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## Using DNA-Stable Isotope Probing to Identify MTBE- and TBA-Degrading Microorganisms in Contaminated Groundwater

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### Abstract

Although the anaerobic biodegradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) has been documented in the laboratory and the field, knowledge of the microorganisms and mechanisms involved is still lacking. In this study, DNA-stable isotope probing (SIP) was used to identify microorganisms involved in anaerobic fuel oxygenate biodegradation in a sulfate-reducing MTBE and TBA plume. Microorganisms were collected in the field using Bio-Sep® beads amended with <sup>13</sup>C<sub>5</sub>-MTBE, <sup>13</sup>C<sub>1</sub>-MTBE (only methoxy carbon labeled), or <sup>13</sup>C<sub>4</sub>-TBA. <sup>13</sup>C-DNA and <sup>12</sup>C-DNA extracted from the Bio-Sep beads were cloned and 16S rRNA gene sequences were used to identify the indigenous microorganisms involved in degrading the methoxy group of MTBE and the *tert*-butyl group of MTBE and TBA. Results indicated that microorganisms were actively degrading <sup>13</sup>C-labeled MTBE and TBA in situ and the <sup>13</sup>C was incorporated into their DNA. Several sequences related to known MTBE- and TBA-degraders in the Burkholderiales and the Sphingomonadales orders were detected in all three <sup>13</sup>C clone libraries and were likely to be primary degraders at the site. Sequences related to sulfate-reducing bacteria and iron-reducers, such as *Geobacter* and *Geothrix*, were only detected in the clone libraries where MTBE and TBA were fully labeled with <sup>13</sup>C, suggesting that they were involved in processing carbon from the *tert*-butyl group. Sequences similar to the *Pseudomonas* genus predominated in the clone library where only the methoxy carbon of MTBE was labeled with <sup>13</sup>C. It is likely that members of this genus were secondary degraders cross-feeding on <sup>13</sup>C-labeled metabolites such as acetate.

### INTRODUCTION

Methyl *tert*-butyl ether (MTBE) has been widely used as a fuel oxygenate. It was first added to gasoline in the late 1970s as tetra-ethyl lead was phased out. To reduce air emissions and comply with the Clean Air Act Amendments passed in 1990, MTBE use as a fuel additive increased with concentrations in gasoline ranging between 11% and 15% by volume (Fiorenza and Rifai 2003). While the addition of MTBE did result in cleaner-burning fuel, it

proved to be a significant contributor to groundwater pollution at sites where gasoline had been leaked or spilled. Due to these environmental issues, MTBE is now banned in several states, and it has largely been replaced with ethanol. MTBE is 20 times more water soluble (50µg/L at room temperature) and less volatile than benzene, toluene, ethylbenzene, and xylene (BTEX), so it rapidly migrates in groundwater and creates larger contaminant plumes than BTEX (Davis and Erickson 2004). Since MTBE also has a low adsorption onto organic matter, adsorption to the sediments does not significantly retard the contaminant's migration. tert-Butyl alcohol (TBA) is frequently found as a co-contaminant with MTBE. TBA may be present as an added component or an impurity in gasoline, and it is also a key intermediate in MTBE biodegradation.

MTBE and TBA are capable of being degraded through aerobic respiration (Salanitro et al. 1994; Hanson et al. 1999; Deeb et al. 2001; Wilson et al. 2001; Bradley et al. 2002), denitrification (Bradley et al. 2001, 2002), iron (III) reduction (Landmeyer et al. 1998; Bradley et al. 2001; Finneran and Lovley 2001), and sulfate reduction (Bradley et al. 2001, 2002; Somsamak et al. 2001, 2006). The biodegradation of MTBE by methanogenesis has been documented, but TBA appears to be recalcitrant under methanogenic conditions (Mormile et al. 1994; Wilson et al. 2000; Bradley et al. 2001, 2002; Somsamak et al. 2006). The persistence of these compounds in several studies suggests that the necessary microorganisms are not ubiquitous in the subsurface (Suflita and Mormile 1993; Kane et al. 2001). The ether bond and the tert-butyl group are difficult for most microorganisms to metabolize, which has contributed to the recalcitrance of MTBE and TBA (Suflita and Mormile 1993; White et al. 1996; Fiorenza and Rifai 2003).

Although anaerobic degradation pathways for MTBE have not been determined in detail, all reports of anaerobic MTBE biodegradation indicate that MTBE is initially O-demethylated to TBA, and TBA has been documented as an intermediate in anaerobic field and laboratory research (Schmidt et al. 2004; Wilson et al. 2005; Youngster et al. 2008). Acetogenic bacteria are known to O-demethylate aryl aromatic ethers, and it is likely that the initial step in MTBE degradation is mediated by acetogens (Kuder et al. 2005; Mackay et al. 2007). Acetogens can further degrade the methyl group through acetogenesis, and the acetate produced can be readily used by a variety of microorganisms in the subsurface community (Weber et al. 1984; Youngster et al. 2010). Youngster et al. (2008) examined the role of acetogens in MTBE degradation and found that MTBE-degrading enrichment cultures could degrade other aromatic ethers and that the addition of aromatic ethers as co-substrates increased MTBE degradation rates. Youngster et al. (2008) also found that MTBE biodegradation was inhibited by propyl iodide. This provided more evidence for an initial O-demethylation mechanism because propyl iodide has been shown to inhibit O-demethylation dependent on a corrinoid-containing protein acting as a methyl acceptor (Choi et al. 1994; Naidu and Ragsdale 2001).

Since oxygen is rapidly depleted in hydrocarbon-impacted groundwater, understanding the microorganisms involved in anaerobic MTBE degradation and the conditions necessary for sufficient biodegradation rates are important areas of research. No anaerobic MTBE-degrading microorganisms have been isolated to date, but the composition of microbial consortia has recently begun to be investigated. In a study characterizing anaerobic MTBE-

degrading cultures, 16S-rDNA-based amplified ribosomal DNA restriction analysis indicated the presence of novel microorganisms that were not closely related to any known genera or species (Wei and Finneran 2009). Raynal et al. (2010) analyzed 16S rRNA from three microbial consortia utilizing either iron (III), sulfate, or a combination of the two as electron acceptors, and identified microorganisms that were related to known MTBE degraders at the genus or species level, including *Achromobacter*, *Pseudomonas* spp., *Rhodococcus*, and *Sphingomonas*. One of the 16S rRNA gene sequences was 99% similar to *Ochrobactrum cytisi*, a known MTBE degrader. In another study, anaerobic enrichment cultures established from three sources, each of which had retained the ability to degrade MTBE for over a decade, were characterized using terminal-restriction fragment length (T-RFLP) analysis of 16S rRNA genes (Youngster et al. 2010). The community profiles of the three cultures were considerably different, suggesting that multiple species were involved in MTBE biodegradation. Deltaproteobacteria, Firmicutes, and Chloroflexi were the dominant phylotypes indicated by T-RFLP analysis.

DNA-based stable isotope probing (SIP) has also been used to identify MTBE- and TBA-degrading microorganisms. DNA-SIP is a method that traces the fate of a carbon-13 (<sup>13</sup>C)-amended contaminant as it is biodegraded and incorporated into DNA (Radajewski et al. 2000, 2003; Dumont and Murrell 2005; Madsen 2006; Neufeld et al. 2007a, 2007b). DNA-SIP can provide an important link between metabolic activity and phylogeny, which is often lacking in the study of mixed cultures. In a recent DNA-SIP study of MTBE degradation in methanogenic microcosms, bacteria from the Ruminococcaceae family and the *Sphingopyxis* genus and archaea from the *Methanosarcina* and *Methanocorpusculum* genera had <sup>13</sup>C-enriched DNA and were identified as the dominant MTBE degraders in a culture from a wastewater treatment plant (Sun et al. 2012). In a study of TBA-degraders cultured from aerobic granulated activated carbon bioreactors, all microorganisms detected using DNA-SIP belonged to the Burkholderiales order of the Betaproteobacteria. Four functional genes that have been associated with TBA oxidation in pure cultures were also detected in the <sup>13</sup>C-metagenomic DNA (Aslett et al. 2011).

Most SIP studies have been performed on enrichment cultures in microcosms where variables such as carbon sources and electron acceptor availability can be controlled. However, microcosms can never completely replicate in situ conditions, and it is estimated that only a fraction of the microorganisms in the environment are currently capable of being cultured (Amann et al. 1995; Hugenholtz et al. 1998). While SIP field studies are inherently less controlled than laboratory studies, they can be used to document biodegradation by indigenous microorganisms under actual aquifer conditions. Numerous field studies have been conducted using bio-traps containing Bio-Sep® beads (University of Tulsa, Tulsa, OK, USA) amended with a <sup>13</sup>C-labeled compound deployed in monitoring wells (Geyer et al. 2005; Busch-Harris et al. 2006, 2008a; Fiorenza et al. 2009; Chiang et al. 2012). After an incubation period, the bio-traps are retrieved and PLFA, dissolved inorganic carbon, and/or methane are extracted for stable isotope analysis. MTBE and TBA biodegradation has been documented in anaerobic aquifers using this method (Busch-Harris et al. 2008a). However, PLFA cannot provide the taxonomic information that DNA can.

The objective of this study was to identify indigenous microorganisms involved in degrading the methoxy group and the tert-butyl group of MTBE and TBA in the first DNA-SIP field study to use bio-traps for these chemicals. Bio-Sep beads amended with  $^{13}\text{C}$ -MTBE,  $^{13}\text{C}$ -MTBE (methoxy carbon labeled), or  $^{13}\text{C}$ -TBA were incubated in a sulfate-reducing MTBE and TBA plume at Vandenberg Air Force Base in Lompoc, CA.  $^{13}\text{C}$ -DNA and  $^{12}\text{C}$ -DNA extracted from the Bio-Sep beads were cloned and sequenced to identify the microorganisms involved in MTBE and TBA biodegradation.

## SITE DESCRIPTION

Site 60 at Vandenberg Air Force Base (VAFB) in Lompoc, California, is the location of a former gas station, and has been described previously (Mackay et al. 2006, 2007; North et al. 2012). In 1994, a leak was responsible for the release of an estimated 572 gallons (2165 L) of gasoline. Underground storage tanks and piping were excavated in 1995, and the area was backfilled with gravel, sand, and site sediment. A site map including the excavated area and the monitoring wells used in this study is shown in Figure 1. Downgradient from the backfill, the subsurface is composed of layers of silt and clayey silt interspersed with several thin, horizontal layers of sand. Site groundwater in the S3 aquifer occurs between approximately 2.5 and 3.5 m below-ground surface (bgs) and was estimated to flow to the northeast during the study period. Sulfate was the primary electron acceptor in site groundwater, and no dissolved oxygen was detectable in the plume.

