

## ABSTRACT

ASLETT, LOLA DENISE. Stable Isotope Studies of Fuel Oxygenate Biodegradation. (Under the direction of Michael R. Hyman.)

Stable isotope studies have been used to improve our understanding of how microorganisms interact in complex environments such as soil and groundwater. They have been particularly relevant for the field of bioremediation in identifying and characterizing microbial populations which biodegrade environmental contaminants. The fuel oxygenates methyl *tertiary* butyl ether (MTBE) and *tertiary* butyl alcohol (TBA) are widely detected in soil and groundwater throughout the United States. In this work, multiple stable isotope methods have been used in novel approaches to characterize MTBE and TBA biodegradation in various environmental systems.

Remediation options for treating MTBE and TBA impacted groundwater often involve the use of bioreactors which are inoculated with indigenous microorganisms present in the groundwater. DNA Stable Isotope Probing (DNA-SIP) was used to identify the microbial population necessary to support aerobic TBA oxidation in two geographically dispersed bioreactors. Microbial diversity identified in the reactors was limited, but the potential for TBA oxidizing activity appears to be widespread in the environment.

Soil environments are associated with complex microbial interactions and are often difficult to study with traditional methods. DNA-SIP was used to analyze MTBE biodegradation in a soil system and both cometabolic and commensal microbial interactions involving alkane- and TBA-utilizing organisms were required to completely degrade MTBE.

*Nitrosomonas europaea* is a chemolithoautotrophic soil organism that has been shown to oxidize a wide range of organics. In this work, we have shown that *N. europaea* also oxidizes MTBE to TBA. Nuclear magnetic resonance was used to show that TBA is the sole multicarbon metabolite generated by MTBE oxidation.

Compound specific isotope analysis (CSIA) was used to compare  $^{13}\text{C}$  and  $^2\text{D}$  enrichments associated with MTBE oxidation by different biological cultures. Findings suggest that different mechanisms are used in the initial reaction of MTBE oxidation. CSIA was also used to determine the  $^{13}\text{C}$  enrichment associated with TBA metabolism by cultures of two TBA-oxidizing organisms.

Stable Isotope Studies of Fuel Oxygenate Biodegradation

by  
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## BIOGRAPHY

Lola Denise Morgan Aslett was born in North Carolina and grew up in the small, rural town of Angier. She worked in the family farming and landscaping businesses while growing up and eventually attended North Carolina State University (NCSU), where she earned a Bachelor of Science in Industrial Engineering. For the next few years, she worked in industry. One summer, while vacationing at Yellowstone National Park, Denise learned about the microorganisms living in the park hot springs. She learned about *Thermus aquaticus* and how its heat stable polymerase is useful in the study of molecular biology. Acquiring this knowledge turned out to be a life changing experience. After returning from vacation, Denise's new interests led her to enroll part-time at NCSU, where she began pursuing various undergraduate life science classes. During this time, a project on *Pyrococcus furiosus* in Dr. Amy Grunden's lab solidified her interest in microbiology research and she eventually attended graduate school at NCSU. There she was fortunate to work in Dr. Michael Hyman's lab where she learned about environmental microbiology and used stable carbon isotopes to study biodegradation of the groundwater and soil contaminants MTBE and TBA. To learn a new dimension of microbiology, Denise is pursuing post doctoral training in the lab of Dr. Ed Breitschwerdt at the NCSU School of Veterinary Medicine. Denise still enjoys living in Angier with her husband, Harry, and their golden retriever, Duncan.

