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Impacts of microbial community composition on isotope fractionation during reductive dechlorination of tetrachloroethylene

Yiran Dong · Elizabeth C. Butler · R. Paul Philp · Lee R. Krumholz

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Abstract Isotope fractionation has been used with increasing frequency as a tool to quantify degradation of chlorinated aliphatic pollutants in the environment. The objective of this research was to determine if the electron donor present in enrichment cultures prepared from uncontaminated sediments influenced the extent of isotope fractionation of tetrachloroethylene (PCE), either directly, or through its influence on microbial community composition. Two PCE-degrading enrichment cultures were prepared from Duck Pond (DP) sediment and were incubated with formate (DPF) or H_2 (DPH) as electron donor. DPF and DPH were significantly different in both product distribution and extent of isotope fractionation. Chemical and isotope analyses indicated that electron donors did not directly affect the product distribution or the extent of isotope fractionation for PCE reductive dechlorination. Instead, restriction fragment length polymorphism (RFLP) and sequence analysis of the 16S rRNA clone libraries of DPF and DPH identified distinct microbial communities in each enrichment culture, suggesting that differences in microbial communities were responsible for distinct product distributions and isotope fractionation between the two cultures. A dominant species identified only in DPH was closely related to known dehalogenating species (*Sulfurospirillum multivorans* and *Sulfurospirillum halorespirans*) and may be responsible for PCE degradation in DPH. Our study suggests that different dechlorinators exist at the same site and can be preferentially stimulated by different electron donors, especially over the long-term (i.e., years), typical of in-situ ground water remediation.

Keywords Tetrachloroethylene · Reductive dechlorination · Microbial community composition · Isotope fractionation · Electron donors

Present Address:
Y. Dong
Energy and Bioscience Institute, University of Illinois-Urbana Champaign, Urbana, IL 61801, USA

Y. Dong · E. C. Butler
School of Civil Engineering and Environmental Science, University of Oklahoma, Norman, OK 73019, USA

R. P. Philp
School of Geology and Geophysics, University of Oklahoma, Norman, OK 73019, USA

L. R. Krumholz (✉)
Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA
e-mail: krumholz@ou.edu

Introduction

Improper disposal and storage have led to widespread contamination of ground water resources by chlorinated aliphatic contaminants, such as tetrachloroethylene (PCE). Due to their toxicity and suspected carcinogenic properties, monitoring the remediation of this group of contaminants has gained wide public

and academic interest. 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; Elsner et al. 2004; Aeppli et al. 2009; Morrill et al. 2009; Schmidt et al. 2010). Isotope ratio analysis of in situ contaminants involves the use of enrichment factors, generally obtained from lab-scale studies, to quantitatively estimate the fraction of contaminants transformed at a site (Elsner et al. 2005; Van Breukelen et al. 2005; VanStone et al. 2005; Nijenhuis et al. 2007). In order to avoid significant uncertainties in this process, it is important to choose enrichment factors appropriate for specific field sites and to understand the factors that influence this parameter.

A variety of anaerobic bacteria are capable of reductively dechlorinating PCE to less chlorinated compounds, such as trichloroethylene (TCE), dichloroethylene (DCE) isomers, vinyl chloride (VC) and/or ethylene via dehalorespiration or cometabolism (Bradley 2003; Bhatt et al. 2007). Different levels of isotope fractionation have been reported during microbial reductive dechlorination of PCE with enrichment factors ranging from −0.42 to −16.8‰ (Hunkeler et al. 1999; Slater et al. 2001; 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). In addition to the differences among dehalogenating species, the extent of isotope fractionation during microbial metabolism is also influenced by factors such as reaction pathways (e.g., aerobic vs. anaerobic reactions) (Hirschorn et al. 2004, 2007), structure of functional enzymes (Nikolausz et al. 2006), relative accessibility of the enzyme to the substrate (Nijenhuis et al. 2005), and availability of trace elements (e.g., iron for aerobic biodegradation of toluene by *Pseudomonas putida* (Mancini et al. 2006) and cobalt for reductive dechlorination of chlorinated ethenes catalyzed by Vitamin B12 or its analogues as coenzymes (Slater et al. 2003; Nijenhuis et al. 2005; Cichocka et al. 2007)). In addition, the availability and types of electron donors (Bruechert 2004; Hoek et al. 2006; Hoek and Canfield 2007) have also been shown to influence the extent of isotope fractionation. There are likely to be interactions among geochemical parameters (e.g., electron acceptors and electron donors) and the composition of the microbial community (Petrie et al. 2003; Gu et al. 2004; Freeborn et al. 2005), so it is difficult to separate these factors during field experiments. Our current knowledge regarding isotope fractionation during microbial reductive dechlorination has been mainly derived from studies focused on isolated dehalogenating bacteria or enrichment cultures without identified microbial composition (Nijenhuis et al. 2005, 2008; Cichocka et al. 2007; Lee et al. 2007; Liang et al. 2007), but whether changes in microbial communities can affect isotope fractionation is not well understood. The objective of this study is to evaluate whether the major factors influencing isotope fractionation in a dechlorinating enrichment are related to the electron donor or whether they are specifically related to the composition of the microbial community that is present.

