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CHEMICAL, ISOTOPE AND MOLECULAR ANALYSIS OF MICROBIAL REDUCTIVE DECHLORINATION OF TETRACHLOROETHYLENE AND TRICHLOROETHYLENE

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CHEMICAL, ISOTOPE AND MOLECULAR ANALYSIS OF MICROBIAL REDUCTIVE DECHLORINATION OF TETRACHLOROETHYLENE AND TRICHLOROETHYLENE

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ABSTRACT

Tetrachloroethylene (PCE) and trichloroethylene (TCE) are among the most commonly detected groundwater contaminants in the U. S. Microbial reductive dechlorination of this group of contaminants was investigated in different reaction systems, including isolated pure cultures, enrichment cultures and microcosms. Chemical, isotope and molecular analyses were performed to evaluate the feasibility of stable carbon isotope fractionation to quantitatively monitor microbial PCE and TCE reductive dechlorination and the potential factors that may lead to uncertainties of this monitoring technique.

Microbial PCE and TCE reductive dechlorination was first analyzed and the product distribution and stable carbon isotope fractionation were determined in two isolated pure cultures (*Sulfurospirillum multivorans* and *Desulfuromonas michiganensis* Strain BB1) and one mixed culture, Bio-Dechlor Inoculum (BDI™). *S. multivorans* and *D. michiganensis* Strain BB1 produced *cis*-DCE when PCE or TCE was used as the parent substrate, while the *Dehalococcoides*-containing BDI was able to completely dechlorinate PCE and TCE to ethylene. Different extents of isotope fractionation were observed among the three cultures. Generally, weaker isotope fractionation occurred during PCE reductive dechlorination (ε_{bulk} = -1.33 to -7.12 ‰) than that during TCE transformation (ε_{bulk} = -4.07 to -15.02 ‰). The different levels of fractionation by individual species/culture might be due to their diversity in the structure of functional

enzymes (e.g., reductive dehalogenase), cofactors or rate-limiting steps before enzymatic reactions.

In order to evaluate potential impacts of environmental factors (e.g., electron donors and pH) and microbial diversity on isotope fractionation during microbial reductive dechlorination of chlorinated ethylenes, two enrichment cultures (DPF and DPH) stimulated from the same source but in the presence of different electron donors were investigated. These two cultures showed significantly different product distribution and isotope fractionation. Chemical and isotope analyses indicate that electron donors and pH do not directly change the product distribution and only slightly changed extents of isotope fractionation. However, phylogenetic analysis of the 16S rRNA clone libraries of DPF and DPH suggests that electron donors might indirectly influence extents of isotope fractionation by leading to a shift in microbial community composition.

At contaminated sites, microbial and abiotic reductive dechlorination may simultaneously occur. To understand the relative contributions of these remediation processes and to evaluate the feasibility of applying isotope fractionation to monitor potentially parallel microbial and abiotic transformation processes, PCE and TCE reductive dechlorination was carried out in a series of well-defined microcosms. Alternative electron accepting processes, e.g., iron-, sulfate-reduction and methanogenesis, were developed to vary contents of biogenic iron and sulfide minerals; electron donors were spiked to stimulate indigenous dechlorinating bacteria. Our results

showed that microbial reductive dechlorination was dominant in 21 out of 24 PCE microcosms and 5 out of 8 TCE microcosms. Isotope analysis indicated that weak isotope fractionation occurred in most microcosms, while some of them had very negative ε_{bulk}. All of them were within the range of or comparable with the ε_{bulk} of microbial reductive dechlorination of PCE and TCE that have been published so far. In addition, compared to the isotope fractionation during PCE and TCE abiotic reductive dechlorination by FeS, the extents of isotope fractionation observed in these microcosms was generally weaker. Higher environmental pH was suggested to be unfavorable for growth of dechlorinating bacteria. Meanwhile, the comparable levels of microbial and abiotic dechlorinated products were only observed in the microcosms with slow microbial reductive dechlorination, suggesting that abiotic dechlorination might be important only when microbial reductive dechlorination is slow. Comparison of geochemical conditions with abiotic product recoveries suggests that high concentrations of Fe(II) and S(-II) solid species produced under sulfate- and iron-reducing conditions are likely important for abiotic reductive dechlorination to occur.

In general, the different levels of isotope fractionation during microbial PCE and TCE reductive dechlorination observed in our pure culture, enrichment cultures and microcosm experiments, indicate to us that a number of factors need to be considered in applying isotope fractionation to quantitatively monitor bioremediation of this group of contaminants in the field. This includes whether the appropriate conditions have been

selected for development of model enrichment cultures, potential indirect impacts of environmental factors (e.g., pH and electron donors) and impacts of different transformation pathways (e.g., abiotic versus microbial) on the extents of isotope fractionation.

Key words: tetrachloroethylene, trichloroethylene, reductive dechlorination, isotope fractionation, geochemical conditions, electron donors, phylogenetic analysis, alternative electron accepting processes

CHAPTER 1

Introduction

Chlorinated solvents (e.g., PCE and TCE) are a group of major contaminants in many environments, especially in the subsurface. Past practices (e.g., source excavation, thermal methods, pump-and-treat systems, surfactant treatment etc.) are costly, lengthy and even ineffective (Pankow et al., 1996; Sinke and Hecho, 1999; USEPA, 2001a). Monitored Natural Attenuation (MNA) has been discussed as a promising and cost-effective option to remove chlorinated solvents (Grandel and Dahmke, 2004). Application of MNA to remediate contamination by chlorinated contaminants requires understanding of different transformation pathways, their relative contributions and effective approaches to monitor the transformation processes. This dissertation is focused on microbial reductive dechlorination of PCE and TCE. This chapter is provides some background and is organized into four sections: (1) motivation for this research; (2) reductive dechlorination of PCE and TCE; (3) stable carbon isotope fractionation during reductive dechlorination; and (4) overview of this study.

1.1. Motivation for This Research

PCE and TCE have been widely used as degreasing agents and solvents in many industrial and aviation applications (Häggblom and Bossert, 2003; Grandel and Dahmke, 2004). Due to improper waste disposal, spills and careless handling, they

are among the contaminants most commonly detected at USEPA superfund sites (Moran et al., 2007; ATSDR, 2008). Between 1998 and 2001, the total on- and off-site releases of PCE and TCE were about 4 and 33 million pounds, respectively (USEPA, 2001b). The special physico-chemical properties of PCE and TCE (e.g., poor water solubility, high specific density and high vapor pressure) allow them to be transported in subsurface via several processes (e.g., dissolved in groundwater, volatilized in the unsaturated zones or sorbed onto soil matrix). Since they can also exist as dense non-aqueous-phase-liquids (DNAPL), they can form widespread contaminant plumes (Häggblom and Bossert, 2003; Grandel and Dahmke, 2004). Considering their toxicity and recalcitrance (Mackay and Cherry, 1989; Massachusetts Department of Public Health, 1996), the USEPA has set Maximum Contaminant Levals (MCLs) for both contaminants in drinking water at 5 μg/L (USEPA, 2004).

Monitored Natural Attenuation (MNA) is an approach that relies on physical, chemical and microbial processes under favorable conditions to decrease the mass, toxicity, mobility, volume or concentration of contaminants, and thus is promising and cost-effective (USEPA, 1999; Grandel and Dahmke, 2004). Physical processes in MNA, including sorption, dispersion and volatilization, do not degrade the contaminants but alter their distribution among different phases (e.g., aqueous, solid and gaseous phases) (Sinke and Hecho, 1999; USEPA, 1999; Wiedemeier et al., 1999). However, microbial and chemical processes can transform PCE and TCE under proper conditions, and thus significantly contribute to remediation of dissolved chlorinated solvents and achieve remediation goals at a low cost within reasonable

periods of time (Holliger, 1995; Sinke and Hecho, 1999; USEPA, 1999; Wiedemeier et al., 1999; Bossert et al., 2003; Bradley, 2003; Holliger et al., 2003; Bhatt et al., 2007).

1.2. Reductive Dechlorination of Chlorinated Solvents

1.2.1. Microbial Reductive Dechlorination of PCE and TCE

Bacteria have evolved strategies to degrade chlorinated solvents. Microbial reductive dechlorination can be carried out via dehalorespiration, during which bacteria use chlorinated contaminants (e.g., PCE and TCE) as the terminal electron acceptors coupling the reduction of the solvent to oxidation of organic compounds or hydrogen and allowing energy to be obtained (Bossert et al., 2003; Bradley, 2003; Bhatt et al., 2007). Alternatively, other bacteria rich in reduced transition-metal coenzymes (e.g., Vitamin B_{12}, cofactor F430 or hemes) are able to fortuitously cometabolize chlorinated ethenes in a process that does not involve energy conservation (Holliger et al., 2003). Reductive dechlorination typically follows hydrogenolysis, during which chlorine atoms in the molecules are sequentially substituted with hydrogen (Gossett and Zinder, 1997). Thus, sequentially dechlorinated products, such as TCE (for PCE), dichloroethylene isomers (DCEs), vinyl chloride (VC) and/or ethylene can be produced as the intermediates or final products.

In the last decade, diverse anaerobic bacteria have been identified, isolated and studied for their capacity to transform PCE and TCE, including *Desulfitobacterium*

spp., *Desulfuromonas spp.*, *Enterobacter spp.*, *Sulfurospirillum spp.*, *Dehalococcoides spp.*, *Geobacter lovleyi* and *Clostridium bifermentans* (Chang et al., 2000; Holliger et al., 2003; Loeffler et al., 2003; Sung et al., 2006). Except for *Dehalococcoides spp.* (Maymo-Gatell et al., 1997; Zinder, 2001; He et al., 2003; He et al., 2005), all dechlorinating species that have been isolated so far accumulate partially dechlorinated products (e.g., TCE and DCEs) during reductive dechlorination of PCE and TCE (Chang et al., 2000; Holliger et al., 2003; Loeffler et al., 2003; Sung et al., 2006).

Microbial reductive dechlorination via dehalorespiration is catalyzed by a group of functional enzymes, designated as reductive dehalogenases (Rdhs) (Fetzner and Lingens, 1994; Holliger et al., 2003). This group of enzymes contains tetrapyrrole-cofactors, such as corrinoids, iron porphyrins or coenzyme F_{430} (Ni et al., 1995; Neumann et al., 1996; Magnuson et al., 1998; Neumann et al., 1998; Louie and Mohn, 1999; Suyama et al., 2002b). One widely accepted mechanism for enzyme catalyzed reductive dechlorination is that the reactions are initiated by transferring one dissociated electron from the reduced corrinoid (e.g., Co(I)-corrinoid) to the chlorinated compounds. This results in formation of a chlorinated compound radical, which sequentially combines with a proton (H^+) formed by hydrogenase after elimination of a chloride anion (Schumacher et al., 1997; Louie and Mohn, 1999).

Values of standard Gibbs free energy ($\Delta G°'$) of reductive dechlorination (hydrogenolysis) of PCE and TCE range from -56.0 (for PCE) to -52.1 (for TCE) kJ/mol per chlorine removed, corresponding to a redox potential (E'_o) of +540 (for

TCE) to +580 (for PCE) mV (Norris and Matthews, 1993). These redox potentials are literally comparable to the redox couple of NO_3^-/ NO_2^- (E'_o= + 433 mV) and are substantially higher than the values for sulfate reduction (SO_4^{2-}/HS^-) and methanogenesis (HCO_3^-/SH^-) (Norris and Matthews, 1993; Bossert et al., 2003), which suggests that dechlorinating organisms will out-compete sulfate reduction and methanogenesis. In the practical experience, competition of reductive dechlorination of with other terminal electron acceptor processes (e.g., nitrate-, sulfate reduction and methanogenesis) depends on the concentrations of alternative electron acceptors (other than chlorinated compounds) and availability of indigenous microbial communities. Therefore, PCE and/or TCE reductive dechlorination have been reported to occur under methanogenic, sulfate reducing and nitrate reducing conditions or when the alternative electron acceptors are consumed to the concentrations so enough so that reductive dechlorination becomes more thermodynamically favorable (Bossert et al., 2003; Bradley, 2003). In addition, some sulfate reducers (e.g., *Desulfovibrio spp.*, *Desulfomonile tiedjei*), nitrate reducers (e.g., *Desulfovibrio spp.*, *Desulfuromonas spp.*) and methanogenic bacteria (e.g., *Methanosarcina spp.*) (Fathepure et al., 1987; Fathepure and Boyd, 1988a, b; Cabirol et al., 1998; Holliger et al., 2003) can reductively transform chlorinated ethylenes via dehalorespiration or cometabolism.

Diverse electron donors have also been found to be involved in microbial reductive dechlorination. With a few exceptions (e.g., *Desulfuromonas ethenogenes* and *Desulfuromonas michiganensis* strain BB1) (Krumholz et al., 1996; Krumholz,

1997; Sung et al., 2003), H_2 is generally regarded as a direct electron donor for reductive dechlorination (Loeffler et al., 2003). In addition, many dechlorinating microorganisms are able to couple reductive dechlorination and fermentation of a variety of organic compounds, including fatty acids, alcohols, esters and yeast extract (Neumann et al., 1994; Utkin et al., 1994; Utkin et al., 1995; Gerritse et al., 1996; Sharma and McCarty, 1996b; Loeffler et al., 1997; Gerritse et al., 1999; Chang et al., 2000; Sung et al., 2003; Milliken et al., 2004; Sung et al., 2006). In some cases, dechlorinating bacteria form syntrophic association, in which one species lives off the products of another species. In the syntrophic systems, fermenting bacteria produce low concentrations of H_2 or small organic compounds used by the dechlorinating bacteria as electron donors (Drzyzga et al., 2001; Becker et al., 2005; Villemur et al., 2006; He et al., 2007).

1.2.2. Abiotic Reductive Dechlorination of PCE and TCE

Abiotic reductive dechlorination is also an important pathway for transformation of PCE and TCE, and is generally carried out by a variety of iron- and/or sulfide-containing minerals (e.g., iron sulfide (FeS), pyrite (FeS_2), magnetite (Fe_3O_4), various green rusts and dithionite treated soils) (Sivavec and Horney, 1996, 1997; Sivavec et al., 1997; Butler and Hayes, 1999, 2001; Lee and Batchelor, 2002b, a, 2004a, b). Reactive minerals can be formed via biogeochemical processes (Brown et al., 2006; Kennedy et al., 2006a; Kennedy et al., 2006b; Pasakarnis et al., 2006; Shen and Wilson, 2007; Dong et al., 2009) or corrosion of zero-valent-iron as the

packing material in permeable reactive barriers (PRBs) (Matheson and Tratnyek, 1994). Abiotic reductive dechlorination of PCE and TCE typically proceeds via reductive β-elimination (elimination of two halogens and increase in bonding order) and produces acetylene as the major product (Sivavec and Horney, 1996, 1997; Butler and Hayes, 1999, 2001; Lee and Batchelor, 2002b, a; Jeong et al., 2007). The transformation rates of abiotic dechlorination are dependent on a series of geochemical parameters, including pH (Klausen et al., 1995; Butler and Hayes, 1998, 2001; Pecher et al., 2002; Danielsen and Hayes, 2004), surface associated iron and sulfide species (Pecher et al., 2002; Elsner et al., 2004; Lee and Batchelor, 2004a, b), transition metals (Lee et al., 2000; Jeong and Hayes, 2003; O'Loughlin et al., 2003; Maithreepala and Doong, 2004a, b; Choi et al., 2009) and natural organic matter (NOM) (Doong and Wu, 1992; Butler and Hayes, 1998; Hanoch et al., 2006).

1.3. Isotope Fractionation During Reductive Dechlorination

The degradation of PCE and TCE has been determined by monitoring decreasing concentrations of contaminants over time and with distance from the source or appearance of dechlorinated products (Alleman and Leeson, 1997; Jang et al., 2003). However, as discussed above, both physical transport and chemical/biochemical transformation can lead to decreasing concentrations of PCE and TCE (Sinke and Hecho, 1999; USEPA, 1999; Wiedemeier et al., 1999), which may result in overestimation of the fraction of contaminants transformed. This dilemma can be circumvented by monitoring isotope fractionation. While negligible changes in

isotope fractionation are observed during physical processes (Slater et al., 2000a; Slater et al., 2001), significant fractionation have been found during chemical/biochemical transformation processes (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2000b; Slater et al., 2001; Slater et al., 2002; Song et al., 2002; Schuth et al., 2003; Slater et al., 2003; VanStone et al., 2004; Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009; Liang et al., 2009).

In organic compounds, heavier carbon isotopes (^{13}C) make up about 1.1 % of all the natural carbon (Hoefts, 1997). The relative abundance of ^{13}C and lighter isotope (^{12}C) is expressed by their ratio R, where:

$$R = \frac{^{13}C}{^{12}C} \tag{1.1}$$

Such isotope ratios can be measured by gas chromatography connected with isotope ratio mass spectrometry (GC-IRMS) and the $\delta^{13}C$ reported as the difference in per mil with respect to an international reference standard (Clark and Fritz, 1997):

$$\delta^{13}C = (\frac{R - R_{ref}}{R_{ref}}) \times 1000 \tag{1.2}$$

Reductive dechlorination of PCE and TCE causes a shift in the isotope ratio of parent substrates due to the stronger molecular bonds formed by ^{13}C than those by ^{12}C (Bigeleisen and Wolfsberg, 1958). Hence, accumulation of heavier stable isotopes in the residual parent substrates leads to the ratio of pseudo-first order rate constants of ^{12}C and ^{13}C ($^{12}k/^{13}k$), designated as Kinetic Isotope Effect (KIE), to be more than unity (Elsner et al., 2005) when the molecules are transformed. In the lab-scale studies,

extents of isotope fractionation can be quantitatively described as enrichment factor (ε_{bulk}), based on an assumption that isotope fractionation associated with degradation of organic pollutants follows the Rayleigh Model (Mariotti et al., 1981).

$$R_p = R_{p,0} f^{\left(\frac{\varepsilon_{bulk}}{1000}\right)} \quad (1.3)$$

where R_p and $R_{p,0}$ are the isotope ratios of the parent compound at each time point and at time zero, respectively; f is the fraction of parent compound remaining at a given time (i.e. C/C_0). This equation indicates that the more negative the values of ε_{bulk}, the stronger the extent of isotope fractionation.

Substantial isotope fractionation was observed during microbial degradation of PCE and TCE in the lab-scale studies (Bloom et al., 2000; Zwank, 2004; Elsner et al., 2005; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009) and in contaminated sites (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Slater et al., 2000b; Song et al., 2002; Morrill et al., 2005; VanStone et al., 2005; Nijenhuis et al., 2007). ε_{bulk} ranges from -0.42 to -16.7 ‰ for PCE and -2.5 to -20.9 ‰ TCE microbial reductive dechlorination, respectively; The values for abiotic PCE and TCE reductive dechlorination ranges from -14.7 to -30.2 ‰ and -10.1 to -33.4 ‰, respectively) (Hunkeler et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Elsner et al., 2005; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008)

Extents of isotope fractionation during enzymatic reactions can be influenced by biochemical pathways (e.g., anaerobic versus aerobic reactions) (Hirschorn et al.,

2004; Hirschorn et al., 2007), structural variability of functional enzymes (Nikolausz et al., 2006) or molecular size of reactants (Abe et al., 2009b). In addition, some rate-limiting steps prior to enzymatic reactions (e.g., transport of the substrate into the cells and formation of the enzyme-substrate complex) do not result in isotope fractionation. However, they can limit the supply of reactants for enzymatic reactions (O'Leary, 1988) and thus weaken the extents of isotope fractionation (Nijenhuis et al., 2005). Other mass transport processes (like dissolution of a NAPL to the aqueous phase) may also mask isotope fractionation if they occur more slowly than biodegradation (Meffre et al., 2008).

Considering isotopes for different elements in PCE and TCE molecules, there are four forms of C-Cl bonds, including ^{12}C-^{35}Cl, ^{13}C-^{37}Cl, ^{13}C-^{35}Cl and ^{12}C-^{37}Cl. Although it is known that lighter isotopes are preferred during chemical and biochemical reactions, chlorine isotopes are not expected to significantly influence the fractionation of carbon isotopes. It is because that $\delta^{37}Cl$ in the natural environment ranges only several per mil (usually 0-3 ‰) compared to much wide extents of $\delta^{13}C$ and $\delta^{2}H$. This means that regardless of the potential chemical and biochemical reactions that happen, the ratio of chlorine isotopes are relatively constant. Therefore, it is not expected that weak isotope fractionation of chlorine isotopes will significantly influence isotope fractionation of other elements (e.g. stable carbon isotopes of PCE and TCE). This is also supported by the much weaker chlorine isotope fractionation for reductive dechlorination than carbon isotope fractionation (Abe et al., 2009a).

1.4. Objectives of This Research

Despite the abundance of studies to date, important questions, such as the uncertainties of applying isotope technique to quantitatively monitor natural attenuation of chlorinated solvents (e.g., PCE and TCE), still exist. This is due to the complexity of contaminated sites, such as various levels of isotope fractionation by different dechlorinating bacteria, potential impacts of environmental factors or microbial diversity on observed isotope fractionation and simultaneously different transformation processes (e.g., microbial and abiotic reductive dechlorination). The general objective of this study is to evaluate the potential impacts of these factors on isotope fractionation during PCE and TCE reductive dechlorination.

This research is organized as follows. Chapter 2 presents the results of chemical and isotope analyses during PCE and TCE reductive dechlorination in two isolated pure cultures (*Sulfurospirillum multivorans* and *Desulfuromonas michiganensis* Strain BB1) and a mixed enrichment culture (BDI). Their product distribution was determined. ε_{bulk} values were calculated based on Rayleigh Model and the possible reasons resulting in different ε_{bulk} by different species/cultures were discussed. Chapter 3 focuses on the potential effects of microbial diversity and environmental factors (e.g., electron donors and pH) on isotope fractionation. These factors were evaluated on two enrichment cultures stimulated from the same source but that significantly differed in product distribution and isotope fractionation, or on *Sulfurospirillum multivorans* as a simplified model system. Chapter 4 addresses relative contributions of microbial versus abiotic reductive dechlorination in a series

of well-defined microcosms in which dechlorinating bacteria and reactive minerals might be coexistent. Chemical analysis identified the distinct dechlorinated products for either/both dechlorination pathway(s) in the microcosms and isotope fractionation was obtained from the reactive microcosms. The geochemical factors that might be favorable for microbial or abiotic reductive dechlorination were discussed. Finally, Chapter 5 summarizes the conclusions from Chapter 2 to 4. Based on this research, suggestions for future work in both fundamental studies and practical projects are also included.

CHAPTER 2*

Stable Carbon Isotope Fractionation of Tetrachloroethylene and Trichloroethylene during Reductive Dechlorination by Dechlorinating Bacteria

2.1. Introduction

Tetrachloroethylene (PCE) and trichloroethylene (TCE) are widespread groundwater contaminants in the US due to their improper disposal and storage (Pankow et al., 1996; Moran et al., 2007; ATSDR, 2008). Microbial reductive dechlorination by anaerobic bacteria is an important alternative to remediate this group of contaminants in the field (Bradley, 2003; Bhatt et al., 2007).

In addition to the conventional methods to identify and quantify the chlorinated parent substrates and dechlorinated products, stable carbon isotope fractionation is increasingly applied to monitor the fate of chlorinated solvents (e.g., PCE and TCE) (Hunkeler et al., 1999; Bloom et al., 2000; Slater et al., 2000b; Slater et al., 2001; Song et al., 2002; Hunkeler et al., 2004; Zwank, 2004; Elsner et al., 2005; Morrill et al., 2005; Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007; Nijenhuis et al., 2007; Cichocka et al., 2008). This technique takes advantage of the fact that isotope ratio negligibly changes during physical transport processes (e.g., dilution,

* This chapter consists of part of the paper by Liang et al. (2007). This chapter was rewritten from "Environmental Science & Technology 41, Liang, X.; Dong, Y.; Kuder, T.; Krumholz, L.R.; Philp, R.P.; Butler, E.C. Distinguishing abiotic and biotic transformation of tetrachloroethylene and trichloroethylene by stable carbon isotope fractionation, 7094-7100, Copyright (2007) American Chemical Society". Y. D. was responsible for microbial reductive dechlorination of PCE and TCE and the corresponding isotope fractionation.

dispersion, adsorption and evaporation) (Slater et al., 2000a; Hunkeler et al., 2004), while significant changes occur during microbial or chemical transformation which result in changes of molecular structure (Clark and Fritz, 1997; Hoefts, 1997; Elsner et al., 2005). Isotope fractionation results as weaker bonds formed by lighter isotopes (e.g., ^{12}C) are more easily cleaved than those by heavier isotopes (e.g., ^{13}C) and thus leads to accumulation of heavier isotopes in the residual parent substrate (Hoefts, 1997). Extents of isotope fractionation can be expressed as enrichment factor (ε_{bulk}), based on an assumption that isotope fractionation associated with degradation of organic pollutants follows the Rayleigh Model (Mariotti et al., 1981).

Significant isotope fractionation has been reported during microbial reductive dechlorination of PCE and TCE by a variety of isolated dechlorinating bacteria, microbial consortia and even at contaminated sites (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2000b; Slater et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009). Values of ε_{bulk} during these processes range from -0.42 to -16.8 ‰ for PCE and -3.3 to -20.9 ‰ for TCE, respectively (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Elsner et al., 2005; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008).

In addition to identifying occurrence of reductive dechlorination, recent studies have proposed to apply the ε_{bulk} obtained from lab-scale studies and the isotope ratio measured in the field to quantitatively monitor fraction of contaminants that has been

degraded (Van Breukelen et al., 2005; VanStone et al., 2005). This prediction requires reliable and representative fractionation data. The objective of this study is to identify variability of isotope fractionation during microbial PCE and TCE reductive dechlorination by different dechlorinating bacteria. Two pure cultures, *Sulfurospirillum multivorans* (*Sm*) and *Desulfuromonas michiganensis* strain BB1 (BB1) and one mixed culture, BioDechlor INNOCULUM (BDI) were investigated in this study. Chemical and isotope analyses were performed. The potential factors that might influence isotope fractionation were discussed to explain different ε_{bulk} obtained from different cultures.

2.2. Materials and Methods

2.2.1. Chemicals

PCE (99%), TCE (99.5%), *cis*-1,2-dichlorethylene (*cis*-DCE) were obtained from Sigma-Aldrich (St. Louis, MO). Chemicals used for microbiological medium preparation were purchased from Fisher Scientific (Pittsburgh, PA). Ethylene (1026 ppm in N_2) and vinyl chloride (VC) (1019 ppm in N_2) were supplied by Scott Specialty Gases (Houston, TX). All aqueous solutions were prepared with Nanopure water (18.0 MΩ cm resistivity, Barnstead Ultrapure Water System, IA).

2.2.2. Sample Preparation

Two isolated pure cultures, *Sm*, BB1 and one mixed culture, BDI, were kindly provided by Dr. Frank R. Löffler at the Georgia Institute of Technology. All the

cultures were prepared in 1 L PYREX^{TM} bottles modified by a glassblower (G. Finkenbeiner Inc., Waltham, MA) to accommodate a 20 mm septum stopper (Bellco Biotechnology). All the manipulations of culture preparation were performed under a stream of sterile N_2/CO_2 gas unless mentioned specifically. A reduced anaerobic basal salts medium was prepared (Sung et al., 2003) with a vitamin solution (Dworkin et al., 2006) and trace metals (Hurst et al., 2002). The medium was boiled and cooled under a stream of N_2/CO_2 (80/20, v/v) and the pH was adjusted to 7.2 by adding 2.52 g/L $NaHCO_3$. Serum bottle cultures were sealed with Teflon-lined rubber stoppers (West Pharmaceutical Services) and aluminum seals before sterilization. L-cysteine (0.2 mM), Na_2S (0.5 mM) and electron donors were added from sterile anaerobic solutions. The electron donors were (all 5 mM): lactate (BDI), acetate (BB1) and pyruvate (*Sm*). Cultures were inoculated using a 1:50 dilution ratio. Initial concentrations of PCE and TCE in the microcosm experiments were approximately 117 μM and 108 μM, respectively. Microcosms were prepared in duplicate and incubated still in the dark at room temperature.

2.2.3. Analytical Methods for Quantifying Reactants and Products.

Concentrations of PCE, TCE, *cis*-DCE, VC and ethylene were determined by headspace analysis with a Shimadzu GC-17A/flame ionization detector (GC/FID) and an Agilent GS-GASPRO capillary column (30 m × 0.32 mm). The injector temperature and detector temperature were 220 °C and 270 °C, respectively. Fifty microliters of headspace were withdrawn with a gas tight syringe (Hamilton Co.,

Reno, NV) and manually injected into the GC/FID using a split ratio of 1:1. The oven temperature was isothermal at 35 °C for 5 min, ramped to 190 ^{o}C at 30 ^{o}C/min, and then isothermal at 190 ^{o}C for 5 min. Five point external calibration curves were prepared daily. Relative standard deviations for samples and standards using this method were typically less than 5 %.

2.2.4. Isotope Measurements

At each time point, 1 mL culture was withdrawn with a sterile 1 mL syringe and diluted in approximately 23 mL nanopure water acidified with a 2 N HCl solution to a final pH less than 3 to inhibit microbial metabolisms because dechlorinating bacteria can hardly grow under such acidic condition (Neumann et al., 1994; Sung et al., 2003). One ml of N_2/CO_2 was added into the culture bottle to keep pressure constant. Before measurements, the dilution was stored at 4 °C in 20 mL EPA vials (nominal volume about 24 mL) with minimal headspace. Samples were analyzed by purge and trap (PT) coupled with a GC and Isotope Ratio Monitoring Spectrometer (GC-IRMS) for compound-specific isotope ratio analysis (CSIA) with the method previously described (Kuder et al., 2005). Pure CO_2 (instrument grade) was used as the reference gas for isotope fractionation. CO_2 was developed as the standard for detection calibration and the quality of CO_2 was cross-calibrated with alkanes (C15-C20), which have been calibrated against the international standard material, Vienna PeeDee Belemnite (V-PDB) (Coplen et al., 2006). The isotope ratio ($\delta^{13}C$) of the CO_2 as the reference gas may vary significantly depending on suppliers and the

raw compounds used for CO_2 production (e.g., carbonate minerals versus microbially formed organic compounds), the range of $\delta^{13}C$ from -3 to -43 ‰ has been observed in the previously calibration. In order to obtain maximum accuracy, CO_2 working standard was used daily to calibrate the mass spectrometer daily. Parallel standard compounds (e.g., PCE or TCE with known isotope ratio values) were used as in-house working standard to evaluate the potential influence of some physical processes (e.g. extraction, purge and trap) prior to isotope measurement. About 20 % of the samples were measured in duplicate. The standard deviation (n=2) of these duplicate $\delta^{13}C$ values did not exceed 0.6 ‰, and typically did not exceed 0.3 ‰ (Kuder et al., 2005).

2.2.5. Calculation and Definitions

The carbon isotope ratio (R) is reported in δ-notation (‰) relative to a CO_2 standard as described in Equation 1.1 and 1.2 (Chapter 1). The extents of isotope fractionation during the microbial reductive dechlorination were expressed as enrichment factor (ε_{bulk} in ‰) by applying the Rayleigh Model (Equation 1.3, Chapter 1). Apparent Kinetic Isotope Effect (AKIE) was calculated using the equation 2.1 (Zwank et al., 2005):

$$AKIE = \frac{1}{1 + (z \times n \times \varepsilon_{bulk} / (1000 \times x))} \qquad (2.1)$$

where n is the number of atoms of the molecule of a selected element, x is the number of reactive positions, the element(s) directly connected with the reacting bond(s); z is

the number of positions in intramolecular competition between the equivalent sites (e.g., two carbons of PCE molecules).

In order to compare the impact of rate-limiting steps (e.g., transport of substrates through cell membrane or binding of substrates to functional enzymes) on isotope fractionation, rate limitations of single steps preceding the biochemical dechlorination reactions are calculated as a Partitioning Factor (P), which measures the rate of the step that results in isotope fractionation relative to the rates of all preceding steps in the reaction sequence. Assuming the preceding reaction steps are rate limiting steps to some extent but not associated with a significant isotope effect, P is quantified as follows (O'Leary and Yapp, 1978):

$$P = \frac{\alpha_{(kin)} - \alpha_{(1)}}{\alpha_{(1)} - 1} \tag{2.2}$$

where $\alpha_{(kin)}$ and $\alpha_{(1)}$ are the fractionation factors of the purely chemical reactions carried out by proposed cofactor (e.g. Vitamin B12) for reductive dehalogenase and biochemical reactions by different dechlorinating bacteria, respectively (Krasotkina et al., 2001; Maillard et al., 2003; Nijenhuis et al., 2005). α is not directly measured but can be calculated from ε_{bulk} as follows:

$$\alpha_i = \frac{1}{1 + \varepsilon_{bulk,i}/1000} \tag{2.3}$$

where α_i means $\alpha_{(1)}$ or $\alpha_{(kin)}$, which can be calculated from the the corresponding ε_{bulk} for Vitamin B12 and dechlorinating bacteria, respectively. Detailed calculation of AKIE, P and their uncertainties is described in Appendix B.

2.3. Results and Discussion

2.3.1. Isotope Fractionation in the Course of PCE and TCE Reductive Dechlorination

PCE and TCE reductive dechlorination by *Sm*, BB1 and BDI are shown in Figure 2.1. As expected, *Sm* and BB1 dechlorinated PCE and TCE to *cis*-DCE (Neumann et al., 1994; Sharma and McCarty, 1996a; Sung et al., 2003), while ethylene was formed via *cis*-DCE and VC by BDI (Ritalahti et al., 2005). As a mixed culture, BDI contains several strains of *Dehalococcoides* species (e.g., *Dehalococcoides* sp. BAV1 and *Dehalococcoides* sp. FL2) and three ethene producing, PCE-reducing enrichment cultures, such as H7-PCE, H5-PCE and FMC-PCE (Sung, 2006). Thus, one or multiple *Dehalococcoides* species may be responsible for PCE and/or TCE reductive dechlorination in BDI.

$\delta^{13}C$ versus *f* of PCE and TCE microbial reductive dechlorination are shown in Figure 2.2 and the enrichment factors (ε_{bulk}) calculated with Equation 1.3 are listed in Table 2.1. Isotope fractionation was measured for duplicate BDI samples at each time point. The results showed that although transformation rates in the duplicate samples considerably varied (as shown as the big error bars in Figure 2.1 (PCE/BDI)), their isotope fractionation was within 95 % confidence intervals (Figure B1). Therefore, for the other cultures, only one microcosm of replicates was measured for isotope fractionation at each time point.

The values of ε_{bulk} obtained from *Sm*, BB1 and BDI in this study are within the range of the previously published values for microbial PCE and TCE reductive

dechlorination (Hunkeler et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Cichocka et al., 2008). In this study and previously published work (Hunkeler et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Cichocka et al., 2008), two general trends were observed: 1) stronger isotope fractionation occurs during TCE reductive dechlorination than that for PCE by the same bacteria/enrichment cultures; 2) for the same chlorinated substrate, variability in isotope fractionation exists among different dechlorinating species. For PCE reductive dechlorination, similar and weak isotope fractionation was observed by *Sm* and BB1, while stronger isotope fractionation was detected in BDI (Table 2.1 and Figure 2.2).

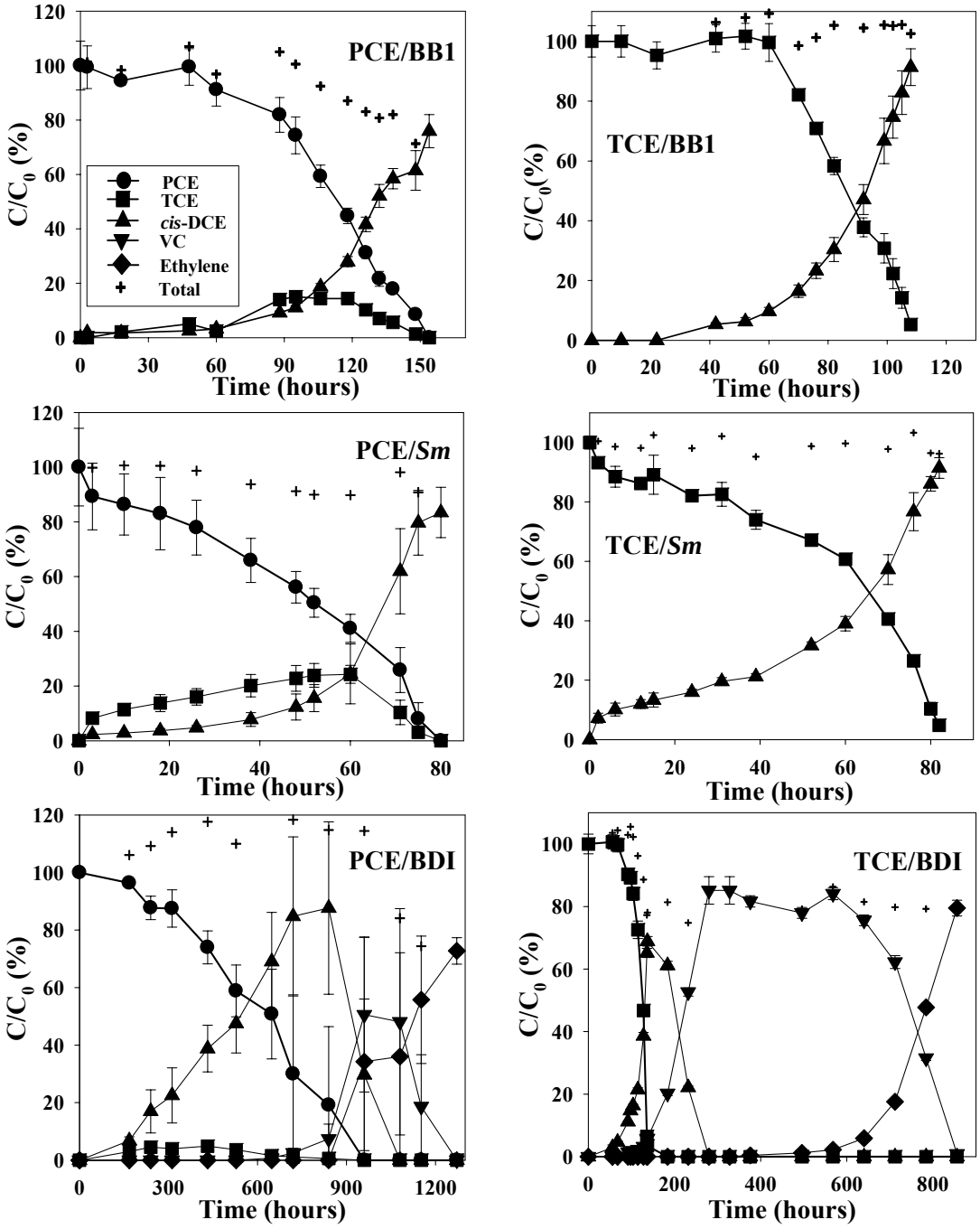

Figure 2.1. PCE and TCE reductive dechlorination by *D. michiganensis* Strain BB1 (BB1), *S. multivorans* (*Sm*) and BDI. The error bars indicate standard deviation of triplicate samples.

