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Natural Attenuation in Streambed Sediment Receiving Chlorinated Solvents from Underlying Fracture Networks

Burcu Imirali^{†,‡,||,⊥}, Jun Yan^{‡,||,⊥,#,§}, Jeongdae Im[▽], Duane Graves[○], Frank E. Löffler^{*,†,‡,||,⊥,§}

[†]Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, Tennessee 37996, United States

[‡]Center for Environmental Biotechnology, University of Tennessee, Knoxville, Tennessee 37996, United States

[§]Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996, United States

^{||}Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

[⊥]Joint Institute for Biological Sciences (JIBS), Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

[#]Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, Liaoning 110016, China

[▽]Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002, United States

[○]Geosyntec Consultants, Knoxville, Tennessee 37922, United States

Abstract

Contaminant discharge from fractured bedrock formations remains a remediation challenge. We applied an integrated approach to assess the natural attenuation potential of sediment that forms the transition zone between upwelling groundwater from a chlorinated solvent-contaminated fractured bedrock aquifer and the receiving surface water. In situ measurements demonstrated that reductive dechlorination in the sediment attenuated chlorinated compounds before reaching the water column. Microcosms established with creek sediment or in situ incubated Bio-Sep beads degraded C₁–C₃ chlorinated solvents to less-chlorinated or innocuous products. Quantitative PCR and 16S rRNA gene amplicon sequencing revealed the abundance and spatial distribution of known dechlorinator biomarker genes within the creek sediment and demonstrated that multiple dechlorinator populations degrading chlorinated C₁–C₃ alkanes and alkenes co-inhabit the sediment. Phylogenetic classification of bacterial and archaeal sequences indicated a relatively uniform distribution over spatial (300 m horizontally) scale, but *Dehalococcoides* and *Dehalobacter* were more abundant in deeper sediment, where $5.7 \pm 0.4 \times 10^5$ and $5.4 \pm 0.9 \times 10^6$

*Corresponding Author: Phone: (865) 974-4933; fax: (865) 974-4007; frank.loeffler@utk.edu.

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Supporting Information

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Additional details, figures and tables relating to methods, site characterization, and electron donor demand calculations (PDF) (XLS)

16S rRNA gene copies per g of sediment, respectively, were measured. The microbiological and hydrogeological characterization demonstrated that microbial processes at the fractured bedrock–sediment interface were crucial for preventing contaminants reaching the water column, emphasizing the relevance of this critical zone environment for contaminant attenuation.

Graphical Abstract

INTRODUCTION

Chlorinated solvents have been widely used in a variety of industrial, military, and household applications since the 1940s.^{1,2} Their extensive use, improper handling and disposal practices, as well as accidental spills resulted in widespread subsurface and groundwater contamination.^{1,3} Common chlorinated solvents including tetrachloroethene (PCE), trichloroethene (TCE), carbon tetrachloride (CT), and 1,1,1-trichloroethane (TCA)³ tend to form dense nonaqueous-phase liquids (DNAPLs), which move gravitationally along interconnected fractures and form pools in low points in fractured bedrock formations. A significant mass of chlorinated solvents in a fractured rock site diffuses into low-permeability zones,^{4–6} and back-diffusion into water-bearing fractures serves as a long-term source of groundwater contamination.^{5,7,8}

In the last two decades, various in situ remediation technologies, including bioremediation and thermal and chemical treatments have been successfully applied to treat chlorinated solvent contamination in porous medium aquifers;^{3,9} however, the remediation of fractured bedrock formations remains challenging due to difficulties in characterizing complicated fracture networks, the back-diffusion of contaminant from low-permeability zones, and the challenge of targeted delivery of remedial fluids.^{2,4,10–12} An alternate remedial approach focuses on treatment at the fractured bedrock–sediment interface, where contaminated groundwater discharges to surface waters. Recent studies have shown that such hyporheic

zones are “hotspots” of microbial activities playing relevant roles for contaminant attenuation.^{13–16}

Organohalide-respiring bacteria (OHRB) use chlorinated hydrocarbons as terminal electron acceptors, and a number of species belonging to different genera (e.g., *Geobacter*, *Dehalobacter* (*Dhb*), *Desulfitobacterium*, and *Sulfurospirillum*) have been demonstrated to couple reductive dechlorination of PCE to TCE or *cis*-1,2-dichloroethene (*cis*-DCE) with energy conservation.⁹ In contrast, complete reductive dechlorination to environmentally benign ethene appears to be restricted to some strains of the species *Dehalococcoides mccartyi* (*Dhc*).⁹ *Dhb* are involved in dechlorination of a range of chlorinated compounds including chlorinated aromatics,¹⁷ chlorinated ethanes, and chlorinated methanes,^{19,20} as well as PCE.²¹ *Dehalogenimonas* spp. have been implicated in dehalogenation of chlorinated alkanes²² and, recently, in reductive dechlorination of *trans*-1,2-dichloroethene (*trans*-DCE) to VC.²³ Studies demonstrated significant correlations between the abundance of OHRB and observed in situ dehalogenation activities.⁹ Therefore, 16S rRNA genes and reductive dehalogenase (RDase) genes from known OHRB serve as biomarkers to assess in situ bioremediation activity and potential.^{9,24}

