Bacterial community analysis of shallow groundwater undergoing sequential anaerobic and aerobic chloroethene biotransformation

Todd R. Miller¹, Mark P. Franklin¹ & Rolf U. Halden¹,²

¹Department of Environmental Health Sciences, Center for Water and Health, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, USA; and ²Department of Geography and Environmental Engineering, Whiting School of Engineering, Johns Hopkins University, Baltimore, USA

Correspondence: Rolf U. Halden, 615 N. Wolfe Street, Rm E6618, Baltimore, MD 21205, USA. Tel.: +410 955 2609; fax: +410 955 9334; e-mail: rhalden@jhsph.edu

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Abstract
At Department of Energy Site 300, beneficial hydrocarbon cocontaminants and favorable subsurface conditions facilitate sequential reductive dechlorination of tetrachloroethene (TCE) and rapid oxidation of the resultant cis-dichloroethene (cis-DCE) upon periodic oxygen influx. We assessed the geochemistry and microbial community of groundwater from across the site. Removal of cis-DCE was shown to coincide with oxygen influx in hydrocarbon-containing groundwater near the source area. Principal component analysis of contaminants and inorganic compounds showed that monitoring wells could be differentiated based upon concentrations of TCE, cis-DCE, and nitrate. Structurally similar communities were detected in groundwater from wells containing cis-DCE, high TCE, and low nitrate levels. Bacteria identified by sequencing 16S rRNA genes belonged to seven phylogenetic groups, including Alpha-, Beta-, Gamma- and Deltaproteobacteria, Nitrospira, Firmicutes and Cytophaga–Flexibacter–Bacteroidetes (CFB). Whereas members of the Burkholderiales and CFB group were abundant in all wells (10⁴–10⁹ 16S rRNA gene copies L⁻¹), quantitative PCR showed that Alphaproteobacteria were elevated (> 10⁶ L⁻¹) only in wells containing hydrocarbon cocontaminants. The study shows that bacterial community structure is related to groundwater geochemistry and that Alphaproteobacteria are enriched in locales where cis-DCE removal occurs.

Introduction
Chloroethenes are ubiquitous pollutants found at many priority (Superfund) cleanup sites owned and operated by private, industrial, and Federal entities, including the US Department of Energy (DOE). Among the various chloroethenes, trichloroethene (TCE) is of particular importance due to this toxicant’s extensive usage as a degreasing agent and as a heat exchange fluid either in pure form or in a mixture with silicon-based lubricants of tetraalkoxy silane structure (Vancheswaran et al., 1999). Chloroethenes are known or suspected carcinogens and their concentrations in drinking water are regulated at the federal level (Chu & Milman, 1981; Moore & Harrington-Brock, 2000; Rhomberg, 2000). Thus, removal of these contaminants from groundwater is of great importance for public health.

In situ bioremediation is one strategy for the removal of chloroethenes from groundwater. It frequently relies on the degradative activity of microorganisms native to the site. Under anaerobic conditions, dehalorespiring microorganisms are known to utilize chlorinated ethenes as terminal electron acceptors by replacing chlorines with hydrogen, using naturally occurring, intentionally added, or cocontaminating compounds as electron donors (Vogel & McCarty, 1985; de Bruin et al., 1992; Holliger et al., 1993; Holliger & Schumacher, 1994). In this process, tetrachloroethene (PCE) is sequentially dechlorinated to TCE, cis-DCE, vinyl chloride and finally to the nonhazardous compounds, ethene and ethane which in turn can be oxidized to carbon dioxide under aerobic conditions (de Bruin et al., 1992; Magnuson et al., 1998). Incomplete reduction of PCE and TCE is common, resulting in the accumulation of less chlorinated, though toxic derivatives, including cis-DCE and vinyl chloride. This common scenario is usually caused by a lack of suitable electron donors and/or the absence of microbial species able to carry out complete dechlorination (Harkness et al., 1999; Lendvay et al., 2003). While addition of alternate electron donors and bioaugmentation with laboratory-cultivated PCE-to-ethene respiring populations has proven successful in some instances, such an approach may be
complicated by other factors including the periodic influx of oxygen which can preclude growth of obligate anaerobes, and the presence of indigenous dehalorespirers that may outcompete bioaugmented species for reducing equivalents (Becker, 2006). For this reason it is advantageous to fully characterize geochemical parameters and microbial species on a site-by-site basis.

