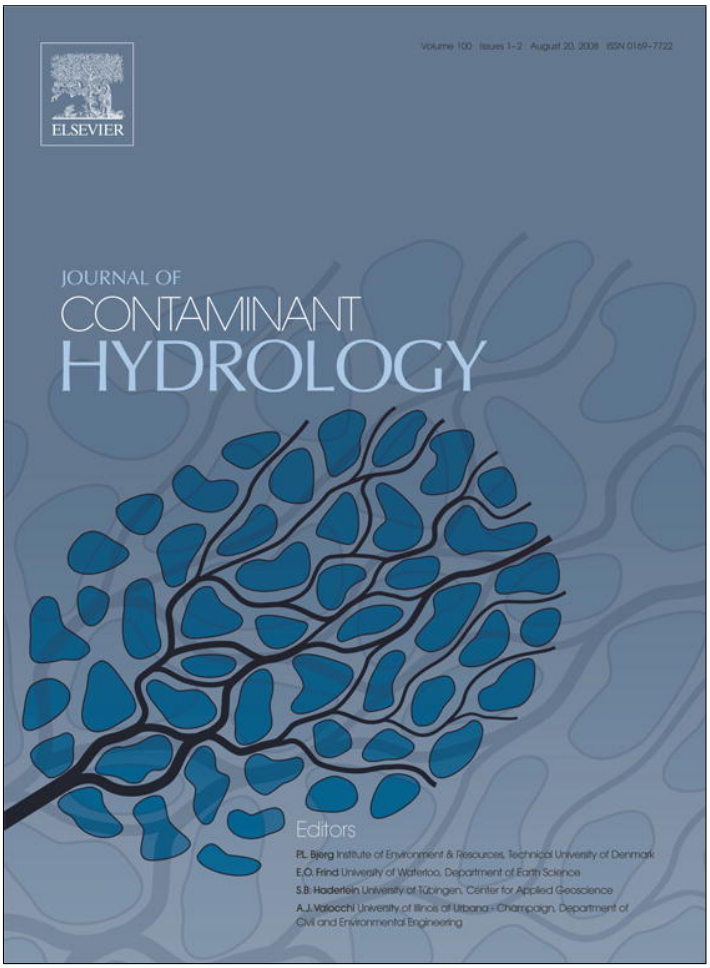

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Continuous-flow column study of reductive dehalogenation of PCE upon bioaugmentation with the Evanite enrichment culture

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ABSTRACT

A continuous-flow anaerobic column experiment was conducted to evaluate the reductive dechlorination of tetrachloroethene (PCE) in Hanford aquifer material after bioaugmentation with the Evanite (EV) culture. An influent PCE concentration of 0.09 mM was transformed to vinyl chloride (VC) and ethene (ETH) within a hydraulic residence time of 1.3 days. The experimental breakthrough curves were described by the one-dimensional two-site-nonequilibrium transport model. PCE dechlorination was observed after bioaugmentation and after the lactate concentration was increased from 0.35 to 0.67 mM. At the onset of reductive dehalogenation, cis-dichloroethene (c-DCE) concentrations in the column effluent exceeded the influent PCE concentration indicating enhanced PCE desorption and transformation. When the lactate concentration was increased to 1.34 mM, c-DCE reduction to vinyl chloride (VC) and ethene (ETH) occurred. Spatial rates of PCE and VC transformation were determined in batch-incubated microcosms constructed with aquifer samples obtained from the column. PCE transformation rates were highest in the first 5 cm from the column inlet and decreased towards the column effluent. *Dehalococcoides* cell numbers dropped from ~73.5% of the total Bacterial population in the original inocula, to about 0.5% to 4% throughout the column. The results were consistent with estimates of electron donor utilization, with 4% going towards dehalogenation reactions.

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1. Introduction

Anaerobic bioremediation technologies have been intensively applied for chlorinated ethene remediation in recent years (AFCEE, 2004). Interest in using anaerobic treatment has increased since the discovery that chlorinated ethenes could be completely dechlorinated to ethene (ETH) *in situ* by either indigenous microorganisms or by cultures that have been bioaugmented into the subsurface (Ellis et al., 2000; Aulenta et al., 2005; Morrill et al., 2005). This is an effective process for highly chlorinated ethenes, since the microbes can obtain energy for growth and maintenance by using them as electron acceptors via dehalorespiration (Holliger et al., 1997). Several studies have demonstrated that the bioaugmentation of reductive dehalogenating cultures can result in complete dehalogenation of tetrachloroethene (PCE) and trichloroethene (TCE) to ETH (Lendvay et al., 2003; Major et al., 2002). Specifically the bioaugmentation with microbial consortia that contain phylogenetic relatives of *Dehalococcoides ethenogenes* has promoted the complete dehalogenation of PCE or TCE to ETH (Richardson et al., 2002). Engineered bioremediation would consist of the addition of soluble electron donors, nutrients, and potentially microorganisms via wells in injection/extraction well recirculation systems or through the creation of reactive barriers through the injection of insoluble slow fermenting substrates (Löffler and Edwards, 2006). Cost analysis studies have shown that both approaches would yield lower costs than pump-and-treat remediation (Lee et al., 1998).

Studies of anaerobic dehalogenation have been performed in continuous-flow columns to evaluate the biotransformation

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of high concentrations of PCE (Isalou et al., 1998; Sleep et al., 2006); to study the bioaugmentation of a culture to stimulate TCE dechlorination (Harkness et al., 1999), and to biologically-enhance removal of PCE from non-aqueous liquid (NAPL) source zones (Cope and Hughes, 2001; Sleep et al., 2006). The column studies show dynamic changes both spatially and temporally in chlorinated aliphatic hydrocarbon (CAH) distributions and provide a reasonable means of mimicking processes that would be encountered in the field.

Molecular methods to characterize the microbial population and enumerate populations of *Dehalococcoides sp.* has recently been developed and employed in field studies (Yang and Zeyer, 2003). Various phylogenetic relatives of *Dehalococcoides ethenogenes* exhibit differences in their ability to transform vinyl chloride (VC) to ETH, a very important step in the reductive dechlorination process since VC is a known carcinogen and ETH is non-toxic (He et al., 2003a,b). The original isolate of *Dehalococcoides*, strain 195, completely dehalogenates PCE to ETH, but the transformation of VC to ETH is a slow cometabolic process (Maymo-Gatell et al., 1997). More recently, *Dehalococcoides* species that halorespire VC to ethene in mixed cultures with faster transformation kinetics have been reported (He et al., 2003a,b). Since these microbes can grow using VC as an electron acceptor, very effective reduction of VC to ethene at low concentrations is possible. It is not currently possible to differentiate using 16S rDNA based methods, *Dehalococcoides* strains that cometabolize VC to ethene from those that can grow on VC as an electron acceptor (Duhamel et al., 2004). For example, *Dehalococcoides* strain 195 and strain FL2 can grown on TCE, and c-DCE as electron acceptors, while the dechlorination of VC to ETH is cometabolic (He et al., 2005). Strains VS and GT utilize TCE, c-DCE, and VC as electron acceptors but do not reduce PCE (Sung et al., 2006). Strain BAV1 utilizes c-DCE isomers and VC as electron acceptors, but not higher chlorinated ethenes (He et al., 2005).

