_i^{\ast} \) can be expressed as

\[ \frac{\partial C_i^{\prime}}{\partial t} = K_a \left( C_i^{\ast} - C_i^{\prime} / K_i^{\prime \rightarrow \ast} \right). \quad (2.6) \]

where the lumped mass transfer rate constant, \( K_a (s^{-1}) \) is a product of the mass transfer coefficient and the interfacial area between the NAPL and aqueous phase per bulk volume of the porous medium (14). The mass transfer coefficient is a function of the chemical's aqueous diffusivity and the thickness of the stagnant layer at the NAPL interface. Phenomenological models and their variations have been developed to characterize the dissolution of NAPLs from porous media, including dissolution at low NAPL volumetric fractions, as presented in references (10,11,13-19).
Biodegradation of a NAPL constituent is represented by the term \( r \), in equation 2.4. Microorganisms represent a sink for contaminant removal, but may not influence aqueous phase concentrations if rates of dissolution are faster than rates of degradation. This would be observed in cases of instantaneous dissolution. In the opposite case, where mass-transfer effects are significant, biodegradation may affect the driving force for dissolution by decreasing the bulk phase concentration. Thus, the overall impact microorganisms might exert on NAPL removal would be dependent upon rates of degradation, rates of dissolution (i.e., availability of contaminant to bacteria), and the presence of other factors, such as electron donors, necessary to sustain biological activity.

References


Chapter Three: LITERATURE REVIEW

The purpose of this chapter is to provide an overview of research efforts addressing anaerobic microbial transformation of chlorinated ethenes. The review begins with a general overview of the process of reductive dechlorination and includes a chronological summary of research efforts published in this field during the last two decades. The remaining review is divided into two sections. The first contains discussions on mechanisms and kinetics of PCE dechlorination, the role of electron donor in enriching and sustaining dechlorination activity, and an overview of the phylogenetic and metabolic diversity among PCE halorespirers. The last section provides an overview of current research efforts regarding the use of dechlorinating microorganisms for source zone treatment.

Reductive Dechlorination of Chlorinated Ethenes
Overview

In situ bioremediation of chlorinated ethenes has received much attention following the discovery of PCE and TCE transformation in mixed, anaerobic cultures (1-4). The pathway of anaerobic transformation, referred to as reductive dechlorination, is depicted in Figure 3.1. PCE is sequentially reduced to TCE, one or more of the dichloroethene (DCE) isomers (usually cis-DCE, as depicted in Figure 3.1), vinyl chloride (VC), and then to ethene (4.5). Anomalies to this pathway have included reported cases of VC oxidation to CO₂ under methanogenic (3.6) and iron reducing
Figure 3.1 Pathway of microbial PCE transformation under anaerobic conditions.

$\text{C}_2\text{Cl}_4 \rightarrow \text{C}_2\text{HCl}_3 \rightarrow \text{C}_2\text{H}_2\text{Cl}_2 \rightarrow \text{C}_2\text{H}_3\text{Cl} \rightarrow \text{C}_2\text{H}_4$
conditions (7.8), and ethene transformation to ethane (9). PCE transformations under aerobic conditions have not been observed.

Much of the research conducted on anaerobic reductive dechlorination of chlorinated ethenes in mixed and pure cultures has been summarized in Table 3.1. Inoculum source, electron donor, and major conclusions are listed for each publication. The following sections provide detailed overviews of research efforts central to this research.

Mechanisms of PCE Dechlorination: Cometabolism vs. Halorespiration

Until recently, PCE dechlorination was believed to be only a cometabolic process. Cometabolism is defined as a fortuitous transformation of the compound of interest by either a biological enzyme or cofactor, in which the compound does not support growth of the microorganism (10). Pure cultures of methanogens have been shown to dechlorinate PCE to TCE, but at slow rates (11-13). At this time, it was believed that energy flow during methanogenesis was being diverted to PCE via a reduced electron carrier (13). Several acetogenic bacteria have also been shown to dechlorinate PCE (as reviewed in (10,14,15)). Cometabolism of PCE is characterized by slow rates of dechlorination that result in the formation of TCE only.

Chlorinated ethenes halorespiration was discovered in 1993 when researchers isolated an anaerobe, Dehalobacter restrictus, that was capable of coupling the reductive dechlorination of PCE to growth (16,17). Five additional isolates, Dehalospirillum multivorans (18), strain TEA (19), Desulfuromonas chloroethenica (20,21), Desulfitobacterium sp. strain PCE1 (22), and Dehalococcoides ethenogenes strain 195
Table 3.1 Summary of Research Efforts Regarding Chlorinated Ethenes Dechlorination by Anaerobes in Mixed and Pure Cultures

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Year</th>
<th>Source of Inoculum</th>
<th>Electron Donor(s)</th>
<th>Results and Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouwer and McCarty (40)</td>
<td>1981</td>
<td>Laboratory digester containing a methanogenic mixed culture</td>
<td>None, except methanol used in PCE and TCE solutions</td>
<td>The degradation of PCE and TCE (10-200 μg/L) in anaerobic and aerobic cultures was investigated. Both compounds were recalcitrant under aerobic conditions, and appreciable degradation under anaerobic conditions was not observed within 16 weeks.</td>
</tr>
<tr>
<td>Bouwer and McCarty (1)</td>
<td>1983</td>
<td>Laboratory culture used by (40)</td>
<td>Acetate</td>
<td>Under methanogenic conditions, PCE (&lt;100 μg/L) was reductively dechlorinated to TCE. Addition of 2-bromoethanesulfonic acid (BES), an inhibitor of the final step in methanogenesis, caused a reduction in acetate utilization but did not affect the extent of PCE dechlorination. The role of acetohydroxyacid synthetase in PCE dechlorination remained uncertain.</td>
</tr>
<tr>
<td>Parsons, Wood, and DeMarco (2)</td>
<td>1984</td>
<td>Muck and surface water from the Everglades</td>
<td>None, except methanol used in PCE and TCE solutions</td>
<td>Muck and surface water from an aquifer recharge basin contaminated with PCE and TCE were used in static microcosms to determine the products of biotransformation. Cis- and trans-DCE and VC were identified as dechlorination end products.</td>
</tr>
<tr>
<td>Bouwer and McCarty (41)</td>
<td>1985</td>
<td>Laboratory methanogenic biofilm</td>
<td>Acetate</td>
<td>Biofilm modeling was used to estimate the rate of secondary utilization of PCE and other halogenated aliphatics by a methanogenic, acetate-fed biofilm. The k/K, value for PCE was found to be 0.08 L·mg⁻¹·day⁻¹ for an influent concentration of 15 μg/L.</td>
</tr>
<tr>
<td>Kleopfer, Easley, Haas, Jr., Deihl, Jackson, and Wurrey (42)</td>
<td>1985</td>
<td>Soil from a TCE spill site in Des Moines, Iowa</td>
<td>Soybean meal</td>
<td>Authors demonstrated that TCE was biologically reduced to the 1,2-DCE isomers using radiolabeled TCE in soil microcosms.</td>
</tr>
<tr>
<td>Vogel and McCarty (3)</td>
<td>1985</td>
<td>Laboratory culture used by (40)</td>
<td>Acetate</td>
<td>PCE was dechlorinated in fixed film methanogenic columns. Reduced end products were TCE, the DCE isomers, and VC. Twenty-four percent of the initial PCE added was reported to be</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
</tr>
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<td>-----------------------------------------------</td>
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<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Barrio-Lage, Parsons, Nassar, and Lorenzo (43)</td>
<td>1986</td>
<td>Muck and surface water from the Everglades</td>
<td>Organics in the sediment and water from sampling site</td>
<td>Investigated the biotransformation of DCE isomers in microcosms under anaerobic conditions. VC was detected in all spiked microcosms. Chloroethane was detected in microcosms fed cis-DCE only.</td>
</tr>
<tr>
<td>Barrio-Lage, Parsons, Nassar, and Lorenzo (44)</td>
<td>1987</td>
<td>Muck from the Everglades, calcareous rock, sandy, and organic soil from Vero Beach, FL</td>
<td>Organics in the sediment and in some cases, sodium acetate</td>
<td>Differences in TCE transformation based on inoculum source were investigated. TCE was dechlorinated to cis-DCE in the Vero Beach microcosms within one year. Microcosms containing rock had less biomass, and the initial TCE spike was depleted within 24 months. Depletion of the TCE spike in the muck microcosms occurred within 21 months. Addition of sodium acetate did not have an effect on the dechlorination rate, indicating that acetoclastic methanogens were not responsible for the dechlorination observed. Denitrifying bacteria were likewise not involved in TCE dechlorination as nitrate concentrations in the microcosms remained constant.</td>
</tr>
<tr>
<td>Fathepure, Nengu, and Boyd (11)</td>
<td>1987</td>
<td>Pure cultures of acetoclastic methanogens</td>
<td>Methanol and acetate</td>
<td>Pure cultures of anaerobic bacteria capable of dechlorinating PCE were identified. DCB-1, a chlorobenzoate fed anaerobe, and two strains of <em>Methanosarcina</em> dechlorinated PCE. DCB-1 stoichiometrically converted PCE to TCE. When DCB-1 was mixed with the two <em>Methanosarcina</em> strains, higher rates of PCE dechlorination were achieved and TCE was degraded.</td>
</tr>
<tr>
<td>Fathepure and Boyd (12)</td>
<td>1988</td>
<td>Sewer sludge and acetoclastic methanogens in pure culture</td>
<td>Methanol and acetate</td>
<td>Dechlorination was stimulated in two different sources of sewage sludge. PCE dechlorination and methanogenesis were significantly inhibited in sludges by the addition of BES. Acetoclastic methanogens, <em>Methanosarcina</em> sp. and <em>M. mazei</em> were shown to dechlorinate PCE in pure cultures. These results were in contrast to Barrio-Lage, et. al (1987) who concluded that acetoclasts were not directly involved in PCE dechlorination. These authors concluded that a relationship between PCE dechlorination and methanogenesis existed.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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</tr>
<tr>
<td>Fathepure and Boyd (13)</td>
<td>1988</td>
<td>Pure culture of <em>Methanosarcina</em> sp. strain DCM</td>
<td>Methanol, acetate, methylvamine, and trimethylamine</td>
<td><em>Methanosarcina</em> sp. strain DCM was shown to dechlorinate PCE to TCE while growing on methanol, acetate, methylvamine, and trimethylamine. PCE dechlorination was observed only during methanogenesis, and was found to be contingent upon methanol utilization. Authors suggested that electrons generated during methane synthesis were diverted &quot;... to PCE by a reduced electron carrier ...&quot;, and that stimulating methanogenesis could enhance PCE dechlorination.</td>
</tr>
<tr>
<td>Freedman and Gossett (4)</td>
<td>1989</td>
<td>Laboratory anaerobic digester seeded with digested sludge from an Ithaca, NY, waste water treatment plant</td>
<td>Methanol, hydrogen, formate, acetate, and glucose</td>
<td>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.</td>
</tr>
<tr>
<td>Scholz-Muramatsu, Szewydk, Szewydz, and Gaiser (45)</td>
<td>1990</td>
<td>Laboratory biofilm reactor</td>
<td>Benzoate</td>
<td>BES inhibited methanogenesis and benzoate degradation in batch cultures not amended with PCE. Cultures that received PCE and BES metabolized benzoate to acetate and dechlorinated PCE to DCE. The authors concluded that dechlorination could occur in the absence of methanogenesis provided that a fermentable substrate was available. This was the first report of PCE dechlorination to DCE in a mixed anaerobic culture without methanogenesis.</td>
</tr>
<tr>
<td>Bagley and Gossett (46)</td>
<td>1990</td>
<td>Laboratory culture used by (4)</td>
<td>Lactate</td>
<td>Demonstrated PCE could be dechlorinated to TCE and <em>cis</em>-DCE under sulfate-reducing conditions. The extent and rate of PCE dechlorination was less than that observed in mixed methanogenic cultures. Very little methanogenic activity was measured in all cases. Methanogenesis did not occur in cultures inhibited with BES, although PCE dechlorination was similar to that of</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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</tr>
<tr>
<td>Sewell and Gibson (47)</td>
<td>1991</td>
<td>Aquifer solids</td>
<td>Toluene</td>
<td>uninhibited cultures. PCE dechlorination was enhanced and methanogenesis was minimal in cultures inhibited by fluoroacetate, an acetate consumer inhibitor. Although the microorganisms responsible for dechlorination were not identified, inhibitor studies indicated that microorganisms other than methanogens were responsible for PCE dechlorination.</td>
</tr>
<tr>
<td>DiStefano, Gossett, and Zinder (5)</td>
<td>1991</td>
<td>Laboratory culture used by (4)</td>
<td>Methanol</td>
<td>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.</td>
</tr>
<tr>
<td>Gibson and Sewell (26)</td>
<td>1992</td>
<td>Aquifer solids from Traverse City, MI.</td>
<td>Short chain organic acids and alcohols</td>
<td>PCE (55 mg/L) was degraded to ethene (&lt;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 absence of methanogenesis. This observation was inconsistent with previous work, which implicated that methanogens participated in PCE dechlorination. Authors concluded that other organisms, potentially acetogens, played a role in PCE dechlorination.</td>
</tr>
<tr>
<td>de Bruin, Kotterman, Posthumus, Schraa, and Zehnder (9)</td>
<td>1992</td>
<td>Rhine River sediment and anaerobic granular sludge</td>
<td>Lactate</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>PCE (1.5 mg/L) was dechlorinated to ethene, which was further reduced to ethane, in a fixed-bed column fed lactate. When methanogenesis was inhibited by BES, ethene was not reduced to</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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</tr>
<tr>
<td>DiStefano, Gossett, and</td>
<td>1992</td>
<td>Laboratory culture used by (4)</td>
<td>Methanol and hydrogen</td>
<td>Ethane. Authors observed that for PCE dechlorination to be complete, both river sediment and anaerobic granular sludge were needed as inoculum. Two different cultures, one that converted PCE to cis-DCE, and one that converted cis-DCE to ethene, were recovered from the column. Authors hypothesize that more than one microorganism may be necessary for complete dechlorination of PCE to ethene. 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, an euubacterial 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 dechlorinating microorganism.</td>
</tr>
<tr>
<td>Zinder (27)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Holliger, Schraa, Stams,</td>
<td>1993</td>
<td>PER-K23 (Dehalobacter restrictus)</td>
<td>Hydrogen and formate</td>
<td>A gram negative bacterium (PER-K23) was isolated from the inoculum used by (9). PER-K23 was the first isolate found to couple the dechlorination of PCE to cis-DCE to growth. Growth on PCE or TCE could only be sustained using hydrogen or formate. Biomass and dechlorination were found to account for all of the electrons generated from hydrogen and formate utilization.</td>
</tr>
<tr>
<td>and Zehnder (17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gibson, Robertson,</td>
<td>1994</td>
<td>Aquifer solids from Traverse City, MI</td>
<td>Mixtures of lactate,</td>
<td>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 PCE 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</td>
</tr>
<tr>
<td>Russell, and Sewell (48)</td>
<td></td>
<td></td>
<td>acetate, and propionate</td>
<td></td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
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</tr>
<tr>
<td>Fathepure and Tiedje (49)</td>
<td>1994</td>
<td>Enrichment culture seeded with <em>D. tiedjei</em> DCB-1</td>
<td>3-chlorobenzoate</td>
<td>μM·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 PCE reduction to TCE.</td>
</tr>
<tr>
<td>Tandoi, DiStefano, Bowser, Gossett, and Zinder (50)</td>
<td>1994</td>
<td>Laboratory culture used by (4)</td>
<td>Methanol</td>
<td>Biofilm reactor packed with an enrichment culture containing <em>Desulfomonile tiedjei</em> 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 or a similar microorganism was involved in dechlorination. The maximum PCE dechlorination rate was 10.3 μmol·L⁻¹·hr⁻¹.</td>
</tr>
<tr>
<td>Komatsu, Momonoi, Matsuo, and Hanaki (51)</td>
<td>1994</td>
<td>Anaerobic digested sewage sludge</td>
<td>Glucose, yeast extract, propionate, hydrogen, methanol, and acetate</td>
<td>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·mg VSS⁻¹·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 reduction to VC. Trans-DCE conversion to VC was modeled using first order kinetics.</td>
</tr>
<tr>
<td>Neumann, Scholz-Muramatsu, and Diekert (18)</td>
<td>1994</td>
<td><em>Dehalospirillum multivorans</em></td>
<td>Pyruvate, hydrogen, and formate</td>
<td>Cis-DCE dechlorination in anaerobic cultures was supported by glucose, yeast extract, propionate, and to a lesser extent by hydrogen. The rate of cis-DCE dechlorination was slower in methanol-fed cultures than in cultures in which no electron donor was added. This implied that methanol may have had an inhibitory effect on dechlorination.</td>
</tr>
<tr>
<td>Scholz-Muramatsu, Neumann, Meßmer,</td>
<td>1995</td>
<td><em>Dehalospirillum multivorans</em></td>
<td>Hydrogen, pyruvate, lactate, ethanol,</td>
<td><em>Dehalospirillum multivorans</em>, an anaerobe capable of utilizing PCE as its terminal electron acceptor, dechlorinated PCE to cis-DCE. Pyruvate, formate and hydrogen could serve as electron donor. Fumarate was found to be an alternative terminal electron acceptor. The enzyme PCE dehalogenase was recovered in cell free extracts.</td>
</tr>
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<td></td>
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<td></td>
<td>Characterization of <em>D. multivorans</em> showed that the microorganism grew with PCE and hydrogen as its terminal electron acceptor and</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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</tr>
<tr>
<td>Moore, and Dickert (34)</td>
<td></td>
<td>formate, and glycerol</td>
<td>electron donor, respectively. Other electron donors (pyruvate, lactate, ethanol, formate, and glycerol) were found to support growth. Fumarate and nitrate were capable of replacing PCE as terminal electron acceptor. Growth on pyruvate and PCE resulted in the formation of cis-DCE, acetate, lactate, and hydrogen. The dechlorination rate was found to be 50 nmol·min⁻¹·mg cell protein⁻¹.</td>
<td></td>
</tr>
<tr>
<td>Maymó-Gatell, Tandoi, Gossett, and Zinder (52)</td>
<td>1995</td>
<td>Laboratory culture used by (4)</td>
<td>Hydrogen</td>
<td>Using the anaerobic enrichment culture developed by (4), 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 B₁₂, supernatant from an anaerobic digester sludge, and acetate as a source of carbon. Methanol and acetate could not replace hydrogen as electron donor, and the culture did not produce methane or acetate. This suggests that the hydrogen-PCE culture did not contain methanogens or acetogens.</td>
</tr>
<tr>
<td>Sharma and McCarty (24)</td>
<td>1996</td>
<td>Strain MS-1</td>
<td>Glucose, pyruvate, formate, lactate, acetate, yeast extract, amino acids</td>
<td>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. 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 cis-DCE.</td>
</tr>
<tr>
<td>Bradley and Chapelle (7)</td>
<td>1996</td>
<td>Anaerobic aquifer sediments</td>
<td>Vinyl chloride added as substrate.</td>
<td>First demonstration that vinyl chloride can be oxidized to CO₂ under iron (III)-reducing conditions. Transformation was dependent upon the bioavailability of Fe(III), which was added as Fe-EDTA.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
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<tr>
<td>Smatlak, Gossett, and Zinder (28)</td>
<td>1996</td>
<td>Butyrate enrichment culture seeded with culture from (4)</td>
<td>Hydrogen and formate</td>
<td>The half-velocity constants with respect to hydrogen utilization were measured and compared for methanogens and dechlorinators in a mixed, butyric acid-fed culture. The half-velocity constant for dechlorination was 100 nM, and the half-velocity constant for methanogenesis was 1000 nM. Authors concluded that using an electron donor that generates a slow, low-level release of hydrogen would allow dechlorinators to out compete methanogens for hydrogen, thus, “maximizing dechlorination potential”.</td>
</tr>
<tr>
<td>Gerritse, Renard, Gomes, Lawson, Collins, Gottschal (22)</td>
<td>1996</td>
<td>Desulfitobacterium sp. strain PCE1</td>
<td>Lactate, pyruvate, butyrate, formate, succinate, and ethanol</td>
<td>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-phenylacetate as terminal electron acceptors. PCE was dechlorinated to TCE, and cis-DCE and trans-DCE. Hydrogen was found to inhibit dechlorination.</td>
</tr>
<tr>
<td>Krumholz, Sharp, and Fishbain (20)</td>
<td>1996</td>
<td>Strain TT4B</td>
<td>Acetate or pyruvate</td>
<td>Strain TT4B was isolated from stream sediments contaminated with TCE and toluene. The isolate dechlorinated PCE to cis-DCE, and was found to use other terminal electron acceptors (TCE, fumarate, and ferric nitritolacetate).</td>
</tr>
<tr>
<td>Fennell, Gossett, and Zinder (30)</td>
<td>1997</td>
<td>Laboratory culture used by (27)</td>
<td>Butyric acid, ethanol, lactic acid, propionic acid</td>
<td>Investigated several electron donors as potential hydrogen donors for PCE dechlorination. Over long term experiments, dechlorination could be equally sustained and maintained regardless of electron donor fed. Short term experiments demonstrated that there were differences among the four electron donors with respect to hydrogen production and the distribution of electron equivalents between dechlorination and methanogenesis.</td>
</tr>
<tr>
<td>Ballapragada, Stensel, Puhakka, and Ferguson (29)</td>
<td>1997</td>
<td>Laboratory methanogenic consortium</td>
<td>Lactate, acetate, hydrogen, and propionate</td>
<td>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 ($K_v$ values for hydrogen uptake by dechlorinators were reported to be 12-28 ppm).</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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<tr>
<td>Schöllhorn, Savary, Stucki, and Hanselmann (53)</td>
<td>1997</td>
<td>Mixture of anaerobic sludges</td>
<td>Formate, methanol, acetate, hydrogen, and ethanol</td>
<td>Under simulated groundwater conditions (14°C, equivalent ionic strength, pH 7-7.5), compared the suitability of different electron donors for the start-up and completeness of TCE transformation. All electron donors tested supported TCE dechlorination to cis-DCE, although lactate and formate had the shortest lag times (12 and 24 days, respectively).</td>
</tr>
<tr>
<td>Lee, Yoshimi, Ike, and Fujita (54)</td>
<td>1997</td>
<td>Contaminated soil sample</td>
<td>Citrate, pyruvate, succinate, formate, acetate, and acetate with hydrogen</td>
<td>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). 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/mg VSS/hr.</td>
</tr>
<tr>
<td>Gao, Sken, Hooker, and Quesenberry (55)</td>
<td>1997</td>
<td>Sediments taken from several contaminated sites.</td>
<td>Methanol, lactate, acetate, and sucrose</td>
<td>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.</td>
</tr>
<tr>
<td>Bradley and Chapelle (8)</td>
<td>1997</td>
<td>Creek bed sediments obtained near discharge of contaminated groundwater</td>
<td>cis-DCE and VC added as substrates.</td>
<td>cis-DCE and VC mineralization were observed under both methanogenic and Fe(III)-reducing conditions. In the methanogenic microcosms, 5% to 44% of 14VC and 4% to 14% of 14DCE were recovered as 14CO2. Under iron reducing conditions, the recovery of 14CO2 from labeled VC was twice that of the methanogenic microcosms, and similar for labeled cis-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.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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</tr>
<tr>
<td>Maymó-Gatell, Chien, Gossett, and Zinder (23)</td>
<td>1997</td>
<td><em>Dehalococcoides ethanogenes</em> strain 195</td>
<td>Hydrogen</td>
<td>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 an anaerobic digester sludge supernatant for growth. This is the first report of an isolate with the capability of reducing PCE completely to ethene.</td>
</tr>
<tr>
<td>Carr and Hughes (32)</td>
<td>1998</td>
<td>Laboratory enrichment culture seeded from an anaerobic upflow sludge blanket; sediments from a contaminated site in Texas</td>
<td>Methanol, lactate, and hydrogen</td>
<td>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.</td>
</tr>
<tr>
<td>Fennell and Gossett (56)</td>
<td>1998</td>
<td>Laboratory culture used by (30)</td>
<td>Butyric acid, ethanol, lactic acid, and propionic acid</td>
<td>Using data obtained from a laboratory culture (30), 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 compensating for competition of electron donor by adding excess donor eventually led to failure of dechlorination and the development of a predominantly methanogenic population.</td>
</tr>
<tr>
<td>Cabriol, Jacob, Perrier, Fouillet, and Chambon (6)</td>
<td>1998</td>
<td>Methanogenic and sulfate-reducing mixed culture from the Bourg-en-Bresse waste water treatment plant in France</td>
<td>Methanol</td>
<td>Fixed-bed reactor containing a methanogenic and sulfate-reducing enrichment culture was shown to remove up to 98% of influent PCE concentrations of 215 μM. Addition of BES inhibited PCE dechlorination, and a methanogen (<em>Methanosarcina</em> sp. strain FR) was isolated from the culture that transformed radiolabeled PCE to an unknown labeled compound. Mass balances on the labeled PCE demonstrated that PCE was being transformed to CO₂, and</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Source of Inoculum</td>
<td>Electron Donor(s)</td>
<td>Results and Conclusions</td>
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<tr>
<td>Isalou, Sleep, and Liss (57)</td>
<td>1998</td>
<td>Anaerobic digester sludge from Metropolitan Toronto Main Treatment Plant</td>
<td>Methanol</td>
<td>Digester sludge was enriched on methanol and PCE (12-600 μM) in a continuous flow column packed with sand. For the first 29 months of operation, PCE dechlorination resulted in the production of VC. Significant ethene production coincided with the onset of acetogenesis, which became the major pathway for methanol metabolism. Ethene production was sensitive to the methanol:PCE molar ratio, and VC accumulated at ratios less than 5.0.</td>
</tr>
<tr>
<td>Yang and McCarty (31)</td>
<td>1998</td>
<td>Aquifer material from a PCE-contaminated groundwater site in Victoria, TX</td>
<td>Benzoate and propionate</td>
<td>Demonstrated that in batch studies containing benzoate and cis-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.</td>
</tr>
<tr>
<td>Cabriol, Vilemur, Perrier, Jacob, Fouillet, and Chambon (58)</td>
<td>1998</td>
<td>Methanosarcina sp. strain FR</td>
<td>Methanol, acetate, and hydrogen</td>
<td>Methanogenic isolate was shown to dechlorinate at PCE (50-87 μM). Dechlorination of radiolabeled PCE produced an unidentified radiolabeled end product that was not chlorinated. The dechlorination rate, 76 nM·mg protein⁻¹·day⁻¹, was found to be faster than rates previously reported for other methanogens (59).</td>
</tr>
<tr>
<td>Haston and McCarty (60)</td>
<td>1999</td>
<td>Aquifer material from a PCE-contaminated groundwater site in Victoria, TX</td>
<td>Benzoate and hydrogen</td>
<td>The maximum degradation rates and half-velocity coefficients for PCE, TCE, cis-DCE, and VC dechlorination were determined in batch cultures. Degradation rates were highest with PCE (77 μM/day), and degradation rates for cis-DCE and VC were similar (14 μM/day, 13 μM/day, respectively). Half-velocity coefficients for PCE, TCE, cis-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 cis-DCE and/or VC can be partially explained by kinetics.</td>
</tr>
</tbody>
</table>
(23), have since been discovered, and all are capable of utilizing PCE as their terminal electron acceptor. Strain MS-1 (24) has also been shown to dechlorinate PCE in pure culture, but PCE dependent growth has not yet been demonstrated. Of these isolates, all but one. *Dehalococcoides ethenogenes* strain 195, dechlorinates PCE to TCE or *cis*-DCE. *D. ethenogenes* strain 195 dechlorinates PCE completely to ethene. Other microorganisms capable of *cis*-DCE halorespiration have yet to be identified. The discovery of PCE and TCE halorespiring anaerobes is considered an evolutionary marvel (17) since these chlorinated compounds are anthropogenic and have only been in existence for less than a century (25).

