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Communication

Identification of Aerobic ETBE-Degrading Microorganisms in Groundwater Using Stable Isotope Probing

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Abstract

A limited number of microorganisms have been identified with the capability to degrade ethyl *tert*-butyl ether (ETBE) in the environment. Knowledge of the identity and distribution of ETBE-degrading microorganisms is important for the implementation of management measures such as natural attenuation and bioremediation at ETBE-release sites. In this study, DNA-stable isotope probing (SIP) was used to identify microorganisms able to aerobically degrade ^{13}C-labeled ETBE in laboratory microcosms constructed with groundwater and aquifer material from an ETBE-release site. Microorganisms in the Class γ-proteobacteria, Order β-proteobacteriales, Family Burkholderiaceae, and classified as *Methylibium* and *Leptothrix*, respectively, were identified as primary ETBE degraders. Comparisons with ETBE-responsive microorganisms (those which increased in abundance after the addition of ETBE), identified by high-throughput sequencing of microcosms established from the same site, showed that only a small proportion of the ETBE-responsive organisms were primary degraders as determined by SIP. ETBE degraders were taxonomically related to microorganisms able to degrade other gasoline components, but not ETBE, implying that this functionality results from acquisition of the *eth* gene cluster by these organisms. These ETBE degraders could also be identified at ETBE-release sites, but at low relative abundance and generally only in those locations from which the microcosms had been established. Therefore, we recommend that molecular investigations of ETBE-contaminated sites focus on functional genes (i.e., the *eth* gene cluster) rather than specific taxa.

Introduction

Ethyl *tert*-butyl ether (ETBE) is a gasoline ether oxygenate (GEO) that is added to gasoline formulations to increase the octane rating and reduce vehicle emissions. ETBE is predominantly used in European markets to meet the requirements of EU directive 2009/28/EC, whereas MTBE (methyl *tert*-butyl ether) is currently the most commonly used GEO worldwide (Thornton et al. 2020). While the fate of MTBE in the subsurface environment is well understood (see reviews by Deeb et al. 2000; Fiorenza and Rifai 2003; Schmidt et al. 2004; Stupp et al. 2012), ETBE is relatively new to the gasoline market and consequently there is limited knowledge of ETBE biodegradation potential in groundwater (Hyman 2013; Thornton et al. 2020). Accidental releases of GEOs from storage tanks and other distribution infrastructure can result in these compounds entering the subsurface environment, including groundwater (Shih et al. 2004; Fayolle-Guichard et al. 2012; Bombach et al. 2015; van der Waals et al. 2018). Where releases of GEOs have occurred, in situ bioremediation is one of the most cost-effective remediation strategies (Fiedler and Berman 2003; Fiorenza and Rifai 2003; Davis and Erickson 2004). This process relies on a microbial community with the metabolic capability to degrade the organic compounds. However, monitoring the effectiveness of these processes requires knowledge of the microbial communities involved, their functional contribution to biodegradation and distribution in aquifers (Nicholls et al. 2021; van der Waals et al. 2024).

A limited number of microorganisms have been identified with the capability to degrade ETBE, either partially to the intermediate metabolite *tert*-butyl alcohol (TBA), or completely to CO_2, as reviewed by Thornton et al. (2020). *Rhodococcus ruber* IFP 2001 (formerly *Gordona terrae*) and *Rhodococcus equi* IFP 2002 were isolated from activated sludge and degraded ETBE to TBA, the TBA then being degraded by other community members (Fayolle et al. 1998). Similarly, Le Digabel et al. (2013) isolated *Rhodococcus* sp. IFP2042 from contaminated groundwater that was able to degrade ETBE to TBA, with another isolate

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Bradyrhizobium sp. IFP2049, able to degrade the TBA to CO_2. Some organisms such as *Aquincola tertiaricarbonis* L108 (a member of the Order Burkholderiales) can fully degrade ETBE (Müller et al. 2008). The best characterized route for ETBE biodegradation involves the *ethRABCD* gene cluster (Chauvaux et al. 2001). The *ethABCD* genes encode a cytochrome P450 system regulated by *ethR*, although the latter is not always present. The *ethB* gene encodes the cytochrome P450 itself and is often used as a diagnostic marker of ETBE biodegradation ability under aerobic conditions. Other biodegradation pathways have been proposed but are not well characterized. For example, *Rhodococcus* sp. IFP 2042 does not contain a detectable *ethB* gene but is proposed to degrade ETBE via alkane hydroxylases (Le Digabel et al. 2013).

Although the culturing and isolation of ETBE-degrading microbes is a valuable tool with which to study the mechanisms involved, isolates are often unrepresentative of complex microbial communities and some organisms cannot be readily cultured. An alternative approach is DNA-stable isotope probing (DNA-SIP), where stable isotope-labeled substrates are added to complex microbial consortia. Biodegradation of the substrate and incorporation of the heavy isotope into the DNA of actively dividing degraders increases its density, allowing isolation by CsCl centrifugation (Neufeld et al. 2007). While incorporation of the ^{13}C label leads to a change in buoyant density, sharp bands and full separation of unlabeled and labeled fractions are typically not achieved when labeling complex microbial communities. The position of DNA fragments in the CsCl gradient depends on their GC content (Schildkraut et al. 1962), which varies between sequences derived from a single genome and between genomes of different organisms (Uhlík et al. 2009). Also, labeling is unlikely to be complete. This leads to distributions of labeled fragments down the gradient and modeling approaches are required to identify labeled organisms (Youngblut et al. 2018; Barnett and Buckley 2020).

DNA-SIP was used by Key et al. (2013) to study MTBE and TBA degraders in contaminated groundwater. They deployed Bio-Sep beads amended with isotopically-labeled MTBE or TBA into monitoring wells for 41 d before retrieval and isolation of DNA from recovered biomass. Members of the Burkholderiales and Sphingomonads were identified as likely primary degraders of MTBE and TBA. Sulfate and iron reducers were proposed as degraders of the *tert*-butyl group, with a *Pseudomonad* implicated in the utilization of the methoxy carbon of MTBE. A related method was used by Bombach et al. (2015), who placed [$^{13}C_6$]-ETBE loaded BACTRAP®s into groundwater monitoring wells for 119 d and then analyzed isotope incorporation into total lipid fatty acids (TLFA). They found significant isotope enrichment into fatty acids 16:0 and 16:ω17, the latter a biomarker of gram-negative bacteria. Further taxonomic assignment was not possible using this approach. Aslett et al. (2011) used ^{13}C-TBA and DNA-SIP to identify TBA-degraders in microcosms prepared from oxygenated activated carbon reactors used for TBA bioremediation. ^{13}C-enriched DNA was analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) and contained *Cupravidus*, *Polaromonas*, *Rhodoferax*, and *Methylibium* species, among others. van der Waals et al. (2019) used $^{13}C_6$-labeled ETBE and DNA-SIP to examine ETBE biodegradation in a batch-fed mixed algal/microbial reactor but did not detect significant differences in the microbial communities of the ^{12}C- and ^{13}C-enriched fractions.