Materials and methods

Sources of chemical reagents

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): PCE (99%), TCE (99.5%), *cis*-1,2-dichloroethylene (*cis*-DCE) (97%), *trans*-1,2-dichloroethylene (*trans*-DCE) (98%), 1,1-dichloroethylene (1,1-DCE) (99%) and 2,2,4,4,6,8,8-heptamethylnonane (98%). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Preparation of enrichment cultures and subcultures

Sediment-free enrichment cultures were developed from microcosms constructed using anaerobic sediments from a local pond (Duck Pond, DP) adjacent to the University of Oklahoma, Norman, OK, as previously described (Dong et al. 2009). No PCE or TCE contamination has been reported at this site. During sampling and storage, anaerobic conditions were maintained by storing sediments in 1 L canning jars with the headspace flushed with sterile N_2 and CO_2 mixed gas (80:20, v:v). The jars were then quickly closed with screw caps, sealed with vinyl tape and stored at 4°C before use. After the initial incubation period, subcultures were transferred (1:10, v/v) into 25 ml serum bottles containing 5 ml freshly prepared

basal salts medium (Hurst et al. 2002) at intervals of 45–60 days by using N_2 flushed syringes. PCE reductive dechlorination activity was maintained in this way for more than 4 years. In both microcosms and sediment-free enrichment cultures, 0.22 μm filter-sterilized anaerobic digester sludge from an activated sludge unit at the Norman Wastewater Treatment Plant, Norman, OK was added at a final concentration of approximately 10% (v/v). The soluble substrates in activated sludge (including peptides, amino acids, vitamins, and other soluble cell components) may act as sources of carbon, nitrogen, and/or trace components for stimulation of the growth of dechlorinating bacteria (Kastner 1991; Stasinakis et al. 2004). H_2 was added as an electron donor to DP-H_2 (DPH) enrichment cultures from N_2:H_2 (80:20, v:v). Based on the total amount of H_2 added and its distribution between the aqueous and gaseous phases (the Henry's Law constant equals 1.91×10^{-2} at 25°C) (Sangster 2003), the equilibrium aqueous concentration of H_2 was approximately 150 μM. Formate (10 mM) was used as the electron donor for DP-formate (DPF) enrichment cultures. Both enrichment cultures received 0.5 ml 50 mM PCE stock solution prepared by dissolving PCE in 0.22 μm filter sterilized 2,2,4,4,6,8,8-heptamethylnonane. Bromoethanesulfonate (BESA) was added (1 mM) to inhibit methanogenic Archaea from competing for electron donors with dehalogenating Bacteria (Loeffler et al. 1997). The cultures were sealed with Teflon-lined butyl rubber septa (West Pharmaceutical Services, PA) and incubations were held static at room temperature in the dark unless otherwise indicated. After about 10 transfers, the enrichment cultures were inoculated into 120 ml serum bottles containing 100 ml of the aqueous medium described above, with PCE added from an aqueous stock solution to a final concentration of about 100 μM for kinetic and isotope experiments. In order to assess whether microbial processes were responsible for PCE reductive dechlorination in the kinetic experiments, two bottles of each enrichment culture were pasteurized at 85°C for 15 min after inoculation, but before addition of PCE stock solution (Ballerstedt et al. 2004) and products were quantified.

In order to test whether electron donors were directly responsible for the different product distribution and isotope fractionation in DPH and DPF, cultures of DPH and DPF were incubated until the PCE was completely transformed. Cultures (30 ml) were transferred into N_2/CO_2 flushed Falcon™ conical tubes and then centrifuged at $12{,}000 \times g$ at 4°C for 5 min. The cell pellets were washed with the same anaerobic medium twice before being resuspended in 6 ml fresh medium containing the other electron donor (i.e., formate for DPH and H_2 for DPF). These cultures are called "DPH + formate" and "DPF + H_2". Parallel controls were prepared by inoculating DPF and DPH in the medium with the same electron donor (formate for DPF and H_2 for DPH) and they were called "DPH + H_2" and "DPF + formate", respectively.

Analytical techniques

Concentrations of PCE, TCE and *cis*-DCE were determined by manual injection headspace analysis with a Shimadzu GC-17A equipped with a flame ionization detector (GC/FID) and an Agilent GS-GASPRO capillary column (30 m × 0.32 mm) (J&W Scientific, Folsom, CA, USA) (Liang et al. 2007). The same method was used to measure the concentration of methane to be sure that methanogenesis was completely inhibited. In addition, 1 ml of culture was withdrawn and prepared for isotope analysis (Dong et al. 2009) at each time point. 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). The combustion reactor temperature was 940°C. The reactor was an alumina tube packed with Ni and Pt wires, run with an auxiliary stream of oxygen (1–2 V measured on the 44 detector cup). Precision and accuracy of the isotope ratio measurements were controlled by PCE standards and by duplicates of the samples. For PCE standard assessment, the stock solution (4 μg/l) was prepared from pure phase PCE (Sigma-Aldrich, HPLC grade, purity 99.9%) of known isotopic composition identified by conventional dual inlet bulk isotope ratio mass spectrometry (IRMS) after off-line combustion on CuO. At least one such standard was run daily under the same condition as for samples, and the reproducibility was evaluated. The maximum standard deviations of the lowest and the highest $\delta^{13}C$ of any standard run for a specific sample set did not exceed 0.5‰, with most values not exceeding 0.2‰. The analyte was also