**Kinetics of PCE Dechlorination**

The kinetics of PCE dechlorination have been determined in both mixed and pure cultures, although inconsistencies in the manner in which the dechlorination rates were determined have made comparisons between studies difficult. Table 3.2 lists rate coefficients for various cultures and includes the temperature at which the study was conducted. The disparity in dechlorination rates between cometabolic and halorespiratory processes is demonstrated by the organisms *Methanosarcina* sp. and *Dehalospirillum multivorans*, whose rates of dechlorination are $3.5 \times 10^{-5}$ and 11.4 $\mu$mol·mg protein$^{-1}$·hr$^{-1}$, respectively. In this particular case, rates of halorespiration are over 5 orders of magnitude greater than that of cometabolism. Although these determinations were not made at the same temperature, it is still evident that PCE dechlorination as a result of halorespiration is much faster than that of cometabolism. In mixed cultures, rates of


<table>
<thead>
<tr>
<th>Reference</th>
<th>Culture</th>
<th>Temperature (°C)</th>
<th>Rate of Dechlorination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fathepure et al. (11)</td>
<td><em>Methanosarcina</em> sp.</td>
<td>37</td>
<td>$3.5 \times 10^{-3}$ μmol·mg protein⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Distefano et al. (5)</td>
<td>enrichment culture</td>
<td>35</td>
<td>11.5 μmol·L⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>de Bruin. et al. (9)</td>
<td>fixed-bed column</td>
<td>20</td>
<td>3.7 μmol·L⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Carter and Jewell (61)</td>
<td>attached film expanded bed reactor</td>
<td>15</td>
<td>1.34 μmol·mg VS⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Tandoi. et al. (50)</td>
<td>enrichment culture</td>
<td>35</td>
<td>0.19 μmol·mg VSS⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Gerritse. et al. (62)</td>
<td>enrichment culture</td>
<td>30</td>
<td>14.2 μmol·L⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Sharma and McCarty (24)</td>
<td>strain MS-1</td>
<td>NA³</td>
<td>0.5 μmol·mg cell⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Lee. et al. (54)</td>
<td>enrichment culture</td>
<td>30</td>
<td>0.4 μmol·mg VSS⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Eisenbeis. et al. (63)</td>
<td><em>Dehalospiillum multivorans</em> in fluidized bed reactor</td>
<td>20</td>
<td>3.3 μmol·mg protein⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Eisenbeis. et al. (63)</td>
<td><em>Dehalospiillum multivorans</em> in batch culture</td>
<td>30</td>
<td>11.4 μmol·mg protein⁻¹·hr⁻¹</td>
</tr>
<tr>
<td>Cabriol. et al. (6)</td>
<td>fixed-bed reactor</td>
<td>37</td>
<td>3 μmol·L⁻¹·hr⁻¹</td>
</tr>
</tbody>
</table>

³ Not available.
dechlorination and the resulting end product are used to estimate whether PCE dechlorination is cometabolic or respiratory.

**Role of Electron Donor in Sustaining PCE Dechlorination**

One of the main topics under investigation with regards to the process of reductive dechlorination is whether there are particular electron donors best suited to support this activity. A large majority of the research regarding reductive dechlorination of chlorinated ethenes has been conducted in mixed cultures. Numerous electron donors, ranging from simple substrates, such as hydrogen, to organic acids and alcohols, have been shown to support dechlorination in these systems (see Table 3.1).

At one time, hydrogen was believed to serve as the final electron donor for dechlorination. Gibson and Sewell (26) observed that substrates whose metabolism produced large amounts of hydrogen, such as lactate and ethanol, were able to support dechlorination while those that did not produce high levels of hydrogen could not (i.e., methanol and acetate). DiStefano, *et al.* (27) reported that hydrogen produced by methanol acetogenesis sustained PCE dechlorination in a mixed culture. The authors observed that the addition of vancomycin inhibited acetogenesis in both methanol- and hydrogen-fed cultures, but that dechlorination was only inhibited in the methanol-fed culture. Both reports support the role of hydrogen as the final electron donor for dechlorination, although they are contradictory with respect to the amount of hydrogen production necessary to sustain this activity. Other investigations, however, have demonstrated that hydrogen-based dechlorination is not universal to all cultures. Acetate,
whose metabolism usually does not result in the formation of hydrogen (10), has been shown to support dechlorination in both mixed (1.3.4) and pure (20.21) cultures.

For environments in which dechlorination is hydrogen-based, competition for electron donor between methanogens, acetogens, and dechlorinators may become important. Several investigators have recently examined this issue (28.29). In both reports, the half-velocity constants with respect to hydrogen, $K_v(H_2)$, were measured for dechlorinators and methanogens in a mixed culture. $K_v(H_2)$ values were found to be approximately an order of magnitude lower for dechlorinators than for methanogens (see Table 2.3). Based on the difference between the half-velocity constants, the authors concluded that dechlorinators could out compete methanogens for hydrogen when hydrogen levels were low (approximately 10-100 nM). The authors suggested that the best method for providing a competitive advantage to dechlorinators would be to use an electron donor whose metabolism resulted in the slow release of low hydrogen levels.

Microbial competition for hydrogen produced through the fermentation of organic substrates has been investigated by Fennel, et al. (30) and Yang and McCarty (31). In the study by Fennel, et al., butyric acid, ethanol, lactic acid, and propionic acid were tested with regard to their ability to serve as hydrogen donors for PCE dechlorination. The authors observed that in long-term batch studies, no differences in dechlorination activity were observed among the four electron donors. However, in short-term batch studies, methane formation was reduced or absent in culture bottles containing electron donors that maintained low levels of hydrogen (i.e., propionic acid and butyric acid). Although the distribution of reducing equivalents to dechlorination was higher in propionic- and
butyric acid-fed cultures, dechlorination was not substantially improved. As a case in point, lactic acid-fed cultures exhibited the greatest extent and rate of dechlorination.

Yang and McCarty (31) conducted a comparative study of benzoate and propionate as hydrogen donors for cis-DCE dechlorination. As in the study by Fennel et al., the authors observed that dechlorinators, when fed propionate under batch conditions, could maintain a hydrogen level below that which could support both methanogenesis and acetogenesis. In batch studies fed benzoate, elevated hydrogen concentrations were formed, methanogenesis, acetogenesis, and dechlorination were observed, and ethene formation was reduced. Under continuous feed conditions, however, benzoate-fed dechlorinators were able to maintain a constant hydrogen concentration that was poised below that which would sustain methane and acetate production. These observations lead to the conclusion that in hydrogen-based dechlorinating cultures, one can give dechlorinators a competitive advantage by either using an electron donor whose metabolism results in the formation of ideal hydrogen concentrations that exclude the growth of other hydrogenotrophic organisms, or by adjusting the dose and delivery rate of any hydrogen precursor (31).

To date, no studies have demonstrated that high hydrogen partial pressures have resulted in the exclusion of dechlorination in favor of methanogenesis or acetogenesis. In fact, two reports (29,32) have demonstrated that rates and extents of dechlorination improved over time in systems containing both high hydrogen partial pressures and an active methanogenic community. In the study by Carr and Hughes (see Chapter 4, reference (32)), lactate, methanol, and hydrogen were fed to a mixed, methanogenic
culture in equivalent amounts and were found to equally sustain PCE dechlorination over a period exceeding one year. No differences were observed with respect to extent or rate of dechlorination, and it was concluded that cost and method of delivery should dictate electron donor selection for in situ bioremediation systems. Similar observations were made by Ballapragada, et al. (29), who reported that rates of PCE, TCE, and cis-DCE dechlorination increased with increasing hydrogen partial pressures produced in a lactate-fed methanogenic consortium.

Collectively, these studies indicate that many electron donors can sustain dechlorination either individually or by serving as a hydrogen precursor. There is no question that hydrogen is a key electron donor in many dechlorinating cultures, although certain exceptions are known to exist. As for electron donor competition, it can be concluded that PCE and cis-DCE dechlorinators can out compete methanogens and acetogens for hydrogen when present at low partial pressures, thus giving dechlorinators a competitive advantage. High hydrogen partial pressures, on the other hand, have not been found to result in the exclusion of dechlorination in favor of methanogenesis or acetogenesis and in several instances have been correlated to improved dechlorinator performance (i.e., improved rates and extents of dechlorination). The latter findings indicate that the dynamics of electron donor competition in an anaerobic mixed culture can not be simplified through one comparative analysis (i.e., half-velocity constants). Many factors, such as limiting concentrations of other growth substrates, interspecies relationships, and other physiological properties of the consortium constituents (i.e., true
cell yields. maximum specific utilization rates) will influence electron donor usage and potential competition effects.

**Phylogenetic and Metabolic Diversity among PCE-Halorespiring Bacteria**

The isolation and characterization of PCE-halorespiring anaerobes should prove useful for determining the conditions necessary to enrich and sustain this activity *in situ*. To date, the information gathered from isolation work has indicated that these bacteria are phylogenetically and metabolically very diverse. This information has been summarized in the following two tables: Table 3.3 lists the original source of each isolate, morphological characteristics, and phylogeny as determined by 16S rDNA gene sequencing; Table 3.4 lists the electron donor and nutritional requirements of each isolate as well as relevant growth properties. Although PCE dependent growth has not yet been demonstrated for strain MS-1, this organism has been included for discussion because it exhibits properties both unique and comparable to the known PCE-halorespiring isolates.

PCE-halorespiring anaerobes have been found in a variety of environments, the majority of which were sites with known histories of chlorinated solvent contamination (see Table 3.3). Interestingly, other bacteria have also been isolated from laboratory enrichment cultures inoculated with anaerobic sludges with no previous exposure to chlorinated ethenes, indicating that either these organisms are quite ubiquitous in nature, or some bacteria can quickly adapt to PCE halorespiration. Although all of these microorganisms are eubacteria, they are not closely related phylogenetically (with the exception of *Dehalobacter restrictus* and strain TEA, which share 99.7% 16S rDNA sequence identity).
<table>
<thead>
<tr>
<th>Property</th>
<th><em>Dehalobacter restrictus</em> (17,33)</th>
<th><em>Dehalospirillum multivorans</em> (18,34)</th>
<th><em>Desulfovibrio chloroethenica</em> (20,21)</th>
<th><em>Desulfotignum sp.</em> strain PCE1 (22)</th>
<th><em>Dehalococcoides ethenogenes</em> 195 (23,52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>original source of inoculum</td>
<td>Rhine river sediment mixed with anaerobic granular sludge</td>
<td>Activated sludge with no previous exposure to chlorinated ethenes</td>
<td>Aquifer material from PCE contaminated site in Victoria, TX</td>
<td>Anaerobic charcoal reactor used to remediate PCE and TCE contaminated groundwater</td>
<td>Freshwater stream sediments from contaminated soil in Massachusetts</td>
</tr>
<tr>
<td>morphology</td>
<td>rod</td>
<td>spirillum</td>
<td>rod</td>
<td>rod</td>
<td>curved rod</td>
</tr>
<tr>
<td>size, μm (D × L)</td>
<td>0.3-0.5 × 2-3</td>
<td>0.45 × 2-5</td>
<td>0.8-1.0 × 2-3</td>
<td>0.2-0.3 × 2-5</td>
<td>0.6 × 1.0-1.7</td>
</tr>
<tr>
<td>gram stain</td>
<td>gram-negative</td>
<td>gram-negative</td>
<td>gram-negative</td>
<td>gram-negative</td>
<td>gram-positive</td>
</tr>
<tr>
<td>motility</td>
<td>+ (one lateral flagellum)</td>
<td>+ (some strains non-motile)</td>
<td>+ (one to four lateral flagella)</td>
<td>+ (one subpolar flagellum)</td>
<td>+ (four lateral flagella)</td>
</tr>
<tr>
<td>phylogeny</td>
<td>gram-positive bacteria with low G+C content</td>
<td>Epsilon subdivision of Proteobacteria</td>
<td>Family Enterobacteriaceae</td>
<td>Delta subdivision of Proteobacteria, Genus <em>Desulfovibrio</em></td>
<td>gram-positive bacteria with low G+C content, Subphylum Clostridia, Genus <em>Desulfitobacterium</em></td>
</tr>
<tr>
<td>nucleotide sequence accession number</td>
<td>GenBank #U84497</td>
<td>EMBL Data Library #X82931</td>
<td>GenBank #1.43508</td>
<td>EMBL Data Library #Y10164</td>
<td>GenBank #134771</td>
</tr>
</tbody>
</table>

*4 Not available.*
### Table 3.4 Metabolic Properties of Anaerobic Bacteria that Demonstrate PCE Halorespiration

<table>
<thead>
<tr>
<th>Property</th>
<th><em>Dehalobacter restrictus</em> (17,33)</th>
<th><em>Dehalospirillum multivorans</em> (18,34)</th>
<th>strain MS-1 (24)</th>
<th><em>Desulfuromonas chloroethenica</em> (20,21)</th>
<th><em>Desulfotahacterium</em> sp. strain PCE-1 (22)</th>
<th><em>Dehalococcoides ethenogenes</em> 195</th>
</tr>
</thead>
<tbody>
<tr>
<td>electron donors</td>
<td>hydrogen, formate</td>
<td>hydrogen, formate, pyruvate, lactate, ethanol, glycerol</td>
<td>hydrogen</td>
<td>pyruvate, acetate</td>
<td>formate, pyruvate, lactate, butyrate, succinate, ethanol</td>
<td>hydrogen</td>
</tr>
<tr>
<td>additional nutritional requirements</td>
<td>arginine, histidine, threonine; acetate as carbon source</td>
<td>acetate as carbon source</td>
<td>NA</td>
<td>acetate and/or carbon dioxide as carbon source</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>fermentative growth</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>cellular material obtained from sonicated mixed dechlorinating cultures</td>
</tr>
<tr>
<td>terminal electron acceptors&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PCE, TCE</td>
<td>PCE, TCE, fumarate, nitrate</td>
<td>PCE, TCE&lt;sup&gt;c&lt;/sup&gt;, TCE&lt;sup&gt;c&lt;/sup&gt;, oxygen, nitrate</td>
<td>PCE, TCE</td>
<td>PCE, TCE, fumarate, ferric nitritocetate, polysulfide</td>
<td>PCE, TCE, fumarate, ortho-chlorinated phenols, sulfite, thiosulfate</td>
</tr>
<tr>
<td>dechlorination end product</td>
<td><em>cis</em>-DCE</td>
<td><em>cis</em>-DCE</td>
<td><em>cis</em>-DCE</td>
<td><em>cis</em>-DCE</td>
<td>TCE</td>
<td>ethene</td>
</tr>
<tr>
<td>maximum PCE concentration</td>
<td>200 µM (33 mg/l.)</td>
<td>300 µM (50 mg/l.)</td>
<td>1 mM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
<td>47 µM (7.8 mg/l.)</td>
<td>NA</td>
</tr>
<tr>
<td>doubling time&lt;sup&gt;e&lt;/sup&gt;</td>
<td>19 hr</td>
<td>2.5 hr</td>
<td>NA</td>
<td>NA</td>
<td>48-96 hr</td>
<td>58 hr</td>
</tr>
<tr>
<td>Yield&lt;sup&gt;e&lt;/sup&gt; (g protein/mol CT)</td>
<td>2.1</td>
<td>1.4</td>
<td>NA</td>
<td>0.1-0.25</td>
<td>0.15</td>
<td>1.6</td>
</tr>
<tr>
<td>optimum temperature (°C)</td>
<td>25-35</td>
<td>30</td>
<td>37</td>
<td>NA</td>
<td>21-31</td>
<td>34-37</td>
</tr>
<tr>
<td>optimum pH</td>
<td>6.8-7.6</td>
<td>7.0-7.5</td>
<td>7.0</td>
<td>NA</td>
<td>7.4</td>
<td>7.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not available.  
<sup>b</sup> Terminal electron acceptors that are utilized with non-fermentable substrates.  
<sup>c</sup> Proposed terminal electron acceptor. PCE dependent growth has not yet been demonstrated.  
<sup>d</sup> Nominal concentration, unclear whether reported concentration was corrected for aqueous-gas partitioning.  
<sup>e</sup> Data taken from Table 1 in (33).
The electron donor requirements of the PCE halorespiring isolates are also quite diverse (see Table 3.4). Three organisms can only utilize hydrogen (19.23) or hydrogen and formate as electron donor (33). while one, *Desulfuromonas chloroethenica*, can not use either (20.21). The remaining isolates, including *D. chloroethenica*, couple the reduction of PCE with the oxidation of organic acids or short chain fatty acids (18.21.22.34). These inconsistencies in energy requirements among the known PCE-halorespiring bacteria complicate the selection of electron donor for *in situ* bioremediation.

