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Molecular characterization of microbial populations at two sites with differing reductive dechlorination abilities

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Abstract

This study compares three molecular techniques, including terminal restriction fragment length polymorphism (T-RFLP), RFLP analysis with clone sequencing, and quantitative PCR (Q-PCR) for surveying differences in microbial communities at two contaminated field sites that exhibit dissimilar chlorinated solvent degradation activities. At the Idaho National Engineering and Environmental Laboratory (INEEL), trichloroethene (TCE) was completely converted to ethene during biostimulation with lactate. At Seal Beach, California, perchloroethene (PCE) was degraded only to *cis*-dichloroethene (cDCE) during biostimulation but was degraded to ethene after bioaugmentation with a dechlorinating culture containing *Dehalococcoides* strains. T-RFLP analysis showed that microbial community composition differed significantly between the two sites, but was similar within each site among wells that had low or no electron donor exposure. Analysis of INEEL clone libraries by RFLP with clone sequencing revealed a complex microbial population but did not identify any *Dehalococcoides* strains. Q-PCR targeting the 16S rRNA gene of *Dehalococcoides* strains – known for their unique capability to dechlorinate solvents completely to ethene – revealed a significant population at INEEL, but no detectable population at Seal Beach prior to bioaugmentation. Detection of *Dehalococcoides* by Q-PCR correlated with observed dechlorination activity and ethene production at both sites. Q-PCR showed that *Dehalococcoides* was present in even the pristine well at INEEL, suggesting that the difference in dechlorination ability at the two sites was due to the initial absence of this genus at Seal Beach. Of the techniques tested, Q-PCR quantification of specific dechlorinating species provided the most effective and direct prediction of community dechlorinating potential.

Introduction

Chlorinated ethenes are significant groundwater contaminants with a range of toxic and carcinogenic effects (Lash 2000). One method for treating groundwater contaminated with chlorinated solvents is bioremediation by microorganisms capable of anaerobic reductive dechlorination, a microbial process that successively dechlorinates solvents such as perchloroethene (PCE) and trichloroethene (TCE) to *cis*-dichloroethene (cDCE), vinyl chloride (VC), and finally ethene. Both PCE and TCE have been shown to serve as electron acceptors in anaerobic respiration (Holliger et al. 1993; El Fantroussi et al. 1998), and field sites contaminated with both solvents have been treated

by injection of electron donor substrates that stimulate degradation by indigenous microorganisms (Song et al. 2002; Lendvay et al. 2003).

Unfortunately, many microorganisms are incapable of completely dechlorinating solvents to ethene, resulting in the build-up of hazardous chlorinated intermediates, such as cDCE and VC, a known carcinogen. To date, only members of the genus *Dehalococcoides* have been shown to completely degrade chlorinated solvents to the nontoxic end-product, ethene (Maymo-Gatell et al. 1997; Cupples et al. 2003; He et al. 2003). Bioremediation efficacy at a given site is largely dependent on the indigenous microbial populations and the redox potential of the soil environment (Lee et al. 1998; Hendrickson et al. 2002). The presence of *Dehalococcoides* strains appears to be required for robust and complete dechlorination activity, though other uncharacterized species with similar enzymatic activities may exist (Duhamel et al. 2002; Major et al. 2002; Richardson et al. 2002; Godsy et al. 2003).

This study compares the subsurface microbial communities at two sites, the Idaho National Engineering and Environmental Laboratory (INEEL) and the Installation Restoration Program Site 40, Naval Weapons Station Seal Beach, California (Seal Beach), that were undergoing similar sodium lactate injection strategies to enhance reductive dechlorination of TCE and PCE, respectively. Because biostimulation at Seal Beach produced only incomplete dechlorination, the lactate injection regimen was followed by bioaugmentation with a mixed community that included *Dehalococcoides* strains. The molecular characterization of the microbial communities described herein was used to assess the effectiveness of each remediation strategy in promoting the growth of organisms capable of achieving degradation to ethene.This work compares the molecular techniques of terminal restriction fragment length polymorphism analysis (T-RFLP), RFLP analysis with clone sequencing, and quantitative PCR (Q-PCR) for surveying and tracking the ability of microbial communities to degrade chlorinated solvents. These three techniques were chosen because they cover a spectrum of resolution: TRFLP provides a broad picture of the microbial community composition but cannot always identify specific species, clone libraries and RFLP analysis can identify specific species but are unlikely to detect all species present, and Q-PCR can give very accurate quantifiable information but only about the chosen target or targets. By applying these techniques to two sites with differing dechlorination profiles and remediation strategies, this study compares the utility of these techniques as predictors of dechlorination capability.

Materials and methods

Sampling and GC analysis

Details of well sampling methods and chlorinated ethene analysis at INEEL are given by Song et al. (2002). Briefly, sample wells were purged using low-flow sampling principles (Puls & Barcelona 1996) before groundwater collection using dedicated submersible pumps and Teflon-lined polyethylene tubing. Chlorinated ethenes, acetate, propionate and butyrate concentrations were quantified at INEEL by gas chromatography using a flame ionization detector. Lactate analyses were performed by ion exclusion chromatography with conductivity detection (Song et al. 2002).

At Seal Beach, groundwater samples were taken weekly for redox condition monitoring and analyzed on-site as previously described (French et al. 2003). Samples were periodically analyzed off-site for dissolved gases and organic acids (at Performance Analytical, Simi Valley, California and INEEL, respectively). Groundwater samples were collected monthly (method described in French et al. (2003)) and sent to U.C. Berkeley for microbial DNA analyses.