diluted to similar concentrations (i.e., in the range of 4 μg/l) to avoid potential problems due to different loads of PCE from run to run (Sherwood Lollar et al. 2007). Meanwhile, a portion of the samples (about 30%) 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; Liang et al. 2007). The extent of isotope fractionation was then expressed as the bulk enrichment factor (ε_{bulk}), based on the Rayleigh Model (Mariotti et al. 1981):

$$\frac{\delta^{13}C + 1000}{\delta^{13}C_0 + 1000} = f^{\left(\frac{\varepsilon_{bulk}}{1000}\right)} \quad (1)$$

where $\delta^{13}C_0$ and $\delta^{13}C$ are the standardized isotope ratios measured at time zero and at different time points, respectively; f is the fraction of parent compound remaining at a given time (i.e. C/C_0).

DNA extraction and 16S rRNA gene analysis

Duplicate daughter cultures, inoculated with replicates of cultures subject to isotope analysis, were sacrificed when 70–80% of PCE had been transformed. The cells were centrifuged at 13,000×g for 5 min. Cell pellets were then resuspended in sterile PBS and the washing procedure was repeated. Washed cells were stored at −20°C until DNA extraction was performed. Total community DNA was extracted using the Easy-DNA kit (Invitrogen, Co., CA) according to the manufacturer's instructions. Purity and concentration of extracted genomic DNA were determined spectrophotometrically.

Bacterial 16S rRNA genes were amplified from community DNA with primers 27F (5′-AGAGTTTG ACMTGGCTCAG-3′) and 1492R (5′-GGYTACC TTGTTACGACTT-3′) (Lane 1991). PCR amplification was performed with the *Taq* DNA polymerase kit (Fermentas Inc., MD). The reaction mixture (50 μl) contained 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 μM of each oligonucleotide primer, 1.25 U *Taq* polymerase and 1 μl appropriately diluted template DNA (ca. 50 ng). Reaction mixtures were amplified in an iCycler thermal cycler (Bio-Rad Laboratories, France) 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™ 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). A total number of 256 clones (98 for DPF and 148 for DPH) were randomly picked and grown in 1 ml LB medium amended with 50 μg/ml of ampicillin.

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). The digested fragments were separated by agarose gel electrophoresis (0.8%) and visualized by staining with ethidium bromide and UV illumination. Restriction fragment length polymorphism (RFLP) patterns for each library were grouped visually. A total of 70 representative clones (35 for each sample) were selected based on distinct RFLP patterns, purified with the GenCatch™ 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 more than 3 replicates with similar RFLP patterns, two to nine clones were sequenced to confirm that they represented identical species.

All the sequences were 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) (Cole et al. 2005). Clones were grouped into Operational Taxonomic Units (OTUs) at a level of sequence similarity ≥97%. 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. In order to assess whether the clone libraries developed were representative of the microbial communities in DPH and DPF, saturation of OTUs was analyzed to estimate microbial diversity of the two PCE-degrading enrichment cultures by using an extrapolation method (Colwell and Coddington 1994) with the program SigmaPlot. 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).

GenBank accession numbers

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

Results

Effect of electron donors on reaction products and bulk enrichment factors

Over 4 years, both DPH and DPF cultures were transferred into fresh medium about 25 times to keep their PCE reduction activity. DPH maintained its capacity to transform PCE to *cis*-DCE (Fig. 1a), while DPF lost its capacity to transform TCE to *cis*-DCE after about 2.5 years (about 15 transfers) (Fig. 1b). Since 1,1-DCE and *trans*-DCE never exceeded 3% of the initial concentration of PCE during initial analyses, these two compounds were not included during subsequent chemical analyses. Isotope fractionation was measured after DPF lost its capacity to transform TCE to *cis*-DCE. In our previous study (Liang et al. 2007), although transformation rates in duplicate samples varied considerably, there was no statistically significant difference in ε_{bulk} values calculated from a single microcosm (six data points) versus pooled duplicate microcosms (11 sampling points). Therefore, for the enrichment cultures in this study, only one of two replicate bottles was measured for isotope fractionation at each time point. The results showed that compared to DPH ($\varepsilon_{bulk} = -1.98 \pm 0.16‰$), much stronger isotope fractionation was observed in DPF ($\varepsilon_{bulk} = -10.29 \pm 0.47‰$) (Fig. 2).