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## **CHAPTER 1**

### **Literature Review**

## I - Stable Isotope Methods

### 1.0 Background

The word isotope reportedly comes from the Greek *isos* (equal) and *topos* (place) (26, 44). Its use was first proposed in 1913 in a letter to *Nature* by Frederick Soddy (3, 21, 159, 160). Soddy's proposal emerged from the idea that multiple atomic variations of the same element all reside in the same location within the Periodic Table of Elements (21, 26, 44, 159). The atomic nucleus for an element is comprised of protons and neutrons, the sum of which is the element's atomic weight. The number of protons fixes an element's position within the Periodic Table and this number is always the same for an individual element. The number of neutrons, however, can vary. It is the difference in the number of neutrons that gives rise to isotopes. For example, the element carbon has six protons, but can have as few as two and as many as 14 neutrons (69). Like carbon, each element has multiple isotopes, most of which are designated as radioactive because they are unstable and can rapidly decay into more stable forms. However, 81 elements (190) also have at least one stable isotope that either does not decay or decays at such a slow rate that any associated radioactivity has not been detected (21). Some elements have multiple stable isotopes (69). For example, carbon has two stable isotopes, designated  $^{12}\text{C}$  and  $^{13}\text{C}$ , in which the number represents the isotope's atomic weight. Stable isotopes have various distributions throughout the environment. For some elements, like carbon and nitrogen, the lighter stable isotopes are more abundant in nature. For other elements, like boron and lithium, the heavier stable isotopes are

more prevalent (44). Stable isotopes and their relative abundance levels for some selected biologically relevant elements are shown in Table 1.0.a. Stable isotopes can be employed in a variety of ways to study natural and environmental processes and these studies usually can be thought of in terms of two broad categories: isotopic fractionation studies and labeling studies (8).

**Table 1.0.a - Stable Isotopic Mass and % Natural Abundance for Selected Elements.** Mass data is from Audi, et al. 2003. The AME2003 atomic mass evaluation. Nuclear Physics A 729: 337-676. Percent natural abundance data is from IUPAC Technical Report, Atomic Weights of the Elements: Review 2000. by J.R. De Laeter, et al. 2003. Pure Appl Chem 75(6):683-800

Element	Symbol	Mass of Atom (u)	% Abundance
Hydrogen	$^1\text{H}$	1.007825	99.99
Deuterium	$^2\text{H}$	2.014102	0.01
Carbon	$^{12}\text{C}$	12.000000	98.93
	$^{13}\text{C}$	13.003355	1.07
Nitrogen	$^{14}\text{N}$	14.003074	99.64
	$^{15}\text{N}$	15.000109	0.36
Oxygen	$^{16}\text{O}$	15.994915	99.76
	$^{17}\text{O}$	16.999132	.04
	$^{18}\text{O}$	17.999160	.20

Early isotopic fractionation studies focused on elemental cycling and comparison of isotopic ratios contained within natural compounds and are usually thought of as natural abundance studies. This type of research takes advantage of the isotopic fractionation that occurs during chemical and biological reactions. The small mass differences between isotopes (see Table 1.0.a) contained within natural compounds can result in mass-dependent isotopic fractionation as those compounds are transformed. One isotope may promote faster reactions or become preferentially

incorporated over another. Biological processes in particular are usually associated with the preferential use of light isotopes over heavy isotopes (8) and this observation provides a way to distinguish between biological and chemical, abiotic processes. Mass-dependent isotopic fractionation effects can be measured and used to describe substrate sources, fixation pathways, trophic relationships, environmental and physiological conditions, or other general patterns (8) that enhance our understanding of biogeochemical and environmental processes. According to Peterson and Fry, "isotopic compositions change in predictable ways as elements cycle through the biosphere"(126). These predictable patterns have been used to understand global element cycles (126) and much of this understanding has evolved from isotopic fractionation analyses of important natural processes like plant photosynthesis (34), sulfate reduction (53, 54), nitrogen fixation (86, 166, 196), methanogenesis (18, 89), acetogenesis (48), aerobic oxidation of short chain gaseous alkanes (83), and microbial autotrophy (116).

Unlike isotopic fractionation studies which rely on the natural distribution of stable isotopes, labeling studies start with an isotopically enriched compound, like  $^{13}\text{CO}_2$ ,  $^{15}\text{NH}_4\text{Cl}$ , or  $\text{H}_2^{18}\text{O}$  so that reactions involving those compounds are forced to utilize the heavy isotopes. Various techniques can then be employed to isolate the heavy reaction products and analyze them for information regarding potential reaction pathways and enzymes. Stable isotope labeling studies may also combine isotopic enrichment with biomarker analysis to study groups of organisms involved in a particular reaction. Biomarkers can be proteins, phospholipids, and nucleic acids.