The ability to use terminal electron acceptors other than PCE and TCE has been observed in *D. multivorans*, *D. chloroethenica*, and *Desulfotobacterium* sp. strain PCE1 (18.21.22.34). Interestingly, those isolates which are restricted to PCE and TCE as sole terminal electron acceptors are also the most restricted with respect to electron donor. i.e., they can only utilize hydrogen or formate. Such metabolic restrictions could significantly impact the ability of these microorganisms to compete for electron donor and sustain PCE halorespiration in mixed cultures. Conversely, those organisms with more diverse metabolic capabilities may only dechlorinate PCE when other more thermodynamically favorable terminal electron acceptors are absent. This phenomenon has been observed in strain MS-1, which preferentially utilized oxygen or nitrate before PCE (24).

To sustain PCE-halorespiring bacteria in mixed cultures, other growth factors, such as carbon source and trace nutrients, become important. Only in a few cases have these elements been identified. Both *Dehalobacter restrictus* and *Dehalospirillum multivorans* are heterotrophic, requiring an external source of acetate for growth (33.34).
*Dehalobacter restrictus* also requires the addition of the amino acids arginine, histidine, and threonine, and the vitamins cyanocobalamin and thiamine (33). Dechlorination could not be sustained in pure cultures of *Dehalococcoides ethenogenes* 195 without the addition of cellular material from mixed, dechlorinating cultures (23). The necessary constituent in this case has yet to be identified. Understanding the nutritional factors essential for PCE halorespiration is important for applications in the field, where these determinants may or may not be supplied by the metabolic activities of other microorganisms.

**Dehalogenase Enzymes Associated with PCE Halorespiration**

Recent work has focused on purifying and characterizing dehalogenases associated with PCE halorespiration. Dehalogenase enzymes have been found in the cytoplasm of *D. multivorans* (35) and in the cell membranes of *D. restrictus* (36) and strain PCE-S (37). Although respiration chains for these bacteria have not yet been determined, models have been proposed and are covered in recent review articles by Wohlfarth and Diekert (36), El Fantroussi. *et al.* (14), and Middeldorp. *et al.* (10).

**Reductive Dechlorination as a Mechanism for Enhancing Removal Rates of PCE-Containing NAPLs**

The ability to enrich and sustain dechlorination in the presence of PCE-containing NAPLs is dependent upon the ability of dechlorinating microorganisms to grow at potentially high concentrations of PCE. Several of the PCE-halorespiring isolates grow at concentrations of 30-50 mg/L PCE (18,33,34), effectively one-sixth to one-fourth of the saturation concentration, under batch conditions. Sharma and McCarty (24) isolated a
facultative aerobe that dechlorinated PCE up to 1 mM concentrations (166 mg/L). Under continuous flow conditions, the culture used in this research has sustained dechlorination at PCE concentrations of 86 mg/L (32). These reports demonstrate the potential of dechlorinating bacteria to survive at high PCE concentrations and indicate that dechlorination within the source zone may be possible.

The impact of dechlorinating microorganisms on NAPL longevity has not been extensively researched. Peyton, et al. (38) conducted an experiment in which columns containing DNAPL contaminated soil were inoculated with dechlorinating microorganisms. The resulting effluent PCE concentrations were compared to abiotic control columns. The authors reported that the effluent PCE concentration was reduced to 17 µg/L in the inoculated columns as compared to 26 mg/L in the abiotic controls.

Nielsen and Keasling (39) reported PCE and TCE dechlorination in the presence of pure phase NAPLs. In both cases, PCE and TCE were dechlorinated to vinyl chloride and ethene. Although limited in details, this report and that of Peyton et al. indicate that dechlorination can be sustained near NAPLs. More quantitative studies on the effect of dechlorination on NAPL longevity are needed, as well as studies that elucidate how dechlorination can impact NAPL composition.

References


Chapter Four: ENRICHMENT OF HIGH-RATE PCE DECHLORINATION AND COMPARATIVE STUDY OF LACTATE, METHANOL, AND HYDROGEN AS ELECTRON DONORS TO SUSTAIN ACTIVITY

The contents of Chapter 4 have been published and are reproduced with permission from *Environmental Science and Technology*, 1998, 32(12), 1817-1824. Copyright 1998 American Chemical Society. After publication, the paper was the subject of a formal Comment and Response to the editor of *Environmental Science and Technology*. The comment and response are reproduced in the appendix.
The influence of three electron donors on the ability to sustain rapid tetrachloroethene (PCE) dechlorination in mixed cultures was investigated. Experiments were performed in recycle columns containing either a high-rate PCE dechlorinating culture developed from granular sludge or a (1:1) mixture of this culture with an enrichment culture derived from soil cores taken from a chlorinated ethene contaminated site. Columns were fed PCE (aqueous concentration of 5 mg/L) and either lactate, methanol, or H₂ as their electron donor. Comparisons between electron donors were formulated using the observed rates of PCE disappearance and the extent of dechlorination achieved in each column. Over a period exceeding 1 year, the observed PCE half-life in each column was found to decrease from approximately 13 h to slightly less than 2 h, regardless of electron donor fed. Although initial mass balance studies demonstrated that the extent of dechlorination varied between inoculum and electron donor, over time, the recycle columns behaved similarly, and PCE was recovered as approximately 80% vinyl chloride and 20% ethene in all systems. Rates and extents of PCE dechlorination were improved over time in systems containing high H₂ partial pressures (0.8 atm) in the presence of an active methanogenic community.

Materials and Methods

Chemicals. The following chemicals were obtained in liquid form: tetrachloroethene (ACS reagent, 99.5+%; Sigma-Aldrich); trichloroethene (HPLC grade, 99.9+%; Sigma-Aldrich); and cis-1,2-dichloroethene (cis-DCE) (99% purity; Supelco). Vinyl chloride (7.99%) in nitrogen was purchased from Air Liquide. Fluorobenzene, 2000 mg/mL in methanol, was purchased from Supelco. Sodium lactate (60 wt % aqueous solution), pentane (HPLC grade), and methanol (certified ACS, spectrally pure) were obtained from Acros Organics, Fisher Scientific. Methane (99.0%), propane (99.5%), and ethene (99.5%) were procured from Scott Specialty Gases. Custom-made gases, N₂/CO₂ (90/10%, v/v) and H₂/CO₂ (80/20%, v/v), were prepared by TriGas.

Nutrient Medium. Reagent-grade chemicals were used in nutrient medium preparation. The nutrient medium consisted of the following: 400 mg/L NH₄Cl, 400 mg/L KCl, 400 mg/L MgCl₂·6H₂O, 80 mg/L (NH₄)₂HPO₄, 25 mg/L CaCl₂·2H₂O, 10 mg/L NaNO₃, 10 mg/L (NaPO₄)₂, 2.5 mg/L KI, 2.5 mg/L CoCl₂·6H₂O, 0.5 mg/L MnCl₂·4H₂O, 0.5 mg/L NH₄VO₃, 0.5 mg/L ZnCl₂, 0.5 mg/L Na₂MoO₄·2H₂O, 0.5 mg/L H₃BO₃, 0.5 mg/L NiCl₂.
6H₂O, 200 mg/L yeast extract, and NaHCO₃ as buffer when needed. Except where noted, the nutrient medium was made anaerobic by the addition of 300 mg/L Na₂S·9H₂O and 40 mg/L FeCl₃·4H₂O, and 1 mg/L resazurin was added as a redox indicator.

**PCE Saturated Aqueous Solution, PCE₉**. Two milliliters of PCE was added to 100 mL of deionized water in a 120 mL serum bottle. After sealing with a Teflon-lined butyl rubber septum and aluminum crimp cap, the PCE/water solution was vigorously shaken, then allowed to sit quiescently for a period of 24 h. The resulting aqueous-phase PCE concentration was consistently measured to be 200 mg/L (the aqueous solubility of PCE at ca. 25 °C). The aqueous phase was used for PCE addition in selected experiments as described in the following sections.

**Analytical Methods**. A variety of gas chromatography techniques were established to quantify and identify chlorinated ethenes, nonchlorinated end products, and methane. Quantification of chlorinated ethenes and ethene required correction for partitioning between aqueous and gas phases. Dimensionless Henry’s Gas Law constants (Hₑ) reported for PCE, TCE, the DCE isomers, and VC at 24.8 °C were used (25). A dimensionless Henry’s Gas Law constant for ethene was measured in the lab as outlined in ref 25 and was found to be 7.75 ± 0.46 (95% confidence interval). Throughout this text, all reported aqueous and gas-phase concentrations have been corrected for partitioning.

Two methods were used for PCE and TCE quantification. The first method allowed for low level analysis of PCE, TCE, and DCE isomers. The second method was used for samples with high concentrations of PCE and TCE, but was not effective for the analysis of DCE isomers.

In the first method (method I), aqueous samples were analyzed by a gas chromatograph (GC) (Hewlett-Packard) equipped with a mass selective detector (MS). Samples were introduced to the GC following concentration by purge and trap (P&T analytical) equipped with a VOCARB 3000 trap (Supelco). Separation was achieved with a 60 m × 0.25 mm i.d., 1.5 mm film VOCOL capillary column (Supelco). The detector and injection port temperatures were 275 and 220 °C, respectively. The oven temperature program was as follows: 40 °C, hold 4 min, 4 °C/min to 190 °C, no hold. Helium, the carrier gas, was split 13:1. Nominal detection limits for PCE, TCE, and cis-DCE were 48, 22, and 46 μg/L, respectively. The program for the purge and trap was as follows: purge 6 min, dry purge 7 min, desorb 4 min at 250 °C, and bake 20 min at 270 °C. Ultrahigh purity helium was used as the purge gas. All samples were obtained in a 5 mL purge and trap syringe (Hamilton). Fluorobenzene was used as the internal standard. The purge and trap/GC-MS system was calibrated daily.

In the second method (method II), aqueous samples (1 mL) were extracted in 5 mL of pentane and analyzed by direct injection (1 μL) with a GC (Hewlett-Packard) equipped with an electron capture detector (ECD). The oven temperature program was as follows: 40 °C, hold 4 min, 4 °C/min to 190 °C, no hold. Helium (8 mL/min) was used as the carrier gas, and nitrogen (56 mL/min) was used as the auxiliary and anode purge gas. Standards were prepared by adding known volumes of methanol containing PCE and TCE to vials containing 5 mL of pentane and 1 mL of deionized water. The extraction efficiencies of PCE and TCE were determined to be 92.2 ± 10% and 95 ± 7%, respectively. Nominal detection limits for PCE and TCE were 12 and 59 μg/L, respectively.

Headspace analysis was used to quantify VC, ethene, and propane. Headspace samples (100 μL) were injected into a GC (Hewlett-Packard) equipped with a flame ionization detector and a packed column (6 ft × 1/8 in. o.d.) containing 60/80 Carbopack B/1% SP-1000 (Supelco). The oven program was as follows: 40 °C, hold 2 min, 20 °C/min to 150 °C, no hold. 10 °C/min to 200 °C, hold 10 min. The detector and injection port temperatures were 275 and 200 °C, respectively. The gas flow rates were 12 mL/min helium, 40 mL/min H₂, and 460 mL/min zero air. Standards were prepared in serum bottles (60 mL) closed with aluminum crimp caps and Teflon-lined butyl rubber stoppers. The liquid-to-gas ratio in the serum bottles was consistent with the liquid-to-gas ratio in the systems being evaluated. Known volumes of VC, ethene, and propane were added to the serum bottles using gastight, locking syringes (Dynatech). Gas standards were kept inverted to minimize loss through the septum.

Analyses of methane and H₂ were conducted by direct injection of headspace samples (100 μL) into a GC (EG&G Chandler Engineering) equipped with a thermal conductivity detector (TCD) and a molecular sieve 5A column (6 ft × 1/4 in. o.d. 8100 mesh, EG&G Chandler Engineering). The oven was operated isothermally at 102 °C. Nitrogen was the carrier gas, and the flow rate was 30 mL/min. Standards were prepared by volumetric dilutions of certified gas standards.

The pH of cultures was routinely checked with a pH meter and probe (Accumet). The pH meter was calibrated daily using a certified buffer solution of pH 7 (Fisher Scientific).

**Culture Enrichment**. Two PCE-dechlorinating methanogenic mixed cultures were developed and used in further studies. One was enriched on methanol as its primary electron donor and is referred to as the methanol/PCE enrichment culture. The other culture was derived from aquifer solids, referred to as the aquifer-solids-derived suspended growth enrichment culture, and fed both lactate and H₂ as its primary electron donors. The inoculum for the methanol/PCE enrichment culture (a gift from Shell Development, Westhollow Research Center, Houston, TX) was an anaerobic granular sludge obtained from an upflow sludge blanket reactor used for the treatment of wastewater "generated" during the synthesis of polyester with no previous exposure to chlorinated ethenes (water is a product of the reaction between monomeric subunits of polyester and is condensed with ethylene glycol as a wastewater, thus, chlorinated solvents were never introduced into the system). The aquifer solids used in the aquifer-solids-derived suspended growth enrichment culture were obtained from a chlorinated ethene contaminated site in Texas. These samples were stored frozen by Groundwater Services, Inc., Houston, TX. The following section describes how both cultures were developed and maintained.

For the methanol/PCE enrichment culture, anaerobic granular sludge was packed into an airtight, glass column (8.1 cm in diameter, 35 cm long, empty bed volume of 1800 mL) fitted with three sampling ports at 5, 17, and 24 cm from the base. Nutrient medium (without Na₂S·9H₂O, FeCl₃·4H₂O, and resazurin) was continuously delivered to the base (2 mL/min) via a peristaltic pump (Cole Parmer). The resulting empty bed hydraulic retention time was 15 h. A syringe pump (Harvard Apparatus) continuously dispensed a methanol/PCE solution to the bulk medium flow near the base of the column. Influent PCE concentrations, reported after the mixing of the methanol/PCE solution with the nutrient medium, were incrementally increased from an initial concentration of 0.5 mg/L (2.8 μM) to 86 mg/L (483 μM) as dechlorination activity increased. During days 357–455, PCE was replaced by an equimolar amount of TCE (483 μM) to confirm TCE dechlorination. After this time, the influent chlorinated ethene was changed back to PCE. The concentration of methanol fed to the column (after mixing with medium) was 65 mM until the influent PCE concentration was increased to 86 mg/L and then was raised to 52 mM. To avoid adsorption losses, inert tubing (i.e., stainless steel and...
Henry's constant, ethene, were added to a recycle column along with deionized water (60 mL). Sorptive losses of PCE were quantified over a 4 day period. The initial PCE concentration was 1.51 mg/L [number of observations (n) = 1], and the average concentration (n = 3) over the next 96 h was 1.51 mg/L (standard deviation = 0.24). The percent recovery of ethene in the recycle column after 24 h was determined to be 102% (n = 3), indicating that the recycle column system was not subject to volatile losses.

Three columns were inoculated with 12 mL of methanol/PCE enrichment culture, and the other three were inoculated with 6 mL of methanol/PCE enrichment culture and 6 mL of aquifer-solids-derived suspended growth enrichment culture. At the time of inoculation, the methanol/PCE enrichment culture and the aquifer-solids-derived suspended growth enrichment culture had been enriched on PCE for a period of 645 and 111 days, respectively. One column from each inoculum subset was fed methanol, lactate, or H2 as electron donor. The methanol-, lactate-, and H2-fed columns containing the methanol/PCE enrichment culture only were named using the suffix "I" (i.e., MeOH-I, Lact-I, and H2-I, respectively). The three columns containing the methanol/PCE enrichment culture and the aquifer-solids-derived suspended growth enrichment culture were named likewise using the suffix "II" (i.e., MeOH-II, Lact-II, and H2-II).

Nutrient medium (60 mL) was added to the medium reservoir of all six columns. The headspace in the medium reservoir of the lactate- and methanol-fed columns was purged with N2/C02, while H2/C02 was used to purge the headspace in the H2-fed columns. H2 (as H2/C02) was added daily as needed in the H2-fed columns to relieve the negative pressure in the medium reservoir. The average uptake of H2 in H2-I and H2-II was determined during the initial startup of the columns and was found to be 8.5 mequiv/day (130 mL of H2/C02). The average H2 uptake was checked again 1 month later and was found to be consistent. The methanol- and lactate-fed columns were fed 17 mequiv of methanol and lactate, respectively, every 2 days to ensure that electron donor additions were equivalent in all systems [equivalent calculations were based on the conversion of electron donor to CO2, (28)]. Yeast extract was not considered a source of reducing equivalents since control studies demonstrated that, in systems where only yeast extract was added (i.e., no lactate, methanol, or H2), dechlorination activity was indistinguishable from systems where no electron donor was added (i.e., no yeast extract, lactate, methanol, or H2). For this reason, it was observed to be consistent over the next 472 days, except for occasions when the pH dropped below 6.8. On these days, the amount of H2 added was less than 8.5 mequiv/day. The average daily consumption from days 0 to 472 was 4.7 and 5.3 mequiv for H2-I and H2-II, respectively. Every day, the positive pressure in the methanol- and lactate-fed columns was measured and released.

PCE was added to the recycle columns systems as PCE5 eq every 4 days. The target PCE concentration in the columns was 5 mg/L (approximately 6.7 µmol or 0.034 mequiv), resulting in an electron donor to PCE ratio (ED:PCE) of 830:1 mequiv. The initial PCE concentration was not able to be measured in the column due to the rapid dechlorination by the cultures. Therefore, at every PCE addition, the initial PCE concentration was estimated using a set of four serum bottles, each containing deionized water at the same gas-to-liquid ratio as the recycle column system. In two serum bottles, a corresponding volume of PCE5 eq was added to achieve the equivalent PCE concentration targeted in the column. The remaining serum bottles were maintained as blanks, each amended with the same volume of deionized water as PCE5 eq. All four bottles were stored inverted for a period of 24 h. Aqueous samples (1 mL) from each bottle were analyzed by method 1. After correcting for background
PCE found in the blanks. The PCE concentrations in the other two serum bottles were averaged to reflect the initial PCE concentrations in the columns.

On a daily basis, medium was removed (1 mL) and analyzed for PCE, TCE, and DCE isomers using method I. Headspace samples were routinely analyzed for VC and ethene, and pH was checked periodically (if needed, the pH was increased to 6.8—7.2 by adding NaHCO₃). Every fourth day, the headspace was purged with either N₂/CO₂ (methanol-and lactate-fed systems) or H₂/CO₂ (H₂-fed systems), nutrient medium was completely removed via the syringe port, and fresh nutrient medium was added. Propane (290 μL, 1 atm) was used as an internal standard to evaluate volatile losses from the system. The cultures were enriched on their respective ED and PCE for nearly 100 days before detailed experiments commenced.