DNA extraction

Field samples were collected in 1.0 l amber glass bottles and shipped on ice within 48 h to the laboratory at UC Berkeley, CA and stored at 4 °C for same day processing. Cells in 500 ml of groundwater from each sample were concentrated by filtration through a 0.2 μm membrane (Fisher 09-719-2B). The membrane was then transferred to a tube containing 10 ml of filtrate and vortexed vigorously for 5 min. The membrane was removed, and the cells were pelleted by centrifugation at 21,000 *g* for 30 min. Supernatant was discarded, and the pellet was immediately processed for DNA extraction as described by Dojka

et al (1998). Briefly, cells were incubated first with lysozyme and then with sodium dodecyl sulfate and proteinase K, followed by homogenization by bead beating. DNA was extracted with a phenol: chloroform: iso-amyl alcohol (25:24:1) solution, precipitated with cold isopropanol, and resuspended in 40 μl sterile water. Final DNA concentrations were quantified using the PicoGreen® assay (Molecular Probes, Eugene, Oregon) on a TD-700 fluorometer (Turner Designs, Sunnyvale, California).

16S rDNA Amplification and Clone Library Construction

PCR amplification of the 16S rRNA gene sequence was carried out using universal bacterial primers 8F (5′-AGAGTTTGATCCTTGGCTCAG-3′) and 1492R (5′-GCYTACCTTGTTACGACTT-3′) as described by Dojka et al., except that Amplitaq, rather than *pfu*, was used as the DNA polymerase in all reactions (Dojka et al. 1998). Six clone libraries were constructed from July and August INEEL samples by cloning 16S rDNA, amplified from genomic DNA, using the TOPO TA Cloning Kit (Invitrogen Corp.) according to manufacturer's protocol. A more extensive library was constructed using well INEEL-25 groundwater in November 2001 as described previously (Macbeth et al. 2004).

T-RFLP analysis

Whole community 16S rDNA from the July and October 2001 time points was amplified as before using the 8F and 1492R universal bacterial primers except that the 5′ end of the 8F primer was labeled with the fluorescent tag 6-FAM (6-carboxyfluorescein) as described by Liu et al. (1997). Mixed bacterial PCR products were digested with *Msp*1 and resulting fragments were analyzed with the ABI 377 Sequencer (Applied Biosystems) and GeneScan software, version 3.1 (Applied Biosystems).

RFLP analysis and clone sequencing

For RFLP analysis, the cloned 16S rDNA insert was amplified with primers 8F and 1492R and then digested with restriction enzyme *Msp*1 (Gibco) as suggested by the manufacturer. In order to visualize the RFLP banding patterns, digested DNA was analyzed on an Agilent Bioanalyzer using DNA 7500 chips (Panaro et al. 2000). The most frequently occurring clones, and those showing RFLP similarity with previously encountered organisms (Richardson et al. 2002), were partially sequenced with the ABI 377 Sequencer (Applied Biosystems) using the Big Dye™ Terminator Kit (Applied Biosystems) and the T7 primer. In some cases full sequencing of 16S rDNA PCR product was accomplished with the T7, M13R, and 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) primers. For sequencing reactions, the polymerase Amplitaq Gold (Perkin Elmer) was used. Sequences were aligned using AutoAssembler software, version 2.1 (Applied Biosystems). Sequence matches and nearest cultured neighbors of clones were determined using BLAST software at the Genbank website (Altschul et al. 1990; Benson et al. 2000). In cases where several organisms shared similarly high scores, sequence affiliations were identified with the "classifier" software available at the Ribosomal Database Project II (Cole et al. 2005), and were considered valid when having a >90 % bootstrap confidence estimate.

Quantitative PCR (Q-PCR)

TaqMan™ primers and probes for Q-PCR were designed using Primer Express™ software version 2.0 as recommended by Applied Biosystems. Sequences (forward primer: 5′-GGTAATACG TA GGAAGCAAGCG-3′ and reverse primer: 5′-CCG GTTAAGCCGGGAAATT-3′) were specific to the *Dehalococcoides ethenogenes* strain 195 16S rDNA sequence (Seshadri et al. 2005) and span base pairs 467 to 566. Fluorogenic probe (5′- ACAT CCAA CTTGAAAGACCACCTACGCTCACT-3′) spanning base pairs 513–545 was synthesized by Applied Biosystems and was conjugated to a 5′ reporter molecule (6-FAM) and a 3′ quencher molecule (TAMRA). This primer and probe set targeted a variety of *Dehalococcoides* strains capable of metabolizing PCE, TCE and their daughter products cDCE and VC. Primer and probe specificity were confirmed by BLAST analysis (Altschul et al. 1990).

To generate a Q-PCR standard curve (log DNA concentration versus an arbitrarily set cycle threshold value [C_t]), a pure laboratory culture of

D. ethenogenes strain 195 kindly provided by Steve Zinder at Cornell University was briefly sustained in defined medium developed for the ANAS culture (Richardson et al. 2002) so that DNA could be extracted from actively degrading cells. A fragment spanning positions 8–1417 of the *D. ethenogenes* strain 195 16S rDNA gene (Seshadri et al. 2005) was cloned out of this culture using the TOPO TA Cloning Kit (Invitrogen Corp.) according to manufacturer's instructions. Plasmid DNA was purified, quantified fluorometrically, and used to create a dilution series spanning seven orders of magnitude. Based on the known size of plasmid and insert, DNA concentrations were converted to insert copy numbers.