The aim of this study was to identify aerobic ETBE-degrading microorganisms in a microcosm experiment using DNA-SIP analysis. ETBE-degrading microcosms, established with groundwater and aquifer material from a well-characterized field site (Nicholls et al. 2020; Nicholls et al. 2021), were amended with ETBE isotopically labeled on the ethanol moiety. Comparisons were made between microorganisms identified as ETBE degraders in these microcosms and microorganisms that could be isolated from the microcosms. To determine if the SIP-identified ETBE degraders could be identified at the original ETBE-release site, comparisons were made, using high-throughput sequencing of 16S rRNA amplicons, with field samples from the original field site.

Materials and Methods

Microcosm Design and Isotope Labeling

The inoculum for the microcosms used for SIP analysis came from microcosms described by Nicholls et al. (2020). In this previous study, groundwater from ETBE-impacted monitoring wells (I=impacted) was overlaid on aquifer sediment to create microcosms to which 3 additions of ETBE were added (microcosms I-X-E1/2/3). By the time of the third addition, ETBE was degraded relatively rapidly (~11 d) with a short (<3 day) lag. The original groundwater from well I was aerobic (dissolved O_2 [DO] 500 µg/L) and the microcosms were maintained in an aerobic state (Nicholls et al. 2020). When well I was resampled, the DO was 2100 µg/L with a redox potential of −43 mV.

These microcosms were subdivided into sterile 50 mL glass tubes containing 20 mL of groundwater and 20% (w/v) aquifer material, forming a "slurry," and sealed with a crimp cap. Four identical microcosms were prepared and 3000 µg/L unlabeled ETBE or ^{13}C-labeled ETBE was added once (Day 0) or three times (Days 0, 14, 17). Unlabeled ETBE was purchased from Sigma-Aldrich Ltd., UK. ^{13}C-ETBE was synthesized from *tert*-butanol and ^{13}C-labeled ethanol by FaraPack Polymers Ltd. (Sheffield, UK), producing $^{13}C_{5,6}$-labeled ETBE. Purity was checked by gas chromatography mass spectrometry (GCMS) and nuclear magnetic resonance. Microcosms were incubated at 12 °C in darkness to reflect aquifer conditions.

The biodegradation of ETBE was determined by GCMS as described in Nicholls et al. (2020), withdrawing 1 mL aliquots of the aqueous phase every 2 to 3 d. Microcosms were removed from the incubator and allowed to warm to room temperature for 1 h prior to sampling. The detection limits were 0.025 µg/L for ETBE and 3.6 µg/L for TBA.

DNA Extraction

A slurry sample (groundwater and suspended aquifer material) was filtered through a polycarbonate 0.2-µm membrane filter (Whatman) using a plastic syringe. Samples were stored at −80 °C prior to DNA extraction. DNA was extracted using FastDNA Spin kit for Soil (MP Bio-

medicals, UK) according to the manufacturer's instructions, with an additional 10-min incubation at 65 °C prior to homogenization. DNA quantification was performed using Qubit dsDNA HS Assay according to the manufacturer's instructions (ThermoFisher, UK).

DNA-Stable Isotope Probing

Ultracentrifugation and Gradient Fractionation

Ultracentrifugation and gradient fractionation was performed as described by Neufeld et al. (2007). Briefly, DNA extracts were added to a CsCl solution, giving a final density of 7.125 g/mL. The solution was transferred to a 5.1-mL quick-seal polypropylene ultracentrifuge tube (Beckman) and sealed. The sample tubes were loaded into a NVT 65.2 rotor (Beckman) and run at 144,000*g* at 20 °C for 66 h with the brake turned off. Gradient fractions were collected by pumping sterile distilled water colored with bromophenol blue dye into the top of the ultracentrifugation tube, at a flow rate of 425 µL/min using a syringe pump. A hole was made at the bottom of the tube using a sterile syringe, allowing fractions to be collected in sterile microcentrifuge tubes. Twelve fractions were collected from each tube and weighed to validate gradient formation.

DNA was precipitated from each fraction by adding 20 µg linear polyacrylamide and 2 volumes of 30% polyethylene glycol 6000, 1.6 M NaCl according to Neufeld et al. (2007). After 2 h incubation at room temperature, DNA was collected by centrifugation at 13,000*g* for 30 min, washed with 70% (v/v) ethanol and air dried. Samples were resuspended in 50 µL 10 mM Tris, 1 mM EDTA pH 8.0. DNA was quantified using Qubit dsDNA HS Assay, according to the manufacturer's instructions (ThermoFisher, UK).

qRT-PCR

The copy number of 16S rRNA and *ethB* genes were quantified by quantitative real time polymerase chain reaction (qRT-PCR), as described in Nicholls et al. (2020). Standards were made containing a known number of copies of the *ethB* gene (produced by PCR amplification). *Escherichia coli* DNA was added to the standards as amplification efficiency is affected by sample complexity. Quantification of each gene was performed using 1 µL of DNA extract and SensiFast SYBR No-ROX (Bioline), according to the manufacturer's instructions.

16S rRNA Gene Sequencing and Analysis

The 16S rRNA gene was amplified as described by Nicholls et al. (2020). Briefly, 16S rRNA gene fragments were amplified from each sample in triplicate using universal bacterial primers, Bakt341 and Bakt805 (Klindworth et al. 2012). The amplification products were mixed, cleaned using Agencourt AMPure XP beads (Beckman) and indexed using Nextera XT Index Kit Set A v2 (Illumina). Amplicons were sequenced using an Illumina MiSeq producing 250 base pair (bp) reads. Data have been deposited at the European Nucleotide Archive under Project PRJEB52346.

