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Elucidating carbon uptake from vinyl chloride using stable isotope probing and Illumina sequencing

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Abstract Vinyl chloride (VC), a known human carcinogen, is a common and persistent groundwater pollutant at many chlorinated solvent contaminated sites. The remediation of such sites is challenging because of the lack of knowledge on the microorganisms responsible for in situ VC degradation. To address this, the microorganisms involved in carbon assimilation from VC were investigated in a culture enriched from contaminated site groundwater using stable isotope probing (SIP) and high-throughput sequencing. The mixed culture was added to aerobic media, and these were amended with labeled (^{13}C-VC) or unlabeled VC (^{12}C-VC). The cultures were sacrificed on days 15, 32, and 45 for DNA extraction. DNA extracts and SIP ultracentrifugation fractions were subject to sequencing as well as quantitative PCR (qPCR) for a functional gene linked to VC-assimilation (*etnE*). The gene *etnE* encodes for epoxyalkane coenzyme M transferase, a critical enzyme in the pathway for VC degradation. The relative abundance of phylotypes was compared across ultracentrifugation fractions obtained from the ^{13}C-VC- and ^{12}C-VC-amended cultures. Four phylotypes were more abundant in the heavy fractions (those of greater buoyant density) from the ^{13}C-VC-amended cultures compared to those from the ^{12}C-VC-amended cultures, including *Nocardioides*, *Brevundimonas*, *Tissierella*, and *Rhodoferax*. Therefore, both a previously identified VC-assimilating genus (*Nocardioides*) and novel microorganisms were responsible for carbon uptake. Enrichment of *etnE* with time was observed in the heavy fractions, and *etnE* sequences illustrated that VC-assimilators harbor similar *Nocardioides*-like *etnE*. This research provides novel data on the microorganisms able to assimilate carbon from VC.

Keywords SIP · Vinyl chloride · *etnE* · *Nocardioides* · *Brevundimonas* · *Tissierella* · *Rhodoferax*

Introduction

Groundwater contamination by vinyl chloride (VC) remains an important environmental problem and human health concern worldwide. VC is a known human carcinogen (Bucher et al. 2005) and has been found at many US Environmental Protection Agency National Priority List sites in soils, surface water, and groundwater. VC pollution in groundwater originates primarily from the higher-chlorinated ethenes including tetrachloroethene (PCE) and trichloroethene (TCE) (Bradley 2003). Leakage of these chemicals to aquifers, followed by their reduction by certain anaerobic bacteria (e.g., *Dehalococcoides* spp.), yields the lesser chlorinated ethenes, *cis*-1,2-dichloroethene and VC. These secondary pollutants are often more problematic than the parent compounds because they tend to accumulate.

Biological degradation is becoming an increasingly common remediation method for groundwater contaminants, either through natural attenuation or enhanced bioremediation. Microbial VC degradation can occur under both anaerobic and aerobic conditions and by co-metabolism or direct VC-assimilation (Mattes et al. 2010). However, linking specific

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microorganisms and enzymes to in situ activities remains a major challenge. Several bacteria belonging to the phyla *Actinobacteria* and *Proteobacteria* are capable of direct aerobic VC consumption and have been isolated from various environments or have been obtained in the laboratory (Coleman et al. 2002b; Danko et al. 2004; Elango et al. 2006; Fathepure et al. 2005; Hartmans and Debont 1992; Hartmans et al. 1985; Jin et al. 2010; Jin and Mattes 2008; Taylor et al. 2007; Verce et al. 2000). While these organisms have greatly contributed to our understanding of VC metabolism, the limitations associated with culture-based methods has likely resulted in an incomplete understanding of VC-assimilating microorganisms at field sites.

To overcome the limitations associated with culture-based methods, molecular testing during groundwater monitoring has become popular. For example, a quantitative PCR (qPCR) assay has been developed (Jin and Mattes 2010, 2011) and applied (Atashgahi et al. 2013; Jin et al. 2010; Patterson et al. 2013) to detect VC-oxidizing bacteria at contaminated sites. This qPCR method targets the functional genes *etnC* and *etnE*, which encode the alkene monooxygenase (AkMO) alpha subunit and the epoxyalkane coenzyme M transferase (EaCoMT), respectively. In VC-assimilators, AkMO is responsible for the initial attack on VC to convert it to VC epoxide. The epoxide is then conjugated to CoM by EaCoMT, which is a critical step to the central metabolic pathway. Therefore, presence of *etnE* gene indicates the potential for VC-assimilation.

