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RESEARCH ARTICLE

Natural Biodegradation of Vinyl Chloride and *cis*-Dichloroethene in Aerobic and Suboxic Conditions

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Abstract

Chlorinated ethene (CE) groundwater contamination is commonly treated through anaerobic biodegradation (i.e., reductive dechlorination) either as part of an engineered system or through natural attenuation. Aerobic biodegradation has also been recognized as a potentially significant pathway for the removal of the lower CEs *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC). However, the role of aerobic biodegradation under low oxygen conditions typical of contaminated groundwater is unclear. Bacteria capable of aerobic VC biodegradation appear to be common in the environment, while aerobic biodegradation of cDCE is less common and little is known regarding the organisms responsible. In this study, we investigate the role of aerobic cDCE and VC biodegradation in a mixed contaminant plume (including CEs, BTEX, and ketones) at Naval Air Station North Island, Installation Restoration Site 9. Sediment and groundwater collected from the plume source area, mid-plume, and shoreline were used to prepare microcosms under fully aerobic (8 mg/L dissolved oxygen (DO)) and suboxic (< 1 mg/L DO) conditions. In the shoreline microcosms, VC and cDCE were rapidly degraded under suboxic conditions (100% and 77% removal in < 62 days). In the suboxic VC microcosms, biodegradation was associated with a > 5 order of magnitude increase in the abundance of functional gene *etnE*, part of the aerobic VC utilization pathway. VC and cDCE were degraded more slowly under fully aerobic conditions (74% and 30% removal) in 110 days. High-throughput 16S rRNA and *etnE* sequencing suggest the presence of novel VC- and cDCE-degrading bacteria. These results suggest that natural aerobic biodegradation of cDCE and VC is occurring at the site and provide new evidence that low (< 1 mg/L) DO levels play a significant role in natural attenuation of cDCE and VC.

Keywords Aerobic · Vinyl chloride · Etheneotroph · Chlorinated solvents · Aquifer sediment

Introduction

Chlorinated solvents, including the chlorinated ethenes (CEs), tetrachloroethene (PCE), and trichloroethene (TCE), are some of the most frequently detected groundwater contaminants (Moran et al. 2007). Enhanced anaerobic bioremediation (EAB) using CE-dechlorinating bacteria (e.g., *Dehalococcoides mccartyii*) is widely used for treating CE contamination. Although complete dechlorination of CEs to ethene is possible using EAB, the process frequently stalls in the field, due to unfavorable conditions, such as a lack of electron donor or low pH, resulting in an accumulation of *cis*-1,2-dichloroethene (cDCE) or vinyl chloride (VC) (Blázquez-Pallí et al. 2019a, b; Choi et al. 2021; Cox 2012; Tiehm and Schmidt 2011). Because cDCE and VC are much more water soluble than higher CEs, they tend to form larger, more dilute plumes (e.g., 10–100 µg/L) than PCE and TCE and thus may migrate more rapidly away from the source area and escape anaerobic treatment.

Natural biodegradation, as part of monitored natural attenuation (MNA), a groundwater remediation strategy involving the characterization of natural mitigation

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mechanisms and subsequent monitoring (Wiedemeier et al. 1998), is an attractive option for treating these more diffuse plumes. Like EAB, MNA can rely on reductive dechlorination to fully reduce CEs to ethene, but this may not be useful if the plume extends beyond an anaerobic source area (Wiedemeier et al. 1998). Technical guidance from the USEPA indicates that reductive dechlorination is possible when the dissolved oxygen (DO) concentration is < 0.5 mg/L and oxidation reduction potential (ORP) is < 50 mV (Wiedemeier et al. 1998). Beyond this original guidance, molecular biological tools (MBTs) such as quantitative polymerase chain reaction (qPCR) or high-throughput 16S rRNA gene amplicon sequencing, have been widely used to assess reductive CE dechlorination processes in the field (Cupples 2008; Hendrickson et al. 2002; ITRC 2011; Krajmalnik-Brown et al. 2007; Loeffler and Edwards 2009). More recently, MBTs are increasingly integrated with stable isotope methods to provide multiple lines of evidence for anaerobic MNA of CEs (Blázquez-Pallí et al. 2019a; Murray et al. 2019).

Aerobic CE biodegradation pathways could be another important MNA mechanism. Aerobic biodegradation of VC and cDCE by either metabolic or cometabolic processes is well known under laboratory conditions (Atashgahi et al. 2013; Coleman et al. 2002b; Danko et al. 2004; Hartmans and De Bont 1992; Schmidt et al. 2010; Tiehm et al. 2008; Verce et al. 2000) and has been observed in the field (Bradley and Chapelle 1998; Wiedemeier and Wilson 1997). Several aerobic ethene- and VC-degrading bacteria, known as etheneotrophs, have been isolated (Coleman et al. 2006, 2002b; Danko et al. 2004; Hartmans and De Bont 1992; Verce et al. 2000). A few studies have demonstrated aerobic cDCE metabolism in mixed cultures (Bradley and Chapelle 2000; Schmidt et al. 2010; Tiehm et al. 2008), but it is not as common as with VC and only a single aerobic cDCE-utilizing organism has been isolated (Coleman et al. 2002a). The USEPA provides little guidance on the aerobic biodegradation of CEs, other than recognizing that it may play a role in MNA of VC when DO > 5 mg/L (Wiedemeier et al. 1998). More recent technical guidance suggests that aerobic biodegradation of cDCE and VC may occur when DO > 2 mg/L (Stroo et al. 2013), and recognizes the possibility of aerobic biodegradation at lower DO concentrations (Lebron et al. 2015), but still offers little guidance on conditions favoring aerobic biodegradation. This updated guidance still leaves a wide gap between conditions thought to be favorable for anaerobic dechlorination and aerobic biodegradation of CEs.

However, there is mounting evidence that aerobic biodegradation may play an important role in the CE attenuation under suboxic conditions (DO < 1 mg/L). There has been some recognition of the potential role of aerobic processes when DO is below the field standard for anaerobic conditions (0.1–0.5 mg/L) (Bradley 2011; Bradley and Chapelle 2011). Laboratory studies have shown that etheneotrophs can degrade VC under very low (≥ 0.02 mg/L) DO conditions (Coleman et al. 2002b) and aerobic VC oxidation has even been observed under apparently anaerobic conditions when a known low flux of oxygen was provided (Gossett 2010). More recently, genes involved in aerobic VC biodegradation have been found to be abundant under seemingly anaerobic conditions (Liang et al. 2017b; Richards et al. 2019) and a VC-utilizing strain was isolated from anaerobic groundwater (Fullerton et al. 2014). Certain bacteria, known as microaerophiles, are adapted to life under low oxygen conditions. Microaerophiles utilize molecular oxygen as a terminal electron acceptor, but either grow poorly or will not grow under normal atmospheric conditions (21% oxygen)(Krieg and Hoffman 1986). The role of microaerophiles in bioremediation is poorly understood, but the groundwater contaminants BTEX and 2,3,4,6-tetrachlorophenol have been shown to be biodegraded at similar rates under suboxic and fully aerobic conditions (Benedek et al. 2021; Männistö et al. 2001).