In this study, we bioaugmented aquifer material with the Evanite (EV) culture (Yu et al., 2005), a *Dehalococcoides*-containing enrichment culture that completely dechlorinates PCE to ethene, with apparent halorespiration during all dechlorination steps. The EV culture has been kinetically characterized by Yu et al. (2005). PCE transformation was studied in a continuous flow column packed with non-sterile aquifer solids from the Hanford DOE site (Washington) and bioaugmentation with the EV culture. Electron balances were calculated using chemical reaction equations for reduction of the chlorinated ethenes, lactate utilization as electron donor, acetate and propionate production from lactate frementation, and sulfate and iron reduction. At the end of the column test, the column solids were destructively sampled and analyzed for microbial composition using 16S rDNA based molecular methods. Additionally, transformation rates for PCE and VC were measured in microcosms constructed from the column solids to investigate the spatial correlation between transformation rates and microorganism abundance. The study illustrates how chemical monitoring, microcosm testing, and the application of 16S rDNA based molecular methods can be integrated to evaluate the response to bioaugmentation and electron donor addition under conditions that mimic subsurface bioremediation.

2. Materials and methods

2.1. Column material

The column was packed with aquifer material obtained from the Hanford formation at a quarry near Pasco, WA and consisted of an alluvial deposit of sands and gravels of basaltic and granitic origin. It contained approximately 6 wt% total Fe, less than 0.001 wt% organic matter, and the grain density was 2.9 g/cm^3 (Lindsey and Jaeger, 1993). The dry aquifer materials were sifted with a 60 mesh sieve (0.175 mm openings) to remove fine particulates (Tyler Standard Screen Scale, Mentor, Ohio).

2.2. Column construction and operation

Aquifer material was packed in a glass chromatography column (30 cm L×4.5 cm ID; Kontes CHROMAFLEX chromatography column, Vineland, NJ) sealed with Teflon end caps. PEEK tubing (1/8" OD) (Upchurch Scientific, Oak Harbor, WA) was used to connect the reservoirs, pump, column, and sampling valves. PEEK tubing was chosen because of its rigidity and low O_2 permeability. The column was packed with about 1 kg of aquifer material yielding a porosity of 0.35 and a pore volume of 192 mL, based on weight and volume measurements.

The experimental schematic of the column system is provided in Fig. 1. The column was amended with synthetic Hanford groundwater supplemented with trace elements, vitamins, PCE, and lactate. Synthetic groundwater was prepared to approximate the chemistry of Hanford groundwater as reported by Last et al. (1991). Trace elements and vitamins were added in the form of anaerobic medium used to grow the EV culture, as described by Yu and Semprini (2004). The media was diluted by a factor of five with the synthetic groundwater and the pH of final solution was about 7.45. Solutions were pumped through the column at a flow of 0.1 mL/min using a Dionex HPLC gradient pump (Sunnyvale, CA). The system had two reservoirs (5 L Kimble GL-45 media bottles), one containing PCE-saturated solution and nutrients and other containing synthetic groundwater and lactate as an electron donor. The reservoirs were purged with nitrogen gas from a tube furnace (2100 tube furnace, Barnstead/Thermolyne, IA) to remove oxygen and were pressurized to 5 psi to prevent air from leaking into the reservoirs during column operation.

The PCE solution was prepared by filling one reservoir with 5 L of synthetic groundwater, purging with N_2, and adding 0.19 mM Na_2S to make the reservoir stay anaerobic conditions. 2.0 mL of pure PCE was added to the reservoir and the solution was vigorously stirred with a magnetic stir bar for 3–4 h to make a saturated aqueous PCE solution (1 mM). Non-aqueous PCE remained at the bottom of the reservoir thus indicating the solution was saturated, which was confirmed by analytical measurements of the influent solution added to the column. The stirrer was left on at a low speed (~30 rpm) throughout the experiment to ensure the contents in the bottle remained well mixed. The reservoirs were re-pressurized if the pressure dropped below 1 psi.

2.3. Bioaugmentation culture

The column was bioaugmented with the EV mixed bacterial culture capable of complete dechlorination of PCE

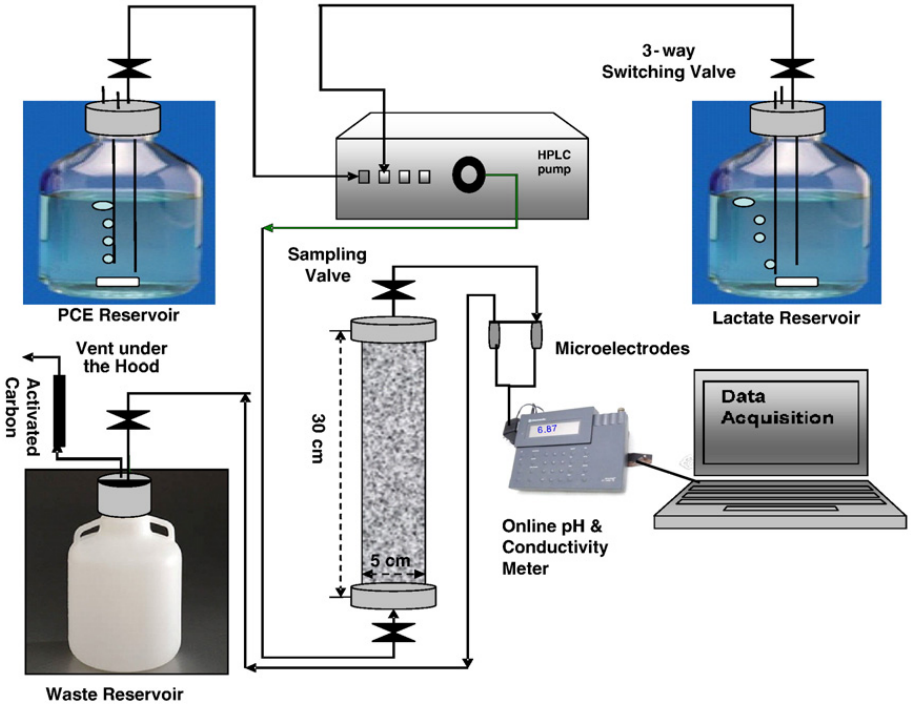

Fig. 1. Schematic of experimental setup and column design. The column was made from pyrex and connected with PEEK tubing.

to ETH under strictly reduced conditions using butanol or lactate as fermenting electron donors. Transformation rate kinetics for the complete dechlorination of PCE have been studied in detail by Yu and Semprini (2004) and Yu et al. (2005). Based on these studies TCE should be the most rapidly transformed, while VC should be the most slowly transformed. Prior to bioaugmentation the EV culture was maintained in batch-fed reactors supplemented with PCE and butanol as previously described by Yu et al. (2005).