To understand the diversity of mixed cultures and associated functional genes, molecular methods can often more accurately target the key active microorganisms. The stable isotope probing (SIP) method is valuable because it can identify the active microorganisms responsible for carbon or nitrogen uptake from the amended substrates (Radajewski et al. 2000). The method involves sample exposure to a stable-isotope-labeled compound and DNA extraction over time. The DNA is then subject to ultracentrifugation, fractionation (to separate label incorporated DNA from the unlabeled DNA), and community analysis (Dumont and Murrell 2005; Lueders et al. 2004; Luo et al. 2009; Madsen 2006; Singleton et al. 2005; Sun et al. 2010; Sun and Cupples 2012).

The overall objective was to determine the dominant microorganisms involved in carbon uptake from VC from a mixed culture derived from contaminated site groundwater. For this, SIP fractions were subjected to high-throughput sequencing. The relative abundance of phylotypes in fractions obtained from ^{13}C-VC- and ^{12}C-VC-amended mixed cultures were compared. The phylotypes enriched in the fractions from the ^{13}C-VC-amended cultures compared to the fractions from the ^{12}C-VC-amended cultures are considered responsible for incorporating ^{13}C from VC (or VC degradation products). This is the first study to combine SIP and high-throughput sequencing to examine carbon uptake from VC. Along with the information provided by qPCR and clone library analysis of *etnE*, this study contributes to our understanding of VC degradation in mixed communities and at contaminated sites.

Materials and methods

Site information and groundwater collection

Groundwater was collected in accordance with USEPA/540/S-95/504 on June 9, 2009 from several monitoring wells (RB46D, RB73, RB52I, RB60, RB64I, RB63I, and RB58I) at a site in Carver, MA. The site was contaminated by disposal of material containing PCE at a landfill in 1986, which ultimately resulted in a large, dilute VC plume. Remediation efforts for the plume have involved oxygen and ethene injections and have been described previously (Begley et al. 2009, 2012; Chuang et al. 2010; Jin and Mattes 2010). The groundwater from these wells was composited; 1 L was shipped to the University of Iowa, where it was stored at 4 °C in the dark.

Development of VC-degrading enrichment cultures

Triplicate VC enrichment cultures were constructed in August 2009 by mixing composite groundwater with sterile minimal salts medium (MSM; prepared as described previously (Coleman et al. 2002b)) (1:1), placing 72 mL of the mixture into a 160-mL serum bottle (Wheaton, Millville, NJ), sealing with a butyl rubber stopper and aluminum crimp cap. Approximately 40 μmol VC (99.5 % from Fluka) was added and the bottles were monitored by gas chromatography with flame ionization detection (GC-FID) as described previously (Mattes et al. 2005). VC oxidation commenced after a 63-day lag period. Live culture samples were sent to MSU in January 2010 for the SIP experiment described below.

Analytical methods

Headspace samples of VC (100 μL) were analyzed via gas chromatography (Perkin Elmer) with flame ionization detection and a capillary column (J&W Scientific, DB-624, diameter 0.53 mm). The peak areas were compared to an external standard for VC quantification. Aqueous phase VC concentrations were calculated using a previously reported Henry's law constant (1.069) (Gossett 1987).

SIP experimental design, DNA extraction, ultracentrifugation, and fractionation

The SIP experimental setup consisted of sterile serum bottles (160 mL), mixed culture (5 mL), minimal salts medium (67 mL MSM) (Hartmans et al. 1985), and VC (~47 mg L^{-1} or 120 μmol) (the initial liquid and headspace volumes were

72 and 88 mL). These microcosms were prepared as previously described (Coleman et al. 2002a). Based on the calculated amount of oxygen required for VC oxidation, and on previous VC studies (Coleman et al. 2002b), oxygen limitation was not expected under these conditions. Three abiotic microcosms (controls, obtained via autoclaving) and nine live microcosms were amended with unlabeled VC (hereafter referred to as ^{12}C-VC) (99 %, Specialty Gases of America) (to control for heavy GC microorganisms). An additional nine live microcosms were amended with labeled VC (hereafter referred to as ^{13}C-VC) ($^{13}C_2$ VC, 99 %, Cambridge Isotope Laboratories). The microcosms were sealed, protected from light, and incubated at room temperature (21–23 °C), with shaking (200–300 rpm). VC concentrations were monitored over 45 days and DNA was extracted (from ^{13}C-VC-amended and ^{12}C-VC-amended microcosms) at three time points during the experimental period (days 15, 32, and 45).