The predominant contaminant along the centerline of the plume emanating from the former source area from 1995 through 2004 was MTBE (Mackay et al. 2006). During that time, the MTBE plume extended at least 520 m whereas the BTEX extended no further than 25 m from the source. By 2004, BTEX were at very low concentrations; for example TPHg was less than 0.04 mg/L. After 2004, the predominant contaminant within the aquifer at and downgradient of B1 (Figure 1) was TBA. The most likely cause of the shift in plume composition was in situ transformation of MTBE to TBA stimulated by experimental injection of ethanol into the aquifer starting in 2004 (Mackay et al. 2007; Rasa et al. 2011). Even after cessation of ethanol addition in 2005, TBA continued to be the dominant volatile organic carbon (VOC) in the plume, suggesting the transformation of MTBE to TBA was initiated somehow by ethanol addition but persisted even after ethanol addition ceased (Rasa et al. 2011). A subsequent injection experiment in 2005 to 2006 included injection of MTBE- and ethanol-spiked groundwater in one lane and TBA spiked groundwater into wells upgradient of B1 at an average concentration of 2.8 mg/L (unpublished results). BTEX concentrations decreased below detectable levels, but MTBE and TBA continued to emanate from the source area, with concentrations decreasing slowly (Rasa et al. 2011). These observations suggested that BTEX biodegradation processes have been more ubiquitous and effective than the processes responsible for the biodegradation of MTBE and TBA.

In 2008, bio-traps were deployed in the following 2-in (5.1-cm) monitoring wells: B2, B5-1, B5-2, and B5-3. In early July 2008, groundwater sampling of wells B1-1, B1-2, and B1-3 the three wells immediately upgradient from B2, indicated that TBA concentrations in these wells were 32  $\mu\text{g/L}$ , 138  $\mu\text{g/L}$ , and 5  $\mu\text{g/L}$ , respectively, and MTBE concentrations were 12  $\mu\text{g/L}$ , 46  $\mu\text{g/L}$ , and 4  $\mu\text{g/L}$ , respectively. In August 2008 no MTBE was detected in the three

wells in transect EJ (Figure 1) that were approximately 7.6 m immediately downgradient of the B5 wells, but TBA concentrations in these wells ranged between 117 µg/L and 192 µg/L. Contaminant concentrations in the B5 wells were not monitored before or during the study, but they are near the plume centerline and thus likely to have had very similar concentrations as observed in transects downgradient, as was the case for other monitoring events for months before and after this study.

## MATERIALS AND METHODS

### Bio-Sep Bio-Traps

All bio-traps consisted of a 15.24-cm long, 2.54-cm diameter Schedule 40 PVC tubes with two rows of 1.6-mm slots 3 mm apart along the entire tube length to allow groundwater contact with the Bio-Sep beads. The tubes were sealed at both ends with PVC end caps that were held in place with stainless steel set screws. All PVC parts were washed with methanol prior to use. The bio-traps were filled with approximately 450 Bio-Sep beads either amended with 13C5-MTBE (fully labeled with 13C), 13C1-MTBE (methoxy carbon labeled only), or 13C4-TBA (fully labeled with 13C) (99 + %; Sigma Aldrich, Miamisburg, Ohio). Bio-Sep beads were amended with MTBE or TBA by vapor-phase adsorption under reduced pressure as described previously (Geyer et al. 2005). The fully labeled MTBE amendment was 100% 13C5-amended MTBE, and the total MTBE concentration on the beads was  $0.46 \pm 0.025$  mg/bead (mean  $\pm$  standard deviation,  $n = 3$ ). The MTBE amendment with only the methoxy carbon labeled was 100% 13C1-amended MTBE, and the total MTBE concentration on the beads was  $0.59 \pm 0.026$  mg/bead. The fully labeled TBA amendment was 100% 13C4-amended TBA, and the total TBA concentration on the beads was  $1.03 \pm 0.06$  mg/bead. The bio-traps were assembled under sterile conditions and stored in sterile Whirl-pak® bags (Nasco, Fort Atkinson, Wisconsin) at 4 °C until deployed.

### Bio-Trap Deployment

In July 2008, two 13C1-MTBE-amended bio-traps were deployed in well B2. The top trap was installed 30.5 cm below the water table, and the second trap hung 30.5 cm below the first. At the same time, wells B5-1 and B5-3 received one 13C4-TBA-amended bio-trap each, both suspended 30.5 cm below the water table. Two 13C4-TBA-amended bio-traps were installed in well B5-2. The two bio-traps in well B5-2 were separated by 30.5 cm, and the top of the first bio-trap was 30.5 cm below the water table. After a 41-d incubation period, all bio-traps were retrieved and shipped overnight, on ice to Microbial Insights Inc. (Knoxville, Tennessee) for DNA extraction and separation into 12C- and 13C-DNA fractions. On the same day, two 13C5-MTBE-amended bio-traps were deployed in well B2. The two bio-traps were separated by 30.5 cm, and the top of the first bio-trap was installed 30.5 cm below the water table. After a 49-d incubation period, the bio-traps were retrieved and shipped overnight, on ice to Microbial Insights for DNA extraction and separation into 12C- and 13C-DNA fractions.

### Bio-Sep Bead Analysis

**Bio-Sep Bead MTBE and TBA Analysis**—Bio-Sep beads were shipped from Microbial Insights to the University of Tulsa (Tulsa, Oklahoma) for gas chromatography-mass

spectrometry (GC-MS) analysis of MTBE and TBA concentrations. The MTBE and TBA concentrations in Bio-Sep beads were determined by extraction with methylene chloride (1 mL/bead), followed by GC-MS analysis. Analysis was performed on a GC-MS QP5000 (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) using a 5-point calibration curve. 13C5-MTBE, 13C1-MTBE, and 13C4-TBA concentrations in extracts were determined by selected ion monitoring using ions (m/z) of 77, 74, and 63, respectively (Busch-Harris et al. 2008a). Contaminant concentration in Bio-Sep beads are reported here as the mean  $\pm$  standard deviation (n = 3).

**Separation of 13C-DNA and 12C-DNA:** DNA was extracted from the Bio-Sep beads using a MO BIO Power Soil DNA kit (MO BIO Laboratories Inc., Carlsbad, California). 13C-DNA was isolated from 12C-DNA using cesium chloride (CsCl) density gradient centrifugation (Radajewski et al. 2000; Gallagher et al. 2005). Ultracentrifuge tubes were filled with 1.0 g of CsCl dissolved in 1000  $\mu$ L of TE buffer (10 mM TRIS, 1 mM EDTA, pH 7.5), 20  $\mu$ L of ethidium bromide (10 mg/mL), and 600 ng of DNA. The tubes were sealed and centrifuged at 75,000 rpm for 48 h at 20 °C. Two bands spaced about 9 to 12 mm apart were visible in the tubes after centrifugation, and the bands were withdrawn separately using sterile 18 gauge needles. To remove the ethidium bromide, 10 to 20 volumes of 1-butanol were mixed with each band, and the organic layer was discarded. This process was repeated 4 to 5 times until all ethidium bromide was removed. One-tenth TE buffer was added to bring the volume of the extracted DNA up to 500  $\mu$ L, and 50  $\mu$ L of 3 M sodium acetate (pH 5.2), 1  $\mu$ L of glycogen, and 1000  $\mu$ L of cold 100% isopropanol were added. The DNA fractions were stored at -20 °C for at least an hour and then centrifuged at 13,000 rpm for 30 min. The supernatant was poured off, and 500  $\mu$ L of cold 70% ethanol was added. The DNA fractions were centrifuged at 13,000 rpm for 5 min two times, with the supernatant discarded after each time. After the pellets of DNA were dry, the isolated DNA fractions were resuspended in 50  $\mu$ L of 1/10 TE buffer. The DNA fractions were sent to the University of Oklahoma (Norman, Oklahoma) for polymerase chain reaction (PCR) amplification and cloning.

### DNA Cloning and Sequencing

DNA cloning was performed by Dr. Kathleen Duncan at the University of Oklahoma (Norman, Oklahoma). Six clone libraries were created, a 13C-DNA and a 12C-DNA library from each of the three types of bio-traps: 13C5-MTBE, 13C1-MTBE, and 13C4-TBA. Primers (e.g., 27 F and 1391R) and PCR reaction conditions as recommended by the DOE Joint Genome Institute ([http://my.jgi.doe.gov/general/protocols/SOP\\_16S18S\\_rRNA\\_PCR\\_Library\\_Creation.pdf](http://my.jgi.doe.gov/general/protocols/SOP_16S18S_rRNA_PCR_Library_Creation.pdf)) were used to amplify approximately 1350 to 1400 base pairs (bp) of the eubacterial 16S rRNA gene sequence. The PCR products were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, California), and the manufacturer's instructions were followed to maximize the diversity of sequences cloned. Transformed colonies were inoculated into a 96-well plate containing tryptone-yeast extract glycerol broth with ampicillin (Elshahed et al. 2003). The colonies were grown overnight at 37 °C and stored at -80 °C until sequencing. DNA sequencing was performed by Microgen (Oklahoma City, Oklahoma) using an ABI 3730 Capillary Sequencer and M13F and M13R primers.

## Sequence Analysis

The SEQUENCHER program (Gene Codes Corp., Ann Arbor, Michigan) was used to view the chromatograms and assemble consensus sequences from the contigs. The greengenes NAST-aligner was used to align the sequences, and Bellerophon (Version 3, [http://greengenes.lbl.gov/cgi-bin/nph-bel3\\_interface.cgi](http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi)) was used to detect and remove chimeras. The DOTUR program (Schloss and Handelsman 2005) used distance matrices (DeSantis et al. 2006) created from the 16S rRNA gene sequence libraries to group sequences sharing 97% similarity into operational taxonomic units. One representative sequence was selected from each operational taxonomic unit, and taxonomic affiliations were determined by the Ribosomal Database Project Classifier (Wang et al. 2007). BLASTN (Altschul et al. 1997) was used to identify the closest match to representative sequences in the GenBank database. Good's method (1953) was used to estimate the percentage library coverage at the 97% level of similarity. The representative sequences determined in this study have been submitted to the GenBank database and assigned nucleotide sequence accession numbers KC758898 to KC758957. Sequences of type strains, obtained from the SEQMATCH program (Cole et al., 2008) that most closely matched representative sequences and outgroup sequences were aligned using ClustalX (Version 1.81) (Thompson et al. 1997). The neighbor-joining method of Saitou and Nei (1987) was used to demonstrate the phylogenetic relationships among selected sequences, as implemented in ClustalX (Thompson et al. 1997). The support for the tree branches was estimated from 1000 bootstrap replicates (Felsenstein 1985). Only representative sequences for two or more sequences were used for construction of the phylogenetic trees.