No difference in product distribution was observed between DPH and DPH + formate (Fig. 1a, a′), or between DPF and DPF + H_2 (Fig. 1b, b′). While small differences in ε_{bulk} values between DPH and DPH + formate (Fig. 2a), and between DPF and DPF + H_2 (Fig. 2b) were observed, there was a general trend of weak isotope fractionation in DPH and DPH + formate versus stronger isotope fractionation in DPF and DPF + H_2 (Fig. 2). For the control samples (DPF + formate and DPH + H_2), both product distribution and dechlorination rates were similar to their parent cultures (DPF and DPH) (data not shown).

Influence of microbial community composition on isotope fractionation during microbial reductive dechlorination

Molecular analysis was performed to identify the potential dechlorinating species in the two 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. As expected, 1 mM treatment with BESA completely inhibited methanogenic reactions but did not change the dechlorinating capacity and dechlorination rates (data not shown), confirming that Bacteria were more important than methanogenic Archaea (Loeffler et al. 1997) in the cultures studied here.

RFLP analysis was performed on a total of 256 clones of the 16S rRNA clone libraries developed for DPF and DPH. Subsequent sequencing of representative RFLP groups indicated the presence of 11 OTUs in DPF and 10 OTUs in DPH. The phylogenetic affiliation of OTUs and their closest sequence matches are shown in Tables 1 and 2. As shown in Fig. 3, the observed accumulation of OTUs was fitted to a Michaelis–Menten Equation curve by using non-linear regression (Gu et al. 2004) and bacterial richness was calculated and expressed as N_{max} (Fig. 3). The maximum numbers for bacterial richness (number of different OTUs) are 14 for DPF and 10 for DPH. Therefore, the OTUs identified by RFLP and sequencing in this study accounted for approximately 79% and close to 100% of the expected maximum number of OTUs (N_{max}) for DPF and DPH, respectively.

Taxonomic classification showed that both cultures include *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, *Spirochaetes* and some unidentified species were only detected in DPF (Tables 1, 2). The phylogenetic relationship of these 16S rRNA gene clone sequences and their close relatives and type strains are illustrated in Fig. 4. The only identical OTUs shared by DPF and DPH were members of *Bacteroidetes* (Table 2; Fig. 4), while the other OTUs varied between these two enrichment cultures, although they were developed from the same source. In comparison, no

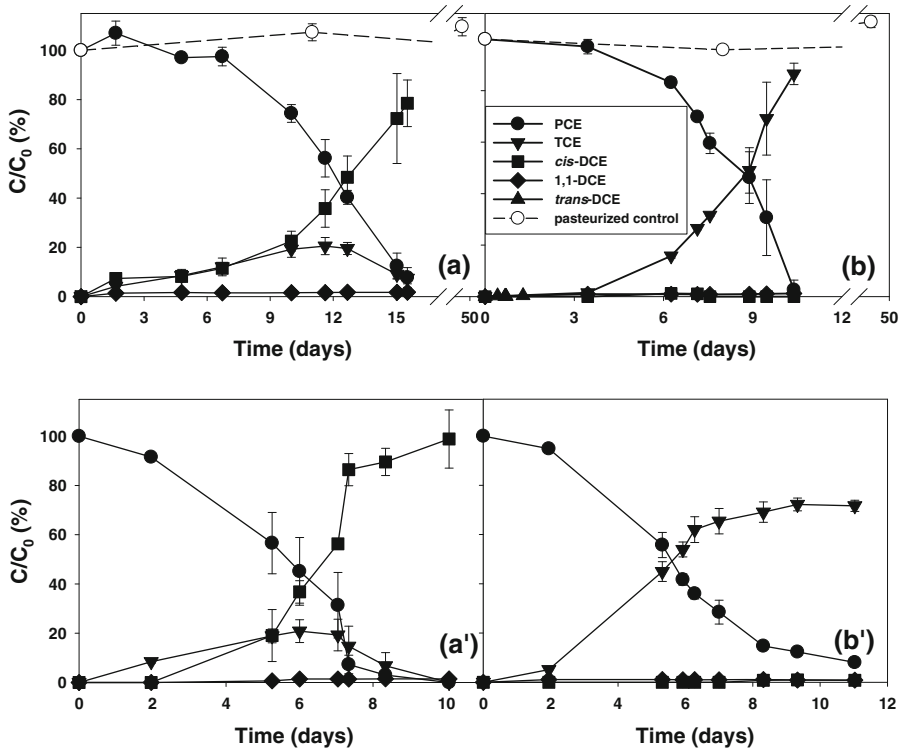

Fig. 1 PCE reductive dechlorination. Panels (**a**) and (**b**) show reductive dechlorination in DPH and DPF, respectively. Panels (**a**′) and (**b**′) show reductive dechlorination in DPH + formate and DPF + H_2, respectively. The pasteurized controls in (**a**) and (**b**) show PCE concentration for up to 48 days (negative controls). Data points are average values and *error bars* indicate the standard deviation of triplicate samples