Evaluation of H₂ as the Final Electron Donor for PCE Dechlorination. After 474 days of operation, an experiment was performed to evaluate whether H₂ could serve as the primary electron donor for dechlorination in the methanol- and lactate-fed columns. All four columns were operated normally until gas production ceased; nutrient medium was replaced, and the medium reservoirs were flushed with H₂/CO₂. The columns were then spliced with PCEₐ to a concentration of 5 mg/L and operated in the same manner identical to H₂-I and H₂-II.

PCE Dechlorination at High H₂ Partial Pressures. An experiment was performed to compare the flux of H₂ between methanogens and dechlorinators in homogenous batch systems at high H₂ partial pressures. For this experiment, the H₂-I column was sacrificed (on day 472) and used as inoculum for batch cultures.

In an anaerobic chamber (100% N₂), the column contents were placed in an Erlenmeyer flask (flask had syringe port and sampling port) and diluted with anaerobic medium (60 mL). The flask was sealed from the atmosphere and purged with H₂/CO₂. The culture was stored on a stir plate and homogenized. For a period of 1 week, H₂ was fed daily and PCE was added on a 4 day interval (analogous to recycle columns). After 8 days, aliquots (5 mL) of the homogenized culture were transferred to duplicate serum bottles (120 mL) containing anaerobic medium (55 mL) sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. Headspace were flushed with H₂/CO₂ to remove any residual PCE or reduced end products. Propane (50 μL, 1 atm) was added as an internal standard to assess any volatile losses, and cultures were spliced with PCEₐ to a concentration of 5 mg/L.

Fifteen minutes prior to headspace and aseptic sampling, H₂/CO₂ was added as needed to maintain an atmospheric pressure of 1 atm within the bottles. Cultures were sampled approximately every 3 h, and the liquid and gas samples were analyzed for methane, H₂, PCE, and TCE (method I), and other dechlorination end products. After 14 h, the cultures were sacrificed to compare biomass levels. Cultures were filtered with 47 mm glass fiber filters (Gelman Scientific), and the filtered residue was dried to a constant weight at 120 °C.

Results

Methanol/PCE Enrichment Culture. Figure 1 shows the influent and effluent PCE and TCE concentrations from the methanol/PCE enrichment culture during the first 100 days of operation. Although this culture had not previously been exposed to PCE, dechlorination commenced within 24 h as indicated by the production of TCE. TCE concentrations increased for the first 20 days as effluent PCE concentrations dropped. Then TCE concentrations decreased rapidly to approximately 0.5 μM. Effluent concentrations fluctuated for the next 40 days, but on day 62, both PCE and TCE concentrations dropped below detectable limits. For the remaining 38 days, effluent concentrations of PCE and TCE were typically below detection limits. Elevated PCE effluent concentration on day 70 was caused by a failure in the peristaltic pump system that caused PCE to flow into the column undiluted.

The influent PCE concentration was maintained at 2.97 μM (0.5 mg/L aqueous concentration) to day 165, after which it was incrementally increased. Changes in PCE influent concentration and resulting effluent PCE and TCE concentrations for the culture are shown in Figure 2, panels a and b, respectively. Effluent PCE concentration was increased by a factor of more than 160 within a period of 81 days with no effect on the effluent PCE and TCE concentrations. Occasional increases in PCE effluent concentrations above 5 μM corresponded to days in which problems with the peristaltic pump system occurred.
During days 358–456, TCE was substituted for PCE in equimolar amounts (483 μM) to confirm TCE dechlorination by the methanol/PCE enrichment culture. Effluent TCE concentrations during this time were below detectable limits. On day 457, the influent contaminant was returned to PCE (519 μM). The influent concentration remained at this level, as further increases in concentration were limited by the aqueous solubility of PCE.

Periodic headspace sampling of gas trapped in the first Erlenmeyer flask showed that cis-DCE and VC were the major reduced end products being formed. Ethene was observed in appreciable quantities. Because the column was maintained as an open system, completing a mass balance on all products from this system was not attempted.

Rate and Extent of PCE Dechlorination in Recycle Columns. PCE dechlorination was observed in all systems throughout the experimental period (ca. 474 days), although its rate of disappearance and the distributions of dechlorination products changed during the study. Columns were monitored throughout the period to ensure dechlorination activity and to evaluate dechlorination products. Occasionally, studies were conducted to obtain rate information and quantify product distributions. Results of these more extensive monitoring studies are presented in Tables 1 and 2.

Table 1 presents the observed PCE half-lives \( t_{1/2} \) in all columns throughout the experimental period. Half-lives were calculated from observed first-order rate coefficients. Pearson's \( r^2 \) values obtained from the linearization of PCE disappearance data are shown along with the number of observations \( n \) used for the analysis. In early experiments, PCE disappearance was slower, allowing for more data to be used in approximating \( t_{1/2} \). Over time, the observed PCE disappearance rates increased such that PCE concentrations dropped below detectable limits within several hours after PCE addition. In these experiments, there are three or fewer data points. Half-lives are not shown for day 427 because PCE concentrations decreased to below detectable levels before samples were taken, corresponding to an estimated \( t_{1/2} \) value of 2 h or less.

The distribution of PCE and reduced end products recovered in these experiments after 4 days of incubation are presented in Table 2. On day 98, the primary products of dechlorination were cis-DCE and VC. In columns inoculated from the methanol/PCE enrichment culture column only (e.g., MeOH-I, H2-I, and Lact-I), cis-DCE was the primary reduced end product in systems fed methanol and H2, while VC was the primary reduced end product in Lact-I. On day 98, VC was observed in all columns inoculated with both enrichment cultures, and only MeOH-II had measurable levels of cis-DCE. By day 120, VC was observed in all columns, without detectable levels of ethene, and then by day 129.

Table 2. Percent Molar Distribution of Dechlorination End Products in Recycle Columns

<table>
<thead>
<tr>
<th>Column</th>
<th>Ethene</th>
<th>Day 98</th>
<th>Day 106</th>
<th>Day 120</th>
<th>Day 129</th>
<th>Day 427</th>
<th>Day 474</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH-I</td>
<td>% PCE</td>
<td>0.0</td>
<td>2.2</td>
<td>2.4</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% TCE</td>
<td>0.0</td>
<td>0.0</td>
<td>0.8</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% cis-DCE</td>
<td>100.0</td>
<td>97.8</td>
<td>97.5</td>
<td>69.5</td>
<td>68.6</td>
<td>64.6</td>
</tr>
<tr>
<td></td>
<td>% VC</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% ethene</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% cis-DCE</td>
<td>44.4</td>
<td>29.9</td>
<td>28.9</td>
<td>25.5</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>% VC</td>
<td>36.3</td>
<td>34.5</td>
<td>38.6</td>
<td>70.3</td>
<td>77.4</td>
<td>77.4</td>
</tr>
<tr>
<td></td>
<td>% ethene</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lact-I</td>
<td>% PCE</td>
<td>26.7</td>
<td>3.3</td>
<td>4.3</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
<tr>
<td></td>
<td>% TCE</td>
<td>3.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% cis-DCE</td>
<td>7.0</td>
<td>8.7</td>
<td>8.7</td>
<td>8.7</td>
<td>8.7</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>% VC</td>
<td>95.9</td>
<td>96.7</td>
<td>96.7</td>
<td>96.0</td>
<td>96.0</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td>% ethene</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% cis-DCE</td>
<td>4.9</td>
<td>4.9</td>
<td>4.9</td>
<td>4.9</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>% VC</td>
<td>95.1</td>
<td>94.3</td>
<td>93.5</td>
<td>95.1</td>
<td>95.1</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td>% ethene</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% cis-DCE</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>% VC</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Percent molar distribution of PCE, TCE, cis-DCE, VC, or ethene. The average percent molar recovery and standard deviation for the experiments reported above were MeOH-I = 74.1% ± 15.9; MeOH-II = 66.4% ± 22.2; Lact-I = 89.5% ± 18.9; Lact-II = 95.1% ± 6.4; H2-I = 82.8%; ± 20.0; H2-II = 108.9% ± 19.3. On day 474, experiments were performed in lactate- and methanol-fed columns with H2 added as electron donor.
sampling. The amount of H₂ added was used to calculate the amount of H₂ consumed between sampling. Changes in the mass of H₂ in the headspace that occurred between sampling is shown in Figure 3c. Using Henry's Law constant for H₂, \(7.06 \times 10^{-4}\) atm/mol fraction at 25 °C, changes in the aqueous H₂ concentration were calculated. Throughout the duration of the experiment, the calculated aqueous H₂ concentration ranged from 645 to 306 μM.

**Discussion**

The rapid onset of PCE dechlorination (within 24 h after PCE addition) observed in the methanol/PCE enrichment culture was not anticipated since the culture had no prior history of chlorinated ethene exposure. It is likely that this activity was due to cometabolic processes. Over time, the culture exhibited an increase in the extent of dechlorination observed. For a period of approximately 2 years, the primary dechlorination end product in the methanol/PCE enrichment culture was cis-DCE, with very little or no VC or ethene being detected. After this time, VC was observed followed by the production of small amounts of ethene. Production of end products not associated with the process of reductive dechlorination but reported in other cases, e.g., carbon dioxide (19, 20) and ethane (18), was not investigated, although the formation of these compounds cannot be ruled out. Dechlorination was sustained in the methanol/PCE enrichment culture throughout the experimental period, and dechlorination rates were rapid enough to accept large increases in the influent PCE concentration and maintain PCE/TCE concentrations below detection limits in the effluent. Throughout this time, daily gas production levels fluctuated from near zero (it never ceased indefinitely) to greater than 500 mL. Interestingly, no changes in dechlorination were observed during periods without gas production.

Dechlorination was observed to commence immediately upon PCE addition in the aquifer-solids-derived suspended growth enrichment culture. Like the methanol/PCE enrichment culture, it had a significant population of methanogens as indicated by the production of methane. Observed dechlorination end products included TCE, cis-DCE, and VC.

At the time of the recycle column inoculation, the methanol/PCE enrichment culture was not producing detectable levels of VC or ethene. Thus, the aquifer-solids-derived suspended growth enrichment culture was seeded as an additional inoculum to the second set of recycle columns since it was capable of this higher degree of dechlorination. Although VC production did commence earlier in systems inoculated with both cultures, over the duration of the experiments all of the columns developed the ability to dechlorinate beyond cis-DCE with similar levels of VC and ethene formation. (Six months after the inoculation of the recycle columns, the methanol/PCE enrichment culture also began producing large amounts of vinyl chloride, indicating that the culture was still undergoing the process of acclimation.) Rates of PCE disappearance were initially higher in the recycle columns containing only the methanol/PCE enrichment column, but by day 474, the half-life of PCE in all six columns was approximately 2 h. Thus, inoculum was not found to be particularly influential in either the extent or the rate of PCE dechlorination.

Methanol (12–15) and fatty acids (12, 17–19, 23, 27–30) have been shown to sustain dechlorination in various types of experimental systems. Results from several investigations have implied that H₂, produced by the metabolism of fermentative and acetogenic substrates, served as the final electron donor for dechlorination (14, 23). This study represents a long term, comparative analysis of electron donors for sustaining PCE dechlorination in which H₂ was fed directly. Results from recycle column experiments.
demonstrated that electron donor did not influence the extent or the rate of dechlorination. The H₂-fed column, H₂-I, had smaller half-lives for PCE initially, but over time, the half-lives in all six columns converged to approximately 2 h. The extent of dechlorination achieved was not dependent upon electron donor added either, since PCE was recovered as approximately 80%/VC and 20%/ethene with all three electron donors on day 427, with the MeOH-II column lagging slightly behind in ethene production. Thus, with time, rates of PCE disappearance increased collectively, and the extent of dechlorination progressed to favor less chlorinated ethenes with all three electron donors. The average percent recovery of PCE and dechlorination products for all experiments in the recycle columns is presented at the bottom of Table 2. The percent of mass recovered for individual four day experiments varied between columns and throughout the study. In some of the early experiments with methanol-fed columns (days 98 and 106), only 30–50% recovery was obtained. Analytical problems were not believed to be attributed to the low mass recovery since all of the mass was recovered in Lact-II and H₂-II on the same days. Although the reason for this occurrence is not known, it is possible that these particular cultures may have been dechlorinating PCE to a product not analyzed (e.g., CO₂, ethane). The percent of mass recovered was also affected by the loss of volatiles during gas release in methanol- and lactate-fed cultures. Lastly, mass balances were observed to get better over time, i.e., recovery would increase significantly from day 2 to day 4, possibly due to uptake and release by biomass in the system.

The inability of these cultures to completely dechlorinate all of the PCE fed to ethene may have been indirectly caused by the design of the recycle columns. Possibly, the large headspace volume in the medium reservoir may have led to contact limitations between VC and the culture in the column. The Henry's Law constant for VC is relatively high. Thus, the majority of the VC mass remained in the headspace and only that which was dissolved in the aqueous phase came into contact with the culture. This phenomenon may have led to the slow development of a culture capable of VC dechlorination.

Data obtained in the recycle columns supports the results obtained by Fennell et al. (24), who investigated several electron donors as potential H₂ donors for PCE dechlorination. The authors concluded that over long-term experiments, dechlorination could be equally sustained and maintained regardless of electron donor fed. In their research, however, the electron donors (ethanol, butyric acid, lactic acid, and propionic acid) were added in equivalent amounts according to the number of H₂ equivalents that would be released upon fermentation, whereas in the research presented here, electron equivalent calculations were based upon the complete oxidation of each electron donor.

The role of H₂ as the final electron donor for PCE dechlorination was investigated by adding H₂ to the methanol- and lactate-fed recycle columns. The extent and rate of PCE dechlorination were relatively unchanged, indicating that the dechlorinating organisms in these cultures were capable of utilizing H₂ for energy purposes. The average uptake of H₂ in these four columns (1.9 mequiv/day MeOH-I; 2.2 mequiv/day MeOH-II; 2.3 mequiv/day Lact-I; and 2.5 mequiv/day Lact-II) during this experiment was considerably less than that of the recycle columns H₂-I and H₂-II, although it continued to increase daily. The low but increasing H₂ uptake observed may have been attributed to an increase in the growth of H₂-utilizing, nondechlorinating microorganisms stimulated by the increase in hydrogen availability. As a result, these data indirectly support the role of H₂ as the final electron donor for dechlorinators in systems fed organic substrates, although it is possible that when fed methanol or lactate, these cultures utilized an electron donor other than H₂.

To date, no attempts have been made to isolate a PCE-respiring bacterium from cultures used in these studies, but the rapid rates of dechlorination and the products observed are similar to mixed cultures from which PCE-respiring organisms have been isolated (15). In mixed culture systems exhibiting high dechlorination rates, others have reported the potential for methanogens to out-compete dechlorinating bacteria for H₂ under conditions of high H₂ partial pressures (17, 22). In recycle column systems fed H₂, this was not observed (e.g., rate and extent of dechlorination increased with time). A possible reason that this competition was not observed in H₂-fed column systems—compared to suspended growth systems—is the spatial heterogeneity of H₂ and/or PCE in column systems that may have resulted in the spatial stratification of individual communities and organisms. For example, growth of dechlorinating organisms could have been favored at the top of the column, where aqueous H₂ concentrations would have been lower than at the base, or the concentrations of PCE fed could have been inhibitory to methanogens at the base of the column yielding the inverse. Thus, the purpose for homogenizing one of the H₂-fed column systems, and comparing the flux of H₂ equivalents to dechlorination and methanogenesis at high H₂ partial pressure with results from column studies, was to evaluate this hypothesis.

During the experiment, dechlorination and methanogenesis occurred simultaneously, and neither process appeared to be inhibited by the other. In the batch systems, it was calculated that less than 1% (0.4%) of the H₂ equivalents consumed (uptake as measured by GC) was utilized for dechlorination, and that 69% of the H₂ equivalents was accounted for by methane production. The remaining 30% was presumably shunted to cell growth and/or acetogenesis. The small percentage of H₂ equivalents being used for dechlorination is consistent with results from the H₂-I column, in which it was determined that 0.36% of H₂ equivalents added were being utilized for dechlorination (based on cumulative moles of H₂ and PCE added). The electron donor to PCE ratio in batch systems was less than that in the recycle column system (1.14 mequiv H₂/I mequiv PCE—due to the smaller headspace), but resulting aqueous-phase H₂ concentrations were similar in both cases. The influence of H₂-utilizing acetogens on methane production or dechlorination was not evaluated.

For a period exceeding 1 year, it was demonstrated that PCE dechlorination in mixed cultures could be sustained equally using equivalent amounts of methanol, lactate, or H₂. Furthermore, it was observed that dechlorination was not impacted by competition for electron donor at high H₂ partial pressures by other H₂-utilizing microorganisms, particularly methanogens. These results imply that issues such as cost and method of delivery may dictate the electron donor (i.e., fermentable substrates vs H₂) selected for stimulation of anaerobic in situ bioremediation systems.

Acknowledgments
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Chapter Five: ECOLOGICAL AND NUTRITIONAL CHARACTERIZATION OF A HIGHLY PURIFIED PCE-DECHLORINATING ENRICHMENT CULTURE

Abstract
A purified PCE-dechlorinating enrichment culture was obtained through liquid serial dilutions of the methanol/PCE enrichment culture presented in Chapter 4. The purified enrichment culture dechlorinated PCE to cis-DCE when hydrogen, pyruvate, ethanol, glucose, or yeast extract was added as electron donor and acetate was added as carbon source. Sustained dechlorination required the addition of filter-sterilized cell extract from the parent culture, indicating an undefined nutritional dependency of the dechlorinator(s) with other organisms present in the parent culture. Isolation of the dechlorinator(s) was not achieved using a variety of isolation techniques, but preliminary molecular studies on the purified enrichment culture indicated several organisms were still present. One impurity that did not dechlorinate PCE was isolated and found to be related phylogenetically to the fermentative organism, *Lactosphaera pasteurii*.

Introduction
The prevalence of tetrachloroethene (PCE) and trichloroethene (TCE) in the environment has driven the need to develop remediation strategies capable of effecting the complete removal of these compounds. One such strategy, anaerobic *in situ* bioremediation, has shown promise as a remediation process based on cost, the potential for complete transformation of contaminants to a non-toxic species (i.e., ethene) (1-4), and recent evidence demonstrating microbially enhanced removal of chlorinated ethenes-
containing nonaqueous phase liquids (see Chapter 6, reference (5)). No other remediation technology currently holds such promise for this class of compounds.

Microbial transformation of chlorinated ethenes occurs through dechlorination, a process that is either cometabolic (6-11) or respiratory (12-20). Because PCE- and TCE-halorespiring organisms exhibit dechlorination rates orders of magnitude greater than that of cometabolism (21), much interest has developed in isolating and characterizing these organisms. To date, six PCE-halorespiring bacteria have been isolated. Only one organism, *Dehalococcoides ethenogenes*, can dechlorinate PCE completely to ethene (20). The other isolates dechlorinate PCE to TCE (17) or cis-DCE (13-16,18,19). No nutritional homogeneities exist among the six halorespiring isolates other than their ability to utilize PCE as a terminal electron acceptor (see Chapter 3 for a review).

The lack of information regarding the nutritional requirements and metabolic capabilities of PCE-halorespiring bacteria has limited the use of anaerobic *in situ* bioremediation. For example, one of the primary concerns regarding this technology is the potential for incomplete dechlorination that results in the accumulation of vinyl chloride, a known carcinogen. To further the use of dechlorination in the field, more information on chlorinated ethenes halorespiration and halorespiring organisms are needed.

The objective of this research was to isolate and characterize the PCE-dechlorinating organism(s) from the methanol/PCE enrichment culture. High rates of PCE dechlorination (see Chapter 4) indicated that dechlorination in the methanol/PCE enrichment culture was occurring via halorespiration. Efforts were made to isolate the
dechlorinator through serial dilutions in liquid and solid medium. A highly purified PCE-dechlorinating enrichment culture was developed that could not be purified further. Information gained on the nutritional requirements of this culture is presented. Preliminary molecular studies were conducted to characterize the phylogeny of the purified enrichment culture are also included.

**Materials and Methods**

**Chemicals.** The following chemicals were obtained in liquid form: tetrachloroethene (99+%; Acros); trichloroethene (99.5%; Aldrich); cis-dichloroethene (97%; Acros); and tridecane (99%; Sigma). Custom made gases. N₂/CO₂ (90/10%, v/v) and H₂/CO₂ (80/20%, v/v), were prepared by TriGas. Reagent grade chemicals were used in medium preparation and chemicals for molecular work were of molecular/electrophoresis grade.

**Source of Inoculum.** The inoculum consisted of effluent from the packed column containing the methanol/PCE enrichment culture, as described previously in Chapter 4 (22). The packed column was continuously fed methanol (52 mM) and PCE (maximum aqueous concentration of 86 mg/L) for a period exceeding 5 years, and PCE was consistently dechlorinated to vinyl chloride and ethene.