## RESULTS AND DISCUSSION

Prior to deployment, the Bio-Sep beads were amended with 13C5-MTBE, 13C1-MTBE, or 13C4-TBA. A comparison of the initial and post-incubation MTBE and TBA concentrations is shown in Table 1. In each case, 13C-labeled oxygenate was lost from all bio-traps during the incubation period, possibly in part due to leaching, but isotopic analysis of DNA provided evidence that some loss was due to biodegradation. Although contaminant concentrations were not measured after the first deployment of bio-traps, groundwater samples collected from well B2 1 d after the 13C5-MTBE bio-traps were removed showed significant MTBE and TBA concentrations (23.6 mg/L and 9.6 mg/L, respectively). Most of the MTBE was presumably from the MTBE-amended bio-traps from the first and second deployments, and the TBA was likely a biotransformation product of the MTBE. In an ex situ study of 13C5-MTBE and 13C4-TBA bio-traps at the VAFB Site 60, researchers found that approximately 90% of the TBA and 45% of the MTBE desorbed from the Bio-Sep beads during a 6-week time period, with the majority of the mass lost during the first few days of the study (North et al. 2012).

Evidence of 13C incorporation into microbial DNA was found in all three types of bio-traps, indicating that microorganisms present at the site were capable of degrading MTBE and TBA. Six clone libraries were developed from the 13C- and 12C-DNA extracted from the 13C5-MTBE, 13C1-MTBE, and 13C4-TBA bio-traps. A total of 205 sequences from the 6 clone libraries were analyzed and grouped into 60 operational taxonomic units based on



97% sequence similarity. Libraries ranged from 26 to 41 sequences. Library coverage was estimated by Good's method (Good 1953). The 13C1-MTBE clone library had the highest percent coverage (93%), suggesting that the majority of the microorganisms in this sample were represented in the clone library. The 12C clone library from the 13C5-MTBE bio-trap had the lowest percent coverage (58%), indicating that additional sequencing of clones from this sample would be expected to yield additional phylotypes. The number of sequences and operational taxonomic units found in each clone library and library coverage are shown in Table 2.

A variety of microorganisms had incorporated 13C from the labeled MTBE or TBA into their DNA. Thirty-nine operational taxonomic units were identified in the 13C-DNA clone libraries, 19 of which were unique to the 13C-DNA and not detected in any of the 12C-DNA libraries. The number of operational taxonomic units shared by any two samples is summarized in Table 3. The dissimilarity between the 12C- and 13C-clone libraries suggested that the 13C-DNA sequences represented a sub-sample from the total microbial population of microorganisms involved in MTBE and TBA biodegradation. The three 13C-clone libraries were also dissimilar from each other, suggesting that different microorganisms were involved in processing the methoxy carbon and the tert-butyl group of MTBE and TBA.

A summary of the most abundant class, order, and genus for each of the clone libraries based on Ribosomal Database Project Classifier results is shown in Table 4, and graphs of affiliations for the 13C- and 12C-DNA clone libraries are shown in Figures 2 through 5. Complete Ribosomal Database Project Classifier and BLASTN results for each operational taxonomic unit are included in Table S1(Supporting Information) and the phylogenetic affiliation of the operational taxonomic units to those of bacterial type strains are shown in Figure 6A and 6B. Three relevant features of this dataset emerge when comparing clone libraries: (1) Gammaproteobacteria, specifically the genus *Pseudomonas*, predominated in the 13C1-MTBE clone library; (2) Deltaproteobacteria were detected in the 13C5-MTBE and 13C4-TBA clone libraries but were absent from the 13C1-MTBE clone library, and (3) multiple operational taxonomic units related to known MTBE- and TBA-degraders were identified in all three 13C clone libraries.

As shown in Table 4, the 13C1-MTBE clone library was primarily composed of Gammaproteobacteria (78%), and 19 of these sequences were most similar (99% over 1396 nucleotides) to *Pseudomonas fluorescens* (CP003041.1) (Loper et al. 2012). This suggested that this operational taxonomic unit (KC758942) was involved in processing the 13C-labeled methoxy carbon. This operational taxonomic unit was also detected in the 13C4-TBA library (4 sequences), the 12C library from the 13C1-MTBE bio-trap (7 sequences), and the 12C clone library from the 13C4-TBA bio-trap (2 sequences) (Figures 4 and 5). *Pseudomonas* is a large genus known for its ubiquity in the environment and ability to metabolize a variety of organic compounds (Lessie and Phibbs 1984; Kukor and Olsen 1990; Ahn et al. 1998; Abuhamed et al.2004; Wu et al. 2011). It is unclear whether this *Pseudomonas* operational taxonomic unit was a primary degrader of MTBE or if it was feeding on the resulting metabolites since cross-feeding of isotopically labeled metabolites can occur during DNA-SIP experiments (Radajewski et al. 2003).

*P. fluorescens* has been observed to grow on acetate (Kornberg 1958), so this operational taxonomic unit may be outcompeting other microorganisms for the acetate intermediate of MTBE degradation. Research has shown that acetogenic microorganisms are likely involved in the initial O-demethylation of MTBE, and a variety of microorganisms are capable of assimilating the resulting acetate (Kuder et al. 2005; Mackay et al. 2007; Youngster et al. 2010). *Pseudomonas* species that have been directly linked to MTBE degradation have been most frequently reported degrading MTBE co-metabolically with hydrocarbons, which would not result in  $^{13}\text{C}$  being directly incorporated into the microorganism's DNA (Garnier et al. 1999; Smith and Hyman 2004; Schmidt et al. 2004).

In future studies, retrieving bio-traps at different time periods may provide a better picture of the sequence of carbon flow through the microbial population. In a DNA-SIP microcosm study of MTBE-degrading enrichment cultures conducted by Youngster (2009), T-RFLP profiles of samples collected from six cultures at six time-points indicated that some microorganisms incorporated  $^{13}\text{C}$  earlier than others, presumably the microorganisms directly involved in MTBE biodegradation. After 35 d, T-RFLP profiles from  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA were highly similar, which suggested that the  $^{13}\text{C}$  was spreading through the community through cross-feeding.

Deltaproteobacteria were detected in the  $^{13}\text{C}5$ -MTBE and  $^{13}\text{C}4$ -TBA clone libraries but were absent from the  $^{13}\text{C}1$ -MTBE clone library (Figure 2). This class includes several iron-reducers and sulfate-reducing bacteria (SRB). Sequences with 100% similarity over 1413 nucleotides to the iron-reducing genus *Geobacter* (KC758938, KC758939) were detected in both the  $^{13}\text{C}4$ -TBA (4 sequences) and  $^{13}\text{C}5$ -MTBE (8 sequences) clone libraries. Clones related to *Geobacter* have been identified in previously characterized MTBE-degrading enrichment cultures (Raynal et al. 2010; Youngster et al. 2010). The  $^{13}\text{C}4$ -TBA clone library also contained a sequence similar (100% over 1397 nucleotides, KC758898) to *Geothrix*, an iron-reducing microorganism in the Acidobacteria class (Nevin and Lovley 2002). The  $^{13}\text{C}4$ -TBA clone library included sequences with 100% similarity to the following SRB: *Desulfobulbus* (2 sequences, KC758932, 1418 nucleotides), *Desulfovibrio* (4 sequences, KC758934, 1409 nucleotides), *Desulfuromonas* (3 sequences, KC758936, 1416 nucleotides), and an unclassified bacterium from the *Desulfobulbaceae* family (1 sequence, KC758933, 1403 nucleotides). The  $^{13}\text{C}5$ -MTBE clone library also contained one sequence (KC758936) similar (100% over 1416 nucleotides) to *Desulfuromonas*. These results suggested that iron reducers and SRB were involved in processing the carbon from the degradation of TBA or the tert-butyl group of MTBE since they were not detected in the  $^{13}\text{C}1$ -MTBE clone library where only the methoxy carbon was labeled.

The opposite trend was observed in a previous SIP study where SRB appeared to be more involved in processing the methoxy carbon of MTBE than the tert-butyl group (Busch-Harris et al. 2008b). In the study, anaerobic sediments from MTBE-contaminated aquifers were added to sulfate-amended microcosms containing Bio-Sep beads amended with either  $^{13}\text{C}5$ -MTBE,  $^{13}\text{C}1$ -MTBE (methoxy carbon labeled),  $^{13}\text{C}3$ -MTBE (tert-butyl group labeled), or  $^{13}\text{C}4$ -TBA. After 4 months, PLFA was extracted from the Bio-Sep beads and analyzed for  $^{13}\text{C}$  enrichment.  $^{13}\text{C}$ -labelling was evident in PLFA from the  $^{13}\text{C}5$ -MTBE and  $^{13}\text{C}1$ -MTBE microcosms, and 10me16:0, a biomarker associated with the sulfate-reducer

Desulfobacter (Dowling et al. 1986; Kohring et al. 1994), was highly enriched with  $^{13}\text{C}$ . However, the Desulfobacter biomarker from the  $^{13}\text{C}_4$ -TBA microcosm was less  $^{13}\text{C}$ -enriched, and no  $^{13}\text{C}$ -labeling was detected in PLFA from the  $^{13}\text{C}_3$ -MTBE microcosm. One possible explanation the differences in metabolic activity exhibited by SRB in the PLFA-SIP study and this study is that some SRB involved in biodegradation may not have been identified by PLFA-SIP, since PLFA analysis cannot identify taxonomy with the precision of DNA sequencing. Also, Desulfobacter was the microorganism associated with the degradation of the methoxy group in the PLFA-SIP study, but it was not among the SRB genera detected in this study.

$^{13}\text{C}$ -DNA from the  $^{13}\text{C}_5$ -MTBE or  $^{13}\text{C}_4$ -TBA clone libraries was more likely to represent primary degraders than  $^{13}\text{C}$ -DNA from the  $^{13}\text{C}_1$ -MTBE clone library since TBA is more persistent in the environment than metabolites from the methoxy group, such as acetate, and TBA accumulation has been previously documented at VAFB Site 60 (Mackay et al. 2007). Several sequences from the  $^{13}\text{C}_5$ -MTBE or  $^{13}\text{C}_4$ -TBA clone libraries were related to known aerobic MTBE and TBA degraders or microorganisms that had been previously identified in MTBE-degrading enrichment cultures. Since wells were sealed at the surface and the aquifer was relatively shallow, aerobic microorganisms may have been supported by oxygen diffusing from the surface or from unimpacted groundwater outside of the plume.

Burkholderiales was the most abundant order (24%) in the  $^{13}\text{C}_5$ -MTBE library (Table 4), and four sequences in the  $^{13}\text{C}_4$ -TBA clone library and five sequences in the  $^{13}\text{C}_1$ -MTBE clone library also belonged to this order (Figure 4). Genera identified as members of order Burkholderiales included Hydrogenophaga, Rhodoferax, Aquabacterium, and Achromobacter. Three sequences from the  $^{13}\text{C}_4$ -TBA library and three sequences from the  $^{13}\text{C}_5$ -MTBE library (KC758925) had 100% similarity to Hydrogenophaga over 1388 nucleotides, and six sequences from the  $^{13}\text{C}_5$ -MTBE library (KC758926) were 95% similar to Rhodoferax over 1392 nucleotides. An operational taxonomic unit with 99% similarity over 1390 nucleotides to Aquabacterium was detected in the  $^{13}\text{C}_1$ -MTBE and  $^{13}\text{C}_5$ -MTBE libraries (KC758924). Four sequences from the  $^{13}\text{C}_1$ -MTBE clone library (KC758923) were 100% similar to Achromobacter over 1390 nucleotides. The  $^{13}\text{C}_4$ -TBA clone library contained an operational taxonomic unit of unclassified genus (KC758927) with 99% similarity over 1393 nucleotides to the Oxalobacteraceae family in the Burkholderiales order.