Amplification and detection of DNA by Q-PCR was performed with the ABI 7000 Sequence Detection System (Applied Biosystems). All reagents and materials were purchased from Applied Biosystems. Reaction volumes of 25 μl contained forward and reverse primers at a concentration of 700 nM, probe at a concentration of 100 nM, 1× TaqMan Universal PCR Master Mix, and 5 μl of sample DNA. Default settings for cycle number and reaction conditions were used for all runs (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Standards and unknowns were run in triplicate to ensure reproducibility. Samples showing amplification in only a single well were designated detectable, but not quantifiable.

Results

Physical sites

The two solvent contaminated field sites, INEEL and Seal Beach, are shown schematically in Figures 1a, b. INEEL has a TCE plume several thousand meters long with aqueous concentrations in the source area up to 5 mg/l. Samples were taken from representative wells ranging from within the plume source (INEEL-25) to downstream with lower TCE concentrations (INEEL-29) to pristine wells outside the plume area (INEEL-10A). Significant concentrations of cDCE were found in the plume prior to lactate injection, indicating some intrinsic reductive dechlorination activity (Sorenson et al. 2000). Initially, the site was characterized as being mildly reducing, and became even more so after initiation of biostimulation, particularly within a radius of approximately 15 m from the lactate injection wells (INEEL-25, 26, and 31) (Sorenson et al. 2000). Down-gradient locations (INEEL-28, 29, and 10A) were not significantly impacted by the lactate injection (Sorenson et al. 2000).

Seal Beach was contaminated with PCE at maximum observed historical concentrations of 4 mg/l, with a plume above 10 μg/l extending only a few hundred meters. Seal Beach wells described here were chosen to vary both in the distance downstream and radial direction from the source. Like INEEL, conditions at Seal Beach were predominantly reducing (French et al. 2003). Due to its coastal location, the Seal Beach aquifer was influenced by salt-water intrusion, resulting in naturally high initial sulfate concentrations of several hundred milligrams per liter. After lactate injection, however, sulfate concentrations dropped to below the field method detection limit of 50 mg/l in all sample wells.

From January 1999 to February 2000, water containing 16,700 kg of sodium lactate was injected into the INEEL site at an average concentration of 24 g/l. The maximum organic acids concentration recorded near the injection well was 6 g/l, and the concentration 15 m up-gradient (INEEL-31) was 0.85 g/l. Lactate was injected again in early July, September, and November 2001. T-RFLP samples were taken after the July injection, when concentrations down gradient (INEEL-25) were as high as 1.5 g/l, and again in October 2001, when organic acid concentrations had dropped below detection limits.

At Seal Beach, water containing approximately 6300 kg of sodium lactate at 30 g/l was injected over a period of 240 days from August 2001 to March 2002. The first T-RFLP samples were taken prior to lactate injection. Organic acid concentrations in October 2001, when the second T-RFLP samples were taken, ranged from 1.5 g/l at well Seal Beach-25, near the injection well, to 0.25 g/l at Seal Beach-26, 6 m away. Organic acids were degraded in a similar manner at both Seal Beach and INEEL; lactate was rapidly converted to predominantly acetate and propionate with a small amount of butyrate within a short distance of injection wells.

Due to the incomplete dechlorination observed during biostimulation at Seal Beach, lactate injections were discontinued from March 2002 until