**Anaerobic Nutrient Medium.** The composition of the anaerobic nutrient medium was as follows (per liter of medium): 10 mL basal salts solution (containing in grams per liter of medium: KCl, 0.4; MgCl₂·6H₂O, 0.4; NH₄Cl, 0.4; KH₂PO₄, 0.14; CaCl₂·2H₂O, 0.025; NaHCO₃, 7.5; Na₂S·6H₂O, 0.24); 10 ml trace element solution I (containing in milligram per liter of medium: FeCl₂·4H₂O, 15; ZnCl₂, 0.5; MnCl₂·4H₂O, 0.5; H₃BO₃, 0.5; CoCl₂·6H₂O, 2.5; NiCl₂·6H₂O, 0.5; Na₂MoO₄·2H₂O, 0.5); 10 mL trace element solution
II (containing in milligram per liter of medium: (NaPO₃)₁₆, 10; KI, 2.5; NH₄VO₃, 0.5); 1 ml of vitamin solution (containing in milligram per liter of medium: 4-aminobenzoic acid, 0.04; D(+)-biotin, 0.01; nicotinic acid, 0.1; Ca-D(+)-pantothenate, 0.05; pyridoxamine dihydrochloride, 0.15; thiamine hydrochloride, 0.1; cyanocobalamin, 0.05); 10 mM filter-sterilized bromoethanesulfonic acid (BES); and 1% (w/v) filter-sterilized cell extract (FSCE: see next section). Prepared anaerobic nutrient medium was adjusted to pH 7.0.

**Preparation of FSCE.** The cellular extract in the anaerobic nutrient medium was prepared from effluent of the packed column. Cells were harvested by centrifugation (25,400 x g, 9 min, 4°C), the supernatant was discarded, and cell pellets were resuspended in nutrient medium (excluding Na₂S·9H₂O and FeCl₂·4H₂O) and centrifuged again. Pellets were washed in this manner a total of three times. After washing, pellets were weighed, homogenized manually using tissue grinders, and resuspended in nutrient medium to a final concentration of 10% (w/v). Cells were disrupted using ultrasonication (Heat Systems Ultrasonics, Inc.). Because of the granular nature of the cells, it was necessary to pass the sonicated cellular mixture through a French Press (1000-1500 psig, SLM Aminco) to ensure complete disruption. The resulting mixture was centrifuged (30,000 x g, 9 min, 4°C) and the supernatant was sterilized by filtration (0.2 μm, Sycamore, Nalgene). FSCE was stored at -20°C until use.

**PCE/Tridecane and TCE/Tridecane Mixtures.** Tridecane was autoclaved for 1 hour at 121°C (15 psi). Filter sterilized (0.2 μm, GVWP, Millipore) PCE or TCE was added to the tridecane to a final concentration of 0.48 M and 0.09M, respectively.
**Chlorinated Ethenes Quantification.** PCE, TCE, and cis-DCE were quantified using gas chromatography. Standards were prepared in anaerobic culture tubes (27 mL) containing deionized water (10 mL) and tridecane (100 μL). Aliquots of neat PCE, TCE, and cis-DCE were added to the tridecane, and tubes were capped with Teflon-lined butyl rubber septa and aluminum crimp caps. Following vigorous shaking for one hour, tubes were allowed to sit quiescently for phase separation and equilibration. Gas samples (100 μL) were removed from the tubes using a gas-tight locking syringe (VICI Precision Sampling, Inc.) and analyzed on a Hewlett Packard 5890 gas chromatograph (GC). The GC was equipped with a flame ionization detector and a packed column (6 ft x 1/8 in OD) containing 60/80 Carbopack B/1% SP-1000 (Supelco). The oven program was as follows: 40°C. hold 2 min. 20°C/min to 150°C. no hold. 10°C/min to 200°C. hold 10 min. The detector and injection port temperatures were 275°C and 200°C, respectively. The gas flow rates were 12 mL/min helium, 40 mL/min H₂, and 460 mL/min zero air.

**Microscopy.** Phase-contrast photomicrographs were prepared using a Nikon Microphot-FX microscope. 600X total magnification. Colonies grown in agar were viewed with a Zeiss Stemi 1000 stereoscope.

**Development of Purified Enrichment Culture.** A highly purified enrichment culture was obtained through serial liquid dilutions. Effluent from the packed column was serially diluted in sterile anaerobic nutrient medium containing 2 mM acetate and 100 μL PCE/tridecane (aqueous PCE concentration was approximately 24 mg/L based on a tridecane/water partition coefficient of 3.060 (5)). Dilutions were conducted using either pyruvate (10 mM final concentration) or hydrogen as electron donor, and were prepared
in anaerobic culture tubes (Bellco) closed with Viton stoppers (Wheaton) and aluminum crimp caps. Before capping, the culture tube headspace was replaced with sterile H₂/CO₂ or N₂/CO₂ (pyruvate). Culture tubes were stored on a shaker table (150 rpm) at room temperature (25°C). Headspace samples were periodically analyzed by GC for dechlorination end products, and once active dechlorination was observed, serial dilutions were repeated using the highest dilution demonstrating dechlorination activity.

Further isolation attempts were made in solid medium. The highly purified enrichment culture was used to inoculate a series of agar shake dilutions containing 1% noble agar, anaerobic nutrient medium, 100 μL PCE/tridecane, 2 mM acetate, and either H₂ or 10 mM pyruvate. Single colonies were removed from the highest dilutions containing TCE and cis-DCE (determined by GC) and resuspended in anaerobic nutrient medium or another series of agar shake dilutions.

**Isolation of Contaminants.** Contaminants (i.e., nondechlorinating organisms present in the purified enrichment culture) were isolated on Wilkins-Chalgren agar plates. Plates were stored at 25°C in GasPak jars (BBL, Becton Dickinson) under an atmosphere of hydrogen and carbon dioxide. Individual colonies were transferred two or more times to obtain a pure culture. To test whether cells would dechlorinate PCE or TCE, purified colonies were resuspended in anaerobic broth (MIC) or in anaerobic nutrient medium (H₂/CO₂ headspace in both cases) containing either PCE/tridecane (100 μL) or TCE/tridecane (100 μL).

**DNA Extraction.** Aliquots (500 μL) from liquid cultures and colonies from Wilkins-Chalgren plates (resuspended in 250 μL Tris/EDTA (TE) buffer) were centrifuged (3,000
× g. 1 minute) to remove debris. and the clarified supernatant was centrifuged at 13,000 × g for 20 minutes. Cell pellets were microwaved (960 W) for 30 seconds, at 15 second intervals, and resuspended in TE buffer containing sodium dodecyl sulfate (SDS, 3% w/v) and 100 ng/μL proteinase K. Following incubation at 45°C for 45 minutes, lysates were extracted with phenol-chloroform-isoamyl alcohol. Nucleic acids were precipitated from the aqueous layer by the addition of 2 volumes ice-cold ethanol containing 0.3 M sodium acetate. Samples were centrifuged (13,000 × g, 60 minutes) and pellets were allowed to air dry before being resuspended in deionized water (15 μL). DNA was quantified using UV spectrophotometry (as outlined in (23). Gilford Response UV/VIS Spectrophotometer) or a DNA DipStick Kit (Invitrogen).

PCR and Cloning. Ribosomal DNAs (rDNAs) (0.1–0.4 ng) from the highly purified enrichment culture and Wilkins-Chalgren contaminants were amplified by polymerase chain reaction (PCR) in 1× PCR buffer containing 200 μM each deoxynucleoside triphosphate, 400 nM each forward and reverse primer, 0.01% (v/v) Igepal, 0.6 mg/ml bovine serum albumin (BSA), and 0.02 U of Taq DNA Polymerase (Perkin Elmer) per μL reaction mixture. Additional MgCl₂ (3.5 mM MgCl₂ final concentration) was added to most reaction mixtures to increase the yield of amplified products (23). Bacteria-specific oligonucleotide primers (Life Technologies) were 8F (5′- AGAGTTTGATCCTGCTCAG-3′) and 1492R (5′-GGTTACCTTGTACGACTT-3′). The PCR reaction conditions were as follows: 94°C for 5 min; 30 cycles of 92°C for 1 min, 55°C for 1 min, 72°C for 1 min; and an extension period of 15 min at 72°C.
PCR products (1 μL) were cloned into pCR2.1 vectors as outlined by the manufacturer (Invitrogen). Positive clones, determined through blue/white screening, were cultured overnight (37°C) in LB medium containing 50 μg/mL kanamycin. Plasmids were extracted using Wizard Plus SV Minipreps (Promega).

**Restriction Fragment Length Polymorphism (RFLP) Analysis.** Cloned rDNA inserts (0.4 ng) were reamplified by PCR using primers specific to the plasmid (i.e., M13F and M13R). The composition of the PCR reaction mixture was as described previously, with the exception that primer concentrations were 200 nM. Reamplified rDNA PCR products (15 μL) were digested (20 μL total volume) with 1U each of restriction endonucleases *HinP1* and *Msp1* (New England Biolabs) in 1× NEB2 buffer containing 0.01% triton X-100. Following a 3 hour digest at 37°C, samples were incubated at 65°C for 20 minutes to inactivate restriction endonucleases. Digested products were separated by agarose (2% MetaPhor; FMC Bioproducts) gel electrophoresis. Gels were stained with ethidium bromide and viewed under UV illumination.

**Sequencing of Contaminant 16S rDNA.** Pure cultures obtained on Wilkins-Chalgren agar were submitted to MIDI Labs, Inc. (Newark, DE) for 16S rDNA sequencing. The 16S rDNA gene was PCR-amplified using primers corresponding to *E. coli* positions 5 and 531 and sequenced by automated fluorescent cycle sequencing. Bacterial identification was made by comparing sample sequences against sequences found in GenBank (National Center for Biotechnology Information, National Institutes of Health) and the Ribosomal Database Project (RDP), Michigan State University.
Dechlorination in the Presence and Absence of FCSE. The dependency of dechlorination activity on the presence of FSCE was evaluated in anaerobic culture tubes containing the highly purified enrichment culture and pyruvate (20 mM) as electron donor. Cultures were incubated on a shaker table at 25°C. PCE and dechlorination end products were quantified by GC.

Carbon Source in Hydrogen-Fed Cultures. The purified enrichment culture grown on hydrogen was tested to determine whether the addition of acetate was necessary for dechlorination. The culture was transferred (10% dilution) either into anaerobic nutrient medium containing 2 mM acetate or into medium without acetate. Dechlorination was monitored by GC.

Electron Donor Selectivity of Purified Enrichment Culture. Experiments were performed to determine which electron donors could sustain dechlorination activity in the highly purified enrichment culture. Electron donors tested included hydrogen, formate, pyruvate, acetate, acetaldehyde, lactate, propionate, butyrate, methanol, ethanol, glucose, and yeast extract.

Aliquots (1 mL) of the highly purified enrichment culture were transferred into culture tubes (27 mL) containing anaerobic nutrient medium (9 mL). Electron donors formate, pyruvate, acetate, acetaldehyde, lactate, propionate, butyrate, methanol, ethanol, and glucose were added to a final concentration of 20 mM. For hydrogen-fed tubes, hydrogen was added as H₂/CO₂ to the headspace. Yeast extract was added at a concentration of 0.2% (w/v) in tubes in which this substrate served as electron donor. Acetate (5 mM final concentration) was added as carbon source to all tubes. Electron
donors lactate, propionate, and butyrate were also tested in the absence of 5 mM acetate. To test whether acetate and FSCE contributed to PCE dechlorination, controls were prepared containing 5 mM acetate and no electron donor. All tubes were prepared in duplicate and were purged with N₂/CO₂ (organic electron donors and control) or H₂/CO₂ following the addition of PCE/tridecane (100 µL). Tubes were capped and stored at 25°C on a rotary shaker table.

Headspace samples (100 µL) were analyzed for dechlorination end products on days 7, 14, and 21 post inoculation. Cultures exhibiting dechlorination were transferred (10% dilution) with the respective electron donor two or more times to ensure the electron donor could support dechlorination after multiple transfers.

Results
Development of Purified Enrichment Culture. Serial dilutions were conducted over a period of several months with both hydrogen and pyruvate as electron donor. Within the first four transfers, it became evident that transference of dechlorination activity was dependent upon the addition of FSCE. Filter-sterilized spent medium (10% v/v) from the packed column could not replace the FSCE, nor could autoclaved cell extract (1% w/v), yeast extract (0.2% v/v), or bacto-peptone (0.2% w/v). Loss of cis-DCE dechlorination was also observed in the first dilution series (10,000-fold dilution). After this point, the culture consistently dechlorinated PCE or TCE to cis-DCE. No differences were observed between the hydrogen- and pyruvate-fed series with regard to dechlorination activity or morphologies of organisms present (based on microscopic analysis). A photomicrograph of the highly purified enrichment culture taken after 10 months of serial
liquid dilutions is shown in Figure 5.1a. Two dominant morphologies were present: curved, motile rods (3-4 μm long, 0.5-1 μm wide), and cocci (1-1.5 μm in diameter) that were often observed singularly, in pairs, or in clusters.

Attempts to further separate the culture in solid medium were unsuccessful. When transferred into agar, colonies would appear rapidly (1-5 days in the highest dilution), but dechlorination end products were slow to develop (in excess of 3 or more weeks). Colonies were transferred to liquid or solid medium when dechlorination was observed. Individual colonies were fuzzy in appearance and found to contain curved rods, short rounded rods or cocci, and straight, motile rods. Neither liquid nor solid medium transfers would dechlorinate PCE even after 4 months of incubation.

**Isolation of Contaminants.** A contaminant from the purified enrichment culture was isolated on Wilkins-Chalgren agar plates (contaminant was isolated from both the hydrogen- and pyruvate-fed cultures). Off-white colonies of circular form and undulate lobular margins appeared within 24 hours and grew to a diameter of 2 mm within 7 days. Microscopic evaluation revealed the colonies consisted of cocci (see Figure 5.1b). The contaminant could not dechlorinate PCE or TCE, but it was observed that growth of the contaminant was substantially greater when the anaerobic broth was amended with FSCE (1% w/v).
Figure 5.1 Photomicrographs of the purified enrichment culture (a) and contaminant isolated on Wilkins-Chalgren agar (b). The purified enrichment culture (fed pyruvate) was 14 days old at the time the photomicrograph was taken. In (b), the contaminant was resuspended in anaerobic broth (MIC) after three weeks of incubation on Wilkins-Chalgren agar. The bar represents 10 μm.
Analysis of 16S rDNA libraries from Purified Enrichment Culture. Kit cloning efficiencies for rDNAs from the contaminant and purified enrichment culture were determined to be approximately 10% in both cases. One rDNA insert was obtained from the contaminant recombinant clones (sample C9) and 8 inserts were recovered from the purified enrichment culture recombinant clones (samples PC5, PC15, PC9, PC10, PC33, PC34, PC49, and PC412). Results from the digest of reamplified rDNA inserts with *HinP1* and *Msp1* are shown in Figure 5.2. Digests yielded 3-11 bands in the 100-1500 bp size range. Of the nine samples, 7 distinct RFLP patterns were observed visually. Samples PC15 and PC33 exhibited similar RFLP patterns, as did PC10 and PC34. The RFLP pattern for the contaminant rDNA insert was not observed among the 8 samples from the purified enrichment culture.

**Phylogenetic Analysis of the Contaminant.** The 16S rDNA sequence (500 bp) for the Wilkins-Chalgren contaminant is shown in Table 5.1. Searches on GenBank and the Ribosomal Database Project determined that the contaminant was closely related (98% sequence identity match, GenBank; 0.95 similarity rank, RDP) to *Lactosphaera pasteurii*. 

**Dechlorination in the Presence and Absence of FSCE.** Results from an experiment in which the purified enrichment culture was grown in the presence and absence of FSCE are shown in Figure 5.3. In the absence of FSCE, dechlorination was not observed for a period of 16 days. No trace TCE or *cis*-DCE was formed during this time. Cultures containing FSCE started dechlorinating after a 1 day lag phase. TCE production in these cultures was minimal, and the majority of the reduced PCE was present as *cis*-DCE.
Figure 5.2 Electrophoresis gel from RFLP analysis. Lanes 1 and 12 contain 100 bp DNA ladder, and lane 2 contains a negative control. Samples C9, PC5, PC15, PC9, PC10, PC33, PC34, PC49, and PC412 are in lanes 3-11, respectively. DNA markers are shown to the left.
Table 5.1 Sequence* (500 bp) Data from Wilkins-Chalgren Contaminant

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGAGAGGrTTTGATCTCTGGCTCAGGACGAACGCTGCGTGCCCTAATACAT</td>
<td></td>
</tr>
<tr>
<td>GCAAGTCGAACGGTCTTTTCTATGGAAGCTTGGCTTCCACTGAGAAGATAGTG</td>
<td></td>
</tr>
<tr>
<td>GCGAACCAGGCTGAGTAACACGGTGTTAACCTGCCATAGAGGGAGGGAATAAACA</td>
<td></td>
</tr>
<tr>
<td>TCCGGAACCGGGTGCTAATACCGCATAGTTTTCTGGATCGCATGATyGrGAAA</td>
<td></td>
</tr>
<tr>
<td>GAAAGACGGCCCTTTGTGCTGTCGCTTTATGGATGAGACCGCCCACCCGTTATTAGT</td>
<td></td>
</tr>
<tr>
<td>TAGTTGGTGAGGTAAACGGCTCACCAAGACGATGATACGTAGCCGACCTGAGA</td>
<td></td>
</tr>
<tr>
<td>GGGTGATCGGCCACATTGGGACTGAGACACGGCCCACAACGTACGGGAGGC</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>GTTGGAGAGTAACCTGCTCCAGCCTTGACGGTATCTGACGAAAGCCACGCT</td>
<td></td>
</tr>
<tr>
<td>AACTACGTGCCAGCAGCCGCGGT</td>
<td></td>
</tr>
</tbody>
</table>

*a“r” indicates either base A or G possible. “y” indicates either base C or T possible.*
Figure 5.3  PCE dechlorination in the presence and absence of FSCE. Solid symbols represent data taken from cultures in which FSCE was added, and hollow symbols represent data taken from cultures with no FSCE. In both cases, data shown are averages from duplicate sets of tubes.
Carbon Source in Hydrogen-Fed Cultures. Dechlorination in the hydrogen-fed purified enrichment culture was found to lag over a 16 day period in the absence of 2 mM acetate (as compared to tubes containing acetate). Addition of acetate to the former tubes resulted in a marked increase in dechlorination activity (mass of TCE and cis-DCE doubled within a period of 48 hours). Subsequent transfers demonstrated continued increases in dechlorination activity in the presence of acetate. Acetate was considered a carbon source rather than electron donor since control tubes grown in the absence of hydrogen with acetate and FSCE present (see following section) would not dechlorinate.

Electron Donor Selectivity of Purified Enrichment Culture. Results from tubes in which dechlorination was sustained are presented in Table 5.2. Control tubes (also listed in Table 5.2) did not demonstrate extensive dechlorination, measured as the mass of TCE and cis-DCE produced, over a 21 day period. Although some dechlorination activity was observed (3 μmoles of TCE and cis-DCE were produced after 21 days), subsequent transfers of the control culture resulted in the loss of dechlorination activity. Hydrogen, pyruvate, glucose, ethanol, and yeast extract were shown to support dechlorination in the purified enrichment culture. In cultures fed glucose or ethanol, dechlorination was not observed until after 7 days of incubation. Dechlorination initiated much more rapidly in tubes containing hydrogen, pyruvate, or yeast extract. Although all five substrates could sustain dechlorination after multiple transfers, extent of dechlorination was greatest with glucose, pyruvate, or hydrogen. Mass balance data indicated that the majority of the initial PCE added could be accounted for as residual PCE, or TCE and cis-DCE.
Table 5.2 Extent of PCE Dechlorination Sustained in the Purified Enrichment Culture According to Electron Donor Added

<table>
<thead>
<tr>
<th>electron donor</th>
<th>day 7 (μmol)</th>
<th>day 14 (μmol)</th>
<th>day 21 (μmol)</th>
<th>mass balance(^b) (％)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (control)</td>
<td>2.3(^a)</td>
<td>2.8</td>
<td>3.0</td>
<td>92</td>
</tr>
<tr>
<td>glucose</td>
<td>1.9</td>
<td>2.5</td>
<td>16.4</td>
<td>100</td>
</tr>
<tr>
<td>ethanol</td>
<td>2.1</td>
<td>5.2</td>
<td>9.1</td>
<td>104</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5.9</td>
<td>8.6</td>
<td>9.4</td>
<td>91</td>
</tr>
<tr>
<td>pyruvate</td>
<td>8.7</td>
<td>12.7</td>
<td>15.9</td>
<td>97</td>
</tr>
<tr>
<td>hydrogen</td>
<td>9.6</td>
<td>14.7</td>
<td>20.7</td>
<td>77</td>
</tr>
</tbody>
</table>

\(^a\) All reported values are based on results from duplicate samples. \(^b\) The average mass of PCE added (41.6 μmoles, standard deviation = 3.61) was used to calculate the reported mass balance.
Dechlorination was not observed in cultures fed acetate, acetaldehyde, formate, lactate, propionate, butyrate, or methanol. In these cultures, the extent of dechlorination was equal to or less than that observed in the controls. The presence or absence of acetate in lactate-, propionate-, and butyrate-fed tubes did not have any influence on dechlorination.