Many known aerobic MTBE and TBA degraders belong to Burkholderiales, including *Methylibium petroleiphilum* PM1 (Hanson et al. 1999), *Aquincola tertiaricarbonis* L108 (Müller et al. 2008), and *Hydrogenophaga flava* ENV735 (Steffan et al. 1997; Hatzinger et al. 2001). In a DNA-SIP study conducted on TBA-utilizing microorganisms from oxygenated granulated activated carbon reactors, all  $^{13}\text{C}$ -DNA clones belonged to Burkholderiales, including a sequence similar to *Hydrogenophaga taeniospiralis* (98.5%) and one similar to *Rhodoferax ferrireducens* (99.1%) (Aslett et al. 2011). These two genera belong to Comamonadaceae, which also contains *M. petroleiphilum* PM1. *Aquabacterium* has also been reported as a close relative of *M. petroleiphilum* PM1 (Bruns et al. 2001). An *Achromobacter* clone was detected in MTBE-degrading cultures under both sulfate- and

iron-reducing conditions (Raynal et al. 2010), and *Achromobacter xylosoxidans* MCM1/1 is reported to be capable of MTBE degradation (Eixarch and Constantí 2010).

Sequences with 100% similarity to *Sphingobium* (class Alphaproteobacteria, family Sphingomonadaceae) were detected in the 13C4-TBA (1 sequence) and 13C1-MTBE libraries (2 sequences, KC758922, 1347 nucleotides). In a DNA-SIP study of MTBE degradation in methanogenic microcosms, 18 clones enriched with 13C were related to *Sphingopyxis* of the Sphingomonadaceae family (Sun et al. 2012). The *Sphingomonas* genus in this family has also been associated with MTBE degradation. A *Sphingomonas* strain able to grow aerobically with only MTBE as a carbon source has been isolated (Okeke and Frankenberger 2003), and *Sphingomonas* has been identified in MTBE-degrading consortia in bioreactors (Pruden et al. 2001; Zein et al. 2004). Additionally, a clone 98% similar to *Sphingomonas* was detected in an iron-reducing microcosm of an MTBE-degrading enrichment culture (Raynal et al. 2010).

In contrast to the operational taxonomic units classified as Proteobacteria, the sequences affiliated with Spirochaetes, Bacteroidetes, Firmicutes, and other groups often were only distantly related to described strains of bacteria (Figure 6B). Hence, although such sequences were relatively abundant in our samples and in other subsurface bioremediation sites (see Supporting Information), their role, if any, in MTBE degradation remains unknown.

## CONCLUSIONS

Results from this study demonstrated that microorganisms were actively degrading 13C-labeled MTBE and TBA in bio-traps in situ and the 13C was incorporated into their DNA. The most frequently detected operational taxonomic unit in the 13C1-MTBE clone library was 99% similar to *P. fluorescens*. Although some *Pseudomonas* species have been reported to co-metabolically degrade MTBE with hydrocarbons (Garnier et al. 1999; Smith and Hyman 2004; Schmidt et al. 2004), it is likely that this species was cross-feeding on 13C-labeled metabolites and was a secondary degrader. *P. fluorescens* has been reported to grow on acetate (Kornberg 1958), a product associated with the O-demethylation and subsequent acetogenesis of MTBE. The operational taxonomic units in the 13C1-MTBE clone library related to *Aquabacterium*, *Achromobacter*, and *Sphingobium* are more likely to be involved in the initial step of MTBE degradation since these genera are related to previously documented MTBE degraders.

Operational taxonomic units from the 13C5-MTBE and 13C4-TBA libraries were likely to represent primary degraders since TBA is much more persistent in the environment than the intermediates resulting from the O-demethylation of MTBE. Several operational taxonomic units from these clone libraries were related to known MTBE and TBA degraders or microorganisms identified in MTBE-degrading enrichment cultures, especially the four operational taxonomic units related to the Burkholderiales order. Many operational taxonomic units related to SRB and iron-reducing genera were also detected in the 13C5-MTBE and 13C4-TBA libraries, suggesting that they were involved in processing carbon from the tert-butyl group.

Additional microorganisms involved in the carbon flow from MTBE and TBA degradation may not have been identified by DNA-SIP due to its low sensitivity. Slow growth and degradation rates have been reported for many MTBE-degrading isolates (Hanson et al. 1999; Deeb et al. 2001; François et al. 2002). MTBE degraders also grow more slowly on 13C-MTBE than 12C-MTBE. For example, an MTBE-degrading enrichment culture degraded 300 µM 12C-MTBE in approximately 50 d, while only about 150 µM 13C-MTBE had been degraded after 60 d (Youngster 2009). It is possible that slow-growing MTBE- and TBA-degraders had not incorporated enough 13C-DNA or were not present in high enough concentrations to be detected or to dominate the sequences analyzed in this study. In the future, longer bio-trap incubation times may lead to increased detection of any slow-growing organisms.

While microorganisms capable of degrading MTBE and TBA were detected through DNA-SIP, these contaminants have persisted at VAFB Site 60 for over a decade. The low concentrations (4 to 46 µg/L MTBE and 5 to 138 µg/L TBA) present in groundwater prior to this study may not have been high enough to induce MTBE and TBE biodegradation. Pre-deployment concentrations in the Bio-Sep beads (460 µg/bead 13C5-MTBE, 590 µg/bead 13C1-MTBE, and 1000 µg/bead 13C4-TBA) were higher than aquifer concentrations, and leaching from the beads significantly increased contaminant concentrations in the monitoring well. These higher concentrations may have stimulated members of the microbial community that were previously dormant (Hyman 2012). Most DNA-SIP studies use concentrations of 13C-labeled materials that are higher than environmentally relevant because DNA is the least sensitive biomarker used in SIP studies. Cell division is required for 13C to be incorporated into DNA, and DNA must contain a minimum of 15 to 20% 13C to be separated from 12C-DNA (Radajewski et al. 2003). In future studies, Bio-Sep beads could be amended with MTBE and TBA in concentrations that are more representative of concentrations at the site. Bio-Sep beads could also be pre-leached in sterile water prior to deployment to remove the most labile fraction of MTBE or TBA and avoid any regulatory issues associated with contaminant releases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

- Abuhamed T, Bayraktar E, Mehmeto lu T, Mehmeto lu Ü. Kinetics model for growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation. *Process Biochemistry*. 2004; 39(8):983–988.
- Ahn I-S, Ghiorse WC, Lion LW, Shuler ML. Growth kinetics of *Pseudomonas putida* G7 on naphthalene and occurrence of naphthalene toxicity during nutrient deprivation. *Biotechnology and Bioengineering*. 1998; 59(5):587–594. [PubMed: 10099376]

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST a new generation of protein database search programs. *Nucleic Acids Research*. 1997; 25(17):3389–3402. [PubMed: 9254694]
- Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*. 1995; 59(1):143–169. [PubMed: 7535888]
- Aslett D, Haas J, Hyman M. Identification of tertiary butyl alcohol (TBA)-utilizing organisms in BioGAC reactors using <sup>13</sup>C-DNA stable isotope probing. *Biodegradation*. 2011; 22(5):961–972. [PubMed: 21286787]
- Bradley PM, Landmeyer JE, Chapelle FH. TBA biodegradation in surface-water sediments under aerobic and anaerobic conditions. *Environmental Science and Technology*. 2002; 36(19):4087–4090. [PubMed: 12380079]
- Bradley PM, Chapelle FH, Landmeyer JE. Effect of redox conditions on MTBE biodegradation in surface water sediments. *Environmental Science and Technology*. 2001; 35(23):4643–4647. [PubMed: 11770765]
- Bruns MA, Hanson JR, Mefford J, Scow KM. Isolate PM1 populations are dominant and novel methyl tert-butyl ether-degrading bacteria in compost biofilter enrichments. *Environmental Microbiology*. 2001; 3(3):220–225. [PubMed: 11321538]
- Busch-Harris J, Sublette K, Roberts KP, Landrum C, Peacock AD, Davis G, Ogles D, Holmes WE, Harris D, Ota C, Yang X, Kolhatkar A. Bio-traps coupled with molecular biological methods and stable isotope probing demonstrate the in situ biodegradation potential of MTBE and TBA in gasoline-contaminated aquifers. *Ground Water Monitoring & Remediation*. 2008a; 28(4):47–62.
- Busch-Harris, J.; Sublette, K.; Wilson, JT.; Adair, C.; Yang, X.; Kuder, T.; Philip, P.; Davis, G.; McElroy, D.; Holmes, WE.; Harris, D. International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Abstract K-036. Monterey, California: Battelle Press; 2008b. Stable isotope probing with <sup>13</sup>C-MTBE- and <sup>13</sup>C-TBA-amended Bio-Sep® beads in sulfate-reducing microcosms.
- Busch-Harris J, Jennings EM, Sublette KL, Roberts KP, White DC, Peacock AD, Davis G, Ogles D, Holmes WE, Yang X, Kolhatkar A, Beckmann D, Kolhatkar R. Monitoring subsurface microbial ecology and demonstrating in situ biodegradation potential using Bio-Sep® bio-traps. *Ecological Chemistry and Engineering*. 2006; 13:349–372.
- Chiang S-YD, Mora R, Diguseppi WH, Davis G, Sublette K, Gedalanga P, Mahendra S. Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools. *Journal of Environmental Monitoring*. 2012; 14(9): 2317–2326. [PubMed: 22825917]
- Choi S-C, Chase T, Bartha R. Metabolic pathways leading to mercury methylation in *Desulfovibrio desulfuricans* LS. *Applied and Environmental Microbiology*. 1994; 60(11):4072–4077. [PubMed: 16349435]
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*. 2008; 37:D141–D145. no. Database issue: [PubMed: 19004872]
- Davis LC, Erickson LE. A review of bioremediation and natural attenuation of MTBE. *Environmental Progress*. 2004; 23(3):243–252.
- Deeb RA, Hu H-Y, Hanson JR, Scow KM, Alvarez-Cohen L. Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. *Environmental Science and Technology*. 2001; 35(2):312–317. [PubMed: 11347603]
- DeSantis TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research*. 2006; 34(suppl 2):W394–W399. [PubMed: 16845035]
- Dowling NJE, Widdel F, White DC. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. *Journal of General Microbiology*. 1986; 132(7):1815–1825.