At a former metal manufacturing facility located adjacent to Third Creek, a Tennessee River tributary in Knoxville, TN (Figure 1), chlorinated solvents (primarily PCE, TCE, TCA, and CT) were released and penetrated the underlying fractured bedrock formation. Although no DNAPL source zones could be identified, dissolved-phase concentrations of total chlorinated volatile organic compounds (cVOCs) exceeded 10 mg/L in bedrock monitoring wells indicative of free-phase chlorinated solvents (Table S1). Spent solvents are the primary source of groundwater contamination, and, as such, the solvents would be co-contaminated with the oils and grease from the cleaning operation. Additionally, machining and lubricating oils and mineral spirits were used throughout the history of the facility but in much smaller quantities than the chlorinated solvents. These limited, and often localized, sources of hydrocarbons were not quantified during site assessments but are thought to have supported the modest and incomplete microbial transformation of contaminants observed in the fractured bedrock (Table S1), indicating the need for alternative remedies at the Third Creek site. This study evaluated the role of the streambed sediment as a natural barrier preventing contaminant discharge into Third Creek surface water. Integrated efforts characterizing the hydrogeological (e.g., flow paths) and microbiological site conditions at the Third Creek site demonstrated efficient natural attenuation in the sediment.

MATERIAL AND METHODS

Chemicals

Chlorinated compounds were of >99% purity. PCE and CT were purchased from ACROS Organics (Morris Plains, NJ), TCE was obtained from Fisher Scientific (Pittsburgh, PA), and *cis*-DCE, vinyl chloride (VC), ethene, TCA, dichloromethane (DCM), chloroform (CF), chloromethane (CM), 1,2-dichloropropane, 1,1-dichloroethane (DCA), and chloroethane (CA) were obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

Site Characterization

The manufacturing site is bounded by Third Creek on its west side (Figure 1). The facility used chlorinated solvents as degreasers from the mid-1930s to the late 1990s. During the course of manufacturing activities, chlorinated solvents, primarily PCE, TCE, and, to a smaller extent, TCA and CT, were released resulting in contamination of the underlying groundwater-bearing fractured bedrock (Figure S1–2). Direct observation of soil and bedrock cores failed to locate DNAPL although contaminant concentrations as high as 120 mg/L were measured in nonflowing fractures (Figure S3). The presence of *cis*-DCE, VC, DCA, and CF in several bedrock monitoring wells indicated that some contaminant transformation occurred; however, high concentrations of parent compounds and no ethene and ethane formation indicated limited dechlorination capacity within the fracture network (Table S1). Groundwater seepage rates were measured with leak-tested seepage meters.²⁵ Horizontal and vertical bedrock groundwater flow direction was evaluated using seasonal groundwater elevations from monitoring wells screened at multiple depths and staff gauges in the creek. The Supporting Information provides additional details about the site characterization efforts.

Sediment Pore Water Diffusion Sampling

To measure concentrations of cVOCs, methane, ethene, and ethane, as well as geochemical parameters, sediment pore water diffusion samples were collected with depth-discrete diffusion samplers loaded with 40 mL glass vials. Before the placement of the samplers into the sediment, the 40 mL glass vials were filled with deionized water and covered with either polyethylene film for cVOC sampling or a porous, nonwoven fabric for the measurement of geochemical parameters. The samplers were installed in the same locations as the seepage meters 0–0.55 m (0–1.8 ft) below the sediment surface and left in the creek for 2 weeks to achieve equilibrium with the pore water. After recovery of the samplers, the vials were immediately sealed and shipped to analytical laboratories (SiREM, Guelph, Canada and Microbac, Maryville, TN) for volatile fatty acids (VFAs), cVOC, and anions measurements.

Sediment Collection

Grab sediment samples and cores were collected from location numbers (nos.) 1, 2, and 3 (Figure 1, left panel). These locations were chosen on the basis of the observed sediment cVOC concentrations and the site's hydrogeological characteristics. Top-layer sediment samples were collected using autoclaved, sealable glass containers (Mason jars). Deeper sediment layers were obtained using direct push tools (AMS, Inc., American Falls, ID). The plastic liners and caps were wiped with 70% ethanol before use. All other materials (spatulas, containers, etc.) were autoclaved, and aseptic techniques were applied to the extent feasible. All core samples were immediately transferred to sterile Mason jars, filled completely with creek water to exclude air, capped, and placed in a cooler with ice packs.

Depth-Resolved Sediment Collection

Depth-discrete diffusion samplers were employed to collect sediment at multiple depths at location number (no.) 3. The diffusion sampler was loaded with 40 mL glass vials evenly spaced over a length of 99 cm (3.2 ft). The vial openings were covered with plastic mosquito

netting (1 mm mesh size) held in place with rubber bands. The loaded sampler was pushed into the sediment to a depth of about 55 cm (1.8 ft). A second sampler loaded with customized Bio-Trap samplers (about 200 Bio-Sep beads per sampler, Microbial Insights, www.microbe.com) was placed in the sediment in the same location. After a 1 month incubation period, the samplers were removed, and the glass vials with sediment material were immediately closed with sterile Teflon-lined rubber septa. The vials and the Bio-Sep bead cartridges were placed individually in Ziploc plastic bags, immediately transferred to a cooler with ice, and transported to the laboratory for microcosm setup and DNA extraction.