For DNA extraction, at each time point, the entire volume from each mixed culture bottle was centrifuged. Following this, the pellet was washed with MSM and the sample was centrifuged again. The pellet was then resuspended in Tris-EDTA (TE) buffer and DNA was extracted using the Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's procedure. Quantified DNA extracts (~10 μg) were loaded into Quick-Seal polyallomer tubes (13 by 51 mm, 5.1 mL; Beckman Coulter (Brea, CA) along with a Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8)-CsCl solution for ultracentrifugation. Prior to sealing (cordless Quick-Seal tube topper; Beckman), the density was determined with a model AR200 digital refractometer (Leica Microsystems Inc., Buffalo Grove, IL) and adjusted by adding small volumes of CsCl solution or TE buffer with a final value of 1.730 g mL^{-1}. The tubes were ultracentrifuged at 178,000×*g* (20 °C) for 48 h in a StepSaver 70 V6 vertical titanium rotor (8 by 5.1 mL capacity) within a Sorvall WX 80 Ultra Series centrifuge (Thermo Scientific, Waltham, MA). Following ultracentrifugation, the tubes were placed onto a fraction recovery system (Beckman Coulter), and fractions (~20, 150 μL) were collected. The buoyant density of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation. The DNA concentration in each fraction was quantified using the Qubit assay (Quant-iT™ dsDNA High-Sensitivity Assay Kit using the Qubit® 2.0 Fluorometer). The abundance of *etnE* was determined in ultracentrifugation fractions using qPCR. For this, for each time point (days 15, 32, and 45), fractions from one ^{12}C-VC-amended culture and one ^{13}C-VC-amended culture were investigated. In addition, for days 32 and 45, fractions from one ^{13}C-VC-amended culture and fractions from one ^{12}C-VC-amended culture were subjected to Illumina sequencing.

MiSEQ Illumina sequencing and SIP fraction analysis

In all, 94 samples were subjected to high-throughput sequencing (MiSEQ Illumina Sequencing at Michigan State University's Research Technology and Support Facility, RTSF). This included four DNA extracts (before ultracentrifugation) from the ^{12}C-VC- and ^{13}C-VC-amended cultures at day 32 and at day 45. Ninety additional samples were submitted for Illumina sequencing, and these included thirty individual SIP fractions (following ultracentrifugation) sequenced in triplicate. This involved eight fractions from each of the ^{13}C-VC- and ^{12}C-VC-amended cultures at day 32 (8 fractions×2 treatments×triplicates=48). Seven fractions from each of the ^{13}C-VC- and ^{12}C-VC-amended bottles from day 45 were also sequenced (7 fractions×2 treatments×triplicates=42).

The fractions were sequenced to determine which organisms were enriched in the heavy fractions and were therefore responsible for VC-assimilation. The fractions were selected based on their buoyant density in comparison to previous SIP studies which illustrated label uptake in fractions with buoyant density values ranging from 1.74 to 1.77 g mL^{-1} (Sun et al. 2012; Sun and Cupples 2012). In the current study, the fractions selected for sequencing ranged from 1.74 to 1.785 (day 32) and 1.744 to 1.797 g mL^{-1} (day 45).

PCR and Illumina sequencing were performed at RTSF using a previously reported protocol (Caporaso et al. 2011). Briefly, this involved the amplification of the V4 region of the 16S ribosomal RNA (16S rRNA) gene using a set of multiplex indexed primers. Following amplification, individual reactions were quantified (Picogreen assay), a pool of equimolar amounts of each was made, and these were purified using Ampure XP beads. A final gel purification step was included to ensure that non-specific products were eliminated. The combined library was loaded onto the Illumina MiSEQ Platform using a standard MiSEQ paired end (2×250 bp) flow cell and reagent cartridge.

Sequencing data obtained from the MiSEQ platform Laboratory Information Management System were analyzed using Mothur v.1.33.2 (Schloss 2009) using the MiSEQ standard operating procedure (Kozich et al. 2013). The sequence data in the fastq format were processed to remove the barcodes, and these were then aligned to form contiguous sequences. The data were checked for sequencing errors (removing ambiguous bases) and read length (275 bp). The sequences were then aligned to the SILVA database (SILVA version 119) (Pruesse et al. 2007). Additional steps included setting the maximum homopolymer length to 8, checking for chimeras using UCHIME (Edgar et al. 2011), classifying with the Bayesian classifier, and removing unwanted lineages. The sequences were clustered into OTU using a 0.03 cutoff level. Mothur was also used to generate information on phylotypes.

To determine which phylotypes were responsible for label uptake, the most abundant phylotypes in the heavy fractions

from the ^{13}C-VC-amended samples from day 32 were determined using the sort function in Excel. These values were then compared to relative abundance of these phylotypes in the fractions from the ^{12}C-VC-amended samples from day 32. Additionally, the relative abundance of these phylotypes was determined in the ^{13}C-VC and ^{12}C-VC fractions from day 45. The relative abundance values were normalized to the mass of DNA in each fraction (Quant-iT™ dsDNA High-Sensitivity Assay Kit using the Qubit® 2.0 Fluorometer) by multiplying the relative abundance by the total mass of DNA in each fraction.