At DOE Site 300 near Livermore, CA, spillage of TCE together with tetrakis(2-ethylbutoxy)silane (TKEBS) has created favorable subsurface conditions previously shown to support the incomplete reductive dechlorination of TCE to cis-DCE under anaerobic conditions by organisms closely related to *Dehalobacter restrictus* (Lowe et al., 2002). This is consistent with reports indicating that this bacterium converts PCE and TCE to cis-DCE during anaerobic growth in the presence of molecular hydrogen, although it does not further dechlorinate the resultant cis-DCE (Holliger et al., 1993). Potential electron donors for dehalogenases are dihydrogen and fumarate, both common end-products produced by fermentative mixed microbial communities. At Site 300, fermentation of 2-ethylbutanol and n-butanol, released continuously during slow hydrolysis of silicon lubricants, has been shown to support reductive dechlorination of TCE to cis-DCE (Vancheeswaran et al., 2003). The accumulation of cis-DCE under anaerobic conditions is problematic; however, it may biodegrade aerobically under favorable conditions (Bradley & Chapelle, 1998; Azizian et al., 2005).

In the presence of oxygen, cometabolism of chloroethenes by nonspecific oxygenases may occur. A variety of species have been shown to produce mono- and dioxygenases that have relaxed substrate specificities, supporting the oxidation of primary growth substrates and of a number of chlorinated ethenes serving as cosubstrates (Folsom et al., 1990; Mcclay et al., 1995; Shim et al., 2001). For example, methanotrophic species of the genera *Methylocystis*, *Methylosinus*, *Methyloomonas* and *Methylococcus* possess soluble methane monooxygenases that rapidly transform aliphatic and aromatic substrates, including chlorinated ethenes to less toxic compounds in the presence of suitable electron donor, carbon and nitrogen sources (Koh et al., 1993; Grosse et al., 1999). *Methylococcus capsulatus* also aerobically degrades methyl bromide (Oremland et al., 1994). Other TCE- and DCE-cometabolizing species, including *Burkholderia*, *Ralstonia*, *Pseudomonas*, *Mycobacterium* and *Xanthomonas*, can utilize ammonia, toluene, ethylene, propylene, or other hydrocarbons as growth substrates [reviewed in (Arp et al., 2001)].

In this study, we observed removal of anaerobically accumulated cis-DCE from shallow perched groundwater at DOE Site 300 when oxygen was introduced due to soil vapor extraction activities and/or rainfall events. To begin to understand what members of the microbial community might be involved in this removal process, we assessed the geochemistry and microbial community composition in groundwater from subsurface strata where cis-DCE removal occurs in the presence of cocontaminating hydrocarbons. Whereas previous microbial ecological studies of the site employed 16S rRNA gene cloning and focused on two monitoring wells in pristine and highly contaminated locations (monitoring wells T5 and D3, respectively) (Lowe et al., 2002), the present study utilized denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) to explore the microbial community within a larger geochemical gradient intersected by a total of five sampling locations containing multiple contaminants.

**Materials and methods**

**Study site description and sample collection**

Site 300 is an explosives test facility located in the Altamont Hills of CA, 100 km southeast of San Francisco. It is operated by the Department of Energy and listed as an EPA Superfund site. The site is used as an Operable Unit known as the Building 834 complex (B834) where TCE and TKEBS were spilled from faulty piping systems over the course of several decades. This chemical mixture is contaminating a shallow perched water-bearing zone in which chloroethenes are migrating in a southwesterly direction. Monitoring wells provide access to groundwater outside and inside of the chloroethene plume, representing pristine to highly contaminated conditions with varying amounts of TCE, TKEBS and cis-DCE. In addition, diesel is present in groundwater from well U1, situated in a location previously occupied by a fuel tank.

For chemical analyses, water was pumped to the surface using an *in situ* pump and stored in glass bottles certified for volatile organic carbon analysis (1-Chem, Rockwood, TN). Microorganisms were sampled by passing 4 L of groundwater through sterile 0.2 µm Mini capsule filters (Pall Corporation, MI). Both water samples and filters collected in May of 2002 were immediately placed on ice for transport back to the laboratory, where they were stored at −20°C.

**DNA extraction**

Genomic DNA was extracted directly from filters as previously described by Giovannoni et al. (1996), with minor modifications. Each unit received 2 mL of lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 sucrose and 50 mM Tris HCl pH 9.0) and were incubated at 37°C for 15 min. Proteinase K (0.2 g mL⁻¹) and 1% SDS was added and the units incubated at 55°C for 60 min. The lysate was recovered and extracted twice with equal volumes of phenol–chloroform–iso amyl alcohol (25:24:1; pH 8) followed by a single extraction with an equal volume of chloroform. The
aqueous phase was removed and the DNA precipitated using isopropanol and sodium acetate, washed with 70% ethanol and resuspended in 300 μL of sterile distilled water.