**Discussion**

Although attempts at isolating the halorespiring organism(s) were unsuccessful, important information was gained through these studies. For example, information on the complex nutritional requirements required to sustain dechlorination activity in purified cultures was obtained. The necessary addition of FSCE indicated that the dechlorinators possessed an undefined nutritional dependency on one or more organisms in the parent culture. Because the medium was consistent with that used in the packed column, the inability to transfer dechlorination after four transfers could only be explained by the loss of an essential organism supplying an undefined growth factor. Although very little information is known regarding the composition of FSCE, it was shown that the constituent required for dechlorination was heat liable, and was either absent or present in unsatisfactory concentrations in spent medium, yeast extract, or bacto-peptone. The requisite addition of an undefined growth factor from a parent culture, such as filter-sterilized spent medium, has been observed in the isolation of other PCE-halorespiring bacteria (20.24).

The loss of cis-DCE dechlorination may have been due to the addition of BES to the medium or due to a low number of cis-DCE dechlorinators in the inoculum. In
separate experiments not presented here, it has been observed that the addition of 10 mM BES has an inhibitory affect on cis-DCE dechlorination in purified cultures of cis-DCE dechlorinators prepared from the same parent packed column (25). The inhibitory effect of BES on dechlorination in the absence of methanogenesis has also been reported by others (26). When transferred in the absence of BES cis-DCE dechlorination did not resume in the purified enrichment culture, indicating that at least two organisms are responsible for PCE dechlorination to ethene in the parent packed column. Complete dechlorination via two dechlorinating organisms, one degrading PCE to cis-DCE and the other degrading cis-DCE to ethene, is a common observation in many cultures (2.12.16.27.28), and in only one instance has an organism been found to dechlorinate PCE completely to ethene (20).

Isolation of the dechlorinating organism from the purified enrichment culture was confounding and could not be achieved with multiple liquid serial dilutions. It was not determined whether impurities in the purified enrichment culture remained due to nutritional dependencies by the dechlorinator(s) and/or because they were present in numbers exceeding that of the dechlorinator(s) and could not be eliminated by serial dilution. Microscopic evaluation of high dilution tubes that did not dechlorinate indicated that the latter was an important factor. Because the coccus isolated on Wilkins-Chalgren did not dechlorinate, the other dominant morphology in the purified enrichment culture (curved, motile rod) was assumed to be the dechlorinating organism. The appearance of colonies in prepared agar shake dilutions prior to the detection of dechlorination end products indicated that the impurities in the culture grew more quickly than the
dechlorinators. This is supported by the overnight presence of contaminant growth on Wilkins-Chalgren agar. Colonies pulled from dechlorinating agar shake tubes were never observed to be pure and consisted of several morphotypes. It is not known why transferred colonies would not dechlorinate in liquid or solid medium. Two possible explanations would be exposure to oxygen during the transfer process or transferring the wrong colony. Efforts were made to minimize oxygen exposure and to transfer colonies of varying appearance, but neither approach resulted in dechlorinating transfers.

The contaminant isolated on Wilkins-Chalgren agar resembled the morphology of the cocci from the highly purified enrichment culture. Because Wilkins-Chalgren contains several undefined nutritional supplements demonstrated to support the growth of a wide variety of anaerobes (29), it can be assumed that any other contaminants, if present, possessed nutritional requirements not met by this medium. Phylogenetic analysis of the isolated coccus indicated that this organism shared 98% 16S rDNA sequence identity (based only on a 500 bp analysis) with Lactosphaera pasteurii, a novel genus and species described in 1995 (30). L. pasteurii is a strictly anaerobic coccus (1-1.5 μm in diameter) isolated from anaerobic digester sludge from a municipal sewage treatment plant in Konstanz, Germany (31). Like the impurity in the purified enrichment culture, L. pasteurii occurs singularly, in pairs, or small clusters. Of the electron donors tested in this research, L. pasteurii has been reported to utilize pyruvate and glucose, and can not use formate, acetate, lactate, ethanol, or yeast extract (31). L. pasteurii can also ferment many sugars, L-tartrate, citrate, and oxaloacetate (30), yielding lactate, acetate, formate, ethanol, and carbon dioxide as end products (30, 31). Growth on hydrogen has
not been reported. Based on the 0.95 similarity rank through RDP match, the contaminant in the purified enrichment culture is probably a new species of *Lactosphaera*.

At this time, it can not be determined whether the presence of the contaminant was necessary to support dechlorination in the purified enrichment culture. Of the reported fermentation products listed for *L. pasteurii* (which may not be consistent in the contaminant), only acetate and ethanol were found to be associated with sustained dechlorination (acetate as carbon source in hydrogen-fed cultures, and ethanol as electron donor). To ascertain a possible syntrophic relationship between the contaminant and dechlorinator, more information on the metabolic activities and nutritional requirements of both organisms are required.

Results from the RFLP analysis of cloned rDNA inserts indicated that the purified culture may have consisted of more than the two dominant microorganisms. It is not known whether more than one dechlorinating organism was present, or if multiple contaminants existed. To further analyze the results from the cloning and RFLP experiment, the 16S rDNA inserts should be sequenced. It is possible that chimeric sequences (i.e., incorrect artifacts generated when PCR-mediated recombination occurs between two or more separate genes) were formed. The two repeated patterns observed in rDNA inserts from the purified enrichment culture indicated a high probability that these inserts were indigenous to the culture and not experimental artifacts. Cloning efficiencies were very low for both the contaminant and purified culture, and may have been due to the large size of the insert (1500 bp). To better describe the ecology of the
purified culture and to compensate for PCR and cloning biases. more rDNA inserts should be cloned to delineate dominant species as well as less dominant species that may not have been represented in the 8 potential clones found here.

Nutritional studies using the purified enrichment culture indicated that the dechlorinator required acetate as a source of carbon (note that at this time no other carbon sources have been tested). Electron donor usage was found to vary in the culture. In the absence of a pure culture, it could not be determined which electron donors listed in Table 5.2 were used directly by the dechlorinator or indirectly following transformation of the electron donor to a usable substrate by another organism. The initial absence of dechlorination activity in glucose- and ethanol-fed tubes suggested these substrates were not used directly. Hydrogen may have served as the final electron donor for dechlorination in this culture, but more information on dechlorinators and/or the substrates used and metabolic products formed by nondechlorinating organisms would be required to resolve this issue.

The variety of substrates shown to sustain dechlorination in both the parent culture (see Chapter 4) and highly purified culture indicate that microbial community structure and potential symbiosis among constituents may play a more important role in enriching and sustaining PCE dechlorination in situ than electron donor selection. Results from recycle column experiments presented in Chapter 4 revealed that lactate, methanol, and hydrogen sustained dechlorination equally in the parent culture and that yeast extract did not serve as a source of reducing equivalents. Purifying the culture eliminated lactate- and methanol- sustained dechlorination, indirect proof that a separate
organism in the parent culture may have converted these substrates into a form usable by dechlorinating bacteria. Dechlorination in the highly purified culture in the presence of yeast extract was an unexpected and interesting observation. In initial serial dilutions as well as in the recycle columns, yeast extract was not observed to enhance dechlorination. It is probable that the FSCE added during the purification process selected for organisms capable of yeast extract degradation since the two substrates have the potential to be compositionally similar (yeast extract is produced during the autolysis of baker’s yeast and is composed of water-soluble vitamins, amino acids, peptides, and carbohydrates (23)). Also, the culture of Chapter 4 was much more biologically diverse than the purified culture presented here. Thus, the flow of potential reducing equivalents from yeast extract degradation to dechlorination may have been less in the former.

Through attempts to isolate a halorespiring organism from the PCE-dechlorinating enrichment culture described in Chapter 4, it was determined that the PCE-dechlorinating organism(s) possessed undefined nutritional requirements that were met at least partially through either the addition of filter-sterilized cell extract or through the metabolic products of another organism(s) that required FSCE for growth. Experiments conducted in agar shake dilutions may indicate that dechlorinatror(s) had additional nutritional dependencies on other organisms not met through the addition of FSCE, since the dechlorinator(s) could not be isolated from the culture. Several substrates supported dechlorination in the purified enrichment culture, suggesting that electron donor selection may be less important in mixed cultures than the interactions and syntrophic relationships
between dechlorinators and other microorganisms. Further work is required to
characterize these relationships and to characterize the phylogeny of species present.

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Chapter Six: EFFECT OF DECHLORINATING BACTERIA ON THE LONGEVITY AND COMPOSITION OF PCE-CONTAINING NONAQUEOUS PHASE LIQUIDS UNDER EQUILIBRIUM DISSOLUTION CONDITIONS

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Effect of Dechlorinating Bacteria on the Longevity and Composition of PCE-Containing Nonaqueous Phase Liquids under Equilibrium Dissolution Conditions

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The influence of dechlorinating microorganisms on PCE and its reduced end products in the presence of a PCE-containing nonaqueous phase liquid (NAPL) was investigated. Experiments were conducted in continuous-flow stirred-tank reactors (CFSTRs) containing a mixed PCE dechlorinating culture and a model NAPL consisting of PCE and tridecane. Comparisons between biotic and abiotic CFSTRs demonstrated that dechlorination resulted in a factor of 14 increase in PCE removal rates from the NAPL. The formation of dechlorination daughter products trichloroethene and cis-dichloroethene were observed, and cis-dichloroethene was not dechlorinated further. Partitioning of daughter products between phases caused temporal changes in the chlorinated ethenes distribution within the NAPL. The combined effects of dissolution and dechlorination on the removal of chlorinated ethenes from the NAPL were described using a mathematical model that approximated dechlorination as a pseudo-first-order process. Pseudo-first-order dechlorination rate coefficients for PCE and TCE were determined and were 0.18 and 0.27 h⁻¹, respectively. It was determined that total chlorinated ethenes removal from the NAPL would be achieved in 13 days in biotic CFSTRs, as compared to 77 days in the abiotic CFSTRs, corresponding to an 83% reduction in longevity of the chlorinated ethenes component of the NAPL.

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are among the most frequently detected contaminants in groundwater (1, 2). Because of their limited aqueous solubility and miscibility in other organic solvents, PCE and TCE contamination are often associated with mixed organic nonaqueous phase liquids (NAPLs). Areas in which NAPLs are present, referred to as source zones, represent long term sources of groundwater contamination (3), and their presence greatly complicates the ability to restore contaminated aquifers. Pump-and-treat has long been recognized as an ineffective method for source zone restoration (4), and the removal of chlorinated ethenes containing-NAPLs, especially those that are denser than water, is considered to be a technical challenge “...unprecedented in the field of ground-water engineering” (3). Currently, all accepted remediation technologies focus on source containment and do little, if anything, to reduce the longevity of source zones.

Although microbial reductive dechlorination of PCE and TCE has been well documented (5–15) and is currently being employed to treat chlorinated ethenes contaminated groundwater (16, 17), dechlorination-based source zone restoration has not been rigorously evaluated. A primary concern for source zone bioremediation is the potential toxicity of high concentrations of contaminants found near the NAPL (3). Studies have demonstrated, however, that dechlorination can be sustained at high PCE concentrations including saturation (15, 18–20). Furthermore, source zones may represent an ecological niche for certain dehalorespiring microorganisms capable of withstanding high PCE concentrations (21–24). For these organisms, a PCE-containing NAPL represents a continuous source of terminal electron acceptor.

The extent to which dechlorination can impact source zone longevity (i.e., the time required to exhaust the mass of chlorinated ethenes from the NAPL) could prove to be an important consideration in assessing the duration of natural attenuation scenarios or in the implementation of engineered bioremediation processes in chlorinated ethenes source zones. In the absence of dechlorination, the longevity of the PCE component of the NAPL is controlled strictly by the rate of PCE dissolution. Dechlorination has the potential to increase PCE removal rates by depleting PCE from the aqueous phase in the direct vicinity of the NAPL, thereby increasing the overall mass-transfer of PCE into solution. If dechlorination rates in source zones are sufficiently rapid, it may be possible to substantially reduce the longevity of the PCE component of the NAPL and minimize the time required for site restoration.

A complicating factor in assessing the impact of dechlorination on source zone longevity is the interaction of dechlorination end products with the NAPL itself. PCE dechlorination results in the formation of TCE, cis-dichloroethene (cis-DCE), and vinyl chloride (VC). These daughter products will partition into the existing NAPL, causing temporal changes in NAPL composition and affecting the overall longevity of the source. Because dechlorination daughter products are contaminants themselves, all chlorinated ethenes must be removed from the source for restoration to be considered complete.

The objectives of this research were to determine whether dechlorinating bacteria can reduce the longevity of PCE in a PCE-containing NAPL, and to evaluate the transient effect of dechlorinating bacteria on the chlorinated ethenes distribution between the NAPL and aqueous phase. Experiments were conducted in continuous-flow stirred-tank reactors (CFSTRs) containing a PCE-dechlorinating enrichment culture. Results demonstrated that dechlorination could substantially reduce the longevity of PCE in NAPLs as compared to dissolution alone and that the overall impact of dechlorination within a source zone could be described mathematically using a combined dissolution-dechlorination model.

Materials and Methods

Chemicals. The following chemicals were obtained in liquid form: tetrachloroethene (99.9±%; Acros); trichloroethene (99.5%; Aldrich); cis-dichloroethene (97%; Acros); 1,1,1-trichloroethane (99.5%; Aldrich); tridecane (99%; Sigma); pentane (HPLC grade; Acros); methanol (certified ACS; Fisher); and formic acid (88%; Fisher). Vinyl chloride (8%).
balance N₂, propane (99.95%), and nitrogen (99.999%) were prepared by TriGas. Ethene (99.5%) was procured from Scott Specialty Gases.

Nutrient Medium. Nutrient medium was prepared as outlined previously (15), with the following exceptions: (NH₄)₂HPO₄ was replaced with 140 mg/L KH₂PO₄; 0.01 M phosphate buffer was substituted for bicarbonate buffer; no reducing agents were added; and vitamins were included (final concentration of 0.04 mg/L 4-aminozenoic acid, 0.01 mg/L D(+)-biotin, 0.1 mg/L nicotinic acid, 0.05 mg/L Ca-D(+)pantothenate, 0.15 mg/L pyridoxamine dihydrochloride, 0.1 mg/L thiamine hydrochloride. 0.05 mg/L cyanocobalamin). Formate served as electron donor and was added to nutrient medium (pH 7) to a final concentration of 10 mM.

Analytical. Gas chromatography was used to determine aqueous phase concentrations of all chlorinated ethenes. For PCE and TCE quantification, aqueous samples (150 µL) were extracted in pentane (5 mL) and injected (1 µL) into a gas chromatograph (GC) (Hewlett-Packard) equipped with an electron capture detector (ECD). This method has been described previously, as outlined by Carr and Hughes (15). All samples were amended with 1,1,1-trichloroethane (final concentration of 390 µg/L) as an internal standard prior to analysis. Nominal detection limits for PCE and TCE were 12 and 59 µg/L, respectively.

Headspace analysis was used to quantify cis-DCE, VC, and ethene. Aqueous samples (5 mL) were added to 70 mL serum bottles capped with a Teflon-lined butyl rubber septum and aluminum crimp cap. Propane (50 µL) was added to each serum bottle as an internal standard. Following equilibration between the aqueous phase and headspace, headspace samples (100 µL) were directly injected onto a GC (Hewlett-Packard) equipped with a flame ionization detector (FID) and packed column (6 ft. x 1/8 in OD) containing 60/80 Carbopack B/1% SP-1000 (Supelco). GC operating parameters were identical to those described previously for this analytical method (15). Standards were prepared by adding methanol dissolved cis-DCE stock solutions and known volumes of VC and ethene to 70 mL serum bottles containing 5 mL of deionized water. Nominal detection limits for cis-DCE and VC were 200 and 50 µg/L, respectively.

Determination of Partition Coefficients. Dimensionless partition coefficients (K(OC)) for PCE, TCE, and cis-DCE in tridecane were measured independently. A known mass of each chlorinated ethene was diluted in a known volume of tridecane. The mixture was added to duplicate serum bottles (15 mL) containing deionized water. The final oil-to-water ratio was approximately 0.005 (v/v) to be consistent with oil-to-water ratios used in experimental systems. The serum bottles were capped with Teflon-lined butyl rubber septa and aluminum crimp caps and were stored on a shaker table at room temperature (24 °C ± 0.57) for a period of no less than 24 h. Bottles were removed from the shaker table and allowed to sit quiescently for phase separation. Duplicate aqueous phase samples (150 µL for PCE and TCE analyses) and 5 mL for cis-DCE analysis) were removed from each bottle using gastight syringes and analyzed by GC.

Reactor Design. Experiments were conducted in CFSTRs as depicted in Figure 1. The reactors were composed of glass bottles (600 mL) that had been modified on one side to include a glass stopcock with Teflon plug for sampling. The reactors were capped with stainless steel plugs (Rice University Support Shop) kept in place by open-top screw caps and Viton O-rings (American Packing and Gasket Co.). The effluent line extended to the bottom of the reactor to prevent NAPL washout.

Aqueous mobile phases (see protocol section) were continuously pumped into the reactors via a peristaltic pump (Cole Parmer). Reactors were operated with zero headspace.

FIGURE 1. Schematic of CFSTRs used in experiments.

The flow rate was maintained at 0.14 mL/min, resulting in a hydraulic retention time (HRT) of 3 days. Reactor effluent was routed via a two-way stainless steel valve to either a sampling port or to a bottle for effluent collection.

Start-up of Biotic Reactors. Effluent from an up-flow column containing a PCE-dechlorinating enrichment culture was used to inoculate the biotic CFSTRs. The PCE-dechlorinating culture, previously described as the methanol/PCE enrichment culture (19), had been enriched on methanol and PCE for a period of over 5 years and had consistently dechlorinated PCE (at an influent concentration of 86 mg/L) to VC and ethene. Effluent from the column was collected, purged with N₂, and divided into two CFSTRs. Prior to the onset of experiments, each culture was maintained under continuous flow, completely mixed conditions for a period of 17 days. Nutrient medium was pumped through the systems (HRT = 3 days), and neat PCE (10 µL) was added directly to the contents of both reactors approximately every 72 h to maintain dechlorination activity. The cultures were monitored daily for dechlorination activity, extent of dechlorination, and pH.

NAPL Preparation. A mixture of tridecane and PCE was prepared to simulate a PCE-containing NAPL consisting of an insoluble and recalcitrant organic fraction. PCE was diluted in tridecane to a final weight fraction of 0.12 g PCE/g NAPL (0.13 mol PCE/mol NAPL) based on an average molecular weight of 182 g/mol. The density of the NAPL was 0.81 g/mL.

Protocol for CFSTR Experiments. Abiotic and biotic CFSTRs were run in duplicate. The contents and mobile phase of the abiotic reactors were a 0.005 M CaCl₂ solution. Following the initial start-up period, biotic reactors were maintained in the same fashion as the abiotic reactors with the exception of having nutrient medium as the mobile phase. NAPL was added gravimetrically to each reactor (Abiotic 1: 1.63 g; Abiotic 2: 1.67 g; Biotic 1: 1.60 g; Biotic 2: 1.65 g) at the onset of the experiment. Equilibrium dissolution was promoted by vigorous stirring, and flow through the reactors commenced immediately following NAPL addition. Equilibrium dissolution was verified in separate experiments, and it was determined that aqueous PCE concentrations reached equilibrium with the concentration of PCE in the NAPL in less than 10 min. All reactors were operated at 24 °C.

For aqueous PCE and TCE samples, flow and stirring were temporarily halted to allow NAPL to accumulate at the top of the reactor. Samples were taken through the side port using gastight syringes fitted with 6 in. stainless steel needles. Because larger sample volumes were required for cis-DCE, VC, and ethene quantification, these samples were collected from the sampling port at the top of the reactor during normal...
TABLE 1. Dissolution-Dechlorination Model

\[ C_1 = C_{1.0}e^{-k_1t} \]  \hfill (2)

\[ C_2 = \frac{K_1}{K_2 - K_1}C_{1.0}(e^{-k_1t} - e^{-k_2t}) \]  \hfill (3)

\[ C_3 = \frac{K_1K_2}{(K_3 - K_2)(K_1 - K_2)(K_1 - K_3)}C_{1.0}(K_3 - K_2)e^{-k_1t} + \frac{K_1K_2K_3}{(K_1 - K_3)(K_3 - K_2)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - (K_1 - K_3)e^{-k_4t}) \]  \hfill (4)

\[ C_4 = \frac{K_1K_2K_3}{(K_2 - K_1)(K_3 - K_2)(K_4 - K_1)}C_{1.0}(e^{-k_1t} - e^{-k_4t}) + \frac{K_1K_2K_3K_4}{(K_1 - K_3)(K_3 - K_2)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - e^{-k_4t}) + \frac{K_1K_2K_3K_4}{(K_1 - K_3)(K_2 - K_3)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - e^{-k_4t}) \]  \hfill (5)

\[ C_5 = \frac{K_1K_2K_3K_4}{(K_2 - K_1)(K_3 - K_2)(K_4 - K_1)}C_{1.0}(e^{-k_1t} - e^{-k_4t}) + \frac{K_1K_2K_3K_4K_5}{(K_1 - K_3)(K_3 - K_2)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - e^{-k_4t}) + \frac{K_1K_2K_3K_4K_5}{(K_1 - K_3)(K_2 - K_3)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - e^{-k_4t}) + \frac{1}{1 - |K_2 - K_1|} \left[ \frac{1}{(K_2 - K_1)(K_3 - K_2)(K_4 - K_1)} \right] + \frac{1}{1 - |K_2 - K_1|} \left[ \frac{1}{(K_2 - K_1)(K_3 - K_2)(K_4 - K_1)} \right] \frac{K_1K_2K_3K_4K_5}{(K_1 - K_3)(K_2 - K_3)(K_4 - K_3)}C_{1.0}(e^{-k_3t} - e^{-k_4t}) \]  \hfill (6)

* Subscript "0" represents the initial condition. Subscripts "1-5" correspond to PCE, TCE, cis-DCE, VC, and ethene, respectively. \( K_i \) and \( K_0 \) are defined as follows: \( K_i = (r + k_i)R_i; K_0 = k_0R_0 \).