The sequencing results were delivered as Fastq files and processed using Qiime2 (Bolyen et al. 2019). Primer sequences were removed, sequences filtered for quality, errors corrected, paired, and chimeras removed using dada2 (Callahan et al. 2016). This analysis produces a set of Amplified Sequence Variants (ASVs) that are sequences which represent the diversity of organisms present in the sample. ASV sequences were aligned using MAFFT (Katoh et al. 2002) and aligned using FastTree2 (Price et al. 2010). Taxonomies were created by comparison with the SILVA132 database at 99% identity (Quast et al. 2013). All subsequent analyses were performed in R (R Core Team 2021) and the *phyloseq* package (McMurdie and Holmes 2013). Differences between communities were determined by PERMANOVA using the *adonis* function of the *vegan* package (Oksanen et al. 2020).

The impact of incorporation of ^{13}C from the labeled ETBE was determined by simulation. Utilization of the ^{13}C-labeled acetate moiety released in the first step of ETBE degradation was calculated as leading to a shift of one gradient fraction in the labeled DNA. To simulate this effect, the relative abundance of each ASV was multiplied by the total amount of DNA in each ^{12}C-labeled fraction to calculate an absolute abundance (this was not performed for fractions with very low DNA concentrations as the calculations became unreliable). For each ASV in turn, 1 was added to the fraction number, the new amount of DNA in that fraction calculated and a new relative abundance determined. The simulated profile was then compared with the measured ^{13}C-labeled profile.

ETBE-Degrading Isolates

Serial dilutions of microcosm slurry samples were plated onto mineral salts media (MSM) (Rohwerder et al. 2006), with the addition of 20 mg/L ETBE. Colonies were picked and streaked onto MSM plates. Individual colonies were selected for metabolite testing in liquid MSM containing either 200 mg/L ETBE or molar equivalents of sodium acetate, TBA, or MTBE. Cells were grown in ETBE, centrifuged, and resuspended at an optical density at 600 nm (OD_{600}) of 0.05. Then 0.25 mL of cell suspension was added to 10 mL of MSM medium with the appropriate addition. The remaining inoculum was pelleted at 5000*g* for 5 min and washed with phosphate-buffered saline before DNA extraction using UltraClean Microbial Kit (Qiagen), according to the manufacturer's instructions. DNA was quantified using a Qubit dsDNA HS Assay, according to the manufacturer's instructions.

The 16S rRNA gene was amplified from the DNA extracts using MyTaq DNA polymerase (Bioline) with the universal prokaryotic primers Bakt799 and Bakt1193 (Bodenhausen et al. 2013). PCRs were carried out using 2 µL of DNA extract, 0.2 µL of 20 µM primers and the manufacturer's buffer, with a final reaction volume of 40 µL, under the following conditions: 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C 30 s, and 72 °C for 2 min using a Prime thermocycler (Techne). Amplification was confirmed by gel electrophoresis using a 1.5% (w/v) agarose gel. Samples were submitted to the Core Genomics Facility, University of Sheffield for sequencing.

Field Samples

For comparison with field samples and previous microcosm experiments, high-throughput 16S rRNA amplicons were re-analyzed from Nicholls et al. (2020, 2021) using

the SILVA bacterial taxonomy. These samples (originally collected in 2016) provided microbial community profiles from the original field sites (time point 0, N = non-impacted, I = ETBE impacted) at 3 depths (x = 8 to 9 m below-ground level, y = 9 to 10 mbgl, z = 13 to 14 mbgl). The effect of ETBE, MTBE, or ETBE + MTBE additions to microcosms prepared from these sites was provided by time points 33 to 202 (I) and 77 to 202 (N) days. The field site was re-visited in 2019 and the monitoring wells re-sampled at depth x (8 to 9 m). As Nicholls et al. (2021) had shown that ETBE-degrading organisms were preferentially attached to aquifer sediment, samples were collected into sterile 2 L Duran bottles after purging 1, 3, and 6 borehole volumes from each well using a submersible pump and stored at 4 °C. Samples were filtered through a 5 µm membrane to collect dislodged aquifer sediment and attached microorganisms, and the flow-through collected on a 0.2 µm membrane to collect planktonic microorganisms. DNA was extracted directly from the membranes as described in (Nicholls et al. 2021).

Results

ETBE Biodegradation in Microcosms

To identify primary ETBE degraders, new microcosms were created from the microcosms described in Nicholls et al. (2020), containing both a liquid phase and 20% (w/v) aquifer sediment. ETBE (^{12}C or ^{13}C) was added to a concentration of 2.5 mg/L, and degradation monitored at intervals (Figure 1). A mixed slurry of groundwater and sediment was sampled for SIP analysis. For microcosms where a single addition of ETBE was made, samples were collected after 12 d, when ETBE concentrations had fallen to ~1 mg/L. A small amount (~80 to 100 µg/L) of TBA was present at this time. TBA concentrations were below detection before 12 d. Further additions of ETBE led to much more rapid ETBE degradation and the accumulation of TBA to concentrations 1000 to 1600 µg/mL. The headspace in the microcosms contained sufficient oxygen to ensure that the microcosms remained aerobic throughout the experiment.

Molecular Analysis of ETBE-Degrading Organisms

DNA extracted from the groundwater/sediment mixtures was subjected to CsCl gradient ultracentrifugation. The resulting gradient was divided into 0.425 mL fractions, DNA extracted and quantified. Figure 2 shows the distribution of DNA within these fractions.

High-throughput sequencing of 16S rRNA amplicons from each fraction produced between 26,137 and 97,827 high-quality paired reads per fraction. Rarefaction analysis (Figure S1) showed that this was sufficient to capture most of the bacterial diversity.