Sample Handling

Grab samples and sediment core samples from the same locations were combined and mixed inside an anoxic glovebox filled with H₂/N₂ (3%:97%, vol/vol). Approximately 100 g of sediment material from each of the three sampling locations was transferred to sterile plastic tubes and stored at –80 °C. The remaining sediment materials were kept at 4 °C and microcosms were established within 1 week of sample collection. Sediment materials collected in the 40 mL glass vials from eight different depths were individually transferred to sterile plastic tubes, homogenized, and stored at –80 °C for molecular analyses. The Bio-Trap samplers collected from eight depths were opened inside the glovebox, and about 200 Bio-Sep beads per depth sample were transferred to a sterile plastic tube for DNA extraction and microcosm experiments. All procedures used strictly aseptic techniques. To prevent cross-contamination, sediment samples from different locations were not handled simultaneously.

Microcosm Setup

For sediment microcosm setup inside the anoxic glovebox, approximately 4 g (wet weight) of the sediment from location nos. 1, 2, or 3 were transferred to sterile 60 mL glass serum bottles. A total of 26 mL of anoxic, bicarbonate-buffered mineral salts medium²⁷ amended with 5 mM lactate was added to each bottle before the vessels were sealed with autoclaved butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK). Neat PCE, TCE, *cis*-DCE, VC, TCA, DCA, 1,2-dichloropropane, CT, CF, DCM, and CM were added to triplicate microcosms with 5 or 10 μ L Hamilton glass syringes (Hamilton 85925 and 80370) to achieve initial aqueous-phase concentrations of approximately 0.2 mM (12.5–33.1 mg/L). A single microcosm for each treatment was autoclaved for 60 min at 121 °C. An additional set of live control microcosms received all amendments except the cVOC additions.

To obtain depth-resolved information about microbial reductive dechlorination activity, additional microcosms were established with in situ incubated Bio-Sep beads collected at location no. 3. Inside the glovebox, five beads were transferred to sterile 60 mL glass serum bottles containing 30 mL of reduced mineral salts medium amended with 5 mM lactate. The bottles were closed with butyl rubber stoppers and PCE, TCE, *cis*-DCE, VC, or TCA were added to reach aqueous-phase concentrations of approximately 0.2 mM. Initially, cVOCs could not be measured due to sorption to the Bio-Sep beads, but quantitative analysis was possible in transfer cultures. After a 2 month incubation period, 3% (v/v) culture suspension without beads was transferred to fresh medium amended with 5 mM lactate and 0.2 mM of the respective cVOC. Enrichment cultures that showed no reductive dechlorination received

6 mL of H₂ to ensure that electron donor availability was not a limitation. All microcosms and enrichment cultures were incubated statically at room temperature in the dark and monitored over a 20-month incubation period.

DNA Isolation, qPCR, and 16S rRNA Gene Amplicon Sequencing

To assess the spatial distribution of known dechlorinators in relation to the three different sampling locations and sediment depth at location no. 3, PCR, quantitative PCR (qPCR), and 16S rRNA gene fragment amplicon sequencing were performed. DNA was extracted from 0.25 g of wet sediment using the MoBio PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA). DNA extraction from Bio-Sep beads (~160 beads per depth) was performed by Microbial Insights using established procedures.²⁸ Published primer sets and PCR conditions were used to amplify total bacterial,²⁹ *Dhc*,³⁰ *Dhb*,³¹ *Dehalogenimonas*,²² and *Geobacter lovleyi* strain SZ³² 16S rRNA genes, and *dcpA* encoding a 1,2-dichloropropane-to-propene RDase.³³ For increased sensitivity of detection, a nested PCR approach was applied³⁴ (see the Supporting Information for details). qPCR to enumerate total bacterial, *Dhc*, and *Dhb* 16S rRNA genes, as well as the *bvcA*, *vcrA*, *tceA*, and *cfrA* RDase genes used published primers and probes and followed established protocols (Table S2).

To compare the microbial communities at location nos. 1, 2, and 3, amplification of the hypervariable V1–V3 and V3–V5 regions of the 16S rRNA gene and subsequent pyrosequencing of the PCR amplicons was performed with barcoded-primers^{35–38} (Table S2). Library preparation was performed as described³⁸ with minor modifications (see the Supporting Information), and pyrosequencing was performed on a 454 FLX Life Sciences Genome Sequencer (Roche Diagnostics) according to the manufacturer's instructions. Sequence data analyses followed established procedures (see the Supporting Information).

Analytical Methods

cVOCs, ethene, ethane, propene, and methane were monitored using an Agilent 7890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-624 capillary column (60 m by 0.32 mm with a film thickness of 1.80 μ m) as described.³⁹ Details of the method for cVOCs measurements by SiREM Laboratory are given in the Supporting Information.

RESULTS

Third Creek Site Hydrological Features

Available hydrogeological data sets from 46 sampling locations on both sides of Third Creek consistently indicated that groundwater has an upward, vertical gradient, and horizontal gradients sloping toward the creek from both sides. The groundwater seepage meters placed in creek sediment confirmed, as predicted by vertical (Figure 1) and horizontal gradients (not shown), that the creek received groundwater with maximum, mean and median seepage rates of 3380, 721, and 205 mL/m²/day, respectively, at location no. 3. The broad range of measured seepage rates were likely influenced by the creek stage, sampling location, and groundwater elevation. The lowest mean and median seepage rates of 47 and 5.4 mL/m²/day, respectively, were measured near location no. 1, and occasionally, negative values (i.e.,

losing water) were observed. Assuming the highest rate of seepage (3380 mL/m²/day), a sediment depth of 0.52 m, a sediment porosity of 20%, and no retardation of cVOC migration in the sediments, the shortest retention time for groundwater seeping through the sediment into the creek was calculated to be approximately 30 days.