2.4. Experimental design

The chronology of the column study was broken down into 7 test phases (Table 1). Bromide tracer and PCE transport tests were performed with deionized water. Aqueous solutions of 0.6 mM bromide and 0.09 mM PCE were continuously pumped through the column and breakthrough concentrations of bromide and PCE were measured in the column effluent. PCE was continuously injected for 42 days in order to achieve steady-state effluent concentrations. The column was then inoculated with 20 mL of the EV culture (~1.5 mg protein) directly harvested from a batch growth reactor and directly injecting to the column bottom inlet using a 25 mL gas tight syringe. Continuous lactate addition was initiated at a concentration of 0.34 mM. After 51 days and 105 days of column operation, the lactate concentration was increased to 0.67 mM and 1.34 mM, respectively. After 170 days of operation the flow was stopped and the column was destructively sampled in an anaerobic glove box.

2.5. Analytical methods

The EPA 8000 purge-and-trap method was used to determine the concentrations of PCE and its transformation products, including ETH using the method previously described by Azizian et al. (2007). Hydrogen was measured by injecting 100 μL headspace samples into an HP-5890 GC series II with a thermal conductivity detector (TCD), operated isothermally at 220 °C. Chromatographic separation was achieved using a Carboxen 1000 column (15 ft.×1/8 in., Supelco, Bellefonte, PA) and argon as the carrier gas. Methane concentrations were determined by placing liquid samples (1-mL) into 2-mL vials sealed with Teflon-lined silicon septa. After equilibrating the vials with agitation for 1 min at 20 °C, 100-μL headspace was injected onto the GC equipped with a GS-Q column (30 m×0.53 μm I.D., J&W Scientific, Folsom, CA) and subsequent detection using a FID detector. The hydrogen detection limit was 20 nM (aqueous concentration). Bromide,

Table 1
The chronology of the treatment phases of the study

Phase	Activity	Day	Cumulative pore volumes
I	Bromide tracer (0.6 mM) and PCE (0.09 mM) transport tests	0–14	0–10
II	PCE (0.09 mM) injected to reach steady-state concentration	14 – 28	10 - 21
III	PCE (0.09 mM) amended with Hanford synthetic groundwater	28–42	21–32
IV	Inoculation with 20 mL Evanite culture (~ 1.5 mg protein) and addition of 0.34 mM lactate	42–51	32–38
V	Increase of lactate concentration from 0.34 mM to 0.67 mM	51–105	38–80
VI	Increase of lactate concentration from 0.67 mM to 1.34 mM	105–170	80–128
VII	Termination of column experiment and sampling for microcosm study and molecular analysis	170	128

chloride, and sulfate, were determined with a Dionex DX-500 ion chromatograph (Sunnyvale, CA) equipped with an electrical conductivity detector and a Dionex AS14 column. Organic acids, including lactate, acetate, and propionate were measured with a Dionex-500 HPLC chromatograph equipped with UV/VIS detector and an Alltech Prevail Organic acid column. When lactate concentrations were high the samples were diluted in order to separate lactate and acetate during the HPLC analysis. Reducible iron oxide concentrations of column aquifer solids were measured by sequential extractions using the method described by (Peltier et al., 2005). The aqueous Fe(II) concentration was determined using the o-phenanthroline method (APHA, 1995).

2.6. Column aquifer solids sampling

After 170 days of continuous operation, flow was stopped and the column was destructively sampled inside an anaerobic glove box. Column solids were sampled for nucleic acid extraction and for microcosms construction. Anaerobic conditions during column sampling were maintained by placing the column in an anaerobic glove box with an atmosphere of 5% H_2 and 95% N_2. The column was split into 6 sections, each 5 cm in length, beginning at the inlet end of the column. Samples were scooped from the center of each section with an autoclaved spatula and placed in an autoclaved sample container and homogenized.

2.7. Microcosm studies

The microcosms were constructed in the anaerobic glove box using procedures described by Yu and Semprini (2002). Approximately 20 g of homogenized solids were added to autoclaved 156 mL serum bottles. Three microcosms were constructed from each section of the column to perform kinetic tests on PCE dechlorination, VC dechlorination, and lactate fermentation. After distributing solids into the microcosms, 75 mL of synthetic groundwater with diluted media was added, leaving approximately 55 mL of headspace in the PCE and VC microcosms. Lactate microcosms were filled up to the 125 mL mark, since aqueous sampling was to be performed.

Lactate (1-mL of stock solution, 160 mM) was added to each microcosm as an electron donor, resulting in lactate concentrations of 1.6 mM. The lactate stock solution was prepared by diluting lactate (60% syrup, J.T. Baker, NJ) 1:50 with N_2-sparged autoclaved deionized water. The headspace of the PCE and VC microcosms was further amended with 2 and 3 mL of H_2, respectively to ensure dehalogenation was not electron donor limited. An anaerobic PCE-saturated solution was prepared in a 156 mL Wheaton bottle (Wheaton Glass Co., Millville, NJ) using procedures previously described. 1 mL of PCE-saturated water was added to each PCE microcosm to achieve an aqueous concentration of about 10.9 μM. The anaerobic stock of VC gas was made by adding 10 mL of pure VC to an N_2-sparged 156 mL Wheaton bottle. 0.9 mL of anaerobic VC gas stock solution was added to each microcosm to achieve an aqueous concentration of approximately 16 μM. Concentrations of CAHs and ethene in the headspace samples (100 μL) were analyzed by gas chromatography using the method previously described by Yu et al. (2005).