CFSTR operation. Sampling was initiated 2 h following startup, and thereafter the biotic reactors were sampled every 12 to 24 h. The biotic reactors were sampled more frequently (every 8-24 h) to monitor dechlorination activity.

**Determination of Microbial Influences on Partitioning Behavior.** At the end of the experiment, partition coefficients were measured in the biotic reactors to determine whether changes in partitioning behavior had occurred as a result of microbial activity. Aqueous phase concentrations of chlorinated ethenes were quantified, and then 20 mL of additional tridecane was added to each reactor. The reactor contents were vigorously stirred to allow the chlorinated ethenes to equilibrate between the aqueous and nonaqueous phases. Concentrations of chlorinated compounds remaining in the aqueous phase were determined by GC, and partition coefficients were calculated.

**Description of the Dissolution-Dechlorination Model.** The reactors were modeled as completely mixed systems where dissolution of NAPL was rapid and could be described by the equilibrium condition, \( C_i = K_i^{-1}C_{eq} \) (mol/L) represent the concentration of species \( C_i \) in the oil (o) and water (w) phases, respectively. Reductive dechlorination of PCE to the end product ethene was modeled by sequential pseudo-first-order reactions (i.e., constant dechlorination activity was assumed) that accounted for the production and decay of the intermediates TCE, cis-DCE, and VC. With this approach, a mass balance for any chlorinated ethene species \( i \) in the reactor yields the equation

\[ R_i \frac{dC_{wi}}{dt} = -\tau C_{wi} + k_{i-1}C_{wi-1} - k_i C_{wi} \]  \hfill (1)

\( \tau = \frac{Q}{V^o} \), \( R_i = 1 + \frac{V^o}{V^w}k_i^{-1} \)

\( \tau \) represents time (h); \( \tau \) is the hydraulic retention time (h); \( Q \) is the flow through the reactor (L/h); \( V^o \) and \( V^w \) are the volume (L) of the oil and aqueous phases, respectively; and \( k_i \) (h\(^{-1}\)) represents the pseudo-first-order dechlorination rate coefficients. \( R_i \) (dimensionless) represents the retardation coefficient of a particular chlorinated ethene, and is a function of the partition coefficient and the NAPL to water ratio (\( V^o/V^w \)) in the CFSTR. Equation 1 includes removal of species \( i \) by dissolution (\( -\tau C_{wi} \)), generation of \( i \) due to transformation of the preceding \( i \)-1 species (\( +k_{i-1}C_{wi-1} \)), removal of \( i \) via dechlorination (\( -k_i C_{wi} \)), and partitioning of the various species between the NAPL and aqueous phase (\( R_i \)).

As PCE and daughter products are removed from the NAPL, the volume of the NAPL would be expected to change. Because a maximum NAPL volume change of less than 6% (based on the initial volume percent of PCE in the NAPL mixture) occurred in experiments presented herein, \( V^o \) was considered to be constant and temporal changes in the retardation coefficient were neglected. Using eq 1, rate equations for all chlorinated ethenes were solved as coupled differential equations with the initial condition that the only chlorinated ethene present at \( t = 0 \) was PCE. The resulting analytical solutions to eq 1 for temporal variations in aqueous concentrations of PCE, TCE, cis-DCE, VC, and ethene comprise the dissolution-dechlorination model and are presented in Table 1. Equations 2-6 (see Table 1) were used.
to fit experimental data, and values for the pseudo-first-order dechlorination rate coefficients were obtained. All model calculations were performed using SigmaPlot (version 4.0).

Results

Determination of Partition Coefficients. Dimensionless partition coefficients for PCE, TCE, and cis-DCE in tridecane were determined experimentally at 24 °C. The average partition coefficients from duplicate bottles were as follows: $K_{\text{PCE}}^{\text{tridecane}} = 3060$, $K_{\text{TCE}}^{\text{tridecane}} = 395$, and $K_{\text{CDE}}^{\text{tridecane}} = 94$. Theoretical partition coefficients were calculated using Raoult's Law (NAPL activity coefficients were assumed to be unity in all cases) and aqueous solubilities taken from literature (25). Theoretical partition coefficients obtained from these calculations were as follows: $K_{\text{PCE}}^{\text{theoretical}} = 3.706$, $K_{\text{TCE}}^{\text{theoretical}} = 51.7$, and $K_{\text{CDE}}^{\text{theoretical}} = 115$.

Start-up of Biotic Reactors. Cultures initially dechlorinated PCE to VC, as observed in the original enrichment culture. However, cis-DCE became the major reduced end product after several days of operation. By the end of the 17-day start-up period, dechlorination consistently stopped at the level of cis-DCE and VC was no longer detected.

CFSTR Experiments. Results from the CFSTR experiments have been summarized in Figure 2 and Table 2. Following NAPL addition in the biotic CFSTRs, a lag phase (1 day) was observed, and limited transformation of PCE occurred. Rapid dechlorination did not commence until after 24 h of operation.

Temporal aqueous phase concentrations of PCE, TCE, and cis-DCE are presented for the abiotic and biotic reactors in Figure 2A. The first 6 days of data have been plotted for all four CFSTRs. Aqueous PCE concentrations in the abiotic reactors demonstrated that equilibrium dissolution was maintained throughout the experiment. Decreases in aqueous PCE concentrations in the abiotic systems occurred as the mole fraction of PCE in the NAPL decreased. In the biotic reactors, aqueous PCE concentrations decreased at a faster rate than observed in the abiotic systems. Aqueous TCE concentrations, which started to rise slightly after 18 h, increased steadily until hour 54 and then decreased thereafter. Both PCE and TCE concentrations were at or below detection limits in samples taken after 135 h. Aqueous concentrations of cis-DCE peaked at approximately 96 h, remained near constant for a period of 24 h, and then began to decrease as dissolution and washout occurred. VC production was not observed.

The dechlorination rate coefficient in eq 2 was set to zero for calculating the predicted removal of PCE in the abiotic CFSTRs. A value of 0.79 was ascertained for the biotic CFSTRs based on differences between the model predictions and the average observed aqueous concentrations from the two abiotic CFSTRs. Pseudo-first-order dechlorination rate coefficients were calculated by obtaining a least-squares fit of the model to the average concentrations from the two biotic reactors. Because of the initial lag period, the rate coefficients were determined using data from hours 24 to 135 (i.e., 24 h data corresponds to $t = 0$ in eqs 2–6). The pseudo-first-order dechlorination rate coefficients obtained for PCE and TCE were $k_1 = 0.18$ h$^{-1}$ ($r^2 = 0.97$) and $k_2 = 0.27$ h$^{-1}$ ($r^2 = 0.42$), respectively. The cis-DCE pseudo-first-order dechlorination rate coefficient ($k_3$) was zero in eq 4 since no cis-DCE dechlorination was observed. Therefore, the model prediction for removal of cis-DCE ($r^2 = 0.89$) only included the production and dissolution of this compound.

Figure 2B presents the cumulative moles of chlorinated ethenes removed from the abiotic and biotic reactors. Data are shown for all four CFSTRs and calculations for cumulative moles removed (represented by lines in Figure 2B) were made by integrating the area under the model curves in Figure 2A. For the biotic cumulative model, mass removed over the first 24 h through dissolution and limited dechlorination activity were included to obtain the calculated line in Figure 2B. Despite the initial lag phase, chlorinated ethenes removal was slightly higher in the biotic reactors as compared to the abiotic reactors after 48 h (average cumulative chlorinated ethenes was 0.11 and 0.074 nmol, respectively). PCE and cis-DCE were the main constituents of biotic reactor effluent during this time (see Figure 2A). After this period, cis-DCE production rapidly increased, and the total cumulative moles removed from the biotic reactors exceeded that of the abiotic reactors by 50% to over 150%. Because cis-DCE dechlorination was not observed, the experiments were considered complete once PCE and TCE concentrations in the biotic reactors approached their detection limit (135 h). At this point, the experiments were stopped, and mass balances were performed in all reactors. Abiotic CFSTRs, which had been operating slightly longer than the biotic CFSTRs, were stopped at 144 h.

Temporal changes in NAPL chlorinated ethenes composition are shown in Figure 2C. NAPL composition was calculated using temporal aqueous phase concentrations and measured partition coefficients. In the abiotic CFSTRs, the mole fraction of PCE in the NAPL decreased 20–24% over the duration of the experiment. In both biotic reactors, the mole fraction of PCE in the NAPL decreased from 0.13 mol PCE/mol NAPL to approximately 0.01 mol PCE/mol NAPL. Partitioning of cis-DCE in these systems resulted in a maximum cis-DCE mole fraction of 0.01 mol cis-DCE/mol NAPL.

Mass balance data for these experiments are presented in Table 2. In the abiotic reactors, 16–17% of the initial PCE mass was recovered in the effluent after 144 h of operation. The mass of PCE remaining in the reactors was determined for both phases, and 96% of the initial PCE mass was accounted for in Abiotic 1 and 101% in Abiotic 2. Mass balances in the biotic reactors were calculated in the same manner, and the mass recovery was 96% and 104% for Biotic 1 and Biotic 2, respectively.

After 135 h, the percent PCE removal in the biotic CFSTRs ranged from 89 to 91%, as compared to abiotic reactors in which percent PCE removal ranged from 16 to 17% (see Table 2). The percent chlorinated ethenes removal (i.e., PCE, TCE, and cis-DCE) in the biotic systems was 47–50%. Based on the initial mass of PCE added and the calculated pseudo-first-order dechlorination rate coefficients, the average time required to achieve 90% mass removal of total chlorinated ethenes in the biotic systems was 13 days including the 1 day lag phase. According to model predictions for the abiotic CFSTRs, 90% PCE removal would have been achieved after 77 days of continuous operation.

Determination of Microbial Influences on Partitioning Behavior. At the end of the experiment, the partition coefficient for cis-DCE was determined in both biotic reactors. $K_{\text{DCE}}^\text{tridecane}$ values were determined to be 102 in Biotic 1 and 110 in Biotic 2, which were not statistically different (95% confidence interval) from the independently measured partition coefficient reported earlier.

Discussion

The objective of this research was to demonstrate the effects of dechlorinating bacteria on the fate of PCE and its reduced end products in the presence of PCE-containing NAPLs. An integral part of this experiment was the determination of tridecane/water partition coefficients for PCE, TCE, and cis-DCE. The partition coefficients were critical in understanding and predicting compositional changes of NAPL in biotic systems as well as in obtaining mass balance data. Measured and theoretical partition coefficients were found to compare favorably, although some nonideality was observed for all three compounds. Partition coefficients decreased as the
FIGURE 2. Temporal aqueous phase concentrations (a), cumulative moles of chlorinated ethenes collected from abiotic and biotic CFSTRs (b), and changes in NAPL chlorinated ethenes composition (c). In all panels, shaded symbols represent data from Biotic 1 and Abiotic 1, and hollow symbols represent data from Biotic 2 and Abiotic 2. In panels (a) and (c), PCE (•), TCE (◆), and cis-/DCE (□) values are presented for biotic reactors, and PCE (Φ) is shown for abiotic reactors. In panel (b), biotic reactors are represented by (Φ), and abiotic reactors are represented by (◊). Model predictions are represented by lines.
chlorinated ethene became more reduced (i.e., $K_{PCE} > K_{TCE} > K_{Cl}$), due mainly to their increasing aqueous solubilities. Thus, dechlorination in the presence of a PCE-containing NAPL will yield reduced species that partition more strongly into the aqueous phase, and an increase in the total chlorinated ethenes removal rate from the NAPL is possible.

The prepared NAPL consisted of PCE and a conservative hydrocarbon to imitate a mixed organic waste. This composition was chosen based on the observation that pure phase NAPLs are not commonly encountered and that many chlorinated solvent spills occur in the presence of fuels or other organics such as mineral spirits (2, 4, 26). BTEX and other constituents that are commonly found in mixed NAPLs and that may serve as an electron donor were omitted from the NAPL as their dissolution would have significantly increased the complexity in describing observed results and transient NAPL composition. However, experiments with NAPLs containing an internally supplied electron donor are of great field relevance and warrant investigation.

The CFSTR experiments were designed to quantitatively determine the effect of dechlorination on the rate of chlorinated ethenes removal from a PCE-containing NAPL as compared to dissolution alone. The experiment was conducted under completely mixed conditions to achieve instantaneous equilibrium dissolution of PCE and to eliminate mass-transfer limitations. Under these conditions, optimal mass depletion of PCE from the NAPL was achieved via dissolution. Dechlorination, which should not exceed the rate of dissolution in this case, represented an additional source of chlorinated ethenes removal (as indicated by the $-k_cC_{PCE}$ term in the dissolution-dechlorination model). Under mass-transfer limited conditions, as is commonly observed in porous media, dissolution rates may be slower allowing dechlorination to affect the driving force for dissolution by decreasing the bulk phase PCE concentration. Because the experiments and modeling presented herein are for completely mixed systems and do not take into account mass-transfer limitations, they should not be extrapolated to field sites. Experiments taking into account mass-transfer resistances resulting from flow through porous media are currently in progress.

In the biotic CFSTRs, rapid dechlorination commenced after a 24 h lag period, and aqueous PCE concentrations continuously dropped below that which was observed in abiotic controls. The dechlorinating microorganisms were able to achieve 90% PCE removal within 135 h, a removal rate approximately 14 times faster than that determined for the abiotic systems. As dechlorination occurred in the aqueous phase, TCE and cis-DCE were formed and equilibrated with the NAPL.

Although the biotic CFSTRs were inoculated with a PCE-dechlorinating culture that routinely dechlorinated PCE to VC and ethene, the cultures lost the ability to dechlorinate beyond the level of cis-DCE during the 17 day start-up period. The reason for this loss in dechlorination activity is not known. Slow growth rates have been observed in highly purified enrichment cultures of cis-DCE dechlorinators in this laboratory (data not shown), and it is presumed that the short hydraulic retention time in the CFSTRs resulted in the washout of these bacteria from the reactors. Toxicity of high concentrations of cis-DCE was not a probable explanation for the loss of cis-DCE dechlorination since the initial PCE-dechlorinating culture was continuously fed much higher concentrations of chlorinated ethenes than used in CFSTRs.

For modeling purposes, PCE and TCE dechlorination were modeled as pseudo-first-order processes assuming the absence of dechlorinating microorganisms was constant throughout the experiment. This approach was useful in estimating removal rates of chlorinated ethenes from non-aqueous phase liquids but cannot be used to derive microbial growth kinetics in these systems. Model calculations were found to fit PCE removal ($r^2 = 0.97$) throughout the experiment. Initial TCE and cis-DCE concentrations did not coincide with their respective model calculations due to the limited dechlorination that took place during the lag phase. The TCE model did not accurately fit TCE formation during 32–48 h, after which TCE concentrations were consistent with model calculations. Similar observations were made between measured cis-DCE concentrations and the cis-DCE model, which overpredicted cis-DCE formation until approximately 72 h. The reason for the apparent variation in dechlorination activity is not known. One possible explanation is that dechlorination rate coefficients were changing during these periods and eventually became constant. As a whole, however, model calculations were found to be representative of PCE and TCE removal and cis-DCE formation and dissolution. In abiotic systems, data demonstrated that effluent aqueous phase concentrations were at equilibrium with PCE concentrations in the NAPL and that measured concentrations were found to closely match ($r^2 = 0.79$) the temporal calculated values.

The cumulative millimoles of chlorinated ethenes removed in the abiotic vs biotic CFSTRs clearly demonstrates the potential impact that dechlorinating bacteria can impart on removal rates of these contaminants from NAPLs. After approximately 6 days of operation, the total chlorinated ethenes removal was approximately three times greater in the presence of a dechlorinating culture as compared to equilibrium dissolution alone. At this time, the NAPL in the biotic reactors contained cis-DCE as the primary chlorinated component. Because the cis-DCE tridecane/water partition coefficient is 30 times smaller than that for PCE, cis-DCE removal is comparatively much more rapid. Thus, the time required to achieve a 90% reduction in total chlorinated ethenes was 77 days in the abiotic CFSTRs and only 13 days in the biotic CFSTRs (including the 1 day lag phase)—corresponding to an 83% reduction in longevity of the chlorinated ethenes component of the source.
It is important to note that the maximum obtainable enhancement of chlorinated ethenes removal is based upon both the rate of dechlorination and the partitioning behavior of the terminal chlorinated ethene. Had TCE been the terminal chlorinated ethene in the CFSTR experiments, the reduction in source longevity would have been diminished. The opposite is true for VC, which has a calculated tridecane/water partition coefficient \( K_{OCW}^{tridecane} \) of approximately 33 (using 8400 mg/L as the aqueous solubility). Any production of VC would have decreased the longevity of the source, as VC partitions more strongly into the aqueous phase and is washed out more quickly.

Mass balance data was able to account for the majority of mass in all CFSTRs (96–104%), demonstrating that losses due to sorption or volatilization were minimal. To determine that dechlorination activity was solely responsible for the increase in chlorinated ethenes removal and that microbial activity had not influenced tridecane/water partition coefficients, \( K_{OCW}^{tridecane} \) values were measured at the end of the experiment. Measured \( K_{OCW}^{tridecane} \) values in the biotic reactors compared favorably with the value previously determined in the batch experiment \( (K_{OCW}^{tridecane} = 94) \). Thus, dechlorination was assumed to be the sole contributor to the increase in observed removal rates.

In continuously stirred, continuous flow systems, it was demonstrated that dechlorinating microorganisms can impact the longevity of NAPLs as a source of chlorinated ethenes contamination by two distinct mechanisms: first, by depleting PCE from the aqueous phase, causing an increase in the overall mass-transfer of PCE from the NAPL into solution; and last, by reducing PCE to species that partitioned more strongly into the aqueous phase. It was determined that the partitioning behavior of the terminal chlorinated ethene was a key factor in assessing the longevity of the chlorinated ethenes component of the NAPL and that source longevity was diminished as dechlorination daughter products became more reduced. Because dechlorination can substantially impact removal rates of chlorinated ethenes from NAPLs, this process may be an important factor in evaluating the duration of natural attenuation and may lead to bioremediation strategies focused on source zone treatment.

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Literature Cited


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Chapter Seven: CONCLUSIONS

The following specific and general conclusions have been drawn regarding the characterization of PCE-dechlorinating microorganisms based on research performed using the methanol/PCE enrichment culture and the purified enrichment culture:

- Dechlorination of chlorinated ethenes can be enriched in cultures with no known previous exposure to chlorinated solvents. Acclimation and contact between the chlorinated compounds and the methanol/PCE enrichment culture appeared to be the most influential factors in determining the extent of dechlorination achieved.

- PCE dechlorination can be enriched and sustained equally, as determined by rate and extent of dechlorination, using lactate, methanol, or hydrogen (fed at high partial pressures, 0.8 atm) as an electron donor.

- High hydrogen partial pressures were not a strong selective pressure favoring methanogenesis and acetogenesis over dechlorination. Loss of dechlorination activity under these conditions (i.e., high hydrogen partial pressures) was not observed, as predicted by other researchers based on half-velocity constants with respect to hydrogen. Rates and extents of dechlorination improved with time in cultures containing high hydrogen partial pressures. From these observations, it can be concluded that competition between dechlorinators and other organisms in mixed, anaerobic cultures can not be simplified using only kinetic and energetic analyses.

- Attempt to isolate the PCE-halorespiring organism(s) from the methanol/PCE enrichment culture revealed that the dechlorinator possessed undefined nutritional
requirements not met by the anaerobic nutrient medium used. Syntrophic relationships involving the transfer of growth factors between dechlorinators and other members of the culture were requisite for dechlorination activity, as evidenced by the dependence of dechlorination on the presence of FSCE in the purified enrichment culture. Understanding these relationships may be necessary to isolate the PCE-halorespiring bacterium.