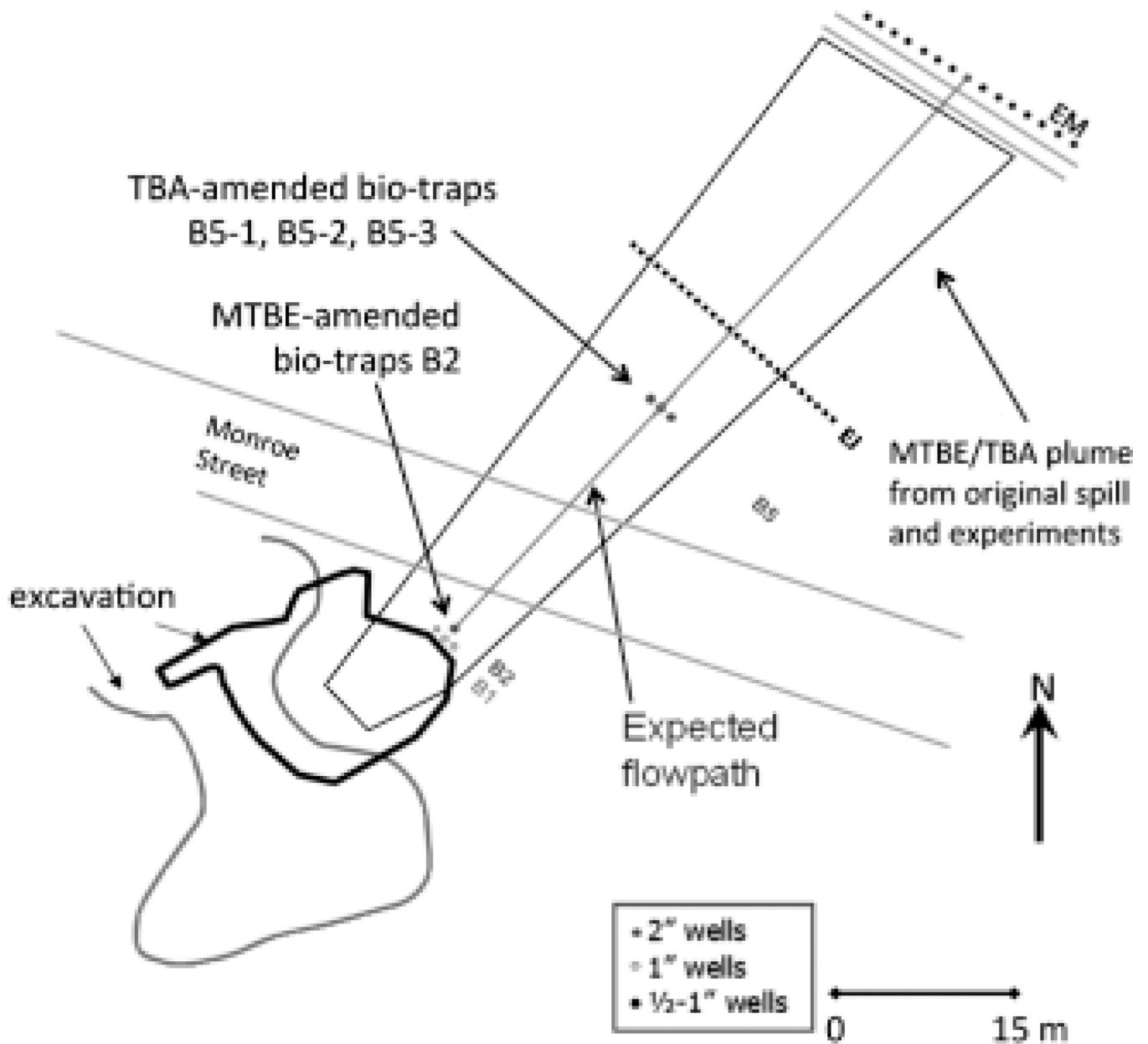
- Dumont MG, Murrell JC. Stable isotope probing—Linking microbial identity to function. *Nature Reviews Microbiology*. 2005; 3(6):499–504.
- Eixarch H, Constantí M. Biodegradation of MTBE by *Achromobacter xylosoxidans* MCM1/1 induces synthesis of proteins that may be related to cell survival. *Process Biochemistry*. 2010; 45(5):794–798.
- Elshahed MS, Senko JM, Najar FZ, Kenton SM, Roe BA, Dewers TA, Spear JR, Krumholz LR. Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Applied and Environmental Microbiology*. 2003; 69(9):5609–5621. [PubMed: 12957951]
- Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. 1985; 39:666–670.
- Finneran KT, Lovley DR. Anaerobic degradation of methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA). *Environmental Science and Technology*. 2001; 35(9):1785–1790. [PubMed: 11355193]
- Fiorenza S, Rifai HS. Review of MTBE biodegradation and bioremediation. *Bioremediation Journal*. 2003; 7(1):1–35.
- Fiorenza S, McClary AC, Lummus S. Use of bio-traps with multiple substrates and electron acceptors to optimize remediation. *Remediation Journal*. 2009; 20(1):69–85.
- François A, Mathis H, Godefroy D, Piveteau P, Fayolle F, Monot F. Biodegradation of methyl tert-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Applied and Environmental Microbiology*. 2002; 68(6):2754–2762. [PubMed: 12039730]
- Gallagher E, McGuinness L, Phelps C, Young LY, Kerkhof LJ. 13C-carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Applied and Environmental Microbiology*. 2005; 71(9):5192–5196. [PubMed: 16151104]
- Garnier PM, Auria R, Augur C, Revah S. Cometabolic biodegradation of methyl t-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Applied Microbiology and Biotechnology*. 1999; 51(4):498–503. [PubMed: 10341433]
- Geyer R, Peacock AD, Miltner A, Richnow HH, White DC, Sublette KL, Kastner M. In situ assessment of biodegradation potential using biotrap amended with 13C-labeled benzene or toluene. *Environmental Science and Technology*. 2005; 39(13):4983–4989. [PubMed: 16053100]
- Good II. The population frequencies of species and the estimation of population parameters. *Biometrika*. 1953; 40(3–4):237–264.
- Hanson JR, Ackerman CE, Scow KM. Biodegradation of methyl tert-butyl ether by a bacterial pure culture. *Applied and Environmental Microbiology*. 1999; 65(11):4788–4792. [PubMed: 10543787]
- Hatzinger PB, McClay K, Vainberg S, Tugusheva M, Condee CW, Steffan RJ. Biodegradation of methyl tert-butyl ether by a pure bacterial culture. *Applied and Environmental Microbiology*. 2001; 67(12):5601–5607. [PubMed: 11722912]
- Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*. 1998; 180(18):4765–4774. [PubMed: 9733676]
- Hyman M. Biodegradation of gasoline ether oxygenates. *Current Opinion in Biotechnology*. 2012
- Kane SR, Beller HR, Legler TC, Koester CJ, Pinkart HC, Halden RU, Happel AM. Aerobic biodegradation of methyltert-butyl ether by aquifer bacteria from leaking underground storage tank sites. *Applied and Environmental Microbiology*. 2001; 67(12):5824–5829. [PubMed: 11722940]
- Kohring LL, Ringelberg DB, Devereux R, Stahl DA, Mittelman MW, White DC. Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA sequence similarities among dissimilatory sulfate-reducing bacteria. *FEMS Microbiology Letters*. 1994; 119(3):303–308. [PubMed: 7519575]
- Kornberg HL. The metabolism of C2 compounds in micro-organisms. I. The incorporation of [2-14C] acetate by *Pseudomonas fluorescens*, and by a *Corynebacterium*, grown on ammonium acetate. *Biochemical Journal*. 1958; 68(3):535–542. [PubMed: 13522656]

- Kuder T, Wilson JT, Kaiser P, Kolhatkar R, Philp P, Allen J. Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: Microcosm and field evidence. *Environmental Science and Technology*. 2005; 39(1):213–220. [PubMed: 15667097]
- Kukor JJ, Olsen RH. Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c. *Journal of Bacteriology*. 1990; 172(8):4624–4630. [PubMed: 2115872]
- Landmeyer JE, Chapelle FH, Bradley PM, Pankow JF, Church CD, Tratnyek PG. Fate of MTBE relative to benzene in a gasoline-contaminated aquifer (1993–98). *Ground Water Monitoring & Remediation*. 1998; 18(4):93–102.
- Lessie TG, Phibbs PV Jr. Alternative pathways of carbohydrate utilization in pseudomonads. *Annual Review of Microbiology*. 1984; 38:359–88.
- Loper JE, Hassan KA, Mavrodi DV, Davis EW 2nd, Lim CK, Shaffer BT, Elbourne LD, Stockwell VO, Hartney SL, Breakwell K, Henkels MD, Tetu SG, Rangel LI, Kidarsa TA, Wilson NL, van de Mortel JE, Song C, Blumhagen R, Radune D, Hostetler JB, Brinkac LM, Durkin AS, Kluepfel DA, Wechter WP, Anderson AJ, Kim YC, Pierson LS 3rd, Pierson EA, Lindow SE, Kobayashi DY, Raaijmakers JM, Weller DM, Thomashow LS, Allen AE, Paulsen IT. Comparative genomics of plant-associated *Pseudomonas* spp.: Insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genetics*. 2012; 8(7):e1002784. [PubMed: 22792073]
- Mackay D, de Sieyes N, Einarson M, Feris K, Pappas A, Wood I, Jacobson L, Justice L, Noske M, Wilson J, Adair C, Scow K. Impact of ethanol on the natural attenuation of MTBE in a normally sulfate-reducing aquifer. *Environmental Science and Technology*. 2007; 41(6):2015–2021. [PubMed: 17410799]
- Mackay DM, de Sieyes NR, Einarson MD, Feris KP, Pappas AA, Wood IA, Jacobson L, Justice LG, Noske MN, Scow KM, Wilson JT. Impact of ethanol on the natural attenuation of benzene, toluene, and o-xylene in a normally sulfate-reducing aquifer. *Environmental Science and Technology*. 2006; 40(19):6123–6130. [PubMed: 17051810]
- Madsen EL. The use of stable isotope probing techniques in bioreactor and field studies on bioremediation. *Current Opinion in Biotechnology*. 2006; 17(1):92–97. [PubMed: 16378724]
- Mormile MR, Liu S, Suflita JM. Anaerobic biodegradation of gasoline oxygenates: Extrapolation of information to multiple sites and redox conditions. *Environmental Science and Technology*. 1994; 28(9):1727–1732. [PubMed: 22176376]
- Müller RH, Rohwerder T, Harms H. Degradation of fuel oxygenates and their main intermediates by *Aquicola tertiarycarbonis* L108. *Microbiology*. 2008; 154(5):1414–1421. [PubMed: 18451050]
- Naidu D, Ragsdale SW. Characterization of a three-component vanillate o-demethylase from *Moorella thermoacetica*. *Journal of Bacteriology*. 2001; 183(11):3276–3281. [PubMed: 11344134]
- Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC. DNA stable-isotope probing. *Nature Protocols*. 2007a; 2(4):860–866.
- Neufeld JD, Wagner M, Murrell JC. Who eats what, where and when? Isotope-labelling experiments are coming of age. *ISME Journal*. 2007b; 1(2):103–110. [PubMed: 18043620]
- Nevin KP, Lovley DR. Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix* fermentans. *Applied and Environmental Microbiology*. 2002; 68(5):2294–2299. [PubMed: 11976100]
- North KP, Mackay DM, Annable MD, Sublette KL, Davis G, Holland RB, Petersen D, Scow KM. An ex situ evaluation of TBA- and MTBE-baited bio-traps. *Water Research*. 2012; 46(12):3879–3888. [PubMed: 22621895]
- Okeke BC, Frankenberger WT Jr. Biodegradation of methyl tertiary butyl ether (MTBE) by a bacterial enrichment consortia and its monoculture isolates. *Microbiological Research*. 2003; 158(2):99–106. [PubMed: 12906382]
- Pruden A, Suidan MT, Venosa AD, Wilson GJ. Biodegradation of methyl tert-butyl ether under various substrate conditions. *Environmental Science and Technology*. 2001; 35(21):4235–4241. [PubMed: 11718336]
- Radajewski S, McDonald IR, Murrell JC. Stable-isotope probing of nucleic acids: A window to the function of uncultured microorganisms. *Current Opinion in Biotechnology*. 2003; 14(3):296–302. [PubMed: 12849783]