The seepage measurements and vertical and horizontal groundwater gradients suggest that Third Creek receives groundwater from underlying fractures downstream of location no. 1 (Figure 1). The elevation data suggest that the creek transitions from a losing to a gaining creek along the perimeter of the contaminated area near location no. 1 (see the Supporting Information for details). Based on the average creek widths of 7.3 m between location nos. 1 and 2 and 10.4 m between location nos. 2 and 3, segment lengths of 226.5 m between location nos. 1 and 2 and 77.1 m between location Third Creek between location nos. 1 and 3 was estimated at 42 000 to 170 000 L/year using median and mean seepage rates, respectively.

Third Creek Site Geochemical Characteristics

At location no. 3, sediment pore water measurements detected *cis*-DCE, VC, ethene, ethane, and methane in the deep sediment, whereas significantly lower cVOC concentrations were measured near the sediment–surface water interface. Concentrations varied with depth (Figure 2), and maximum ethene, ethane and methane concentrations of 0.25, 0.08, and 5.2 mg/L, respectively, were measured near the sediment–surface water interface. This observation suggests the formation of ethene and ethane as reductive dechlorination products with their further degradation occurring in shallower sediment layers. Using location no. 3 ethene, ethane, and cVOC data, the change in ethene and ethane concentrations from 0.52 to 0.22 m depth accounts for approximately 92% of the loss in cVOCs on a mole basis. cVOC concentrations were lower near the sediment–surface water interface and the *cis*-DCE and VC concentrations were less than the method quantification limit of <0.01 mg/L. Maximum *cis*-DCE and VC concentrations of 0.78 and 0.33 mg/L, respectively, were observed in the deeper sediment at location no. 3. The lack of cVOC detections at the sediment–surface water interface could be the result of dilution with the creek water; however, the upward flow of water into the creek, the lack of cVOC detections below the sediment–water interface, and the presence of methane in the shallow sediment indicate that the influence of surface water on pore water cVOC concentrations is inconsequential. With the exception of *cis*-DCE (0.03 mg/L) in the deep sediment at location no. 1, no cVOCs were detected in sediment pore waters collected at location nos. 1 and 2 (Table S3). TCE concentrations were generally below 0.01 mg/L in the deep sediment, but TCE was occasionally detected near the sediment–surface water interface (Figure 2 and Table S3). In addition, concentration gradients of VFAs, sulfate, and chloride were observed in the sediment pore water at location no. 3. VFA concentrations of 374 mg/L in the deep sediment pore water decreased to 4 mg/L in pore water collected near the sediment–surface water interface, and sulfate concentrations increased from <0.8 mg/L at depth to 12 mg/L in the upper sediment layers at location no. 3. Maximum sulfate concentrations of 130 mg/L were detected at location no. 2. At a deeper sediment depth of 0.45 m at location no. 3, the consistently gaining section of the stream, chloride concentrations of 14 mg/L were measured. The chloride concentration increased to 28 mg/L at the shallower 0.14 m sediment depth and then decreased slightly to

24 mg/L just below the sediment–surface water interface. These observations suggest a possible correlation between chloride content in pore water and reductive dechlorination activity, although the amount of chlorinated solvents presumably degraded is not stoichiometrically balanced with the observed chloride concentration. Tables S3 and S4 summarize sediment pore water cVOC, ethene, ethane, and methane concentrations along with geochemical parameters observed at the three sampling locations.

Reductive Dechlorination in Microcosms

In sediment microcosms, reductive dechlorination of chlorinated ethenes started within 1 week, and stoichiometric conversion to ethene was achieved within 4–5 weeks regardless of sampling locations. The rate of dechlorination observed in the laboratory is very similar to the shortest residence time of 30 days for cVOCs in the stream sediment, i.e., the time available for dechlorination before the cVOCs from the deepest pore water samples pass through the sediment and reach the surface water. In microcosms amended with TCA, sequential reductive dechlorination to DCA and CA occurred; however, ethane was not detected (Table 1). In both live and killed CT-amended microcosms, CT disappeared and no more than 40% of CT), DCM formed transiently, but CM, the reductive dechlorination daughter product of DCM, was not observed. In CM-amended microcosms, CM was not degraded. Microcosms amended with 1,2-dichloropropane produced propene without intermediate formation of monochlorinated propanes, suggesting a dichloroelimination reaction occurred. Methane was produced in all live microcosms except in those amended with CT. No dechlorination daughter product formation and no methane formation occurred in control microcosms (except for the limited, abiotic CT-to-CF transformation observed in the CT-amended microcosms).

Table 1 also summarizes the major dechlorination end products in microcosms established with in situ incubated Bio-Sep beads collected from eight discrete depth intervals. While PCE to TCE dechlorination occurred in cultures derived from all depth horizons, complete reductive dechlorination of PCE to ethene was observed in cultures derived from only two of the eight depth horizons. However, VC-to-ethene reductive dechlorination occurred in cultures derived from all discrete depth horizons. In contrast to the sediment microcosms, TCA reductive dechlorination did not occur in any of the Bio-Sep bead-derived enrichment cultures. The addition of H₂ to transfer cultures that showed no or limited activity did not stimulate reductive dechlorination, indicating that electron donor availability was not a limiting factor (data not shown). Methane production occurred in all Bio-Sep bead microcosms and transfer cultures.