- Indirect evidence from Chapters 5 and 6 suggest that PCE dechlorination to VC and ethene in the methanol/PCE enrichment culture is effected via two or more microorganisms.

- The large number of electron donors shown to support dechlorination in both the highly purified and methanol/PCE enrichment cultures indicates that electron donor selection may be less influential in sustaining dechlorination in mixed cultures than method and frequency of electron donor delivery or obligate syntrophic relationships between dechlorinators and other microorganisms. Because the culture became more discriminating with respect to electron donor when purified, one can infer that electron donor selection becomes more important when the culture is less biologically diverse.

The following conclusions were derived from the research investigating the ability of PCE-dechlorinating bacteria to influence removal rates of PCE-containing NAPLs:

- Dechlorination can impact the longevity of chlorinated ethenes-containing NAPLs by acting as a source of contaminant removal and by reducing contaminants to species that partition more strongly into the aqueous phase. Under equilibrium dissolution
conditions, dechlorination resulted in a factor 14 increase in PCE removal from a PCE-containing NAPL as compared to dissolution. Total mass removal (defined as 90%) of all chlorinated ethenes was predicted to be 13 days in dechlorinating systems and 77 days in abiotic systems, which represented an 83% reduction in the chlorinated ethenes component of the NAPL.

- The partitioning behavior of the terminal chlorinated ethene was a key factor in assessing the longevity of the chlorinated ethenes component of the NAPL. NAPL/water partition coefficients for PCE, TCE, cis-DCE, and VC were observed to decrease as the chlorinated ethene became more reduced. Had VC been the terminal chlorinated ethene in this research, the reduction in the longevity of the chlorinated ethenes component of NAPL would have been greater due to higher rates of VC washout from the system (assuming rates of cis-DCE dechlorination were comparable to the rates of PCE and TCE dechlorination observed).
Chapter Eight: ENGINEERING SIGNIFICANCE

Much of the work presented in this thesis has been directed towards understanding PCE-dechlorinating organisms, their ability to dechlorinate PCE using various electron donors, and their nutritional requirements. Perhaps one of the most significant findings from this research has been the reaffirmation that hydrogen and a large number of organic substrates can be used to stimulate dechlorination in mixed cultures. Moreover, the theory that electron donor delivery must be engineered to maintain low levels of hydrogen (i.e., nM range) via fermentation of organic electron donors was unsubstantiated. It was demonstrated that high concentrations of hydrogen (645 μM aqueous phase concentration) led to improved dechlorination over time and did not result in the exclusion of dechlorination. In comparing the two approaches to hydrogen addition (i.e., direct hydrogen addition vs. low level hydrogen addition via fermentation of organic substrates) it is important to note that the low level hydrogen method may not generate enough electron donor to support extensive dechlorination in heavily contaminated areas such as source zones. Collectively, these results indicate that the selection of electron donor for in situ bioremediation systems should be based on cost, method, and frequency of delivery rather than substrate.

The ability to utilize dechlorination for the remediation of PCE-containing nonaqueous phase liquids is a novel concept that has not been previously investigated. It has been estimated that over 60% of all sites listed on the US Superfund National Priority List (NPL) have a medium to high likelihood of having DNAPLs, the majority of which
contain chlorinated solvents (1). The prevalence of chlorinated solvent containing-NAPLs and their associated risk to public health and the environment necessitate the development of low cost technologies capable of effecting their removal. To date, only emergent technologies have been developed to address NAPL remediation, and no accepted technologies exist. The ability of dechlorinating bacteria to substantially reduce the longevity of PCE-containing NAPLs, as shown in this research, may prove to be an important consideration in the design of source zone remediation. Unlike many of the emergent technologies in this area, bioremediation is associated with contaminant mass destruction rather than transformation to another form or phase requiring further treatment, as in surfactant flooding. Engineered bioremediation systems within the source zone may also have the advantage of being cheaper to operate. For these reasons, the influence of dechlorinating bacteria on chlorinated ethenes-containing NAPLs should be investigated further for strategies focused on source zone treatment.

Reference

Chapter Nine: FURTHER RESEARCH

Results from research presented in the previous chapters indicate that further research is warranted in specific areas. A better understanding of the growth requirements of dechlorinating microorganisms can only be gained through research using highly purified or pure cultures. Interestingly, isolation of the dechlorinator in the enrichment culture used in these experiments appears to be contingent upon first understanding its nutritional dependencies on other organisms present in the culture. Components of the FSCE should be analyzed and fractionated by molecular weight to determine the essential growth factor(s) that support dechlorination. Likewise, information gained through the isolation and characterization of contaminants could be used to further purify the culture by specifically targeting contaminant removal (e.g. addition of BES for methanogens) and/or supplementing the nutrient medium with the growth factor excreted from the contaminant. Identification of contaminants and dechlorinator(s) present in the purified enrichment culture should be performed by 16S rDNA phylogenetic analysis. More efficient cloning of rDNA inserts may be possible using a newly developed TOPO TA cloning kit (Invitrogen) designed specifically for cloning large inserts (>1000 bp). For statistical purposes, 200 or more clones should be analyzed. Furthermore, the lack of information available on cis-DCE and VC dechlorinators necessitates their isolation and characterization.

Results from the studies investigating microbial influences on NAPL removal indicate that in situ dechlorination may prove to be a promising technology for source zone remediation. To extrapolate the findings from the CFSTR experiments to field sites,
similar experiments need to be conducted in columns containing porous medium.

Because column experiments would contain a much greater mass of chlorinated ethenes by design, studies on electron donor loading rate and delivery must be conducted to avoid substrate limitations. Investigations coupling dechlorination with multicomponent NAPLs composed of a mixture of chlorinated solvents or a source of electron donor (e.g., BTEX and PCE NAPL) would also be of benefit since the majority of NAPLs present in the environment are not pure phases.
APPENDIX

The Comment and Response to Comment regarding the publication reprinted in Chapter 4 are presented in the appendix. The Comment is reprinted with the author's permission from *Environmental Science and Technology*. 1999, 33(15), 2681-2682. Copyright 1999 American Chemical Society. The Response to Comment is reprinted with permission from *Environmental Science and Technology*. 1999, 33(15), 2683-2684. Copyright 1999 American Chemical Society.
Comment on "Enrichment of High-Rate PCE Dechlorination and Comparative Study of Lactate, Methanol, and Hydrogen as Electron Donors To Sustain Activity"

SIR: Carr and Hughes (1) investigated the relative abilities of the electron donors lactate, methanol, and H₂ to sustain reductive dechlorination of tetrachloroethene (PCE) in mixed cultures. They concluded that dechlorination was sustained equally, regardless of the donor administered. This is at odds with our published studies (2, 3), from which we concluded that donors (such as butyrate or propionate) which can generate only low concentrations of H₂ are superior to those (such as ethanol) which tend to cause accumulation of high H₂ concentrations. We explained our conclusions in terms of microbial competition—specifically, differences in H₂ access thresholds and biokinetics that favored dechlorinators over competing methanogens at low H₂ concentrations.

It is important to explain the apparent discrepancy between the two studies, so that readers will not be confused. There were several differences between the electron-donor experiments of Carr and Hughes (1) and our own (2, 3). Chief among these was the ratio of electron donor to PCE employed.

We envision three scenarios involving hydrogenotrophic dechlorination, based on PCE concentration and donor:PCE ratio. In the first scenario, PCE concentration is not inhibitory to competing organisms (<50 ppm), but available electron-donor level is very high—orders of magnitude higher than stoichiometrically required for dechlorination. In this case any donor added will—through fermentation and the maintenance of a large, endogenously decaying biomass pool—produce enough trace, scavangeable H₂ to stimulate dechlorination of small quantities of chloroethenes (4). This can be true even of a donor such as methanol, which is directly used by methanogens. The second scenario is one where PCE is present at high levels (>50 ppm) that are inhibitory to competing methanogens. In this case, relatively low ratios of donor may be applied, because most of the reducing equivalents will be available to the dechlorinators—the competition is eliminated (5, 6). The third scenario is an "intermediate" situation where a significant amount of PCE is present (1–50 ppm), and donor is available at a similar level (2, 3). Under these conditions, competition for limited donor by nondechlorinators such as methanogens could dictate whether dechlorination is complete. Since dechlorinators have a significantly lower half-velocity coefficient (and probably threshold) for H₂ use than do methanogens (7–9), H₂ donors fermented slowly, and under low H₂ partial pressures selectively contribute H₂ to dechlorinators, while minimizing that available for methanogens (2, 3, 9).

The two studies differed in their applicable donor:PCE scenarios. We examined the "intermediate" scenario where ethanol, lactate, butyrate, and propionate were added at 1:1 and 2:1 donor:PCE ratios (H₂ basis, Table 1). If expressed in terms of complete oxidation to CO₂ (as Carr and Hughes have done), our donor:PCE ratios were 2.3:1 to 10:1. These ratios are 1–2 orders of magnitude lower than those used by Carr and Hughes (1). In their recycle-column experiments where H₂, methanol, and lactate were compared, a donor:PCE ratio (CO₂ basis) of 630:1 was used; and in their high-H₂ batch systems, the initial donor:PCE ratio was 114:1. In our systems, depending upon the donor added, 38–100% of the donor (H₂ basis) was consumed by dechlorination. In contrast, in H₂-fed systems described by Carr and Hughes, only 0.4% of the donor was consumed by dechlorination.

<table>
<thead>
<tr>
<th>TABLE 1. Equivalent Conversions for Selected Electron Donors</th>
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</thead>
<tbody>
<tr>
<td>donor</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>butyric acid</td>
</tr>
<tr>
<td>ethanol</td>
</tr>
<tr>
<td>lactic acid</td>
</tr>
<tr>
<td>propionic acid</td>
</tr>
<tr>
<td>methanol</td>
</tr>
<tr>
<td>H₂</td>
</tr>
</tbody>
</table>

When defining equivalents, we have usually done so on the basis of H₂ produced from fermentation of the donor. It has been expedient to do so, since our work has involved a mixed culture known to contain a hydrogenotrophic dechlorinators, and our donor:PCE ratios were low enough that secondary reducing equivalents (e.g., from biomass decay and/or fermentation) are scavangeable metabolic intermediates—rather than significant contributors. However, when reduction is primarily affected from such secondary sources—as when 100-fold excess of a non-H₂-producing donor such as methanol are employed—then it is sensible to express equivalents on a total oxidation (to CO₂) basis, as Carr and Hughes have done. It also makes sense to define electron donor equivalents differently if H₂ is not directly used by the particular dechlorinators under consideration.

Our study documented head-to-head competition for limited donor (H₂) between dechlorinators and methanogens—the third scenario described above—with dechlorinators competing most successfully for H₂ formed at low partial pressures by the addition of propionate or butyrate. In contrast, the study of Carr and Hughes is a case of the first scenario described above—low amounts of PCE in the presence of large amounts of donor and the huge biomass pool that it supports. The dechlorinators scavenge for—and subsist on—a trace amount of donor that is an insignificant fraction of the total applied. In that case, the specific donor added is, indeed, irrelevant to the question of how completely or at what rate dechlorination is carried out.

We also reported results from long-term studies in which there were no significant differences among the various donors, in terms of completeness of dechlorination. The primary reason for this (not mentioned by Carr and Hughes in their reference to our study) is that prefermented yeast extract, added as a required nutritional amendment, also provided significant amounts of propionate and butyrate (2, 3), Their subsequent fermentation supplied low levels of H₂ that were used by dechlorinators, but not by methanogens, long after the primary donor (e.g., ethanol or lactate) had been consumed. In short-term batch tests, where yeast extract could be omitted without nutritional effect, the aforementioned differences among donors were observed.

We do agree with the conclusion of Carr and Hughes that when donor is added at a very high level (i.e., 630-fold) relative to the amount of chloroethenes to be degraded, it makes very little difference what specific donor is added. However, we believe it is a mistake to conclude, generally, that the identity of the supplied electron donor is irrelevant. For in situ bioremediation systems, it may not always be prudent to add a large amount of donor, even if cost is not an issue. If it is feared that well or aquifer biofouling may become problematic, or if regulatory issues dictate that donor must not be added in greatly excessive amounts, it may be necessary to add donor at lower donor:PCE ratios. In this case, it is important that the donor be chosen wisely.

An additional and very important factor is that these low-H₂-generating, slowly degraded donors persist. This is a highly
desirable characteristic in in-situ bioremediation, where transport of the donor past the highly biologically active injection point and throughout the aquifer is critical to successfully stimulating dechlorination throughout the treatment zone. Substrates such as H₂ or ethanol may be consumed so rapidly at the point of injection that their distribution in the subsurface becomes difficult.

Literature Cited


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Response to "Comment on "Enrichment of High-Rate PCE Dechlorination and Comparative Study of Lactate, Methanol, and Hydrogen as Electron Donors To Sustain Activity""

SIR: We thank Fennell and Gossett for their analysis of our work and regret that any confusion may have resulted from comparisons between our research and theirs. We appreciate the opportunity to address the issues they have raised in their Comment and to resolve any confusion that may exist. In their Comment to the editor, the correspondents focus on the difference between electron donor to PCE ratios (ED:PCE) used in the two studies. They also comment on the omission of our article regarding the role of prefermented yeast extract (FYE) in their long-term studies and introduce an important discussion of relevant engineering design issues for the addition of electron donors to the subsurface. We have addressed these comments in the order in which they are raised.

In both our work (1) and the work of Fennell et al. (2), experiments were conducted to evaluate the ability of several electron donors to sustain PCE dechlorination in mixed, methanogenic cultures. Despite the similarity in the central objective of these studies, several important differences must be recognized. One difference, as pointed out by the correspondents, was the ED:PCE ratio. Perhaps a more important difference was the electron donors employed, since lactate was the only common substrate between the two studies. Fennell et al. used butyric acid, ethanol, lactic acid, and propionic acid, while we used methanol, lactic acid, and hydrogen. Hydrogen could be considered a common electron donor in both studies, since it is produced through fermentation of organic electron donors. However, the partial pressures of hydrogen in our systems in which hydrogen was fed directly were orders of magnitude greater than would be expected from fermentation processes. Other differences included inoculum, experimental design (batch vs recycle column), duration of experiments, and experimental temperature. Each of these factors may impose some degree of incongruity in the interpretation of findings between the two reports.

Fennell and Gossett raise an interesting point in the evaluation of our systems regarding ED:PCE ratios. We elected not to focus on this issue in our manuscript as electron donor consumption data was available only for the hydrogen-fed recycle columns. For those studies we reported an ED:PCE ratio of 630:1 based on a PCE addition of 0.054 mequiv and the amount of hydrogen that was consumed over a 4-day period (34 mequiv total or 8.5 mequiv per day). At the beginning of the study, dechlorination was not complete (e.g., PCE, TCE, and cis-DCE were present without vinyl chloride or ethene production) within the 4-day feeding cycle, and the 630:1 ratio accurately described the operation of the systems. Over time, dechlorination rates increased to the point that dechlorination to vinyl chloride and ethene was complete within a few hours after PCE addition, and the observed daily hydrogen consumption averaged 5 mequiv per day. Thus, by day 474, the ratio of hydrogen equivalents consumed to PCE equivalents fed was approximately 15:1 during active dechlorination. In other words, the observed ratio of hydrogen consumption to PCE dechlorination was not constant throughout the study, due to the continuous increase in dechlorination activity.

We believe that this finding is important, since there has been considerable concern regarding the ability of dechlorinators to compete with other microorganisms (i.e., methanogens and acetogens) at high hydrogen partial pressures. The high partial pressure of hydrogen in our hydrogen-fed systems would almost certainly saturate the rates of all competing processes (based on K values) and provide the ideal opportunity for competition effects to be observed. Despite the fact that PCE was present for only a few days every 4 days and that high partial pressures of hydrogen (relative to a fermentation-based system) were available throughout the 474 days of column operation, the extent and rate of dechlorination increased throughout the experimental period. If high hydrogen partial pressures were a strong selective pressure favoring dechlorinators, the opposite observation would be expected.

Fennell and Gossett introduce several hypothetical scenarios involving competition for hydrogen between methanogens and dechlorinators under various concentrations of PCE. While it is interesting to investigate all possible scenarios as presented, we feel it is important to focus on "typical" conditions at contaminated sites. As a general rule, source areas (i.e., regions where nonaqueous-phase liquids exist) contain dissolved phase contaminant concentrations at or slightly above 1% of the effective aqueous solubility of the contaminant (3). In the case of PCE, this would be approximately 1–2 mg/L (higher concentrations certainly exist at the water–DNNAPL interface or may occur in media where dispersion processes are significantly impeded). For this reason, the second scenario presented in their commentary is uncommon. Recognizing that required levels of remediation demand that dechlorination result in mg/L concentrations and that the stoichiometry of dechlorination is quite favorable (i.e., over 20 mg of PCE can be dechlorinated to ethene by 1 mg of hydrogen), the first scenario presented is more likely to be observed in anaerobic remediation systems. This is further supported by the fact that the addition of excess electron donor represents the only safety factor to ensure complete dechlorination. We do agree that ratios as high as 630:1 are probably excessive, but it is difficult at this time to determine a priori appropriate levels of electron donor addition required to obtain complete dechlorination.

In the discussion section of our manuscript we draw comparisons between our long-term tests to those of Fennell et al. We did not address the role of FYE in these studies as noted by the correspondents. Our reason for neglecting this point in our manuscript is based on a comment from their discussion. That is, Fennell et al. reported that in long-term study controls, fed only FYE, dechlorination was incomplete with "significant amounts of remaining PCE and TCE." This statement was inconsistent with their conclusion that "...the addition of FYE significantly influenced the outcome of the long-term tests"—a conclusion resulting from short-term experiments evaluating the role of FYE in sustaining dechlorination. We were unclear as to reason for the apparent discrepancy between the long-term and short-term study results and were most interested in the long-term studies as they were more closely related to our own experiments. For that reason, we chose to neglect the matter in our discussion.

The last point raised in the correspondence of Fennell and Gossett focuses on engineering considerations for electron donor delivery. We agree with the correspondents that biofueling is a management concern for any liquid delivery system. From our limited experience there are also regulatory issues that complicate the permitting of liquid delivery systems if contaminated water is reinjected. We agree...
TABLE 1. Estimates of True Yields from Various Organic Substrates and Hydrogen

<table>
<thead>
<tr>
<th>Substrate</th>
<th>True yield* (mg cells-eq substrate~1)</th>
<th>True yield* (mg cells-eq hydrogen~1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>propionate</td>
<td>0.271</td>
<td>0.633</td>
</tr>
<tr>
<td>butyrate</td>
<td>0.266</td>
<td>1.328</td>
</tr>
<tr>
<td>ethanol</td>
<td>0.610</td>
<td>1.831</td>
</tr>
<tr>
<td>lactate</td>
<td>0.723</td>
<td>2.170</td>
</tr>
<tr>
<td>methanol</td>
<td>1.187</td>
<td>3.560</td>
</tr>
<tr>
<td>H2 (heterotrophic)</td>
<td>1.011</td>
<td>1.011</td>
</tr>
<tr>
<td>H2 (autotrophic)</td>
<td>0.237</td>
<td>0.237</td>
</tr>
</tbody>
</table>

* Free energy of formation data was taken from ref 4 or calculated from Table A1.1 in ref 5. pH = 7.0. For calculation of mg cells produced, ammonia was used as the nitrogen source and the chemical formula for biomass was assumed to be C6H12O6N. * Moles of hydrogen produced by the fermentation of organic substrates (per mole basis) to acetate were the following: propionate, 3; butyrate, 2; ethanol, 2; lactate, 2; and methanol, 1.

that it may be possible to minimize fouling concerns through electron donor selection—while maintaining adequate electron donor dose—using substrates that result in low yields of nondechlorinating organisms, in particular fermentors and methanogens. Table 1 presents calculated true yields of mixed methanogenic cultures (i.e., methanogens and fermentors) for a range of substrates. Yields were normalized to net hydrogen production during organic substrate fermentation (shown in the right most column) assuming that dechlorination is supported by hydrogen and not the organic substrates themselves. From this analysis, it is clear that electron donor selection may influence the dose allowed to avoid biofouling concerns. For example, the substrates butyrate, ethanol, lactate, and methanol result in more biomass production per equivalent of hydrogen produced than propionate or the direct addition of hydrogen.

It is also important to note that a variety of electron donor delivery systems are being evaluated that do not require liquid injection, in part to avoid certain practical concerns (including biofouling) and regulatory issues that influence reinjection. Alternative methods include hydrogen-based biosparging, in situ cathodic hydrogen production, and "slow release hydrogen" materials that can be injected into the formation. Iron slurry walls may serve as electron donor delivery systems in addition to their ability to dechlorinate via abiotic mechanisms. Other systems of which we are currently unaware may also be in development. Any comments regarding the extent to which any of these processes would be effective in mitigating biofouling would be premature. However, it is important to note that alternative electron donor delivery systems may offer methods to provide adequate electron donor delivery and minimize the specific issues raised.

In closing, we would like to thank Drs. Fennell and Gossett again for their thorough and thoughtful comments. We hope that any confusion resulting from our manuscript has been resolved through this process.

Literature Cited


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