MBTs have also been developed for etheneotrophs (Jin and Mattes 2010). Two etheneotroph functional genes, *etnC* and *etnE*, and their transcripts have been used as biomarkers to determine the abundance and activity of etheneotrophs in soil and groundwater (Jin and Mattes 2010; Liang et al. 2017a, 2017b; Patterson et al. 2013; Richards et al. 2019). Both genes are part of the aerobic ethene and VC biodegradation pathway; *etnC* encodes the alpha subunit of an alkene monooxygenase which catalyzes the first step in aerobic ethene or VC biodegradation and *etnE* encodes an Epoxyalkane:Coenzyme M (CoM) transferase involved in shuttling ethene or VC-derived carbon to central metabolism (Coleman and Spain 2003). Carbon stable isotope analysis has also been used to detect VC isotopic enrichment associated with aerobic VC biodegradation in both laboratory (Chartrand et al. 2005) and field (Patterson et al. 2013) applications.

Despite these advances, the role of etheneotrophs in facilitating CE biodegradation in the field is still poorly understood. Linking MBTs to biodegradation is critical for expanding their use in bioremediation, particularly in MNA. This study has two primary goals. The first goal is to use etheneotroph MBTs in conjunction with a conventional microcosm study to evaluate the link between functional gene abundance and biodegradation potential. The second goal is to evaluate natural attenuation of cDCE and VC under both aerobic and suboxic conditions to help fill the gaps in the current technical guidance for CE biodegradation. To our knowledge, this is the first such study to combine in situ MBTs with microcosm studies to evaluate aerobic biodegradation of VC and cDCE. This is also the first study to use these techniques to evaluate natural attenuation of VC and cDCE under suboxic conditions.

Methods and Materials

Site description and field sampling

Naval Air Station North Island (NASNI) is an active military installation adjacent to San Diego Bay in California, USA. Installation Restoration Site 9, the "Site," is a mixed contaminant plume originating from four chemical waste disposal trenches that were used at NASNI between the 1940s and 1970s (Brown and Caldwell 1983)(Figure S1). Known contaminants include CEs (PCE and TCE), petroleum hydrocarbons (BTEX), and ketones (NAVFAC 2019a). Site investigations have not discovered dense non-aqueous phase liquid (DNAPL), but contaminant concentrations suggest that cryptic DNAPL is present (NAVFAC 2019a). Natural reductive dechlorination is occurring at the site, demonstrated by an accumulation of the daughter products cDCE and VC (NAVFAC 2019a). The plume extends from the source area to San Diego Bay (NAVFAC 2019a). The site geology is characterized by alternating layers of clean sand and less permeable silty sand and clay (Figure S2)(NAVFAC 2019b). Groundwater is shallow (approximately 4 ft bgs) and tidally influenced (NAVFAC 2019b).

Aquifer sediment cores used in the aerobic and suboxic microcosm studies consisted primarily of fine-to-medium grained sand and were collected using a hollow stem auger from three locations along the plume representing the source area, mid-plume, and shoreline. From each of these cores, samples were taken from layer 1 (L1) (12–21 ft below ground surface (bgs)) and layer 3 (L3) (35–41 ft bgs). Corresponding groundwater samples were collected from nearby monitoring wells via low flow sampling. Temperature, pH, conductivity, and dissolved oxygen were measured using a YSI Pro DDS handheld meter. VOCs were analyzed by EPA method 8260, and TOC was measured by SM5310b at EMAX Laboratories, Inc. (Torrance, CA). Nitrate and ferrous iron were not determined. Biomass samples were also collected in the field by filtering groundwater (up to 1 L) through 0.2-µm Sterivex filters (MilliporeSigma, Burlington, MA). The filters were shipped on ice and stored at -80 °C prior to processing.

Aerobic and suboxic microcosm setup and sampling

Twelve sets of microcosms were prepared representing three sampling locations (source area, mid-plume, and shoreline), two depths (layers L1 and L3), and two contaminants of interest (VC and cDCE) as shown in Table S1. Every set included triplicate live microcosms and killed controls. Each microcosm consisted of a 240-ml serum bottle sealed with a chlorobutyl septa containing approximately 30 g of sediment and 50 ml of groundwater supplemented with 50 ml of minimal salts media to provide pH buffering and trace nutrients (Coleman et al. 2002b). Filter-sterilized VC gas (> 99%, Synquest Laboratories, Alachua, FL) or 0.01 M cDCE (97%, Sigma-Aldrich, St. Louis, MO) in deionized water was added to initial aqueous concentrations of 100 µM. Sediment and groundwater used in the controls was autoclaved at 121 °C and 15 psig for 60 min in separate containers. Killed controls were amended with sodium azide (50 mM final concentration) (Sigma-Aldrich) to further suppress biological activity. Suboxic microcosms were prepared using the L3 sediment and groundwater in an anaerobic glove box under a nitrogen atmosphere. Filter-sterilized ultra-high-purity (UHP) oxygen (Praxair, Cedar Rapids, IA) was added to the suboxic microcosms by syringe for an initial DO concentration of 1 mg/L. Aerobic microcosms were prepared using the L1 sediment and groundwater under atmospheric air.

Microcosms were sampled every two weeks for 16 weeks. Gasses and cDCE were measured in the headspace as described below. Aqueous samples (2 ml) were collected and analyzed for pH, ORP, and anions. Additional 2 ml aqueous samples were collected at the initial and final sampling points for DNA to be used in qPCR and sequencing.

Permeation tube microcosms

Selected suboxic microcosms, which exhibited the highest aerobic biodegradation capacity in the suboxic study, were continued into a second experiment utilizing permeation tubes to provide a continuous slow flux of oxygen (details in SI). These permeation tubes consisted of a piece of low-density polyethylene (LDPE) tubing (5.5 cm long, 0.64 cm ID, 0.95 cm OD) (Freelin-Wade part #1 J-074–10, McMinnville, OR) sealed at the ends with #00 butyl rubber stoppers (Gossett 2010)(Figure S3). One end of each tube was sealed, and the tube was flushed with UPH oxygen using a gassing cannula with the remaining stopper loosely in place. The cannula was quickly withdrawn as the second stopper was pushed into place. The microcosms were momentarily opened under stream of UHP nitrogen, and two permeation tubes were added to each bottle. The microcosms were re-sealed and the headspace was flushed with UHP nitrogen for 5 min to remove any trace oxygen. Permeation tubes were assumed to be at atmospheric pressure, each containing a calculated 1.74 ml or 7.2×10^{-2} mmol of oxygen (0.15 mmol/bottle).

Chemical analyses

All gasses and VOCs were measured in headspace samples collected with gas-tight syringes (VICI Precision Sampling,

Baton Rouge, LA). Methane, ethene, and VC samples (100 µl) were analyzed using a gas chromatograph with a flame ionization detector (GC-FID) equipped with a 1% SP-1000 on CarboPack B column (60/80 mesh, 6 ft × 1/8 in dia.) with isothermal separation at 90 °C (Richards et al. 2019). Oxygen samples (200 µl) were analyzed using a GC with a thermal conductivity detector equipped (GC-TCD) with a 5A molecular sieve column (5 ft × 1/8 in dia.) at 70 °C (Richards et al. 2019)(detection limit 0.02 mg/L DO). cDCE samples (100 µl) were analyzed by GC with mass spectrometry detection (GC–MS) with a Restek Rtx-VMS column (30 m, 0.32 mm dia., 1 µm film). The column was held at 35 °C for 1 min and then heated to 180 °C at 30 °C/min. Dissolved gas and VOC concentrations were calculated by Henry's law using the coefficients listed in Table S2. Anions were filtered (0.2 µm) and quantified by ion chromatography using a AS18 column (Dionex, Sunnyvale, CA) and isocratic separation (35 mM KOH eluent) (Richards et al. 2019). pH was measured by EPA method 100.1 using a Thermo Orion 9107BN probe. ORP was measured by using FisherBrand Accumet platinum pin electrode.