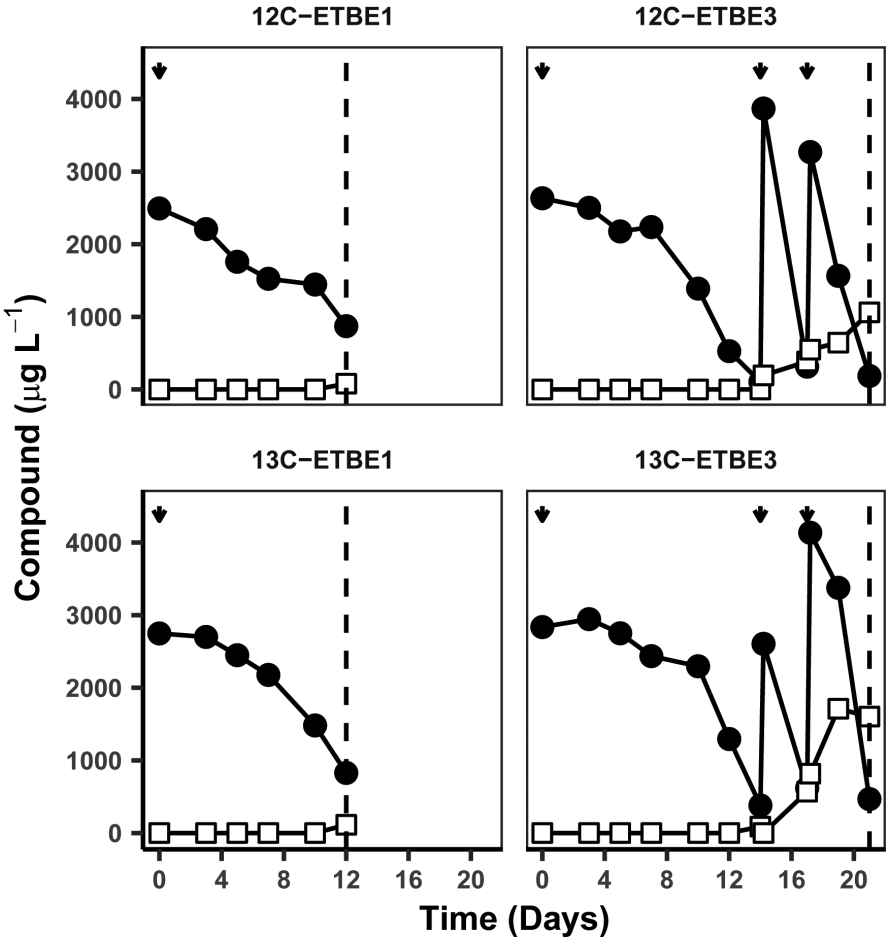

Figure 1. ETBE (filled circles) and TBA (open squares) concentrations in microcosms. ETBE was unlabeled (12C) or ^{13}C labeled (13C) and added 1 or 3 times. Arrows indicate when ETBE was added. The dotted line shows when microcosms were sampled.

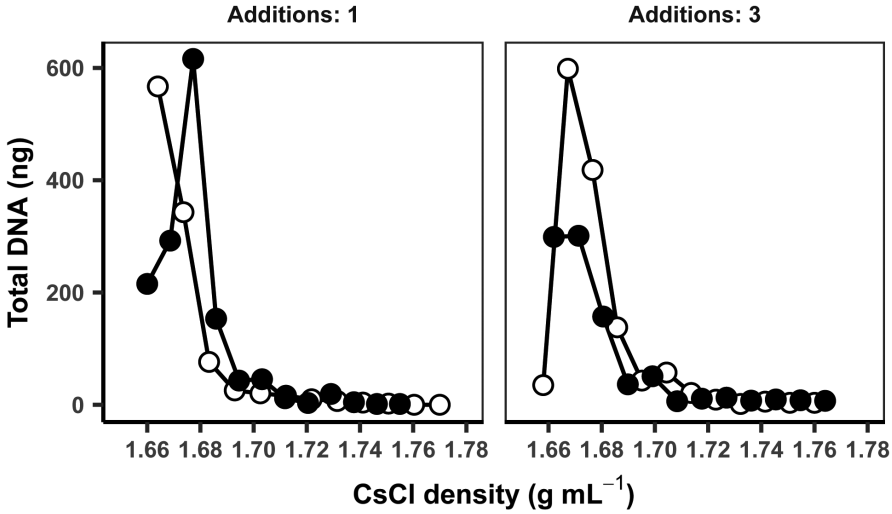

Figure 2. Distribution of DNA within the CsCl gradient. ^{12}C-ETBE (open circles), ^{13}C-ETBE (closed circles) from microcosms with 1 or 3 ETBE additions. Each fraction was 0.425 mL.

Figure 3 shows the relative abundance of the bacterial samples in the different fractions at the Family taxonomic level. The samples were dominated by Proteobacteria, with the greatest contributions from members of the Burkholderiaceae, Methylophilaceae, Methylococcaceae, and Methylomonaceae. PERMANOVA analysis showed that there was no significant difference between communities that received unlabeled or ^{13}C-labeled ETBE ($p=0.186$) but that there was a significant difference between microcosms receiving 1 or 3 additions ($p<0.001$). The mean Shannon diversity index fell from 5.72 in microcosms with a single addition to 4.63 in those receiving 3 additions.

To identify ETBE-degrading organisms, the expected distribution following ^{13}C-labeling was determined by modeling (see Materials and Methods). For each ASV, the absolute abundance was calculated by multiplying the relative abundance by the amount of DNA in that fraction, then the fraction number was increased by 1 (the change in fraction number expected from ^{13}C labeling). The relative abundance was then recalculated and compared with the actual profile obtained with ^{13}C labeling (Figure 4). Incorporation of unlabeled ^{12}C from the TBA moiety would reduce heavy isotope enrichment, while incorporation of labeled ^{13}C into both DNA strands would increase heavy isotope enrichment. Despite these uncertainties, the simulation allowed the effect of ^{13}C labeling to be separated from other factors that influence amplicon distribution within the CsCl gradient.

In total, 1165 ASVs were identified in these samples and the simulated distributions calculated. Many ASVs were of low relative abundance with 81 ASVs accounting for 50% of the total community (see Figure S2). Inspection of these models identified four ASVs where the distribution of relative abundances in the ^{13}C-labeled samples was similar to, or exceeded, that calculated in the simulations. All 4 were members of the Burkholderaceae. However, the relative abundances of two of these were very low.

Figure 4 shows the top 20 ASVs (ranked by overall relative abundance) and the two rare ASVs which also showed labeling, their distribution within unlabeled and ^{13}C-labeled microcosms and simulated ^{13}C distributions following addition of ETBE. Note that these calculations show relative abundance and hence the ASVs appear distributed along the gradient.