2.8. DNA extraction

Total DNA from the Hanford soil column matrix was prepared according to SDS-based DNA extraction methods (Zhou et al., 1996). Up to 0.5 g of soil was mixed with 0.25 mL of 1× TE buffer (100 mM Tris–HCl, 100 mM EDTA, pH 8.0). A spatula tip of acid washed glass beads (0.1 to 0.15 μm in diameter, Sigma-Aldrich, Inc.) and SDS to final concentration of 2% were added to the soil slurry. The samples were vortexed and incubated in boiling water for 2 min. Following the incubation in boiling water samples were immediately frozen in liquid nitrogen and kept on ice until they were completely thawed. The thawed samples were amended with 0.05 mL of a 10% BSA solution and vortexed for 10 min. After centrifugation at 4 °C for 3 min at 12,000 RCF the soil slurry (~0.85 mL) was combined with 0.43 mL extraction buffer (0.8 M NaCl, 500 mM Na acetate, pH 5.5). Soil samples were split in half and DNA was extracted at least twice with 1.5 volumes of phenol:chloroform:isoamylalcohol (25:24:1 vol, pH 8.0, Sigma-Aldrich, Inc.). DNA in the aqueous phase was precipitated with 2.5 volumes of absolute ethanol at −20 °C. The precipitated DNA was washed in 75% ethanol, centrifuged and dried for 15 min at room temperature. DNA was dissolved over night at 4 °C in 40 μL nuclease-free water and aliquots were stored at −20 °C.

2.9. Real-time quantitative PCR

The relative quantification of *Dehalococcoides sp.* in the aquifer solids was performed using iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA). Primers targeting the 16S rRNA gene of all known members of the genus *Dehalococcoides* and general bacterial 16S rRNA gene primers were used. The general bacterial 16S rRNA gene primers were those of Muyzer et al. (1993). Each sample had a 30 μL reaction volume containing, 1×iQ SYBR Green Supermix, forward and reverse primer at a concentration of 500 nM, and 6 μL of the prepared DNA. PCR amplification and detection were conducted in an iCycler (BioRad Laboratories, Hercules, CA) under the following conditions: 3 min 95 °C followed by 40 cycles of 10 s at 95 °C and 45 s at 61.5 °C. The amplification program was succeeded by a melt curve analysis starting with the incubation of the PCR product for 1 min at 95 °C and 1 min at 55 °C followed by 80 ten

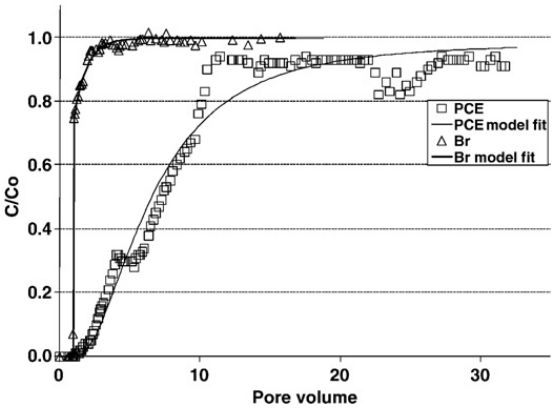

Fig. 2. Bromide and PCE breakthrough curves and model simulations using a two-site non-equilibrium CXTFIT model. Symbols represent experimental data and solid lines represent bromide and PCE CXTFIT model fits.

second cycles in which the temperature was increased by 0.5 °C each cycle. Each reaction was performed in triplicate and triplicate measurements were repeated twice with template DNA from individual extractions. The relative *Dehalococcoides* population abundance of the bioaugmentation culture was determined by real-time PCR to be 73.5±6.1% of the total bacteria prior to column inoculation.

3. Results

3.1. Column performance

The normalized breakthrough curves for bromide and PCE in the phase I tests are shown in Fig. 2. The bromide breakthrough curve indicated a 32 h (~1.3 day) hydraulic residence time in the column, corresponding to a total pore volume of 192 mL, which was in agreement with the values determined from weight and volume estimates. PCE transport through the column was significantly retarded due to sorption onto the aquifer solids. A PCE retardation factor (R) of 8.4 was estimated using the one-dimensional two-site-nonequilibrium CXTFIT version 2.1 transport model (Toride et al., 1995). PCE sorption resulted in a PCE retention time of about ten days, indicating that responses to changes would be on the order of days to weeks. During the initial period phase I and II testing (0–28 days), only small amounts of TCE were observed, while c-DCE, VC, and ETH concentrations were all below detection limits (~0.5 μM). The normalized PCE concentration (C/C_o) approached 1.0 indicating minimal transformation of PCE during this period. The model simulation shows a slow approach toward unity that likely resulted from mass transfer limitations in the sorption process.