Molecular biological tools

DNA was extracted from the field-sampled Sterivex filters using a Qiagen PowerWater Sterivex kit (Qiagen, Germantown, MD) following the manufacturer's instructions. DNA samples from the microcosms (2 ml sediment/water slurry) were extracted using the same kit, following a modified protocol (Richards and Mattes 2021). The concentrations of the etheneotroph functional genes *etnC*, *etnE*, and total bacterial 16S rRNA genes were determined by qPCR using SYBR Green chemistry as previously described (Liang et al. 2017b). The primer sets used in this study for PCR, qPCR, and sequencing are included in Table S3. Samples were analyzed using an Applied Biosystems QuantStudio 7 at the Iowa Institute for Human Genomics (IIHG). The quantification limit for both qPCR and reverse transcription (RT)-qPCR was 30 gene copies per well, which corresponded to a Ct of 26.2–26.5. Melting curve analysis was used to check qPCR product specificity. No amplification was observed in the no template controls for any of the target genes. Additional qPCR information, including qPCR quantification limits and quality control parameters, is provided in Table S4 per guidelines on the Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE)(Bustin et al. 2009).

Duplicate RNA samples (5 ml, sediment/water slurry) were collected from selected permeation tube microcosms and extracted using a RNeasy Powersoil Total RNA Kit according to the manufacturer's instructions. After the lysis step, luciferase mRNA (1 ng; GenBank accession No. X65316, Promega, Madison, WI) was added to the samples to serve as an internal standard (Mattes et al. 2015). Contaminating DNA was removed from the RNA with the TURBO DNA-Free Kit (Thermofisher Scientific, Waltham, MA). The RNA samples were purified with the Zymo Direct-zol RNA MiniPrep Plus kit (Zymo Research Corporation, Irvine, CA) with the addition of on-column DNase treatment. RNA concentrations were measured with the Qubit RNA high-sensitivity assay kit. To obtain cDNA for qPCR, reverse transcription was performed with the SuperScript IV First Strand Synthesis System (Invitrogen, Carlsbad, CA) using the random hexamer protocol. RNA quality and cDNA concentrations were measured with a NanoDrop 2000. Once RNA was converted to cDNA, the cDNA was used for RT-qPCR analysis as described above for qPCR. Any observed cDNA amplification with a determined Ct value greater than 26.2–26.5 was deemed detected, but not quantifiable.

A small-clone library was prepared with *etnE* sequences from the suboxic microcosms exhibiting VC biodegradation. Near full-length *etnE* genes were amplified by PCR using the primer pair Com-F1L/R2E. PCRs (25 µl) consisted of 12.5 µl Taq PCR Master Mix (Qiagen, Germantown, MD), 1 µl of template DNA, and 1 µM of each primer, and nuclease-free water. PCR products were evaluated by agarose gel electrophoresis and purified using a Qiagen gel clean-up kit. The purified products were cloned using an Invitrogen TOPO TA Cloning Kit with TOPO 2.1 vector and TOP10 *E. coli* (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. Plasmid DNA was harvested from clones using an Invitrogen Plasmid Miniprep kit and Sanger sequenced at IIHG using M13 F/R primers. The six-cloned *etnE* sequences were searched against the NCBI nr/nt database using BLASTn. The cloned sequences were aligned with BLAST hits in MegaX using MUSCLE, and the aligned sequences were used to prepare a neighbor joining tree demonstrating their phylogenetic relationship (Edgar 2004; Kumar et al. 2018).

Sequencing of 16S rRNA gene amplicons from selected microcosms exhibiting VC or cDCE degradation was performed at MR DNA (Shallowwater, TX) using Illumina Miseq (150 bp PE) and the 515F/806R primer set (Table S3). Sequences were analyzed using the MR DNA pipeline. Briefly, paired sequences were joined, and short or ambiguous sequences were removed. The remaining sequences were quality filtered, denoised, and assigned to an amplicon sequence variant (ASV)(Callahan et al. 2017). ASV taxonomy was determined by BLAST using a curated database.

The high-throughput partial 16S rRNA gene sequencing data generated in this study were deposited in the GenBank Sequence Read Archive under BioProject number PRJNA756259. The *etnE* genes retrieved from sediment samples are deposited under GenBank accession numbers OK574327-OK574335.

Results and Discussion

Groundwater chemistry and MBTs

Groundwater was sampled and analyzed from several wells to characterize the site shortly before collecting aquifer sediment and groundwater samples for the microcosm study. Oxygen and ORP were variable across the site. Only a single well was screened in L1 in the regions of the plume investigated in this study, located in the source area. Despite the shallow depth, there was little DO and the ORP was slightly reducing (-18 mV) (Table S5). L3 in the source area also had little or no DO, and the ORP was generally reducing (-18 to -210 mV). Sulfate was present in relatively low quantities compared to downgradient wells (1.8–590 mg/L), and methane concentrations were comparatively very high (960–15,000 µg/L). One well in L3, S9-MW-48, appeared slightly oxidizing (0.12 mg/L DO, + 61 mV ORP, 1500 mg/L sulfate). The reducing conditions seen in most samples are likely a result of microbial activity fueled by the high total organic carbon (TOC) and BTEX (particularly toluene) concentrations across much of the site (Table S6). Downgradient from the source area, groundwater reaeration should result in a shift to more oxidizing conditions. Wells in the mid-plume and shoreline also had low DO (< 1 mg/L) and negative ORP values (-31 to -190 mv), but a substantial increase in the sulfate (130–2300 mg/L in the mid-plume and shoreline) and decrease in methane concentrations (78–510 µg/L in the mid-plume and 2.8–45 µg/L in the shoreline) may indicate some reaeration, but it was insufficient to overcome the prevailing reducing conditions. Conductivity was variable, ranging from 4.3 to 63 mS/cm, and tended to be higher in L3 than L1 and increased from the source area to the shoreline. The high conductivity and sulfate concentration likely reflect seawater intrusion.

Groundwater samples had relatively little PCE (≤ 110 µg/L), but a greater amount of TCE was seen in some of the wells in both the source are and mid-plume (≤ 7600 µg/L). However, much greater concentrations of cDCE were present in all but one well. cDCE concentrations in the source area ranged from 8.8×10^3 to 5.5×10^5 µg/L. cDCE concentrations were drastically lower in the mid-plume and shoreline (1300–4800 µg/L), but still well above the maximum contaminant limit of 70 µg/L (USEPA 1991). VC concentrations were also very high in some wells in the source area and mid-plume, as much as 5.5×10^4 µg/L, but they attenuate as the plume approaches the shoreline (1.2–82 µg/L).

The typical conceptual model of a contaminant plume is of an anaerobic core in the source area with a smooth transition through a series of sharp redox gradients as the plume expands downgradient and more favorable electron acceptors become available. However, redox heterogeneity and non-equilibrium in the mid-plume and shoreline open the possibility that both aerobic and anaerobic processes may be occurring throughout a large portion of the plume. This heterogeneity is best illustrated by well S9-MW-48, which is in L3 and the source area. This well is surrounded by strongly reducing conditions, and yet several redox indicators show conditions favorable for aerobic biodegradation in this well. High-resolution characterization of other sites has also noted that heterogeneity can cause variable redox conditions throughout a contaminant plume (McMillan et al. 2018; Richards et al. 2019). Biomarkers for aerobic and anaerobic CE degrading bacteria have also been found to coexist at high concentrations in both sediment and groundwater (Liang et al. 2017b; Richards et al. 2019).