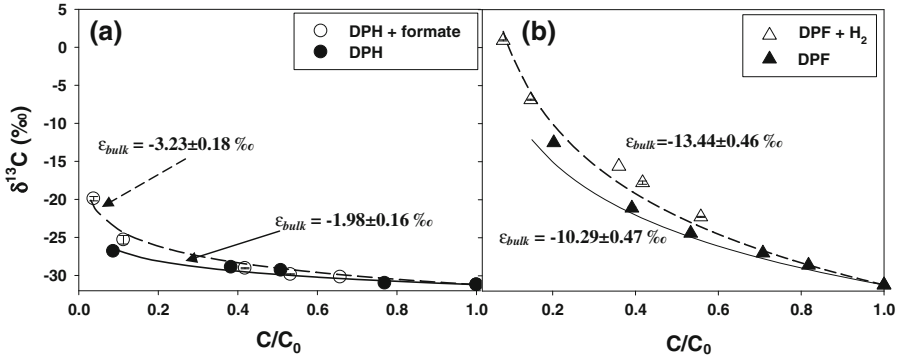

Fig. 2 Isotope fractionation during microbial reductive dechlorination of PCE in DPH and DPH + formate (**a**) and DPF and DPF + H_2 (**b**). The *solid* and *dashed curves* are Rayleigh Model fits. The coefficients of determination (R^2) for Rayleigh Model fits range from 0.95 to 0.98. About 30% of the samples were measured in duplicate and the *error bars* show the standard deviation for duplicate measurements. Some *error bars* are too small to be visible

dominant species was observed in DPF, but an OTU classified as *Sulfurospirillum* species accounted for 63% of the clone library for DPH.

Discussion

Negligible PCE and TCE reductive dechlorination in the pasteurized controls (Fig. 1) indicates that dechlorination in these cultures was due to microbial activity rather than indirect reductive dechlorination by extracellular agents (e.g., porphyrins, metalloproteins, or proteins) (Novak et al. 1998) that has been observed in some systems.

When we took bacteria from cultures grown for long periods with either formate or H_2 as the electron donor (i.e., DPF and DPH, respectively), then added these bacteria to serum bottles containing the opposite

Table 1 Phylum level distribution of clones from DPF and DPH

	DPF		DPH	
	Number of sequenced clones[a]	Fraction[b] (%)	Number of sequenced clones[a]	Fraction[b] (%)
Firmicutes	11	43.2	7	7.6
Proteobacteria				
δ-Proteobacteria	6	17.9	1	0.8
ε-Proteobacteria	ND	ND	9	63.0
Spirochaetes	3	6.0	ND	ND
Bacteroidetes	9	29.9	12	28.6
Unidentified	2	3.0	ND	ND

ND denotes not detected

[a] The number of sequences is the sum of all the successfully sequenced clones in one phylum/class; chimeras have been deleted

[b] The fraction is the percentage of clones classified to each phylum/class among the total number of clones picked in a clone library

electron donor (i.e., DPF + H_2 and DPH + formate, respectively) and immediately spiked with PCE, there were no dramatic changes in product distributions or ε_{bulk} values (Figs. 1, 2). This differs from previous studies in which electron donors and their concentrations were reported to influence isotope fractionation during microbial metabolism (e.g., dissimilatory sulfate reduction) (Bruechert 2004; Hoek and Canfield 2007), possibly by induction of different electron donor flow pathways (e.g., complete or incomplete oxidation) or rates of electron transfer along the electron transport chains (Bruechert 2004). Instead, the results reported here suggest that the electron donors may have indirectly influenced product distributions and ε_{bulk} values by selecting, over long time periods, for bacteria or communities that metabolize PCE differently.

Analysis of 16S rRNA genes reveals distinct microbial community composition in DPF and DPH. The percentages listed in Table 2 may not represent the actual microbial abundance in the enrichment cultures because different factors (e.g., potential PCR bias, cloning bias, and variable copy numbers of 16S rRNA genes in different bacteria) may result in deviation from the actual population distribution (Becker et al. 2000; Acinas et al. 2004). However, the absence of certain OTUs in one culture or the other indicates that electron donors significantly affected the microbial community composition in DPF and DPH (Table 2; Fig. 4). Although *Bacteroidetes* have been widely observed in microbial communities capable of reductively dechlorinating chlorinated ethylenes or PCBs (Boyle et al. 1999; Richardson et al. 2002), no isolates belonging to this phylum have been identified as dechlorinators. In addition, this phylum is known for fermentation of a variety of carbohydrates and simple sugars to produce smaller fatty acids and bicarbonate (Madigan et al. 2006). Thus, *Bacteroidetes* species detected in both cultures may act as fermenters and provide small organic molecules or H_2 for growth of dehalogenating bacteria.