Detection and Quantification of Dechlorinator 16S rRNA and RDase Genes

Nested PCR detected *Dhc* and *Dhb* 16S rRNA genes in all sediment samples from location nos. 1, 2, and 3, including the depth-discrete sediment samples and the Bio-Sep beads collected at location no. 3. Similarly, *Dehalogenimonas* 16S rRNA genes were detected in the majority of samples tested. *G. lovleyi* 16S rRNA genes and the *dcpA* gene were present in most of the sediment samples tested but found in only one of the Bio-Sep bead samples (Table S5). Direct PCR did not generate reproducible target gene-specific amplicons. With qPCR, $5.2 \pm 0.7 \times 10^5$, $5.6 \pm 0.4 \times 10^5$, and $1.8 \pm 0.5 \times 10^5$ *Dhc* 16S rRNA gene copies per

g of wet sediment were measured at sampling location nos. 1, 2, and 3. Somewhat greater variability was observed with *Dhb* 16S rRNA genes, and $1.7 \pm 0.1 \times 10^6$, $2.1 \pm 0.5 \times 10^5$, and $6.4 \pm 0.7 \times 10^4$ copies were measured per gram of wet sediment at locations nos. 1, 2 and 3, respectively. *Dhc* 16S rRNA genes were distributed throughout the sediment column, although they were generally more abundant in deeper sediments layers, with cell numbers exceeding 8.0×10^4 per g of wet sediment or 4.0×10^4 per g of wet Bio-Sep beads (Figure 2). The *bvcA* gene was present in numbers exceeding 7.0×10^5 per g of wet sediment or 4.0×10^5 per g of Bio-Sep beads (wet weight) and could be quantified in all sediment depth horizons. The *vcrA* gene was distributed throughout the sediment but was present in lower abundances and could be quantified in only two sediment depths. The *tceA* gene was present in numbers exceeding 1.0×10^5 per g of wet sediment or 9.0×10^4 per g of Bio-Sep beads throughout the sediment column. *Dhb* 16S rRNA genes were also detected throughout the sediment column but in greater abundance in deeper sediment horizons. The CF and TCA RDase gene *cfrA* was not detected in any of the sediment samples, and only one Bio-Sep bead sample (0.45 m depth, $1.7 \pm 0.2 \times 10^3$ gene copies per g of wet Bio-Sep beads) tested positive.

Community Analysis

Many dechlorinators, such as the organohalide-respiring Chloroflexi, strictly depend on the microbial community to supply essential nutrients (e.g., corrinoid, H_2).²⁴ To link microbial dechlorination activity with phylogeny at a natural attenuation site, barcoded pyrosequencing of bacterial and archaeal 16S rRNA gene amplicons from Third Creek sediment samples collected at location nos. 1, 2, and 3 was performed. A total of 53 815 high-quality bacterial and archaeal 16S rRNA gene amplicon sequences were obtained after processing. On the basis of the Chao1 index, the highest bacterial operational taxonomic unit (OTU) richness was associated with location no. 1 sediment, whereas location no. 3 showed the lowest richness (Figure S4). A total of 47 bacterial phyla could be assigned across all samples on the basis of the analysis of bacterial V3–V5 region sequences. All bacterial OTUs recovered from Third Creek sediment samples are summarized in Table S6. The majority of the OTUs belonged to the phyla Proteobacteria, Bacteroidetes, and Chloroflexi, which contributed on average 65%, 15% and 6% of all V3–V5 bacterial sequences, respectively (Figure 3, Table S6). OTUs belonging to the class Dehalococcoidia represented up to 4% of the total bacterial sequences; however, *Dehalococcoides* sequences were detected in lower abundances (<0.1% of the total bacterial OTUs), and none of the amplicon sequences affiliated with the genus *Dehalogenimonas*. *Dhb* OTUs were detected only at location nos. 1 and 2 in low abundances (<0.1% of the total bacterial 16S rRNA sequences). At the genus level, *Sulfuricurvum* (36% and 27% of total V1–V3 and V3–V5 bacterial 16S rRNA gene sequences, respectively) was the most-abundant genus in the sediment sample collected from location no. 3, whereas this group contributed to less than 1% of the total OTUs in the other two sampling locations. In sediment samples from location nos. 1 and 2, methanotrophs including members of the family *Methylococcaceae* contributed no more than 0.2% of total bacterial OTUs, and this group was not detected in location no. 3 sediment samples. Sulfate reducers including members of the orders Desulfobacterales, Desulfovibrionales, and Syntrophobacterales contributed up to 4% of total bacterial sequences. *Geobacter* OTUs exceeded 3% at location nos. 1 and 2 but contributed less than

0.1% of the bacterial amplicon sequences at location no. 3. The analysis of bacterial V1–V3 region sequences contributed two additional phyla (*ABY1_OD1* and *Lentisphaerae*), both with average abundances of less than 0.1% of total bacterial sequences. Across all samples analyzed, the V3–V5 amplicon analysis contributed 11 phyla with total sequence abundances not exceeding 0.5%, which were not detected among the V1–V3 region amplicons. Despite these differences, taxonomic classification of bacterial amplicons based on V1–V3 and V3–V5 sequence analysis revealed similar results when OTUs that contributed at least 1% of the total sequences were included in the analysis (Table S7 and Figure S5).