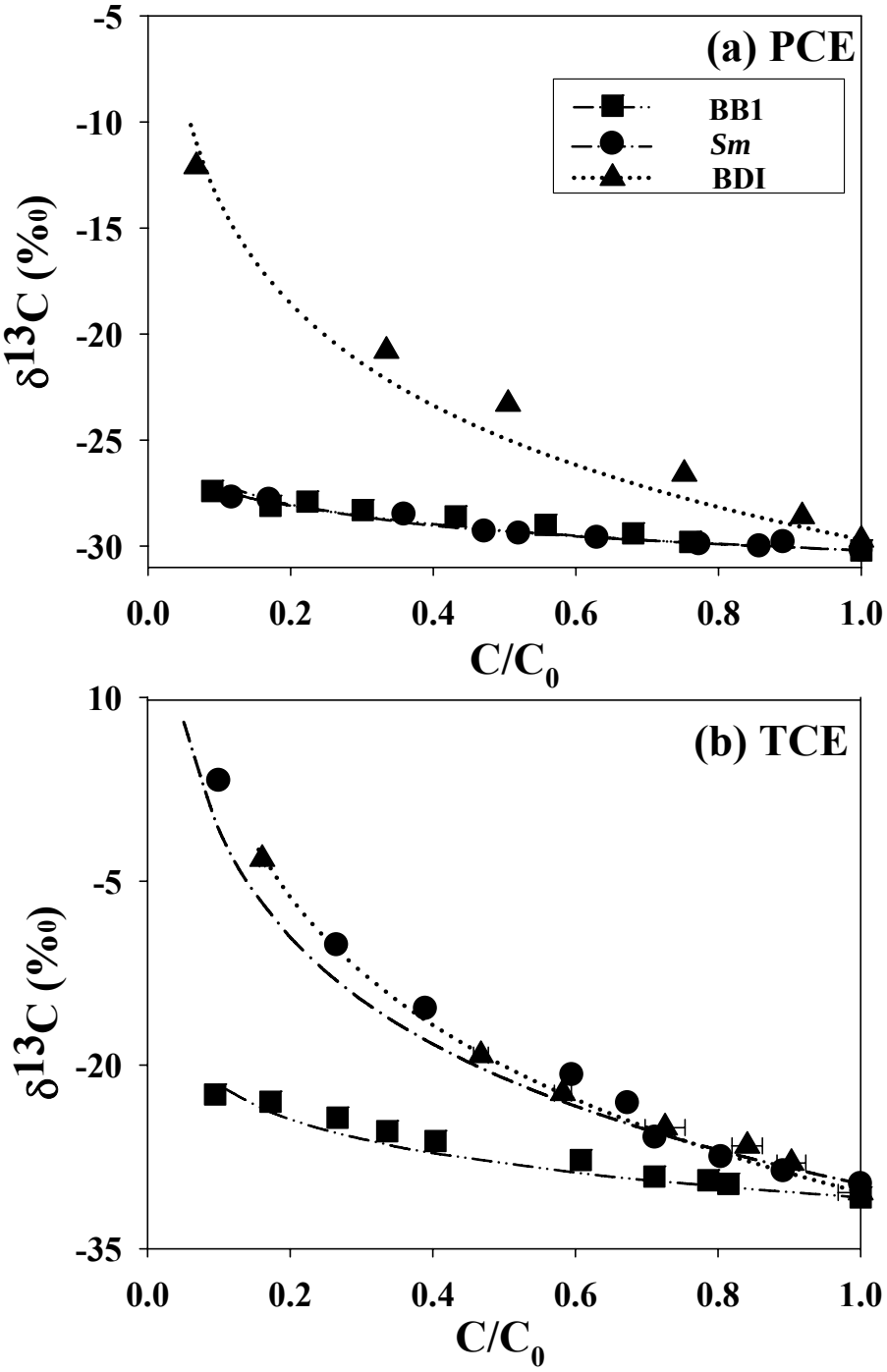

Figure 2.2. Isotope fractionation during PCE (a) and TCE (b) reductive dechlorination by *Sm*, BB1 and BDI. The error bars indicate standard deviation of duplicate samples.

Table 2.1. Stable carbon isotope fractionation enrichment factor (ε_{bulk}), apparent kinetic isotope effect (AKIE) and partitioning factor (P) for reductive dechlorination of PCE and TCE by Sm, BB1 and BDI

Compound	Cultures	ε_{bulk} (‰)[a]	AKIE	P[b]
PCE	BB1	-1.39 ± 0. 21	1.00278 ± 0.00043	8.5±1.8
	Sm	-1.33 ± 0.13	1.00266 ± 0.00027	8.9±1.5
	BDI	-7.12 ± 0.72	1.0145 ± 0.0015	0.84±0.28
TCE	BB1	-4.07 ± 0.48	1.0082 ± 0.0010	2.76±0.28
	Sm	-12.8 ± 1.6	1.0262 ± 0.0034	0.18±0.22
	BDI	-15.27 ± 0.79	1.0315 ± 0.0017	NA[c]

[a] Uncertainties are 95% confidence intervals calculated from non-linear regression; [b] The partitioning factor-P was calculated versus cyanocobalamin as described in Appendix B2 (Slater et al., 2003; Nijenhuis et al., 2005) using equation 2.2 and uncertainties are 95% confidence intervals; [c] Not reported because the extent of isotope fractionation during microbial TCE reductive dechlorination by BDI was slightly stronger than that by cobalamin (Cichocka et al., 2007), resulting in negative value of P here.

The ε_{bulk} for *Sm* in this study was consistent with the previous reported values by the same species (Nijenhuis et al., 2005; Cichocka et al., 2007) and was close to that of *Sulfurospirillum halorespirans* (ε_{bulk} = -0.46 ‰) and *Geobacter lovleyi* Strain SZ (ε_{bulk} close to zero) (Cichocka et al., 2008). However, it was lower than that for *Desulfitobacterium sp.* PCE-S (ε_{bulk} = -4.6 ‰) (Nijenhuis et al., 2005; Cichocka et al., 2007). The stronger isotope fractionation by BDI is comparable to that by *Dehalococcoides ethenogenes* Strain 195 (ε_{bulk} = -6.0 ‰) (Cichocka et al., 2008) and KB-1, a *Dehalococcoides* containing enrichment culture (ε_{bulk} = -5.5 ‰) (Slater et al., 2001). The strongest isotope fractionation for microbial PCE reduction that has been published so far was by a TCE accumulating species, *Desulfitobacterium sp.* Viet1 (ε_{bulk} = -16.7 ‰) (Cichocka et al., 2008).

In comparison, TCE reductive dechlorination led to more variable and stronger isotope fractionation (Figure 2.2 and Table 2.1). Relatively weak isotope

fractionation by BB1 (ε_{bulk} = -4.07 ‰) was comparable to that by *Dehalobacter restrictus* strain PER-K23 (ε_{bulk} = -5.5 ‰) and KB-1 (ε_{bulk} = -2.5~-6.6 ‰) (Bloom et al., 2000; Lee et al., 2007; Cichocka et al., 2008); stronger isotope fractionation detected in *Sm* and BDI was close to that during TCE reductive dechlorination by *Dehalococcoides ethenogenes* Strain 195 (ε_{bulk} = -9.6 to -13.7 ‰), *Dehalococcoides* Strain BAV1 (ε_{bulk} = -16.9±1.4 ‰), *Sulfurospirillum halorespirans* (ε_{bulk} = -18.9 ‰), *Desulfitobacterium* sp. Strain PCE-S (ε_{bulk} = -12.2 ‰) and ANAS, an enrichment culture (ε_{bulk} = -16.0 ‰) (Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009)..

2.3.2. Potential Factors that might Influence Isotope Fractionation During Microbial Reductive Dechlorination

Microbial enzyme-catalyzed reactions can be typically simplified as:

$$S_{out} \underset{(1)}{\rightleftharpoons} S_{in} + E \underset{(2)}{\rightleftharpoons} E{\cdot}S_{in} \underset{(3)}{\rightarrow} E{\cdot}P_{in} \underset{(4)}{\rightleftharpoons} E + P_{in} \underset{(5)}{\rightleftharpoons} P_{out}$$

where the numbers indicate: (1) transport of the substrate from outside to inside the cell; (2) formation of the enzyme-substrate complex; (3) bond cleavage and formation of enzyme-product complex; (4) dissociation of enzyme-product complex; and (5) transport of the product from inside to outside the cell (O'Leary and Yapp, 1978; Zwank, 2004; Nijenhuis et al., 2005). Among all the steps, only Step (3) irreversibly changes the conformation of a chemical bond and thus leads to kinetic isotope fractionation (Morasch et al., 2001). Isotope fractionation during bond cleavage and

formation of enzyme-product complex in Step 3 can be influenced by biochemical pathways/mechanisms (Hirschorn et al., 2004; Hirschorn et al., 2007), structural variability of functional enzymes (Nikolausz et al., 2006) or cofactors (Estep et al., 1978; Ivlev et al., 1996; Igamberdiev et al., 2001). On the other hand, although Step 1 and 2 do not lead to isotope fractionation because no bond cleavage is involved (Morasch et al., 2001), these rate-limiting steps can suppress isotope fractionation due to the limits on substrate supply for enzymatic reactions (O'Leary, 1988; Nijenhuis et al., 2005). The impacts of these non-fractionation steps on isotope fractionation is also expressed as "commitment to catalysis" (Northrop, 1977). If commitment to catalysis is high for the catalytic step (Step 3), the bond changes involving an element are fast in comparison to the reverse steps of all the preceding processes (e.g., Step 1 and 2) and thus lead to weak isotope fractionation.

2.3.2.1 Potential impacts of biochemical pathways and rate-limiting steps on isotope fractionation

The pathway of microbial reductive dechlorination may not be an important factor to determine extents of isotope fractionation during PCE and TCE reductive dechlorination because only one pathway, hydrogenolysis, is followed (Gossett and Zinder, 1997). Instead, some other steps or factors may be more important to influence isotope fractionation during enzyme-catalyzed PCE and TCE transformation.

AKIE was calculated from ε_{bulk} following the detailed protocol as described in

the Appendix B1 (Elsner et al., 2005). The calculated AKIE values (Table 2.1) were then compared with the value of Kinetic Isotope Effects (KIE) (Huskey, 1991) to determine whether the rate limiting processes in the overall transformation reaction involved bond cleavage. The theoretical KIE for C-Cl bond is 1.03 assuming 50 % bond cleavage at transition state (Elsner et al., 2005). If bond cleavage is rate limiting in the enzymatic reactions, the AKIE and KIE values should be close (Elsner et al., 2005). In our study, the values of AKIE for microbial PCE reductive dechlorination by all the three cultures were generally less than 1.03 for C-Cl bond cleavage (Table 2.1). This suggests that bond cleavage and formation of enzyme-product complex (Step 3) is not the rate-limiting step during enzymatic PCE reductive dechlorination. On the contrary, for *Sm* and BDI, their AKIE values are higher than the theoretical KIE for TCE reductive dechlorination (Table 2.1). It suggests that the rate of TCE dechlorination by these species is more strongly influenced by the rate of C-Cl bond cleavage (Step 3).

The suppression of isotope fractionation by rate-limiting steps prior to enzymatic reactions is quantified as partitioning factor (P) (Table 2.1) following the protocol in the Appendix B2. The non-fractionating rate-limiting steps include transport of substrates through membrane or binding of the chlorinated substrate to the catalytic center of enzyme (Nijenhuis and Zinder, 2005; Cichocka et al., 2007). As shown in Table 2.1, the calculated values of P was relatively higher for the three cultures during PCE reductive dechlorination (P = 0.84 to 8.9). In comparison, negligible partitioning ($P \leq 2.76$ for all the three cultures) was observed in TCE

reductive dechlorination, consistent to a previously published study by Cichocka et al. (2008). This suggests the rate-limiting steps may "suppress" extents of isotope fractionation by limiting the supply of PCE as the parent substrate. However, the study of TCE reductive dechlorination with whole cells, crude extracts and purified Rdhs showed no statistical difference in isotope fractionation among the dechlorinating bacteria with different integrity (Cichocka et al., 2008). Our study and previously published studies provide extra evidence to support that TCE reductive dechlorination may be more influenced by the rate of C-Cl bond cleavage (Step 3) rather than the steps not related to bond cleavage (Nijenhuis et al., 2005; Cichocka et al., 2007).

2.3.2.2 Effects of structural variability of functional enzymes on isotope fractionation

The different levels of isotope fractionation observed during PCE and TCE transformation among *Sm*, BB1 and BDI may be influenced by structure of Rdhs involved in reductive dechlorination by the three cultures. Changes of amino acids in protein sequences may drastically influence enzymatic properties if the active centre is modified (Nikolausz et al., 2006), and thus impact isotope fractionation. This may be due to the changes in the properties of active center and binding site (e.g., activation energy required to cleave a bond or reaction kinetics in the transition state before the biochemical reaction becomes irreversible) (Srinivasan et al., 1993; Lightstone et al., 1997; Brazeau and Lipscomb, 2001). Unfortunately, no crystal

structure of Rdhs has been reported, so it is difficult to compare their structure and physiological property. In addition, among the three cultures in this study, the information of reductive dehalogenase was only available for *Sm* (Neumann et al., 1998; Neumann et al., 2002). However, comparison and analysis of the previously studied Rdhs in different dechlorinating bacteria provide clues to understand the diversity of this group of functional enzymes in different dechlorinators.

Alignments of protein sequences of reductive dehalogenase (e.g., PceA and TceA) of different dechlorinating bacteria (e.g., *Sulfurospirillum spp.*, *Geobacter lovleyi*, *Desulfitobacterium spp.*, *Dehalococcoides spp.*, *Dehalobacter restrictus* and *Clostridium bifermentans*) are shown in Figure B2 (Miller et al., 1998; Neumann et al., 1998; Magnuson et al., 2000; Okeke et al., 2001; Suyama et al., 2002a; Seshadri et al., 2005b; Tsukagoshi et al., 2006; Krajmalnik-Brown et al., 2007). It indicates that the Rdhs in different dechlorinating bacteria are only relatively conserved or identical in the order and composition of amino acids in two electron transfer mediator (e.g., iron-sulfide clusters) binding motifs (shown as grey boxes in Figure B2) (Holliger et al., 2003), while the remaining regions appear to be highly variable. In addition, Rdhs for the species belonging to the same genus share higher homogeneity than those from different genera**,** such as the similar sequences among *Desulfitobacterium spp.* but significant difference between *Desulfitobacterium* and *Sulfurospirillum spp.* (Figure B2) (Miller et al., 1998; Neumann et al., 1998; Suyama et al., 2002b). This observation coincides with the similar trend in isotope fractionation by different dechlorinating bacteria. For example, Rdhs of *Sm* shares 91 % identity with the

catalytic enzyme of a close relative *Sulfurospirillum halorespirans* (Neumann et al., 1998). The enrichment factors for *Sm* are -0.42 to -1.33 ‰ (Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007) for PCE and -12.8 to -18.7 ‰ for TCE (Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007). These values of ε_{bulk} by *Sm* are close to those by *Sulfurospirillum halorespirans* (-0.46 ‰ for PCE and -18.6 ‰ for TCE) (Cichocka et al., 2008). In contrast, Rdhs of *Sm* and *Desulfitobacterium* sp. Strain PCE-S are only 27 % in identity and the ε_{bulk} for the latter species range from -5.18 to -5.4 ‰ for PCE (Nijenhuis et al., 2005; Cichocka et al., 2007) and -12.2 ‰ for TCE (Nijenhuis et al., 2005; Cichocka et al., 2007). Based on this observation and the fact that dechlorinators studied in the three cultures in our study are not phylogenetically closely related, we infer that their reductive dehalogenases may differ in structure and/or rate limiting steps, thus result in different levels of isotope fractionation.

2.3.2.3 Other factors that might influence isotope fractionation during microbial PCE and TCE reductive dechlorination

Structure of cofactor may also limit rates of the dechlorination reaction and thus affects the fractionation (Nijenhuis et al., 2005). Impacts of cofactors on isotope fractionation may be achieved by modifying the topology of active sites or stable ternary substrate-enzyme complex (Estep et al., 1978; Ivlev et al., 1996; Igamberdiev et al., 2001). These changes could conceivably alter reaction rates and perhaps even reaction mechanisms (e.g., changes in the contribution of rate limiting steps to the

overall reaction rates), which result in a new kinetic isotope effect (Estep et al., 1978; Ivlev et al., 1996; Igamberdiev et al., 2001). For *Sm*, its cobalamin cofactor of the PCE dehalogenase is a novel and unusual type of corrinoid, which has been identified to be norpseudo-B12 (Kraeutler et al., 2003). Thus, its special cofactor may contribute to the distinct isotope fractionation by this species compared to the two other cultures in this research.

Also, it worth mentioning that PCE and TCE reductive dechlorination is carried out by a single Rdhs in *Sm* (Neumann et al., 2002; Cichocka et al., 2007). However, *Dehalococcoides sp* Strain 195 uses a two-component enzyme pathway for PCE and TCE transformation, with its PCE-reductive dehalogenase (PceA) transforming PCE to TCE and the TCE-reductive dehalogenase (TceA) catalyzing TCE to ethylene (Magnuson et al., 1998). Although it is not known whether other *Dehalococcoides spp.* follow the same mechanism, multiple reductive dehalogenase genes detected in genomes of *Dehalococcoides spp.* (Kube et al., 2005; Seshadri et al., 2005a; Nonaka et al., 2006) suggest that different reductive dehalogenases might be involved in PCE and TCE reductive dechlorination by BDI. This might explain the significantly different enrichment factor by BDI compared to *Sm* and BB1 during PCE reductive dechlorination, since Rdhs with different structure and substrate specialties may be involved in the enzymatic reactions.

2.4. Implication for the Assessment of *In Situ* Natural Attenuation of Chlorinated Solvents Using Isotope Fractionation

In this study, we observed variable extents of isotope fractionation during microbial reductive dechlorination of PCE and TCE that were dependent on the specific microorganisms involved. The results indicate that although the same pathway (hydrogenolysis) was followed for the enzymatic reactions, their stable isotope fractionation may differ significantly, which might be due to the structure of enzymes, rate limiting steps before enzymatic reactions or cofactors. A better understanding of the enzymatic reactions (e.g., structure of Rdhs, cofactors or impacts of the pre-enzymatic reaction steps) is necessary. Thus, when using isotope techniques to quantitatively monitor transformation of contaminants in the fields, precautions must be taken for the different extents of isotope fractionation by individual dechlorinating species. For a practical point of view, selection of more negative enrichment factors will result in conservative estimation (e.g. an underestimation) about the fraction of contaminants transformed in the field.

CHAPTER 3

Impacts of Microbial Community Composition and Environmental Factors on Isotope Fractionation during Microbial Reductive Dechlorination of Chlorinated Ethenes

3.1. Introduction

Improper disposal and storage have led to widespread contamination by chlorinated contaminants (e.g. tetrachloroethylene (PCE) and trichloroethylene (TCE)) in the U. S. (Moran et al., 2007; ATSDR, 2008). Due to their toxicity and suspected carcinogenic properties, monitoring remediation of this group of contaminants has gained wide public and academic interest. A variety of anaerobic bacteria are capable of reductively dechlorinating PCE or TCE to less chlorinated compounds, such as TCE (for PCE), dichloroethylene isomers (DCEs), vinyl chloride (VC) and/or ethylene via dehalorespiration or cometabolism (Bradley, 2003; Bhatt et al., 2007). Hydrogen has been proposed to be the direct electron donor for most dechlorinating bacteria, while some species can also ferment a variety of organic substrates or coexist with fermenting bacteria, which produce low concentrations of hydrogen or acetate from organic substrates (Bradley, 2003; Becker et al., 2005; He et al., 2007).

Microbial community structure has been studied and dechlorinators have been identified within reactive PCE and TCE dechlorinating consortia (Rosner et al., 1997; Flynn et al., 2000; Loeffler et al., 2000; Fennell et al., 2001; Richardson et al., 2002; Gu et al., 2004; Freeborn et al., 2005; Bedard et al., 2006; Rahm et al., 2006). Researchers have shown that microbial communities are dynamic systems, whose microbial composition and metabolic pathways are not only dependent on availability

of indigenous species (Fennell et al., 2001; Rahm et al., 2006) but also change over time (Avrahami et al., 2003) and in response to the presence of different electron acceptors (Flynn et al., 2000; Gu et al., 2004), electron donors (Flynn et al., 2000) and pH (Dodin et al., 2000; Inagaki et al., 2006). Thus, studies on microbial community composition in the presence of different environmental factors help to understand the ecological and complex interactions in reactive dechlorinating microbial consortia.

In addition to the conventional methods focusing on identification and quantification of parent substrates and dehalogenated products, compound specific isotope analysis (CSIA) has been increasingly applied to monitor transformation of chlorinated contaminants (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2000b; Slater et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009). Fractionation of isotopes during degradation of contaminants occurs as reactions involving the stronger bonds formed by heavier isotopes (e.g. ^{13}C) are slower than those involving lighter isotopes (e.g. ^{12}C) and thus lead to accumulation of heavier isotopes in the residual parent substrate (Hoefts, 1997). Different levels of isotope fractionation have been reported during microbial reductive dechlorination of PCE with the enrichment factors ranging from -0.42 to -16.8 ‰ (Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007). The reasons for variability in enrichment factors by different dechlorinating bacteria has not been fully explained but have been suggested to be due to diverse mechanisms (e.g. aerobic vs. anaerobic reactions) (Hirschorn et al., 2004; Hirschorn et al., 2007), structural variability of functional enzymes (Nikolausz et al., 2006) or nutrient conditions (e.g. concentrations of cobalamin) (Mancini et al., 2006). In addition, in our previous study (Dong et al., 2009), two sets of microcosms prepared with the sediment from the same site but at

different pH values (7.2 versus 8.2) showed significantly different isotope fractionation during microbial PCE reductive dechlorination (-10.68 ‰ for L-meth-pH 7.2 versus -16.78 ‰ for L-meth-pH 8.2). It has been found that pH may change the kinetic parameters of enzymatic reactions by titrating functional groups responsible for reactant binding, catalysis or altering the conformation of the enzymes (Karsten and Cook, 2006), suggesting potential impacts by environmental factors (e.g. pH) on isotope fractionation in our microcosms. Recently, a number of research groups have proposed to use the Rayleigh Model along with isotope ratio analysis of *in situ* contaminants and enrichment factors obtained from lab-scale studies to quantitatively monitor the fraction of transformed contaminants at a site (Elsner et al., 2005; Van Breukelen et al., 2005; VanStone et al., 2005; Nijenhuis et al., 2007). Thus, in order to avoid significant uncertainties during *in situ* assessment, it is important to choose representative enrichment factors for individual field sites and to understand the factors that may potentially influence this parameter.

Most studies to date have not focused in detail on potential influence on isotope fractionation by heterogeneous bacterial distribution and shift in microbial communities due to the environmental factors (e.g. electron donors and pH) or over a long-term remediation scenario (Sherwood Lollar et al., 1999; Bloom et al., 2000; Zwank, 2004; Elsner et al., 2005; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008; Dong et al., 2009). The objective of this study is to evaluate whether microbial communities and different environmental factors (e.g. electron donors and pH) influence observed isotope fractionation during microbial reductive dechlorination. Two enrichment cultures showing different product distribution and enrichment factors during PCE reductive dechlorination were studied. Product distribution, isotope fractionation and

microbial community structures were analyzed in these two cultures inoculated from the same source but grown with different electron donors.

3.2. Materials and Methods

3.2.1. Sources of Chemical Reagents

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): PCE (99%), TCE (99.5%), *cis*-1,2-dichlorethylene (*cis*-DCE), *trans*-1,2-dichlorethylene (*trans*-DCE), 1,1-dichlorethylene (1,1-DCE) and 2,2,4,4,6,8,8-heptamethylnonane. Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). All aqueous solutions were prepared with nanopure water (18.0 MΩ cm resistivity, Barnstead Ultrapure Water System, IA).

3.2.2. Microbial Consortia and Subculture Enrichment Protocol

All the enrichment cultures were derived from anaerobic sediment taken from a pond in Brandt Park, Norman, OK (Duck Pond or DP) in December 2004 by using the sampling and storage methods as described previously (Dong et al., 2009). We are not aware of any contamination by chlorinated compounds in this sampling area.

Within three days after sampling, microcosms were prepared inside an anaerobic glove box (Coy Laboratory Products Inc., MI) filled with a mixture of 95-97 % N_2 and up to 5 % H_2. Five milliliters of sterile, anaerobic and reduced mineral medium containing 3.5 g/L $NaHCO_3$ (Dworkin et al., 2006) was seeded with approximately 2 g of Duck Pond sediment. One milliliter of anaerobic sludge from the Norman Wastewater Treatment Plant, OK, was passed through a 0.22 μm filter (Whatman Inc., UK) and spiked as an extra source of C, N, P, Fe and trace elements (Dionisi et al., 2005). Formate and H_2 were used as the electron donor for Duck Pond-Formate

(DPF) and Duck Pond-H_2 (DPH), respectively. Formate was spiked from a sterile and anaerobic stock solution to yield the final concentration of 20 mM, while H_2 was added by flushing the headspace of butyl rubber stopper sealed serum bottles with sterile cotton filtered H_2/CO_2 (80/20, v/v) pressurized to 1.3 atm for 3-5 minutes. The medium was reduced with $Na_2S{\cdot}9H_2O$ with the final concentration approximately 0.5 mM. In order to inhibit methanogenic microorganisms from competing for electron donors with dechlorinating bacteria, 2-bromo-ethanesulfonate (BESA) was added. Its concentration was 1 mM to minimize the inhibition on microbial reductive dechlorination (Loeffler et al., 1997). All the samples received 0.5 mL 50 mM PCE stock solution prepared by dissolving PCE in 0.22 μm filter sterilized 2,2,4,4,6,6,8,8-heptamethylnonane. After that, subcultures were repeatedly transferred into freshly prepared basal salt medium (1:10, v/v) (Dworkin et al., 2006) containing the same amendments at intervals of 45 to 60 days. After about 10 transfers, the cultures were transferred into 120 mL (nominal volume) serum bottles with 100 mL medium, in which PCE was added from an aqueous stock solution to a final concentration about 100 μM.

Sulfurospirillum multivorans was kindly provided by Dr. Frank R. Löffler at the Georgia Institute of Technology and the medium was prepared as previously described (Sung et al., 2003). The medium was buffered with 2.52 g/L $NaHCO_3$ equilibrated with CO_2/N_2 (20/80, v/v) and the pH was adjusted with 1 N HCl or 1 N NaOH to 7.2 or 8.2 before sterilization. Following TCE reductive dechlorination, pH was measured again and the changes were within ± 0.3 pH units compared to the initial values.

All microcosms and cultures were sealed with Teflon-lined butyl rubber septa (West Pharmaceutical Services, PA) and aluminum seals. Incubations were static at room temperature and in the dark unless otherwise indicated.

Sterile controls were prepared to assess whether microbial reductive dechlorination was responsible for the PCE transformation in DPF and DPH. For the cultures to be used as sterile controls, they were pasteurized at 85 ± 5 °C for 15 min after inoculation but before addition of PCE stock solution (Ballerstedt et al., 2004). Meanwhile, in order to test whether electron donors were directly responsible for the different product distribution and isotope fractionation in DPH and DPF, subcultures from both were prepared with switched electron donors. Basically, 30 mL cultures were transferred into N_2/CO_2 flushed Falcon™ conical tubes and then centrifuged at 12,000 ×g for 5 minutes. The pellets were washed with the same anaerobic medium twice before they were resuspended in 6 mL medium. The DPH suspension was inoculated into the medium with formate as the electron donor and the DPF suspension was inoculated into the medium flushed with H_2/N_2 (80/20, v/v)).

In order to evaluate whether different dechlorinators were involved DPH and DPF, dechlorination rates for PCE degradation were compared between the cultures with and without a "PCE-starving" pretreatment. DPH and DPF were prepared as six replicate samples and the replicates were divided into two groups. Group 1 was inoculated and spiked with PCE stock solution at day 0, while Group 2 was inoculated at day 0 but spiked with PCE at day 15. All the samples spiked with PCE at day 15 had additional electron donors added prior to PCE addition to eliminate the possibility of electron donors being limiting factors. Here, the rate of degradation was quantified as the mass of dechlorinated products formed within the same period of

time. After PCE was added, the dechlorinated products were identified and quantified after the same period of time (day 15 for Group 1 and day 30 for Group 2).

3.2.3. Analytical Techniques

Concentrations of PCE, TCE and *cis*-DCE were determined by manual injection headspace analysis with a Shimadzu GC-17A/flame ionization detector (GC/FID) and an Agilent GS-GASPRO capillary column (30 m × 0.32 mm) (J&W Scientific, Folsom, CA, USA). The injector temperature and detector temperature were 220 °C and 270 °C, respectively. Fifty microliters of headspace were withdrawn with a gas tight syringe (Hamilton Co., Reno, NV) and manually injected into the GC/FID using a split ratio of 1:1 (Liang et al., 2007). The oven temperature ramped from 80 ^{o}C to 190 ^{o}C at 30 ^{o}C/min, and then isothermal at 190 ^{o}C for 7 min. Five point external calibration curves were prepared and the relative standard deviations for samples and standards using this method were typically less than 5 %.

Additionally, 1 mL of culture was withdrawn and prepared for isotope analysis at each time point by using the method described (Dong et al., 2009). Carbon isotope ratios were measured against a CO_2 standard with aqueous samples using an O.I. Analytical - Model 4560 purge and trap system interfaced with a Varian 3410 GC with Finnigan MAT 252 mass spectrometer (Kuder et al., 2005). Approximately 15 % of samples were run in duplicate and the typical standard deviation for $\delta^{13}C$ values from duplicate measurements was 0.2-0.3 ‰ or better (Kuder et al., 2005). The results were then combined to calculate enrichment factors (ε_{bulk}) by using Rayleigh Model (Clark and Fritz, 1997).

3.2.4. DNA Extraction and 16S rRNA Gene Analysis

After about 20 transfers, cultures from duplicate samples were collected for DNA extraction when about 70-80 % of the PCE had been transformed. The cultures were centrifuged at about 13,000 ×g for 5 minutes. The cell pellets were resuspended in sterile phosphate buffered saline (PBS) buffer solutions (Hurst et al., 2002) and the washing procedure was repeated. Cells were then stored at -20 °C until DNA extraction was performed. Total community DNA was extracted using the Easy-DNA^{TM} kit (Invitrogen, Co., CA) according to the manufacturer's instruction. Purity and concentration of extracted genomic DNA were measured with a Thermo Scientific NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific Inc., U. S.).

Bacterial 16S rRNA genes were amplified from community DNA with primers 27F (5'-AGAGTTTGACMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3') (Lane, 1991). PCR amplification was performed with the *Taq* DNA polymerase kit (Fermentas Inc., MD). The reaction mixture (50 μl) contains 2.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.4 mM each oligonucleotide primer, 1.25 U *Taq* polymerase and 1 μl appropriately diluted template DNA (ca. 50 ng). Reaction mixtures were incubated in an iCycler thermal cycler (Bio-Rad Laboratories, France) and the PCR amplification was performed under the following conditions: 95°C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 52°C for 30 s and extension at 72 °C for 90 s, with a final extension at 72 °C for 20 min. PCR products were separated and visualized by agarose gel (0.8 %) electrophoresis. The band corresponding to the product (~ 1.4 kb) was excised and purified with the GenCatch^{TM} gel extraction kit (Epoch Biolabs, Inc., TX). Prior to cloning, the purified PCR product was amplified for one cycle as described above with a final

extension at 72°C for 20 min. Afterwards, the PCR products were cloned with a TOPO TA cloning kit (Invitrogen Co., CA) in accordance with the manufacturer's instructions. Selected clones were grown in 1 mL LB medium amended with 50 μg/mL of ampicillin (Hurst et al., 2002).

The 16S rRNA inserts from recombinant clones were reamplified with M13 primers and then digested independently with restriction endonucleases *Msp* I and *Alu* I (Fermentas Inc., MD) as suggested by the manufacturer. The digested fragments were separated by agarose gel electrophoresis (0.8 %) and visualized by staining with ethidium bromide and UV illumination. RFLP patterns for each library were grouped visually. Representative clones were selected based on unique RFLP patterns, purified with the GenCatch^{TM} PCR Clean-up Kit (Epoch Biolabs, Inc.) and sent to Oklahoma Medical Research Foundation (OMRF) for sequencing using ABI 3730 capillary sequencers. In cases where there were many replicates with similar RFLP patterns, multiple clones were sequenced to confirm that they represent identical species.

All the sequences were initially aligned with Clustal W, visually examined and adjusted to allow maximal alignment by referring to representative bacterial sequences from the Ribosomal Database Project II (RDP) database (Cole et al., 2005). Clones were grouped into Operational Taxonomic Units (OTUs) at a level of sequence similarity ≥ 97 %. The possible chimera were checked with Bellerophon software (Huber et al., 2004) and Pintail (Ashelford et al., 2005) and chimera were excluded from the phylogenetic analysis. Phylogenetic affiliations of the sequences of approximately 1.4 kb were estimated by BLAST and the classification function in Greengenes (DeSantis et al., 2006). Neighbor joining trees were constructed with ARB (Ludwig et al., 2004).

3.2.5. GenBank accession numbers

Sequences determined in this study have been deposited into the GenBank database and the accession numbers are under GQ377111 to GQ377131.

3.3. Results and Discussion

3.3.1. PCE Dechlorination and Isotope Fractionation in DPF and DPH

During the first 15 feeding cycles, both DPF and DPH produced *cis*-DCE from PCE (Figure C1). The ability of DPH to transform PCE to *cis*-DCE has not changed (Figure 3.1a). However, after about 2.5 years, DPF lost its capacity to transform TCE to *cis*-DCE and thus accumulated TCE during PCE degradation (Figure 3.1 (b)). The stable PCE concentration in pasteurized controls indicated that dechlorination was due to microbial activity in both enrichment cultures rather than abiotic reductive dechlorination by extracellular agent (e.g., porphyrin, a metalloprotein or proteins) (Novak et al., 1998).

In DPF, isotope fractionation was measured after loss in dechlorination capacity from TCE to *cis*-DCE was observed. As shown in Figure 3.2, weak isotope fractionation occurred in DPH (ε_{bulk} = -1.98±0.16 ‰), close to the previously published enrichment factors by *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* Strain BB1, *Sulfurospirillum halorespirans*, *Geobacter lovleyi* Strain SZ and some enrichment cultures (e.g., KB-1 and TP) with the enrichment factors ranging from -0.42 to -2.6 (Slater et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007; Cichocka et al., 2008). However, much stronger isotope fractionation with the enrichment factor -10.29±0.47 ‰ was observed in DPF. Similar to DPF, relatively stronger isotope fractionation during PCE microbial reductive dechlorination has been reported for some TCE-accumulating cultures and

microcosms, including *Desulfitobacterium sp* Viet 1 (ε_{bulk} = -16.7 ‰) and some microcosms in our previous experiments (e.g. L-Meth-pH 7.2, L-Meth-pH 8.2 and AAFB-SR-12-pH 7.2) (ε_{bulk} = -8.5 to -16.78) (Cichocka et al., 2008; Dong et al., 2009).

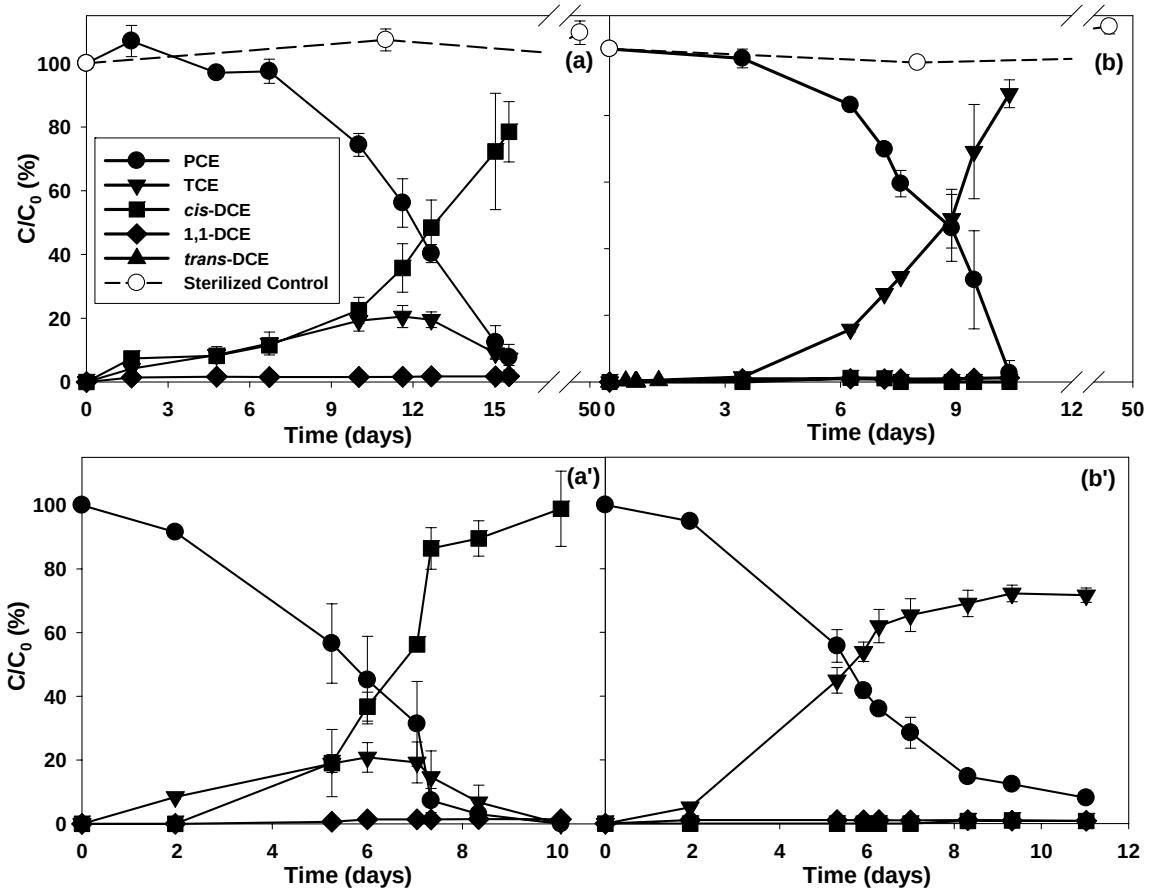

Figure 3.1. PCE reductive dechlorination by DPH and DPF. (a) and (b) show reductive dechlorination by DPH and DPF with H_2 and formate as the electron donor, respectively. (a') and (b') show PCE reductive dechlorination in (DPH+formate) and (DPF+H_2). The open circles and dashed lines in (a) and (b) show PCE concentration for up to 48 days in the controls treated by pasteurization before PCE was added. The points show average values and error bars indicate standard deviation of triplicate samples.