Functional gene (*etnE*) qPCR and clone libraries

Fractions from all three time points (15, 32, and 45 days) from both the ^{13}C-VC- and ^{12}C-VC-amended cultures were used in the *etnE* qPCR analysis. This resulted in 78 DNA fractions (buoyant density range = 0.981–1.799 g mL^{-1}) being analyzed by qPCR for *etnE*. An ABI 7000 Sequence Detection System (Applied Biosystems) with a 96-well plate was used for qPCR, as described previously (Jin et al. 2010). Reaction mixtures (25 μL) contained 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), 750 nM *etnE* qPCR primers RTE_F (5′-CAGAAYGGCTGYGACATYATCCA-3′) and RTE_R (5′-CSGGYGTRCCCGAGTAGTTWCC-3′) (Jin and Mattes 2010), and 2 μL of DNA extract. Each fraction was analyzed in duplicate.

Standard curves were developed in triplicate using *etnE* from *Nocardioides* sp. strain JS614 (Jin et al. 2010) amplified using the CoMF1L (5′-AACTACCCSAAYCCSCGCTGGT ACGAC-3′) and CoMR2E (5′-GTCGGCAGTTTCGGTG ATCGTGCTCTTGAC-3′) (Coleman and Spain 2003). Reactions (25 μL) contained 12.5 μL Qiagen PCR Master Mix, 0.2 μM of each primer, and 2 ng of total DNA. Genes per microliter of PCR product were estimated using a previously reported equation (Jin and Mattes 2010). ABI 7000 System SDS software (Applied Biosystems) was used to analyze real-time PCR fluorescence data using the auto baseline function. The following information is provided in accordance with MIQE guidelines (Bustin et al. 2009): The fluorescence threshold was set manually (at 0.05505) to optimize qPCR efficiency (102.5 %) and obtain a linear fit of the standard curve (>0.9976). The Y-intercept of the standard curve was 31.96.

A light fraction (denoted as L9, buoyant density = 1.734 g mL^{-1}) and a heavy fraction (denoted as L4, buoyant density = 1.773 g mL^{-1}) from day 45 were selected as representative fractions for the *etnE* clone libraries. The L4 and L9 fractions were purified as stated above, and *etnE* was amplified using a touch-down PCR protocol. Reaction mixtures (25 μL) contained Qiagen Taq Core Kit 10X buffer (12.5 μL), 25 mM Mg^{2+} solution (0.5 μL), 10 mM dNTPs (0.5 μL), Taq polymerase (0.2 μL), the CoM-F1L/CoM-R2E primer set (0.2 μM)(Coleman and Spain 2003), and 1 μL DNA. The thermocycling protocol consisted of an initial denaturation step (94 °C, 5 min), followed by a touch-down phase (20 cycles of 94 °C for 30 s, 65 °C for 45 s (0.5 °C decrease of each cycle), and 72 °C for 2 min), a secondary amplification (10 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min), and a final extension (72 °C for 15 min). PCR products (891 bp) from L4 and L9 were purified with QIAquick PCR Purification Kit (Qiagen) and cloned with the Invitrogen TA Cloning Kit with an overnight ligation at 4 °C into the pCR®2.1 vector. A 1:1 molar insert to vector ratio was used. Ligations were transformed into One Shot® TOP10 Chemically Competent *E. coli*. Transformants were analyzed according to the cloning kit instructions. Plasmids were extracted using QIAprep Spin Miniprep Kit and PCR-screened with M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. Those with the appropriately sized inserts were Sanger-sequenced at the University of Iowa Institute of Human Genetics Genomics Division with the M13F primer. Sequences with good quality (9 sequences from L4 and 10 sequences from L9) were used for alignment via Clustal W (Thompson et al. 2002) and further phylogenetic analysis with MEGA 5 (Tamura et al. 2011).

Genbank accession numbers

The *Nocardioides* sp. partial 16S rRNA gene was deposited in the NCBI Genbank Database (Accession Number: KJ509930.1). The *etnE* sequences were also placed in this database (Accession Numbers: KJ509928-KJ509936, KM245084, KM245085). Illumina sequencing data was deposited in the NCBI Sequence Read Archive under BioProject Number SAMN03202071.

Results

VC degradation and total microbial community characterization

VC degradation occurred in both of the ^{13}C-VC-amended and ^{12}C-VC-amended mixed cultures but not in the abiotic controls, confirming biological removal (Supplementary Fig. S1). The DNA extracted during this period (days 15, 32, and 45) was subjected to ultracentrifugation, and the heavy fractions (from day 32 and 45) were submitted for Illumina sequencing. Following Mothur analysis, 9,029,943 sequences were obtained. On average, each sample generated 99,230±36,815 sequences (total sequences, not unique sequences).