Archaeal V1–V3 sequences grouped into a total of 1462, 1296, and 1779 OTUs for location nos. 1, 2, and 3, respectively. The archaeal species richness differed between sampling sites and the highest OTU abundance was observed at location no. 1 (Figure S6). The majority (up to 60%) of archaeal OTUs belonged to the Euryarchaeota, followed by the Crenarchaeota (varying between 27 and 44% of the total archaeal sequences) (Table S6 and Figure S7). Rarefaction analysis indicated that the number of unique 16S rRNA gene amplicon sequences from all sediment samples did not reach saturation (Figures S4 and S6), suggesting that the amplicon data may underestimate the true species richness in Third Creek sediment samples.

DISCUSSION

Importance of Critical Zone Interfaces for Contaminant Attenuation

Although microbial activity within fractures has been demonstrated,^{2,40,41} poor matrix continuity, absence of dechlorinators, and unfavorable geochemical conditions often limit natural attenuation capacity.⁴⁰ The hydrogeological characterization of the Third Creek site suggested that in situ dechlorination within the fracture network is insufficient to prevent cVOCs from discharging into Third Creek sediment. Remarkably, contaminant release to surface waters is negligible, suggesting that the sustained presence and activity of OHRB allows the sediment to serve as an effective, natural reactive barrier that achieves dechlorination and prevents cVOCs from reaching surface water. These results emphasize the relevant role of fractured bedrock–sediment interfaces for contaminant attenuation.

Distribution of Dechlorinators in Third Creek Sediment

The Third Creek sampling locations span a distance of approximately 300 m but showed consistent levels of reductive dechlorination biomarkers and activity in microcosm tests. No evidence for localized activity hot spots were obtained, although a finer sampling grid would be required for more accurate delineation of organisms and activities.^{14–16} In this study, the abundances of reductive dechlorination biomarkers were in good agreement with the observed metabolic activities. Both *Dhc* and *Dhb* 16S rRNA genes were more abundant in deeper sediment horizons where higher concentrations of cVOCs and VFAs were measured. Similar correlations between the detection of *Dhc*, organic carbon availability, contaminant type(s), and contaminant concentration(s) in sediments have been observed at other sites.^{16,42,43} For example, spatial variability in biotransformation extent of chlorinated solvents was observed within a 45 m distance test area of Zenne River (Belgium) sediment receiving

cis-DCE-, VC-, and DCA-contaminated groundwater. Within this test area, the distribution of *Dhc* and *Dhb* 16S rRNA genes was highly correlated with organic carbon availability in the sediment,^{42,43} and contaminant attenuation was patchy.^{15,16,42,43}

The microcosm experiments and molecular analyses with Third Creek samples demonstrated the presence of multiple *Dhc* strains (i.e., strains carrying the *tceA*, *bvcA*, *vcrA*, or *dcpA* genes) with different dechlorination capabilities. The presence of multiple *Dhc* strains carrying different RDase genes at contaminated sites is not uncommon and has been observed in other river sediments.⁴⁴ To date, it is unclear whether different *Dhc* strains with different RDase genes have distinct properties, which could affect their dispersal and distribution throughout the sediment column. The distribution of *Dhc* cells is obviously relevant for bioaugmentation applications, and studies have demonstrated that *Dhc* cells disperse following injection;^{45,46} however, it remains to be seen if *Dhc* strains with unique reductive dechlorination capabilities exhibit distinct propensities for attachment and dispersion.

Interestingly, in some of the sediment samples from different depths, *bvcA* and *tceA* gene copies were 5- to 10-fold higher than the *Dhc* 16S rRNA gene copies (Figure 2), a phenomenon that has been observed previously at activity “hot spot” locations.^{42,47} *Dhc* genome analyses demonstrated that individual RDase genes occur as single copy genes,²⁴ and there is no evidence for gene duplications as a possible explanation for the elevated *bvcA* and *tceA* gene copy numbers. These observations indicate that non-*Dhc* hosts may carry these RDase genes and suggest a possible role of lateral gene-transfer events in the dissemination of RDase genes. Indeed, recent comparative genomic analyses suggested that *tceA*^{48,49} and VC RDases (i.e., *bvcA* and *vcrA*)^{50,51} can be horizontally acquired.

Co-occurrence of Multiple Contaminants at the Third Creek Site

CT, CF, and TCA are potent inhibitors of reductive dechlorination of chlorinated ethenes,^{21,52} and thus, a sequential treatment alleviating inhibition has been suggested for successful bioremediation.¹⁸ At the Third Creek site, while the CF and TCA RDase gene *cfrA* was detected only in deep sediment layers, the *Dhc* RDase gene *bvcA* implicated in *cis*-DCE and VC reductive dechlorination was distributed throughout the sediment. Such a stratified distribution of ORHB with distinct dechlorination capabilities can effectively overcome inhibition in mixed contaminant plumes¹⁸ and may be a key feature of mixed contaminant sites with productive natural attenuation capacity.