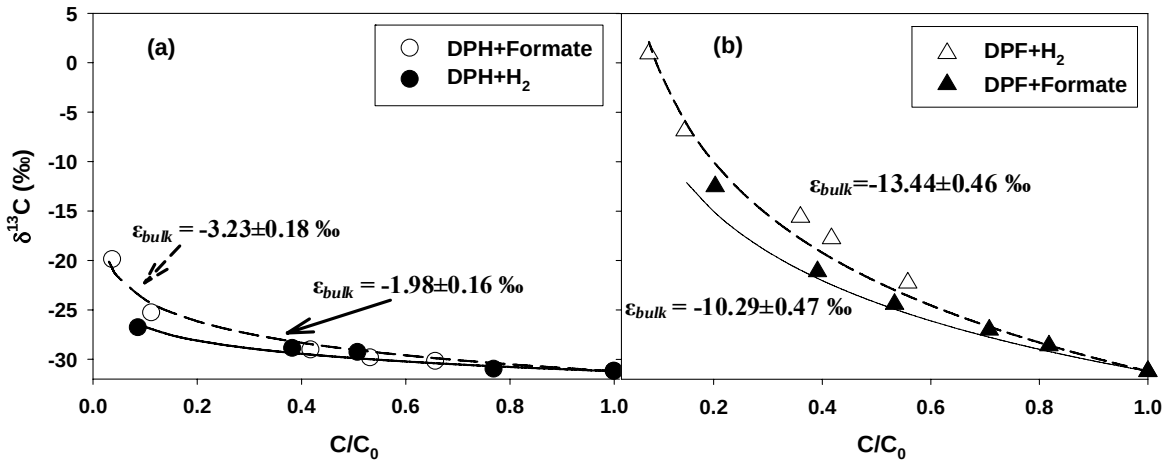

Figure 3.2. Isotope Fractionation of during microbial PCE reductive dechlorination DPH (a) and DPF (b) in the presence of different electron donors. The solid and dashed curves are Rayleigh Model fit for the samples with the original and switched electron donors, respectively.

3.3.2. Impacts of Electron Donors on Isotope Fractionation during Reductive Dechlorination

Electron donor was the major factor that differed between DPH and DPF, so it was important to evaluate whether it was responsible for the significantly different isotope fractionation between the two cultures. Previous studies have indicated impacts of electron donors and their concentrations on isotope fractionation during microbial metabolism (e.g. dissimilatory sulfate reduction) (Bruechert, 2004; Hoek et al., 2006; Hoek and Canfield, 2007). Impacts of electron donors on isotope fractionation might be due to the induction of different electron donor flow pathways (e.g. complete or incomplete oxidation) or rates of electrons transfer along the electron transport chains (Bruechert, 2004). In order to evaluate whether electron donors directly influenced isotope fractionation during microbial dechlorination of PCE, subcultures were prepared from DPF and DPH with switched electron donors. Reductive dechlorination in subcultures of DPF (normally using formate) flushed with H_2 (DPF+H_2) initiated without any lag time and the rate for PCE transformation was

slightly faster than that for DPF (Figure 3.1a'). For the subculture from DPH (normally using H_2) spiked with formate (DPH+formate), the rate of dechlorination was similar to that of DPH. Meanwhile, the product distribution in the subcultures (TCE for DPF+H_2 and *cis*-DCE for DPH+formate) was the same as that with the original electron donors. This suggests that the final dechlorinated products in DPF and DPH may be independent of electron donors. Isotope fractionation obtained from DPF+H_2 and DPH+formate is shown in Figure 3.2. Compared with DPF and DPH, slightly stronger isotope fractionation was observed in the subcultures with switched electron donors. However, the general trend with much stronger isotope fractionation observed in DPF+H_2 than that in DPH+formate was the same as observed with the original electron donors. This observation suggests that electron donors may not be the direct reason for the significantly different extents of isotope fractionation observed between DPH and DPF.

3.3.3. Impacts of pH on Isotope Fractionation During Microbial Reductive Dechlorination

To specifically evaluate the effects of pH on isotope fractionation, TCE reductive dechlorination at two pH values (7.2 and 8.2) was tested. To avoid the potential complexity due to the shift in microbial community composition, a well studied isolated pure culture, *S. multivorans* was studied (Neumann et al., 1994; Neumann et al., 1998; Neumann et al., 2002). These pH values were selected because they represent the neutral and upper limit of typical pH values for groundwater (Kasenow, 2000). In addition, the relatively strong fractionation for TCE degradation (Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007) provides relatively wide space for the potential changes in isotope fractionation. Results showed that changes

in pH from 7.2 to 8.2 significantly slowed either growth or rates of TCE reductive dechlorination (Figure C2a), which is consistent with the reported optimum pH around 7.2 for *Sulfurospirillum multivorans* (Neumann et al., 1994). However, no statistically significant change (at 95 % confidence level) in isotope fractionation was observed. Compared to $\delta^{13}C$ = -12.8±1.6 ‰ at pH 7.2 (Liang et al., 2007), the enrichment factor was -15.1±0.9 ‰ when the pH was raised to 8.2 (Figure C2b).

The lack of difference in isotope fractionation for TCE reductive dechlorination by *Sulfurospirillum multivorans* at different pH might be explained by the stable intracellular pH regardless of the changes in environmental pH. When encountering temporal or drastic fluctuations in the environmental pH, microorganisms maintain intracellular pH to be relatively stable by homeostatic regulation (Padan and Schuldiner, 1986, 1987). Bacteria living in acidic environments typically use primary proton pumps, membrane channels with a reduced pore size that excludes protons and cytoplasmic buffering to maintain a neutral pH within the cells (Padan and Schuldiner, 1987; Baker-Austin and Dopson, 2007). Thus, in the presence of constant intracellular pH, the steps in enzymatic reactions that are related to isotope fractionation (e.g. bond cleavage and formation of enzyme-product complex) will not likely be affected and therefore isotope fractionation will not change as external pH is modified. These results indicate that the significantly different extents of isotope fractionation observed between L-Meth-pH 7.2 and L-Meth-pH 8.2 are more likely due to other factors (e.g. microbial community composition).

3.3.4. Microbial Community Analysis

After the direct influence of electron donors and pH on isotope fractionation

during PCE reductive dechlorination was ruled out, the microbial community was analyzed to reveal the difference between DPF and DPH cultures. DNA was extracted from DPF and DPH to construct clone libraries of nearly full-length bacterial 16S rRNA genes. Archaeal clone libraries were not constructed because all known dechlorinators belong to the domain Bacteria. In addition, treatment with BESA did not change the dechlorinating capacity in this study (Figure C3) with the relative difference at each time point typically less than 15 %, suggesting that bacteria were more important than methanogenic archaea (Loeffler et al., 1997). RFLP analysis was performed on a total of 256 clones and subsequent sequencing of 70 16S rRNA genes of representative RFLP groups revealed 11 OTUs in DPF and 10 OTUs in DPH. Phylogenetic classification of OTUs showed that both enrichments include *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. *Spirochaetes* and some unidentified species were only detected in DPF (Figure 3.3 and Table 3.1). Among the *Proteobacteria*, ε-*Proteobacteria* accounted for a major part of the DPH clone library but was not detected in DPF, while δ-*Proteobacteria* were detected in both DPH and DPF. More importantly, except for *Bacteroidetes*, the fractions of all the phyla differed significantly between DPH and DPF, suggesting a significant difference in the community structure.

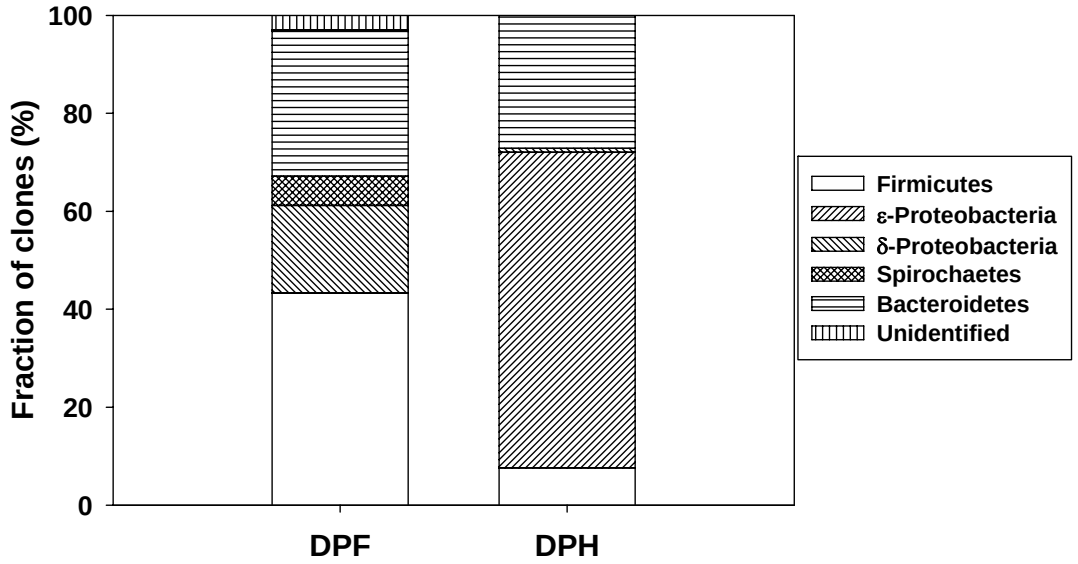

Figure 3.3. Phylum level distribution of clones from DPF and DPH. The *Proteobacteria* is divided into classes of δ-proteobacteria and ε-proteobacteria to indicate different microbial distribution between the two enrichment cultures.

Table 3.1 shows the phylogenetic affiliation of each OTU and their closest sequence matches in the GenBank database as determined by BLAST (Altschul et al., 1990). Figure 3.4 shows the phylogenetic relationship of 16S rRNA gene clone sequences of DPF, DPH and their close relatives and type strains. The percentages listed in Table 3.1 may not represent the actual microbial abundance in the enrichment cultures because different factors (e.g. potential PCR bias, cloning bias and varied copy numbers of 16S rRNA genes in different bacteria) may result in the deviation from the actual population distribution (Becker et al., 2000; Klappenbach et al., 2001; Acinas et al., 2004; Kurata et al., 2004). However, the absence of certain OTUs in one culture or the other indicates that electron donors affect microbial community composition in DPF and DPH (Table 3.1 and Figure 3.4). The only identical OTUs shared by DPF and DPH were members of *Bacteroidetes* (Table 3.1 and Figure 3.4). Although *Bacteroidetes* have been widely observed in reactive microbial communities

capable of reductively dechlorinating chlorinated ethylenes or PCBs (Richardson et al., 2002; Freeborn et al., 2005; Bedard et al., 2006), no members belonging to this phylum have been identified to be dechlorinators. *Bacteroidetes spp.* are known for fermentation of a variety of carbohydrates, and simple sugars to produce smaller fatty acids and bicarbonate (Janssen, 1991; Madigan et al., 2006; Smith et al., 2006). Thus, it is possible that *Bacteroidetes* identified act as the fermentors by degrading organic substrates (e.g. yeast extracts) and provide smaller organic molecules or H_2 for growth of dechlorinating bacteria. Similar functions may also be carried out by *Spirochaetes* detected in DPF, which may ferment carbohydrates to acetate or ethanol and potentially support growth of dechlorinators (Canale-Perola, 1984).

Table 3.1. Phylogenetic Summary of DPH and DPF Based on Clone Library Construction and Sequence Analysis[a]

OTU_{DPF}[b]	Fraction of $Clones_{DPF}$ (%)	OTU_{DPH}[b]	Fraction of $Clones_{DPH}$ (%)	Phylogenetic group	Closest GenBank match[c]	Identity[d] (%)
DPF03	25.4	DPHC01	10.8	Bacteroidetes	*Porphyromonadaceae* bacterium JN18, DQ168658.1	99
DPF25	4.5	DPHB03	16.0	Bacteroidetes	Clone E48, EU864431.1	98
		DPHB02	0.8	Bacteroidetes	Clone A25B8, EF644518.1	99
		DPHB06	0.8	Bacteroidetes	*Paludibacter propionicigenes* AB078842.2	95
DPF01	1.5			Clostridia	Clone PL-38B5	98
DPF04	23.9			Clostridia	*Clostridium sticklandii*, L04167.1	97
DPF06	14.9			Clostridia	*Anaerofilum pentosovorans*, X97852	98
DPF35	1.5			Clostridia	Clone 9, FJ534955.1	98
		DPHA07	0.8	Clostridia	Clone Er-LLAYS-35, EU542503-1	97
		DPHE06	1.7	Clostridia	*Clostridium pascui*, X96736.1	99
		DPHB07	0.8	Clostridia	Clone 49, FJ534994.1	93
DPF18	1.5			Erysipelotrichi	Clone M011_60, EU014057	98
		DPHA05	4.2	Bacilli[e]	Clone mle1-9, AF280848.1	98
DPF02	17.9			δ–proteobacteria	*Desulfovibrio desulfuricans* Essex 6, AF192153.1	99
		DPHG05	0.8	δ–proteobacteria	*Desulfovibrio sp*, AJ133797.1	98
		DPHA01	63.0	ε–proteobacteria	*Sulfurospirillum deleyianum*, AB368775.1	97
DPF05	6.0			Spirochaetes	*Spirochaeta sp*. Buddy; AF357916	99
DPF029	1.5			Unclassified	Clone CLONG36, DQ478740.1	99
DPF033	1.5			Unclassified	Clone E1, EU864437.1	99

[a] Clone libraries were constructed using bacterial primers 27F and 1492R. [b] DNA was extracted from the 20th generation enrichments when about 80 % PCE has been transformed; Fraction of $Clones_{DPF}$ and Fraction of $Clones_{DPH}$ indicate the percentage of each OTUs in the clone libraries of DPF and DPH, respectively. [c] Sequences in database with ≥ 95 % similarity to DPF or DPH clones are listed, along with the sequence unidentified by Classifier in RDP (http://rdp.cme.msu.edu/classifier/classifier.jsp). [d] Identity in the order and composition of nucleiotides; [e] This OTU was classified as Bacilli by Greengenes but classified as Unclassified by the Classife in RDP.

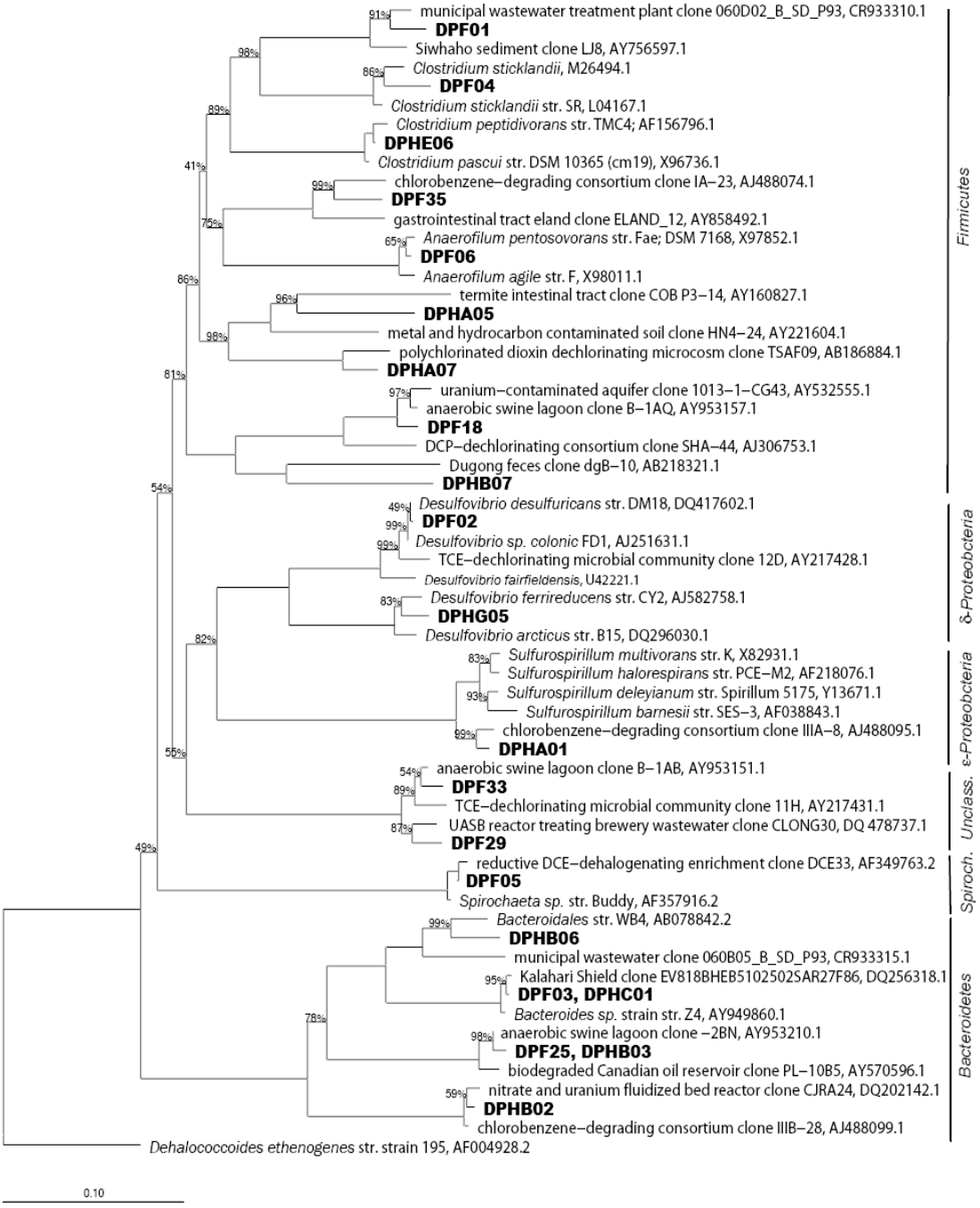

Figure 3.4. Phylogenetic tree of 16S rRNA gene clone sequences (indicated by boldface type) of DPF, DPH and their close relatives and type strains. Calculation was performed by neighbor-joining methods incorporating Jukes-Cantor distance correction. *Dehalococcoides ethenogenes* Strain 195 was used as the out-group. The numbers at the nodes indicate the percentages of times that nodes appeared in 1,000 bootstrap analyses. The scale bar indicates that 0.1 change per nucleotide position. *Unclass*: *Unclassified*; *Spiroch*: *Spirochaetes.*

Different OTUs belonging to *Clostridia* were detected in the two cultures (Table 3.1). Some species belonging to this class (e.g. *Desulfitobacterium spp.*, *Dehalobacter spp.* and *Clostridium bifermentans* DPH-1) have been identified to be dechlorinators for chlorinated ethenes or chlorinated aromatic compounds (Wild et al., 1996; Chang et al., 2000; Holliger et al., 2003; Loeffler et al., 2003; Villemur et al., 2006). Although three OTUs, including DPF35, DPHA07 and DPF18, were closely related to chlorobenzene, polychlorinated dioxin and dichlorophenol (DCP) dechlorinating consortia clones (Figure 3.4), they were not closely related to any PCE or TCE dechlorinating bacterium. The same is true of the other OTUs (DPF01, DPF04, DPF06, DHE06 and DPHB07).

The δ-proteobacteria include some known dechlorinators, such as *Desulfuromonas spp.*, *Desulfomonile tiedjei*, *Desulfovibrio spp.*, *Anaeromyxobacter dehalogenans*, *Geobacter lovleyi* and *Trichlorobacter thiogenes* (Loeffler et al., 2003). Analysis of species closely related to the OTUs detected in DPF and DPH (Clone DPF02 and DPHG05) suggested that they may contribute to the dechlorinating enrichments in two ways. First, *Desulfovibrio spp.* (e.g. *Desulfovibrio sp*. strain SULF1, *Desulfovibrio fructosivorans* and *Desulfovibrio desulfuricans*) and dechlorinating bacteria can form syntrophic association, in which production of hydrogen by oxidation of organic compounds from yeast extract or sewage sludge added to the medium by *Desulfovibrio* species can be transferred to dechlorinating bacteria and thus support microbial reductive dechlorination (Drzyzga et al., 2001; Drzyzga and Gottschal, 2002; He et al., 2007). In our study, the clone DPF02 but

not DPHG05 is about 99 % in identity with *Desulfovibrio desulfuricans* strain Essex 6 (Loubinoux et al., 2000) in 16S rRNA genes. Second, some *Desulfovibrio* species (e.g. *Desulfovibrio sp*. Strain TBP-1 and Strain SF3) are known to reductively dechlorinate halogenated contaminants, such as 2,4,6-tribromophenol and 2-chlorophenol (Boyle et al., 1999; Sun et al., 2000). To the best of our knowledge, reductive dechlorination of chlorinated ethenes by *Desulfovibrio spp.* has not been reported. However, considering the PCE degrading capacity of some degraders of chlorinated aromatic compounds (e.g. *Desulfomonile tiedjei* and *Desulfitobacterium sp.* Strain Viet1) (Cole et al., 1995; Loeffler et al., 1997; Tront et al., 2006), we cannot rule out the potential importance of this genus in PCE reductive dechlorination, especially in DPF, although 16S rRNA of DPF02 and DPHG05 are not highly similar to *Desulfovibrio sp*. Strain TBP-1 or Strain SF3.

DPHA01, the dominant sequence detected in DPH but not in DPF was grouped in the *Sulfurospirillum spp.* of ε-*proteobacteria*. This sequence was 95 % identical with *S. multivorans* and *S. halorespirans* strain PCE-M2, which reductively dechlorinate PCE to *cis*-DCE via dehalorespiration (Neumann et al., 1994; Scholz-Muramatsu et al., 1995; Luijten et al., 2003). It is reported that H_2 and formate can serve as the electron donors for *Sulfurospirillum spp.* (e.g. *S. deleyiamum*, *S. cavolei*, *S. multivorans* and *S. halorespirans* strain PCE-M2) (Neumann et al., 1994; Scholz-Muramatsu et al., 1995; Luijten et al., 2003). This is consistent to the nutrient conditions in DPH and its subculture DPH+formate. During PCE reductive dechlorination, both *S. multivorans* and *S. halorespirans* weakly fractionate with the

enrichment factors ranging from -0.42 to -1.33 ‰ (Nijenhuis et al., 2005; Liang et al., 2007; Cichocka et al., 2008), close to the observed isotope fractionation (-1.98 to -3.23 ‰) by DPH. Although we cannot rule out the possibly that some previously unidentified dechlorinating bacteria are responsible for the PCE reductive dechlorination in DPH and DPF, the high fraction of the OTU closely related to *Sulfurospirillum* in DPH, the same product distribution and physiological properties compared to the previously published dechlorinating *Sulfurospirillum spp.*, close enrichment factors and absence of TCE to *cis*-DCE transformation capacity in the absence of this genus in DPF suggest that *Sulfurospirillum* related species is most likely responsible for PCE reductive dechlorination in DPH.

3.3.5. Response to Absence of Electron Acceptors by DPF and DPH

Chemical, isotope and molecular analyses suggest that different dechlorinating bacteria might be responsible for PCE reductive dechlorination in DPF and DPH as discussed above. Here, dechlorination rates of the cultures with and without "PCE-starving" pretreatment were compared to evaluate the metabolisms of involved dechlorinating species. We hypothesized that if reductive dechlorination is the only terminal electron accepting process for dechlorinators and they obtain energy in the reaction processes (e.g. dehalorespiration), "PCE-starving" may lead to growth of non-dechlorinators and thus result in competition for electron donors when both PCE and electron donors are added afterwards. If this occurred, we would predict lower PCE dechlorination rates in the samples spiked with PCE at day 15 than those added

PCE at day 1. Alternatively, if other electron acceptors in the medium (e.g. yeast extract or organic compounds in activated sludge) can also support metabolism of dechlorinators, their growth may not be dependent on the availability of chlorinated substrates (e.g. PCE). Thus, their dechlorination capacity can be quickly recovered when electron donors and PCE are added and will result in similar or even higher PCE dechlorination rates compared to the samples spiked with PCE at day 1.

Dechlorination rates were quantified as the total amount of dechlorinated products formed within the same period of time (15 days). As shown in Figure 3.5, DPF and DPH presented significantly different patterns. For DPF, the amounts of dechlorinated products in the samples spiked with PCE at different time points were similar. However, for DPH, the amount of dechlorinated products formed in the samples with preincubation without PCE was only about 25 % of that without pretreatment. Based on the above hypotheses, we infer that PCE reductive dechlorination was the terminal electron accepting process for the dechlorinators in DPH, while the comparable dechlorination rates in the DPF subcultures spiked with PCE at day 1 and day 15 suggests that alternative electron accepters in the medium may also support growth of dechlorinators in DPF. Even though accumulation of TCE by DPF is consistent with the product distribution in most cometabolic dechlorinating species (Holliger et al., 2003; Loeffler et al., 2003), indirect evidence suggests that DPF carries out dehalorespiration rather than cometabolism. This evidence includes the fact that no species closely related to any previously published cometabolic PCE degraders (Bradley, 2003; Bhatt et al., 2007) have been identified in

this culture (see below); cometabolism typically results in orders of magnitude slower dechlorination rates than dehalorespiration (Holliger et al., 2003; Loeffler et al., 2003), however the dechlorination rate of DPF was within the same order of magnitude as DPH (Figure 1); dehalogenating bacteria that use H_2 and formate as electron donors during reductive dechlorination of chlorinated ethylenes are typically identified as dehalorespiring bacteria (Holliger et al., 2003), but it is difficult to determine whether dechlorinating species in DPF use formate as the direct electron donor.

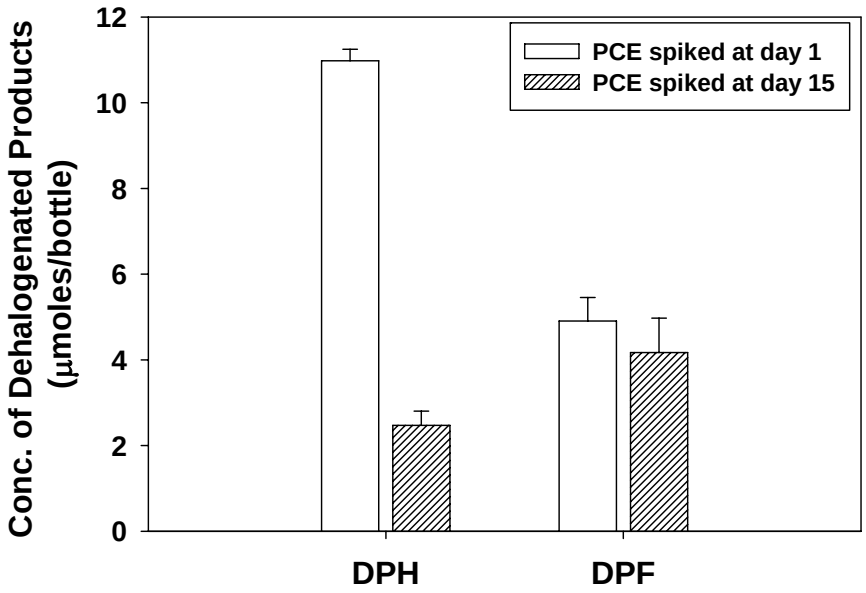

Figure 3.5. Dechlorinated products formed within 15 days during microbial PCE reduction in DPH with versus without "PCE-starving" pretreatment. The dechlorination rates were quantified as the total dechlorinated products (e.g., sum of TCE and *cis*-DCE for DPH and TCE for DPF) produced within the same period of time (15 days). The values of bars are average and the uncertainties indicate standard deviation of triplicate samples.

3.4. Environmental Importance for the Assessment of *In Situ* Bioremediation

The chemical, isotope and RFLP results suggest that different dechlorinating communities and especially dechlorinating species from the same source can vary in

the presence of different environmental factors (e.g., pH and electron donors) after being stimulated for about four years. Hence this study may provide an explanation for why different extents of isotope fractionation were observed for the same enrichment culture (e.g., TCE dechlorination by KB-1) in different studies (Bloom et al., 2000; Slater et al., 2001) or were added with different electron donors (e.g., butyric acid versus ethanol acting as electron donors for PCE dechlorination by TP microbial consortia) (Slater et al., 2001). In this study, a *Sulfurospirillum* relative was proposed to be the dechlorinators in DPH, while it is not clear which species in DPF is involved. We suggest that the species affiliated to *Desulfitobacterium* or *Desulfovibrio* may be responsible for PCE reductive dechlorination in DPF. Regardless, the results indicate that multiple dechlorinating bacteria are likely present at different sites and preferential growth of one or some of the species and the resulting contributions to reductive dechlorination might be indirectly influenced by environmental conditions (e.g. electron donors and pH).

To determine the potential significant uncertainties related to the selection of unrepresentative ε_{bulk} during *in situ* assessment by using isotope fractionation, we used the following equations (Cichocka et al., 2008; Hunkeler et al., 2008), to predicted the extent of biodegradation (B %) (Figure 3.6). We chose both the strongest (ε_{bulk} = -13.44 ‰) and the weakest (ε_{bulk} = -1.98 ‰) isotope fractionation values to determine the upper and lower limits by using the equation as follows.

$$B(\%) = (1-f)\times 100 = \left[1-\left(\frac{\delta^{13}C+1000}{\delta^{13}C_0+1000}\right)^{\frac{1000}{\varepsilon_{bulk}}}\right]\times 100 \qquad (3.1)$$

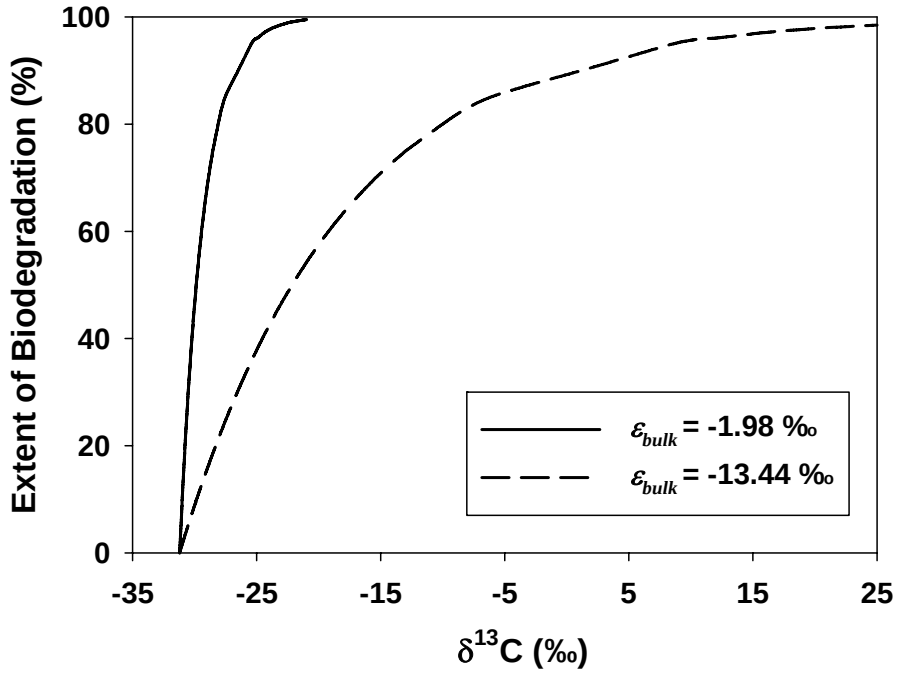

Figure 3.6. Relative influence of different values of enrichment factors (ε_{bulk}) on the calculated extent of PCE microbial reductive dechlorination. The strongest and the weakest extents of isotope fractionation obtained in this study (ε_{bulk} = -13.44 and -1.98 ‰, respectively) were used for estimation.

Assuming we measure $\delta^{13}C$ to equal -25 ‰, about 40 % of the contaminant is predicted to be transformed if ε_{bulk} = -13.44 ‰, while more than 90 % of the contaminant is predicted to be transformed if ε_{bulk} = -1.98 ‰. The significant difference in predicted B stresses the potential uncertainties due to the selection of unrepresentative ε_{bulk} during *in situ* assessment by using isotope fractionation. Therefore, it is recommended that microcosms be developed to directly determine the possible enrichment factors. Microcosm studies should be repeated periodically in long-term bioremediation projects to ensure that potential changes in extents of

isotope fractionation due to the shift of microbial diversity over time can be tracked, especially for the remediation approaches that involve addition of electron donors.

CHAPTER 4*

The Relative Contributions of Abiotic and Microbial Processes to the Transformation of Tetrachloroethylene and Trichloroethylene in Anaerobic Microcosms

4.1. Introduction

Ground water contamination by chlorinated aliphatic contaminants such as tetrachloroethylene (PCE) and trichloroethylene (TCE) is a widespread problem in the United States (Moran et al., 2007). PCE and TCE can be transformed abiotically by reactive Fe(II) and S(-II) minerals (Sivavec and Horney, 1996, 1997; Butler and Hayes, 1999, 2001; Lee and Batchelor, 2002b, a) that are typically formed during microbial reduction of Fe(III) oxides and sulfate (Morse et al., 1987; Fredrickson et al., 1998). Bacteria can also directly degrade PCE or TCE by dehalorespiration or cometabolism (Holliger et al., 1997; Bradley, 2003; Bhatt et al., 2007). Abiotic reductive dechlorination of PCE and TCE typically takes place by reductive β-elimination that results in accumulation of acetylene and other completely dechlorinated products (Sivavec and Horney, 1996, 1997; Butler and Hayes, 1999,

* This chapter consists of the paper by Dong et al. (2009) from "Environmental Science & Technology, 43, Dong, Y., Liang, X., Krumholz, L.R., Philp, R.P., Butler, E.C., 2009. The Relative Contributions of Abiotic and Microbial Processes to the Transformation of Tetrachloroethylene and Trichloroethylene in Anaerobic Microcosms. Copyright (2009) American Chemical Society". The same paper is given in full in Xiaoming Liang's dissertation "Kinetic and Isotope Analysis during Abiotic Transformation of Chlorinated Hydrocarbons by Iron and Sulfur Minerals, 2009, University of Oklahoma" and we did not divide this paper as that in Chapter 2 because any effort of division significantly weakens data interpretation. Y. D. was responsible for the microcosm setup, geochemical analysis of iron species and NOM, sample preparation for SEM, acetylene transformation, identification and quantification of reductive dechlorination in all the PCE microcosms and part of TCE microcosms.

2001; Lee and Batchelor, 2002b, a), while microbial reductive dechlorination occurs via sequential hydrogenolysis that results in accumulation of lesser chlorinated ethenes along the sequence TCE, dichloroethylenes (DCEs), vinyl chloride (VC), and ethene (Brown et al., 2006). The distinct reaction products for abiotic versus microbial PCE and TCE reductive dechlorination can help identify the predominant process in a given environmental system.

Despite knowledge gained from the laboratory (Pasakarnis et al., 2006; Shen and Wilson, 2007) and field (Brown et al., 2006; Kennedy et al., 2006a; Kennedy et al., 2006b), the relative contributions of abiotic and microbial processes to the natural transformation of PCE and TCE is currently a subject of debate. Furthermore, while PCE and TCE transformation by pure mineral species has been well studied in the lab (see refs. in (Liang et al., 2007)), the influence of readily measurable subsurface geochemical parameters, such as weakly and strongly bound Fe(II), acid soluble sulfur, and chromium extractable sulfur (CrES), on PCE and TCE transformation kinetics has not yet been reported. The objectives of this research were to: (1) assess the relative importance of microbial versus abiotic PCE and TCE reductive dechlorination under a variety of geochemical conditions and (2) identify the geochemical conditions for which abiotic PCE and TCE reductive dechlorination are most important. Microcosm studies were conducted using aquifer solids from three locations that were amended to generate iron reducing, sulfate reducing, and methanogenic conditions. We assessed the importance of abiotic and microbial reductive dechlorination in the microcosms by analysis of reaction products and kinetics, utilization of abiotic (killed) controls, comparison of observed half lives to those of laboratory studies using pure minerals, and stable carbon isotope analysis.

4.2. Materials and Methods

The specifications and sources of all chemical reagents are given in the Supporting Information. Solid and liquid samples were collected from three sites, including an anaerobic zone of an aquifer located adjacent to the closed landfill at the Norman Landfill Environmental Research Site (U.S. Geological Survey Toxic Substances Hydrology Research Program), Norman, OK (Norman Landfill or L), a pond in Brandt Park, Norman, OK (Duck Pond or DP), and two permeable reactive barriers containing mulch ("biowalls") at Altus Air Force Base, Altus, OK (AAFB). There have been no reports of PCE or TCE contamination at the first two sites, while the sampling areas at AAFB intersect TCE plumes (Kennedy et al., 2006b; Lu et al., 2008). Two AAFB samples (AAFB 12 and AAFB 14) were from a biowall section that had been modified by addition of magnetite to promote formation of FeS upon microbial sulfate reduction (Parsons, May 2006). Additional details about the sampling locations and procedures are given in the Supporting Information.