ASVs were considered to be degraders when the measured ^{13}C distribution was similar to, or exceeded, that of the simulation. Where the relative abundance of the ASV was responding rapidly to ETBE additions, the profiles may differ between microcosms (e.g. ASV 2 or 7) but there was no evidence of specific labeling. ASVs 8 and 17 showed patterns expected from ^{13}C labeling in microcosms receiving either 1 or 3 ETBE additions. Both were members of the Burkholderiaceae in the Genera *Methylibium* (ASV 8) and *Leptothrix cholodnii* SP-6 (ASV 17) and constituted ~1% of the total microbial community. In total the Burkholderiaceae represented ~7% of the bacterial community. ASVs 260 and 411 also showed evidence of labeling although their relative abundances were very low and the simulations become unreliable. However, ASV260 was also classified as *Leptothrix* at the genus level while ASV411 was classified as *Piscinibacter* (formerly *Methylibium*). These four ASVs are identified in Figure 4 by highlighting in gray.

Laboratory Culturing of ETBE-Degrading Isolates

Laboratory culturing of isolates proved challenging, as growth on MSM with added ETBE was slow with colonies taking 3 to 4 weeks to become visible. Isolates were obtained, tested for the presence of the *ethB* gene, a portion of the 16S rRNA gene sequenced and compared with the ASVs identified by high-throughput sequencing. Of these, one isolate (identified four times) matched ASV 17 perfectly (*Methylibium*) and contained an *ethB* gene. It did not contain amplifiable *mdpA* or *mdpJ* genes associated with MTBE degradation (Hristova et al. 2007). When grown in liquid culture containing ~150 mg/L ETBE, this isolate degraded ETBE with little accumulation of TBA (TBA was below detection limit at 58 d and 0.8 mg/L TBA after 126 d). These cultures also degraded sodium acetate, but not MTBE or TBA when supplied on its own (Figure S3).

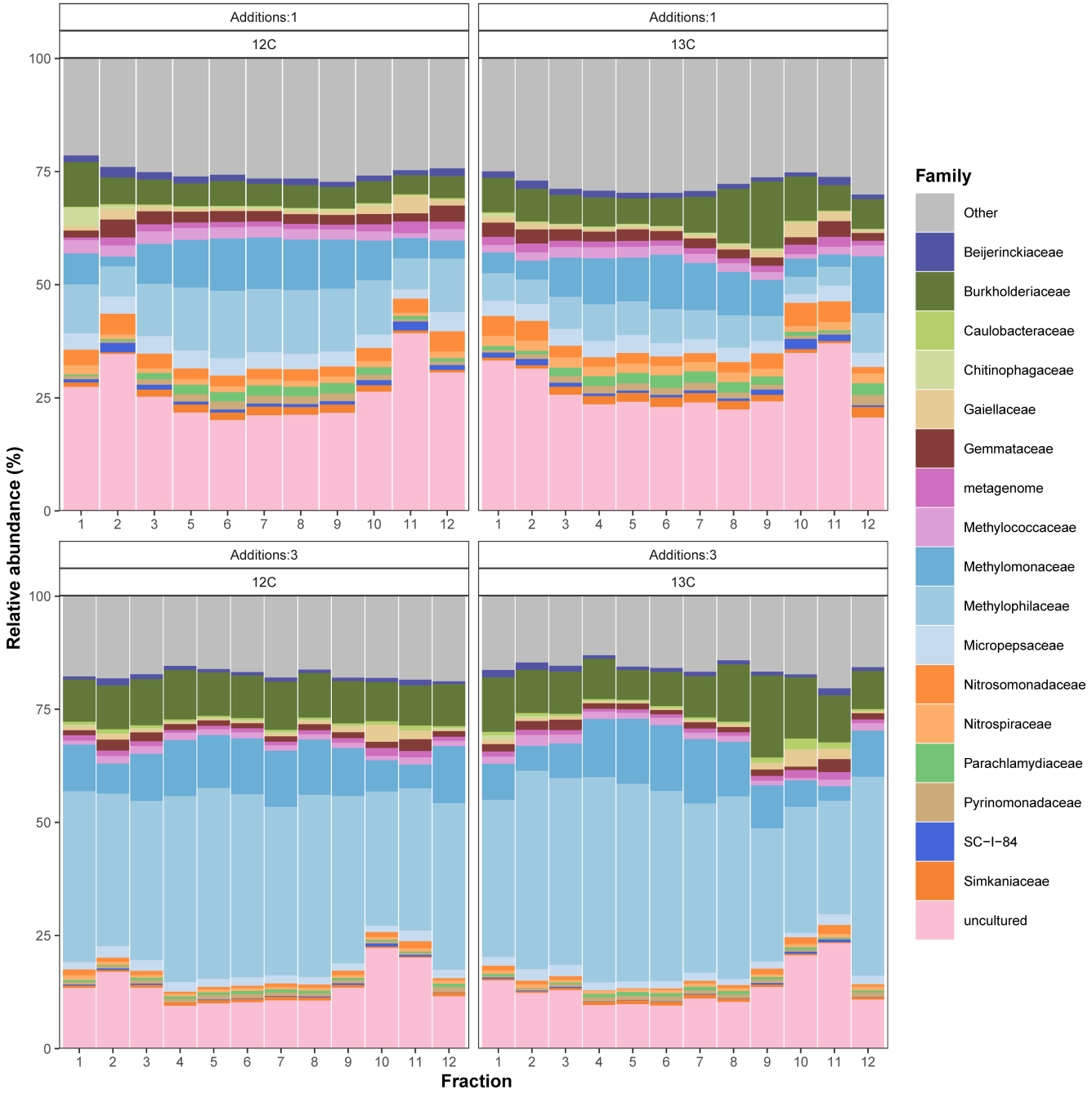

Figure 3. Relative abundance at the Family level of amplicons in CsCl gradient fractions. Amplicons of 16S rRNA sequences were generated from microcosms following one or three additions of unlabeled (^{12}C) or labeled (^{13}C) ETBE. Classes which contributed <2% in any fraction were grouped as "Other" for clarity. "Uncultured" refers to sequences in the Silva database that are only known from environmental samples and have not previously been isolated and grown in culture.

We did not obtain isolates for the other ETBE-degrading ASVs.

Identification of SIP-Labeled Organisms at ETBE-Release Sites

The microcosms used in the current study for SIP were derived from microcosms established using material collected from an ETBE-impacted field site. The original field site is described in Nicholls et al. (2020). Two monitoring wells were studied—one which was not impacted by ETBE (well N) and a second which was impacted by ETBE (well I). Different depths were sampled (x = 8 to 9, y = 9 to 10, z = 13 to 14 m below-ground level). The microcosms for SIP analysis were derived from microcosms I-*x* which had received three additions of ETBE.