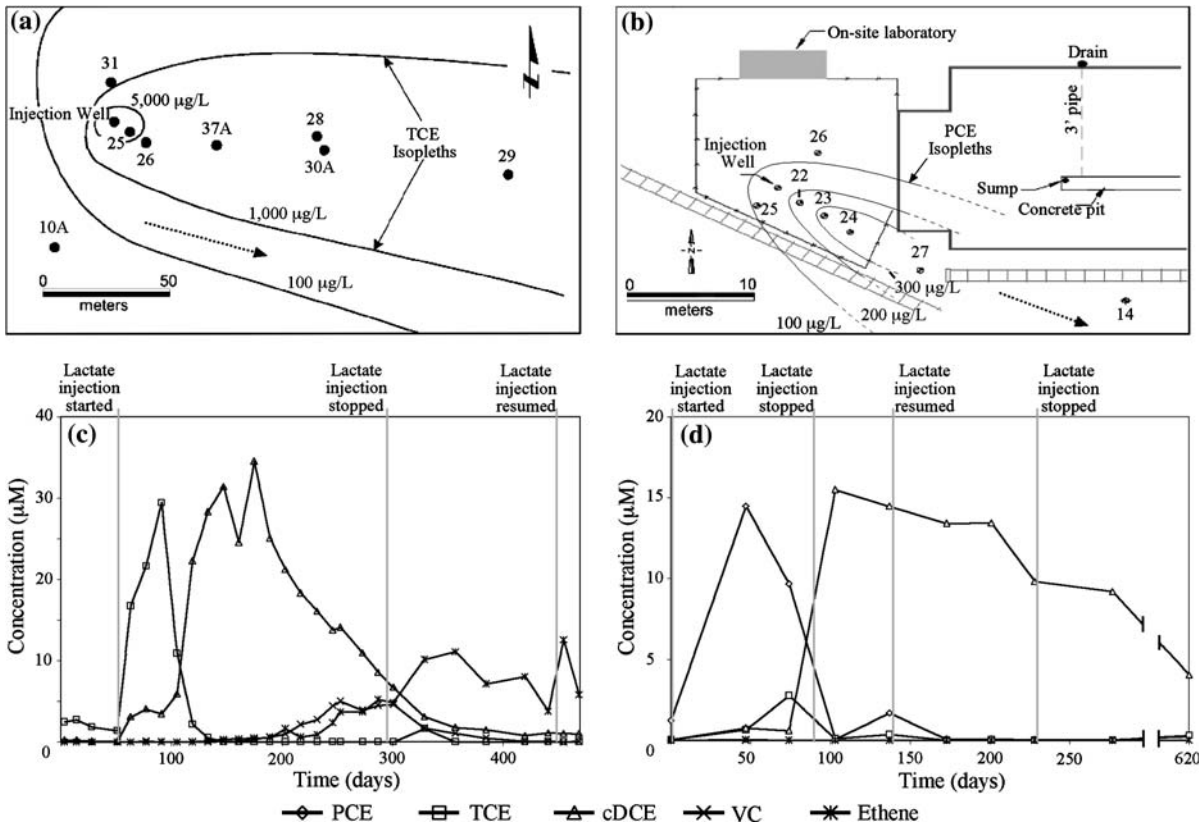

Figure 1. (a) INEEL site plan showing sampling wells and TCE isopleths. (b) Seal Beach site plan showing sampling wells and PCE isopleths. Dotted arrows show the direction of ground water flow. (c) Solvent concentrations at well INEEL-25. (d) Solvent concentrations at well SB-25.

March 2003, when they were resumed in preparation for bioaugmentation with a *Dehalococcoides*-containing culture in April 2003. Approximately 20 l of commercially available KB1 culture (SIREM, Guelph, Ontario – www.siremlab.com) was injected one time into two inoculation points, SB-22 and SB-25, using the procedure outlined in Major et al. (Major et al. 2002), but without injection of reduced water immediately following inoculation. Lactate was injected every few weeks from March 2003 through November 2003, at 30 g/l as before.

Degradation of chlorinated solvents

The two sites exhibited fundamentally different chlorinated ethene degradation patterns in response to lactate injection. At INEEL, aqueous phase TCE was fully dechlorinated to ethene at all wells that had organic acid exposure. A representative INEEL solvent degradation profile (Figure 1c) shows removal of TCE accompanied by a gradually increasing ethene concentration over time. Stable isotope analysis showed that the unexpected increase in TCE concentration after lactate injection resulted from mass transport of TCE from the DNAPL source area caused by the density of the injected lactate solution (Song et al. 2002). At Seal Beach, PCE was dechlorinated only as far as cDCE during biostimulation, leading to accumulation of this product. The dechlorination pattern shown (Figure 1d) is representative of the five wells analyzed, SB-22 through SB-26. A follow up sample taken 620 days after the initial lactate injection verified that no degradation to VC or ethene had occurred. This incomplete dechlorination is typical of chlorinated solvent spill sites where *Dehalococcoides* strains are not detected (Hendrickson et al. 2002). Following bioaugmentation in April 2003, however, VC and ethene were detected in chronological order corresponding to distance from the inoculation points, indicating commencement of complete dechlorination.

T-RFLP analysis

T-RFLP patterns from wells that had low or no exposure to electron donor (INEEL-37A, 28 and 29 and all Seal Beach wells in July 2001, prior to lactate injection) were similar to each other within each site, often dominated by one or two peaks, but differed between sites (Figure 2). Patterns from wells where electron donor concentrations were high (INEEL-25 and Seal Beach-25, 23 and 26 in October 2001, after lactate injection) clearly varied from the low or no exposure wells at both sites.

A comparison of INEEL T-RFLP profiles revealed that, during both sampling events, INEEL-25 community profiles were qualitatively different from those at wells INEEL-37A, 28 and 29, with a greater number of peaks detected in the well with high lactate exposure (Figures 2a, b). In addition, INEEL-25 showed significant profile shifts between the two time points, particularly in the 210–230 bp region (bracket I), the 275–325 bp region (bracket II) and in the 475–560 bp region (bracket III). As the two time points were similar across environmental conditions (i.e. redox, temperature, alkalinity, etc.), population shifts in these regions (I–III) are likely related to the change in lactate concentrations. In fact, prominent fragments of 218, 224, and 300 base pairs in a T-RFLP profile generated from well INEEL-25 during the lactate injection of November 2001 were tentatively assigned to *Acetobacterium* spp. (Macbeth et al. 2004), which have been shown to utilize hydrogen and lactate during acetogenesis (Drake 1994). No distinguishable 513 base pair peak, corresponding to *Dehalococcoides* strains was seen in any INEEL well.