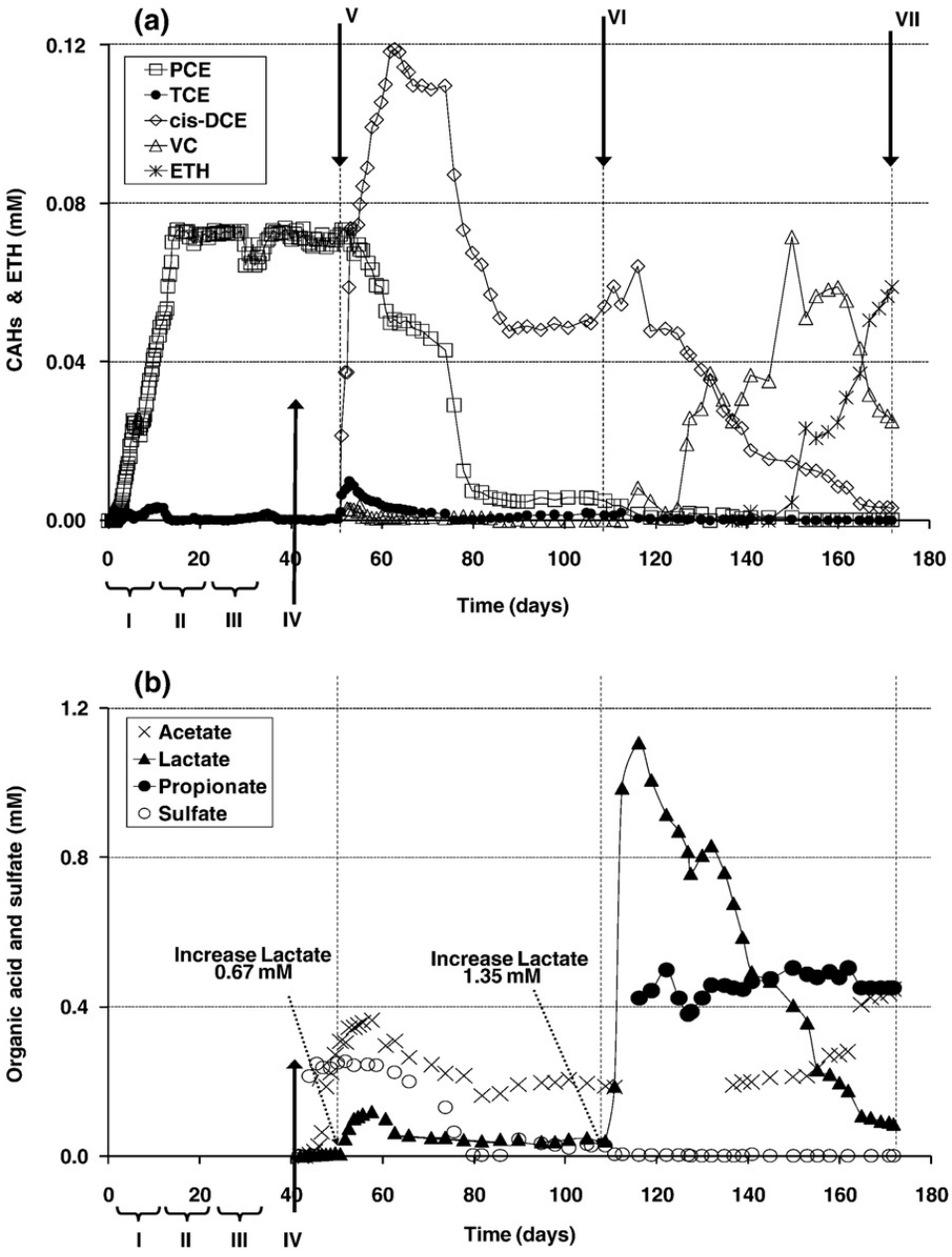

Fig. 3. PCE breakthrough and transformation to TCE, c-DCE, vinyl chloride and ethene during the 170 days of column operation (a). Treatment phases I to VII are indicated by arrows and Roman numerals according to Table 1. Organic acids and sulfate concentrations (b).

The long term concentration histories of the CAHs, ETH, electron donor, fermentation products, and sulfate in the column effluent are provided in Fig. 3. After 28 days, synthetic groundwater and trace elements were added (Table 1, phase III) resulting in a small temporary decrease in PCE concentration in the effluent and a slight accumulation of TCE. This could be due to the abiotic iron reduction, since the PCE solution contained a low concentration of Na_2S (0.19 mM). The EV culture was bioaugmented on day 42 and 0.34 mM lactate addition was started (Table 1, phase IV). Within the first 10 days after bioaugmentation minor amounts of PCE transformation to TCE were observed and sulfate levels remained elevated. On day 51 the lactate concentration was increased to 0.67 mM. PCE dechlorination to TCE and cis-dichloroethene (c-DCE) occurred immediately (Fig. 3a and Table 1, phase V). The increased lactate concentration helped to initiate sulfate reduction as sulfate levels began decreasing around day 60. By day 84, most of the influent PCE was being transformed to c-DCE (Fig. 3a) and effluent sulfate concentrations were approaching the analytical detection limit (~1 μM). Pseudo-steady-state effluent c-DCE, PCE, and lactate concentrations were observed between 84 to 105 days with essentially no production of VC or ETH, possibly indicating electron donor limitations.

On day 105, the influent lactate concentration was increased to 1.34 mM. Effluent lactate concentration increased to approximately 1.1 mM over the next two weeks followed by a gradual decline to about 0.1 mM over the remaining 50 days of column operation (Fig. 3b). During the period of 110–136 days, lactate and acetate co-eluted in the organic acids analysis, due to the high lactate concentration. Reported lactate values, represent a mixture of acetate and lactate. Propionate and acetate were observed at quantifiable levels in the column effluent on days 115 and 137 respectively, with acetate levels increasing with increased lactate removal. Analytical procedures were changed for samples obtained after day 136 (samples were diluted) so lactate and acetate could be separated during HPLC analysis. By day 125, c-DCE reduction to VC began in earnest and by day 150, ETH began being produced. By the end of the test on day 170, ETH and VC were the only products remaining in the column effluent with slightly greater than a 2:1 ETH to VC concentration ratio. Sulfate concentrations decreased to below the detection limit after lactate concentration was increased to 1.34 mM (Fig. 3b). Methane production was never detected in the column effluent and the hydrogen concentration in the column effluent was below detection limit (20 nM) during the entire column operation. Our hydrogen detection limit however, was above the reported hydrogen thresholds for iron reduction and c-DCE and VC dechlorination (Lovely et al., 1994; Cupples et al., 2004). Reduced iron (Fe (II)) was detected in the column effluent only after increasing the lactate concentration to 1.34 mM and Fe (II) concentration increased from 0.07 mM on day 127 to 0.41 mM on day 170.

3.2. Column mass balances

Mass balance calculations were performed for the CAHs, organic acids, and sulfate exiting the column by multiplying the measured effluent concentrations by the fluid flow rate and integrating over time. Table 2 summarizes the mass of PCE, lactate, and sulfate added to the column and the amounts recovered in the column effluent along with the masses of

Table 2
PCE dechlorination, sulfate, lactate, and iron reduction mass balances and estimates of lactate utilization based on the governing chemical reactions (neglecting cell synthesis)

PCE dechlorination, sulfate, and lactate reduction mass balance			Estimates of lactate utilization based on electron reducing equivalents (equations 1–9 below)	
Compound	Quantities	Mass (mmol)	Quantities	Mass (mequiv)[a]
PCE	Added	2.05	Total Lactate Reduced in Column Test	150.1
PCE	Recovered	0.67	Lactate to Reduce PCE to TCE	0.06
TCE	Recovered	0.03	Lactate to Reduce PCE to c-DCE	3.43
c-DCE	Recovered	0.86	Lactate to Reduce PCE to VC	1.73
VC	Recovered	0.29	Lactate to Reduce PCE to ETH	0.84
ETH	Recovered	0.11	Lactate for CAHs dechlorination	4.03%
Total CAHs + Ethene	Recovered	95.2%	Lactate to Reduce SO_4^{2-} to S^{2-}	24.1
Lactate	Injected	17.92	Lactate fermented to propionate	53.4
Lactate	Recovered	5.42	Lactate fermented to acetate	19.3
Total Lactate	Recovered	30.2%	Lactate to reduce Fe^{3+} (Fe^{2+} extracted after	29.5
Sulfate	Injected	4.12	completion of the column experiment)	
Sulfate	Recovered	1.11	Total lactate reducing equivalents	132.3
Total Sulfate	Recovered	26.9%	Reduced lactate accountable	88.2%

Dehalogenation/reduction reactions w/lactate as electron donor dehalogenation and fermentation products