Illumina sequencing of the 16S rRNA gene was also performed on four total DNA extracts (before ultracentrifugation, two from day 32, and two from day 45). The most abundant phylotypes were determined (relative abundance of 1 %) for

each culture (Fig. 1). Similar phylotypes were observed at both time points for the ^{13}C-VC- and ^{12}C-VC-amended mixed cultures. In all four cultures, the phylotype *Gp4* was present at a high relative abundance (9.9–30.8 %). In addition, the phylotypes *Aquabacterium*, *Sediminibacterium*, *Nocardioides*, and unclassified *Comamonadaceae* were all more abundant compared to the other phylotypes. The most abundant phylotypes classified within eight phyla (*Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Flavobacteria*, *Gemmatimonadetes*, and *Verrucomicrobia)*. The most dominant phyla included *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Flavobacteria*.

Identification of VC-assimilators

To identify the microorganisms responsible for the uptake of ^{13}C from VC (or VC degradation products), DNA extracts from two time points (days 32 and 45) were subject to ultracentrifugation, fractioning, and sequencing. For this, the most abundant phylotypes in the heavy fractions from the ^{13}C-VC-amended samples were determined and compared to the relative abundance of these phylotypes in the fractions from the ^{12}C-VC-amended samples (Fig. 2). In all, four phylotypes showed dominance in the ^{13}C-VC-amended cultures compared to the ^{12}C-VC-amended cultures, including *Rhodoferax*, *Nocardioides*, *Tissierella*, and *Brevundimonas*. As stated previously, the Illumina relative abundance data were normalized by the amount of DNA in each fraction. Only low levels of enrichment were noted from the phylotypes *Tissierella* and *Brevundimonas* at both time points. The other two phylotypes (*Nocardioides* and *Rhodoferax*) were enriched at a higher level, with *Rhodoferax* illustrating the highest level of enrichment. The *Nocardioides* sequences from these cultures were compared to those in GenBank (Supplementary Fig. S2).

The relative abundance of each of these four phylotypes was determined from the total DNA extract sequencing data. These data indicate that only *Nocardioides* was a dominant community member (4.1–18.7 %). The other three phylotypes illustrated only a low relative abundance in the community (<0.08 %).

Functional gene (*etnE*) abundance in SIP fractions

To provide evidence that functional genes associated with VC-assimilation were also enriched in the heavy fractions, we quantified *etnE* abundance by qPCR (Fig. 3 and Supplementary Table S1). Indeed, as VC degradation proceeded, increased *etnE* abundance was observed among