At Seal Beach the four profiles from July 2001, 1 month prior to lactate injection, were qualitatively similar to each other and contained roughly the same dominant peaks (Figure 2c). Four months later, however, the site had changed significantly (Figure 2d): a fragment of approximately 215 bp (bracket IV) appeared in SB-25, 23 and 26, and peaks in the 280–300 bp region (bracket V) appeared in SB-25. Additional changes

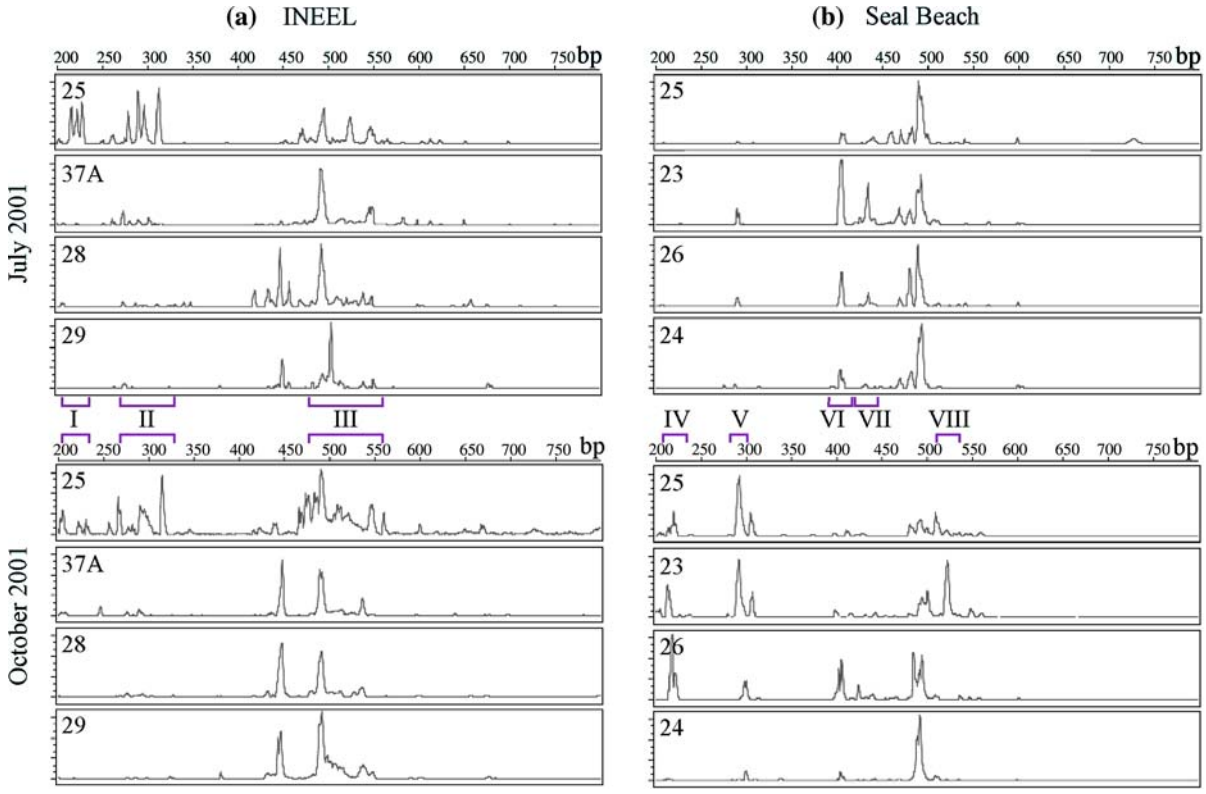

Figure 2. T-RFLP community profiles from (a) INEEL: July 2001 and October 2001 and (b) Seal Beach: July 2001 and October 2001. Peaks discussed in the text are denoted with brackets and roman numerals.

were observed in SB-23, where fragments of 400 and 430 bp (brackets VI and VII) were lost while a fragment of 520 bp (bracket VIII) was detected. This shift in community profile likely also reflects the impact of lactate injection. The 513 base pair peak corresponding to *Dehalococcoides* strains was not detected as a major fragment in any well; however, a minor peak of roughly the correct size was detected in the October sampling of well SB-25.

RFLP analysis and sequencing

T-RFLP data indicated that communities were fairly homogeneous among wells with little electron donor exposure. However, RFLP analysis after *Msp*1 digestion did not support this conclusion. Specifically, 215 of 240 RFLP patterns were found only in the well from which they were isolated, indicating little overlap of species between wells (Table 1).

Additionally, INEEL clone libraries were not useful for reliably identifying dominant species detected by T-RFLP. Based on the T-RFLP profiles (Figure 2), between three and ten dominant clones were expected. Instead, none of the RFLP clones were particularly abundant; the most common RFLP clone represented only 6% of the total INEEL library. Also, 32% of all RFLP clones were seen only once, while 74% were found in three instances or less. Because T-RFLP and RFLP analyses were carried out with the same restriction enzyme, the sizes of prominent T-RF's were expected to match bands found within RFLP profiles. Again, this was not the case. Only one RFLP profile contained a fragment that agreed with a dominant T-RFLP peak; in well INEEL-29, five of twenty-six clones (19%) matched a *Legionella* sequence that produced a 500 base pair RFLP fragment consistent with the dominant T-RFLP peak.

Thirty-three clones from the July/August INEEL libraries were chosen for sequencing. None of the sequences, however, corresponded to *Dehalococcoides* strains. Four sequences corresponded to cultured bacteria: the *Legionella* sequence discussed above, a *Bdellovibrio* sequence, and two *Clostridium* species. Predicted RFLP banding patterns for these *Bdellovibrio* and *Clostridium* sequences (Cole et al. 2005) did not match any major observed T-RFLP peaks. Of the remaining 29 clones, 10 were most homologous to unidentified organisms, which have not been phylogenetically placed, and 19 to entries for uncultured organisms, which have been phylogenetically placed by homology but never cultured in a lab. Of these 19, 13 could not be confidently classified into any taxa, 4 could be placed with the clostridia family and 2 with proteobacteria. Some uncultured entries could be affiliated with closely related cultured organisms including *Clostridium propionicum* and *Desulfobacterium indolicum*. Remaining uncultured organisms could not be confidently assigned to any phylogenetic lineage.