**PCR and DGGE**

The 16S rRNA gene was amplified in a PCR using eubacterial primers 907R and GM5F-GC-clamp (Teske et al., 1996). These primers amplify the variable V3 region of the 16S rRNA gene producing 550 bp fragments suitable for phylogenetic comparisons (Muyzer et al., 1995). Each 50 μL reaction mixture contained 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 2% bovine serum albumin (BSA), 100 pmol each dNTP, 50 pmol of each primer, 1 U of Redtaq DNA polymerase (Sigma, Milwaukee, WI) and a total of 1 ng of extracted DNA. After a ‘hot start’ at 94 °C for 5 min the reaction mix underwent a touchdown PCR whereby the initial annealing temperature was set at 65 °C and decreased by 1 °C every two cycles until a touchdown of 55 °C, at which temperature 10 additional cycles were carried out. Amplification was performed at 1 min of denaturation at 94 °C, 1 min of primer annealing, and 1 min of primer extension at 72 °C, followed by 7 min of final primer extension. We also attempted to amplify 16S rRNA genes from known dehalorespiring bacteria using primer sets and PCR conditions previously reported (Löffler et al., 2000; Fagervold et al., 2005). PCR products were analyzed by DGGE using a BioRad Dcode system (BioRad, Hercules, CA). Replicate PCR products were loaded onto multiple 6% (w/v) acrylamide gels [37.5 : 1 acrylamide : N,N’-methylenebis-acrylamide in 20 mM Tris buffer (pH 7.8), 10 mM sodium acetate, and 0.5 mM disodium EDTA] containing linear chemical gradients with a 5% change in denaturant concentration ranging from 35–65% denaturant, where 100% denaturant is equal to 7 M urea and 40% deionized formamide. The gels were run at 100 V for 18 h at 60 °C. Gels were stained with 50 μg mL⁻¹ ethidium bromide and visualized using a UV transilluminator and gel documentation system (Alpha Imager 2000 V5.5, Alpha Innotech Corporation, VA). All visible bands were excised from the gels using sterile pipette tips and eluted from the gel matrix in 100 μL of sterile distilled water overnight at 4 °C. A 1-μL aliquot of the suspension was used for PCR amplification using the same primers as described above and PCR products were reanalyzed by DGGE to confirm the presence of a single band. PCR products were purified with the QIAquick-spin DNA purification system (Qiagen, Valencia, CA) as per manufacturer’s instructions and subjected to cycle sequencing using an Applied Biosystems 3730 DNA Analyzer.

**Analysis of DGGE gels**

DGGE gel band patterns were subjected to computer analysis to compare bacterial community structure. Band migration distances were measured from a common point at the top of the gel to the middle of each band using Adobe Photoshop (San Jose, CA). To facilitate band identification a straight horizontal line was drawn across the middle of each band, and three measurements were made per band. These data were then used to construct a dendrogram according to the unweighted pair group method with arithmetic mean (UPGMA) using the SIMINT and SAHN modules of the NTSYSpc Analysis software package (EXETER software, Setauket, NY).

**Comparative sequence analysis**

All sequences obtained from DGGE bands were analyzed for chimeras in a single batch file using the Bellerophon server (Huber et al., 2004). Putative chimeric sequences were then reanalyzed individually using Pintail (Ashelford et al., 2005), comparing percent base pair mismatches of the putative chimeric sequence to its closest BLAST match using a window size of 25 bp. Sequences were judged to be chimeric based upon the deviation from an expectation statistic calculated by the software. Nonchimeric sequences were then used in a BLAST search (Altschul et al., 1990) to find the top five most similar sequences in GenBank and all sequences were manually aligned according to the 16S rRNA gene secondary structure using Phytid (Chun, 1995) and *E. coli* 16S rRNA gene secondary structure as the template. Additional sequences from reference strains obtained from GenBank were added to delineate representative taxons. Evolutionary trees were generated using the neighbor-joining, Fitch-Margoliash, and maximum parsimony algorithms in the PHYLIP package (Felsenstein, 1989). Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes & Cantor (1969). The confidence in tree topology was evaluated after 1000 bootstrap samplings of the neighbor-joining data, and only values of 500 or higher were shown on the tree.