To determine the prevalence of the ETBE degraders as identified by SIP, we re-analyzed these microcosm data. We also re-visited the field site to collect new material from well I (which, at the time of this second sampling, no longer contained detectable ETBE) and well N (Figure 5). Previous studies have shown that ETBE-degrading organisms are preferentially attached to aquifer material and that collecting mixed groundwater-aquifer sediment (slurry) samples after minimal purging provides a better representation

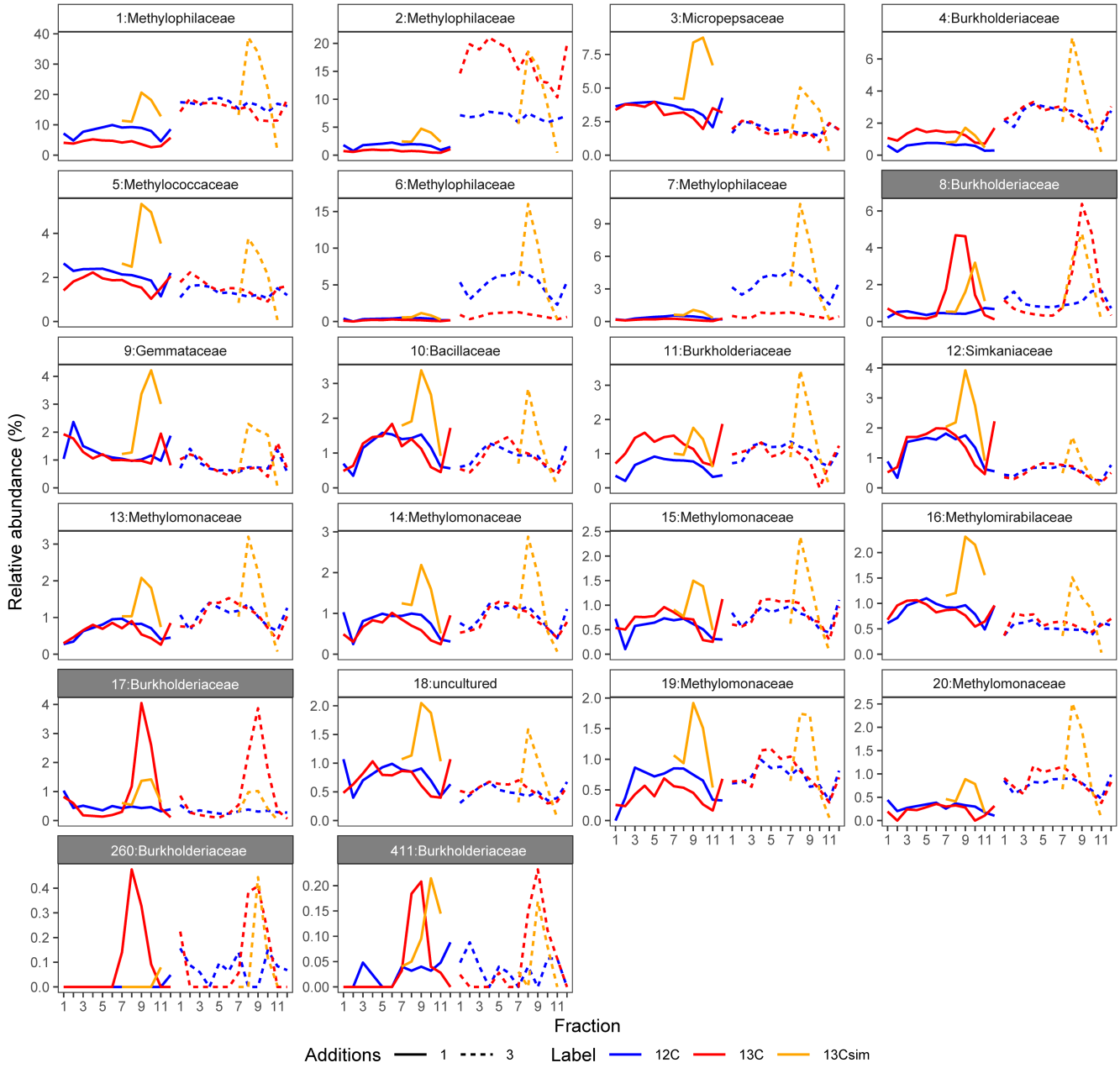

Figure 4. Relative abundance of the top 20 most abundant ASVs in ETBE microcosms. Two additional ASVs (260 and 411) are also shown as these showed distributions indicative of ^{13}C labeling. The relative abundance in each fraction of the CsCl gradient is shown for microcosms supplied with unlabeled (12C blue) or labeled (13C red) ETBE. Simulations (13Csim) of the distributions expected following heavy isotope labeling are shown in orange. Simulations become unreliable at low DNA concentrations (<0.3 ng/µL), and so are not shown for these fractions. Graph titles are ASV number (ranked by mean overall abundance from a total of 1165 ASVs) and Family. ASVs showing 13C enrichment have been highlighted in gray.

of community structure (Nicholls et al. 2021). Therefore, samples were collected at different borehole purge volumes and filtered through a 5 µm filter to collect sediment particles and attached cells. The flow through was collected on 0.2-µm filters. PCR analysis showed that the *ethB* gene was detectable at 0.89 to 5.3×10^7 copies per mL in samples from well I but was below detection limits in samples from well N (Table 1).

In the original groundwater field samples (Figure 5d—Day 0), only *Methylibium* ASV 17 could be detected and its relative abundance was very low (0.005 to 0.014%) (and hence is not visible in Figure 5d). However, GEO additions resulted in *Methylibium* ASV 17 and *Leptothrix* ASV 260 increasing in relative abundance, becoming a significant proportion (~3 to 4%) of the total community. Although *Leptothrix* ASV 8 and *Piscinibacter* ASV 411 could be detected in microcosm samples after GEO additions, their relative abundance remained extremely low (Figure 5d Days >0). When the field site was revisited and resampled, the groundwater ETBE concentration had fallen to below detectable levels (Table 1). However, all four ASVs could be detected in attached communities from well I at depth *x*, and *Methylibium* ASV 17 and *Leptothrix* ASV 260 were also detectable in groundwater samples (Figure 5d). These ASVs were not detected in microcosm samples derived from the non-impacted well N (except for a very low amount of *Lep-*

tothrix ASV 260 in one sample), even though these microcosms developed appreciable ETBE degradation activity after multiple GEO additions (Figure 5d).