A more comprehensive clone library was constructed in November 2001 from well INEEL-25 where, of 93 clones screened, 82% were observed

Table 1. Clone library analysis at INEEL

Sample date	Location	Number of clones	Unique profiles	Most common single clone	Clones found in one well only	Most common taxa identified
July 2001	INEEL-29	40	6 (15%)	7 (18%)		Proteobacteria
	INEEL-31	51	19 (37%)	8 (16%)		Clostridia
August 2001	INEEL-29	26	1 (4%)	5 (19%)		Proteobacteria
	INEEL-31	25	6 (24%)	4 (16%)		Clostridia
	INEEL-10A	53	18 (34%)	7 (13%)		nd
	INEEL-25	45	27 (60%)	3 (7%)		nd
July/August 2001	summary of all wells	240	77 (29%)	14 (6%)	215(90%)	
November 2001	INEEL-25	93	17 (18%)	32 (34%)		Clostridia

For both July and August 2001, each well (Location) was analyzed separately and the number of total clones analyzed for each is given. The number of clones that occurred only once (unique profiles), the number of occurrences of the most common single clone, and the taxa of the few clones that matched known organisms are given. The row "July/August 2001" shows combined results for clone libraries from all wells at these times.

more than once (Table 1); 57 clone sequences matched various *Clostridia*, 32 of which were *Acetobacterium* species. Additionally, one clone sequence matched the dehalogenating organism *Sulfurospirillum multivorans*, and another matched the *Trichlorobacter thiogenes* sequence. Again, no *Dehalococcoides* strains were found. Since a large population of dechlorinating organisms was not identified with the clone library approach, we concluded that it was not an efficient or especially informative method for evaluating dechlorination potential. Consequently, clone library analysis of Seal Beach samples was not performed.

Quantitative PCR (Q-PCR)

At INEEL, where TCE was fully dechlorinated to ethene, *Dehalococcoides* 16S rDNA was found in all wells. The nearly pristine well INEEL-10A contained 1.5×10^4 copies per l suggesting that *Dehalococcoides* strains were naturally present throughout this site. Well INEEL-29, located farthest down gradient from the injection well and beyond the zone of organic acid influence, had 5.1×10^3 copies per L. Wells INEEL-25 and INEEL-37, located immediately down gradient from the injection well and therefore within the zone of organic acid influence had 1.7×10^6 and 4.0×10^6 copies per l, respectively. The difference in population density possibly reflects the distribution of acetate and hydrogen, which serve as carbon source and electron donor for *Dehalococcoides* strains (Fennell et al. 1997; Maymo-Gatell et al. 1997), generated from lactate by fermenting organisms.

At Seal Beach, where incomplete dechlorination resulted in cDCE accumulation, samples yielded no detectable *Dehalococcoides* 16S rDNA in any tested well prior to bioaugmentation (Figure 3a), with a detection limit of $\sim1\times10^3$ copies/l of groundwater. This negative result was validated by a control experiment designed to ensure that recovery and detection of *Dehalococcoides* 16S rDNA was not inhibited by interference caused by Seal Beach ground water. Dilutions of pure *D. ethenogenes* strain 195 culture spanning three orders of magnitude were added to 50 ml of Seal Beach ground water from SB-22 and SB-25. After a 30-min incubation, cells were concentrated and DNA extracted as usual. Samples spiked with *D. ethenogenes* strain 195 showed strong signal, while parallel un-amended field samples again yielded no detectable signal (Figure 3b). The copies per volume of pure culture added was estimated from microscopy (Maymo-Gatell et al. 1997) and the assumption that each cell contains one copy of the 16S rRNA gene per genome. The quantity of added *D. ethenogenes* strain 195 culture agreed well with resulting 16S rDNA copy number. Given the inefficiencies in cell concentration and DNA extraction, the sensitivity of these experiments was limited more by the sample preparation process than by the accuracy of the detection method. Based on DNA recovery during the amendment experiments, the overall sensitivity threshold for Seal Beach samples was shown to be 360–3600 copies per l groundwater.

After bioaugmentation at Seal Beach, VC and ethene appeared at all sampled wells, and was accompanied by detection of *Dehalococcoides* 16S rDNA (Table 2). At SB-22 and 25, the two wells closest to the microbial injection point, both the presence of *Dehalococcoides* 16S rDNA (10^8 copies/l) and production of VC and ethene were detected within one week of inoculation. At SB-23, 2 m down gradient from SB-22, degradation products and *Dehalococcoides* 16S rDNA (10^7 copies/l) were observed within 3 months of inoculation. At SB-24, 4 m down gradient, degradation products and *Dehalococcoides* 16S rDNA (10^5 copies/l) were observed within 4 months.

Discussion

Making the decision between relatively inexpensive biostimulation protocols, which promote dechlorination activity in the indigenous subsurface microbial population by substrate addition alone, and more elaborate and expensive bioaugmentation processes, which deliver exogenous microorganisms with known dechlorination activity to the subsurface contamination zone, is central to the economic and engineering feasibility of bioremediation applications. Often, bioaugmentation is adopted only after biostimulation protocols fail to induce significant remediation, wasting valuable months and incurring additional operating costs. Developing a method to quickly assess whether biostimulation or bioaugmentation is appropriate at a given site has been a long sought after goal.