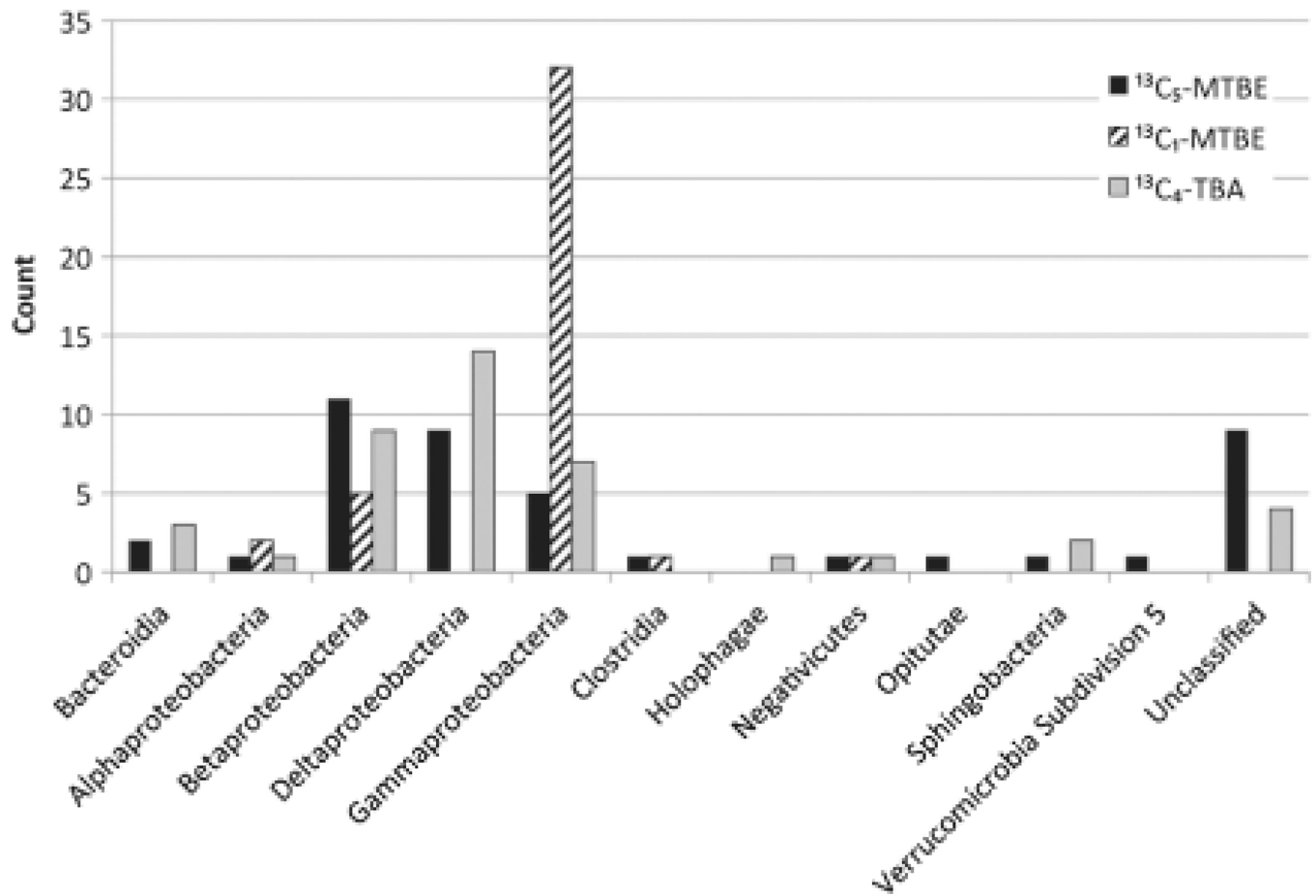
- Radajewski S, Ineson P, Parekh NR, Murrell JC. Stable-isotope probing as a tool in microbial ecology. *Nature*. 2000; 403(6770):646–649. [PubMed: 10688198]
- Rasa E, Chapman SW, Bekins BA, Fogg GE, Scow KM, Mackay DM. Role of back diffusion and biodegradation reactions in sustaining an MTBE/TBA plume in alluvial media. *Journal of Contaminant Hydrology*. 2011; 126(3–4):235–247. [PubMed: 22115089]
- Raynal M, Crimi B, Pruden A. Enrichment and characterization of MTBE-degrading cultures under iron and sulfate reducing conditions. *Canadian Journal of Civil Engineering*. 2010; 37(4):522–534.
- Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 1987; 4:406–425. [PubMed: 3447015]
- Salanitro JP, Diaz LA, Williams MP, Wisniewski HL. Isolation of a bacterial culture that degrades methyl t-butyl ether. *Applied and Environmental Microbiology*. 1994; 60(7):2593–2596. [PubMed: 16349335]
- Schloss PD, Handelsman J. Introducing DOTUR a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology*. 2005; 71(3):1501–1506. [PubMed: 15746353]
- Schmidt TC, Schirmer M, Weiß H, Haderlein SB. Microbial degradation of methyl tert-butyl ether and tert-butyl alcohol in the subsurface. *Journal of Contaminant Hydrology*. 2004; 70(3–4):173–203. [PubMed: 15134874]
- Smith CA, Hyman MR. Oxidation of methyl tert-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and n-octane-grown *Pseudomonas putida* GPo1. *Applied and Environmental Microbiology*. 2004; 70(8):4544–4550. [PubMed: 15294784]
- Somsamak P, Richnow HH, Häggblom MM. Carbon isotope fractionation during anaerobic degradation of methyl tert-butyl ether under sulfate-reducing and methanogenic conditions. *Applied and Environmental Microbiology*. 2006; 72(2):1157–1163. [PubMed: 16461662]
- Somsamak P, Cowan RM, Häggblom MM. Anaerobic biotransformation of fuel oxygenates under sulfate-reducing conditions. *FEMS Microbiology Ecology*. 2001; 37(3):259–264.
- Steffan RJ, McClay K, Vainberg S, Condee CW, Zhang D. Biodegradation of the gasoline oxygenates methyl tert-butyl ether, ethyl tert-butyl ether, and tert-amyl methyl ether by propane-oxidizing bacteria. *Applied and Environmental Microbiology*. 1997; 63(11):4216–4222. [PubMed: 9361407]
- Suflita JM, Mormile MR. Anaerobic biodegradation of known and potential gasoline oxygenates in the terrestrial subsurface. *Environmental Science and Technology*. 1993; 27(5):976–978.
- Sun W, Sun X, Cupples AM. Anaerobic methyl tert-butyl ether-degrading microorganisms identified in wastewater treatment plant samples by stable isotope probing. *Applied and Environmental Microbiology*. 2012; 78(8):2973–2980. [PubMed: 22327600]
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*. 1997; 25:4876–4882. [PubMed: 9396791]
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*. 2007; 73(16):5261–5267. [PubMed: 17586664]
- Weber H, Kulbe K, Chmiel H, Trösch W. Microbial acetate conversion to methane: Kinetics, yields and pathways in a two-step digestion process. *Applied Microbiology and Biotechnology*. 1984; 19(4):224–228.
- Wei N, Finneran K. Microbial community analyses of three distinct, liquid cultures that degrade methyl tert-butyl ether using anaerobic metabolism. *Biodegradation*. 2009; 20(5):695–707. [PubMed: 19340592]
- White GF, Russell NJ, Tidswell EC. Bacterial scission of ether bonds. *Microbiological Reviews*. 1996; 60(1):216–232. [PubMed: 8852901]
- Wilson JT, Adair C, Kaiser PM, Kolhatkar R. Anaerobic biodegradation of MTBE at a gasoline spill site. *Ground Water Monitoring & Remediation*. 2005; 25(3):103–115.
- Wilson GJ, Richter AP, Suidan MT, Venosa AD. Aerobic biodegradation of gasoline oxygenates MTBE and TBA. *Water Science and Technology*. 2001; 43(2):277–284. [PubMed: 11380191]

- Wilson, JT.; Cho, JS.; Wilson, BH.; Vardy, J. Natural Attenuation of MTBE in the Subsurface Under Methanogenic Conditions. Washington, D.C.: Environmental Protection Agency, Office of Research and Development; 2000.
- Wu X, Monchy S, Taghavi S, Zhu W, Ramos J, van der Lelie D. Comparative genomics and functional analysis of niche-specific adaptation in *Pseudomonas putida*. *FEMS Microbiology Reviews*. 2011; 35(2):299–323. [PubMed: 20796030]
- Youngster, L. *Microbiology and Molecular Genetics*. New Brunswick, New Jersey: Rutgers University; 2009. Microbial degradation of the fuel oxygenate methyl tert-butyl ether (MTBE).
- Youngster LKG, Kerkhof LJ, Häggblom MM. Community characterization of anaerobic methyl tert-butyl ether (MTBE)-degrading enrichment cultures. *FEMS Microbiology Ecology*. 2010; 72(2): 279–288. [PubMed: 20180853]
- Youngster LG, Somsamak P, Häggblom M. Effects of co-substrates and inhibitors on the anaerobic O-demethylation of methyl tert-butyl ether (MTBE). *Applied Microbiology and Biotechnology*. 2008; 80(6):1113–1120. [PubMed: 18797864]
- Zein MM, Suidan MT, Venosa AD. MTBE biodegradation in a gravity flow, high-biomass retaining bioreactor). *Environmental Science and Technology*. 2004; 38(12):3449–3456. [PubMed: 15260347]