Overall bacteria were abundant in groundwater samples collected throughout the site (10^4–10^9 copies/L), as determined by total 16S rRNA gene abundance (Figure S4). Etheneotrophs, as measured by the functional genes *etnC* and *etnE*, were relatively abundant in one well in the source area (S9-MW-60-L3, 10^7 copies/L) and one well in the mid-plume (S9-MW-52, 10^6 copies/L) but were present in much lower concentrations (0–10^4 copies/L) in the remaining wells, including all of the shoreline wells.

The high abundance of etheneotroph functional genes in the source area, which is nominally anaerobic (i.e., DO is below detection; 0.1 mg/L for the field probe), is consistent with the finding of other studies (Liang et al. 2017b; Richards et al. 2019). Etheneotroph functional genes have been shown to be better correlated with VC concentrations than with DO or ORP, which may explain their abundance in the source area (Liang et al. 2017b).

Aerobic microcosms

Microcosms with sediment and groundwater collected from L1 were prepared under fully aerobic conditions (DO > 8 mg/L) because this shallow layer should receive oxygen flux from the overlying soil gas. The aerobic VC-fed source area and mid-plume microcosms showed little VC loss over the duration of this experiment (22% and 24% removal in the live microcosms vs. 12% and 16% in the controls, respectively) (Fig.1). The slight downward trend seen in both the live and control microcosms is the result of dilution as air was added into the bottles to compensate for the volume removed at each sampling event. The shoreline microcosms exhibited a slow decrease in VC concentrations with considerable variability between the replicates, averaging 74% VC removal after 110 days. No ethene was observed (data not shown), so this is likely a result of limited aerobic biodegradation. The cDCE-fed source area and

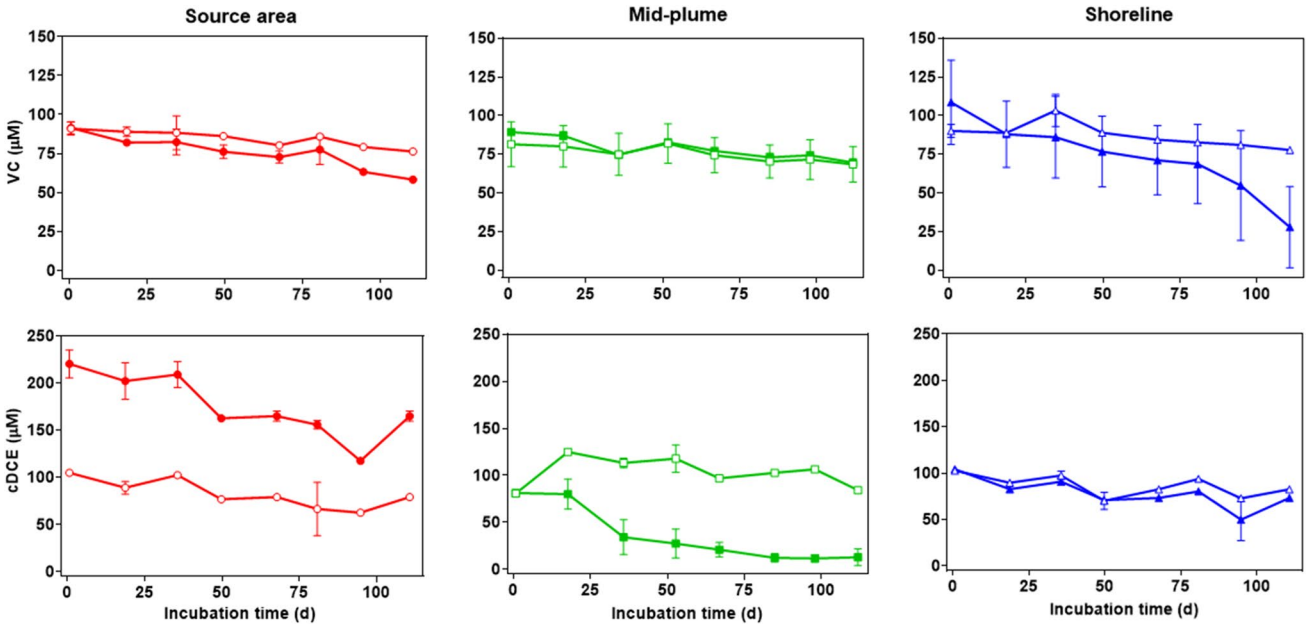

Fig. 1 VC and cDCE concentrations in the aerobic microcosms. Reported values are aqueous concentrations which were calculated from headspace measurements using Henry's law. Error bars represent the standard deviation of triplicate microcosms. Open symbols: killed controls, closed symbols: live

shoreline microcosms showed little change in cDCE concentrations. However, the mid-plume cultures showed a large (85%) decrease in cDCE concentrations. cDCE removal in the mid-plume cultures started rapidly at Day 18, but slowed drastically as the cDCE concentrations decreased, and complete removal was not achieved in the duration of this experiment. The shape of the curve and the incomplete removal of cDCE suggest that the cDCE removal was a cometabolic process. The organisms responsible and the co-substrate are unknown; however, BTEX and methane are both present at the site and are potential co-substrates for CE biodegradation (Alvarez and Illman 2005).

The lack of VC degradation in the source area and mid-plume aerobic microcosms was surprising, since etheneotrophs functional genes were relatively abundant in the groundwater samples collected from this region of the plume. The most likely explanation is preferential consumption of other natural organic matter or other contaminants present at the site (e.g., BTEX) (Table S6). This is clearly the case in the source area, which had very high oxygen demand resulting in oxygen depletion, and highly reducing conditions (Figure S5). The limited VC degradation seen in the aerobic shoreline microcosms may be attributed to a low starting concentration of etheneotrophs, below detection in the qPCR from the field samples, but also much lower amounts of competing electron donor. The lack of cDCE biodegradation in the source area and shoreline microcosms was less surprising. cDCE often is found to resist biodegradation under aerobic conditions, and only the mid-plume may have had the right combination of DO and co-substrate and the appropriate microbial community for cometabolism to occur.