Other than *Bacteroidetes* spp., no OTUs were shared by DPF and DPH, suggesting that different dehalogenating bacteria are responsible for PCE reductive dechlorination in the two cultures. DPHA01, the dominant sequence detected in DPH but not detected in DPF, was grouped within the *Sulfurospirillum* spp. of *ε-proteobacteria* (Table 2; Fig. 4). This sequence is 95% identical to *S. multivorans* and *S. halorespirans* strain PCE-M2, which reductively dechlorinates PCE to *cis*-DCE via dehalorespiration (Neumann et al. 1994; Luijten et al. 2003). It is reported that H_2 and formate can serve as electron donors for *Sulfurospirillum* spp. (e.g., *S. deleyiamum*, *S. cavolei*, *S. multivorans* and *S. halorespirans* strain PCE-M2) (Luijten et al. 2003), which is consistent with the nutrient conditions in DPH and its subculture DPH + formate. In addition, both *S. multivorans* and *S. halorespirans* weakly fractionate during PCE reductive dechlorination ($\varepsilon_{bulk} = -0.42$ to -1.33‰) (Nijenhuis et al. 2005; Liang et al. 2007; Cichocka et al. 2008), close to the observed isotope fractionation ($\varepsilon_{bulk} = -1.98$ to -3.23‰) in DPH. Although we cannot rule out the possibility that other dehalogenating bacteria are responsible for PCE reductive dechlorination in DPH, the above results provide

Table 2 Phylogenetic summary of DPH and DPF based on clone library construction and sequence analysis*

DPF		DPH		Phylogenetic group	Closest GenBank match[c]	Identity[d] (%)
OTU[a]	Fraction[b] (%)	OTU	Fraction (%)			
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
–[e]	–	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	–	–	Mollicutes	Clone M011_60, EU014057	98
–	–	DPHA05	4.2	Bacilli[f]	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
DPF29	1.5	–	–	Unclassified	Clone CLONG36, DQ478740.1	99
DPF33	1.5	–	–	Unclassified	Clone E1, EU864437.1	99

*DNA was extracted from about the 20th generation enrichments when about 80% PCE had been transformed, and clone libraries were constructed using bacterial primers 27F and 1492R

[a] The clones belonging to the same OTUs (≥97% in identity) detected in both samples are listed in the same row

[b] Fraction (%) indicates the percentage of each OTU among the total number of clones picked from one clone library

[c] Type strains with ≥95% similarity to DPF or DPH clones are listed; If no type strains are closely related to the DPF or DPH clones, the closest uncultured clones in database are included

[d] Values of identity in 16S rRNA gene sequences were determined by BLAST

[e] Specific OTU does not exist in this clone library

[f] DPHA05 was classified as Bacilli by Classification package in Greengenes (http://greengenes.lbl.gov) but as unclassified by Classifier in RDP (http://rdp.cme.msu.edu/classifier/classifier.jsp)

strong evidence that *Sulfurospirillum* related species are most likely responsible for PCE reductive dechlorination in DPH.

Different OTUs belonging to *Clostridia* were detected in both cultures (Table 2). Some species belonging to this class (e.g., *Desulfitobacterium* spp., *Dehalobacter* spp. and *Clostridium bifermentans* DPH-1) have been shown to dechlorinate chlorinated ethenes or chlorinated aromatic compounds (Fig. 4; Table 3) (Wild et al. 1996; Holliger et al. 1998; Chang et al. 2000; Villemur et al. 2006). Three OTUs from DPF (DPF35, DPHA07 and DPF18), were closely related to chlorobenzene, polychlorinated dioxin and dichlorophenol (DCP) dechlorinating consortia clones (Fig. 4) while other OTUs belonging to this phylum (DPF01, DPF04, DPF06, DPHE06 and DPHB07) were not closely related to any known dehalogenating species. Hence, the possible contribution of *Clostridia* to dechlorination in DPF cannot be ruled out.

The δ-proteobacteria include some known dechlorinators, including *Desulfuromonas* spp., *Desulfomonile tiedjei*, *Desulfovibrio* spp., *Geobacter lovleyi* and *Trichlorobacter thiogenes* (Loeffler et al. 2003). Analysis of species closely related to the OTUs

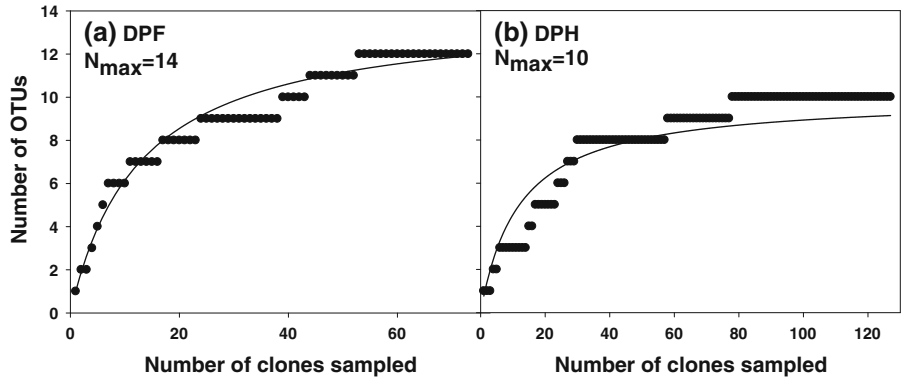

Fig. 3 Observed and estimated richness (OTUs) in DPF and DPH. The maximum richness (N_{max}) was estimated by non-linear regression of observed accumulation data to a Michaelis–Menten equation