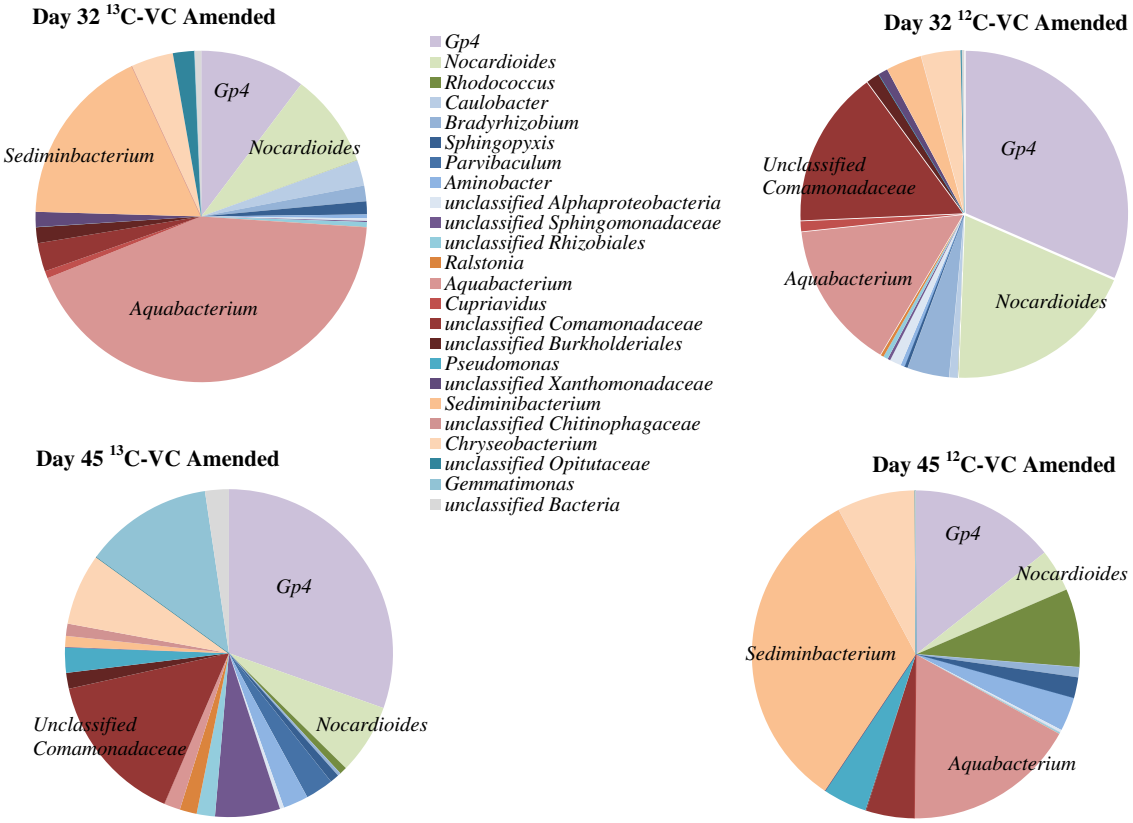

Fig. 1 Microbial composition at the genus level (unless unclassified) of the four VC enrichment cultures. Only those with a relative abundance of 1 % or more in at least one of the cultures are included. The data were obtained from total DNA extracts (before ultracentrifugation)

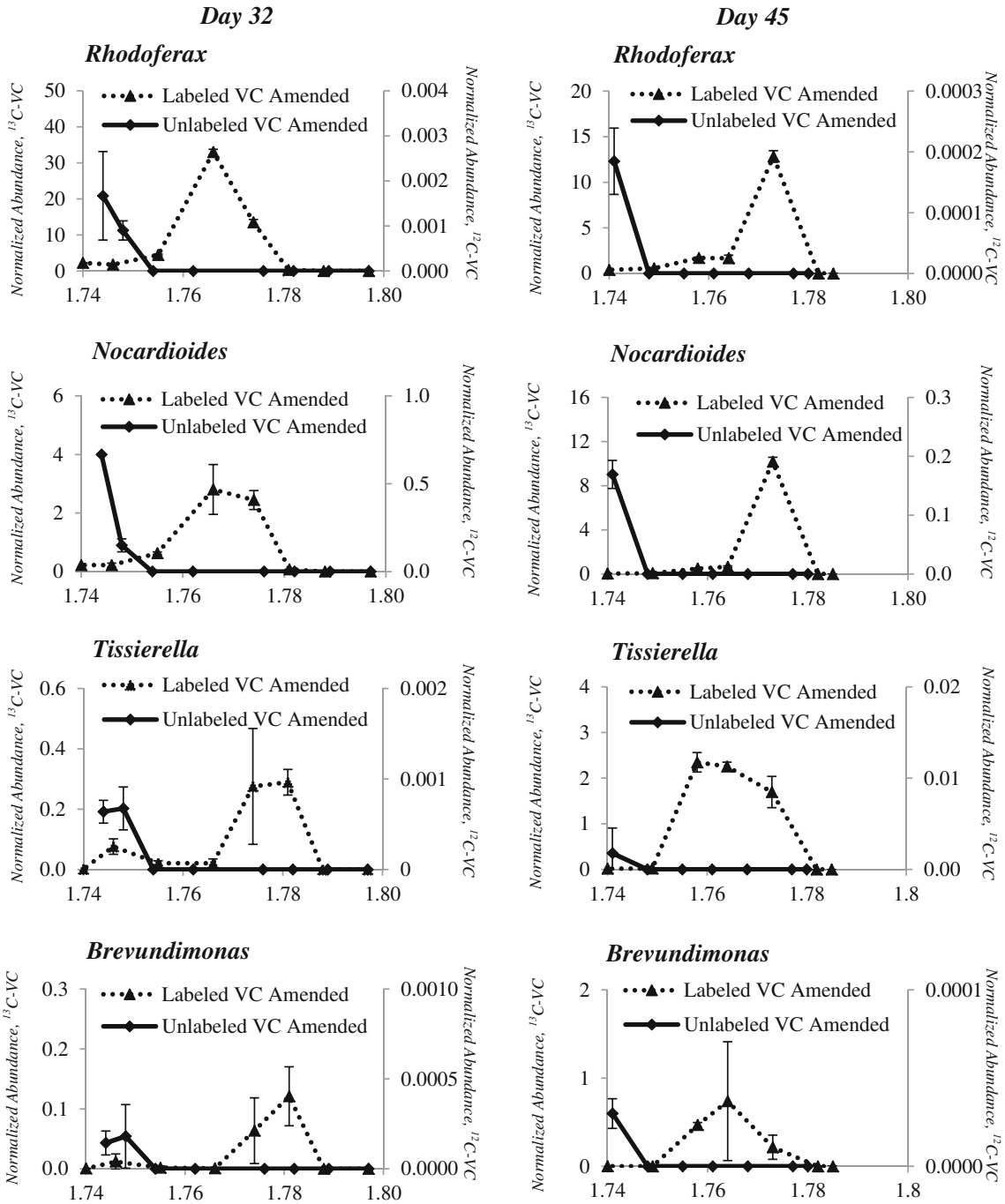

Fig. 2 Normalized abundance of dominant phylotypes in ultracentrifugation fractions from the labeled (^{13}C) VC-amended culture compared to their abundance in the unlabeled (^{12}C) VC-amended culture from day 32 (first column) and day 45 (second column). The axis represents the relative abundance of each phylotype (determined by Illumina sequencing) normalized by the amount of DNA in that fraction (determined by Qubit). *Error bars* represent the standard deviations from triplicate values of Illumina data