Suboxic microcosms

VC and cDCE

Microcosms with sediment and groundwater from L3 were prepared under suboxic conditions (DO ≤ 1 mg/L) because this layer is believed to have limited reaeration resulting from the greater depth below the water table, and the presence of lower permeability sediments between L1 and L3. VC and cDCE concentrations decreased slightly in the suboxic source area and mid-plume microcosms (Fig.2). The decrease in the live bottles was greater than seen in the controls (27–46% removal in live microcosms vs. < 15% removal in controls), suggesting that it was due to limited biodegradation. The shoreline VC microcosms had little change in VC concentration for the first 43 days, but then fully consumed their VC by Day 62. VC was repeatedly added to these microcosms and then fully consumed, usually by the following sampling event. A total of 120 µmol of VC was consumed by each of these bottles. Like with the VC microcosms, the shoreline cDCE microcosms showed little activity for the first 62 days before degradation was evident. cDCE degradation was slower than VC, taking 73 days for the initial cDCE mass to be mostly consumed. A second spike of DCE was mostly consumed by the end

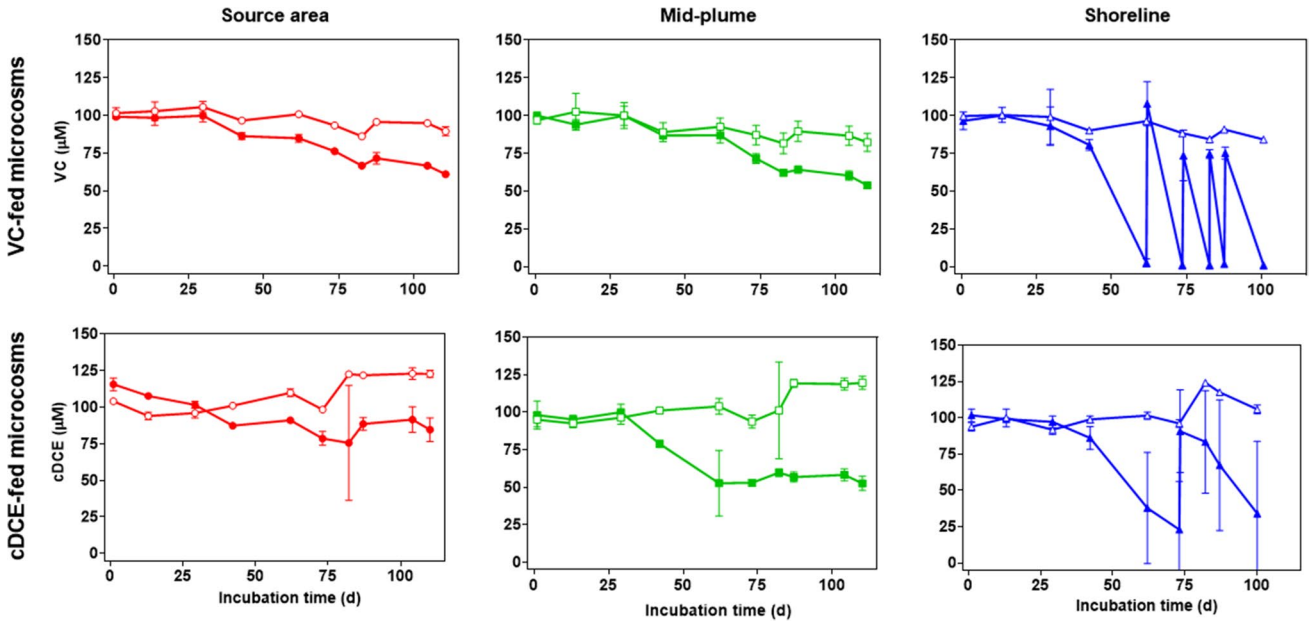

Fig. 2 VC and cDCE concentrations in the suboxic microcosms. Reported values are aqueous concentrations which were calculated from headspace measurements using Henry's law. Error bars represent the standard deviation of triplicate microcosms. Open symbols: killed controls, closed symbols: live

of the experiment (63% average removal). For reasons that are unclear, one of the three triplicate cDCE microcosms degraded cDCE much slower than the others, resulting in large error bars. Traces of VC appeared in the cDCE microcosms on Day 42 and peaked on Day 62, indicative of some reductive dechlorination (Figure S6). However, no ethene was observed, and the VC concentration returned to very low levels (< 6 mM) by Day 73.

The rapid onset of VC consumption in the shoreline microcosms was unexpected because groundwater qPCR did not detect etheneotrophs in the shoreline samples. The slow onset of VC biodegradation in the shoreline microcosms is likely a result of a very small initial etheneotroph population. Etheneotrophs may have also been distracted by other carbon sources initially present in the site material, as they are known to grow on other organic substrates. No ethene was observed in these microcosms, so the consumption of VC was not the result of reductive dechlorination. The aerobic biodegradation of VC under suboxic conditions observed here is consistent with previous work where VC oxidation was observed in microcosms subjected to very low DO conditions (Gossett 2010). A chloride balance was attempted to provide a second line of evidence for VC biodegradation, but high background chloride concentrations (approximately 200 mM, data not shown) made this impractical. Unlike what was seen in the aerobic mid-plume DCE microcosms, the rate of cDCE degradation in the suboxic shoreline bottles accelerated and cDCE was fully consumed in two of the three replicate bottles. This pattern is more suggestive of metabolic cDCE utilization than cometabolism. Reports of aerobic cDCE utilization are rare. There have only been a few reports of aerobic cDCE biodegradation in the absence of co-substrate (Bradley and Chapelle 2000; Schmidt et al. 2010), and only a single cDCE-utilizing bacteria have ever been isolated (Coleman et al. 2002a).

DO and redox indicators in the suboxic microcosms

The suboxic VC-fed control microcosms maintained a DO concentration of approximately 1 mg/L throughout the study (Figure S7), demonstrating that biological activity had been suppressed. All of the live VC-fed cultures had fully consumed their DO within the first day, indicating robust aerobic microbial activity. Oxygen was added to the bottles to return the DO to the target starting concentration of 1 mg/L. By the second sampling point on Day 14, not only had the oxygen been fully consumed, but ORP values had decreased from approximately + 200 mV to nearly -200 mV, sufficient for reductive dechlorination or even methanogenesis (Rittmann and McCarty 2012). Oxygen was repeatedly added and consumed by the subsequent sampling event. ORP remained steady around -200 mV between days 13 and 74, and sulfate concentrations did not drop. After Day 87, ORP values increased to ≥ 0 mV, possibly a result of electron donors becoming depleted. Nitrate was never detected in the suboxic microcosms. Sulfate concentrations were higher in the shoreline microcosms than in the source area or mid-plume, another indication of saltwater intrusion. There was

a noticeable decrease in the sulfate concentration in all of the live microcosms compared to their respective controls (Figure S8), and a black precipitate was observed in soils, both indicating microbial sulfate reduction. This likely occurred while the microcosms became fully anaerobic between sampling events. Sulfate consumption was highest in the first 30 days of the study, after which sulfate concentrations began to rebound, indicating reoxidation of sulfide. This agrees with the ORP data which reached highly reducing levels early in the study before rebounding. Methane concentrations were very low (< 2 µM) throughout the study (data not shown).

DO and ORP in the cDCE-fed suboxic cultures followed a similar trend to the VC-fed cultures. DO was fully consumed in the live cultures at every sampling event, and ORP values decreased from + 200 mV to less than -200 mV by Day 13. However, ORP values seemed to rebound more quickly in the mid-plume and shoreline cDCE cultures than the VC-fed cultures, with ORP values increased to 0 mV by Day 42 (mid-plume) or 87 (source area and shoreline). These microcosms were constructed with the same sediment and groundwater as the VC cultures and should have had approximately the same background oxygen demand. This difference is likely the result of the additional oxygen demand from the repeated VC additions.