Discussion

ETBE-Degraders Identified by DNA-SIP

In this study, $^{13}C_{5,6}$-ETBE DNA-SIP was used to identify ETBE-degrading microorganisms originating from an ETBE-impacted aquifer. The first step of ETBE biodegradation involves the release of acetate and TBA (Müller et al. 2007), which is energetically unfavorable (Thornton et al. 2020), leading to slow biodegradation kinetics. In the microcosms used for the current study, biodegradation rates after the first addition of ETBE were relatively slow, with only small amounts of TBA accumulating, but further additions led to more rapid ETBE biodegradation and accumulation of significant, but non-stoichiometric, amounts of TBA. The same kinetics were seen in the original microcosms used to establish the microcosms for SIP (Nicholls

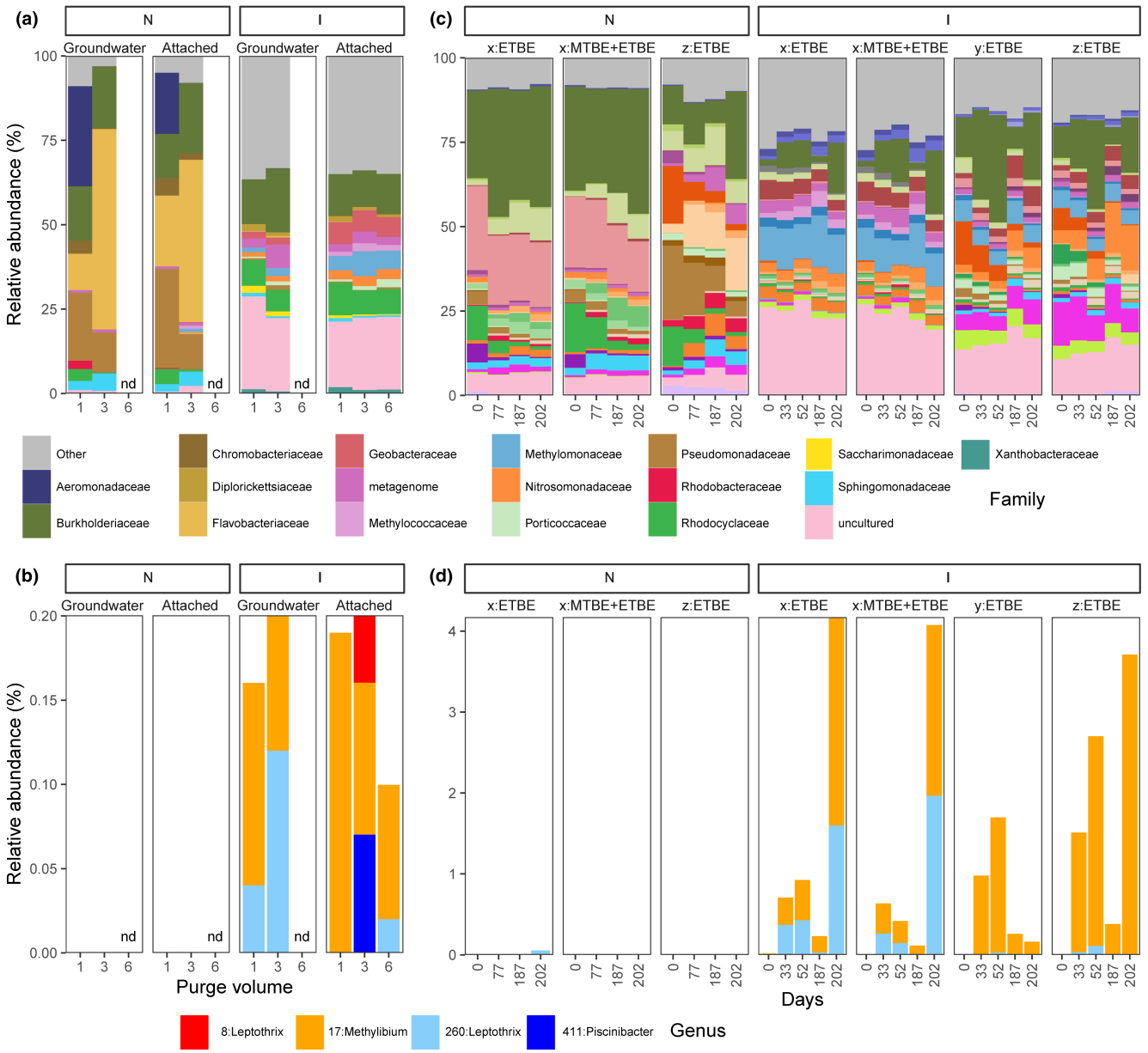

Figure 5. Microbial community composition of samples obtained from the field site (a, b) and microcosms (c, d). Results from two monitoring wells are shown: N—not impacted by ETBE and I—impacted by ETBE. Samples were taken from 8 to 9 m below-ground level (x), 9 to 10 mbgl (y) or 13 to 14 mbgl (z). For (c, d) Day 0 represents groundwater samples as obtained from the field site. Gasoline ether oxygenates were added at Days 1 and 187 (microcosms derived from well N) or Days 1, 33, and 187 (microcosms derived from well I). Microcosms for SIP were derived from microcosm I-X (ETBE). The field site was revisited and samples obtained from well I and N at 8 to 9 mbgl (*x*). The wells were purged for different well volumes. Aquifer sediment and attached cells were collected on 5-µm filters (Attached) and planktonic cells collected by passing the flow-through through a 0.2-µm filter (GW). In some samples, insufficient DNA was extracted for high throughput DNA sequencing (nd, not done). Full details of the samples are provided in the Materials and Methods. Panels (a) and (c) show the bacterial community composition at the Family level. ASVs that are present at less than 2% relative abundance in any samples are shown as "Other" for clarity. Panels (b) and (d) show the prevalence of the four ASVs identified as ETBE-degraders by SIP in the current study.