**Chemical analyses**

Concentrations of chloroethenes, TKEBS and inorganic compounds in groundwater samples were determined by GC/MS using standard methods as previously described (Lowe et al., 2002). Contour maps showing contaminant plumes were constructed from a database of regulatory compliance monitoring data as previously described (Lowe et al., 2002).

**Principal component analysis**

A principal component analysis of organic and inorganic chemical concentrations was performed using NTSYSpc software. TKEBS and diesel contaminants were not included due to infrequent sampling for these compounds. These
data were standardized using the STAND module and the transformed data used to compute a correlation matrix using the SIMINT module. Following calculation of Eigen vectors, both observations and vectors were projected onto the principal component axes using the MXPLOT module.

**Real-time quantitative PCR (qPCR)**

Group-specific primers corresponding to major bacterial groups defined by a phylogenetic analysis were constructed and used in qPCR to estimate group-level abundance in groundwater. All sequences obtained from DGGE bands were analyzed using PRIMROSE computer software (Ashelford et al., 2002) to find unique oligonucleotides within sequences belonging to each phylogenetic group. Pairs of oligonucleotides with similar melting temperatures (55–65 °C) were chosen that could serve as group-specific forward and reverse primers and that amplified a 75–150 bp portion of the 16S rRNA gene. The OLIGOCHECK program (Ashelford et al., 2002) was then used to determine group specificity of each primer pair relative to 16S rRNA gene sequences identified at the site and those within the Ribosomal Database Project (release 8.1) combined (Cole et al., 2003).

Real-time quantitative PCR (qPCR) was performed using a Smart Cycler System (Cepheid, Sunnyvale, CA) as described elsewhere (Monis et al., 2005). Each 25 μL reaction contained 1 × qPCR buffer [50 mM KCl, 10 mM Tris HCl (pH 8.0), 1.5 mM MgCl2, and 2 μM Syto 9 (Molecular Probes, Eugene, OR)], 0.4 μM each deoxynucleotide triphosphate, 5 pmol each primer, 1 U Taq DNA polymerase (New England Biolabs, Ipswich, MA) and 1–20 ng of template DNA. The PCR conditions were an initial denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 95 °C for 30 s, annealing for 30 s and elongation at 72 °C for 30 s. Annealing temperatures were 55, 57, and 61 °C for group I, II and V primer pairs, respectively (Table 1).

Fluorescence of Syto 9 was measured after each elongation group I, II and V primer pairs, respectively (Table 1). 30 s. Annealing temperatures were 55, 57, and 61 °C for group I, II and V primer pairs, respectively (Table 1). 30 s. Annealing temperatures were 55, 57, and 61 °C for group I, II and V primer pairs, respectively (Table 1). 30 s. Annealing temperatures were 55, 57, and 61 °C for group I, II and V primer pairs, respectively (Table 1). 30 s. Annealing temperatures were 55, 57, and 61 °C for group I, II and V primer pairs, respectively (Table 1).

The 16S rRNA gene fragments from known bacteria were excised from DGGE bands, reamplified and used as standards for qPCR reactions. Resulting PCR products were purified using a Qiagen PCR cleanup kit according to the manufacturer’s instructions and a portion analyzed by DGGE to confirm a single band. The concentration of the purified standard was determined by adding a 5 μL aliquot to a 1 : 1000 dilution of Sybr Gold DNA stain in distilled water and fluorescent intensity measured at excitation and emission wavelengths of 490 and 530 nm, respectively, using a SpectraMax Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA). Fluorescent intensity was compared to that of a dilution series of bacteriophage lambda DNA (New England Biolabs, Ipswich, MA), to determine the mass per volume (ng μL−1) of the purified 16S rRNA gene standards. The 16S rRNA gene copy number of each standard was calculated using the following formula: Copies 16S rRNA gene = (Mass of standard ng μL−1)/length of standard (bp) × (1.09 × 10−15) (Feris et al., 2003).

**Nucleotide sequence accession numbers**

The ~550 bp partial 16S rRNA gene sequences from Site 300 groundwater bacteria were deposited in GenBank under accession numbers DQ336570–DQ336606.