The oxygen demand in the suboxic microcosms was higher than anticipated, particularly for the mid-plume and shoreline. Since oxygen consumption did not coincide with VC or cDCE degradation, this is a result of other organic matter present in the sediment and groundwater, whether it is natural organic matter or other contaminants (Table S6). The repeated addition and consumption of oxygen to the suboxic microcosms resulted in cycling and non-equilibrium redox conditions. Although inadvertent, this may mimic conditions in the field, at least for the mid-plume and shoreline samples where reduced chemicals may be transported into more oxidizing groundwater. The complete consumption of oxygen between sampling events makes it unclear how long the bottles were suboxic vs truly anaerobic, and gradients likely formed in the sediment. Another microcosm study found that such gradients may exist even with higher DO concentrations in the overlying water, allowing aerobic and anaerobic VC degradation to occur simultaneously (Atashgahi et al. 2013). The relatively long period between sampling (1–2 weeks) likely increased the length of time before the onset of VC oxidation and slowed the apparent rate. We think that a continuous flux of oxygen, even at very low concentration, would have resulted in higher rates of VC oxidation. Oxygen demand and/or total organic carbon concentrations should be considered when evaluating aerobic MNA potential. The limited data from this study suggest that VC and cDCE oxidation is inhibited when TOC is greater than about 10 mg/L, but further refinement of this number is needed.

Etheneotroph functional genes

The etheneotroph functional genes *etnC* and *etnE* increased in all the VC-fed suboxic microcosms regardless of measurable VC degradation (Fig. 3). The increase in functional gene abundance in the microcosms not exhibiting VC oxidation suggests that etheneotrophs are present but are growing on other carbon sources. The shoreline microcosms demonstrating rapid VC degradation had the highest final concentration

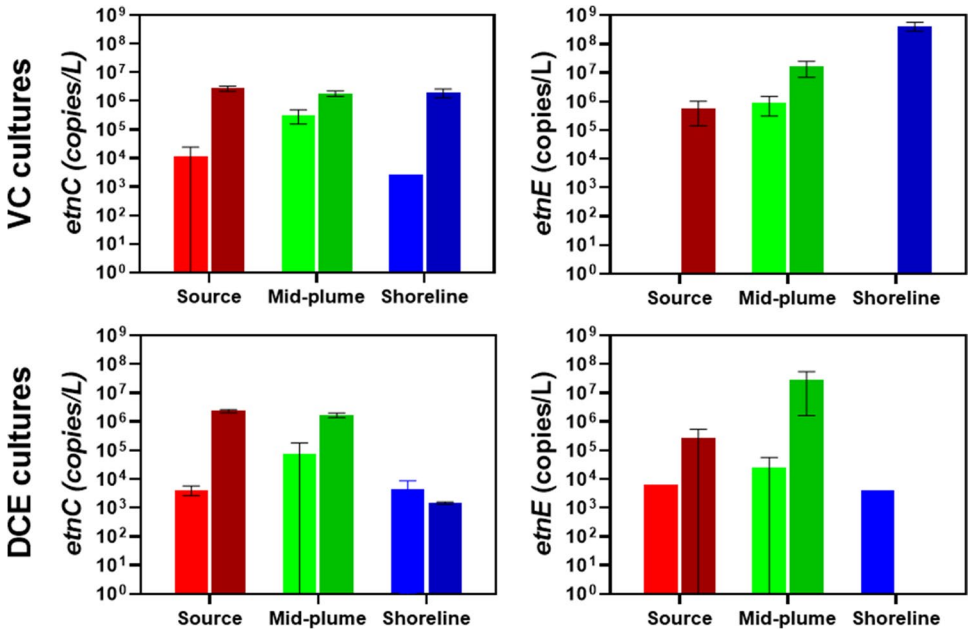

Fig. 3 Initial and final etheneotroph functional gene (*etnC* & *etnE*) concentrations in suboxic microcosms. Initial (left bar) and final (right bar) gene concentrations are shown for each condition. Error bars represent the standard deviation of triplicate samples

of *etnE* (> 10^8 copy/L), but a much lower concentration of *etnC* (10^6 copy/L). *etnC* and *etnE* are normally found at roughly a 1:1 ratio (Liang et al. 2017b; Mattes et al. 2015), but order of magnitude differences in their concentration in groundwater have been noted previously (Liang et al. 2017a). This reason for this discrepancy is unclear; however, the site geochemistry may offer a hint. Marine ethene-utilizing aerobic bacteria belonging to the genus *Haliea* have been isolated which possess *etnE*, but not *etnC*, instead relying on an unrelated particulate monooxygenase (Suzuki et al. 2009). *Haliea* sp. have not been shown to degrade VC, but the unexpectedly high amount of *etnE* in seawater-like groundwater may imply that unknown marine etheneotrophs are present.

The cDCE-fed microcosms also had an increase in both *etnC* and *etnE* concentration in the source and mid-plume conditions. As suggested for the VC microcosms, this indicates that etheneotrophs were growing on other substrates. The shoreline microcosms exhibiting cDCE biodegradation had no increase in etheneotroph functional genes. This suggests that etheneotrophs are not responsible for cDCE biodegradation, which was expected since they are only known to cometabolize cDCE. The aerobic biodegradation pathway for cDCE has only been partially elucidated in one organism, *Polaromonas JS666* (Nishino et al. 2013), and biomarkers specific to cDCE utilization are unavailable (Giddings et al. 2010).

In the microcosms, *etnE* abundance was associated with VC oxidation, but *etnC* abundance was not, suggesting that *etnE* is a more specific indicator of etheneotroph abundance. The discrepancy in *etnC* and *etnE* abundances in the active microcosms, and the lack of known VC degrading genera in the samples both point to the existence of unidentified VC oxidizing strains. It is intriguing that VC and cDCE degradation rates were highest in the shoreline L3 microcosms, in which the groundwater was very similar to seawater. A previous study conducted using offshore sediment from NAS North Island also demonstrated aerobic VC biodegradation in a marine system, but the microbial community was not investigated (Trevet and Geosyntec 2019). To our knowledge, this is the only study investigating aerobic VC biodegradation in a marine system. Marine systems are a largely unexplored source for CE biodegrading strains, which may be important for the remediation of coastal sites such as in this study.

A clone library was prepared with *etnE* sequences recovered from the shoreline suboxic VC microcosms, and a neighbor joining phylogenetic tree was constructed to relate these sequences to known etheneotrophs and clones from other CE-contaminated sites (Figure S9). Most of the clones from the shoreline microcosms were closely related to *Mycobacterium* sp. strain JS619 and *Mycobacterium* JS625, both known VC oxidizers. Two clones from the MW-73 bottles (clones C1 and C13) were distantly related to the other recovered sequences and clustered most closely with the VC-utilizing *Nocardioides* strains JS614 and XL1 (Coleman et al. 2002b; Liu and Mattes 2016). These genes appear to be truncated compared with other sequences, and it is not clear whether they are functional. These clones are of interest because only two VC-utilizing *Nocardioides* strains have ever been isolated, and that although it is most closely related to these two sequences, it shares relatively little sequence similarity with them (75%) and therefore may represent a novel lineage of *etnE* sequences.

16S rRNA gene amplicon sequencing

The microbial communities in the VC and DCE degrading cultures were characterized through 16S rRNA gene amplicon sequencing to identify taxa potentially responsible for biodegradation. Because 16S rRNA gene amplicon sequencing provides a measure of the relative abundance of different taxonomic groups in the sample, a relative increase in the abundance of a particular taxon over time may implicate them in the VC or DCE degradation. However, in these microcosms it is clear from the high oxygen demand that VC and DCE oxidation were not the dominant microbial processes, so any enrichment in VC or DCE degrading organisms was expected to be subtle. Genera associated with VC biodegradation may comprise < 1% of the microbial community at CE-contaminated sites (Liang et al. 2017a; Mattes et al. 2015).