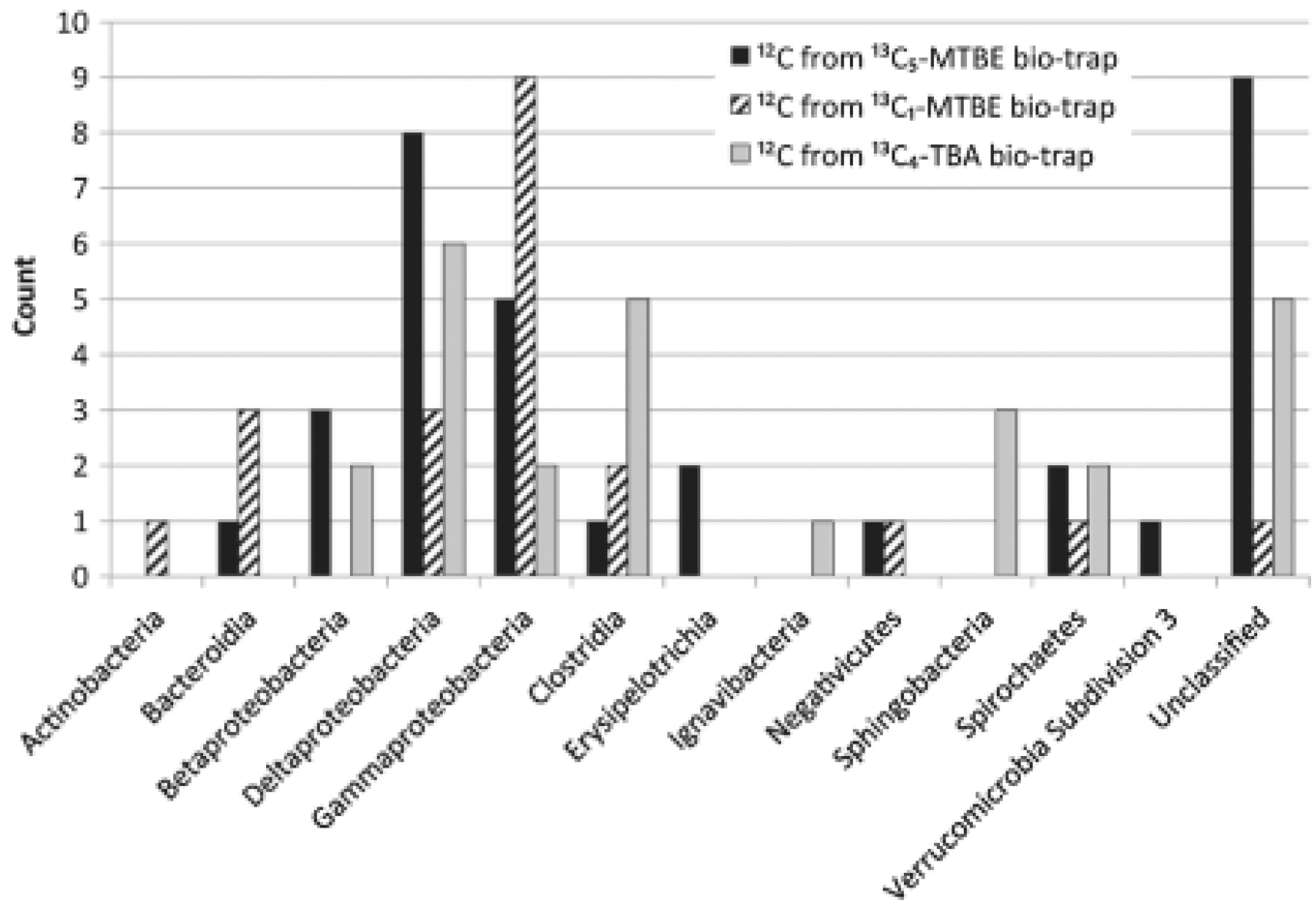


**Figure 1.**

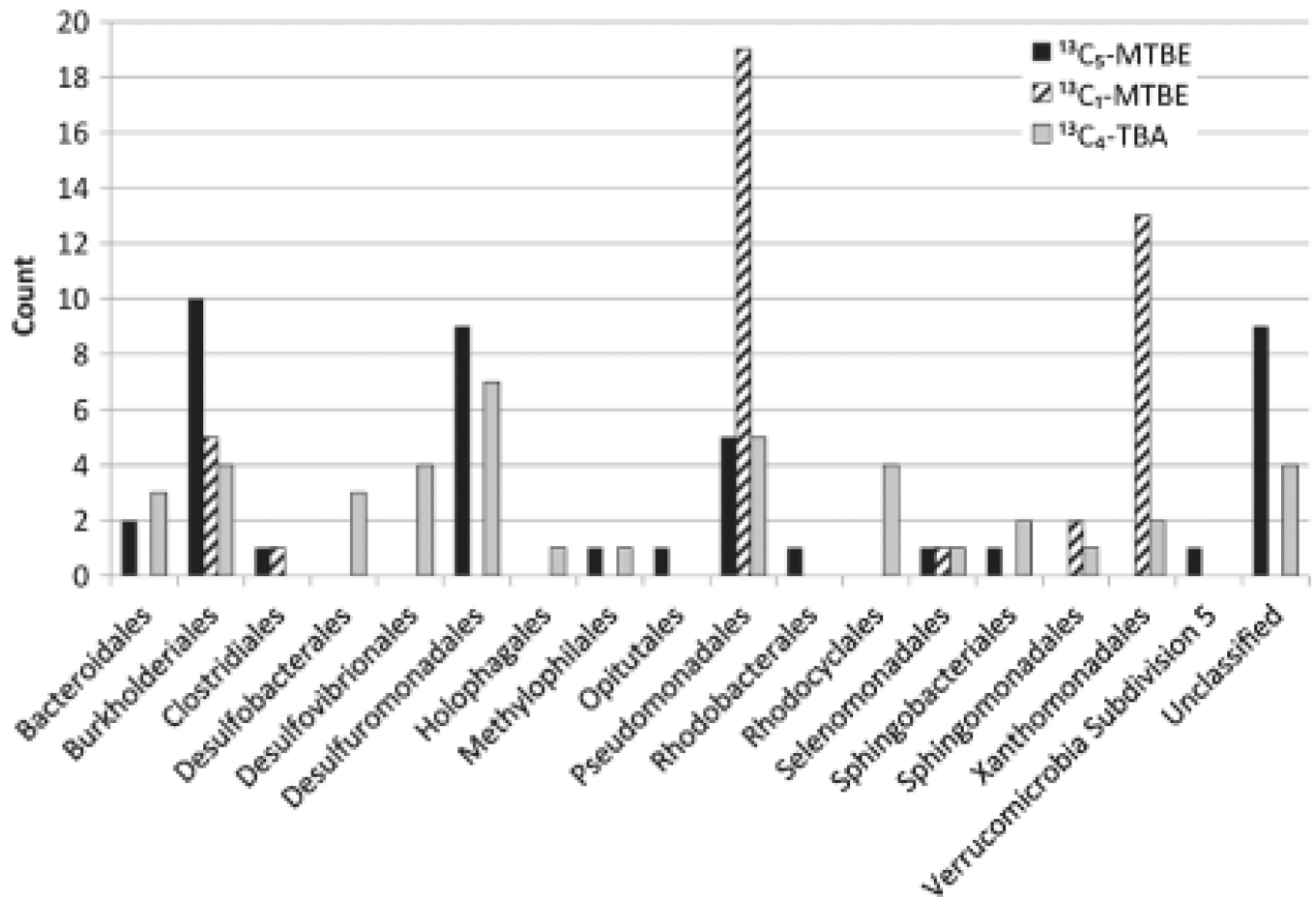
Site 60 map, Bio-traps amended with  $^{13}\text{C}_4$ -TBA were deployed in the three wells in the B5 transect, and  $^{13}\text{C}_5$ -MTBE and  $^{13}\text{C}_1$ -MTBE bio-traps were deployed in well B2. The line extending from the excavation area to the EM transect indicates the best estimate of the groundwater flow direction based on TBA plume monitoring.



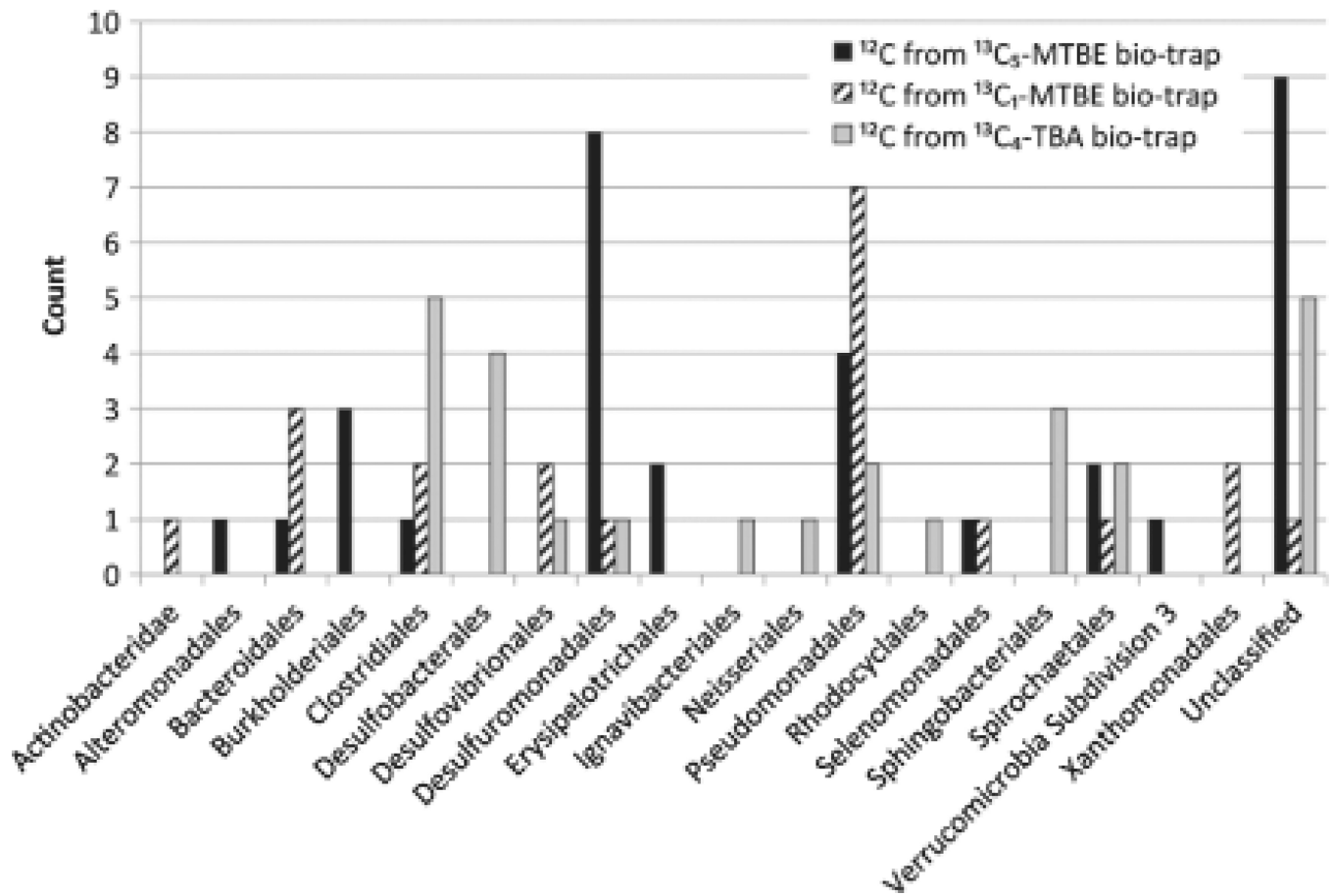
**Figure 2.**  
Higher-level affiliations of  $^{13}\text{C}$ -labeled DNA.