**Results**

**Distribution of contaminants and inorganic compounds**

A contour map showing the distribution of TCE, cis-DCE and TKEBS throughout the study site is shown in Fig. 1. Well D3 is located in a source area where c. 3000 kg of TCE

<table>
<thead>
<tr>
<th>Target product size (bp)</th>
<th>Group most closely affiliated with</th>
<th>Primer name/sequence</th>
<th>$T_m$</th>
<th>Matches to target/nontarget sequences in RDP (%)</th>
<th>$t$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (77)</td>
<td>CFB group</td>
<td>CFB-forward (5'-3') YTYCGTGCCAGSAGCCCGS</td>
<td>62.8</td>
<td>91/7.4</td>
<td></td>
</tr>
<tr>
<td>Group II (76)</td>
<td>Alphaproteobacteria</td>
<td>Alpha-forward (5'-3') ATAAGCGGAGCTAAGCTCG</td>
<td>60.4</td>
<td>86/2.1</td>
<td></td>
</tr>
<tr>
<td>Group V (86)</td>
<td>Burkholderiales</td>
<td>Burk-forward (5'-3') AAGCAACCCGCTAACTAGCGGA</td>
<td>64.2</td>
<td>93/8.4</td>
<td></td>
</tr>
</tbody>
</table>

*16S rRNA gene sequences recovered from groundwater were aligned with each other and unique oligonucleotides within groups of phylogenetically related sequences were identified as primer pairs for qPCR.

Indicates the percent of all target or nontarget sequences in RDP matched by the primer pairs allowing for no more than two mismatches.
were spilled together with similarly substantial but unknown quantities of silicon oil (TKEBS). Longterm monitoring data was used to calculate the average yearly chemical concentrations ($n = 5–32$). Groundwater in this location is characterized by moderately high concentrations of TCE (yearly average and SD of 40 ± 25 mg L$^{-1}$) and relatively high concentrations of the bacterial dechlorination product cis-DCE (23 ± 32 mg L$^{-1}$) (Fig. 2a). This is also the only well containing insoluble quantities of the silicon oil TKEBS (173 ± 274 mg L$^{-1}$). Well U1 is closest to well D3 and contains higher concentrations of TCE (123 ± 28 mg L$^{-1}$) but low concentrations of cis-DCE (2.0 ± 1.8 mg L$^{-1}$). This well is in an area where unknown amounts of diesel fuel were spilled over the years. Marking the northern edge of the TCE contour is well B3, which contains moderately high concentrations of cis-DCE (8.6 ± 4.9 mg L$^{-1}$), and comparatively little TCE (0.35 ± 0.38 mg L$^{-1}$). At the southern end of the TCE contour is well T2, located about 300 m downgradient of the source area. It contains elevated concentrations of TCE (21 ± 6.2 mg L$^{-1}$), no detectable cis-DCE (< 0.005 mg L$^{-1}$), and none of the hydrocarbon contaminants known to be associated with both anaerobic and aerobic transformation of chloroethenes (Vancheeswaran et al., 1999). Another well, T5, located further down gradient, is considered a control well since no contaminants are present.

Select electron acceptors and inorganic nutrients for bacterial growth were also measured in site groundwater (Fig. 2b). Nitrate ($\text{NO}_3^-$) levels varied considerably across the sampling locations. Wells T2 (1.16 μM) and T5 (1.61 μM) had average nitrate concentrations that were 1–2 orders of magnitude higher than those found in wells B3, D3, and U1 (0.18, 0.17, and 0.006 μM, respectively) (Fig. 2b). Sulfate levels were highest in wells B3 and T2, followed by wells...
U1, T5 and D3. Ammonia (NH₄⁺) was low in all wells (0.003–0.02 µM) and nitrite (NO₂⁻) did not vary considerably except for well U1, wherein concentrations were lower (0.06 µM) than measured elsewhere (0.31–0.51 µM). Dissolved oxygen concentrations (data not shown) in wells U1 and D3 are low, and variable (< 0.4–5.1 mg L⁻¹; average and SD of 1.6 ± 0.42 and 1.7 ± 1.59, respectively) while oxygen levels in wells B3 and T2 are slightly higher (2.2 ± 2.2 and 2.6 ± 1.9 mg L⁻¹, respectively) and well T5 is oxygenated year round (6.5 ± 1.0 mg L⁻¹).

**Yearly fluctuations in chloroethenes and dissolved oxygen**

At the source area near well D3, removal of the bacterial dechlorination product cis-DCE occurs simultaneously with oxygen influx. This is exemplified in Fig. 3 which shows typical yearly trends in oxygen and chloroethene concentrations and is representative of a larger database of regulatory compliance monitoring data. During August and September, dissolved oxygen in Well D3 groundwater dropped to 1 mg L⁻¹ while levels of cis-DCE increased from 8.3 to 42.7 mg L⁻¹. In October, a spike in oxygen levels occurred and cis-DCE concentrations dropped to 1.7 mg L⁻¹. In November, the well returned to a low-oxygen state and cis-DCE levels increased from 1.2 to 16.4 mg L⁻¹. Continuous dissolution of dense nonaqueous phase liquid containing chloroethenes, silicon oil and other contaminants led to concentrations remaining above 30 mg L⁻¹, except when oxygen levels dropped below 1 mg L⁻¹ for 2 months or longer, thereby promoting the reductive dehalogenation process known to occur at the site (Lowe et al., 2002).