Samples collected from the VC microcosms at the onset of the experiment were used as controls. The same sediment and groundwater used to prepare the VC and DCE microcosms, so these samples should approximate the original microbial community. The control samples were diverse and included major genera such as *Pseudomonas* (7.0%), *Thiobacillus* (4.5%), *Hydrogenophaga* (18%), *Acidovorax* (22%), *Aquabacterium* (19%), *Streptococcus* (9.2%), and *Porticoccus* (7.0%), as well as many minor taxa (Fig. 4). There was a considerable loss in diversity between the controls and either the VC or DCE-fed microcosms, likely a result of inadvertent selective pressures resulting from laboratory cultivation. Both the VC- and DCE-amended microcosms became dominated by the genera *Pseudomonas* and *Thiobacillus*, which represented 55% and 29% of the VC cultures and 53% and 22% of the DCE cultures, respectively. Pseudomonads are metabolically flexible, often able to grow on a variety of substrates, and are able to use alternative electron acceptors, including sulfate (Brock et al. 2006). Thiobacilli are chemoautotrophs which grow on reduced sulfur compounds and are mostly obligate aerobes (Brock et al. 2006). The rise in Thiobacilli may be a result of cycling redox conditions.

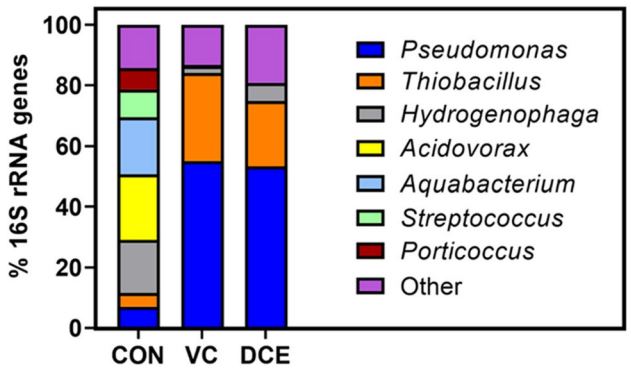

Fig. 4 Relative abundance of major genera determined by 16S rRNA gene amplicon sequencing. Controls (CON) represent the relative abundance of initial community members in the shoreline suboxic microcosms. VC and DCE samples represent the relative abundance of community members after 16 weeks

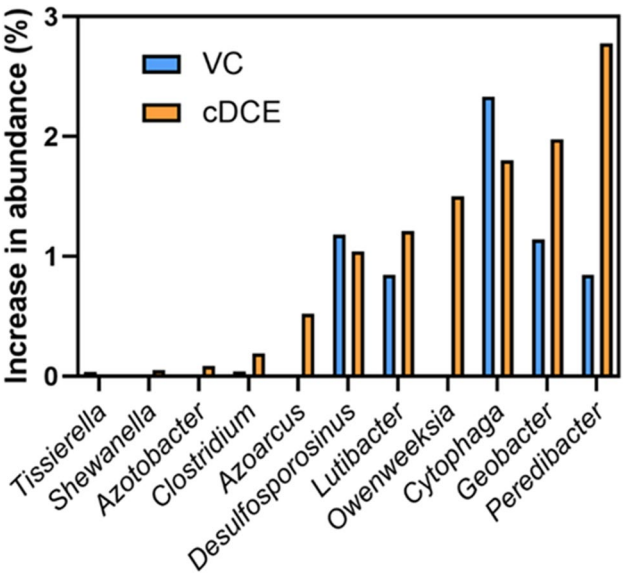

Fig. 5 Absolute increase in abundance of minor genera in suboxic VC and cDCE microcosms as determined by 16S rRNA gene amplicon sequencing. The increase in minor genera was determined by the difference in relative abundance in the control samples from the relative abundance in either VC- or cDCE-utilizing microcosms

Reduced sulfur compounds were formed under anaerobic conditions which could then be utilized when oxygen became available.

The genera typically associated with VC oxidation, *Mycobacteria* or *Nocardioides* (Coleman et al. 2006, 2002b), were completely absent from the VC-fed microcosms, and *Nocardioiodes* comprised only a very small part of the community from the DCE-fed microcosms (0.0011%). Strains of *Pseudomonas* have been shown to oxidize VC (Danko et al. 2006; Verce et al. 2001), but it is unclear whether they are involved in VC oxidation in these microcosms. The similar abundance of *Pseudomonas* in the VC and DCE cultures would suggest that they are utilizing other substrates present in the site material, but this does not preclude them from utilizing VC as well. *Haliea*, a genus which includes the only known marine etheneotrophs (Suzuki et al. 2009), was also absent.

The absence of *Mycobacteria* was unexpected considering the *Mycobacterium*-like *etnE* sequences in the clone library. However, the etheneotroph functional genes are carried on linear plasmids in several characterized strains, and horizontal transfer of these genes has been suggested (Danko et al. 2004; Mattes et al. 2010). These microcosms likely contain previously uncultured etheneotrophs.

Identifying the organisms responsible for cDCE degradation is even more challenging than for the VC. To our knowledge, this is the first study to perform 16S rRNA gene amplicon sequencing on an aerobic cDCE degrading microcosm. Only a single aerobic cDCE-utilizing organism has ever been isolated, *Polaromonas* JS666 (Coleman et al. 2002a), and no members of the *Polaromonas* were identified in these microcosms. However, several minor genera were greatly overrepresented in the DCE culture compared to the VC cultures or the controls (Fig.5). *Owenweeksia* comprised 1.5% of the DCE culture, more than 4.5 times more abundant than in the VC-fed microcosms and was below detection in the controls.

The genus *Owenweeksia* is poorly characterized, with only a single representative species, *Owenweeksia hongkongensis* (Lau et al. 2005; Riedel et al. 2012). The genome of *O. hongkongensis* has been sequenced and found to contain a dioxygenase and haloacid dehalogenases which could be involved in biodegradation of recalcitrant compounds (Riedel et al. 2012). Other enriched genera include *Azoarcus* (0.5% increase), *Azotobacter* (0.090% increase), *Clostridium* (0.19% increase), *Shewanella* (0.052% increase). The reductive dechlorinating bacteria *Dehalogenimonas* were present in the controls at a low level (0.17%) and were enriched in the cDCE microcosms (0.29%), which may explain the transient formation of VC in these bottles.

Nominally anaerobic microcosms

The suboxic shoreline microcosms demonstrated that VC and cDCE could be degraded with relatively low DO concentrations (ca. 1 mg/L). However, the presence of other carbon sources and the complete consumption of oxygen between sampling points made it difficult to determine the minimum oxygen threshold for aerobic VC and cDCE biodegradation. To estimate the minimum oxygen concentration for VC and cDCE biodegradation, the suboxic shoreline microcosms were used in a follow-up experiment in which permeation tubes were used to provide a continuous low flux of oxygen while maintaining nominally anaerobic

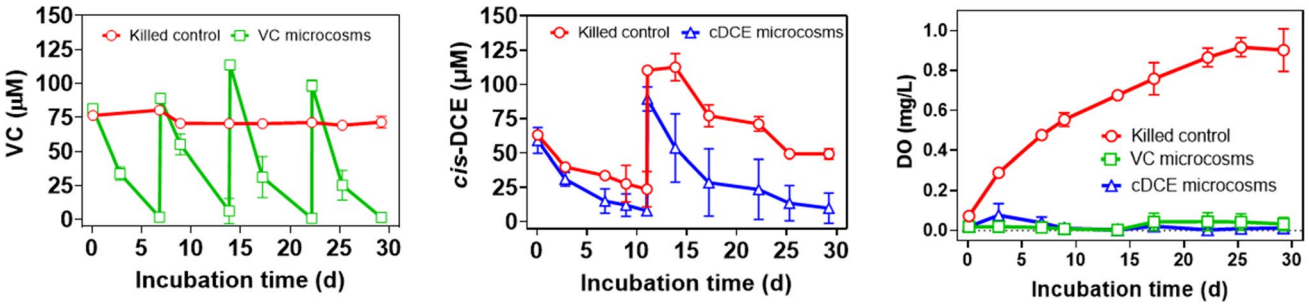

Fig. 6 VC, cDCE, and DO concentrations in microcosms with oxygen permeation tubes. Reported values are aqueous concentrations calculated from headspace measurements using Henry's law. Error bars represent the standard deviation of measurements from triplicate microcosms

conditions, i.e., the DO would be lower than the typical detection limit of a field DO probe (< 0.1 mg/L).