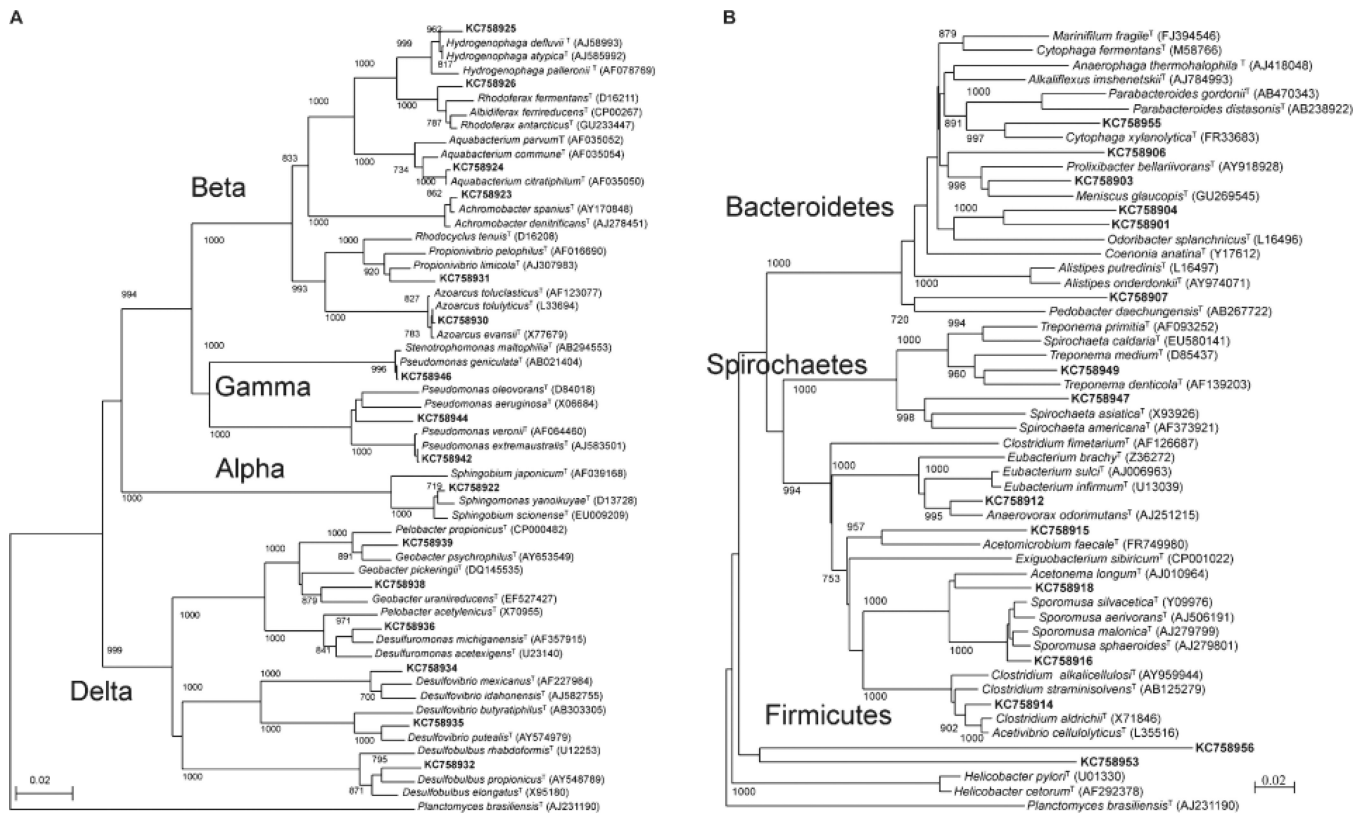
**Figure 3.**  
Higher-level affiliations of  $^{12}\text{C}$ -labeled DNA.



**Figure 4.**  
Order-level affiliations of  $^{13}\text{C}$ -labeled DNA.



**Figure 5.**  
Order-level affiliations of  $^{12}\text{C}$ -labeled DNA.

**Figure 6.**

Phylogenetic relationships of representative 16S rRNA gene sequences cloned from PCR amplicon products of DNA extracted from Bio-Sep beads incubated in a sulfate-reducing MTBE and TBA plume. Sequences were grouped into operational taxonomic units with 97% similarity and one sequence chosen to represent all the sequences in the operational taxonomic unit. Only operational taxonomic units representing two or more sequences were shown. See also Supporting Information. The trees were constructed from nucleotide gene sequences using the neighbor-joining algorithm. One thousand bootstrap replications were performed; only values 700 and greater are shown. Approximately 1320 bp. Bar: 0.02 nucleotide substitutions per nucleotide. (A) Representative sequences of Proteobacteria, with *Planctomyces brasiliensis* as the outgroup sequence. Phylogenetic affiliations of representative sequences of Proteobacteria. (B) Representative sequences from all groups except Proteobacteria, with *Planctomyces* and *Helicobacter* as the outgroup sequences. Phylogenetic affiliations of representative sequences from all groups except Proteobacteria



**Table 1**

## Contaminant Loss from Bio-Sep Beads

Monitoring Well	Chemical	Pre-Deployment (ing/bead)	Post-Incubation (mg/bead)	% Loss
B5-1	100% $^{13}\text{C}_4$ -TBA	$1.03 \pm 0.06$	$0.29 \pm 0.01$	72%
B5-2a	100% $^{13}\text{C}_4$ -TBA	$1.03 \pm 0.06$	$0.25 \pm 0.007$	76%
B5-2b	100% $^{13}\text{C}_4$ -TBA	$1.03 \pm 0.06$	$0.04 \pm 0.001$	96%
B5-3	100% $^{13}\text{C}_4$ -TBA	$1.03 \pm 0.06$	$0.25 \pm 0.018$	76%
B2a	100% $^{13}\text{C}_5$ -MTBE	$0.46 \pm 0.025$	$0.31 \pm 0.03$	33%
B2b	100% $^{13}\text{C}_5$ -MTBE	$0.46 \pm 0.025$	$0.28 \pm 0.016$	39%
B2a	100% $^{13}\text{C}_1$ -MTBE	$0.59 \pm 0.026$	$0.15 \pm 0.004$	75%
B2b	100% $^{13}\text{C}_1$ -MTBE	$0.59 \pm 0.026$	$0.15 \pm 0.004$	75%

**Table 2**

## Sequence Analysis Summary

Clone Library	# Sequences	# Operational Taxonomic Units <sup>a</sup>	% Coverage <sup>b</sup>
<sup>13</sup> C <sub>1</sub> -MTBE	41	7	93%
<sup>13</sup> C <sub>5</sub> -MTBE	42	20	69%
<sup>13</sup> C <sub>4</sub> -TBA	42	23	69%
<sup>12</sup> C from <sup>13</sup> C <sub>1</sub> -MTBE bio-trap	21	10	76%
<sup>12</sup> C from <sup>13</sup> C <sub>5</sub> -MTBE bio-trap	33	20	58%
<sup>12</sup> C-TBA	26	14	77%

<sup>a</sup>Based on 97% sequence similarity

<sup>b</sup>Good's method (Good 1953).

**Table 3**

Number of Operational Taxonomic Units Shared Between Clone Libraries

Clone Libraries Compared		# Operational Taxonomic Units Shared	# Operational Taxonomic Units Total
<sup>13</sup> C <sub>5</sub> -MTBE	<sup>13</sup> C <sub>1</sub> -MTBE	1	26
<sup>13</sup> C <sub>5</sub> -MTBE	<sup>13</sup> C <sub>4</sub> -TBA	7	36
<sup>13</sup> C <sub>1</sub> -MTBE	<sup>13</sup> C <sub>4</sub> -TBA	3	27
<sup>12</sup> C from <sup>13</sup> C <sub>5</sub> -MTBE bio-trap	<sup>12</sup> C from <sup>13</sup> C <sub>1</sub> -MTBE bio-trap	2	28
<sup>12</sup> C from <sup>13</sup> C <sub>5</sub> -MTBE bio-trap	<sup>12</sup> C-TBA	1	33
<sup>12</sup> C from <sup>13</sup> C <sub>1</sub> -MTBE bio-trap	<sup>12</sup> C-TBA	1	23
<sup>13</sup> C <sub>5</sub> -MTBE	<sup>12</sup> C from <sup>13</sup> C <sub>5</sub> -MTBE bio-trap	8	32
<sup>13</sup> C <sub>1</sub> -MTBE	<sup>12</sup> C from <sup>13</sup> C <sub>1</sub> -MTBE bio-trap	3	14
<sup>13</sup> C <sub>4</sub> -TBA	<sup>12</sup> C-TBA	7	30

**Table 4**Ribosomal Database Project Classifier Summary<sup>a</sup>

Clone Library	Most Abundant Class (%)	Most Abundant Order (%)	Most Abundant Genus (%)
<sup>13</sup> C <sub>5</sub> -MTBE	Betaproteobacteria (26%)	Burkholderiales (24%)	<i>Geobacter</i> (19%)
<sup>13</sup> C <sub>1</sub> -MTBE	Gammaproteobacteria (78%)	Pseudomonadales (46%)	<i>Pseudomonas</i> (46%)
<sup>13</sup> C <sub>4</sub> -TBA	Deltaproteobacteria (33%)	Desulfuromonadales (17%)	<i>Desulfobivrio</i> (10%), <i>Geobacter</i> (10%), <i>Pseudomonas</i> (10%)
<sup>12</sup> C from <sup>13</sup> C <sub>5</sub> -MTBE bio-trap	Deltaproteobacteria (24%)	Desulfuromonadales (24%)	<i>Geobacter</i> (21%)
<sup>12</sup> C from <sup>13</sup> C <sub>1</sub> -MTBE bio-trap	Gammaproteobacteria (43%)	Pseudomonadales (33%)	<i>Pseudomonas</i> (33%)
<sup>12</sup> C-TBA	Deltaproteobacteria (23%)	Clostridiales (19%)	<i>Desulfobutbus</i> (15%), <i>Unclassified</i> (15%)
Overall	Gammaproteobacteria (29%)	Pseudomonadales (20%)	<i>Pseudomonas</i> (20%)

<sup>a</sup>The most abundant genus is not necessarily a member of the most abundant class or order.