Microcosms were prepared in an anaerobic chamber (Coy Laboratory Products Inc., MI). Buffered site water (100 mL containing 25 mM HEPES (pH 7.2) or TAPS (pH 8.2)) and 20 g wet sediment or solids were added to 160 mL serum bottles. Experiments were done at pH 7.2 and 8.2 to include the range of pH values found in natural waters. HEPES and TAPS are generally considered suitable for biological systems, and we are not aware of any reports of HEPES or TAPS acting as electron donors for bacteria or exhibiting side effects such as toxicity to dechlorinating bacteria. Strict pH control was required since pH can strongly affect the rates of abiotic reductive dechlorination of PCE and TCE (Hwang and Batchelor, 2000; Butler and Hayes, 2001; Lee and Batchelor, 2002b; Maithreepala and Doong, 2005). Microcosms were either "unamended" (U), which were not preincubated with

electron donors or acceptors before spiking with PCE or TCE and represented baseline geochemical conditions; “amended” (A), which were preincubated with electron acceptors and/or donors in order to increase microbial activity and stimulate reactive mineral formation before spiking with PCE or TCE; or “killed” (K), which were amended and preincubated as described above, then treated by boiling water bath and antibiotics to kill bacteria prior to addition of PCE or TCE. Details about the heat/antibiotic treatment as well as the effect of heat treatment on the concentrations of abiotic mineral fractions are discussed in the Supporting Information.

Except for those that were unamended, microcosms were set up to stimulate iron reduction (IR), sulfate reduction (SR), or methanogenesis (Meth). Electron donors and acceptors were added to the microcosms to increase both the concentrations of potentially reactive biogenic minerals and microbial activity. Duck Pond and Landfill aquifer microcosms were amended with amorphous Fe(III) gel (50 mM) (Cornell and Schwertmann, 2003), $FeSO_4$ (30 mM), or no electron acceptor in order to establish iron reducing, sulfate reducing, or methanogenic conditions, respectively. For AAFB microcosms, only sulfate reducing conditions were stimulated, since this most closely represented site conditions, where dissolved sulfate in the ground water is high (1.4-12.5 mM). Acetate (20 mM), lactate (40 mM), and ethanol (15 mM) were added as electron donors for iron reducing, sulfate reducing, and methanogenic conditions, respectively. While it is possible that the use of different electron donors affected the rate and/or extent of dechlorination in the microcosms, the choice of each electron donor was made to be certain to stimulate microorganisms known to be capable of iron reduction, sulfate reduction, or methanogenesis, respectively.

In order to prevent methanogenic bacteria present in soil and sediment samples

from competing for electron donors and preventing the establishment of iron or sulfate reduction, 1 mM 2-bromo-ethanosulfonic acid was added to the sulfate and iron reducing microcosms before adding electron acceptors and/or donors. This concentration was chosen because it was lower than concentrations reported to inhibit dechlorinating bacteria (2-3 mM) (Loeffler et al., 1997; Chiu and Lee, 2001), but was still sufficient to inhibit methane production. After addition of these amendments, microcosms were preincubated until terminal electron acceptors were consumed in the sulfate and iron reducing microcosms or formation of methane leveled off in the methanogenic microcosms. Then, the solid phase geochemistry was analyzed, microcosms were spiked with PCE or TCE, and monitored for abiotic and microbial transformation. Experiments with PCE were done for all microcosm conditions; experiments with TCE were done for selected conditions (Table 4.1). Sediments from one microcosm (DP-SR-pH 8.2) were imaged by scanning electron microscopy (SEM) to visualize the morphology and surface conditions of biogenic minerals. The images (Figure D1), show rod-shaped bacteria (Figure D1(a) and (b)) and nano- to micrometer scale crystalline precipitates (Figure D1(b)) that could be FeS, Fe_3S_4, and/or FeS_2. Additional details on microcosm setup and analytical techniques are given in the Supporting Information. A summary of all experimental conditions and their abbreviations is given in Table D1 in the Supporting Information. As an example, the abbreviation “DP-Meth-pH 8.2-TCE” is used hereafter for Duck Pond sediments preincubated under methanogenic conditions at pH 8.2 and spiked with TCE.

4.3. Results and Discussion

4.3.1. Equilibrium among the Aqueous, Solid, and Gas Phases in Microcosms

Microcosms contained three phases: gas, aqueous, and solid. Concentrations discussed below and used in calculations ("total concentrations") are equal to the sum of the aqueous, solid, and gas phase masses divided by the aqueous volume. Kinetic parameters were calculated assuming rapid equilibrium of PCE or TCE among the phases relative to kinetic transformation, and kinetic transformation in the aqueous phase only; the approach is described in the Supporting Information.

4.3.2. Relative Importance of Abiotic and Microbial Reductive Dechlorination

Normalized concentrations of PCE and TCE versus time have been plotted for all the microcosm conditions (Figure 4.1) and time courses for representative microcosms, which also show normalized concentrations of detected reaction products, were also plotted (Figure 4.2). Normalized concentrations for antibiotic/heat killed microcosms along with their live counterparts prepared under the same conditions, as well as time courses for all live AAFB microcosms, are shown in the Supporting Information (Figures D2 and D3, respectively). Evidence from these figures indicates that in most cases, reductive dechlorination of PCE and TCE in the microcosms took place primarily by microbial transformation by indigenous dechlorinating bacteria rather than abiotic transformation by reactive minerals. This evidence includes: (1) slow rates and a small extent of PCE transformation in killed microcosms compared to the amended and unamended microcosms prepared under the same conditions (Figure D2 in Appendix); (2) a lag time followed by a rapid pseudo-zero-order (i.e., straight line or constant slope) disappearance of PCE or TCE that is characteristic of microbial transformations, rather than an initial

pseudo-first-order reaction characteristic of abiotic reactions (Figures 4.1, 4.2, D2, and D3); (3) near quantitative accumulation of PCE and TCE hydrogenolysis products, such as TCE (for PCE), *cis* 1,2-DCE, and VC, for all microcosms where there was significant transformation of PCE or TCE (Figure 4.2) (two possible exceptions to this trend, AAFB-12-SR-pH 7.2-PCE and AAFB-14-SR-pH 7.2-PCE, are discussed further below); and (4) the rapid transformation of PCE or TCE after the initial lag period, compared to the relatively slow abiotic transformation of these compounds. For instance, using previously reported mass-normalized rate constants for PCE and TCE transformation by FeS that were corrected for partitioning among the gas, aqueous, and solid phases (D5 in Appendix D, Table D4), we estimated that the half lives for PCE or TCE transformation by the FeS present in our microcosms would be 900-5,000 days (PCE) or 500-1,000 days (TCE) at the highest FeS mass loading (approx. 0.9 g/L) and a median fraction organic carbon (f_{oc}) value of 0.002 (Table D2). (Longer half lives are for pH ≈ 7; shorter half lives are for pH ≈ 8.) While other reactive minerals could have also contributed to abiotic PCE and TCE transformation in the microcosms, their mass loadings and reactivity are likely to be at least the same order of magnitude as those for FeS, so abiotic reactions alone cannot account for the rapid transformation of PCE and TCE following the lag period (Figures 4.1, 4.2, and D3).

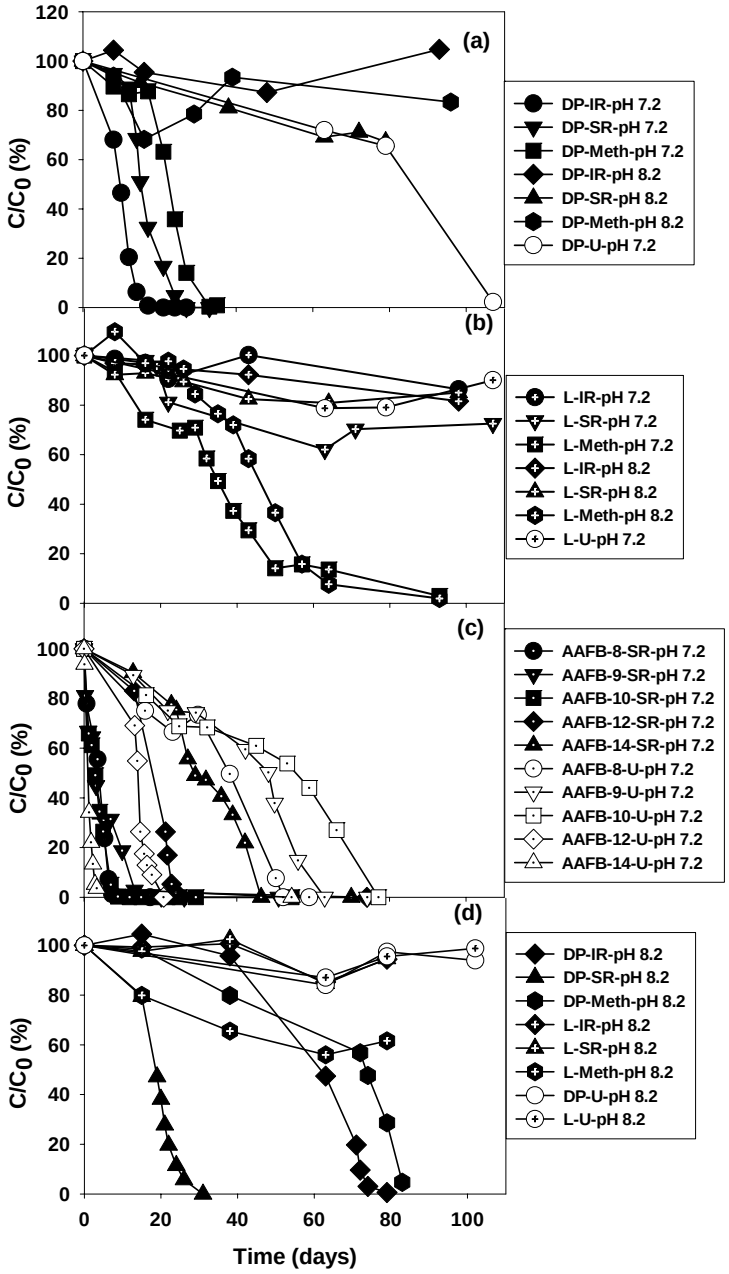

Figure 4.1. PCE reductive dechlorination in the Duck Pond (DP) (a), Landfill (L) (b), and Altus AFB (AAFB) (c) microcosms and TCE reductive dechlorination in selected DP and L microcosms (d), under iron reducing (IR), sulfate reducing (SR), and methanogenic (Meth) conditions. Data points are averages of samples from duplicate or triplicate microcosms.

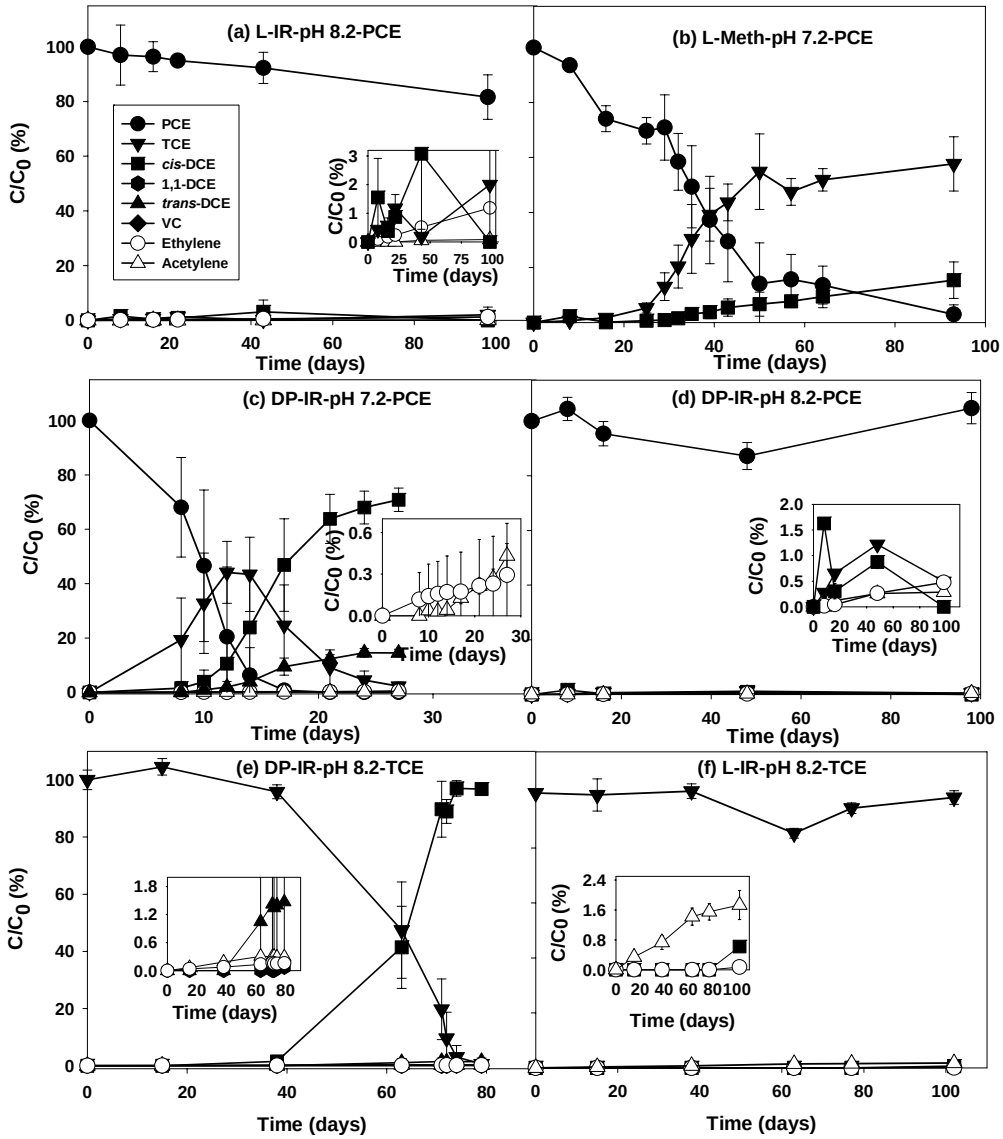

Figure 4.2. Normalized concentrations of PCE (a-d), TCE (e-f), and reaction products in representative microcosms. Reactants and products were normalized by dividing the concentration at any time by the concentration of the reactant at time zero. The insets show reaction products with low concentrations. Error bars are standard deviations of triplicate microcosms. To better show the data points, parts of the error bars were cut off in the insets for (a) and (e). In the inset for (e), the symbols for 1,1-DCE (closed hexagons) are partially covered with ethylene (open circles) and acetylene (open triangles).

To quantify the extent of microbial and abiotic PCE and TCE transformation, we calculated product recoveries for both processes by dividing the summed total concentrations of abiotic or microbial dechlorination products at the last sampling time (see Table 4.1, column 2) by the initial total concentration of PCE or TCE, and

multiplying by 100 % (Lee and Batchelor, 2002a). Calculation details are in the Supporting Information and abiotic and microbial product recoveries are reported in Table 4.1. While product recoveries are not constant with time, their calculation allows comparison of the relative importance of abiotic versus microbial PCE and TCE transformation among microcosms sampled at approximately the same time. For some live AAFB microcosms, we were not able to distinguish whether the ethylene detected in the microcosms came from microbial hydrogenolysis of VC or from abiotic hydrogenation of acetylene (e.g., ref. (Jeong et al., 2007)); in these cases, product recoveries were not calculated. Details are in the Supporting Information.

Table 4.1 shows that abiotic product recoveries were never significantly higher than 1 %. Considering only live microcosms, there were two conditions where the abiotic product recovery exceeded the microbial product recovery, one for PCE transformation (DP-IR-pH 8.2; Figure 4.2d, Table 4.1), and one for TCE transformation (L-IR-pH 8.2; Figure 4.2f, Table 4.1). For these microcosms, both abiotic and microbial transformation were slow (close to 100% of the PCE or TCE remained after approximately 100 days (Table 4.1)), but abiotic products accumulated to a greater extent than did microbial products, suggesting that abiotic processes could be more important for PCE or TCE transformation in subsurface environments under conditions where dechlorinating bacteria are not active. The high pH (8.2) of these microcosms may have inhibited the activity of dechlorinating bacteria. In five other live microcosms (DP-Meth-pH 8.2-PCE; L-IR-pH 8.2-PCE; L-SR-pH 7.2-PCE; L-SR-pH 8.2-TCE; and L-Meth-pH 8.2-TCE), the abiotic and microbial product recoveries were relatively close to each other (within a factor of 10). Four of these five were incubated at pH 8.2, providing additional evidence that, at least in some cases, higher pH values may not be optimal for growth of dechlorinating bacteria. In

all other samples, microbial product recoveries were much higher than abiotic product recoveries.

We considered the possibilities that our low abiotic product recoveries could be due to microbial transformation of abiotic dechlorination products (e.g., acetylene). To test this possibility, we spiked acetylene into the Duck Pond and Landfill microcosms at a total concentration of approximately 2 μM, which was close to the highest concentration of acetylene observed in our microcosms. Figure D4 shows that acetylene was transformed within approximately 2-4 days in the Duck Pond microcosms, but remained essentially constant after more than 40 days in all the Landfill microcosms. We then treated the three Duck Pond microcosms showing the fastest acetylene transformation in a boiling water bath for 15 min and respiked them with acetylene. Following this, no acetylene transformation was observed, indicating that acetylene transformation was microbial, not abiotic. Microbial fermentation of acetylene has been reported previously (Schink, 1985). Despite the loss of abiotically-generated acetylene via microbial transformation in the Duck Pond microcosms, however, there are still several lines of evidence (discussed above) indicating the greater involvement of microbial versus abiotic transformation of PCE and TCE in the microcosms. Consumption of acetylene by indigenous microorganisms cannot account for the low abiotic product recoveries observed for almost every microcosm condition, including the Landfill microcosms, where acetylene transformation was not observed (Figure D4).

Table 4.1. Summary of results for the microcosm experiments[a]

Microcosm ID[b]	Time (days) (PCE/TCE)	Percent remaining (%)		Abiotic product recovery (%) [c]		Microbial product recovery (%) [c]	
		PCE	TCE	PCE	TCE	PCE	TCE
Unamended Microcosms							
DP-U-pH 7.2	107/102	2.26±0.87	94.0±3.9	0	0.1	91	5
L-U-pH 7.2	107/102	90.1±2.0	98.77±0.42	0	0	8	1
AAFB-8-U-pH 7.2	59	0	—[d]	NC[e]	—	119	—
AAFB-9-U-pH 7.2	74	0	—	NC	—	112	—
AAFB-10-U-pH 7.2	77	0	—	NC	—	123	—
AAFB-12-U-pH 7.2	75	0	—	NC	—	105	—
AAFB-14-U-pH 7.2	54	0	—	NC	—	99	—
Amended Microcosms							
DP-IR-pH 7.2	27	0	—	1	—	89	—
DP-IR-pH 8.2	98/79	104.7±5.7	0.607±0.030	1	0.5	0.5	98
DP-SR-pH 7.2	33	0	—	0.1	—	102	—
DP-SR-pH 8.2	79/31	67.3±1.6	0	0.2	0.1	12	111
DP-Meth-pH 7.2	35	0.88±0.63	—	0	—	97	—
DP-Meth-pH 8.2	96/83	83±12	4.72	0.2	0.5	2	106
L-IR-pH 7.2	98	86±16	—	1	—	17	—
L-IR-pH 8.2	98/102	81.6±8.1	98.4±2.5	1	2	2	0.6
L-SR-pH 7.2	107	72±12	—	1	—	8	—
L-SR-pH 8.2	98/102	85±35	104.8±6.5	1	1	21	1.9
L-Meth-pH 7.2	93	2.9±3.5	—	0	—	73	—
L-Meth-pH 8.2	93/102	1.9±2.3	74.9±19.9	0	1	104	8
AAFB-8-SR-pH 7.2	17	0	—	NC	—	67	—
AAFB-9-SR-pH 7.2	51	0	—	NC	—	89	—
AAFB-10-SR-pH 7.2	54	0	—	NC	—	105	—
AAFB-12-SR-pH 7.2	74	0	—	NC	—	NC	—
AAFB-14-SR-pH 7.2	70	0	—	NC	—	NC	—
Killed Microcosms							
L-K-Meth-pH 7.2	53	77.9±3.2	—	0	—	0	—
L-K-Meth-pH 8.2	53	87.08±0.14	—	0	—	0	—
AAFB-8-K-U-pH 7.2	154	71.1±4.3	—	1	—	7	—
AAFB-9-K-U-pH 7.2	149	71.2±1.5	—	1	—	4	—
AAFB-10-K-U-pH 7.2	154	64.8±3.9	—	1	—	3	—
AAFB -12-K-U-pH 7.2	155	79.8±17.4	—	0.5	—	33	—
AAFB -14-K-U-pH 7.2	155	77.5±5.8	—	0.4	—	6	—

[a] Uncertainties are standard deviations of replicate microcosms; [b] Abbreviations: Duck Pond (DP), Norman Landfill (L), Altus AFB (AAFB), unamended (U), killed with heat-treatment and antibiotics (K), iron reduction (IR), sulfate reduction (SR) and methanogenesis (Meth); [c] See Appendix D for discussion of uncertainties; [d] __, samples were not set up under these conditions; [e] NC, not calculated because it was unclear if ethylene came from abiotic or microbial dechlorination. See detailed explanation in Appendix D5.2.

Two possible exceptions to the trend of higher microbial versus abiotic product recoveries are AAFB-12-SR-pH 7.2-PCE and AAFB-14-SR-pH 7.2-PCE (Table 4.1, Figures D3(d) and (e)). In neither case could we determine if the abundant ethylene in these microcosms came from abiotic or microbial processes, or some combination of both. The existence of a lag phase before the onset of pseudo-zero-order PCE disappearance (Figures D3(d) and (e)) and the inhibition of PCE disappearance in killed controls (Figure D2), however, are consistent with a greater role for microbial PCE dechlorination in these microcosms.

4.3.3. Isotope Fractionation during Reductive Dechlorination

Stable carbon isotope fractionation is another tool that may provide information about the predominant process for PCE or TCE transformation, i.e., abiotic or microbial. Several recent articles describe in detail the principles of isotope analysis for environmental applications (Elsner et al., 2005). While a range of ε_{bulk} values has been reported for both abiotic and microbial transformation of PCE and TCE, the range of reported ε_{bulk} values for abiotic PCE transformation in batch systems is generally more negative than that for microbial PCE transformation (Bloom et al., 2000; Slater et al., 2001; Slater et al., 2002; Schuth et al., 2003; Slater et al., 2003; Zwank, 2004; Nijenhuis et al., 2005; Cichocka et al., 2007; Lee et al., 2007; Liang et al., 2007; Cichocka et al., 2008). Thus, very large (in magnitude), negative ε_{bulk} values are suggestive of abiotic PCE transformation while very small (in magnitude), negative ε_{bulk} values are suggestive of microbial PCE transformation. The limitation of this approach lies in the exceptions; specifically, negative ε_{bulk} values that are intermediate in magnitude have been reported for both abiotic and microbial PCE transformation. As just one example, an ε_{bulk} value of -14.7 ‰ was reported for

abiotic transformation of PCE by FeS (Zwank, 2004), while a more negative value of -16.7 ‰ was reported for microbial transformation of PCE (Cichocka et al., 2008). Thus, intermediate ε_{bulk} values such as these are of less value in assessing the predominant reaction pathway for PCE transformation (abiotic or microbial), than are very large or small (in magnitude) values. Also, interpretation of ε_{bulk} values must always be done with caution and in conjunction with other lines of evidence such as those described above (e.g., analysis of reaction order and reaction products). Finally, ε_{bulk} values for abiotic and microbial transformation of TCE are typically closer together than are those for PCE (Zwank, 2004; Liang et al., 2007), making isotope fractionation less useful for differentiating abiotic and microbial transformation of TCE versus PCE.

Plots of $\delta^{13}C$ versus fraction PCE or TCE remaining (C/C_0) for all microcosms for which significant PCE or TCE transformation took place are plotted in Figure 4.3. ε_{bulk} values were calculated using the Rayleigh equation (Mariotti et al., 1981). For PCE, ε_{bulk} values for the Duck Pond and all but one AAFB microcosm showed weak isotope fractionation (these ε_{bulk} values ranged from -0.71 to -3.1 ‰), which is typical of microbial reductive dechlorination of PCE (Bloom et al., 2000; Slater et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007; Liang et al., 2007; Cichocka et al., 2008), and therefore consistent with the other evidence for microbial dechlorination discussed above. Significantly stronger isotope fractionation was measured in the remaining AAFB microcosm (AAFB-14-SR-pH 7.2-PCE; ε_{bulk} = -8.5 ‰) and the Landfill microcosms incubated under methanogenic conditions (ε_{bulk} = -10.68 and -16.78 ‰ for pH 7.2 and 8.2, respectively); thus the isotope data from these microcosms is less useful in distinguishing abiotic from microbial dechlorination. While the first two of these ε_{bulk} values are less negative than previously reported

ranges for abiotic PCE dechlorination, and therefore presumably due to microbial dechlorination, the third value (for L-Meth-pH 8.2-PCE (ε_{bulk} = -16.78 ‰) is close to reported values for both microbial PCE transformation (ε_{bulk} = -16.7 ‰) (Cichocka et al., 2008) and abiotic PCE transformation (ε_{bulk} = -14.7 ‰) (Zwank, 2004). The remaining evidence (discussed above) is, however, consistent with microbial reductive dechlorination for these microcosms.

For TCE, ε_{bulk} values for Duck Pond microcosms incubated with different terminal electron acceptors at pH 8.2 equaled -10.1, -19.4, and -20.9 ‰ for methanogenic, sulfate reducing, and iron reducing conditions, respectively. The first of these values is within the range of previously reported values for microbial TCE reductive dechlorination (Hunkeler et al., 1999; Sherwood Lollar et al., 1999; Bloom et al., 2000; Slater et al., 2001; Zwank, 2004; Lee et al., 2007; Liang et al., 2007). The second two are more negative than previously reported ε_{bulk} values for microbial dechlorination of TCE, but they are close to the value of -18.9 ‰ recently reported by Cichocka et al. (Cichocka et al., 2007). We are reluctant, therefore to interpret these second two ε_{bulk} values as indicative of abiotic reductive dechlorination of TCE. In addition, the remaining evidence for these microcosms (low abiotic and high biotic product recoveries (Table 4.1) and a lag period before the start of TCE degradation (Figures 4.1d and 4.2e)) is consistent with microbial and not abiotic reductive dechlorination.

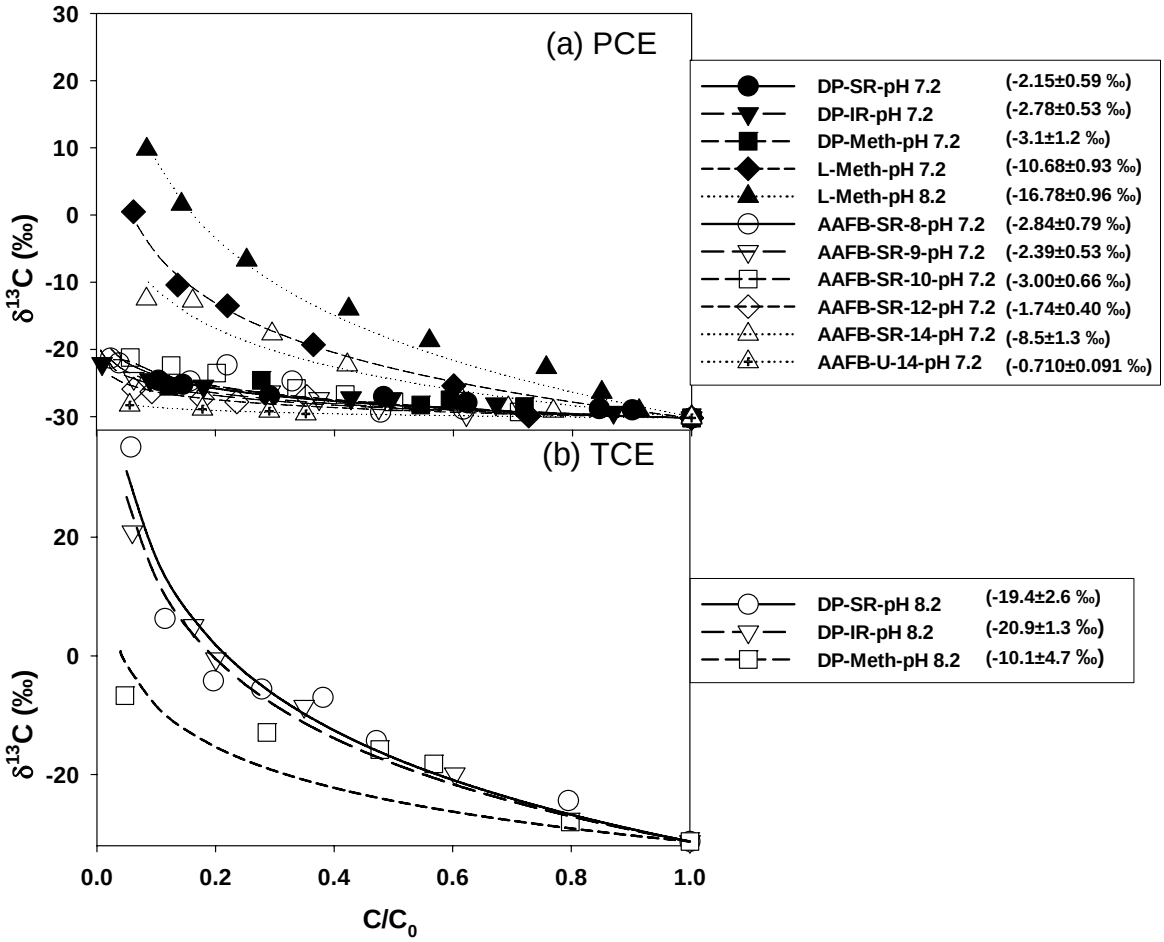

Figure 4.3. Isotope fractionation of PCE (a) and TCE (b) in the microcosms where PCE and TCE were below detection limits at the end of experiment. The values in parentheses are bulk enrichment factors (ε_{bulk} values). Data points are experimentally measured values, and lines represent a fit to the Rayleigh model. Uncertainties are 95 % confidence intervals.

4.3.4. Influence of Geochemical Parameters on Abiotic Reductive Dechlorination.

While microbial transformation of PCE and TCE was typically faster than abiotic transformation in our microcosms, it is possible that abiotic dechlorination may ultimately transform more PCE and TCE under certain conditions, for example where the activity of dechlorinating bacteria is low (e.g., Figures 4.2a, d, and f), for microbial communities that do not completely dechlorinate PCE or TCE, or for soils or sediments that are amended to generate significantly higher mass loadings of reactive minerals or significantly higher pH values as part of a remediation strategy.

For this reason, we analyzed our kinetic and geochemical data to see if there was a relationship between the concentration of one or more geochemical parameters and abiotic product recoveries. Because a number of studies indicate that abiotic reductive dechlorination is a surface and not aqueous phase process (Erbs et al., 1999; Kenneke and Weber, 2003), we considered only solid-associated geochemical species in this analysis. Geochemical data are reported in Table D2 and illustrated in Figure 4.4. The arrows in Figure 4.4 indicate those microcosms where no abiotic PCE or TCE reaction products were detected; this occurred under only three conditions (L-U-pH 7.2, DP-Meth-pH 7.2, and L-Meth-pH 7.2). These three conditions were either unamended (no electron donors or acceptors added), or amended to produce methanogenic conditions (Figure 4.4).

Table D2 and Figure 4.4 show that such microcosms typically had lower concentrations of potentially reactive Fe(II) and S(-II) mineral fractions (presumably due to the absence of iron and sulfate reduction that leads to formation of Fe(II) and S(-II) minerals) than did microcosms incubated under iron reducing or sulfate reducing conditions, suggesting the importance of freshly precipitated Fe(II) and S(-II) minerals in abiotic PCE and TCE dechlorination. It is not possible from Table D2 and Figure 4.4 to identify which mineral fraction is most reactive with respect to PCE and TCE abiotic reductive dechlorination, but Table 4.1 shows similar abiotic product recoveries for microcosms incubated under both iron reducing and sulfate reducing conditions, indicating that both non-sulfur-bearing and sulfur-bearing Fe(II) mineral fractions likely contribute to the slow abiotic reductive dechlorination of PCE and TCE observed in most microcosms.

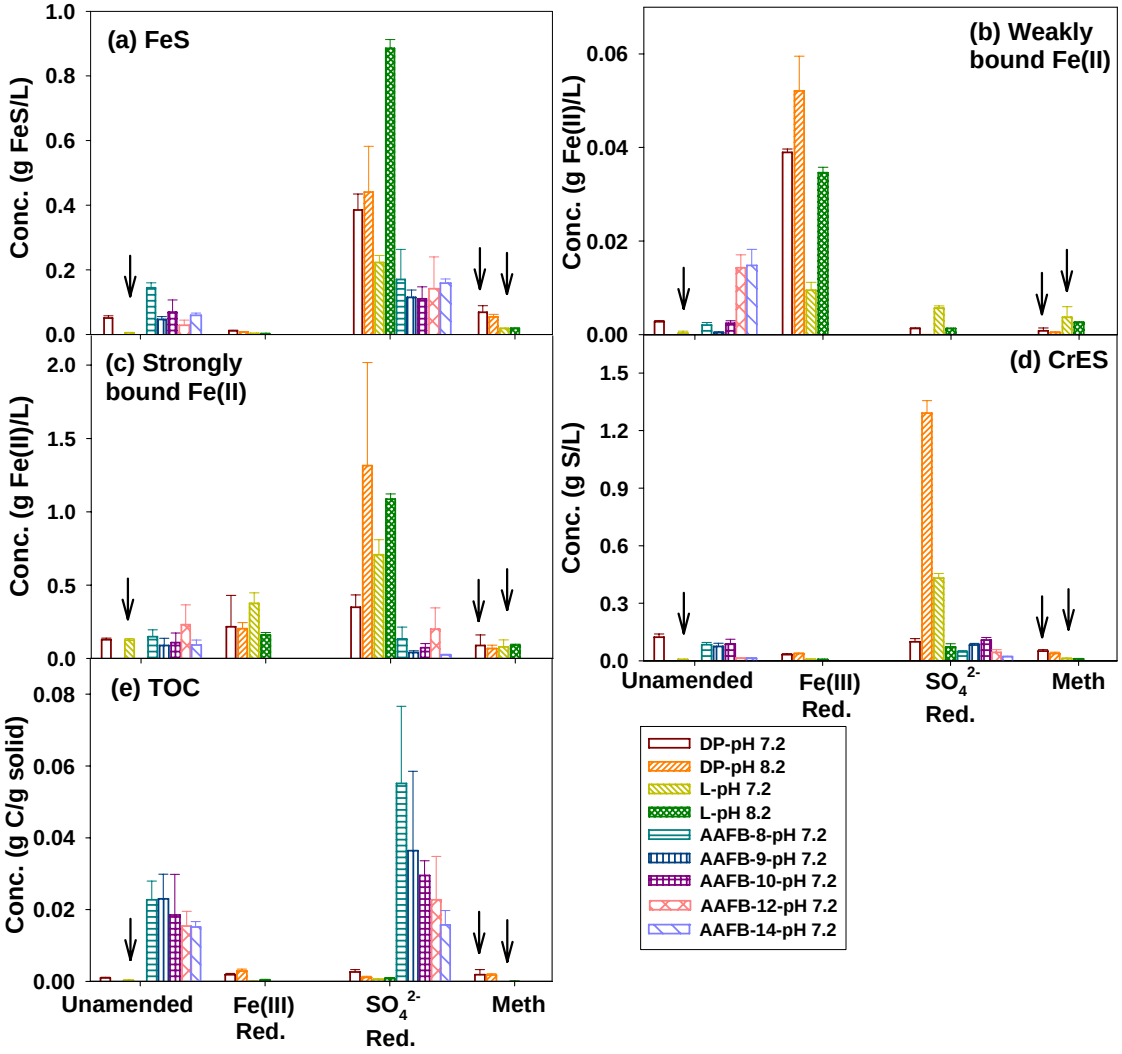

Figure 4.4. Geochemical analyses of the microcosms, including FeS (a), weakly bound Fe(II) (b), strongly bound Fe(II) (c), chromium extractable sulfur (CrES) (d) and TOC (e), under unamended, iron reducing (Fe(III) Red.), sulfate reducing (SO_4^{2-} Red.) or methanogenic (Meth) conditions. Arrows indicate the microcosms where neither PCE nor TCE abiotic reductive dechlorination products were detected. Error bars are standard deviations of triplicate samples from the same microcosm. See the Supporting Information for methods used to quantify the geochemical species shown in this Figure.

4.4. Environmental Significance

Abiotic transformation of PCE and TCE in the microcosms was typically much slower than microbial reductive dechlorination due to the very slow abiotic transformation of PCE and TCE by reactive minerals that were present at concentrations typically below 1 g/L. The microcosms in this study contained 20 g wet soil, 100 mL water, and 50 mL headspace. Assuming a soil water content of

15% and a total volume of wet soil plus water equal to 110 mL, this equals a soil mass loading of 154 g soil/L. Increasing the soil mass loading to a value typical of an aquifer (e.g., 2,000 g soil/L) would have the effect of both increasing the fraction of total PCE or TCE in the sorbed phase and, assuming that the loadings of the soil and its reactive mineral components increased proportionally, increasing the mass loadings of potentially reactive soil minerals. These two phenomena would have opposite effects on apparent rate constants or half lives for abiotic transformation of PCE or TCE by soil minerals. The effect of increasing soil mass loading on rate constants or half lives for microbial PCE or TCE transformation would depend on the fraction of dechlorinating bacteria associated with aquifer solids and would likely result in a commensurate increase in the rate constant. Shen and Wilson (2007) assessed the relative contributions of abiotic and microbial transformation of TCE in a system with a higher mass loading, in which groundwater flowed through laboratory columns constructed from OU1 biowall materials (samples AAFB-8, -9, and -10 were obtained from the OU1 biowall, see Supporting Information) and concluded that the predominant TCE transformation process was abiotic. Further testing will be needed to assess the relative contribution of abiotic and microbial reductive dechlorination under field conditions.

Bacteria capable of dechlorinating PCE or TCE were present under almost all microcosm conditions, and microbial PCE and TCE dechlorination had a typical half life (after the lag phase) of 10 days (Table 4.1). Such half lives are shorter than those reported in most studies of abiotic transformation of PCE and TCE by minerals (Sivavec and Horney, 1996, 1997; Butler and Hayes, 1999, 2001; Lee and Batchelor, 2002b, a), even for conditions where mass loadings of reactive minerals were much higher than those in the microcosms studied here (Table D2). From this we conclude

that microbial processes have the potential for the most rapid transformation of PCE and TCE in the field and should be exploited for this purpose where appropriate. Abiotic processes also have the potential to contribute to the transformation of PCE and TCE in cases where significantly higher mass loadings of reactive minerals are generated in situ as part of a remediation technology or where the activity of dechlorinating bacteria is low (e.g., Figures 4.2a, 4.2d and 4.2f). Abiotic processes can also play a significant role in cases where complete microbial degradation of PCE or TCE to ethene does not occur (e.g., Figure 4.2b), since mineral-mediated dechlorination of *cis*-DCE and VC to ethane, ethylene, and/or acetylene has been shown (Lee and Batchelor, 2002b, a). Under these conditions, although slow, abiotic processes may still contribute to the complete transformation of PCE and TCE to benign products at contaminated sites.