Sediment versus Bio-Sep Bead Analysis

While PCR analysis detected *Dhc*, *Dhb*, and *Dehalogenimonas* 16S rRNA genes in both sediment- and bead-derived DNA, *G. lovleyi* 16S rRNA genes were detected in most sediment samples but in only one Bio-Trap. Similarly, *Dhb*-mediated TCA-dechlorinating activity was readily established in microcosms initiated with sediment but not with Bio-Sep beads. Possible reasons for these findings are organism-specific differences in attachment behavior, or that the 30-day in situ incubation period was insufficient for all sediment populations to colonize the beads.^{53,54} Independent of the causes, these observations suggest that site assessment relying only on in situ enrichment can bias the results, and an integrated

assessment approach that combines in situ (i.e., Bio-Sep bead) and ex situ (i.e., microcosms) cultivation as well as molecular tools will provide the most comprehensive information.

Microbial Community and Heterogeneity in Third Creek Sediment

Although present in low abundance, the recovery of sequences representing members of the Methylococcaceae suggests the potential for aerobic, co-metabolic contaminant transformation. Methanotrophs have been observed in oxic–anoxic transition zones in chlorinated solvent-contaminated sediments and groundwater plumes and implicated in co-metabolic cVOC degradation.^{55–57} Sequences representing ammonia oxidizers of the family Cenarchaeaceae⁵⁸ were abundant despite the low concentrations of nitrate and nitrite measured in sediment pore water samples (Table S4). A possible explanation is ammonium-laden urban runoff, although it is possible that these organisms utilize a broader suite of substrates, as indicated by the genome of the ammonia-oxidizer “Candidate Nitrososphaera gargensis”.⁵⁹ An interesting observation was the high abundance of sequences (i.e., 27–36% of total bacterial sequences, Table S6) belonging to *Sulfuricurvum* in the sediment sample collected at location no. 3. *Sulfuricurvum* spp. are facultative anaerobes capable of oxidizing inorganic sulfur compounds (i.e., S^0 , $S_2O_3^{2-}$, and H_2S) with NO_3^- or O_2 as electron acceptors.^{60–63} The groundwater sulfate concentrations ranged between 10 and 60 mg/L but were generally lower in the deeper sediment pore water, indicative of sulfate reduction activity. Sulfate concentrations increased toward the oxic–anoxic transition zone (Table S4), suggesting the oxidation of sulfur compounds, presumably mediated by *Sulfuricurvum* spp. Additional DNA sequencing efforts performed 25 months later revealed much-lower *Sulfuricurvum* abundances (i.e., < 0.02% of total sequences, data not shown) at location no. 3, suggesting that the population size is undergoing dynamic changes. The first sampling event may have captured a *Sulfuricurvum* bloom, emphasizing that a single sampling event is merely a snapshot in time and not sufficient for determining the community structure at a specific Third Creek sediment sampling location.

Potential Electron Donors Supporting Reductive Dechlorination

A major challenge for successful bioremediation of chlorinated solvents is the supply of fermentable substrates to maintain H_2 fluxes that do not constrain the activity of hydrogenotrophic OHRB. High concentrations of VFAs and methane, as well as depletion of sulfate in deeper sediment horizons, suggested the infiltration of organic material from underlying fractures. Petroleum compounds (“grease oil”) are often a major component of chlorinated solvent DNAPLs, and open-top cleaning technologies in metal manufacturing industries generated approximately 750 kg of chlorinated solvents per 100 kg of petroleum hydrocarbons removed.⁶⁴ Assuming that about two-thirds of the chlorinated solvent volatilized,⁶⁴ the spent metal-degreasing fluid that penetrated the subsurface consisted of a chlorinated solvent/petroleum hydrocarbon mixture of 2.5:1 (w/w). Based on the measured groundwater discharge of 170 000 L/year and the recent highest measured cVOC concentration of 95 mg/L (Table S1), the total annual cVOC mass discharged into Third Creek sediment would be 16.1 kg (Supporting Information, Step 5). Assuming a chlorinated solvent-to-petroleum hydrocarbon mixture ratio of 2.5:1 in the NAPL phase and an electron donor demand of 1 mol of H_2 to release 1 mol of chloride in reductive dechlorination, of the release of 63 mol of H_2 during the degradation of 1 kg of grease oil can explain the extensive

reductive dechlorination of cVOCs observed in the sediment (see the Supporting Information for details). Due to hydrocarbon dissolution and fermentation, the cVOC-to-petroleum hydrocarbon ratio will likely increase over time. In addition, H₂ is a preferred electron donor for other respiratory processes, including ferric iron reduction, sulfate reduction, and methanogenesis, and not all H₂ produced in petroleum hydrocarbon fermentation will be available for reductive dechlorination. Based on these assumptions, the amount of fermentable petroleum hydrocarbons will probably be insufficient to maintain a long-term H₂ flux to sustain reductive dechlorination of the cVOC mass residing in the fractures. Thus, lasting natural attenuation must rely on organic materials (e.g., plant debris) settling through the water column and fermentation processes occurring in the upper layers of the creek sediment. In contrast to the current situation, fermentation reactions will increase H₂ flux in the upper sediment horizons (i.e., the prediction is that inverted H₂ concentrations gradients will establish), suggesting that the zone of active reductive dechlorination may transition to shallower sediment zones. Such a scenario may interrupt the physical separation of stratified, incompatible reductive dechlorination processes and may result in contaminants reaching the water column. Although the current situation is stable and the infiltration of chlorinated solvents into the Third Creek water column does not occur, careful monitoring is warranted to ensure microbiological activity is sustained and chlorinated solvent concentrations remain below regulatory limits in the water column.