**Principal component analysis**

To determine overall geochemical relationships among the wells, concentrations of TCE, cis-DCE, nitrate, nitrite, ammonia and sulfate were subjected to a principal component (PC) analysis. Approximately, 50%, 26% and 15% of the variability in the dataset could be explained by PC1, PC2, and PC3, respectively. Figure 4 shows the first two axes of the PC analysis along with the vectors (monitoring wells) and observations (chemical measurements). The amount of scattering of observations away from the origin indicates chemical variability among all wells while the angle between two vectors (dotted lines) indicates the level of correlation between two wells. The first PC (PC1) clearly differentiates wells on a horizontal plane, based upon TCE concentration, whereas the second PC (PC2) separates the wells by cis-DCE along the vertical axis. The third PC (not shown) separates wells by nitrate levels along a z-axis. Accordingly, well B3 contains cis-DCE and no TCE, which places it in an almost...
completely vertical orientation away from well T5, the pristine control location. Wells T2 and U1 are separated by a small angle. Both contain a small amount of cis-DCE and comparatively significant levels of TCE, which places them on a horizontal plane. Well D3 contains both DCE and TCE, causing a trajectory falling between well B3 and the T2/U1 grouping. Sulfate, nitrite and ammonia are not as important for variations in geochemistry among the wells, as evidenced by the fact that all of these observations are grouped near the origin.

**Bacterial community profiles**

We examined if variations in groundwater chemistry reflected differences in bacterial community structure using DGGE. This technique separates heterogeneous samples of 16S rRNA gene fragments based upon their melting characteristics, and it represents a rapid tool for fingerprinting analysis and preliminary identification of relatively abundant community members (Watts et al., 2001). Representative gel patterns are shown in Fig. 5a. Bacterial 16S rRNA gene fragments amplified from the study site produced 6–11 bands per well in the denaturing gel. The bacterial community from wells U1 and D3 produced the largest number of bands (11 and 10, respectively) followed by well B3 (8 bands) then wells T2 and T5, which produced the least number of bands (7 and 6, respectively). Bands were marked with a horizontal line (Fig. 5b) and band migration distances measured, defining bands with equal distances as operational taxonomic units (OTU). None of the OTUs were shared among all wells. However, a total of seven were shared among at least two wells (closed arrow) while one band is shared among four wells (open arrow).

Fig. 5. This representative DGGE gel shows the microbial community structure in groundwater from five monitoring wells, as determined by separation of fragments of 16S rRNA genes amplified from extracted total DNA (a). Following image annotation (b), the migration distances of individual bands were interpreted by UPGMA cluster analysis to yield a dendrogram (c) indicating considerable species diversity at the site, with similar structure observed in wells T5 and T2, as well as in D3 and U1. Several bands are shared among at least two wells (closed arrow) while one band is shared among four wells (open arrow).
Identification of bacterial community members