heavier fractions (buoyant density = 1.720–1.780 g mL^{-1}) from the ^{13}C-VC-fed culture. Meanwhile, the majority of the *etnE* abundance in the fractions from the unlabeled VC amended cultures occurred within a lower buoyant density range (buoyant density = 1.700–1.740 g mL^{-1}). It is unclear why the *etnE* gene abundance values were lower at day 45 compared to those at days 15 and 32.

Phylogenetic analysis of etnE in SIP fractions

Clone libraries were constructed with *etnE* PCR products from one light fraction (L4) and one heavy fraction (L9) to compare the sequence diversity of ^{13}C-enriched *etnE* with the *etnE* in the unlabeled fraction. An *etnE* phylogenetic tree (Supplementary Fig. S3) revealed that the *etnE* in these two

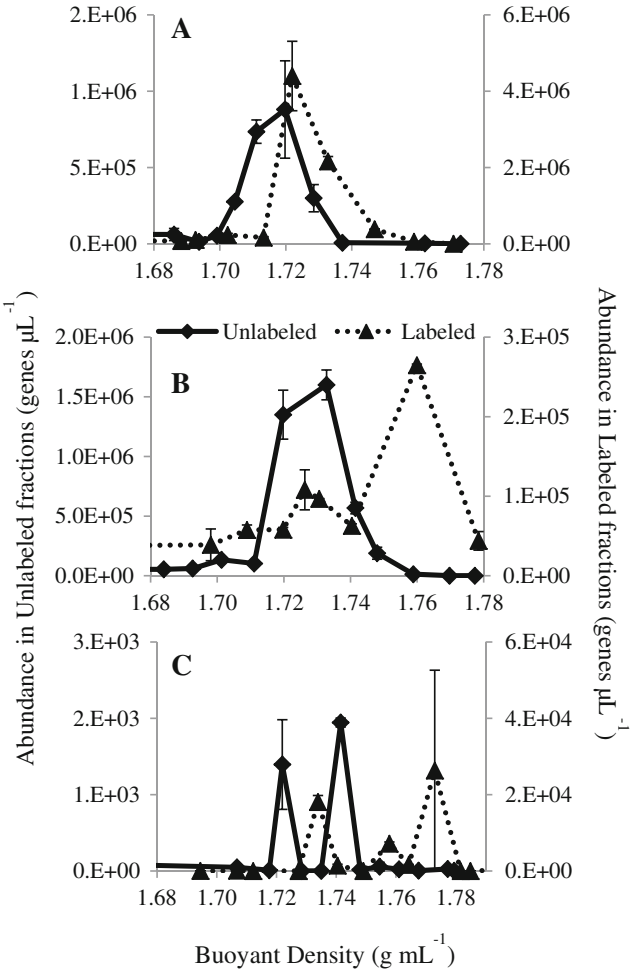

Fig. 3 *etnE* copies after fractionation from ^{13}C-VC-amended (*triangles*, *labeled*) and ^{12}C-VC-amended (*diamonds*, *unlabeled*) cultures at day 15 (**a**), day 32 (**b**), and day 45 (**c**). The *etnE* abundance represents the average of duplicate samples and the *error bars* depict the standard deviation

fractions were similar and that they grouped with the *etnE* from *Nocardioides* sp. JS614 (bootstrap value of 99 %).

A percent identity matrix (Supplementary Table S2) further showed that *etnE* was highly conserved among the light (L4) and heavy (L9) fractions, varying from 94.7 to 100 % identical to each other. An exception is clone L4-10, which was 94.7 to 95.2 % identical to the remaining sequences. Sequences from the light fraction (L4) were 97.8 to 100 % identical to each other, while sequences within the heavy fraction were 99.2 to 99.9 % identical to each other.

Discussion

In this study, VC-assimilating microorganisms were investigated in a mixed culture derived from contaminated site groundwater using SIP, high-throughput sequencing, and qPCR. Although others have isolated VC-assimilating microorganisms (Coleman et al. 2002b; Danko et al. 2004; Elango et al. 2006; Fathepure et al. 2005; Hartmans and Debont 1992; Hartmans et al. 1985; Jin et al. 2010; Jin and Mattes 2008; Taylor et al. 2007; Verce et al. 2000), the microorganisms responsible for VC-assimilation within a mixed culture is more challenging to determine. Such research is important because there is likely a greater diversity of VC-assimilators in the environment than is currently represented in pure culture.