CHAPTER 5

Conclusions and Recommendations

5.1. Conclusions

In this dissertation, isotope fractionation during microbial reductive dechlorination of PCE and TCE was investigated in pure cultures, mixed cultures and microcosms. Impacts of microbial community composition and environmental factors (e.g., electron donors and pH) on isotope fractionation during PCE and TCE reductive dechlorination were evaluated in Chapter 3. Our major findings are summarized as follows:

1. The three cultures studied, including *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* Strain BB1 and Bio-Dechlor INOCULUM (BDI), differed in the extents of isotope fractionation during microbial reductive dechlorination. This might be due to their difference in structure of functional enzymes, rate-limiting steps before enzymatic reactions or commitment of parent substrates to the functional enzymes, which is expressed as ratio between the rates of the catalytic step to the rate of substrate-enzyme dissociation step. In general, relatively weaker isotope fractionation (ε_{bulk} = -1.33 to -7.12 ‰) was observed during PCE transformation than that during TCE reductive dechlorination (ε_{bulk} = -4.07 to -15.27 ‰).
2. Extents of isotope fractionation during microbial reductive dechlorination are generally weaker than that during abiotic reductive dechlorination carried out by reactive minerals (e.g., iron sulfide (FeS) and green rust), which was expressed as less negative ε_{bulk} values.

3. Microbial reductive dechlorination was dominant in most of the microcosms developed with sediments from both uncontaminated and contaminated sites, suggesting wide existence of dechlorinating bacteria. The microcosms prepared with the sediment from Duck Pond, Norman, OK, accumulated *cis*-DCE during PCE and TCE reductive dechlorination, while TCE was the major dechlorinated product in those prepared with soil from Norman Landfill, OK (Landfill); VC and ethylene were the dominant dechlorinated products in the microcosms prepared with the sediment obtained from the biowalls in the Altus Air Force Base (AAFB), OK. The general dechlorination rate in the reactive microcosms was: Duck Pond > AAFB > Landfill, but varied depending on the pH or alternative electron accepting processes (e.g., iron-, sulfate-reduction and methanogenesis) developed in the microcosms.
4. Abiotic reductive dechlorination of PCE and TCE was dominant in the microcosms only when very slow or no microbial reductive dechlorination occurred. Four out of five microcosms showing comparable abiotic and biotic transformation was prepared under pH 8.2. This suggests that high pH (8.2) might be unfavorable for growth of dechlorinating bacteria.
5. Different environmental pH values (7.2 and 8.2) did not significantly influence isotope fractionation during TCE reductive dechlorination by *S. multivorans*.
6. Significantly different product distribution and isotope fractionation was observed between two enrichment cultures (DPF and DPH) stimulated from the same source but in the presence of different electron donors. The major findings based on analyzing the biochemical, isotopic and phylogenetic properties of the two cultures include: 1) environmental factors (e.g., pH and electron donors) do not directly influence the significantly different isotope fractionation observed in the two

cultures; 2) electron donors might indirectly contribute to the different isotope fractionation observed by leading to the changes in microbial community in DPF versus DPH over more than four years of incubation; 3) it is possible that different dechlorinating bacteria exist in the same site and can be preferentially be stimulated in the presence of different environmental conditions (e.g., electron donors).

5.2. Recommendations for Practice

1. Relatively weak isotope fractionation can be used as one line of evidence to identify bioremediation of chlorinated solvents (e.g., PCE and TCE) at field sites. Other approaches (e.g., identification and isotope analysis of dechlorinated products) are also needed to better monitor fate of chlorinated solvents in the field.
2. Variable extents of isotope fractionation by different dechlorinating bacteria suggest that representative enrichment factors should be chosen to avoid uncertainties/errors when applying isotope technique to quantitatively monitor transformation of chlorinated contaminants at different contaminated sites.
3. Based on isotope fractionation by dechlorinating bacteria studied so far, generally stronger extents of isotope fractionation occur when TCE was the final product during PCE reductive dechlorination compared to that when *cis*-DCE or even lower chlorinated products (e.g. VC) were the final product (As shown in Table 5.1). However, no such clear trend was observed during microbial TCE reductive dechlorination (Table 5.1). We also observed some exception that relatively strong isotope fractionation was observed when ethylene was the final product of PCE degradation, e.g. BDI consortium (ε_{bulk} = -7.11 ± 0.72 ‰). However, it is difficult to separate different microorganisms and to identify the contributions of

individual species during the whole degradation reaction. Thus, further work should be performed to provide more conclusive evidence about the relationship between final product(s) and the extents of isotope fractionation, so that final products identified during PCE reductive dechlorination might be helpful to narrow the range of enrichment factors for quantitatively monitoring fraction of microbial reductive dechlorination of PCE in the contaminated sites.

4. Considering that some factors may indirectly influence isotope fractionation (e.g., electron donors as discussed above), a series of microcosms can be developed with the materials (e.g., sediment and groundwater) obtained from the field and in the presence of different electron donors and/or other amendments (e.g., chlorinated compounds as electron acceptors). These microcosm experiments will provide a reasonable range of enrichment factors that are representative of the field site under investigation.
5. For an engineering project to treat a chlorinated solvent contaminated site, it may take a long time to perform complete transformation due to the slow transformation rates. Considering the potential changes in microbial community composition and the resulting shift in isotope fractionation, it is important to monitor isotope fractionation in representative microcosms periodically over time.
6. Relatively weaker extents of isotope fractionation than that of abiotic reductive dechlorination by reactive minerals (e.g., FeS) was observed in our culture and microcosm experiments in which microbial reductive dechlorination was dominant. In the natural environment, microbial and abiotic reductive dechlorination may be significant if the favorable geochemical conditions and dechlorinating bacteria are available. In this case, selection of representative ranges of enrichment factors in the field can be achieved by detection of: 1) dechlorinated products (e.g. acetylene

for abiotic reactions versus hydrogenolysis products for microbial reactions); and 2) geochemical conditions (e.g. pH, alternative electron accepting processes, such as iron reduction and sulfate reduction), which are likely be unfavorable for microbial reductive dechlorination. If both processes are significant, the enrichment factor between the ranges of microbial and abiotic reductive dechlorination will be expected. In this case, further work on microcosms prepared with the local sediment and groundwater will be in need to evaluate whether Rayleigh Model still works for multiple simultaneous transformation processes and to obtain representative enrichment factors if possible. In addition, two-dimensional isotope fractionation can also be used to differentiate different transformation pathways since the ratio of enrichment factors of different elements (e.g. $\varepsilon_{bulk,\ Cl}$ and $\varepsilon_{bulk,\ C}$) are distinct for microbial and abiotic reductive dechlorination.

Table 5.1. Summary of stable carbon isotope fractionation during microbial reductive dechlorination of PCE and TCE[a]

Bacteria	Parent Substrate	Final Product	ε_{bulk} (‰)	Reference
Desulfitobacterium Viet 1	PCE	TCE	-16.7	(Cichocka et al., 2008)
Microcosm L-Meth-pH 7.2	PCE	TCE	-10.68 ± 0.93	(Dong et al., 2009)
Microcosm L-Meth-pH 8.2	PCE	TCE	-16.78 ± 0.96	(Dong et al., 2009)
Sulfurospirillum halorespirans	PCE	*cis*-DCE	-0.50 ± 0.20	(Cichocka et al., 2007)
Sulfurospirillum multivorans	PCE	*cis*-DCE	-0.42 ± 0.08 -1.33 ± 0.13	(Nijenhuis et al., 2005; Liang et al., 2007)
Desulfuromonas michiganensis Strain BB1	PCE	*cis*-DCE	ns[b] -1.39 ± 0. 21	(Liang et al., 2007; Cichocka et al., 2008)
Geobacter lovleyi Strain SZ	PCE	*cis*-DCE	ns	(Cichocka et al., 2008)
Desulfitobacterium sp. Strain PCE-S	PCE	*cis*-DCE	-5.18 ± 0.50	(Nijenhuis et al., 2005)
Microcosm DP-SR-pH-7.2	PCE	*cis*-DCE	-2.15 ± 0.59	(Dong et al., 2009)
Microcosm DP-IR-pH-7.2	PCE	*cis*-DCE	-2.78 ± 0.53	(Dong et al., 2009)
Microcosm DP-Meth-pH 7.2	PCE	*cis*-DCE	-3.1 ± 1.2	(Dong et al., 2009)
BDI consortium	PCE	ethylene	-7.11 ± 0.72	(Liang et al., 2007)
KB-1 consortium	PCE	ethylene	-2.6 to -5.5	(Slater et al., 2001)
TP (butyric acid and ethanol) Consortia	PCE	ethylene	-1.8 to -5.4	(Slater et al., 2001)
Microcosm AAFB-SR-8-pH 7.2	PCE	ethylene	-2.84 ± 0.79	(Dong et al., 2009)
Microcosm AAFB-SR-9-pH 7.2	PCE	ethylene	-2.39 ± 0.53	(Dong et al., 2009)
Microcosm AAFB-SR-10-pH 7.2	PCE	ethylene	-3.00 ± 0.66	(Dong et al., 2009)
Microcosm AAFB-SR-12-pH 7.2	PCE	ethylene	-1.74 ± 0.40	(Dong et al., 2009)
Microcosm AAFB-U-14-pH 7.2	PCE	ethylene	-0.710 ± 0.091	(Dong et al., 2009)
Microcosm AAFB-SR-14-pH 7.2	PCE	ethylene	-8.5 ± 1.3	(Dong et al., 2009)

Bacteria	Parent Substrate	Final Product	ε_{bulk} (‰)	Reference
Desulfuromonas michiganensis Strain BB1	TCE	*cis*-DCE	-3.49 ± 0.20 -4.07 ± 0.48	(Liang et al., 2007; Cichocka et al., 2008)
Geobacter lovleyi Strain SZ	TCE	*cis*-DCE	-8.5 ± 0.6	(Cichocka et al., 2008)
Desulfitobacterium sp. Strain PCE-S	TCE	*cis*-DCE	-12.2 ± 2.2	(Cichocka et al., 2007)
Sulfurospirillum multivorans	TCE	*cis*-DCE	-16.4 ± 1.5 -12.8 ± 1.6	(Lee et al., 2007; Liang et al., 2007)
Sulfurospirillum halorespirans	TCE	*cis*-DCE	-18.9 ± 0.98	(Cichocka et al., 2007)
Microcosm DP-Meth-pH-8.2	TCE	*cis*-DCE	-10.1 ± 4.7	(Dong et al., 2009)
Microcosm DP-SR-pH-8.2	TCE	*cis*-DCE	-19.4 ± 2.6	(Dong et al., 2009)
Microcosm DP-IR-pH-8.2	TCE	*cis*-DCE	-20.9 ± 1.3	(Dong et al., 2009)
Dehalococcoides ethenogenes Strain 195	TCE	ethylene	-9.6 ± 0.4 -13.5 ± 1.8	(Lee et al., 2007; Cichocka et al., 2008)
ANAS consortium	TCE	ethylene	-16.0 ± 0.6	(Richardson et al., 2002; Lee et al., 2007)
KB-1 consortium	TCE	ethylene	-2.5 to -6.6 -13.9 to -15.2	(Bloom et al., 2000; Slater et al., 2001)
Pinellas enrichment	TCE	NA	-7.1	(Sherwood Lollar et al., 1999)

[a] The ε_{bulk} listed do not include the values obtained with crude extracts and purified reductive dehalogenase; [b] ns: fractionation was not significant or the observed shift in isotopic signatures was within the instrumental error; [c]NA: not available.

5.3. Recommendations for Future Research

1. As a highly oxidized compound, PCE is generally reduced by anaerobic bacteria. However, in addition to anaerobic reductive dechlorination, lower chlorinated ethylenes (e.g., TCE, DCEs and VC) can also be oxidized by aerobic bacteria. Oxidation of TCE, DCEs and VC can be carried out by a variety of aerobic bacteria capable of oxidizing methane, methanol, ethene, propane, propene, aromatic compounds, ammonium and isoprene. Oxidation of chlorinated ethylenes may be catalyzed via cometabolism by non-specific oxygenases and CO_2 can be produced as the final product. Some anaerobic bacteria can also oxidize these compounds when alternative electron accepting processes (e.g., iron reduction, sulfate reduction and methanogenesis) occur. Since some of these chlorinated compounds (e.g., TCE, DCEs and VC) are more toxic than PCE and they are commonly accumulated during microbial degradation of PCE if *Dehalococcoides spp.* are absent, monitoring their transformation is also important at fringe and at the discharge point of chloroethene contaminant plumes. So far, isotope fractionation for bioremediation of these substrates has only been studied for anaerobic bacteria. In order to evaluate the role of oxidation of DCEs and VC, studies on product distribution and isotope fractionation during oxidation of lower chlorinated contaminants can be set up by using different isolated pure cultures (e.g., *Mycobacterium aurum* and *Pseudomonas putida* F1) and enrichment cultures. Calculated ε_{bulk} can be compared with the values obtained from anaerobic dechlorinating species. Comparing these results to their anaerobic counterpart will better our understanding about transformation of microbially dechlorinated intermediates or products (e.g. DCEs and VC) in different contaminated field sites and provide a comprehensive enrichment factor pool for applying isotope

technique to monitor their transformation via different pathways (e.g., anaerobic and aerobic transformation).

2. Isotope fractionation during microbial reductive dechlorination has been intensively studied in pure cultures, enrichment cultures and microcosms. Coexistence of different dechlorinating species has been detected in some previously published enrichment cultures. However, isotope fractionation for the systems with multiple dechlorinating bacteria has never been reported. First, it is not known whether competitive, collaborative or independent growth occurs in the systems with multiple dechlorinators. Second, their corresponding relative contribution for isotope fractionation in the observed isotope fractionation during reductive dechlorination under each condition is unknown. In order to understand the microbial reductive dechlorination and isotope fractionation in the systems with multiple dechlorinators, cocultures of different dechlorinating species should be developed. The experiment can be started with the simplified system in which a weakly fractionating species (e.g., *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* Strain BB1, *Sulfurospirillum halorespirans*, *Geobacter lovleyi* Strain SZ) and a species with relatively strong fractionation (e.g., *Desulfitobacterium sp* Viet 1, *Dehalococcoides ethenogenes* Strain 195) are cocultured. In the pure cultures with individual dechlorinators, their normalized transformation rates can be measured (in the unit of transformation rates per unit of cell). The enrichment factors for the species are available in the previously published studies. In the coculture, the abundance of either dechlorinator can be identified with quantitative-PCR (q-PCR) by using specific primers targeting highly variable16S rRNA regions of individual species. Thus, by multiplying the normalized transformation rate, reaction time and abundance of either

dechlorinator, it is possible to quantify fraction of the parent substrate that is transformed by either species in the coculture. Therefore, with the measured fraction of unreacted parent substrate, calculated relative contribution of each species and their enrichment factors, a model can be developed to predict the changes of isotope ratio (e.g., $\delta^{13}C$) at different time points. The predicted $\delta^{13}C$ can be compared with the measured values to evaluate feasibility of the model. If the measured $\delta^{13}C$ values fit those predict by the model, we can apply the model to predict the relative contribution of different dechlorinators in a coculture with other dechlorinating species. This work will help us to understand the transformation patterns (e.g., competitive, collaborative or independent growth) in the system when more than one dechlorinating bacteria are present and their corresponding isotope fractionation. If the work in coculture of two dechlorinators is successful, a more complicated system (e.g., cocultures with more than two dechlorinators) can be developed. We hope this work can be eventually applied in more complicated reaction systems, such as contaminated field sites.

3. Microbial reductive dechlorination is catalyzed by reductive dehalogenases, a group of functional enzymes that contains cofactors (e.g., cobalamin). So far, the reductive dehalogenase genes have been identified in limited number of dechlorinating bacteria that transform chlorinated ethylenes. Further studies to identify this group of enzymes in different dechlorinating bacteria and enzymatic characterization (e.g., substrate specificities, enzymatic kinetics, cofactors, optimum reaction conditions and isotope fractionation) should be done to help understanding their different physiological, biochemical and isotopic properties.
4. In addition to pH and electron donors studied in this dissertation, other environmental factors (e.g., temperature, trace nutrients, bioavailability of parent

substrates and concentrations of electron acceptors) are also important for the performance of microbial metabolism and isotope fractionation. These factors have been studied on dissimilatory sulfate reduction and toluene degradation. However, they have not been evaluated on the dechlorinators that transform chlorinated compounds (e.g., chlorinated ethylene, chlorinated ethane or polychlorinated biphenyls (PCBs)). Thus, it will be interesting to compare the isotope fractionation of different dechlorinating bacteria in the presence of varied environmental factors.

References

Abe, Y., Aravena, R., Zopfi, J., Shouakar-Stash, O., Cox, E., Roberts, J.D., Hunkeler, D., 2009a. Carbon and chlorine isotope fractionation during aerobic oxidation and reductive dechlorination of vinyl chloride and cis-1,2-dichloroethene. Environ Sci Technol 43, 101-107.

Abe, Y., Zopfi, J., Hunkeler, D., 2009b. Effect of molecule size on carbon isotope fractionation during biodegradation of chlorinated alkanes by Xanthobacter autotrophicus GJ10. Isotopes in Environmental and Health Studies 45, 18-26.

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V., Polz, M.F., 2004. Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J Bacteriol 186, 2629-2635.

Alleman, B.C., Leeson, A., 1997. In Situ and On-Situ Bioremediation: Papers from the Fourth International In Situ and On-Situ Bioremediation Symposium. Battelle Press, OH.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., Weightman, A.J., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl Environ Microbiol 71, 7724-7736.

ATSDR, CERCLA Priority List of Hazardous Substances. 2008. Department of Health and Human Services.

Avrahami, S., Liesack, W., Conrad, R., 2003. Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Environ Microbiol 5, 691-705.

Baker-Austin, C., Dopson, M., 2007. Life in acid: pH homeostasis in acidophiles.

Trends Microbiol 15, 165-171.

Ballerstedt, H., Hantke, J., Bunge, M., Werner, B., Gerritse, J., Andreesen, J.R., Lechner, U., 2004. Properties of a trichlorodibenzo-p-dioxin-dechlorinating mixed culture with a Dehalococcoides as putative dechlorinating species. FEMS Microbiol. Ecol. 47, 223-234.

Becker, J.G., Berardesco, G., Rittmann, B.E., Stahl, D.A., 2005. The role of syntrophic associations in sustaining anaerobic mineralization of chlorinated organic compounds. Environ Health Perspect 113, 310-316.

Becker, S., Boger, P., Oehlmann, R., Ernst, A., 2000. PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. Appl Environ Microbiol 66, 4945-4953.

Bedard, D.L., Bailey, J.J., Reiss, B.L., Jerzak, G.V., 2006. Development and characterization of stable sediment-free anaerobic bacterial enrichment cultures that dechlorinate aroclor 1260. Appl Environ Microbiol 72, 2460-2470.

Bhatt, P., Kumar, M.S., Mudliar, S., Chakrabarti, T., 2007. Biodegradation of chlorinated compounds-a review. Crit. Rev. Env. Sci. Technol. 37, 165-198.

Bigeleisen, J., Wolfsberg, M., 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Advances in Chem. Phys. Prigogine, I. Interscience Publishers, Inc., New York, pp. 15-76.

Bloom, Y., Aravena, R., Hunkeler, D., Edwards, E., Frape, S.K., 2000. Carbon isotope fractionation during microbial dechlorination of trichloroethene, cis-1,2-dichloroethene, and vinyl chloride: implications for assessment of natural attenuation. Environ. Sci. Technol. 34, 2768-2772.

Bossert, I.D., Haggblom, M.M., Young, L.Y., 2003. Microbial Ecology of Dehalogenation. In: M., H.M., Bossert, I.D. (Eds.). Dehalogenation: Microbial

Processes and Environmental Applications. Kluwer Academic Publishers, Norwell, MA.

Boyle, A.W., Phelps, C.D., Young, L.Y., 1999. Isolation from estuarine sediments of a Desulfovibrio strain which can grow on lactate coupled to the reductive dehalogenation of 2,4, 6-tribromophenol. Appl Environ Microbiol 65, 1133-1140.

Bradley, P.M., 2003. History and ecology of chloroethene biodegradation: a review. Biorem. J. 7, 81-109.

Brazeau, B.J., Lipscomb, J.D., 2001. Kinetic studies of the two step mechanism of the methane monooxygenase compound Q reaction with substrates. Journal of Inorganic Biochemistry 86, 158-158.

Brown, R.A., Hines, R., Leahy, M.C., Cho, J., 2006. Abiotic and biotic pathways in chlorinated solvent natural attenuation. Remediation of Chlorinated and Recalcitrant Compounds-2006, Proceedings of the International Conference on Remediation of Chlorinated and Recalcitrant Compounds, 5th. Columbus, OH: Battelle Press, 2004, Monterey, CA, United States.

Bruechert, V., 2004. Physiological and ecological aspects of sulfur isotope fractionation during bacterial sulfate reduction. In: Amend, J.P., Edwards, K.J., Lyons, T.W. (Eds.). Sulfur Biogeochemistry, Special Paper 379. Geological Society of America.

Butler, E.C., Hayes, K.F., 1998. Effects of solution composition and pH on the reductive dechlorination of hexachloroethane by iron sulfide. Environ. Sci. Technol. 32, 1276-1284.

Butler, E.C., Hayes, K.F., 1999. Kinetics of the transformation of trichloroethylene and tetrachloroethylene by iron sulfide. Environ. Sci. Technol. 33, 2021-2027.

Butler, E.C., Hayes, K.F., 2001. Factors influencing rates and products in the

transformation of trichloroethylene by iron sulfide and iron metal. Environ. Sci. Technol. 35, 3884-3891.

Cabirol, N., Villemur, R., Perrier, J., Jacob, F., Fouillet, B., Chambon, P., 1998. Isolation of a methanogenic bacterium, Methanosarcina sp. strain FR, for its ability to degrade high concentrations of perchloroethylene. Canadian Journal of Microbiology 44, 1142-1147.

Canale-Perola, E., 1984. The spirochetes. In: Krieg, N.R., Holt, J.G. (Eds.). Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, MD, pp. 38-79.

Canfield, D.E., Raiswell, R., Westrich, J.T., Reaves, C.M., Berner, R.A., 1986. The use of chromium reduction in the analysis of reduced inorganic sulfur in sediments and shales. Chem. Geol. 54, 149-155.

Chang, Y.C., Hatsu, M., Jung, K., Yoo, Y.S., Takamizawa, K., 2000. Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, Clostridium bifermentans DPH-1. Journal of Biotechnology and Bioengineering 89, 489-491.

Chiu, P.C., Lee, M., 2001. 2-bromoethanesulfonate affects bacteria in a trichloroethene-dechlorinating culture. Appl. Environ. Microbiol. 67, 2371-2374.

Choi, J., Choi, K., Lee, W., 2009. Effects of transition metal and sulfide on the reductive dechlorination of carbon tetrachloride and 1,1,1-trichloroethane by FeS. Journal of Hazardous Materials 162, 1151-1158.

Cichocka, D., Imfeld, G., Richnow, H.-H., Nijenhuis, I., 2008. Variability in microbial carbon isotope fractionation of tetra- and trichloroethene upon reductive dechlorination. Chemosphere 71, 639-648.

Cichocka, D., Siegert, M., Imfeld, G., Andert, J., Beck, K., Diekert, G., Richnow, H.-H., Nijenhuis, I., 2007. Factors controlling the carbon isotope fractionation of

tetra- and trichloroethene during reductive dechlorination by *Sulfurospirillum sp*. and *Desulfitobacterium sp*. strain PCE-S. FEMS Microbiol. Ecol. 62, 98-107.

Clark, I., Fritz, P., 1997. Environmental Isotopes in Hydrogeology. Lewis Publications, New York.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., Tiedje, J.M., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33, D294-296.

Cole, J.R., Fathepure, B.Z., Tiedje, J.M., 1995. Tetrachloroethene and 3-chlorobenzoate dechlorination activities are co-induced in Desulfomonile tiedjei DCB-1. Biodegradation 6, 167-172.

Coplen, T.B., Brand, W.A., Gehre, M., Groning, M., Meijer, H.A., Toman, B., Verkouteren, R.M., 2006. After two decades a second anchor for the VPDB delta13C scale. Rapid Commun Mass Spectrom 20, 3165-3166.

Cornell, R.M., Schwertmann, U., 2003. The Iron Oxides Structure, Properties, Reactions, Occurrences, and Uses. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, New York

Cozzarelli, J.M., Suflita, J.M., Ulrich, G.A., Harris, S.H., Scholl, M.A., Schlottmann, J.L., Christenson, S., 2000. Geochemical and microbiological methods for evaluating anaerobic processes in an aquifer contaminated by landfill leachate. Environ. Sci. Technol. 34, 4025-4033.

Danielsen, K.M., Hayes, K.F., 2004. pH dependence of carbon tetrachloride reductive dechlorination by magnetite. Environ. Sci. Technol. 38, 4745-4752.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ.

Microbiol. 72, 5069-5072.

Dionisi, D., Majone, M., Ramadori, R., 2005. Bulking and foaming control methods. In: Tandoi, V., Jenkins, D., Wanner, J. (Eds.). Activated Sludge Separation Problems: Theory, Control Measures, Practical Experiences (Scientific & Technical Report) IWA Publishing (Internatioal Water Association) pp. 62-79.

Dodin, G., Vandergheynst, P., Levoir, P., Cordier, C., Marcourt, L., 2000. Fourier and wavelet transform analysis, a tool for visualizing regular patterns in DNA sequences. J Theor Biol 206, 323-326.

Dong, Y., Liang, X., Krumholz, L.R., Philp, R.P., Butler, E.C., 2009. The Relative Contributions of Abiotic and Microbial Processes to the Transformation of Tetrachloroethylene and Trichloroethylene in Anaerobic Microcosms. Environ. Sci. Technol. 43.

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

Doucette, W.J., 2000. Soil and sediment sorption coefficients. In: Boethling, R.S., Mackay, D. (Eds.). Handbook of Property Estimation Methods for Chemicals: Environmental and Health Sciences. CRC Press LLC, Boca Raton, FL, pp. 141-188.

Drzyzga, O., Gerritse, J., Dijk, J.A., Elissen, H., Gottschal, J.C., 2001. Coexistence of a sulphate-reducing *Desulfovibrio* species and the dehalorespiring *Desulfitobacterium frappieri* TCE1 in defined chemostat cultures grown with various combinations of sulfate and tetrachloroethene. Environ Microbiol 3, 92-99.

Drzyzga, O., Gottschal, J.C., 2002. Tetrachloroethene dehalorespiration and growth of *Desulfitobacterium frappieri* TCE1 in strict dependence on the activity of *Desulfovibrio fructosivorans*. Appl Environ Microbiol 68, 642-649.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., 2006. Bacteria: Firmicutes, Cyanobacteria. The prokaryotes: A Handbook on the Biology of Bacteria. Springer Science&Business Media, LLC, New York.

Elsner, M., Haderlein, S.B., Kellerhals, T., Luzi, S., Zwank, L., Angst, W., Schwarzenbach, R.P., 2004. Mechanisms and Products of Surface-Mediated Reductive Dehalogenation of Carbon Tetrachloride by Fe(II) on Goethite. Environ. Sci. Technol. 38, 2058-2066.

Elsner, M., Zwank, L., Hunkeler, D., Schwarzenbach, R.P., 2005. A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. Environ. Sci. Technol. 39, 6896-6916.

Erbs, M., Hansen, H.C.B., Olsen, C.E., 1999. Reductive dechlorination of carbon tetrachloride using iron(II) iron(III) hydroxide sulfate (green rust). 33, 307-311.

Estep, M.F., Tabita, F.R., Parker, P.L., Van Baalen, C., 1978. Carbon Isotope Fractionation by Ribulose-1,5-Bisophosphate Carboxylase from Various Organisms. Plant Physiol 61, 680-687.

Fathepure, B.Z., Boyd, S.A., 1988a. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by Methanosarcina sp. strain DCM. Appl. Environ. Microbiol. 54, 2976-2980.

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

Fathepure, B.Z., Nengu, J.P., Boyd, S.A., 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. 53, 2671-2674.

Fennell, D.E., Carroll, A.B., Gossett, J.M., Zinder, S.H., 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using

microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. 35, 1830-1839.

Fetzner, S., Lingens, F., 1994. Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. Microbiol Rev 58, 641-685.

Flynn, S.J., Loffler, F.E., Tiedje, J.M., 2000. Microbial community changes associated with a shift from reductive dechlorination of PCE to reductive dechlorination of cis-DCE and VC. Environ. Sci. Technol. 34, 1056-1061.

Fredrickson, J.K., Zachara, J.M., Kennedy, D.W., Dong, H., Onstott, T.C., Hinman, N.W., Li, S.-M., 1998. Biogenic iron mineralization accompanying the dissimilatory reduction of hydrous ferric oxide by a groundwater bacterium. Geochim. Cosmochim. Acta 62, 3239-3257.

Freeborn, R.A., West, K.A., Bhupathiraju, V.K., Chauhan, S., Rahm, B.G., Richardson, R.E., Alvarez-Cohen, L., 2005. Phylogenetic analysis of TCE-dechlorinating consortia enriched on a variety of electron donors. Environ. Sci. Technol. 39, 8358-8368.

Gerritse, J., Drzyzga, O., Kloetstra, G., Keijmel, M., Wiersum, L.P., Hutson, R., Collins, M.D., Gottschal, J.C., 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene by Desulfitobacterium frappieri TCE1. Appl. Environ. Microbiol. 65, 5212-5221.

Gerritse, J., Renard, V., Pedro Gomes, T.M., Lawson, P.A., Collins, M.D., Gottschal, J.C., 1996. Desulfitobacterium sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. Arch. Microbiol. 165, 132-140.

Gibbs, R.J., 1973. Mechanisms of trace metal transport in rivers. Science 180, 71-73.

Gossett, J.M., Zinder, S.H., 1997. Microbiological Aspects Relevant to Natural Attenuation of Chlorinated Ethenes. Proceedings of the Symposium on Natural Attenuation of Chlorinated Organics in Ground Water. U. S. Environmental Protection Agency, Washington, DC, pp. 12-15.

Grandel, S., Dahmke, A., 2004. Monitored natural attenuation of chlorinated solvents: assessment of potential and limitations. Biodegradation 15, 371-386.

Gu, A.Z., Hedlund, B.P., Staley, J.T., Strand, S.E., Stensel, H.D., 2004. Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and cis-DCE. Environ. Microbiol. 6, 45-54.

Häggblom, M.M., Bossert, I.D., 2003. Halogenated organic compounds — a global perspective. In: Häggblom, M.M., Bossert, I.D. (Eds.). Dehalogenation: Microbial Processes and Environmental Applications. Kluwer Academic Publishers, Netherlands, pp. 3-29.

Hanoch, R.J., Shao, H., Butler, E.C., 2006. Transformation of carbon tetrachloride by bisulfide treated goethite, hematite, magnetite, and kaolinite. Chemosphere 63, 323-334.

He, J., Holmes, V.F., Lee, P.K.H., Alvarez-Cohen, L., 2007. Influence of vitamin B12 and cocultures on the growth of *Dehalococcoides* isolates in defined medium. Appl. Environ. Microbiol. 73, 2847-2853.

He, J., Ritalahti, K.M., Yang, K.-L., Koenigsberg, S.S., Loeffler, F.E., 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424, 62-65.

He, J., Sung, Y., Krajmalnik-Brown, R., Ritalahti, K.M., Loffler, F.E., 2005. Isolation and characterization of *Dehalococcoides sp*. strain FL2, a trichloroethane

(TCE)- and 1,2-dichloroethene-respiring anaerobe. Environ. Microbiol. 7, 1442-1450.

Herbert, R.B., Jr., Benner, S.G., Pratt, A.R., Blowes, D.W., 1998. Surface chemistry and morphology of poorly crystalline iron sulfides precipitated in media containing sulfate-reducing bacteria. Chem. Geol. 144, 87-97.

Heron, G., Crouzet, C., Bourg, A.C.M., Christensen, T.H., 1994. Speciation of Fe(II) and Fe(III) in contaminated aquifer sediments using chemical extraction techniques. Environ. Sci. Technol. 28, 1698-1705.

Hirschorn, S.K., Dinglasan-Panlilio, M.J., Edwards, E.A., Lacrampe-Couloume, G., Sherwood Lollar, B., 2007. Isotope analysis as a natural reaction probe to determine mechanisms of biodegradation of 1,2-dichloroethane. Environ Microbiol 9, 1651-1657.

Hirschorn, S.K., Dinglasan, M.J., Elsner, M., Mancini, S.A., Lacrampe-Couloume, G., Edwards, E.A., Lollar, B.S., 2004. Pathway dependent isotopic fractionation during aerobic biodegradation of 1,2-dichloroethane. Environ Sci Technol 38, 4775-4781.

Hoefts, J., 1997. Stable Isotope Geochemistry. Springer-Verlag, Berlin.

Hoek, J., Canfield, D.E., 2007. Controls on isotope fractionation during dissimilatory sulfate reduction. In: Dahl, C., Friedrich, C.G. (Eds.). Microbial Sulfur Metabolism Springer, pp. 273-283.

Hoek, J., Reysenbach, A.-L., Habicht, K.S., Canfield, D.E., 2006. Effect of hydrogen limitation and temperature on the fractionation of sulfur isotopes by a deep-sea hydrothermal vent sulfate-reducing bacterium. Geochim. Cosmochim. Acta 70, 5831-5841.

Holliger, C., 1995. The anaerobic microbiology and biotreatment of chlorinated ethenes. Curr. Opin. Biotechnol. 6, 347-351.

Holliger, C., Gaspard, S., Glod, G., Heijman, C., Schumacher, W., Schwarzenbach, R.P., Vazquez, F., 1997. Contaminated environments in the subsurface and bioremediation: organic contaminants. FEMS Microbiol. Rev. 20, 517-523.

Holliger, C., Regeard, C., Diekert, G., 2003. Dehalogenation by anaerobic bacteria. In: Häggblom, M.M., Bossert, I.D. (Eds.). Dehalogenation Microbial processes and environmental applications. Kluwer Academic Publishers, Netherlands, pp. 115-157.

Howard, P.H., Meylan, W.M., 1997. Physical Properties of Organic Chemicals. CRC, Lewis Publishers, Boca Raton, FL.

Huber, T., Faulkner, G., Hugenholtz, P., 2004. Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20, 2317-2319. .

Huerta-Diaz, M.A., Carignan, R., Tessier, A., 1993. Measurement of trace metals associated with acid volatile sulfides and pyrite in organic freshwater sediments. Environ. Sci. Technol. 27, 2367-2372.

Hunkeler, D., Aravena, R., Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Hunkeler, D., Chollet, N., Pittet, X., Aravena, R., Cherry, J.A., Parker, B.L., 2004. Effect of source variability and transport processes on carbon isotope ratios of TCE and PCE in two sandy aquifers. J. Contam. Hydrol. 74, 265-282.

Hunkeler, D., Meckenstock, R.U., Sherwood-Lollar, B., Schmidt, T.C., Wilson, J.T., A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants using Compound Specific Isotope Analysis (CSIA). 2008. EPA 600/R-08/148.

Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., 2002. Manual of Environmental Microbiology. ASM Press, Washington, D.C. .

Huskey, W.P., 1991. In: Cook, P.F. (Ed.). Enzyme Mechanism from Isotope Effects. CRC Press, Boca Raton, FL, pp. 37-72.

Hwang, I., Batchelor, B., 2000. Reductive dechlorination of tetrachloroethylene by Fe(II) in cement slurries. Environ. Sci. Technol. 34, 5017-5022.

Igamberdiev, A.U., Ivlev, A.A., Bykova, N.V., Threlkeld, C.N., Lea, P.J., Gardestrom, P., 2001. Decarboxylation of glycine contributes to carbon isotope fractionation in photosynthetic organisms. Photosynth Res 67, 177-184.

Inagaki, F., Kuypers, M.M., Tsunogai, U., Ishibashi, J., Nakamura, K., Treude, T., Ohkubo, S., Nakaseama, M., Gena, K., Chiba, H., Hirayama, H., Nunoura, T., Takai, K., Jorgensen, B.B., Horikoshi, K., Boetius, A., 2006. Microbial community in a sediment-hosted CO2 lake of the southern Okinawa Trough hydrothermal system. Proc Natl Acad Sci U S A 103, 14164-14169.

Ivlev, A.A., Bykova, N.V., Igamberdiev, A.U., 1996. Fractionation of carbon (13C/12C) isotopes in glycine decarboxylase reaction. FEBS Lett 386, 174-176.

Jang, Y., Lin, C., Tsai, C., Fan, C., Pang, C., Lin, H., 2003. Monitoring reductive dechlorination in a tetrachloroethene-contaminated site. In: Magar, V.S., Kelley, M.E. (Eds.). In situ and on-site bioremediation - 2003. Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium. Battelle Press, Orlando.

Janssen, P.H., 1991. Fermentation of L-tartrate by a newly isolated gram-negative glycolytic bacterium. Antonie Van Leeuwenhoek 59, 191-198.

Jenkins, R., Snyder , R., 1996. Introduction to X-ray Powder Diffractometry. John Wiley & Sons, Inc., New York, NY.

Jeong, H.Y., Hayes, K.F., 2003. Impact of Transition Metals on Reductive Dechlorination Rate of Hexachloroethane by Mackinawite. Environ. Sci. Technol. 37, 4650-4655.

Jeong, H.Y., Kim, H., Hayes, K.F., 2007. Reductive dechlorination pathways of tetrachloroethylene and trichloroethylene and subsequent transformation of their dechlorination products by mackinawite (FeS) in the presence of metals. Environ. Sci. Technol. 41, 7736-7743.

Karickhoff, S.W., Brown, D.S., Scott, T.A., 1979. Sorption of hydrophobic pollutants on natural sediments. Water Research 13, 241-248.

Karsten, W.E., Cook, P.F., 2006. Substrate and pH dependence of isotope effects in enzyne catalyzed reactions. In: Kohen, A., Limbach, H.-H. (Eds.). Isotope Effects in Chemistry and Biology. CRC Press, Boca Raton, FL, pp. 793-809.