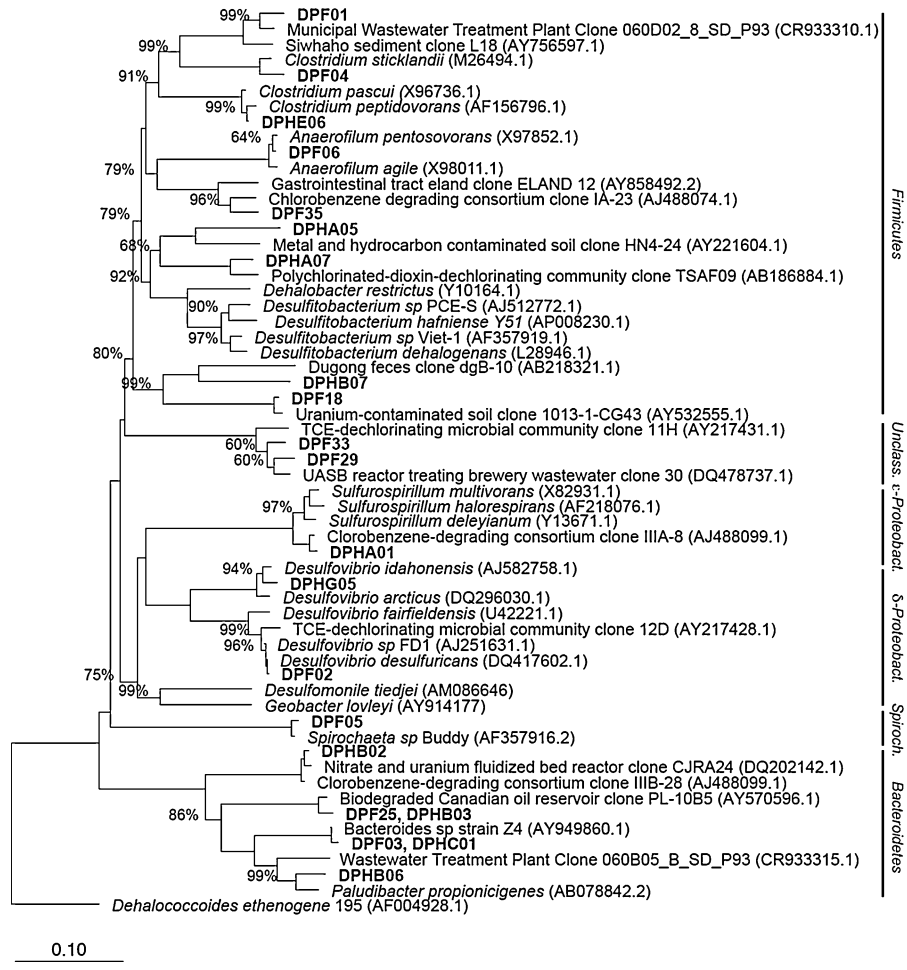

Fig. 4 Phylogenetic tree of 16S rRNA gene clone sequences (indicated by *boldface* type) of DPF, DPH and their close relatives and type strains. Calculation was performed by neighbor-joining methods incorporating Jukes–Cantor distance correction. *Dehalococcoides ethenogenes* strain 195 was used as the 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*; *ε-Proteobact*: *ε-Proteobacteria*; *δ-Proteobact*: *δ-Proteobacteria*; *Spiroch*: *Spirochaetes*

detected in DPF and DPH (Clone DPF02 and DPHG05 are closely related to *Desulfovibrio* spp.) suggest that they may contribute to the dehalogenating enrichments in one of two ways. First, it is possible that members of the *δ-Proteobacteria* may dechlorinate. Some *Desulfovibrio* species (e.g., *Desulfovibrio* sp. strain TBP-1 and strain SF3) are known to reductively dechlorinate halogenated contaminants,

Table 3 Product distribution and enrichment factors for stable carbon isotope fractionation during microbial PCE reductive dechlorination

Bacteria/enrichments	Product	Enrichment factor (ε, ‰)	Reference[a]
DPF consortia	TCE	−10.29 to −13.44	This study
Desulfitobacterium Viet 1		−16.7	Cichocka et al. (2008)
Anaerobic microcosms		−10.68 to −16.78	Dong et al. (2009)
DPH consortia	*cis*-DCE	−1.98 to −3.23	This study
Sulfurospirillum multivorans		−0.42 ± 0.08	Cichocka et al. (2008) and Nijenhuis et al. (2005)
		−1.33 ± 0.13	Liang et al. (2007)
Desulfitobacterium sp. strain PCE-S		−5.18 to −5.4	Cichocka et al. (2008) and Nijenhuis et al. (2005)
Desulfuromonas michiganensis strain BB1		NS	Cichocka et al. (2008)
		−1.39 ± 0. 21	Liang et al. (2007)
Sulfurospirillum halorespirans		−0.46	Cichocka et al. (2008)
Geobacter lovleyi strain SZ		NS	Cichocka et al. (2008)
Anaerobic microcosms		−2.15 to −3.1	Dong et al. (2009)
Dehalococcoides ethenogenes strain 195[a]	Ethene	−6.0	Cichocka et al. (2008)
BDI		−7.11 ± 0.72	Liang et al. (2007)
KB-1 consortium		−2.6 to −5.5	Slater et al. (2001)