Table 1

Field Samples from the ETBE-Release Site

Monitoring Well	Well Purge Volume	ETBE Concentration (mg/L)	*ethB* Copies mL^{-1}
N	1	BDL <0.025 µg/L (previously BDL <0.025 µg/L)	BDL <30
	3		
	6		
I	1	BDL <0.025 µg/L (previously detected at 1.4 mg/L)	5.3×10^7
	3		3.1×10^7
	6		8.9×10^6

Notes: Two groundwater monitoring wells were sampled (N = non-impacted, I = ETBE-impacted) and the ETBE concentration at the time of sampling measured. Aquifer material and attached cells were collected on 5-µm filters after the monitoring well had been purged for 1, 3, or 6 borehole volumes. DNA was extracted and the *ethB* copy number determined by qRTPCR. Results are expressed as copy numbers in mL of groundwater processed. BDL = below detection limit.

et al. 2020). Under these conditions, $^{13}C_{5,6}$-ETBE is expected to label primary degraders, as these will utilize readily degradable acetate. Microorganisms that solely degrade TBA would not be labeled in these experiments, while those that degrade both TBA and acetate at the same rate would be labeled to a lesser extent, due to isotopic dilution.

All of the ASVs identified as primary ETBE-degraders by SIP were members of the Family Burkholderiaceae, which have commonly been implicated in the biodegradation of gasoline components. A wide range of organisms have been reported to degrade ETBE aerobically (see Thornton et al. (2020) for a review) and Gunasekaran et al. (2013) found that a consortium containing *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp., and *Methylovorus* sp. from a gasoline-contaminated site was able to degrade ETBE. *Aquincola tertiaricarbonis* L108 (assigned to the Order Burkholderiales, but the taxonomy is uncertain at the Family level) can fully degrade ETBE (Müller et al. 2008). To our knowledge, no *Leptothrix* isolate has been reported that degrades ETBE, although we were unable to isolate the *Leptothrix* ASVs identified by SIP in this study. Further efforts to isolate this specific Genus using specialized media or iCHIP technologies (Nichols et al. 2010) may prove fruitful. Likewise, inclusion of a solid substrate might aid isolation as recent studies show that *eth*-containing microorganisms from this site show a strong preference for attachment to solid substrata (Nicholls et al. 2021).

The *Methylibium* isolate corresponding to ASV 17 was able to degrade ETBE and contained the *ethB* gene. This isolate readily degraded acetate, a metabolite produced from ETBE degradation and the moiety that was ^{13}C labeled in the SIP analysis. When grown in culture, ETBE was degraded but little or no TBA accumulated. However, this isolate was not able to degrade TBA when supplied on its own indicating that ETBE was necessary for the activation of TBA degradation pathways. The *mdpA* and *mdpJ* genes associated with MTBE degradation were not detectable and the isolate could not degrade this GEO. Related organisms, *Methylibium petroleiphilum* PM1 (Hanson et al. 1999; Kane Staci et al. 2007) and *Methylibium* sp. T29 (Szabó et al. 2015) can degrade MTBE but not ETBE, and do not contain the *eth* gene cluster in their genomes. The *eth* gene cluster is flanked by transposable elements and appears to be readily gained and lost in microbial populations (Chauvaux et al. 2001).

Methylibium ASV 17 was also present, albeit at low relative abundances, in field samples collected prior to this experiment and when the site was re-sampled at the same monitoring well (well I). However, it was neither present at another well at the same site (well N) nor in microcosms derived from that well even though they were able to degrade ETBE. Nicholls et al. (2020), identified 18 ASVs that increased in relative abundance in microcosms supplied with ETBE derived from well I, 8 to 9 mbgl (*x*), 20 from 9 to 10 mbgl (*y*), and 30 from 13 to 14 mbgl (*z*). Only two ASVs were common between these samples but these did include *Methylibium* ASV 17. This is consistent with a relatively wide range of organisms being able to host ETBE genes and their abundance being influenced by their initial prevalence. This finding agrees with that of Kyselková et al. (2019) who created microcosms derived from samples taken upstream or within a gasoline-polluted aquifer. While both sets of microcosms degraded ETBE and contained *ethB* genes, the community structure remained distinct.

Conclusions

In this study, we have used SIP to identify organisms that are able to degrade ETBE and become isotopically enriched from the acetate moiety. Comparisons with ETBE-responsive microorganisms identified by high-throughput sequencing of microcosms established from the same site showed that not all microorganisms were ETBE responsive (i.e., increased in relative abundance following additions of ETBE) and only a small proportion of the ETBE-responsive organisms were identified as primary degraders by SIP. Where isolates were obtained, these were taxonomically related to previously identified organisms that degraded fuel components, but not ETBE, implying that they had acquired the *eth* gene cluster. However, other microcosm community members were not identified as primary ETBE degraders, indicating the utility of the SIP approach to discriminate specific microorganisms involved in ETBE biodegradation. Comparison with the field site demonstrated that these ETBE degraders could be identified directly in field samples, but at low relative abundance and generally only in those locations from which the microcosms had been established. This supports the view that a diversity of organisms is able to host the *eth* gene cluster and that other factors drive the overall community taxonomy. Therefore, we recom-

mend that molecular investigations of ETBE-contaminated sites for bioremediation purposes should focus on functional genes (e.g., the *eth* gene cluster) and whether conditions are favorable for ETBE degradation (e.g., aerobic) rather than quantifying specific taxa.

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Conflict of Interest

M.H. works for Concawe, which represents the European refining industry, whose members may be responsible for managing sites with ETBE impact. S.F.T., H.C.G.N., H.E.M., and S.A.R. have received research funding from Concawe. S.F.T. has received research funding from Concawe member companies, including Shell and TotalEnergies, for research on ETBE and other ether oxygenates in groundwater.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in European Nucleotide Archive at https://www.ebi.ac.uk/ena/browser/home, reference number PRJEB52346.

Supporting Information

Additional Supporting Information may be found in the online version of this article. Supporting Information is generally not peer reviewed.

Figure S1. Rarefaction curves of 16S rRNA sequences. Fractions are from CsCl gradients of amplicons produced in microcosms with additions of unlabeled (12C) or isotopically labeled (13C) ETBE with 1 or 3 ETBE additions. Fraction 1 is the heaviest (lowest), fraction 12 is the lightest (uppermost).

Figure S2. (a) A Pareto plot of cumulative abundance across all samples. (b) The maximum relative abundance of ASVs in any sample plotted on a LOG10 scale.

Figure S3. Degradation of gasoline ether oxygenates or metabolites by isolate 21 (matching ASV 17) in liquid culture. Closed symbols are live cultures, open symbols are uninoculated controls. The panel legend shows the compound added to the microcosm. The symbols show the compounds measured.

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