Kasenow, M., 2000. Applied Ground-water Hydrology and Well Hydraulics. Water Resources Publications, LLC.

Kennedy, L.G., Everett, J.W., Becvar, E., DeFeo, D., 2006a. Field-scale demonstration of induced biogeochemical reductive dechlorination at Dover Air Force Base, Dover, Delaware. J. Contam. Hydrol. 88, 119-136.

Kennedy, L.G., Everett, J.W., Gonzales, J., 2006b. Assessment of biogeochemical natural attenuation and treatment of chlorinated solvents, Altus Air Force Base, Altus, Oklahoma. J. Contam. Hydrol. 83, 221-236.

Kenneke, J.F., Weber, E.J., 2003. Reductive dehalogenation of halomethanes in iron- and sulfate-reducing sediments. 1. reactivity pattern analysis. Environ. Sci. Technol. 37, 713-720.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: the Ribosomal RNA Operon Copy Number Database. Nucleic Acids Res 29, 181-184.

Klausen, J., Troeber, S.P., Haderlein, S.B., Schwarzenbach, R.P., 1995. Reduction of Substituted Nitrobenzenes by Fe(II) in Aqueous Mineral Suspensions. Environ. Sci. Technol. 29, 2396-2404.

Kraeutler, B., Fieber, W., Ostermann, S., Fasching, M., Ongania, K.-h., Gruber, K., Kratky, C., Mikl, C., Siebert, A., Diekert, G., 2003. The cofactor of tetrachloroethene reductive dehalogenase of Dehalospirillum multivorans is norpseudo-B12, a new type of a natural corrinoid. Helv. Chim. Acta 86, 3698-3716.

Krajmalnik-Brown, R., Sung, Y., Ritalahti, K.M., Saunders, F.M., Loffler, F.E., 2007. Environmental distribution of the trichloroethene reductive dehalogenase gene (tceA) suggests lateral gene transfer among Dehalococcoides. FEMS Microbiol. Ecol. 59, 206-214.

Krasotkina, J., Walters, T., Maruya, K.A., Ragsdale, S.W., 2001. Characterization of the B12- and iron-sulfur-containing reductive dehalogenase from *Desulfitobacterium chlororespirans*. J Biol Chem 276, 40991-40997.

Krumholz, L.R., 1997. *Desulfuromonas chloroethenica sp. nov.* uses tetrachloroethylene and trichloroethylene as electron acceptors. International Journal of Systematic Bacteriology 47, 1262-1263.

Krumholz, L.R., Sharp, R., Fishbain, S.S., 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. Appl. Environ. Microbiol. 62, 4108-4113.

Kube, M., Beck, A., Zinder, S.H., Kuhl, H., Reinhardt, R., Adrian, L., 2005. Genome sequence of the chlorinated compound-respiring bacterium Dehalococcoides species strain CBDB1. Nat Biotechnol 23, 1269-1273.

Kuder, T., Wilson, J.T., Kaiser, P., Kolhatkar, R., Philp, P., Allen, J., 2005. Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: microcosm and field evidence. Environ. Sci. Technol. 39, 213-220.

Kurata, S., Kanagawa, T., Magariyama, Y., Takatsu, K., Yamada, K., Yokomaku, T., Kamagata, Y., 2004. Reevaluation and reduction of a PCR bias caused by reannealing

of templates. Appl. Environ. Microbiol. 70, 7545-7549.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

Lee, B.D., Schaller, K.D., Watwood, M.E., Apel, W.A., 2000. Transition metal catalyst-assisted reductive dechlorination of perchloroethylene by anaerobic aquifer enrichments. Biorem. J. 4, 97-110.

Lee, P.K.H., Conrad, M.E., Alvarez-Cohen, L., 2007. Stable carbon isotope fractionation of chloroethenes by dehalorespiring Isolates. Environ. Sci. Technol. 41, 4277-4285.

Lee, W., Batchelor, B., 2002a. Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 1. pyrite and magnetite. Environ. Sci. Technol. 36, 5147-5154.

Lee, W., Batchelor, B., 2002b. Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 2. green rust. Environ. Sci. Technol. 36, 5348-5354.

Lee, W., Batchelor, B., 2004a. Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing phyllosilicates. Chemosphere 56, 999-1009.

Lee, W., Batchelor, B., 2004b. Abiotic reductive dechlorination of chlorinated ethylenes by soil. Chemosphere 55, 705-713.

Liang, X., Dong, Y., Kuder, T., Krumholz, L.R., Philp, R.P., Butler, E.C., 2007. Distinguishing abiotic and biotic transformation of tetrachloroethylene and trichloroethylene by stable carbon isotope fractionation. Environ. Sci. Technol. 41, 7094-7100.

Liang, X., Philp, R.P., Butler, E.C., 2009. Kinetic and isotope analyses of tetrachloroethylene and trichloroethylene degradation by model Fe(II)-bearing

minerals. Chemosphere 75, 63-69.

Lightstone, F.C., Zheng, Y.J., Maulitz, A.H., Bruice, T.C., 1997. Non-enzymatic and enzymatic hydrolysis of alkyl halides: A haloalkane dehalogenation enzyme evolved to stabilize the gas-phase transition state of an S(N)2 displacement reaction. Proc Natl Acad Sci U S A 94, 8417-8420.

Loeffler, F.E., Cole, J.R., Ritalahti, K.M., Tiedje, J.M., 2003. Diversity of Dechlorinating Bacteria. In: Haggblom, M.M., Bossert, I.D. (Eds.). Dehalogenation: Microbial Processes and Environmental Applications. Kluwer Academic Publishers, Norwell, MA, pp. 53-87.

Loeffler, F.E., Ritalahti, K.M., Tiedje, J.M., 1997. Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. Appl. Environ. Microbiol. 63, 4982-4985.

Loeffler, F.E., Sun, Q., Li, J., Tiedje, J.M., 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species. Appl Environ Microbiol 66, 1369-1374.

Loubinoux, J., Mory, F., Pereira, I.A.C., Le Faou, A.E., 2000. Bacteremia caused by a strain of Desulfovibrio related to the provisionally named Desulfovibrio fairfieldensis. Journal of Clinical Microbiology 38, 931-934.

Louie, T.M., Mohn, W.W., 1999. Evidence for a chemiosmotic model of dehalorespiration in Desulfomonile tiedjei DCB-1. J Bacteriol 181, 40-46.

Lovley, D.R., Phillips, E.J.P., 1987. Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl. Environ. Microbiol. 53, 1536-1540.

Lu, X., Wilson, J.T., Shen, H., Henry, B.M., Kampbell, D.H., 2008. Remediation of TCE-contaminated groundwater by a permeable reactive barrier filled with plant mulch (Biowall). J. Environ. Sci. Health, Part A: Environ. Sci. Eng. 43, 24-35.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363-1371.

Luijten, M.L.G.C., de Weert, J., Smidt, H., Boschker, H.T.S., de Vos, W.M., Schraa, G., Stams, A.J.M., 2003. Description of *Sulfurospirillum halorespirans sp. nov.*, an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans comb. nov*. International Journal of Systematic and Evolutionary Microbiology 53, 787-793.

Mackay, D., Shiu, W.Y., Ma, K.-C., Lee, S.C., 2006. Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals. CRC Press LLC, Boca Raton, FL.

Mackay, D.M., Cherry, J.A., 1989. Groundwater Contamination - Pump-and-Treat Remediation .2. Environmental Science & Technology 23, 630-636.

Madigan, M.T., Martinko, J.M., Brock, T.D., 2006. Brock Biology of Microorganisms. Pearson Prentice Hall, Upper Saddle River, N. J. .

Magnuson, J.K., Romine, M.F., Burris, D.R., Kingsley, M.T., 2000. Trichloroethene reductive dehalogenase from Dehalococcoides ethenogenes: Sequence of tceA and substrate range characterization. Appl. Environ. Microbiol. 66, 5141-5147.

Magnuson, J.K., Stern, R.V., Gossett, J.M., Zinder, S.H., Burris, D.R., 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl Environ Microbiol 64, 1270-1275.

Maillard, J., Schumacher, W., Vazquez, F., Regeard, C., Hagen, W.R., Holliger, C., 2003. Characterization of the corrinoid iron-sulfur protein tetrachloroethene reductive dehalogenase of Dehalobacter restrictus. Appl Environ Microbiol 69, 4628-4638.

Maithreepala, R.A., Doong, R.-A., 2004a. Reductive dechlorination of carbon tetrachloride in aqueous solutions containing ferrous and copper ions. Environ. Sci. Technol. 38, 6676-6684.

Maithreepala, R.A., Doong, R.-a., 2004b. Synergistic Effect of Copper Ion on the Reductive Dechlorination of Carbon Tetrachloride by Surface-Bound Fe(II) Associated with Goethite. Environ. Sci. Technol. 38, 260-268.

Maithreepala, R.A., Doong, R.-A., 2005. Enhanced dechlorination of chlorinated methanes and ethenes by chloride green rust in the presence of copper(II). Environ. Sci. Technol. 39, 4082-4090.

Mancini, S.A., Hirschorn, S.K., Elsner, M., Lacrampe-Couloume, G., Sleep, B.E., Edwards, E.A., Lollar, B.S., 2006. Effects of Trace Element Concentration on Enzyme Controlled Stable Isotope Fractionation during Aerobic Biodegradation of Toluene. Environ. Sci. Technol. 40, 7675-7681.

Mariotti, A., Germon, J.C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., Tardieux, P., 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant soil 62, 413-430.

Massachusetts Department of Public Health, Final report of the Woburn environmental and birth study. 1996. Centers for Disease Control and Prevention, M.H.R.I.

Matheson, L.J., Tratnyek, P.G., 1994. Reductive Dehalogenation of Chlorinated Methanes by Iron Metal. Environ. Sci. Technol. 28, 2045-2053.

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

Meffre, S., Large, R.R., Scott, R., Woodhead, J., Chang, Z.S., Gilbert, S.E., Danyushevsky, L.V., Maslennikov, V., Hergt, J.M., 2008. Age and pyrite Pb-isotopic composition of the giant Sukhoi Log sediment-hosted gold deposit, Russia. Geochim. Cosmochim. Acta 72, 2377-2391.

Miller, E., Wohlfarth, G., Diekert, G., 1998. Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. Archives of Microbiology 169, 497-502.

Milliken, C.E., Meier, G.P., Sowers, K.R., May, H.D., 2004. Chlorophenol production by anaerobic microorganisms: transformation of a biogenic chlorinated hydroquinone metabolite. Appl. Environ. Microbiol. 70, 2494-2496.

Moran, M.J., Zogorski, J.S., Squillace, P.J., 2007. Chlorinated solvents in groundwater of the United States. Environ. Sci. Technol. 41, 74-81.

Morasch, B., Richnow, H.H., Schink, B., Meckenstock, R.U., 2001. Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl. Environ. Microbiol. 67, 4842-4849.

Morrill, P.L., Lacrampe-Couloume, G., Slater, G.F., Sleep, B.E., Edwards, E.A., McMaster, M.L., Major, D.W., Sherwood Lollar, B., 2005. Quantifying chlorinated ethene degradation during reductive dechlorination at Kelly AFB using stable carbon isotopes. Environ. Sci. Technol. 76, 279-293.

Morse, J.W., Millero, F.J., Cornwell, J.C., Rickard, D., 1987. The chemistry of the hydrogen sulfide and iron sulfide systems in natural waters. Earth Sci. Rev. 24, 1-42.

Neumann, A., Scholz, M., Diekert, G., 1994. Tetrachloroethene metabolism of

Dehalospirillum multivorans. Arch. Microbiol. 162, 295-301.

Neumann, A., Siebert, A., Trescher, T., Reinhardt, S., Wohlfarth, G., Diekert, G., 2002. Tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans*: substrate specificity of the native enzyme and its corrinoid cofactor. Arch Microbiol 177, 420-426.

Neumann, A., Wohlfarth, G., Diekert, G., 1996. Purification and characterization of tetrachloroethene reductive dehalogenase from Dehalospirillum multivorans. J Biol Chem 271, 16515-16519.

Neumann, A., Wohlfarth, G., Diekert, G., 1998. Tetrachloroethene dehalogenase from *Dehalospirillum multivorans*: cloning, sequencing of the encoding genes, and expression of the pceA gene in Escherichia coli. J Bacteriol 180, 4140-4145.

Ni, S., Fredrickson, J.K., Xun, L., 1995. Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of Desulfomonile tiedjei DCB-1. J Bacteriol 177, 5135-5139.

Nijenhuis, I., Andert, J., Beck, K., Kaestner, M., Diekert, G., Richnow, H.-H., 2005. Stable isotope fractionation of tetrachloroethene during reductive dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S and abiotic reactions with cyanocobalamin. Appl. Environ. Microbiol. 71, 3413-3419.

Nijenhuis, I., Nikolausz, M., Koeth, A., Felfoeldi, T., Weiss, H., Drangmeister, J., Grossmann, J., Kaestner, M., Richnow, H.-H., 2007. Assessment of the natural attenuation of chlorinated ethenes in an anaerobic contaminated aquifer in the Bitterfeld/Wolfen area using stable isotope techniques, microcosm studies and molecular biomarkers. Chemosphere 67, 300-311.

Nijenhuis, I., Zinder, S.H., 2005. Characterization of hydrogenase and reductive dehalogenase activities of Dehalococcoides ethenogenes strain 195. Appl Environ

Microbiol 71, 1664-1667.

Nikolausz, M., Nijenhuis, I., Ziller, K., Richnow, H.-H., Kaestner, M., 2006. Stable carbon isotope fractionation during degradation of dichloromethane by methylotrophic bacteria. Environ. Microbiol. 8, 156-164.

Nonaka, H., Keresztes, G., Shinoda, Y., Ikenaga, Y., Abe, M., Naito, K., Inatomi, K., Furukawa, K., Inui, M., Yukawa, H., 2006. Complete genome sequence of the dehalorespiring bacterium *Desulfitobacterium hafniense* Y51 and comparison with *Dehalococcoides ethenogenes* 195. J Bacteriol 188, 2262-2274.

Norris, R.D., Matthews, J.E., 1993. Handbook of Bioremediation CRC-Press.

Northrop, D.B., 1977. Determining the Absolute Magnitude of Hydrogen Isotope Effects. In: Cleland, W.W., O'Leary, M.H., Northrop, D.B. (Eds.). Isotope Effects on Enzyme Catalyzed Reaction. University Park Press, Baltimore, pp. 122-152.

Novak, P.J., Daniels, L., Parkin, G.F., 1998. Rapid dechlorination of carbon tetrachloride and chloroform by extracellular agents in cultures of Methanosarcina thermophila. Environ. Sci. Technol. 32, 3132-3136.

O'Leary, M.H., 1988. Carbon isotopes in photosynthesis. Bioscience 38, 328-336.

O'Leary, M.H., Yapp, C.J., 1978. Equilibrium carbon isotope effect on a decarboxylation reaction. Biochem. Biophys. Res. Commun. 80, 155-160.

O'Loughlin, E.J., Kemner, K.M., Burris, D.R., 2003. Effects of AgI, AuIII, and CuII on the Reductive Dechlorination of Carbon Tetrachloride by Green Rust. Environ. Sci. Technol. 37, 2905-2912.

Okeke, B.C., Chang, Y.C., Hatsu, M., Suzuki, T., Takamizawa, K., 2001. Purification, cloning, and sequencing of an enzyme mediating the reductive dechlorination of tetrachloroethylene (PCE) from *Clostridium bifermentans* DPH-1. Canadian Journal of Microbiology 47, 448-456.

Padan, E., Schuldiner, S., 1986. Intracellular pH regulation in bacterial cells. Methods Enzymol. 125, 337-352.

Padan, E., Schuldiner, S., 1987. Intracellular pH and membrane potential as regulators in the prokaryotic cell. J. Membr. Biol. 95, 189-198.

Pankow, J.F., Feenstra, S., Cherry, J.A., Ryan, C.M., 1996. Dense chlorinated solvents in groundwater: background and history of the problem. In: Pankow, J.F., Cherry, J.A. (Eds.). Dense Chlorinated Solvents and Other DNAPLs in Groundwater: History, Behavior and Remediation. Waterloo Press, Portland, OR, pp. 1-52.

Parsons, C., May 2006. Construction closeout report bark mulch trench interim corrective action for in-situ anaerobic bioremediation of chlorinated solvents in ground water Altus Air Force Base, Oklahoma. Prepared for Altus AFB Air Education and Training Command and the Air Force Center for Environmental Excellence, Brooks City-Base, Texas. Parsons, Denver, CO.

Pasakarnis, T.S., Gorski, C.A., O'Loughlin, E., Parkin, G.F., Scherer, M.M., 2006. Abiotic reduction of chlorinated ethenes in the presence of anaerobic bacteria. Preprints of Extended Abstracts presented at the ACS National Meeting. American Chemical Society, Atlanta, GA, pp. 957-961.

Pecher, K., Haderlein, S.B., Schwarzenbach, R.P., 2002. Reduction of Polyhalogenated Methanes by Surface-Bound Fe(II) in Aqueous Suspensions of Iron Oxides. Environ. Sci. Technol. 36, 1734-1741.

Rahm, B.G., Chauhan, S., Holmes, V.F., Macbeth, T.W., Sorenson, K.S., Jr., Alvarez-Cohen, L., 2006. Molecular characterization of microbial populations at two sites with differing reductive dechlorination abilities. Biodegradation 17, 523-534.

Richardson, R.E., Bhupathiraju, V.K., Song, D.L., Goulet, T.A., Alvarez-Cohen, L., 2002. Phylogenetic characterization of microbial communities that reductively

dechlorinate TCE based upon a combination of molecular techniques. Environ. Sci. Technol. 36, 2652-2662.

Riehl, J.F., Musaev, D.G., Morokuma, K., 1994. An ab-initio molecular-orbital study of the unimolecular dissociation reactions of dichloroethylene and trichloroethylene. J. Chem. Phys. 101, 5942-5956.

Ritalahti, K.M., Löffler, F.E., Rasch, E.E., Koenigsberg, S.S., 2005. Bioaugmentation for chlorinated ethene detoxification: Bioaugmentation and molecular diagnostics in the bioremediation of chlorinated ethene-contaminated sites. Industrial Biotechnology 1, 114-118.

Rosner, B.M., McCarty, P.L., Spormann, A.M., 1997. In Vitro Studies on Reductive Vinyl Chloride Dehalogenation by an Anaerobic Mixed Culture. Appl. Environ. Microbiol. 63, 4139-4144.

Schink, B., 1985. Fermentation of acetylene by an obligate anaerobe, *Pelobacter acetylenicus* sp. nov. Arch. Microbiol. 142, 295-301.

Scholz-Muramatsu, H., Neumann, A., Meßmer, M., Moore, E., Diekert, G., 1995. Isolation and characterization of Dehalospirillum multivorans gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium Arch. Microbiol. 163, 48-56.

Schumacher, W., Holliger, C., Zehnder, A.J., Hagen, W.R., 1997. Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of Dehalobacter restrictus. FEBS Lett 409, 421-425.

Schuth, C., Bill, M., Barth, J.A.C., Slater, G.F., Kalin, R.M., 2003. Carbon isotope fractionation during reductive dechlorination of TCE in batch experiments with iron samples from reactive barriers. J. Contam. Hydrol. 66, 25-37.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M., 2003. Environmental Organic Chemistry John Wiley&Sons, Inc., Hoboken, NJ.

Seshadri, R., Adrian, L., Fouts, D.E., Eisen, J.A., Phillippy, A.M., Methe, B.A., Ward, N.L., Nelson, W.C., Deboy, R.T., Khouri, H.M., Kolonay, J.F., Dodson, R.J., Daugherty, S.C., Brinkac, L.M., Sullivan, S.A., Madupu, R., Nelson, K.E., Kang, K.H., Impraim, M., Tran, K., Robinson, J.M., Forberger, H.A., Fraser, C.M., Zinder, S.H., Heidelberg, J.F., 2005a. Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science 307, 105-108.

Seshadri, R., Adrian, L., Fouts, D.E., Eisen, J.A., Phillippy, A.M., Methe, B.A., Ward, N.L., Nelson, W.C., Deboy, R.T., Khouri, H.M., Kolonay, J.F., Dodson, R.J., Daugherty, S.C., Brinkac, L.M., Sullivan, S.A., Madupu, R., Nelson, K.T., Kang, K.H., Impraim, M., Tran, K., Robinson, J.M., Forberger, H.A., Fraser, C.M., Zinder, S.H., Heidelberg, J.F., 2005b. Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science 307, 105-108.

Shao, H., Butler, E.C., 2007. The influence of iron and sulfur mineral fractions on carbon tetrachloride transformation in model anaerobic soils and sediments. Chemosphere 68, 1807-1813.

Sharma, P.K., McCarty, P.L., 1996a. Isolation and Characterization of a Facultatively Aerobic Bacterium That Reductively Dehalogenates Tetrachloroethene to cis-1,2-Dichloroethene. Appl. Environ. Microbiol. 62, 761-765.

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

Shen, H., Wilson, J.T., 2007. Trichloroethylene removal from groundwater in flow-through columns simulating a permeable reactive barrier constructed with plant mulch. Environ. Sci. Technol. 41, 4077-4083.

Sherwood Lollar, B., Slater, G.F., Ahad, J., Sleep, B., Spivack, J., Brennan, M.,

MacKenzie, P., 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: Implications for intrinsic bioremediation. Org. Geochem. 30, 813-820.

Siivola, J., Schmid, R.A., 2007. List of mineral abbreviations. In: Douglas, F., Desmons, J. (Eds.). Metamorphic Rocks: A Classification and Glossary of Terms. Recommendations of the International Union of Geological Sciences Subcommission on the Systematics of Metamorphic Rocks. Cambridge University Press, New York, pp. 93-110.

Sinke, A., Hecho, I.l., Monitored Natural Attenuation: Review of Existing Guidelines and Protocols. 1999. USEPA, TNO-MEP-R99/313.

Sivavec, T.M., Horney, D.P., 1996. Reductive dechlorination of chlorinated solvents by zero-valent iron, iron oxide and iron sulfide minerals. Book of Abstracts, 211th ACS National Meeting, New Orleans, LA.

Sivavec, T.M., Horney, D.P., 1997. Reduction of chlorinated solvents by Fe(II) minerals. Book of Abstracts, 213th ACS National Meeting, San Francisco, CA.

Sivavec, T.M., Mackenzie, P.D., Horney, D.P., 1997. Effect of site groundwater on reactivity of bimetallic media: Deactivation of nickel-plated granular iron. Book of Abstracts, 213th ACS National Meeting, San Francisco, pp. ENVR-005.

Slater, G.F., Ahad, J.M.E., Lollar, B.S., Allen-King, R., Sleep, B., 2000a. Carbon Isotope effects resulting from equilibrium sorption of dissolved VOCs. Anal. Chem. 72, 5669-5672.

Slater, G.F., Lollar, B.S., Edwards, E., Sleep, B., Witt, M., Klecka, G.M., Harkness, M.R., Spivack, J.L., 2000b. Carbon isotopic fractionation of chlorinated ethenes during biodegradation: field applications. Natural Attenuation Considerations and Case Studies: Remediation of Chlorinated and Recalcitrant Compounds, International

Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, pp. 17-24.

Slater, G.F., Lollar, B.S., Sleep, B.E., Edwards, E.A., 2001. Variability in carbon isotopic fractionation during biodegradation of chlorinated ethenes: implications for field applications. Environ. Sci. Technol. 35, 901-907.

Slater, G.F., Sherwood Lollar, B., Allen King, R., O'Hannesin, S., 2002. Isotopic fractionation during reductive dechlorination of trichloroethene by zero-valent iron: influence of surface treatment. Chemosphere 49, 587-596.

Slater, G.F., Sherwood Lollar, B., Lesage, S., Brown, S., 2003. Carbon isotope fractionation of PCE and TCE during dechlorination by vitamin B12. Ground Water Monit. Rem. 23, 59-67.

Smith, G.J., Rocha, E.R., Paster, B.J., 2006. The medically important Bacteroides spp. in health and disease. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.). Prokaryotes: a Handbook on the Biology of Bacteria Springer, pp. 381-427.

Song, D.L., Conrad, M.E., Sorenson, K.S., Alvarez-Cohen, L., 2002. Stable carbon isotope fractionation during enhanced in situ bioremediation of trichloroethene. Environ. Sci. Technol. 36, 2262-2268.

Srinivasan, K., Konstantinidis, A., Sinnott, M.L., Hall, B.G., 1993. Large changes of transition-state structure during experimental evolution of an enzyme. Biochem J 291 (Pt 1), 15-17.

Sun, B., Cole, J.R., Sanford, R.A., Tiedje, J.M., 2000. Isolation and characterization of Desulfovibrio dechloracetivorans sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. Appl Environ Microbiol 66, 2408-2413.

Sung, Y., 2006. Isolation and ecology of bacterial populations involved in reductive dechlorination of chlorinated solvents. Georgia Institute of Technology, p. 169 pp.

Sung, Y., Fletcher, K.E., Ritalahti, K.M., Apkarian, R.P., Ramos-Hernandez, N., Sanford, R.A., Mesbah, N.M., Loffler, F.E., 2006. Geobacter lovleyi sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. Appl. Environ. Microbiol. 72, 2775-2782.

Sung, Y., Ritalahti, K.M., Sanford, R.A., Urbance, J.W., Flynn, S.J., Tiedje, J.M., Loffler, F.E., 2003. Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis sp. nov*. Appl. Environ. Microbiol. 69, 2964-2974.

Suyama, A., Yamashita, M., Yoshino, S., Furukawa, K., 2002a. Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium sp* strain Y51. Journal of Bacteriology 184, 3419-3425.

Suyama, A., Yamashita, M., Yoshino, S., Furukawa, K., 2002b. Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium sp.* strain Y51. J Bacteriol 184, 3419-3425.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844-851.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876-4882.

Tront, J.M., Amos Benjamin, K., Loffler Frank, E., Saunders, F.M., 2006. Activity of *Desulfitobacterium sp*. strain Viet1 demonstrates bioavailability of 2,4-dichlorophenol previously sequestered by the aquatic plant Lemna minor. Environ.

Sci. Technol. 40, 529-535.

Tsukagoshi, N., Ezaki, S., Uenaka, T., Suzuki, N., Kurane, R., 2006. Isolation and transcriptional analysis of novel tetrachloroethene reductive dehalogenase gene from *Desulfitobacterium sp* strain KBC1. Applied Microbiology and Biotechnology 69, 543-553.

Ulrich, G.A., Krumholz, L.R., Suflita, J.M., 1997. A rapid and simple method for estimating sulfate reduction activity and quantifying inorganic sulfides. Appl. Environ. Microbiol. 63, 1627-1630.

USEPA, Monitored Natural Attenuation of Chlorinated Solvents. 1999. Agency, U.E.P., EPA/600/F-98/022.

USEPA, Cost Analyses for Selected Groundwater Clearup Projects: Pump and Treat Systems and Permeable Reactive Barriers. 2001a. Agency, U.E.P., EPA 542-R-00-013.

USEPA, Toxics Release Inventory Public Data Release Report. 2001b. Agency, U.E.P., 260-R-03-001.

USEPA, 2004 Edition of the Drinking Water Standards and Health Advisories. 2004. Agency, U.E.P., 822-R-04-005.

Utkin, I., Dalton, D.D., Wiegel, J., 1995. Specificity of reductive dehalogenation of substituted ortho-chlorophenols by *Desulfitobacterium dehalogenans* JW/IU-DC1 [Erratum to document cited in CA122:51008]. Appl. Environ. Microbiol. 61, 1677.

Utkin, I., Woese, C., Wiegel, J., 1994. Isolation and characterization of *Desulfitobacterium dehalogenans gen. nov., sp. nov.*, an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. International Journal of Systematic Bacteriology 44, 612-619.

Van Breukelen, B.M., Hunkeler, D., Volkering, F., 2005. Quantification of

sequential chlorinated ethene degradation by use of a reactive transport model incorporating isotope fractionation. Environ. Sci. Technol. 39, 4189-4197.

VanStone, N., Przepiora, A., Vogan, J., Lacrampe-Couloume, G., Powers, B., Perez, E., Mabury, S., Sherwood Lollar, B., 2005. Monitoring trichloroethene remediation at an iron permeable reactive barrier using stable carbon isotopic analysis. J. Contam. Hydrol. 78, 313-325.

VanStone, N.A., Focht, R.M., Mabury, S.A., Lollar, B.S., 2004. Effect of iron type on kinetics and carbon isotopic enrichment of chlorinated ethylenes during abiotic reduction on Fe(0). Ground Water 42, 268-276.

Villemur, R., Lanthier, M., Beaudet, R., Lepine, F., 2006. The *Desulfitobacterium* genus. FEMS Microbiol Rev 30, 706-733.

Wiedemeier, T.H., Rifai, H.S., Newell, C.J., Wilson, J.T., 1999. Natural Attenuation of Fuels and Chlorinated Solvents in the Subsurface John Wiley and Sons, Inc.

Wild, A., Hermann, R., Leisinger, T., 1996. Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene. Biodegradation 7, 507-511.

Wu, Q., Sowers, K.R., May, H.D., 2000. Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked chlorines, in a defined, sediment-free medium. Appl. Environ. Microbiol. 66, 49-53.

Yalkowsky, S.H., He, Y., 2003. Handbook of Aqueous Solubility Data. CRC Press LLC, Boca Raton, FL.

Yokoyama, K., Fujisawa, G., Yokoyama, A., 1995. The Mechanism of the Unimolecular Dissociation of Trichloroethylene Chcl=Ccl2 in the Ground Electronic-State. J. Chem. Phys. 102, 7902-7909.

Zinder, S.H., 2001. Reductive dechlorination by *Dehalococcoides ethenogenes*. Biologische Abwasserreinigun 15, 31-42.

Zwank, L., 2004. Assessment of the fate of organic groundwater contaminants using their isotopic signatures. Swiss Federal Institute of Technology (ETH), Zurich, Switzerland.

Zwank, L., Berg, M., Elsner, M., Schmidt, T.C., Schwarzenbach, R.P., Haderlein, S.B., 2005. New evaluation scheme for two-dimensional isotope analysis to decipher biodegradation processes: Application to groundwater contamination by MTBE. Environ. Sci. Technol. 39, 1018-1029.

APPENDIX A

Glossary

1,1-DCE	1,1-Dichloroethylene
AKIE	Apparent kinetic isotope effect
BDI	Bio-Dechlor Inoculum
BESA	2-bromo-ethanesulfonate
cis-DCE	*cis*-1,2-dichloroethylene
CI	Confidence interval
DCEs	Dichloroethylene isomers
DNAPL	Dense non-aqueous phase liquid
DP	Duck Pond, Norman, OK
GC-FID	Gas chromatography-flame ionization detector
GC-IRMS	Gas chromatography-isotope ratio mass spectrometer
HEPES	N-(2-hydroxyethyl)-piperazine-N'-3-propanesulfonic acid
KIE	Kinetic isotope effect
MCLs	Maximum contaminant levels
MNA	Monitored natural attenuation
NOM	Natural organic matter
PCE	Tetrachloroethylene
PCR	Polymerase chain reaction
PRB	Permeable reactive barrier
Rdhs	Reductive dehalogenases
RFLP	Restriction fragment length polymorphism
TAPS	[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS).
TCE	Trichloroethylene
TOC	Total organic carbon
trans-DCE	*trans*-1,2-dichloroethylene

USEPA	US Environmental Protection Agency
USGS	US Geological Survey
VC	Vinyl chloride

APPENDIX B

Supporting Information for Chapter 2

This appendix is the supporting information related to chapter 2, including additional data analysis and figures.

B1. Example Calculation of AKIE

In order to calculate AKIE, Eq. 2.1 was used. Hydrogenolysis is the typical pathway for microbial reductive dechlorination (Gossett and Zinder, 1997) and it is consistent to the observed sequentially dechlorinated intermediates/products in our study, so it is the only pathway considered. For PCE, x=2 and z=2, since both C atoms are identical chemically and therefore have equal potential for bond cleavage. For TCE, x=1 and z=1, since the lengths and therefore strengths of the C-Cl bonds vary with C position (Riehl et al., 1994; Yokoyama et al., 1995), and thus the two C atoms have different potentials for cleavage.

Take isotope fractionation of BB1 as an example, its ε_{bulk} for PCE and TCE calculated with the Eq. 1.1 to 1.3 are -1.39 ± 0.21 and -4.07 ± 0.48 ‰, respectively. Therefore, by using Eq. 2.1, AKIE for PCE reductive dechlorination by this species ($AKIE_{PCE,\ BB1}$) is calculated as:

$$AKIE_{PCE,BB1} = \frac{1}{1+(z\times n\times \varepsilon_{bulk}/(1000\times x))} = \frac{1}{1+(2\times 2\times(-1.39)/(1000\times 2))} = 1.00278$$

By using the same method, $AKIE_{TCE,\ BB1}$ is equal to 1.0082 assuming x=1 and z=1 as discussed as above.

The uncertainties for ε_{bulk} are expressed as 95 % confidence interval (CI). CI for AKIE was calculated by using propagation of error considerations shown in the equation as follows:

$$CI_{AKIE} = \sqrt{(\frac{\partial(AKIE)}{\partial(\varepsilon_{bulk})})^2 CI^2_{\varepsilon_{bulk}}} \quad \text{(A2.1)}$$

where CI_{AKIE} and $CI_{\varepsilon bulk}$ are confidence intervals for AKIE and ε_{bulk}, respectively. Based on Eq. 2.1, the differentiation of AKIE with respect to ε_{bulk} can be expressed as:

$$\frac{\partial(AKIE)}{\partial(\varepsilon_{bulk})} = \frac{-500}{(500+\varepsilon_{bulk})^2} \quad \text{(A2.2)}$$

Thus, the values of CI_{AKIE} for PCE and TCE reductive dechlorination by BB1 are 0.00043 and 0.0010, respectively.

B2. Example Calculation of P

Partitioning Factor (P) was calculated using Eq. 2.2. Assuming cobalamin (Vitamin B12) is the cofactor of functional enzymes catalyzing microbial reductive dechlorination (Krasotkina et al., 2001; Neumann et al., 2002; Maillard et al., 2003), the fractionation factors for cobalamin can be designated as $\alpha_{(kin)}$, which is equal to 1.0132±0.0015 (for PCE, $\alpha_{(kin,PCE)}$) or 1.0154±0.0021 (for TCE, $\alpha_{(kin,TCE)}$) (Cichocka et al., 2007). The fractionation factors for certain dechlorinating bacteria with PCE or TCE as the parent substrate are designated as $\alpha_{(1)}$.

Ninety-five percent confidence interval of P (CI_P) can be calculated using the equation as follows:

$$CI_P = \sqrt{(\frac{\partial P}{\partial \alpha_{(kin)}})^2 CI^2_{\alpha_{kin}} + (\frac{\partial P}{\partial \alpha_{(1)}})^2 CI^2_{\alpha_1}} \quad \text{(A2.3)}$$

where $CI_{\alpha_{(kin)}}$ and $CI_{\alpha_{(1)}}$ indicate the 95 % confidence interval of $\alpha_{(kin)}$ and $\alpha_{(1)}$, respectively; $\frac{\partial P}{\partial \alpha_{(kin)}}$ and $\frac{\partial P}{\partial \alpha_{(1)}}$ are the partial derivatives of P with respect to

$\alpha_{(kin)}$ and $\alpha_{(1)}$, respectively. Based on Eq. 2.2, $\frac{\partial P}{\partial \alpha_{(kin)}}$ and $\frac{\partial P}{\partial \alpha_{(1)}}$ can be expressed as:

$$\frac{\partial P}{\partial \alpha_{(kin)}} = \frac{1}{\alpha_{(1)} - 1} \tag{A2.4}$$

$$\frac{\partial P}{\partial \alpha_{(1)}} = \frac{1 - \alpha_{(kin)}}{(\alpha_{(1)} - 1)^2} \tag{A2.5}$$

Substituting Eq. A2.3 with Eq. A2.4 and A2.5, the CI_P can be expressed as:

$$CI_p = \sqrt{(\frac{1}{\alpha_{(1)} - 1})^2 CI_{\alpha_{kin}}^2 + \left[\frac{1 - \alpha_{(kin)}}{(\alpha_{(1)} - 1)^2}\right]^2 CI_{\alpha_1}^2}$$

(A2.6)

During PCE reductive dechlorination by BB1, the ε_{bulk} is -1.39±0.21 ‰, so its calculated fractionation factor ($\alpha_{PCE,BB1}$) is 1.00139±0.00021‰ (Eq. 2.3). Thus, the value of P for BB1 during PCE reductive dechlorination ($P_{PCE, BB1}$) can be calculated as:

$$P_{PCE,BB1} = \frac{\alpha_{(kin,PCE)} - \alpha_{PCE,BB1}}{\alpha_{PCE,BB1} - 1} = \frac{1.0132 - 1.00139}{1.00139 - 1} = 8.5$$

The 95 % confidence interval for $P_{PCE, BB1}$ ($CI_{PCE, BB1}$) is calculated using Eq. A2.6.

$$CI_{PCE,BB1} = \sqrt{(\frac{1}{\alpha_{(PCE,BB1)} - 1})^2 CI_{\alpha_{(kin,PCE)}}^2 + \left[\frac{1 - \alpha_{(kin,PCE)}}{(\alpha_{(PCE,BB1)} - 1)^2}\right]^2 CI_{\alpha_1}^2}$$

$$= \sqrt{(\frac{1}{1.00139 - 1})^2 0.0015^2 + (\frac{1 - 1.0132}{(1.00139 - 1)^2})^2 0.00021^2} = 1.8$$

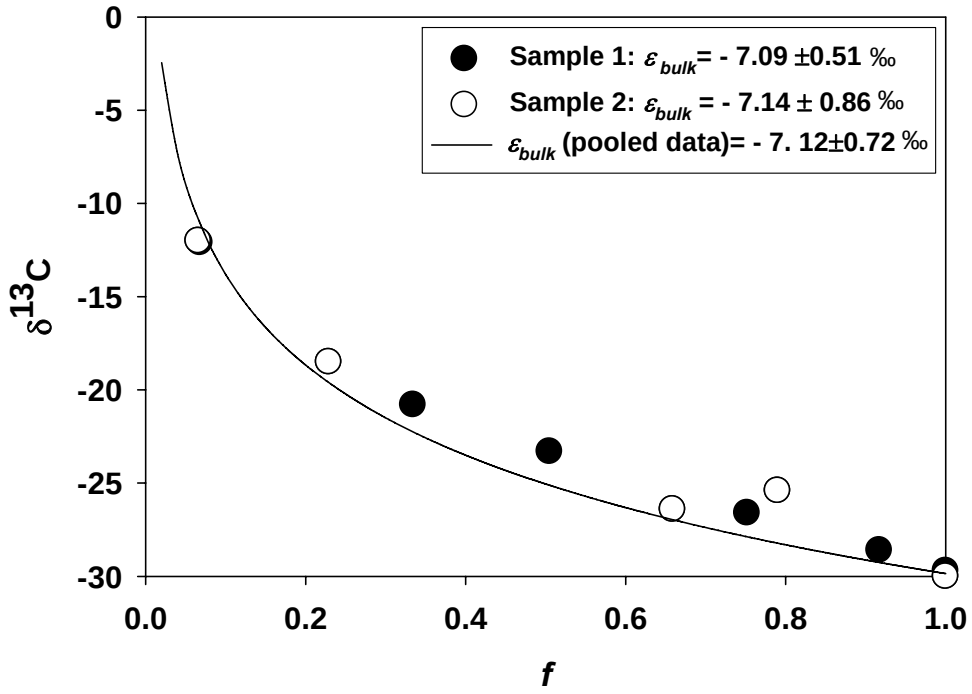

Figure B1. Duplicate experiments showing isotope fractionation for the reductive dechlorination of PCE by BDI. Each experiment was done with a separate microcosm under identical conditions. ε (pooled data) was calculated by nonlinear regression using pooled data from both microcosms.