The detailed hydrological, geochemical, and microbial characterization of the Third Creek site attributed a critical role to the streambed sediment for contaminant attenuation. Phylogenetically distinct groups of OHRB coexist in the sediment, and stratification apparently effectively avoids cVOC toxicity and inhibition resulting in effective degradation of mixed contaminants. A major goal should be to define the environmental conditions that allow a perturbed (i.e., chlorinated solvent spills) system to adapt and sustain a microbial community that effectively detoxifies the contaminants. If the boundary conditions required for a natural attenuation system to emerge can be better-defined and become analytically tractable, monitored natural attenuation can possibly be successfully implemented at many more sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic overview of the Third Creek site (left panel). The solid colored circles show total measured bedrock groundwater cVOC concentrations. Seepage measurements and sediment collection for microbiological analyses occurred at location nos. 1, 2, and 3. The black line (B–B') in the left panel indicates the location of the transect, which is shown in the right panel. Simulated vertical groundwater gradients at the Third Creek site along transect B–B' (right panel). The arrows (unitless) indicate the direction and the relative magnitude of the groundwater flow direction. The vertical red lines in the right panel are permanent well installations near the (B–B') transect. The left and right panels were generated using ArcGIS version 9.1 (Esri, Redlands, CA) and Surfer 6 (Golden Software, LLC, Golden, CO), respectively.

Figure 2. cVOCs, ethene, ethane, and methane concentration profiles (panel A), and dechlorinator 16S rRNA gene and RDase gene abundances in different depths of the sediment at location no. 3. Panel B shows the average gene copies measured in sediment, and panel C shows the gene abundances associated with the Bio-Sep beads. The bars show the bacterial 16S rRNA gene (gray bars, *Bac*), *Dhc* 16S rRNA gene (red bars, *Dhc*), *Dehalobacter* 16S rRNA gene (green bars, *Dhb*), vinyl chloride RDase genes (purple bars, *vcrA*; blue bars, *bvcA*), and TCE RDase gene (yellow bars, *tceA*) abundances. Error bars represent the standard deviation of triplicate measurements. Double asterisks indicate that the gene was detected but was not quantifiable. The numerical data shown in panels B and C are provided in Table S8.

Figure 3.

Bacterial community structures in Third Creek sediment at location nos. 1, 2, and 3 at the class level based on 16S rRNA gene amplicon sequence analysis (V3–V5 region). “Other” refers to lower-abundance OTUs (Table S6b). The category ‘Other_Bacteria’ includes sequences that could not be classified at any taxonomic level. Sequences not classified at the class level are represented with their phylum (p) classification. Loc#1, Loc#2, and Loc#3 represent the location nos. 1, 2, and 3, shown in Figure 1.

Table 1.

Dechlorination Products in Sediment Microcosms and Bio-Sep Bead Enrichment Cultures^a

sediment microcosms	cVOC added and major degradation product(s)							
	PCE	TCE	cis-DCE	VC	TCA	DCA	1,2-D	CF
location no. 1	ethene ^f	ethene ^f	ethene ^f	ethene ^f	CA ^f	ND	propene ^f	CF ^d DCM ^g
location no. 2	ethene ^f	ethene ^f	ethene ^f	ethene ^f	CA ^f	ND	propene ^f	CF ^d DCM ^g
location no. 3	ethene ^f	ethene ^f	ethene ^f	ethene ^f	CA ^f	CA ^f	propene ^f	CF ^d DCM ^g
Bio-Sep bead-derived enrichment cultures, sampling depth (m)	PCE	TCE	cis-DCE	VC	TCA			
location no. 3, 0	cis-DCE, VC, ethene ^d	TCE, cis-DCE, VC, ethene ^e	ethene ^f	VC, ethene ^f	TCA ^f			
location no. 3, 0.07	VC, ethene ^f	ethene ^f	VC, ethene ^e	ethene ^f	TCA ^f			
location no. 3, 0.17	ethene ^f	cis-DCE, VC, ethene ^f	cis-DCE, VC, ethene ^d	VC, ethene ^f	TCA ^f			
location no. 3, 0.22	cis-DCE, VC, ethene ^d	cis-DCE, VC, ethene ^e	ethene ^f	ethene ^f	TCA ^f			
location no. 3, 0.30	cis-DCE, VC, ethene ^d	cis-DCE, VC, ethene ^e	ethene ^f	ethene ^f	TCA ^f			
location no. 3, 0.38	ethene ^f	ethene ^f	ethene ^f	ethene ^f	TCA ^f			
location no. 3, 0.45	cis-DCE, VC, ethene ^e	cis-DCE, VC, ethene ^e	cis-DCE, VC, ethene ^f	ethene ^f	TCA ^f			
location no. 3, 0.52	cis-DCE, VC, ethene ^d	ethene ^f	VC, ethene ^f	ethene ^f	TCA ^f			

^aPCE, tetrachloroethene; TCE, trichloroethene; cis-DCE, cis-1,2-dichloroethene; VC, vinyl chloride; TCA, 1,1,1-trichloroethane; DCA, 1,1-dichloroethane; 1,2-D, 1,2-dichloropropane; CF, carbon tetrachloride; CF, chloroform, DCM, dichloromethane.

^b<10%.

^c10–25%.

^d25–50%.

^e50–75%.

^f75–100% (of total mass/bottle); ND: not determined.

^gDCM was further degraded.