NS not significant

[a] *Dehalococcoides ethenogenes* strain 195 contains a PCE reductive dehalogenase (PCE-RDase) transforming PCE to TCE and a TCE Reductive dehalogenase (TCE-RDase) degrading TCE to ethene (Magnuson et al. 1998)

including 2,4,6-tribromophenol and 2-chlorophenol (Boyle et al. 1999; Sun et al. 2000). In this study, the clone DPF02 is closely related to a clone from a TCE-dechlorinating microbial community (Fig. 4) based on 16S rRNA sequence, which suggests that this species might be involved in PCE reductive dechlorination in DPF. Second, *Desulfovibrio* species may form syntrophic associations with dechlorinating bacteria. Some *Desulfovibrio* spp. (e.g., *Desulfovibrio* sp. strain SULF1, *Desulfovibrio fructosivorans* and *Desulfovibrio desulfuricans*) can also produce hydrogen by transformation of organic compounds added to the medium, which can be transferred to dehalogenating bacteria and thus support microbial reductive dechlorination (Drzyzga et al. 2001; He et al. 2007).

Members of *Spirochaetes* were only detected in DPF. The absence of dechlorinating species in this phylum and the capacity to ferment carbohydrates to acetate or ethanol by many species belonging to this class (Canale-Perola 1984) suggest that the functions of these OTUs in DPF may be similar to those of *Bacteroidetes*.

Chemical, isotope and microbial community analyses suggested that the different extent of isotope fractionation in DPF and DPH prepared from the same sediment might be due to the stimulation of unique dechlorinating communities in the presence of different electron donors. This may explain why differences in levels of isotope fractionation have been observed for the same enrichment culture in prior studies (e.g., TCE dechlorination by KB-1) (Bloom et al. 2000; Slater et al. 2001), or in cultures that were incubated with different electron donors (e.g., butyric acid versus ethanol for PCE dechlorination by TP microbial consortia) (Slater et al. 2001). In this study, a *Sulfurospirillum* relative is proposed to be the dechlorinator in DPH. While it is not clear which species was responsible for PCE reductive dechlorination in DPF, we suggest that one of several possible microbial groups including a species affiliated with *Desulfitobacterium* or *Desulfovibrio* could act as the dechlorinator(s) in DPF. These results suggest that multiple dechlorinating bacteria are likely present at a given site and preferential growth of one or more of these species is

likely influenced by electron donor. Microbial communities, in turn, could strongly influence the ε_{bulk} values measured during PCE reductive dechlorination.

Considering the observed product distribution and the corresponding extents of isotope fractionation, our results (strong isotope fractionation in TCE-producing DPF, versus weak isotope fractionation in *cis*-DCE-producing DPH) are consistent with the general trend that has been reported for microbial PCE transformation by different isolates, enrichment cultures and microcosms (Table 3). This might be due to the different structure of functional enzymes or mechanisms of enzymatic reactions. To the best of our knowledge, there have been no reports on characterization of PCE reductive dehalogenases in TCE-producing dehalogenating bacteria. However, indirect evidence of potential structural differences between the reductive dehalogenase involved in transforming PCE to TCE (PCE reductive dehalogenase) and the one producing lower dechlorinated products (e.g., *cis*-DCE) (TCE reductive dehalogenase) can be derived from the study on a two-component enzyme pathway of *Dehalococcoides ethenogenes* 195 (Magnuson et al. 1998). These two dehalogenases responded to the inhibition by 1-iodopropane and iodoethane in different ways, suggesting their substrate binding pockets to be different sizes (Magnuson et al. 1998).

Others (Cichocka et al. 2008; Hunkeler et al. 2008) have illustrated quantitatively how ε_{bulk} values can significantly influence estimates of biodegradation at contaminated sites using a modified Rayleigh Model. Therefore, it is recommended that microcosms be developed from site-specific sediment and ground water to determine appropriate ε_{bulk} values to be applied at that particular site. Based on this study, identifying final products of reductive dechlorination and performing microbial community analysis can provide information for choosing representative ε_{bulk} values for biodegradation monitoring. In addition, ε_{bulk} values should be periodically re-measured in long-term bioremediation projects to account for the influence of shifts in microbial community structure over time. This is especially important for in-situ remediation approaches that involve addition of electron donors (e.g., molasses, whey and hydrogen release compounds).

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