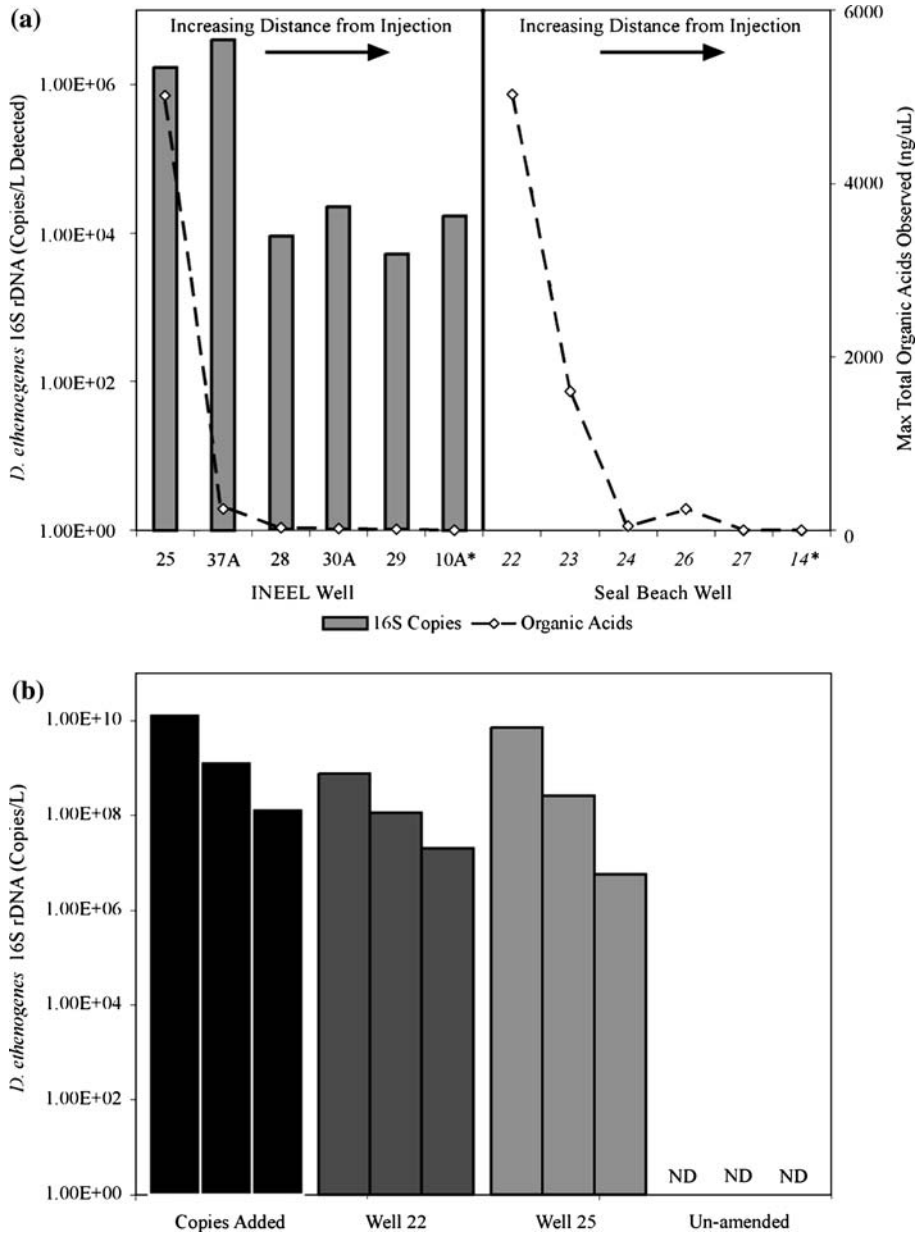

Figure 3. (a) Q-PCR results showing detected copies of *Dehalococcoides* strains and maximum organic acids detected at INEEL (left panel) and Seal Beach (right panel) sampling locations displayed in order of increasing distance from lactate injection. Note that INEEL well 10A and Seal Beach well 14 are outside of the known historic solvent plume areas and thus considered pristine. (b) Black bars represent serial additions of pure *D. ethenogenes* strain 195 culture to Seal Beach ground water from wells 22 and 25. Grey bars show recovery of amended organisms. ND = "None Detected" recovery from un-amended samples.

This study compared three molecular techniques for examining indigenous microbial communities at sites showing anaerobic chlorinated solvent degradation. Results from the application of these molecular techniques, the relative strengths and weaknesses of each, and suggestions for methods to be used at future bioremediation site evaluations are discussed.

T-RFLP was useful for making rapid qualitative comparisons of community diversity over time and space. It was not, however, effective at identifying dechlorinating species, or at providing an

Table 2. Appearance of dechlorination products and *Dehalococcoides* DNA at Seal Beach following bioaugmentation