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                          ....|....|....|....| ....|....| ....|....| ....|....|
                               10        20        30        40        50
D. hafniense St TCE       -----MGEIN RRNFLKVSIL GAAAAAVASA SAVKGMVSPL VADAADIVAP
D. hafniense St Y51       -----MGEIN RRNFLKVSIL GAAAAAVASA SAVKGMVSPL VADAADIVAP
D. restrictus             -----MGEIN RRNFLKASML GAAAAAVASA SAVKGMVSPL VADAADIVAP
D. sp. PCE-S              -----MGEIN RRNFLKASML GAAAAAVASA SVVKGVVSPL VADAADIVAP
D. sp. CR1                -----MGEIN RRNFLKASML GAAAAAVAPA PAVKGTVSPL VAEAADIVAP
D. hafniense DC           ----MKMNLD RRSFLKASLV SVAAVAAASA AAAKETFAPL TAEAAEIIAP
G. lovleyi SZ             --------MD RRDFFRMAAL TSVVAGAAVI SSPQKSSARL VLS-------
S. halorespirans          MEKKKKPELS RRDFGKLIIG AGAAATIAPF GVPGANAAEK EKNAAEIRQQ
S. multivorans            MEKKKKPELS RRDFGKLIIG GGAAATIAPF GVPGANAAEK EKNAAEIRQQ
D. sp. SNR-PCE*           MSEKYHSTVT RRDFMKR--L GLAGAGAGAL GAAVLAENNL PHEFKDVDDL
D. sp. SFR-cis-DCE*       MSEKYHSTVT RRDFMKR--L GLAGAGAGAL GAAVLAENNL PHEFKDVDDL
D. ethenogenes*           MSEKYHSTVT RRDFMKR--L GLAGAGAGAL GAAVLAENNL PHEFKDVDDL
D. ethenogenes            -MLNFHSTLT RKDFLKG--- ------IGMA GAGLGAASAV APMFHDLDEL
D. sp. KBC1               MDREKENTLD QKEEKRSVGI SRRNFFKASG IAAGVAALGL VTKSQPVYAG
C. bifermentans           ---------- ---------- ---------- -------MNR KVLALVIPAL

                          ....|....|....|....| ....|....| ....|....| ....|....|
                               60        70        80        90        100
D. hafniense St TCE       ITETSEFPYK VDAKYQRYNS LKNFFEKTFD PEANKTPIKF HYDDVSKITG
D. hafniense St Y51       ITETSEFPYK VDAKYQRYNS LKNFFEKTFD PEANKTPIKF HYDDVSKITG
D. restrictus             ITETSEFPYK VDAKYQRYNS LKNFFEKTFD PEANKTPIKF HYDDVSKITG
D. sp. PCE-S              ITETSEFPYK VDAKYQRYNS LKNFFEKTFD PEENKTPIKF HYDDVSKITG
D. sp. CR1                ITETSEFPYK VDAKYQRYNC LNNFFEKACD PEANKTPIKF HYDDVSKITG
D. hafniense DC           IRETAEFPYQ VDPKYQRLPA EKLAYLRMFD PEENKGPIKF HFDDVSKITG
G. lovleyi SZ             -KEQDEFPYE ISSDFKGMPQ TNCIFCRVFS ---------- ----------
S. halorespirans          FAMTAGSPII VNDKLERYAQ VRTAFTHPTS ---------- ----------
S. multivorans            FAMTAGSPII VNDKLERYAE VRTAFTHPTS ---------- ----------
D. sp. SNR-PCE*           LSAGKALEGD HANK-VNNHP WWVTTRDHED PTCNIDWSLI KRYSGWNNQG
D. sp. SFR-cis-DCE*       LSAGKALEGD HANK-VNNHP WWVTTRDHED PTCNIDWSLI KRYSGWNNQG
D. ethenogenes*           LSAGKALEGD HANK-VNNHP WWVTTRDHED PTCNIDWSLI KRYSGWNNQG
D. ethenogenes            VASTP----- ----STRNLP WFVKEREHGD PTTPIDWDMI QRR-------
D. sp. KBC1               QESESAIVNF AVQEVDQSPY NLPPFANAEN ---------- ----------
C. bifermentans           LAAGAAHAAE VYNKDGNKLD LYG------- ---------- ----------

                          ....|....|....|....| ....|....| ....|....| ....|....|
                              110       120       130       140       150
D. hafniense St TCE       KKDTGKDLPT LNAERLGIKG RPATHTETSI LFHTQHLGAM LTQRHNETGW
D. hafniense St Y51       KKDTGKDLPT LNAERLGIKG RPATHTETSI LFHTQHLGAM LTQRHNETGW
D. restrictus             KKDTGKDLPT LNAERLGIKG RPATHTETSI LFQTQHLGAM LTQRHNETGW
D. sp. PCE-S              KKDTGKDLPM LNAERLGIKG RPATHTETSI LFHTQHLGAM LTQRHNETGW
D. sp. CR1                KKDTGKDLPT LNAERLGIKG RPATHAETGV LFYTQHIGAM LTQRHNETGW
D. hafniense DC           KKDTGKDLPL LNAESLGIKG RPATLSETGA IFFSHHDGSV LPLREKEMGW
G. lovleyi SZ             ------DKDA VVDEYVQKTY GLTKIDQMGA MLASSKDGFV HPEQHGEPGF
S. halorespirans          ---------- --------MF KPNYKGEVKH WFLSSCDEKV RQIENGENGP
S. multivorans            ---------- --------FF KPNYKGEVKP WFLSAYDEKV RQIENGENGP
D. sp. SNR-PCE*           AYFLPEDYLS PTYTGRRHTI VDSKLEIELQ GKKYRDSAFI KSGIDWMKEN
D. sp. SFR-cis-DCE*       AYFLPEDYLS PTYTGRRHTI VDSKLEIELQ GKKYRDSAFI KSGIDWMKEN
D. ethenogenes*           AYFLPEDYLS PTYTGRRHTI VDSKLEIELQ GKKYRDSAFI KSGIDWMKEN
D. ethenogenes            ----PYTWVR MDPTLPVYDN LKSIGAPVSR WLDWEDKKAE DEILYAKARE
D. sp. KBC1               -----LKRYE LGKNAFYSKE LSMDKFGGNP WHIEAEKYIV KFIKEGVPGY
C. bifermentans           ---------- --KVDGLHYF SDDANSDGDQ TYMRMGFKGE TQVNDMITGY

                          ....|....|....|....| ....|....| ....|....| ....|....|
                              160       170       180       190       200
D. hafniense St TCE       TGLDEALNAG AWAVEFDYSG FNATGGGPGS VIPLYPINPM TNEIAN-EPV
D. hafniense St Y51       TGLDEALNAG AWAVEFDYSG FNATGGGPGS VIPLYPINPM TNEIAN-EPV
D. restrictus             TGLDEALNAG AWAVEFDYSG FNAAGGGPGS VIPLYPINPM TNEIAN-EPV
D. sp. PCE-S              TGLDEALNAG AWAVEFDYSG FNAAGGGPGS AIPLYPINPM TNEIAN-EPV
D. sp. CR1                TGLDEALNAG AWAVEFDYSG FNAAGGGPGS VITPYPINPM TNEIAN-EPV
D. hafniense DC           RALDMALVVA SWSVEYHYNG FTAPGSGPGG VIAHYPFNPM TNETGT-EPV
G. lovleyi SZ             TAVDKALELA GWATNDEFSP YAEFGRR-NS LIGTHIVNPV TGKIAKDKPV
S. halorespirans          KMKAKNVGEA RAGRALEAAG WTLDXNFGGS FGSYYPN--- -------RFS
S. multivorans            KMKAKNVGEA RAGRALEAAG WTLDINYG-- --NIYPN--- -------RFF
D. sp. SNR-PCE*           IDPDYDPGEL GYGDRREDAL IYAATNGSHN CWENPLYGRY EGSRPYLSMR
D. sp. SFR-cis-DCE*       IDPDYDPGEL GYGDRREDAL IYAATNGSHN CWENPLYGRY EGSRPYLSMR
D. ethenogenes*           IDPDYDPGEL GYGDRREDAL IYAATNGSHN CWENPLYGRY EGSRPYLSMR
D. ethenogenes            DFPGWEPGLD GFGDIRTTAL THASEMFSFG NFPTRMNLGG NMVDLVAAVR
D. sp. KBC1               SLMDNAFYDA AWASYKGTPL FSWEPLG--- ---------- ----------
C. bifermentans           GQWEYQVQAN GTEGDKGDSW TRLAFAG--- ---------- ----------
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                    ....|....|....|....| ....|....| ....|....| ....|....|
                            210        220        230        240        250
D. hafniense St TCE   MVPGLYNWDN IDVESVRQQG Q-QWKFE--- -SKEEASKIV KKATRLLGAD
D. hafniense St Y51   MVPGLYNWDN IDVESVRQQG Q-QWKFE--- -SKEEASKIV KKATRLLGAD
D. restrictus         MVPGLYNWDN IDVESVRQQG Q-QWKFE--- -SKEEASKMV KKATRLLGAD
D. sp. PCE-S          MVPGLYNWDN IDVESVRQQG Q-QWKFE--- -SKEEASKIL KKATRLLGAD
D. sp. CR1            MVPGLYNWDN SDVEGVRQQG Q-QWKFK--- -SKEEASKMV KKAACFLGAD
D. hafniense DC       FLAGMYSWDN TKARERREQG R-QWKFE--- -SVEEASRIV KKAARFLGAD
G. lovleyi SZ         FVPGLHTWDN SRAEYEIKRG DGRYQFK--- -DKQEATDRI KRACRYLGAD
S. halorespirans      MLWSGETMLN TQMWATVGLD RRPPDTT--- -DPVELTNYV KFAARMAGAD
S. multivorans        MLWSGETMTN TQLWAPVGLD RRPPDTT--- -DPVELTNYV KFAARMAGAD
D. sp. SNR-PCE*       TMNGINGLHE FGHADIKTTN YPKWEG---- -TPEENLLIM RTAARYFGAS
D. sp. SFR-cis-DCE*   TMNGINGLHE FGHADIKTTN YPKWEG---- -TPEENLLIM RTAARYFGAS
D. ethenogenes*       TMNGINGLHE FGHADIKTTN YPKWEG---- -TPEENLLIM RTAARYFGAS
D. ethenogenes        AAGGYLGSTD SYAGPKMVHT PEEMGGTKYQ GTPEDNLRTL KAGIRYFGGE
D. sp. KBC1           ---------- -VSNIKRAET VGKWEAT--- --PEQNNRYI KKVANEYGSG
C. bifermentans       -KVGDYGSFD YGRNYGVMYD VEGWTDM--- -LPEFGGDSY TKADNFMTGR

                    ....|....|....|....| ....|....| ....|....| ....|....|
                            260        270        280        290        300
D. hafniense St TCE   LVGIAPYD-- ERWTYSTWGR KIYKPCKMP- ---------- NGRTKYLPWD
D. hafniense St Y51   LVGIAPYD-- ERWTYSTWGR KIYKPCKMP- ---------- NGRTKYLPWD
D. restrictus         LVGIAPYD-- ERWTYSTWGR KILKPCKMP- ---------- NGRTKYLPWD
D. sp. PCE-S          LVGIAPYD-- ERWTYSTWGR KIQKPCKMP- ---------- NGRTKYLPWD
D. sp. CR1            XAGIAPYD-- ERWTYSTWGR KIQKPFKMP- ---------- NGRTKLMPWD
D. hafniense DC       MAGIAPYD-- DRWTFSTWCR PNLKPFKLP- ---------- NGRTEYFPTD
G. lovleyi SZ         LVGVTSFERA QKWVYTNWLD RHPMKNTVP- ---------- DGTVKMMTYD
S. halorespirans      LVGVARLN-- RNWVYS---- -----GAVT- ---------- IPD----EQS
S. multivorans        LVGVARLN-- RNWVYS---- -----EAVT- ---------- IPADVPYEQS
D. sp. SNR-PCE*       SVGAIKITDN VKKIFYAKAQ PFCLGPWYT- ITNMAEYIEY PVPVDNYAIP
D. sp. SFR-cis-DCE*   SVGAIKITDN VKKIFYAKAQ PFCLGPWYT- ITNMAEYIEY PVPVDNYAIP
D. ethenogenes*       SVGAIKITDN VKKIFYAKVQ PFCLGPWYT- ITNMAEYIEY PVPVDNYAIP
D. ethenogenes        DVGALELDDK LKKLIFTVDQ YGKALEFGD- ---------- ----------
D. sp. KBC1           DTGVAVLNEQ WFLSQDEKGK PYVFSTEHS- ---------- ----------
C. bifermentans       ANGVATYRNT DFFGLVDGLN VALQYQGAN- ---------- ----------

                    ....|....|....|....| ....|....| ....|....| ....|....|
                            310        320        330        340        350
D. hafniense St TCE   LPKMLSGGGV EVFGHAKFEP DWEKYAGFKP KSVIVFVLEE DYEAIRTSPS
D. hafniense St Y51   LPKMLSGGGV EVFGHAKFEP DWEKYAGFKP KSVIVFVLEE DYEAIRTSPS
D. restrictus         LPKMLSGGGV EVFGHAKFEP DWEKYAGFKP KSVIVFVLEE DYEAIRTSPS
D. sp. PCE-S          LPKMLSGGGV EVFGHAKFEP DWEKYAGFKP KSVIVFVLEE DYEAIRTSPS
D. sp. CR1            LPKILSGGGV EVFGHAKFEP DWEKYAGFKP KSVIVFVLKE DYEAIRTSPS
D. hafniense DC       PFKLMKG-EV EVYGSTSVEA DWEKYAGFTP KSVIAMTFEM DYEAYRTAPS
G. lovleyi SZ         AMEAQKGNCI SAG-YGVSPP DFRAESGFEP KSVITLAWAM DYDAMKTAPS
S. halorespirans      WHKEIEKPIV FKDVPLPIET DDELIIPNTC DNVIVSGIAM NREMLQTAPT
S. multivorans        LHKEIEKPIV FKDVPLPIET DDELIIPNTC ENVIVAGIAM NREMMQTAPN
D. sp. SNR-PCE*       IVFEDIPADQ GHYSYKRFGG DDKIAVPNAL DNIFTYTIML PEKRFKYAHS
D. sp. SFR-cis-DCE*   IVFEDIPADQ GHYSYKRFGG DDKIAVPNAL DNIFTYTIML PEKRFKYAHS
D. ethenogenes*       IVFEDIPADQ GHYSYKRFGG DDKIAVPNAL DNIFTYTIML PEKRFKYAHS
D. ethenogenes        ---------- ---VEECIET PKQVTIPNKC KYIFLWTMRQ PYEWTRRQSG
D. sp. KBC1           ---------- -----KPTIT EEAYYIPKTM NRVIVMLAPM NPNMLKYAPT
C. bifermentans       ---------- ---------- ENQSPEQEGT NNGGDDRNMK NSNGDGFGIS

                    ....|....|....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400
D. hafniense St TCE   VISSATVGKS YSNMAEVAYK IAVFLRKLGY YAAPC-GNDT GISVPMAVQA
D. hafniense St Y51   VISSATVGKS YSNMAEVAYK IAVFLRKLGY YAAPC-GNDT GISVPMAVQA
D. restrictus         VISSATVGKS YSNMAEVAYK IAVFLRKLGY YAAPC-GNDT GLSVPMAVQA
D. sp. PCE-S          VISSATVGKS YSNMAEVAYK IAVFLRKLGY YAAPC-GNDT GISVPMAVQA
D. sp. CR1            VISGAATGKS YSNLAEVAYK IAVFLRKLGY YAAAC-GNDT GLNVPMAVQA
D. hafniense DC       VLQGAAPGKS YSNMGEVAYK VASFLREIGY NAVPS-GNDT GMSVPIAVQA
G. lovleyi SZ         LVAGAAAGEG YSRLAEISYK VSTFLRRLGI KCAPC-GNDT AASIPIAIES
S. halorespirans      SMACATVAFC YSRMGVFDMW LCQFIRYMGY YAIPC-CNTV GQSVALAVEA
S. multivorans        SMACATTAFC YSRMCMFDMW LCQFIRYMGY YAIPS-CNGV GQSVAFAVEA
D. sp. SNR-PCE*       IPMDPCSCIA YPLFTEVEAR IQQFIAGLGY NSMGGGVEAW GPGSAFGNLS
D. sp. SFR-cis-DCE*   IPMDPCSCIA YPLFTEVEAR IQQFIAGLGY NSMGGGVEAW GPGSAFGNLS
D. ethenogenes*       IPMDPCSCIA YPLFTEVEAR IQQFIAGLGY NSMGGGVEAW GPGSAFGNLS
D. ethenogenes        RFEGAATETS YERAYNTKAH FQDFVRGLGY QMISAGNNSL SPAGAWAVLG
D. sp. KBC1           TLSEATVGTE YSQMAESAGK MAEFIRGLGY NAIPMGNDAS -LSVPIAIDA
C. bifermentans       STYDLGMGVS FGAAYTSSDR TNEQVNDS-- TAGGDKADAW TVGLKYDANN
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                          ....|....|....|....| ....|....| ....|....| ....|....|
                                410       420        430        440        450
D. hafniense St TCE       GLGEAGRNGL LITQKFGPRH R-IAKVYTDL ELAPDKPR-K FGVREFCRLC
D. hafniense St Y51       GLGEAGRNGL LITQKFGPRH R-IAKVYTDL ELAPDKPR-K FGVREFCRLC
D. restrictus             GLGEAGRNGL LITQKFGPRH R-IAKVYTDL ELAPDKPR-K FGVREFCRLC
D. sp. PCE-S              GLGEAGRNGL LITQKFGPRH R-IAKVYTDL ELAPDKPR-K FGVREFCRLC
D. sp. CR1                GLGEAGRNGL LITQKFGPRH R-IAKVYTDL ELAPDKPR-K FGVREFCRLC
D. hafniense DC           GLGEAGRSGQ LITQKYGPRV R-IAKVYTDL ELVPDKPI-N IGAREFCRLC
G. lovleyi SZ             GMGEGSRMGM LITEKYGPRV R-LAKIFTDI ELVPDKPR-T FGVKDFCKNC
S. halorespirans          GLGQASRMGA CITPEFGPNV R-LTKVFTNM PLVPDKPI-D FGVTEFCETC
S. multivorans            GLGQASRMGA CITPEFGPNV R-LTKVFTNM PLVPDKPI-D FGVTEFCETC
D. sp. SNR-PCE*           GLGEQSRVSS TIEPRYGSNT KGSLRMLTDL PLAPTKPI-D AGIREFCKTC
D. sp. SFR-cis-DCE*       GLGEQSRVSS TIEPRYGSNT KGSLRMLTDL PLAPTKPI-D AGIREFCKTC
D. ethenogenes*           GLGEQSRVSS IIEPRYGSNT KGSLRMLTDL PLAPTKPI-D AGIREFCKTC
D. ethenogenes            GLGELSRASY VNHPLYGITV RVTWGFLTDM PLPPSRPI-D FGARKFCETC
D. sp. KBC1               GLGELGRHGL LVHPEYGSSV R-ISKVLTDL PIAPDKPI-S FGAAEFCRTC
C. bifermentans           IYLATMYSET RNMTPYGGSN G------SDN TIANKTQN-F EVTAQYQFDF

                          ....|....|....|....| ....|....| ....|....| ....|....|
                                460       470        480        490        500
D. hafniense St TCE       KKCADACPAQ AISHEKDPKV LQ---PEDCE V-----AENP YTEKWHLDSN
D. hafniense St Y51       KKCADACPAQ AISHEKDPKV LQ---PEDCE V-----AENP YTEKWHLDSN
D. restrictus             KKCADACPAQ AISHEKDPKV LQ---PEDCE V-----AENP YTEKWHLDSN
D. sp. PCE-S              KKCADACPAQ AISHEKDPKV LQ---PEDCE A-----SENP YTEKWHVDSE
D. sp. CR1                KKCADACPAQ AISHEKDPKV LQ---PEDCE V-----AENP YTEKWHLDSN
D. hafniense DC           LKCADVCPAQ AISHVKDPWV LQ---PEDCT P-----SENP YTEKWQFDSQ
G. lovleyi SZ             MKCADACPAK AISKDPAQVY KV---GQETS VGKINKSHLA GVEHYYVNAE
S. halorespirans          KKCARECPSK AITEGPRTFE GR---SIHNQ S--------- GKLQWQNDHS
S. multivorans            KKCARECPSK AITEGPRTFE GR---SIHNQ S--------- GKLQWQNDYN
D. sp. SNR-PCE*           GICAEHCPTQ AISHEGPRYD SP---HWDCV S--------- GYEGWHLDYH
D. sp. SFR-cis-DCE*       GICAEHCPTQ AISHEGPRYD SP---HWDCV S--------- GYEGWHLDYH
D. ethenogenes*           GICAEHCPTQ AISHEGPRYD SP---HWDCV S--------- GYEGWHLDYH
D. ethenogenes            GICAENCPFG AINPGEPTWK DDNAFGNP-- ---------- GFLGWRCDYT
D. sp. KBC1               MKCAEACPSE SISKDKDPSD KVACASNNP- ---------- GMKKWYVNTW
C. bifermentans           GLRPEVSFLM SKGKDLGVNG SD-------- ---------- ----------

                          ....|....|....|....| ....|....| ....|....| ....|....|
                                510       520        530        540        550
D. hafniense St TCE       RCGSFWAYNG SPCSNCVAVC SWN--KVETW NHD-VARIAT QIPLLQDAAR
D. hafniense St Y51       RCGSFWAYNG SPCSNCVAVC SWN--KVETW NHD-VARVAT QIPLLQDAAR
D. restrictus             RCGSFWAYNG SPCANCVAVC SWN--KVETW NHD-VARIAT QIPLLQDAAR
D. sp. PCE-S              RCGSFWAYNG SPCSNCVAVC SWN--KVETW NHD-VARVAT QIPLLQDAAR
D. sp. CR1                RCGSFWAYNG GPCNNCVAVC SYN--KVETW NHD-VARVAT QIPLLQDAAR
D. hafniense DC           RCLSFFAYNG GDCGSCIAVC SWN--KIDAW QHD-VARIAT QIPLVQDAAR
G. lovleyi SZ             RCFGYWVATG TTCGTCVAVC PYN--KIDEW HHD-LTKIAT LTPFKP-LLR
S. halorespirans          KCLDYWPESG GNCGTCFAVC PFT--KGNIW IHDGVEWLID NTRFLDPLML
S. multivorans            KCLGYWPESG GYCGVCVAVC PFT--KGNIW IHDGVEWLID NTRFLDPLML
D. sp. SNR-PCE*           KCTN------ --CTICEAVC PFFTMSNNSW VHNLVKSTVA TTPVFNGFFK
D. sp. SFR-cis-DCE*       KCTN------ --CTICEAVC PFFTMSNNSW VHNLVKSTVA TTPVFNGFFK
D. ethenogenes*           KCIN------ --CTICEAVC PFFTMSNNSW VHNLVKSTVA TTPVFNGFFK
D. ethenogenes            KCPH------ --CPICQGTC PFN-SHPGSF IHDVVKGTVS TTPIFNSFFK
D. sp. KBC1               TCLNQWVENG GGCNICLSAC PYN--KPKTW IHDVVKGVSA KTTVFNSTFA
C. bifermentans           ---------- ---------- ---------- -GDQDLVKYA SVGATYYFNK

                          ....|....|....|....| ....|....| ....|....| ....|....|
                                560       570        580        590        600
D. hafniense St TCE       KFDEWFGYNG PVNPDERLES GYVQN-MVKD FWNNPESIKQ ------
D. hafniense St Y51       KFDEWFGYNG PVNPDERLES GYVQN-MVKD FWNNPESIKQ ------
D. restrictus             KFDEWFGYNG PVNPDERLES GYVQN-MVKD FWNNPESIKQ ------
D. sp. PCE-S              KFDEWFGYSG PVNPDERLES GYVQN-MVKD FWNNPESIKQ ------
D. sp. CR1                KFDEWFGYNG PVNPEERLES GYVQN-MVTD FWNNPESIKQ ------
D. hafniense DC           KFDEWFGYNG PVNPEERIES GYIAN-MVKD FWKDTEPTK- ------
G. lovleyi SZ             HLDELFGYGG PLD-KTRSKS KWFKD-AVAD FWNKA----- ------
S. halorespirans          GMDDALGYGA KRNITEIWDG KINTYGLDAD HFRDTVSFRK DRVKKS
S. multivorans            GMDDALGYGA KRNITEVWDG KINTYGLDAD HFRDTVSFRK DRVKKS
D. sp. SNR-PCE*           NMEEAFGYGP RYSPSRDEWW ASENPIRGAS VDIF------ ------
D. sp. SFR-cis-DCE*       NMEEAFGYGP RYSPSRDEWW ASENPIRGAS VDIF------ ------
D. ethenogenes*           NMEGAFGYGP RYSPSRDEWW ASENPIRGAS VDIF------ ------
D. ethenogenes            NMEKTFKYGR KNPATWWDEV DDYPYGVDTS Y--------- ------
D. sp. KBC1               TLDDALGYGT HDKNPKEFWD SDKNVPKWW- ---------- ------
C. bifermentans           NFSTYVDYKI NLLDEDKNFY SQKRHLYR-- ---------- ------
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Figure B2. Alignment of amino acid sequences of different PCE and TCE reductive dehalogenases. This figure is modified from that created by Holliger (Holliger et al., 2003). The TCE reductive dehalogenases (TceA) are marked with * on the name of the species in which they are identified. The black boxes indicate conserved amino acids involved in all the consensus sequence; grey boxes amino acid conserved in consensus sequence motifs, including iron-sulfur cluster binding motifs. Alignment was performed with Clustal W. (Thompson et al., 1997). *D. hafniense* St TCE: Desulfitobacterium hafniense TCE1 (CAD28792); *D. hafniense* St Y51: Desulfitobacterium hafniense Y51 (BAC00915) (Suyama et al., 2002a); *D. restrictus*: *Dehalobacter restrictus* (CAD28790); *D sp*. PCE-S: *Desulfitobacterium sp*. PCE-S (AAO60102) (Miller et al., 1998); *D. sp.* CR1: *Desulfitobacterium sp*. CR1 (BAF57046); *D. sp.* DCB: *Desulfitobacterium hafniense* DCB-2 (YP_002457196); *G. lovleyi* SZ: *Geobacter lovleyi* SZ (YP_001953103); *S. halorespirans*: *Sulfurospirillum halorespirans* (AAG46194); *S. multivorans*: *Sulfurospirillum multivorans* (AAC60788) (Neumann et al., 1998); *D. sp*. SNR-PCE: *Dehalococcoides sp*. SNR-PCE (ABB89707) (Krajmalnik-Brown et al., 2007); *D. sp*. SFR-*cis*-DCE: *Dehalococcoides sp*. SFR-*cis*-DCE (ABB89705) (Krajmalnik-Brown et al., 2007); *D. ethenogenes*: *Dehalococcoides ethenogenes* 195 (YP_180831, NC_002936) (Magnuson et al., 2000; Seshadri et al., 2005b); *D. sp.* KBC1: *Desulfitobacterium sp*. KBC1 (BAE45338) (Tsukagoshi et al., 2006); *C. bifermentans*: *Clostridium bifermentans* (CAC37919) (Okeke et al., 2001). Here, the codes in parentheses are accession numbers of genes in GenBank database.

APPENDIX C

Supporting Information for Chapter 3

This appendix is the supporting information related to chapter 3, including additional figures.

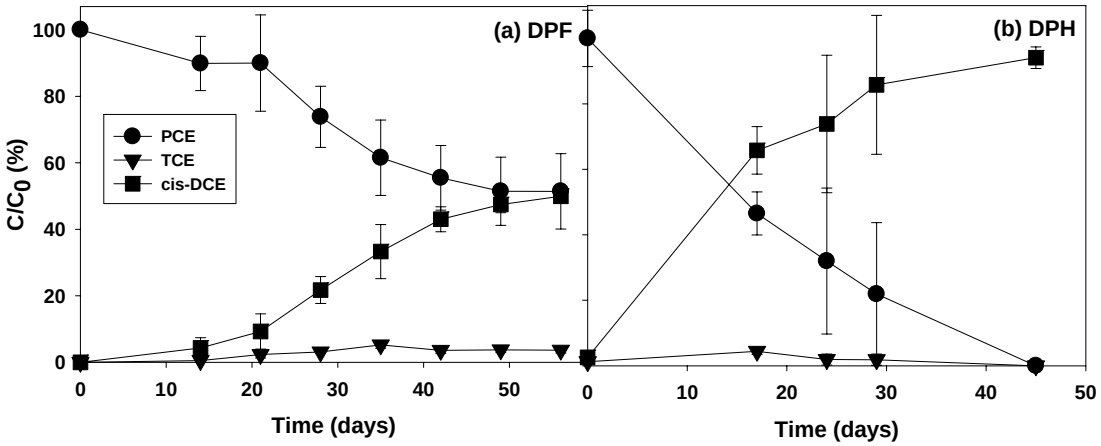


Figure C1. PCE reductive dechlorination by DPF and DPH before DPF lost its capacity to transform TCE to *cis*-DCE. PCE was dissolved in heptamethylnonane before spiked into the cultures (7 mL culture in 25 mL serum bottles) and the final concentration of PCE was about 25 μmoles/bottle. Error bars indicate standard deviation of triplicate samples.

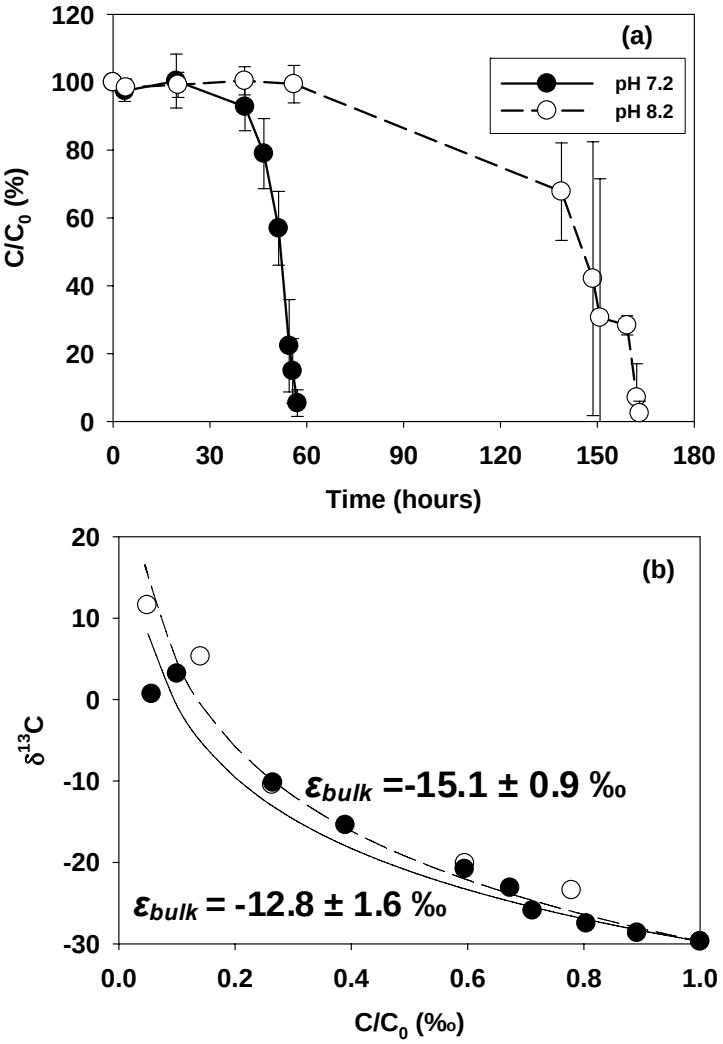


Figure C2. TCE degradation (a) and isotope fractionation (b) by *Sulfurospirillum multivorans* at pH 7.2 versus 8.2. The error bars indicated standard deviation of triplicate microcosms. The uncertainties for enrichment factors indicate 95 % confidence intervals calculated from non-linear regression. The solid line and dash line in (b) indicate Rayleigh Model fit for isotope fractionation of TCE reductive dechlorination at pH 7.2 and 8.2, respectively. The initial concentrations of TCE range from 74.3 to 109.4 μM.

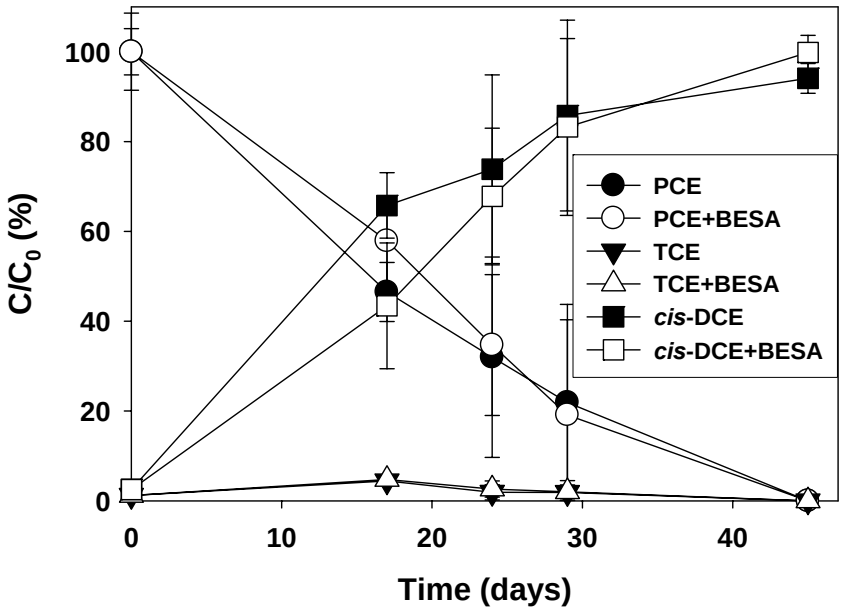


Figure C3. PCE reductive dechlorination by DPH in the presence (open symbols) and in the absence (closed symbols) of BESA. The error bars are standard deviation of duplicate samples. In this experiment, PCE was added as from concentrated stock solution prepared in heptamethylnonane and the initial concentration of PCE ranges from 19.4 to 20.1 μmoles/bottle.

APPENDIX D

Supporting Information for Chapter 4

This appendix is the supporting information related to chapter 4, including experimental setup, analytical methods, and additional data.

D1. Sources of Chemical Reagents

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): PCE (99%), TCE (99.5%), *cis*-1,2-dichlorethylene (*cis*-DCE), *trans*-1,2-dichlorethylene (*trans*-DCE), 1,1-dichlorethylene (1,1-DCE), N-(2-hydroxyethyl)-piperazine-N'-3-propanesulfonic acid (HEPES), and [(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS). Ethylene (1026 ppm in N_2), acetylene (1001 ppm in N_2), and vinyl chloride (VC) (1019 ppm in N_2) were obtained from Scott Specialty Gases (Houston, TX). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). All aqueous solutions were prepared with nanopure water (18.0 MΩ cm resistivity, Barnstead Ultrapure Water System, IA).

D2. Sampling Locations

Norman Landfill (L) soil samples were obtained from approximately 2 m below the ground surface near the No. 35 multilevel well (Cozzarelli et al., 2000) using a Geoprobe® (Geoprobe Systems, KS) and ground water was obtained approximately 3.5 m below the ground surface from the same well using a peristaltic pump. Duck Pond (DP) sediments were taken from the top 3-8 cm of the near shore sediment with a sterile spatula. Duck Pond water was collected in autoclaved 2L Pyrex® medium bottles at the sediment sampling site. Altus AFB (AAFB) biowall

samples were obtained using a Simco earthprobe® (Simco Drilling Equipment Inc. IA) from 3.5-6.2 m deep and approximately 1.5 m south of Well MP 1 (microcosms AAFB-8, AAFB-9 and AAFB-10) inside the biowall in the OU1 area (see map in (Lu et al., 2008)) and from 2.7-5.0 m deep and about 0.9 m east of Well BB04 inside the biowall downgradient of Building 506 in the SS-17 area (microcosms AAFB-12 and AAFB-14) (see map of the area around building 506 in ref. (Kennedy et al., 2006b)). In order to prevent oxidation and loss of fine particles during the sampling process, biowall samples were frozen *in-situ* with liquid nitrogen injected into the ground via a steel tube, extracted from the ground frozen, and then stored on dry ice in a cooler until transport to the laboratory. Ground water at AAFB was pumped from 4.6 m below the ground surface from Wells MP1 and BB05W. All solid and liquid samples were flushed with sterile N_2/CO_2 and stored in the dark at 4 °C before use.

D3. Microcosm Setup

After preparation, microcosms were sealed with sterilized thick butyl rubber stoppers and aluminum crimp seals, removed from the anaerobic chamber and flushed with sterile cotton filtered N_2. Microcosms (except unamended ones) were preincubated until the desired terminal electron accepting process was established. We determined this by monitoring Fe(II) (aq), sulfate, and methane, for iron reducing (IR), sulfate reducing (SR), and methanogenic (Meth) conditions, respectively. During preincubation, microcosms were stored upside down at room temperature in the dark. A summary of the microcosm conditions set up and the abbreviations used to describe them is given in Table D1.