1. C_2Cl_4 (PCE) + 0.17 $C_3H_5O_3^-$ (Lactate) + 0.67 H_2O ===> C_2HCl_3 (TCE) + HCl + 0.33 CO_2 + 0.17 HCO_3^-
2. C_2Cl_4 (PCE) + 0.33 $C_3H_5O_3^-$ (Lactate) + 1.33 H_2O ===> $C_2H_2Cl_2$ (c-DCE) + 2HCl + 0.67 CO_2 + 0.33 HCO_3^-
3. C_2Cl_4 (PCE) + 0.51 $C_3H_5O_3^-$ (Lactate) + 2.01 H_2O ===> C_2H_3Cl (VC) + 3HCl + 1.00 CO_2 + 0.51 HCO_3^-
4. C_2Cl_4 (PCE) + 0.67 $C_3H_5O_3^-$ (Lactate) + 2.68 H_2O ===> C_2H_4 (ETH) + 4HCl + 1.33 CO_2 + 0.67 HCO_3^-
5. $C_3H_5O_3^-$ (Lactate) + 4.0 H_2O ===> HCO_3^- + 2CO_2 + 6H_2
6. $C_2H_3O_2^-$ (Acetate) + 3.0 H_2O ===> HCO_3^- + CO_2 + 4H_2
7. $C_3H_5O_2^-$ (Propionate) + 6.0 H_2O ===> 2HCO_3^- + CO_2 + 7H_2 + H^+
8. $C_3H_5O_3^-$ (Lactate) + 12Fe^{3+} + 4.0 H_2O ===> 2CO_2 + 12Fe^{2+} + HCO_3^- + 12H^+
9. $C_3H_5O_3^-$ (Lactate) + 1.5 SO_4^{2-} (Sulfate) ===> 1.5S^{2-} + 3CO_2 + 2.5H_2O

[a] Assumptions for computation of equivalents (mequiv/mmol): PCE, 8; TCE, 6; c-DCE, 4; VC, 2; H_2, 2; lactate, 12; propionate, 14; acetate, 8; sulfate, 8; iron, 1.

transformation products. The CAHs and ETH recovered in the effluent accounted for 95.2% of the injected PCE, providing a good mass balance. Lactate and sulfate mass recoveries were 30.2% and 26.9%, respectively, indicating their utilization within the column (Table 2).

A CAH balance was performed over the first 51 days of operation by subtracting the amount recovered in the effluent from the amount of PCE injected onto the column. The difference, 0.21 mmol, was likely PCE sorbed to aquifer solids. A mass balance from day 51 to day 81 showed an excess 0.19 mmol of c-DCE produced indicating the enhanced desorption and transformation of PCE. It is unknown whether PCE desorption and subsequent transformation or transformation of sorbed PCE and subsequent desorption of TCE or c-DCE was the dominant mechanism.

Lactate was fermented to products such as hydrogen, acetate and propionate and the electrons provided by lactate and its fermentation products were used to reduce CAHs, iron, and sulfate. An electron balance on lactate addition and the reducing equivalents, required for the above mentioned reactions, was conducted over the 170 days of column operation using the chemical reactions listed in Table 2. Approximately 88.2% of the lactate electron reducing equivalents could be accounted for by the reduction of CAHs, sulfate, iron, and the formation of acetate and propionate. By the end of the column test approximately 4% of the total lactate electron reducing equivalents had been used for dehalogenation reactions.

The total reducible iron extracted from the aquifer solids used to pack the column was about 150 mmol/kg, which would require 12.5 mmol of lactate for complete reduction, using equation 8 (Table 2). Analysis of the aquifer solids upon completion of the column experiment showed the amount of Fe(II) in the aquifer solids was 29.5 mmol, corresponding to an estimated lactate requirement of 2.46 mmol. This represented 19.7% of electron reducing equivalents for lactate utilization (Table 2). These values suggest significant amounts of reducible iron remained in the aquifer solids at the end of the experiment. Recent column studies with the Hanford aquifer solids have shown microbial iron reduction to continue for over 500 days under conditions similar to these tests (data not presented).

3.3. PCE transformation rates

Microcosms prepared on day 170 with column solids acquired from 5-cm spatial sections within the column showed that PCE was rapidly transformed to TCE, c-DCE, VC, and ETH in all of the microcosms and that spatial variability existed in the rates of PCE transformation. Zero-order PCE transformation rates, per gram of aquifer solids, were highest in the 0–5 cm section closest to the column inlet and decreased by an order of magnitude at the column outlet (25–30 cm) (Fig. 4). Zero-order formation rates of the various chlorinated ethenes for the different column sections were also calculated as total mass produced per time per gram of soil (μmol/h/g). TCE and c-DCE formation rates were highest at the inlet end of the column and decreased with increasing distance along the column. The net rates of formation were considered conservative estimates of parent compound transformation since TCE, c-DCE, and VC were being con-

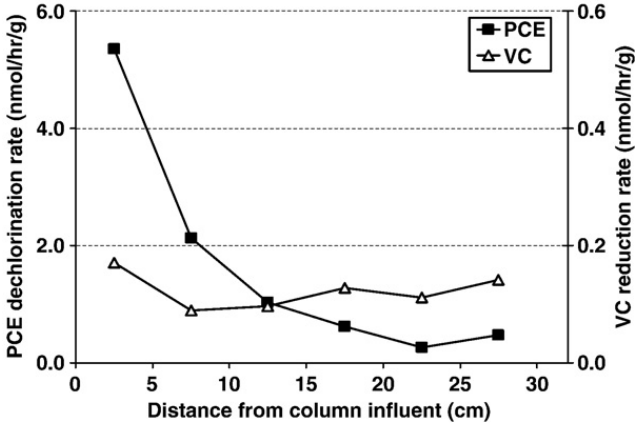

Fig. 4. PCE and vinyl chloride transformation rates in microcosms constructed from aquifer solids from different spatial sections of the column.

currently produced and degraded. ETH formation rates were lower than TCE and c-DCE formation rates in the 0–5 cm section, but similar rates of formation were observed throughout the rest of the column intervals.

3.4. VC transformation rates

VC transformation rates in VC-fed microcosms were determined from rates of ethene formation. A relatively constant rate of VC transformation was observed in the spatial samples with an average rate of 0.12 nmol/h/g. The highest value was observed in the first 5-cm interval (Fig. 4). In contrast, PCE transformation rates decreased markedly along the column length (Fig. 4).

3.5. Lactate fermentation to acetate and propionate

Lactate fermentation to acetate and propionate occurred in all microcosms in the presence and absence of PCE or VC as electron acceptors. Similar rates of lactate fermentation to acetate and propionate were observed in all column sections in the PCE-, VC-, and lactate-fed microcosms. Acetate and propionate formation in all column intervals indicated the presence of lactate fermenting bacteria throughout the column. The ratios of acetate to propionate concentrations were between 1.8 and 2.1 in the 0–5 cm interval and gradually changed to 1.0 towards the column effluent, indicating spatial variation in fermentation reactions and/or fermenting microbial populations.