To better understand these differences in bacterial species composition, 16S rRNA gene fragments in DGGE bands were sequenced and analyzed. A total of 37 nonchimeric, nonredundant sequences were recovered and aligned with GenBank entries (NCBI, Bethesda, MD). In neighbor-joining trees the bacteria clustered into seven well-supported phylogenetic groups including *Cyanophaga–Flexibacter–Bacteroidetes* (CFB), Alpha-, Beta-, Gamma- and Delta-proteobacteria, *Nitrospira* and *Firmicutes* (Fig. 6). Well D3, near the source area, contained 16S rRNA gene sequences related to that of *Acidovorax* sp. UFZ-B517 that grows aerobically on chlorobenzene (clone number, expect value, percent sequence identity; 115-32, E = 0, 98%). This well also contained multiple sequences related to hydrogen oxidizing bacteria within the genus *Hydrogenophaga* (112-23, E = 0, 99% and 115-29, E = 0, 99%) and sequences related to the nitrogen fixing, root symbiont, *Capriavidus taiwanensis* (115-31, E = 0, 99%) (Wen et al., 1999; Probian et al., 1999; Verma et al., 2004). Well U1, which is near well D3, also contained 16S rRNA gene sequences similar to hydrogen oxidizing bacteria within the genus *Hydrogenophaga* (115-41, E = 0, 99%) (Wen et al., 1999). In addition, this well contained sequences related to an aerobic benzene degrading bacterium (112-26, E = 0, 95%), an estradiol degrading *Novosphingomonas tardaugens* ARI-1 (112-43, E = 0, 98%), methylotrophic species (115-38, E = 0, 96%), a denitrifying pivalic acid degrading bacterium (112-28, E = 0, 95%), a thiosulfate oxidizing bacterium (115-39, E = 0, 98%) and bacteria previously found in a uranium contaminated aquifer (115-40, E = 0, 98%) (Rooney-Varga et al., 1999; Fuji et al., 2002; Gihring et al., 2002; Graff & Stubner, 2003; Probian et al., 2003; Lueders et al., 2004). Well B3 contained sequences similar to a manganese oxidizing bacterium, *Leptothrix discophora* strain SP-6 (115-18, E = 1e-133, 93%), a denitrifying pivalic acid degrading bacterium (115-39, E = 0, 97%), hydrogen oxidizing bacteria (112-53, E = 0, 99%) and a type II methane oxidizing bacterium (115-14, E = 0, 98%) (Siering & Ghirose, 1996; Wen et al., 1999; Heyer et al., 2002; Probian et al., 2003). Both wells T2 and T5 contained many sequences related to uncultured and/or uncharacterized bacteria or cloned sequences; however, well T2 contained 16S rRNA gene sequences similar to that of the filamentous bacterium *Halalsomenobacter hydrosis* (110-14, E = 0, 98%), normally associated with wastewater treatment systems, as well as 16S rRNA gene sequences related to a hydrogen oxidizing bacterium (115-72, E = 0, 99%) and a sulfate reducing bacterium (115-76, E = 0, 99%) (Wen et al., 1999; Bade et al., 2000; Schauer & Hahn, 2005).

When grouped by monitoring well, bacterial community structure varied by distance to the source area (well D3). For example, in the proximate wells, D3, U1 and B3, the majority of species identified (67%, 64%, and 80% of species diversity, respectively) were related to the *Burkholderiales*, whereas in well T2 most species were related to *Firmicutes* (30%) or the CFB group (30%).

Abundance of bacterial groups

To estimate bacterial abundance at the group level, qPCR primers were designed to amplify 16S rRNA genes from bacterial clusters identified by the phylogenetic analysis. Primer pairs for qPCR designed specifically for groups I, II and V allowed for two mismatches between primer and template (Table 1). All sequences within each group identified at Site 300 could be amplified by the appropriate primer pair. When aligned with the Ribosomal Database Project, 91%, 86% and 93% of sequences amplified by the group I, II, and V primer pairs were from the CFB group, *Alphaproteobacteria* and *Burkholderiales*, respectively. Within group II, the majority of targets were *Sphingomonas* species (99%). In qPCR reactions using 10^3 to > 10^7 copies of known 16S rRNA gene fragments as template, there was a reciprocal, linear relationship between target template concentration and cycle threshold (Ct) value. This linear relationship occurred in both the presence and absence (Fig. 7a–c, open and closed symbols, respectively) of > 10^7 nontarget fragments, thereby affirming confidence in the group-level specificity of the newly designed primer pairs.

Using these qPCR primers, bacterial abundances in groundwater from different wells were assessed at the group level. Overall, total copy numbers of all groups combined were in the order B3 > U1 > T2 (8.9 > 8.2 > 2.8 × 10^5 copies L^-1), followed by well D3 (3.3 × 10^5 copies L^-1) and then T5 (3.7 × 10^6 copies L^-1). Group II bacteria were most abundant in wells near the source area, starting with well U1 followed by wells D3 and B3; the more remote wells T2 and T5 had the lowest counts (Fig. 7b). Bacteria of group V were most abundant in well B3, followed by well T2, then U1, D3 and T5. Bacteria belonging to group I were most abundant in well T2 and U1, followed by well T5, B3 and D3. These data indicate that of the three groups quantified, group II bacteria showed an increased abundance in wells containing higher levels of chloroethenes and cocontaminants.