Here, four phylotypes were responsible for ^{13}C uptake (Fig. 2). As with many other SIP studies, it is unclear if these phylotypes were involved in label uptake directly from VC or from VC degradation products. VC-assimilators are known to use an alkene monooxygenase to attack VC (Mattes et al. 2010) which forms VC epoxide (chlorooxirane). This intermediate is very unstable in aqueous systems (~1.6 min half-life). It can spontaneously rearrange into chloroacetaldehyde (Barbin et al. 1975). It can also undergo hydrolysis to glycolaldehyde. It is possible that rearrangement or hydrolysis of VC epoxide occurred in the mixed culture and that label uptake by bacteria that are not true VC-assimilators may have also occurred.

From the *Proteobacteria*, the phylotypes *Brevundimonas* (*Alphaproteobacteria*) and *Rhodoferax* (*Betaproteobacteria*) were observed in the heavy fractions from the ^{13}C-VC-amended cultures at both time points. Previously identified VC-assimilators in this phylum include *Pseudomonas* (*Gammaproteobacteria*) (Danko et al. 2004; Verce et al. 2000), *Ochrobactrum* (*Alphaproteobacteria*) (Danko et al. 2004), and *Ralstonia* (*Betaproteobacteria*) (Elango et al. 2006). In the current study, sequences corresponding to the genera *Pseudomonas* and *Ralstonia* were present but were not enriched in the heavy fractions.

From the phylum *Firmicutes*, one phylotype (*Tissierella*) was observed in the heavy fractions from the ^{13}C-VC-amended cultures at both time points. This phylum (*Firmicutes*) has not yet been associated with VC-assimilation but has been previously reported in sites contaminated with chlorinated solvents (Miller et al. 2007).

Similar to previous research (Coleman et al. 2002b), the current study indicated that the phylotype *Nocardioides* (*Actinobacteria*) was responsible for carbon assimilation from VC. This phylotype was observed in the heavy fractions from the ^{13}C-VC-amended cultures at both day 32 and day 45. Additionally, *Nocardioides* was a significant community member in all four cultures (4.1, 6.2, 8.9, and 18.7 %). The other previously reported VC-assimilating phylotype (*Mycobacterium*) within the *Actinobacteria* (Coleman et al. 2002b; Fathepure et al. 2005; Hartmans and Debont 1992; Hartmans et al. 1985; Jin et al. 2010; Jin and Mattes 2008; Taylor et al. 2007) was observed in the culture only at low levels (relative abundance 0.001–0.079 %) and was not found in the heavy fractions, indicating that it was not responsible for

carbon assimilation from VC. Overall, of the four enriched phylotypes, *Nocardioides* is the only microorganism previously linked to VC-assimilation.

The shift in *etnE* abundance toward the heavier fractions during VC degradation indicates the accumulation of ^{13}C in *etnE*. The strongest shift was at day 32; therefore, this time likely represents the greatest label uptake from VC. The shift is less clear at day 45, possibly indicating label cross feeding. Also, the limited shift at day 15 suggests that label uptake was minor early in the incubation. These observations guided the sequencing efforts to day 32 and day 45 samples. Each of the 19 unique *etnE* sequences retrieved from both light (L4) and heavy (L9) fractions formed a clade (a grouping) with the *etnE* from *Nocardioides* sp. JS614. This pattern contrasts with previous studies where VC- or ethene-degrading isolates were primarily *Mycobacterium* spp. (Chuang et al. 2010). The data suggests that potentially greater *etnE* diversity is present within the ethene- and VC-assimilating *Nocardioides* spp. or that *Nocardioides*-like *etnE* sequences are more widely distributed in the environment than *Mycobacterium*-like *etnE*. It is interesting to note that although the VC-assimilators identified by SIP are relatively diverse in comparison to known isolates, the *etnE* genes harbored by these bacteria appear to be relatively conserved.

In summary, the microorganisms responsible for assimilating ^{13}C from VC within a mixed culture derived from contaminated site groundwater were identified. SIP analysis and Illumina sequencing indicated that *Nocardioides* was a dominant culture phylotype as well as a key ^{13}C assimilator. Therefore, both a previously identified VC-assimilating genus (*Nocardioides*) a novel microorganisms (*Rhodoferax*, *Tissierella*, and *Brevundimonas*) were responsible for carbon uptake from VC. Interestingly, *Rhodoferax* was enriched to the highest level and could therefore represent a particularly important novel VC degrader. Despite the diversity of newly discovered VC-assimilators, the functional gene *etnE* associated with VC-assimilation was relatively conserved in this mixed culture.

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Conflict of interest The authors have no conflict of interest with the methods and data described in this manuscript.

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