3.6. Real time quantitative (RT-) PCR

Results of the absolute quantification of *Dehalococcoides* sp. and total bacterial 16S rRNA gene copies ranged from 5×10^8 to 1×10^{10} per gram soil with higher numbers closest to the column inlet where the bioaugmentation culture and media had been injected and the highest PCE transformation rates were found (Fig. 5a). *Dehalococcoides* sp. gene copies decreased slightly from about 1×10^8 cells per gram soil in the first 5 cm to $4.5–6\times10^7$ cells per gram of column matrix throughout the rest of the column (Fig. 5a). When expressed as a percentage of total bacterial gene copies, *Dehalococcoides* gene copies increase from 0.5% in the first 5 cm to about 4% of

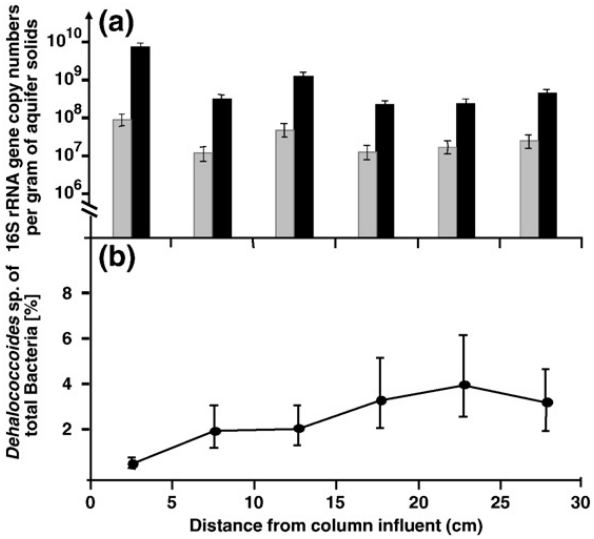

Fig. 5. Relative and absolute quantification of *Dehalococcoides sp.* and total bacterial 16S rRNA gene copies at different locations along the vertical column profile. a) Absolute gene copies per gram aquifer solids, black bars total bacteria, light gray bars *Dehalococcoides sp.* b) *Dehalococcoides* gene copies as percentage of total bacterial gene copies.

all bacteria towards the column outflow (Fig. 5b). This observation fits within expectations that multiple organisms can take advantage of influent electron donors, and PCE or TCE dechlorination, while only *Dehalococcoides* sp are known to anaerobically dechlorinate cDCE and VC.

Presented in Fig. 6a are PCE dechlorination rates normalized to *Dehalococcoides* gene copies numbers. The normalized PCE rates are elevated within the first 10 cm of the column, but become relatively constant from 10 to 30 cm in the column. A similar trend is shown of PCE dechlorination rates normalized to total bacterial numbers (Fig. 6b). These data indicate that multiple species were growing near the influent capable of reductively dechlorinating PCE since higher rates were observed. *Dehalococcoides*, however, likely dominated dechlorination activity farther from the influent where only lesser chlorinated ethenes were present. A more uniform normalized activity for VC dechlorination with respect to *Dehalococcoides* numbers in Fig. 6a is consistent with the fact that *Dehalococcoides* is the only known species to reductively dechlorinate VC. The slightly lower normalized VC dechlorination rate observed in the first 5 cm (Fig. 6a) could be due to the fact that generic *Dehalococcoides* primers were used that would not distinguish between different strains. Thus, it is likely that elevated *Dehalococcoides* DNA levels were detected, but not all the strains were associated with VC dechlorination. The lower normalized VC dechlorination rates at the influent of the column in Fig. 6b are consistent with other data indicating elevated bacterial populations near the influent were performing various fermentation, iron reduction, sulfate reduction, and dechlorination reactions.

4. Discussion

Continuous-flow column experiments conducted with the EV culture bioaugmented onto Hanford aquifer solids, showed PCE being completely transformed to VC and ETH with a fluid residence time of approximately 1.3 days. The results are consistent with batch kinetic studies with the EV culture of Yu and Semprini (2004). PCE dechlorination to TCE and c-DCE was achieved after lactate concentrations were increased from 0.35 to 0.67 mM, potentially indicating electron donor limitations. During this period, iron-reducing organisms may have been competing for hydrogen produced in fermentation reactions. During the initial phase of PCE transformation, effluent c-DCE concentrations exceeded the influent PCE concentrations indicating biologically enhanced PCE desorption from the aquifer solids (Fig. 3a). Aqueous phase mass balances indicated that 91% of the sorbed PCE was recovered as c-DCE, with the remainder possibly being c-DCE sorbed to the aquifer solid, which was not accounted for in the mass balance. The results illustrate how the transformation of a more strongly sorbed contaminant to a less strongly sorbed contaminant might be used to enhance the rate of in-situ treatment. Biologically enhanced dissolution of PCE NAPL has been observed as a result of anaerobic transformations to more soluble products (Yang and McCarty, 2000; Carr et al., 2000; Chu et al., 2003; Sleep et al., 2006). Model simulations of the results presented here have indicated that biological enhanced desorption is possible as a result of transforming more strongly sorbed PCE to less strongly sorbed c-DCE (Mustafa et al., 2007).

TCE did not accumulate as a result of PCE reduction, which was consistent with previous laboratory kinetic studies and modelling analyses by Yu and Semprini (2004) performed in batch systems with the EV culture. The higher TCE maximum utilization rate (k_{max}) combined with the lower TCE half-velocity coefficient (K_s) for the EV culture, compared to those

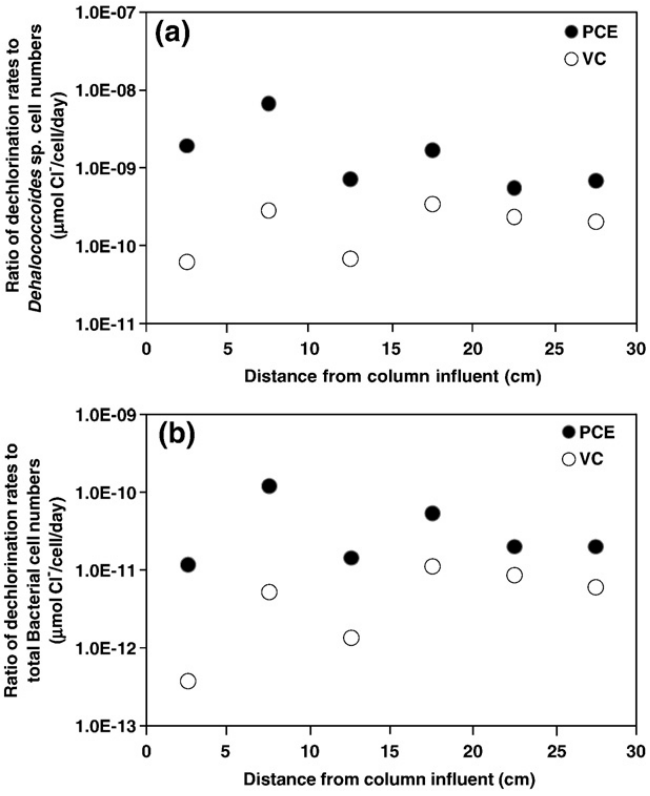

Fig. 6. Transformation rates per cell in the flow column (µmol Cl^-/cell/day). (a). PCE and VC transformation rates to *Dehalococcoides* sp. cell numbers. (b) PCE and VC transformation rates to total bacterial cell numbers.

for PCE and c-DCE (Yu and Semprini, 2004), can explain the lack of TCE accumulation. The lack of TCE accumulation was also observed in the microcosm studies conducted at the end of the column experiment.