After preincubation, some microcosms were killed by placement in a boiling water bath for 15 minutes a total of three times at three day intervals. Then, 100

μg/mL of the wide spectrum antibiotics kanamycin and chloramphenicol were added to completely inhibit microbial metabolism (Wu et al., 2000). Both sulfate reduction and methane production were inhibited in the killed microcosms for up to 155 days.

After preincubation, and, in some cases, heat/antibiotic treatment, butyl rubber septa were replaced with autoclaved Teflon-lined butyl rubber septa (West Pharmaceutical Services, Kearney, NE) inside the anaerobic chamber. Ten milliliters of saturated PCE or TCE stock solution were then spiked into the microcosms to yield total concentration (mass in the aqueous plus gas phases divided by aqueous volume) of 24-103 μM (PCE) or 92-130 μM (TCE) in standards containing no solid phase. At the same time, an additional 5 mM of electron donor was spiked into the microcosms to support microbial reductive dechlorination. After preincubation with electron donors and acceptors (or without preincubation for unamended microcosms), one microcosm for each condition was sacrificed for geochemical analysis using techniques summarized below, and the results are summarized in Table D2. Each geochemical parameter was measured in duplicate. Dissolved Fe(II), sulfate, and methane were also measured to determine whether the desired redox conditions had been established.

All amended microcosms were prepared in triplicate, and unamended and killed microcosms were prepared in duplicate. Except if noted otherwise, reported concentrations, percent remaining values, and product recoveries are means of values measured in replicate microcosms; uncertainties are standard deviations of the mean.

D4. Analytical Techniques

Concentrations of PCE, TCE, *cis*-DCE, *trans* 1,2-dichloroethylene (*trans*-DCE), 1,1-dichloroethylene (1,1-DCE), VC, ethylene, acetylene, and methane

in the microcosms were quantified by gas chromatography (GC) with headspace analysis using methods given in Liang et al. (Liang et al., 2007). No ethane was detected in the microcosms. Carbon isotope ratios were measured against a CO_2 standard with aqueous samples using an O.I. Analytical - Model 4560 purge and trap system interfaced with a Varian 3410 GC with Finnigan MAT 252 mass spectrometer. Approximately 15 % of samples were run in duplicate and the typical standard deviation for $\delta^{13}C$ values from duplicate measurements was 0.2-0.3 ‰ or better (Liang et al., 2007). Isotope analysis was also performed for two samples among duplicate or triplicate microcosms. The results were then combined to calculate ε_{bulk} values. ε_{bulk} values did not differ significantly between replicate microcosms.

Sulfate was quantified using a Dionex ion chromatograph (IC) with an Ion Pac AG 11 guard column (4 × 50 mm) and an Ion Pac AS 11 anion analytical column (4 × 250 mm), coupled with an ED 50 conductivity detector. Solid phase S(-II) was measured using a method adapted from Ulrich et al. (Ulrich et al., 1997) and described in Shao and Butler (Shao and Butler, 2007). FeS was assumed to be equal to the molar concentration of solid phase S(-II), measured as cited above. After S(-II) measurement, the remaining solid was reduced by 1 M Cr(II)-HCl solution for 72 hrs to quantify Cr(II) reducible or Cr(II) extractable sulfur (CrES), which includes S(0), polysulfides, and pyrite (Canfield et al., 1986; Huerta-Diaz et al., 1993).

Ferrous iron species were measured by ferrozine assay as described in Lovely and Phillips (Lovley and Phillips, 1987). For soluble Fe(II), the supernatant of the centrifuged solid/water slurry was acidified with anaerobic 0.5 N HCl at a 1:1 volume ratio prior to Fe(II) measurement. Sequential extractions were then performed to quantify different Fe(II) species in the solid phase (Heron et al., 1994). Five milliliters of solid/water slurry was collected and extracted with 1 M $MgCl_2$ for 5

hours to quantify weakly bound Fe(II) (Gibbs, 1973; Tessier et al., 1979). Extraction with 0.5 N HCl was used to quantify total solid phase Fe(II), including FeS and non-sulfur Fe(II) (Lovley and Phillips, 1987). Non-sulfur solid phase Fe(II) species are referred to as "surface associated Fe(II)". Strongly bound Fe(II) was calculated by subtracting weakly bound Fe(II) from surface associated Fe(II) (Shao and Butler, 2007). Total organic carbon (TOC) in the solid phase was measured with a TOC-5000 analyzer (Shimadzu Corp.) with a solid-sample module (SSM-5050) following the protocols provided by the manufacturer.

To assess the effect of heat treatment on abiotic mineral species that could potentially react with PCE and TCE, the solid phase mineral fractions described above were analyzed for two microcosm conditions (DP-IR-pH 8.2 and AAFB-8-SR-pH 7.2) before and after heat treatment by boiling water bath for 20 minutes. While heat treatment did not significantly affect the concentration of FeS, strongly bound Fe(II), or CrES (as evidenced by overlapping 95% confidence intervals for the concentration of these species before and after heat treatment), it did significantly lower the concentration of weakly bound Fe(II) in the one microcosm (DP-IR-pH 8.2) for which this species was above detection limits (Table D3). Specifically, for DP-IR-pH 8.2, weakly bound Fe(II) decreased by 37% upon heat treatment. While we considered the possibility that this decrease in weakly bound Fe(II) in the killed microcosms could cause us to underestimate the abiotic contribution to PCE or TCE reductive dechlorination, our conclusions about the relative importance of abiotic and microbial reductive dechlorination are in fact based on several lines of evidence—mainly analysis of reaction kinetics and product recoveries in live microcosms. Thus, the 37% decrease in weakly bound Fe(II) upon heat treatment in one representative microcosm (Table D3) does not change our overall conclusions.

For certain microcosms, we identified the more abundant minerals in the solid phase after preincubation by X-ray diffraction (XRD) using a Rigaku DMAX® X-ray Diffractometer (Table D2). Solid/liquid samples were centrifuged at an RCF of 1260 × g for 10 min and the solid was then freeze-dried under vacuum. Transfer to and from the freeze dryer was done in a glass tube with a custom vacuum valve to prevent exposure to the air. Freeze dried samples were then placed in the XRD sample holder inside the anaerobic chamber and mixed with petroleum jelly to retard the diffusion of oxygen to the sample. Quartz was the major mineral identified by XRD in the Landfill and Duck Pond solids and the two solid samples from AAFB that were analyzed (AAFB-12-SR-pH 7.2 and AAFB-14-SR-pH 7.2). We used the Hanawalt search/match method (Jenkins and Snyder 1996) to identify minor mineral species by XRD. First, the peaks associated with quartz were eliminated from the sample pattern. Then the d-spacing value of the strongest peak in the remaining pattern was compared to the d-spacing values of the strongest peaks for iron minerals likely to be present in the natural environment. If a match was found, the sample pattern was searched for the other representative peaks for that mineral (i.e., the second or third strongest peaks). If these additional peaks were matched, then we concluded that that mineral was present in our sample. The whole XRD pattern associated with that mineral was then eliminated and the process restarted with the strongest peak in the remaining XRD pattern. If, however, no match was found for the original strongest peak not associated with quartz, that peak was ignored and the process restarted with the next strongest peak in the sample pattern. All minor mineral species identified in the microcosms using this approach are given in Table D2. In general, only unreactive Fe(III) oxides were identified, with the exception of one microcosm (L-SR-pH 8.2), where mackinawite was identified and two microcosms

(AAFB-SR-12-pH 7.2 and AAFB-SR-14-pH 7.2) where magnetite was identified. As stated in the manuscript, magnetite was added to the biowall area from which the solids used to construct these microcosms were obtained. Other potentially reactive minerals were below XRD detection limits.

One microcosm (DP-SR-pH 8.2) was analyzed using scanning electron microscopy (SEM) with a JEOL JSM-880 High Resolution instrument. This microcosm was chosen because of the high concentration of FeS formed under sulfate reducing reactions (Table D2). The SEM sample was prepared using the method by Herbert and coworkers (Herbert et al., 1998) except that ethanol and not acetone was used for sample dehydration.

D5. Equilibrium Partitioning among the Gas, Aqueous, and Solid Phases

D5.1. Calculation of Total Concentrations

Aqueous concentrations of PCE, TCE, and their dechlorination products ($C_{i,aq}$) were calculated from measured gas concentrations ($C_{i,g}$) using Henry's Law:

$$C_{i,aq} = \frac{C_{i,g}}{H_i} \qquad \text{(D4.1)}$$

where H_i is the dimensionless Henry's Law constant for species i. Henry's Law constants used in these calculations are given in Table D4. Total concentrations ($C_{i,T}$), defined here as the sum of the masses of species i in the gas, aqueous, and solid phases, divided by the volume of the aqueous phase, were calculated using the approach in (Hwang and Batchelor, 2000):

$$C_{i,T} = C_{i,aq}\left(1 + K_{i,s} + H_i \frac{V_g}{V_{aq}}\right) = C_{i,aq}F_i \qquad \text{(D4.2)}$$

where $K_{i,s}$ is the solid-liquid partition coefficient, V_g and V_{aq} are volumes of the gas and aqueous phases (50 and 110 mL, respectively), and the partitioning factor (F_i) is defined as $\left(1 + K_{i,s} + H_i(V_g / V_{aq})\right)$. $K_{i,s}$ was calculated as follows (Hwang and Batchelor, 2000):

$$K_{i,s} = K_{i,d} \frac{m_s}{V_{aq}} \qquad \text{(D4.3)}$$

where $K_{i,d}$ is the solid/water distribution coefficient and m_s is the mass of the solid phase in the microcosm (20 g). $K_{i,d}$ was estimated from the empirical relationship $K_{i,d} = K_{i,oc} f_{oc}$ (Karickhoff et al., 1979), where $K_{i,oc}$ is the solid phase organic matter/water distribution coefficient, and f_{oc} is the weight fraction of organic matter in the solid (i.e., TOC, Table D2). $K_{i,oc}$ was estimated using two empirical equations: (1) for chlorinated aliphatics: $LogK_{i,oc} = 0.57 LogK_{i,ow} + 0.66$ (Schwarzenbach et al., 2003), where $K_{i,ow}$ is the published octanol/water partition coefficients (Howard and Meylan, 1997; Mackay et al., 2006); (2) for ethylene and acetylene: $LogK_{i,oc} = -0.58 LogS_i + 4.24$ (Doucette, 2000), where S_i is the aqueous solubility in μM, obtained from Howard and Meylan (Howard and Meylan, 1997) and Yalkowsky and He (Yalkowsky and He, 2003) (Table D4). Estimated $K_{i,oc}$ values are given in Table D4. The K_{oc} value for TCE in Table D4 is significantly higher than values measured for AAFB biowall solids (14-21 L/kg) (Shen and Wilson, 2007), meaning that we may have overestimated TCE sorption in the AAFB microcosms. We used the K_{oc} values in Table D4, however, so that we could do all calculations with a self consistent set of K_{oc} values.

D5.2. Calculation of Observed Product Recoveries

Observed abiotic and biotic product recoveries (*R*) (Table 4.1) were calculated by dividing the total concentrations of biotic products (i.e., TCE (for PCE), DCE isomers, VC and ethylene) or abiotic products (acetylene, and, except for AAFB microcosms, ethylene) by the total concentration of the reactant (PCE or TCE) at time zero ($C_{r,T,0}$):

$$R(\%) = \frac{\sum C_{p,T}}{C_{r,T,0}} \times 100\% = \frac{\sum C_{p,aq} F_p}{C_{r,T,0}} \times 100\% \qquad \text{(D4.4)}$$

For the live AAFB microcosms, the kinetic data (Figure D3) indicate that, with the possible exceptions of AAFB-12-SR-pH 7.2-PCE and AAFB-14-SR-pH 7.2-PCE (Figures D3d, and e), the majority of ethylene was produced microbially, as evidenced by no co-detection of acetylene, and co-detection with VC. Therefore, we included ethylene in the biotic product recoveries (Table 4.1) for all live AAFB microcosms, except AAFB-12-SR-pH 7.2-PCE and AAFB-14-SR-pH 7.2-PCE. Because it was unclear if ethylene came from abiotic or microbial dechlorination in AAFB-12-SR-pH 7.2-PCE and AAFB-14-SR-pH 7.2-PCE, we calculated neither abiotic nor microbial product recoveries for these microcosms (Table 4.1).

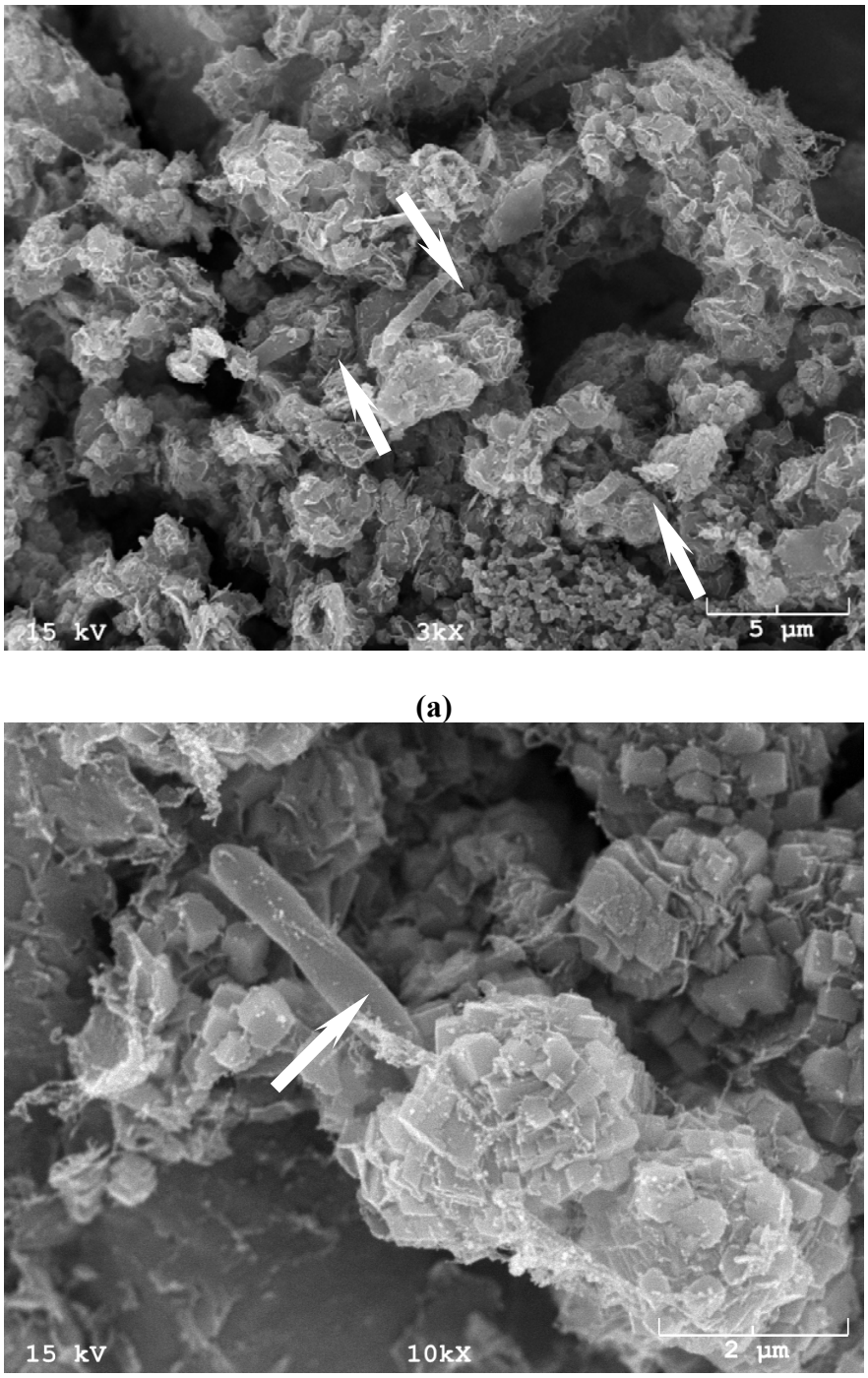
For AAFB killed microcosms, low concentrations of ethylene were observed even when VC was not detected. Thus, ethylene (and, when detected, acetylene) was included in the abiotic product recoveries for killed AAFB microcosms (Table 4.1).

D5.3. Correction of Rate Constants for Partitioning among the Gas, Aqueous, and Solid Phases

Mass normalized rate constants (i.e., rate constants divided by mass loading) for PCE or TCE transformation by FeS, adjusted to or measured in a zero-headspace system (k_m), were taken from the literature (Butler and Hayes, 1999, 2001; Zwank, 2004; Liang et al., 2007). The mass loadings of FeS in Zwank (Zwank, 2004) were estimated from the reported concentrations of reagents used to synthesize the FeS. Rate constants for similar pH values were averaged, yielding the following k_m values ($Lg^{-1}d^{-1}$): PCE at pH 7-7.3: 2.41×10^{-4}; PCE at pH 8-8.3: 1.22×10^{-3}; TCE at pH 7.3: 7.28×10^{-4}; and TCE at pH 8-8.3: 1.95×10^{-3}. Then, we used the approach in Hwang and Batchelor (Hwang and Batchelor, 2000) to correct rate constants to account for partitioning of PCE or TCE among the gas, aqueous, and solid phases ($k_{m,corr}$):

$$k_{m,corr} = \frac{k_m}{F_i} \qquad \text{(D4.5)}$$

where F_i is defined after equation C2, and the subscript "*i*" corresponds to the reactant (PCE or TCE). While V_g and V_{aq} were the same in all our microcosms, $K_{i,s}$ was not, since f_{oc} varied among the microcosms. Values of $k_{m,corr}$ for the case where f_{oc}=0, and therefore $K_{i,s}$ is zero are reported in Table D4. We then multiplied the values in Table D4 by the term $(1 + H_i(V_g / V_{aq}))/F_i$ to yield $k_{m,corr}$ values appropriate for the f_{oc} values of each microcosm. These values of $k_{m,corr}$ were used to estimate half lives for abiotic PCE and TCE transformation based on FeS mass loadings in the microcosms. These values are discussed in the chapter 3.



(a)

(b)

Figure D1. SEM photomicrographs of sediment from Sample DP-SR-pH 8.2. Cells attached to the surface of the minerals are indicated by arrows. Crystalline mineral precipitates are visible on the right side of panel (b).

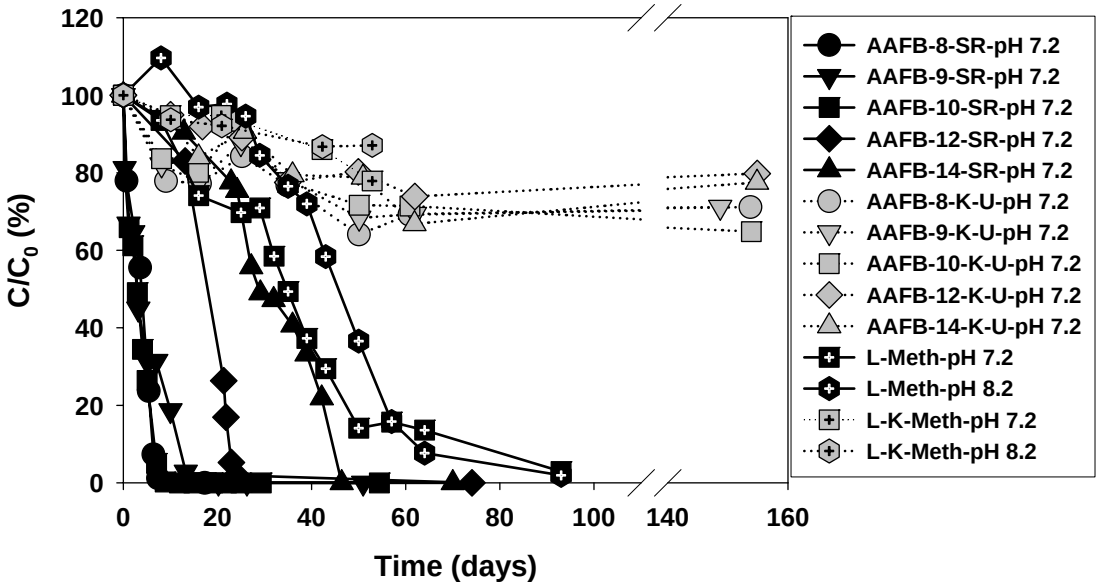


Figure D2. PCE reductive dechlorination in the microcosms with (gray symbols) and without (black symbols) antibiotic and heat treatments.

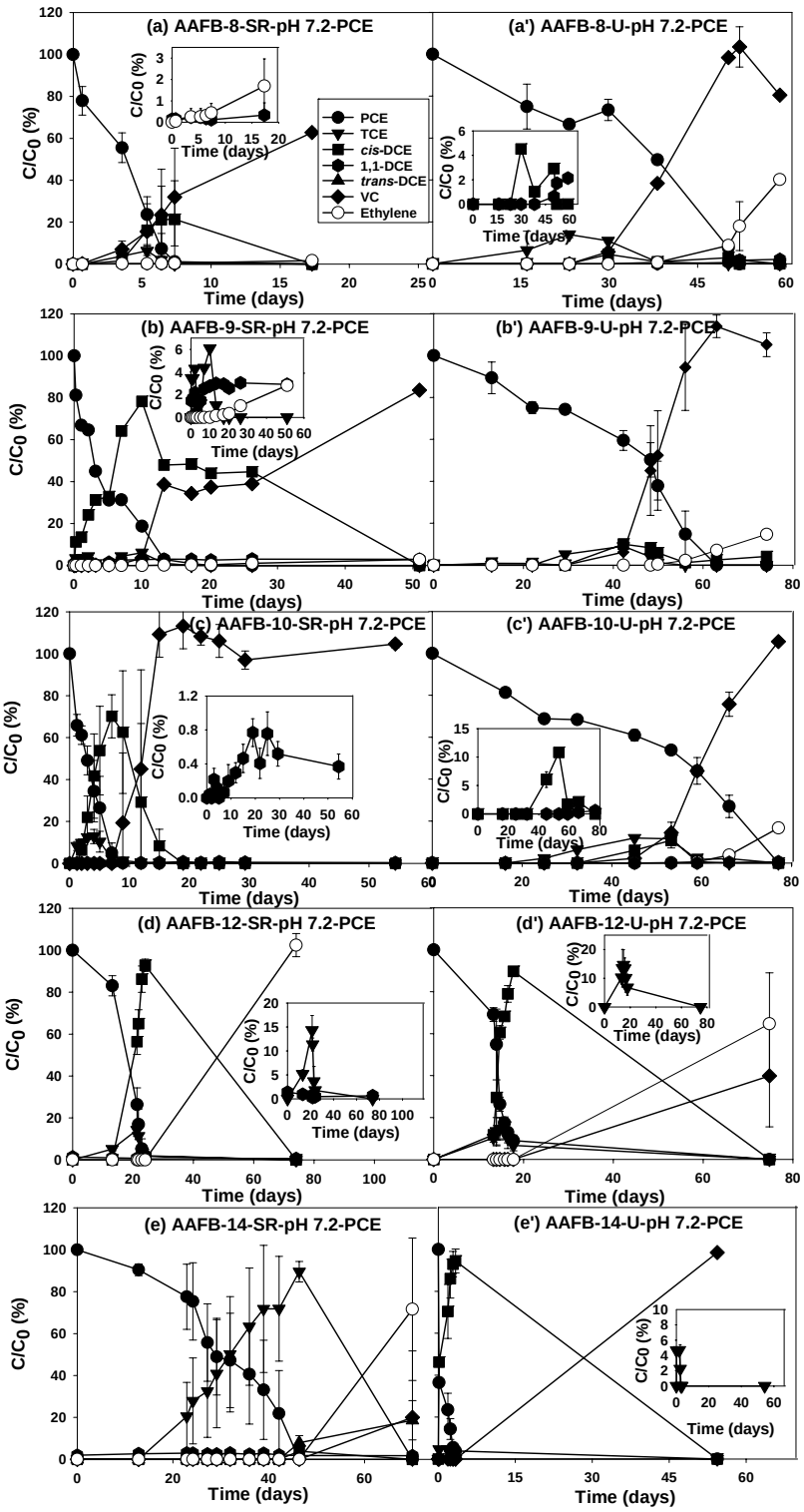


Figure D3. Normalized concentrations of PCE and reaction products in live AAFB microcosms. Reactants and products were normalized by dividing the concentration at any time by the concentration of the reactant at time zero.

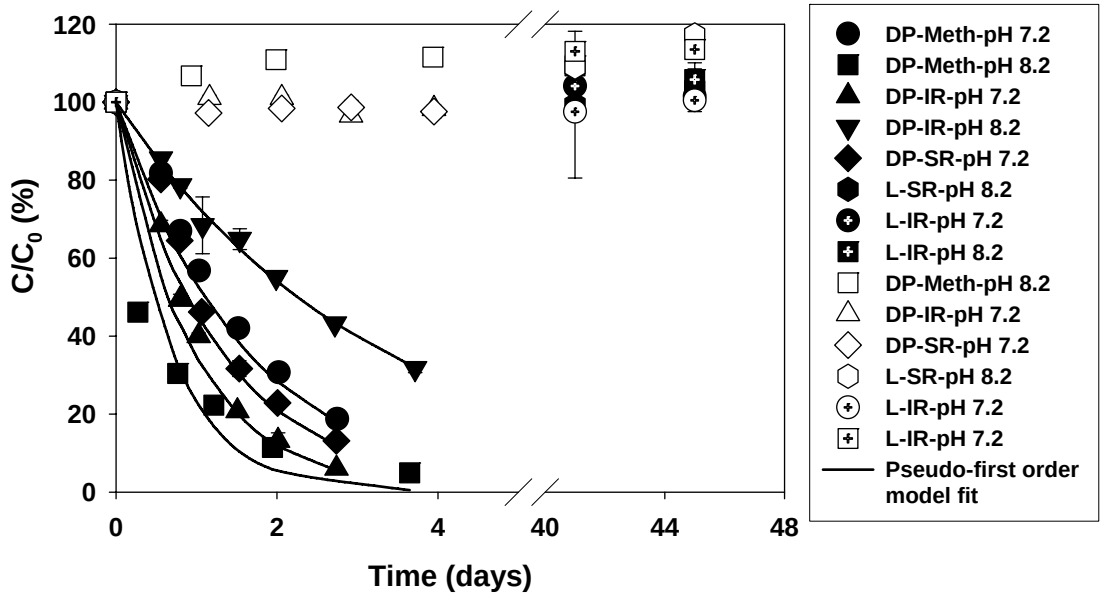


Figure D4. Acetylene transformation in the microcosms. Error bars are standard deviations of the means for duplicate measurements from the same microcosm.

Table D1. Summary of microcosm conditions and abbreviations

Treatment	Geochemical Conditions [b]	pH	Duck Pond		Norman Landfill		AAFB 8-14[a]
			PCE	TCE	PCE	TCE	PCE
Unamended	— [c]	7.2	DP-U-pH 7.2-PCE	DP-U-pH 7.2-TCE	L-U-pH 7.2-PCE	L-U-pH 7.2-TCE	AAFB-*i*-U-pH 7.2-PCE[d]
Amended	Iron reduction	7.2	DP-IR-pH 7.2-PCE	—	L-IR-pH 7.2-PCE	—	—
		8.2	DP-IR-pH 8.2-PCE	DP-IR-pH 8.2-TCE	L-IR-pH 8.2-PCE	L-IR-pH 8.2-TCE	—
	Sulfate reduction	7.2	DP-SR-pH 7.2-PCE	—	L-SR-pH 7.2-PCE	—	AAFB-*i*-SR-pH 7.2-PCE
		8.2	DP-SR-pH 8.2-PCE	DP-SR-pH 8.2-TCE	L-SR-pH 8.2-PCE	L-SR-pH 8.2-TCE	—
	Methano-genesis	7.2	DP-Meth-pH 7.2-PCE	—	L-Meth-pH 7.2-PCE	—	—
		8.2	DP-Meth-pH 8.2-PCE	DP-Meth-pH 8.2-TCE	L-Meth-pH 8.2-PCE	L-Meth-pH 8.2-TCE	—
Killed	Iron reduction	7.2	—	—	—	—	—
		8.2	—	—	—	—	—
	Sulfate reduction	7.2	—	—	—	—	AAFB-*i*-K-U-pH 7.2-PCE
		8.2	—	—	—	—	—
	Methano -genesis	7.2	—	—	L-K-Meth-pH	—	—
		8.2	—	—	L-K-Meth-pH	—	—

[a] The values "8-14" in "AAFB 8-14" refer to the five sampling locations at Altus AFB (AAFB), which were AAFB 8, 9, 10, 12, and 14. [b] acetate, lactate and ethanol were used as the electron donors for iron reducting, sulfate reducing and methanogenic microcosms, respectively. [c] — means that the microcosm was unamended or that no microcosms was prepared for this condition. [d] *i* means 8, 9, 10, 12 or 14, which corresponds to AAFB sampling locations 8, 9, 10, 12, or 14.

Table D2. Geochemical properties of microcosms [a]

Microcosm ID	FeS (g FeS/L)	Weakly bound Fe(II) (g Fe/L)	Strongly bound (g Fe/L)	CrES (g S/L)	TOC (g/g solid)	Iron Minerals Detected by XRD[b]
Unamended Microcosms						
DP-U-pH 7.2	$(5.10\pm0.68)\times10^{-2}$	$(2.76\pm0.23)\times10^{-3}$	$(1.28\pm0.11)\times10^{-1}$	$(1.23\pm0.17)\times10^{-1}$	$(9.6\pm1.7)\times10^{-4}$	—[c]
L-U-pH 7.2	$(5.23\pm0.40)\times10^{-3}$	$(3.3\pm4.0)\times10^{-4}$	$(1.25\pm0.10)\times10^{-1}$	$(6.40\pm0.72)\times10^{-3}$	$(2.6\pm1.8)\times10^{-4}$	—
AAFB-8-U-pH 7.2	$(1.45\pm0.15)\times10^{-1}$	$(2.04\pm0.57)\times10^{-3}$	$(1.49\pm0.46)\times10^{-1}$	$(8.4\pm1.1)\times10^{-2}$	$(2.27\pm0.52)\times10^{-2}$	—
AAFB-9-U-pH 7.2	$(4.66\pm0.87)\times10^{-2}$	$(4.2\pm1.9)\times10^{-4}$	$(8.8\pm5.0)\times10^{-2}$	$(7.5\pm1.6)\times10^{-2}$	$(2.30\pm0.68)\times10^{-2}$	—
AAFB-10-U-pH 7.2	$(6.9\pm3.8)\times10^{-2}$	$(2.42\pm0.54)\times10^{-3}$	$(1.08\pm0.66)\times10^{-1}$	$(8.7\pm2.5)\times10^{-2}$	$(1.9\pm1.1)\times10^{-2}$	—
AAFB-12-U-pH 7.2	$(2.9\pm1.5)\times10^{-2}$	$(1.43\pm0.28)\times10^{-2}$	$(2.3\pm1.4)\times10^{-1}$	$(1.24\pm0.27)\times10^{-2}$	$(1.54\pm0.41)\times10^{-2}$	—
AAFB-14-U-pH 7.2	$(5.95\pm0.69)\times10^{-2}$	$(1.48\pm0.34)\times10^{-2}$	$(9.2\pm3.4)\times10^{-2}$	$(1.27\pm0.31)\times10^{-2}$	$(1.51\pm0.15)\times10^{-2}$	—
Amended Microcosms						
DP-SR-pH 7.2	0.385 ± 0.049	$(1.325\pm0.074)\times10^{-3}$	0.349 ± 0.084	$(9.8\pm1.6)\times10^{-2}$	$(2.58\pm0.67)\times10^{-3}$	Ge, Lep, Fer
DP-SR-pH 8.2	0.44 ± 0.14	$(5.1\pm1.8)\times10^{-5}$	1.31 ± 0.70	1.291 ± 0.065	$(1.11\pm0.28)\times10^{-3}$	Ge, Lep
DP-IR-pH 7.2	$(1.15\pm0.19)\times10^{-2}$	$(3.898\pm0.071)\times10^{-2}$	0.214 ± 0.038	$(3.18\pm0.49)\times10^{-2}$	$(1.85\pm0.34)\times10^{-3}$	Ge
DP-IR-pH 8.2	$(7.5\pm1.1)\times10^{-3}$	$(5.21\pm0.71)\times10^{-2}$	0.203 ± 0.042	$(3.70\pm0.30)\times10^{-2}$	$(2.86\pm0.53)\times10^{-3}$	Ge, Lep
DP-Meth-pH 7.2	$(6.9\pm2.1)\times10^{-2}$	$(8.2\pm5.9)\times10^{-4}$	$(8.9\pm7.2)\times10^{-2}$	$(5.06\pm0.70)\times10^{-2}$	$(1.8\pm1.5)\times10^{-3}$	Ge, Lep, Fer
DP-Meth-pH 8.2	$(5.45\pm0.77)\times10^{-2}$	$(4.7\pm1.3)\times10^{-4}$	$(6.8\pm2.3)\times10^{-2}$	$(3.81\pm0.64)\times10^{-2}$	$(1.76\pm0.35)\times10^{-3}$	Ge
L-SR-pH 7.2	0.223 ± 0.022	$(5.68\pm0.47)\times10^{-3}$	0.71 ± 0.10	0.431 ± 0.024	$(5.8\pm1.1)\times10^{-4}$	Ge
L-SR-pH 8.2	0.885 ± 0.028	$(1.3355\pm0.0012)\times10^{-3}$	1.085 ± 0.036	$(7.2\pm1.6)\times10^{-2}$	$(8.6\pm2.2)\times10^{-4}$	Ge, Lep, Fer, Mgh,
L-IR-pH 7.2	$(3.328\pm0.095)\times10^{-3}$	$(9.5\pm1.7)\times10^{-3}$	0.375 ± 0.071	$(6.2\pm2.6)\times10^{-3}$	$(1.32\pm0.74)\times10^{-4}$	Lep
L-IR-pH 8.2	$(2.63\pm0.26)\times10^{-3}$	$(3.46\pm0.12)\times10^{-2}$	0.158 ± 0.018	$(6.22\pm0.19)\times10^{-3}$	$(3.2\pm2.2)\times10^{-4}$	Ge, Lep, Fer
L-Meth-pH 7.2	$(1.85\pm0.13)\times10^{-2}$	$(3.7\pm2.3)\times10^{-3}$	$(7.9\pm4.8)\times10^{-2}$	$(1.06\pm0.31)\times10^{-2}$	BDL[d]	Ge, Lep
L-Meth-pH 8.2	$(1.85\pm0.14)\times10^{-2}$	$(2.64\pm0.11)\times10^{-3}$	$(8.94\pm0.81)\times10^{-2}$	$(9.3\pm2.3)\times10^{-3}$	$(1.20\pm0.25)\times10^{-4}$	Ge, Lep
AAFB-8-SR-pH 7.2	0.170 ± 0.093	BDL	0.132 ± 0.082	$(4.65\pm0.57)\times10^{-2}$	$(5.5\pm2.2)\times10^{-2}$	—
AAFB-9-SR-pH 7.2	0.115 ± 0.023	BDL	$(4.0\pm1.2)\times10^{-2}$	$(8.22\pm0.78)\times10^{-2}$	$(3.6\pm2.2)\times10^{-2}$	—
AAFB-10-SR-pH 7.2	0.111 ± 0.037	BDL	$(7.4\pm2.8)\times10^{-2}$	$(1.08\pm0.13)\times10^{-1}$	$(2.95\pm0.41)\times10^{-2}$	—
AAFB-12-SR-pH 7.2	0.141 ± 0.099	BDL	0.20 ± 0.15	$(4.4\pm1.4)\times10^{-2}$	$(2.3\pm1.2)\times10^{-2}$	Mag, Mgh, Aka
AAFB-14-SR-pH 7.2	0.159 ± 0.013	BDL	$(2.41\pm0.35)\times10^{-2}$	$(2.07\pm0.31)\times10^{-2}$	$(1.57\pm0.40)\times10^{-2}$	Mag, Mgh, Aka

[a] All measurements, except for weakly bound Fe(II), were carried out with freeze dried solids and the results were corrected by water content to yield values correct for wet solids. [b] Aka: akaganeite, Fer: ferrihydrite, Ge: goethite, Lep: lepidocrocite, Mag: Magnetite, Mgh: maghemite, Mk: mackinawite (Siivola and Schmid, 2007); [c] —, XRD analysis was not performed for this condition. [d] BDL, below detection limits of approx. 8×10^{-6} g/L. Uncertainties are standard deviations of triplicate samples from the same microcosm.

Table D3. Results of geochemical analyses before and after heat treatment

	DP-IR-pH 8.2 (g/L)		AAFB-8-SR-pH 7.2 (g/L)	
	Before[a]	**After**[a]	**Before**[a]	**After**[a]
FeS	0.112±0.014	0.1230±0.0046	0.292±0.046	0.357±0.087
Weakly bound Fe(II)	0.0199±0.0047	0.01253±0.00084	BDL[b]	BDL
Strongly bound Fe(II)	1.72±0.27	1.84±0.16	0.056±0.054	0.076±0.018
CrES	0.114±0.042	0.122±0.014	0.0247±0.0098	0.033±0.012

[a] Uncertainties are 95% confidence intervals of the mean of triplicate samples from the same microcosm.
[b] BDL means below detection limits.

Table D4. Physical-chemical and kinetic properties of reactants and products

	H_i (Dimensionless)[a,b]		Solubility (S_i, µM)[a, c]	$K_{i,oc}$ (25°C, L/Kg)	$k_{m,corr}$ (pH~7) ($Lg^{-1}day^{-1}$)[d]	$k_{m,corr}$ (pH~8) ($Lg^{-1}day^{-1}$)[d]
PCE	0.75	2.99		231.37	(1.8±1.2)	$(9.1\pm1.6)\times10^{-4}$
TCE	0.39	2.67		153.90	$(6.2\pm5.7)\times10^{-4}$	$(1.7\pm1.9)\times10^{-3}$
cis-DCE	0.34	1.86		52.33		
trans-DCE	0.40	2.08		69.62		
1,1-DCE	1.62	2.13		74.83		
VC	5.95	1.53		33.87		
Acetylene	0.93		1.86×10^{7}	4.35		
Ethylene	8.93		1.62×10^{6}	1.05		

[a] Howard and Meylan, 1997; [b] Mackay et al., 2006; [c] Yalkowsky and He, 2003; [d] Calculated for the condition where f_{oc}=0