Discussion

The present study characterized geochemical parameters within a contaminated aquifer and identified abundant bacterial groups associated with *cis*-DCE removal. The data indicate that (1) nitrate, TCE and DCE are principal drivers of variability in groundwater geochemistry across the site, (2) the bacterial community includes seven major phylogenetic groups, and some of these may be related to known
Fig. 6. Neighbor-joining tree showing the phylogenetic relationship of select GenBank entries to the 16S rRNA gene sequences (~330 bp) obtained from site groundwater (accession numbers indicated in parentheses). Bootstrap values (n = 1000) are reported for neighbor-joining analysis values of > 500. An ‘f’ or ‘p’ indicates agreement of the Fitch and parsimony analysis results with those of the neighbor-joining tree (bar represents 0.1 U of evolutionary distance). Names of sequences obtained from Site 300 groundwater in this study are enclosed in boxes.
chloroethene cooxidizing bacteria, and (3) group II bacteria most similar to Alphaproteobacteria are abundant in groundwater at monitoring wells featuring chloroethenes and multiple hydrocarbon contaminants known to support chloroethene degradation. The qPCR data show that group-II bacteria are more abundant in contaminated groundwater near the source area. The combined concentration of cis-DCE and TCE is highest in wells closest to the source area (wells D3, U1 and B3) (Fig. 2a) and both wells D3 and U1 contain cocontaminants including TKEBS and diesel fuel, respectively. One possible explanation for this finding is that group-II bacteria are able to derive energy and biomass from the degradation of cocontaminating hydrocarbons. The group-II primers amplify 16S rRNA genes from Alphaproteobacteria, mainly Sphingomonas species. Many Sphingomonas species are capable of degrading aromatic and aliphatic hydrocarbons, such as those present in well U1 (diesel) and well D3 (TKEBS breakdown products). While Sphingomonas spp. are generally not associated with TCE degradation they have been linked to the cometabolism of TCE in mixed cultures (Lee et al., 2002; Den et al., 2003). Other Alphaproteobacteria in group II may also participate in TCE cometabolism, including type II methanotrophs Methylocystis species (11514-B3) (Nakajima et al., 1992; McDonald et al., 1997; Grosse et al., 1999; Heyer et al., 2002). Some Methylocystis species produce a soluble methane monooxygenase capable of oxidizing aliphatic and aromatic substrates including TCE and other chlorinated ethenes (Grosse et al., 1999; Shigematsu et al., 1999).

The obvious question to answer is: which species cause the rapid cis-DCE loss observed at Site 300 in response to oxygen infiltration in wells near the source area (Fig. 3)? The phylogenetic analysis of rRNA gene sequences accomplished in this study does not conclusively identify bacterial phenotypes. However, the physiology of phylogenetically related organisms combined with group-specific qPCR data provides possible clues. The relative abundance of Alphaproteobacteria in source-area wells and the knowledge that some of these organisms are phylogenetically related to known hydrocarbon degraders (Sphingomonas species) and chloroethene-cometabolizing bacteria (Methylocystis species) suggests these bacteria may be involved in the final step of complete chloroethene degradation at the site. Experiments targeting functional genes, such as soluble methane monoxygenase, butane monooxygenase and/or other mono and dioxygenases whose primary substrate is associated with diesel fuel or silicon oil may provide further evidence.

Accordingly, the hypothesized multi-step process of complete chloroethene degradation is initiated by the established mechanism of 2-ethylbutanol and n-butanol release from the hydrolysis of TKEBS (Vancheeswaran et al., 2003). Excess amounts of these two carbon and energy sources promote oxygen depletion and anaerobic conditions, as shown previously (Vancheeswaran et al., 2003). Fermentation of the alcohols then can provide an ample supply of dissolved molecular hydrogen, as demonstrated in microcosms prepared from site materials (Vancheeswaran et al., 2003). Dehalorespiring organisms may use this energy source to perform the reductive dechlorination of TCE to DCE (Lowe et al., 2002; Vancheeswaran et al., 2003). DCE is not dechlorinated further since organisms potentially capable of performing this task (Maymo-Gatell et al., 1997; He et al., 2005) are absent from this site. This was demonstrated previously (Lowe et al., 2002) and again in the present study,
in which primer pairs suitable for selective amplification of *Dehalococcoides* related organisms (detection limit > 50 copies per reaction) (Fagervold et al., 2005) did not produce an amplicon, whereas controls did (data not shown). The documented periodic influx of oxygen into site groundwater (Fig. 3) may help to explain the absence of *Dehalococcoides* related organisms. Therefore, DCE likely is a dead-end product of microbial metabolism at the site as long as anaerobic conditions prevail. However, upon temporal reaeration of the shallow groundwater, the compound can be removed (Fig. 3). The present study identified group-II bacteria as candidate organisms performing this final and important function of DCE oxidation and succeeded in providing an initial overview of species diversity and spatial microbial distribution at the site. Information gained from this study will guide future experiments focused on the identification of individual species responsible for chloroethene cooxidation at Site 300.

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**References**