When the lactate concentration was increased to 1.34 mM, propionate production was observed from lactate fermentation, and c-DCE reduction to VC occurred (Fig. 3a). The results indicate fermentation reactions at this stage were producing hydrogen at a sufficient rate for c-DCE dehalogenation and for competing electron acceptor reactions, such as iron reduction. VC transformation to ETH was observed once the c-DCE concentration decreased to low levels (Fig. 3a, 148 days). These results are in good agreement with previous laboratory and modeling studies conducted with the EV culture that showed strong inhibition of VC reduction in the presence of TCE and c-DCE (Yu et al., 2005).

Significant quantities of biologically reduced iron in the Hanford aquifer material were measured upon completion of the column test. During the later period of phase V, days 84 to 105, PCE dechlorination was stalled at c-DCE. However, VC production was observed once additional lactate was provided and propionate was detected as a fermentation product, indicating possible hydrogen limitation as a likely reason for the previous lack of c-DCE reduction. The result is consistent with reports by Sleep et al. (2005) that c-DCE was the terminal end product of PCE dechlorination under electron donor limiting conditions. The combination of Fe(III) reduction, sulfate reduction, and PCE and TCE reduction during phase V of the experiment may have reduced the available hydrogen levels below the thresholds required for c-DCE and VC reduction resulting in an accumulation of c-DCE (Fig. 3a). Slow growth of a population of *Dehalococcoides* strains that utilize c-DCE and VC as electron acceptors might also contribute to these observations. EV enrichment culture contained a population of several *Dehalococcoides* strains with all four characterized chloroethene reductive dehalogenases genes (vcrA, bvcA, tceA, and pceA). The *Dehalococcoides* sp. population composition in the EV culture could be fully described by *Dehalococcoides* sp. 16S rRNA gene copy numbers matched the sum of reductive-dehalogenase-homologous genes, indicating that the enrichment culture most likely contains representatives of Dehalococcoides pure cultures (VS, GT, 195, FL2, BAV1, and CBDB1) (Spormann et al., 2007). Iron-reducing organisms have been shown to have potentially lower hydrogen thresholds than dechlorinating organisms that transform c-DCE and VC and may have been successfully competing for hydrogen produced in fermentation reactions during this period (Maurice et al., 2004).

Microcosm results indicated that most of the PCE and TCE transformation was occurring near the column inlet. The estimated electron reducing equivalents from lactate is associated with the fraction of *Dehalococcoides sp*. 16S rRNA gene copies present in the bacterial community. Dehalococcoides sp. gene copy numbers dropped from ~73.5% of the total bacterial gene copy numbers in the original bioaugmentation inocula, to about 0.5% to 4% throughout the column solids at the end of the test. This shift is consistent with the prolonged column operation and the limited electron reducing equivalents for lactate utilization transferred to CAH dehalogenation as a result of the competing electron acceptor reactions. Microcosms supplied with PCE had high TCE and c-DCE formation rates in the sample taken closest to the inlet port and exhibited an essentially exponential decrease of an order of magnitude along the column length. This result is consistent with those reported by Isalou et al. (1998) who observed order of magnitude higher PCE transformation rates in the first 15 cm of a 2 m column fed PCE. VC and ETH formation rates in PCE-fed microcosms were an order of magnitude lower than TCE and c-DCE formation rates at the column inlet and remained more uniform across the column length (Fig. 4). This result was in agreement with microcosm VC transformation rates that were essentially uniform along the column length (Fig. 4). Yu et al. (2005) reported a k_{max} value for VC transformation by the EV culture that was a factor of 1.5 lower and a K_s value 30 times greater than that found for PCE transformation, which is consistent with an order of magnitude lower rate of VC transformation compared to PCE. The uniform spatial distribution of VC transformation activity compared to PCE transformation activity may also have been due in part to the time chosen for column solids analysis. At 170 days, VC transformation to ETH was just being completely developed in the column with more uniform spatial concentrations of VC present, whereas PCE transformation was probably occurring primarily in the first few centimeters. If the solids analysis were conducted on day 70, the result would most likely have reflected a more uniform PCE transformation ability and possibly a lack of VC activity.

5. Conclusions

Bioaugmentation of the EV anaerobic culture to the continuous flow column resulted in complete transformation of PCE to VC and ethene with a fluid retention time of 1.3 days. The combined microbial processes of reductive dehalogenation, lactate fermentation, iron and sulfate reduction were all exhibited within the column. Electron balances performed on the column over the 170 days of operation indicated that approximately 4% of the electron equivalents from lactate were eventually used for dechlorination reactions. This is in general agreement with the microbial community distribution found at the end of the column study which showed Dehalococcoides 16S rRNA gene copies in the total bacterial community were approximately 2–4%.

Microcosm tests revealed large spatial variations in PCE transformation activity, moderate variation in the speciation of lactate fermentation products, and a more spatially uniform ability to transform VC within the column. These microcosms provide a qualitative description of transformation abilities under optimum conditions, such as no electron donor limitation. The results are consistent with those found by other researchers Isalou et al. (1998), Sleep et al. (2005), Yu et al. (2005), Yu and Semprini (2004) and fit the overall conceptual construct of sequential CAH transformation with potential inhibitory influences by parent compounds on daughter product transformation and competition between various microbial populations for electron donors within the system. Further understanding of the transformation abilities of dehalogenating sub-populations, elucidation of the function of currently sequenced reductive dehalogenases and insights into the regulation of dehalogenase gene activity will certainly improve our ability to estimate, evaluate, and stimulate the bioremediation of chlorinated ethenes. In

addition detailed modeling analysis of column studies of this type would provide a more quantitative insight into the processes that are being qualitatively described here. The results provide insights into the combined use of chemical based monitoring, microcosm activity tests and molecular tools to evaluate bioremediation under conditions that mimic those found in the field.

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