All the microcosms had initial DO concentration below detection, as planned (Fig. 6). The abiotic controls had a steady accumulation of DO due to oxygen flux out of the permeation tubes. DO concentrations continued to increase for about 30 days, as expected based on pretests. The final DO concentration was approximately 1 mg/L.

In the VC-fed microcosms, DO concentration remained below detection until Day 17, before rising slightly to about 0.04 mg/L, where it remained for the duration of the experiment. In the cDCE microcosms, there was a small spike in DO concentration on days 3–6, but otherwise followed a trend like the VC-fed microcosms; DO remained below detection until day 25 and then rose to 0.01 mg/L.

Despite the apparent absence of DO, the VC-fed microcosms began degrading VC immediately. By Day 7, all the initial VC had been consumed and additional VC was added. Robust VC degradation continued through the remainder of the experiment, and additional VC was added three times.

The initial cDCE concentration appeared lower than expected, and concentrations in both the controls and the live microcosms declined in a first-order manner for the first 11 days. The shape of the curve and the decrease in cDCE concentration in the abiotic controls suggest that cDCE was adsorbing to the permeation tube. There was a greater decrease in the cDCE concentration in the live bottles (86% removal) than in the controls (63% removal), which suggests that some biodegradation had occurred. Additional cDCE was added, and there was a continued decrease in the cDCE concentration in both the live bottles and the abiotic controls for the duration of the experiment; however, there was greater removal in the live microcosms than the controls (90% vs 55%), indicating continuing biodegradation. The minimum DO concentration for aerobic biodegradation of VC and cDCE was ≤ 0.02 mg/L DO.

The aerobic biodegradation of VC under seemingly anaerobic conditions is consistent with a previous study which used a nearly identical permeation tube system (Gossett 2010). However, the microorganisms responsible for low DO VC biodegradation were not investigated in that study. Here, the qPCR results demonstrate the presence of etheneotrophs, and that growth occurred under suboxic conditions. The duration of the permeation tube experiment was too short to expect significant additional growth. Instead, reverse transcription (RT)-qPCR was used to evaluate etheneotroph activity through the presence of functional gene transcripts. RT-qPCR, which directly measures gene expression, may be more useful for assessing MNA. A small number of studies have used gene transcripts, measured by RT-qPCR, as a measure of etheneotroph activity in groundwater, but to our knowledge it has not been used to evaluate MNA (Liang et al. 2017a, 2017b; Mattes et al. 2015). Directly measuring the activity of etheneotrophs has distinct advantages. RT-qPCR can demonstrate VC-oxidizing activity even if geochemical data (i.e., DO) are ambiguous. RT-qPCR, when done in conjunction with regular qPCR, can specifically demonstrate that etheneotrophs are utilizing VC, not dormant, or utilizing other electron donors (Mattes et al. 2015).

DNA and RNA were extracted from the microcosms on day 15, while oxygen was still being delivered by the permeation tubes. RNA was reverse transcribed to cDNA, and RT-qPCR was performed. Recovery of luciferase rRNA transcripts indicated that RT was successful. Inspection of the melt curves from cDNA amplification was consistent with the melt curves from DNA amplification, which suggests that *etnE* amplification in both cases was specific. Both *etnE* genes and transcripts (in the form of cDNA) were detected in the DNA and cDNA obtained from the suboxic VC microcosms, respectively (Table S7). There was no amplification of *etnE* observed in the RNA extracts prior to RT. Although the *etnE* transcript abundance was below the quantification limit and thus was too low to accurately quantify, this experiment provides qualitative evidence that *etnE* was expressed and that etheneotrophs were aerobically degrading VC under the apparently anaerobic conditions in the bottles. RT-qPCR likely requires larger sample sizes to accurately quantify

gene activity and might not always be practical in microcosm studies.

Conclusions

This microcosm study proved useful in demonstrating aerobic VC and cDCE biodegradation as mechanisms for MNA under aerobic and suboxic conditions. Combining the microcosm study with MBTs clearly demonstrated that etheneotrophs were responsible for VC biodegradation even under nominally anaerobic conditions, but not cDCE biodegradation. This adds to the small number of studies demonstrating that aerobic cDCE biodegradation is a possible mechanism for natural attenuation.

Aerobic biodegradation is thought to be more important near the water table, where oxygen diffusion is highest. The finding that VC and cDCE biodegradation was greater under suboxic than aerobic conditions in the shoreline microcosms suggests that this may not be true. Bacteria involved in VC and cDCE biodegradation in these microcosms may be adapted to low oxygen conditions. Although molecular oxygen is necessary for VC oxidation, neither DO nor ORP was reliable indicators of this process. Both VC and cDCE oxidation occurred under conditions that were apparently anaerobic even to sensitive laboratory analysis, let alone less sensitive field instruments, which often have poor accuracy at low DO concentrations (< 1 mg/L). Better indicators of trace DO are needed. Microcosm studies screening for aerobic biodegradation should be performed under in situ redox conditions, and a full suite of redox analyses should be performed including measurements of dissolved iron and manganese.

Field sampling of the etheneotroph functional genes *etnC* and *etnE* was poor predictors of VC or cDCE biodegradation in the microcosms. This is likely a result of confounding factors, such as high TOC concentrations. The lack of biomarkers for aerobic cDCE biodegradation is a major limitation for assessing MNA. Although aerobic cDCE biodegradation is seemingly rare, it could be a valuable tool for MNA under the right circumstances.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-19755-1.

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Authors' contributions **Patrick M. Richards** helped in conceptualization, methodology, investigation, visualization, writing—original draft preparation, writing—review and editing; **Jessica M. Ewald** contributed to investigation and methodology; **Weilun Zhao** investigated the study; **Heather Rectanus and Dimin Fan** performed conceptualization, methodology, project administration, writing—reviewing and editing; **Neal Durant and Michael Pound** were involved in conceptualization, funding acquisition, writing—reviewing and editing; **Timothy E. Mattes** helped in conceptualization, funding acquisition, methodology, project administration, resources, data curation, supervision, visualization, writing—reviewing and editing;

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Data Availability The high-throughput partial 16S rRNA gene sequencing data generated in this study were deposited in the GenBank Sequence Read Archive under BioProject number PRJNA756259. The *etnE* genes retrieved from sediment samples are deposited under GenBank accession numbers OK574327-OK574335. All other data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate not applicable.

Consent for publication not applicable.

Competing interests The authors declare that they have no competing interests.

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