Location	First appearance of vinyl chloride and ethene	First detection of *Dehalococcoides* DNA	*Dehalococcoides* copies/l
SB-22	April 24	April 24	3.0×10^8
SB-25	April 24	April 24	2.0×10^8
SB-23	June 18	July 16	1.0×10^7
SB-24	September 8	August 13	3.5×10^5

understanding of how community diversity was linked to degradation activity. Profiles of INEEL and Seal Beach suggested that while the microbial communities differed significantly between the two sites, communities shared many similarities within each site in wells that had low or no electron donor exposure. Profiles of wells with significant electron donor exposure revealed observable shifts in community composition at both sites during biostimulation. Several recent studies have used T-RFLP to judge the qualitative effects of environmental perturbations (Mills et al. 2003; Takai et al. 2003). In particular, a recent study by Lendvay et al. successfully applied T-RFLP to measure changes in the microbial community between parallel control/biostimulated and bioaugmented soil plots (Lendvay et al. 2003). Although some studies of simple microbial communities have used T-RFLP to identify expected species (Christensen et al. 2003; Rogers et al. 2003), in general, it is difficult to identify specific organisms from peak lengths without supplementary clone library construction and sequencing steps (Watts et al. 2001). In this study, the 513 base pair peak seen in the October Seal Beach T-RFLP (Figure 2d, well SB-25) did not agree with the absence of observed dechlorination activity or the failure to detect *Dehalococcoides* using the more highly sensitive Q-PCR technique. In complex communities, such problems are likely as many organisms can generate similar peak lengths (Mills et al. 2003).

Clone library construction and RFLP with sequencing analysis at INEEL revealed a complex community with many clone types. Roughly 40 clones were generated for each well, but single-hit sequences were common in most locations and it was not possible to reconcile T-RFLP profiles with sequenced clones. The more comprehensive November INEEL clone library of well 25 was dominated by *Clostridia* species, some of which corresponded to observed T-RFLP peaks, suggesting the presence of a robust fermenting/acetogenic community. Quantitative estimates show the likelihood of identifying dechlorinators by clone library construction to be small. Given an average sample yield of roughly 2.5 μg of DNA per 0.5 l of groundwater, and using a mass of 660 g per mol base pairs and a conservative average genome size of 2×10^6 base pairs, roughly 2×10^9 genomes should have been extracted per l. The average Q-PCR result from INEEL, however, found approximately 10^6 copies of *Dehalococcoides* 16S rDNA per l of groundwater. Thus one *Dehalococcoides* clone could be expected for every 2×10^3 clones analyzed. Given this ratio, it is reasonable that *Dehalococcoides* strains were not detected by clone library analysis. However, this quantity of *Dehalococcoides* cells at INEEL was sufficient to catalyze significant transformation of TCE to ethene, suggesting that techniques with low detection thresholds are necessary to promote effective site management.

Q-PCR measurement of *Dehalococcoides* 16S rDNA provided the most convincing results with respect to evaluating successful bioremediation strategies. The presence of *Dehalococcoides* 16S rDNA at INEEL was coincident with dechlorination beyond cDCE. Similar correlations have been reported previously; Hendrickson et al. (Hendrickson et al. 2002) used PCR to show that the presence of *Dehalococcoides* correlated with dechlorination beyond cDCE at 24 sites. While normal PCR techniques may be adequate for such correlations, more quantitative techniques may be needed to predict whether full degradation to ethene is likely, and if so, how fast such degradation may occur. Q-PCR has been used to demonstrate that observed differences in dechlorination rates between parallel biostimulated and bioaugmented soil plots corresponded to an order of magnitude difference in *Dehalococcoides* cell number (Lendvay et al. 2003). Similarly, dechlorination activity and observed *Dehalococcoides* cell

numbers were higher at INEEL wells located closest to lactate injection (Figure 3a).

Q-PCR has previously been shown to be a reliable and informative assay of dechlorinating organisms in complex communities (He et al. 2003; Smits et al. 2004). Direct characterization of microbial communities in the field, rather than in enrichment cultures, has not been carried out as extensively. It is important to test and validate field-based assays in order to more fully and realistically understand their relationship to bioremediation predictions.

Unsuccessful detection of *Dehalococcoides* at Seal Beach prior to bioaugmentation suggests that the functionally relevant difference between the two sites was the presence/absence of indigenous organisms capable of dechlorinating beyond cDCE. Two observations support this assertion: (1) even the relatively pristine well at INEEL contained detectable numbers of *Dehalococcoides* and (2) the first detection of *Dehalococcoides* after bioaugmentation at Seal Beach corresponded to the generation of vinyl chloride and ethene for the first time at the site. The latter observation is similar to results presented in other studies (Major et al. 2002; Lendvay et al. 2003). It is worth pointing out, however, that some *Dehalococcoides* strains, CBDB1 for example, are not capable of degrading TCE or cDCE (Adrian et al. 2000). Future work should focus on specific functional genes linked to TCE and cDCE dechlorination, thus avoiding possible confusion between *Dehalococcoides* strains.

The techniques used in this work also differ in their susceptibility to PCR bias. T-RFLP, RFLP, and clone library construction suffer from the canonical problem of reporting 16S rDNA copy number without knowing the copy number of 16S rDNA per cell, which may lead to inappropriate conclusions when comparing different results. Additionally, because the target 16S rDNA sequence of each organism differs slightly, differences in PCR efficiency will be exponentially amplified during the experiment. Q-PCR suffers less from the latter of these problems. That is, since Q-PCR can be normalized to plasmid-based standards identical to the target sequence, differences in PCR efficiency between genes can be controlled (Smits et al. 2004).

Given the relatively low cost and experimental ease of Q-PCR for quantification of *Dehalococcoides* 16S rDNA in groundwater samples, it has proven extremely useful as a predictor of the ability of subsurface microbial communities to degrade chlorinated solvents to ethene. This method will continue to become more valuable as sequences for other novel dehalogenating organisms and their functional genes become available.

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