Perhaps the most famous, and arguably the most important stable isotope labeling study conducted to date was performed by Matthew Meselson and Franklin W. Stahl in 1958 when they incubated *Escherichia coli* cells with <sup>15</sup>N-ammonium chloride and used density gradient centrifugation to demonstrate the semi-conservative replication of DNA (114). Since then, DNA and RNA labeling applications have been expanded to the field of microbial ecology and have been used to link environmental functions such as methane and methanol utilization (134), with microbial identities (30, 43, 132, 133, 189).

Stable isotope studies focused on elemental cycling as well as natural ecological and biological processes laid the groundwork for more recent investigations concerning the fate, distribution, and biodegradation of environmental contaminants such as trichloroethylene, toluene, benzene, naphthalene, and the fuel oxygenate methyl *tertiary*-butyl ether (146). Various methods that utilize isotopic fractionation or labeling with isotopically enriched compounds have been employed in these efforts. Three methods that relate directly to the work performed for this dissertation will be further described. Descriptions will focus particularly on applications regarding environmental contaminant biodegradation research.

### **1.1 Compound Specific Isotope Analysis**

Compound Specific Isotope Analysis (CSIA) is a technique that combines Gas Chromatography (GC) with Isotope Ratio Mass Spectrometry (IRMS). Since it became available in the 1990s (112), it has been applied over a wide range (96, 113) of research areas including archaeology, food science, microbiology,

biomedical sciences, and bioremediation. It has found particular popularity in the field of bioremediation because the GC component of the process allows for the separation of multiple compounds that are often found in complex environmental samples (90). Data from this type of analysis are expressed as  $\delta$  values in permil (‰) according to equation (146) shown in Figure 1.1.1. Ratios are used for data evaluations “to allow a correction for mass-discriminating effects in a single instrument and to facilitate the comparison of published GC/IRMS data” (146). The  $\delta$  values can be positive or negative. Positive  $\delta$  values indicate that the sample has a higher content of heavy isotope than the reference and it is said to be “enriched” in the heavy isotope. Similarly, negative  $\delta$  values indicate that the sample contains less heavy isotope than the reference, so it is considered to be enriched in the light isotope. Samples with a  $\delta$  value of 0‰ have no detectable differences in isotopic ratios when compared to the reference.

The application of CSIA to the study of biodegradation of environmental contaminants has grown in recent years. It has been used in laboratory batch as well as field evaluations to provide evidence for biodegradation of aromatic hydrocarbons like benzene, toluene, ethyl-benzene, and *o*-, *m*-, *p*-xylene (38, 106, 169, 203), chlorinated hydrocarbons like trichloroethylene (73, 100) and tetrachloroethene (71, 73), and fuel oxygenates like methyl *tertiary*-butyl ether (MTBE) and *tertiary*-butyl alcohol (TBA) (51, 72, 87, 90, 111, 193). In these types of evaluations, the equation shown in Figure 1.1.1 is used to represent the isotope ratio of a contaminant as compared to an international standard, but some additional

$$\delta_x = \left[ \frac{R_x - R_{ref}}{R_{ref}} \right] \times 1000$$

**Figure 1.1.1 - Equation used to calculate isotope ratios.**  $R_x$  and  $R_{ref}$  represent ratios of the heavy isotope to the light isotope in compound x and an international standard, respectively (146). One international standard for carbon is derived from Peedee Belemnite (PDB), which is a belemnite fossil from the cretaceous Peedee formation in South Carolina (112). An international standard used for hydrogen and oxygen is the Vienna Standard Mean Ocean Water (V-SMOW) (112). The international standard used for nitrogen is derived from air (112).

equations and definitions are necessary. These definitions as they apply to the biodegradation studies described in this dissertation were taken from Kuder, et al. (90) and are shown in Figure 1.1.2.

CSIA has become a particularly useful tool in the field of bioremediation because many sites that are impacted with organic environmental contaminants tend to involve large soil and aquifer subsurface areas with limited oxygen availability (60, 164). As a result, any aerobic biodegradation processes that can benefit from the presence of the contaminating organics quickly consume the available oxygen, leaving large portions of many sites in an anoxic state. Once the sites become primarily anoxic, some biodegradation processes tend to slow considerably (11, 35, 59, 161, 191, 192, 202), making them more difficult to study even with laboratory microcosms consisting of soil and water from the impacted sites. Because biological reactions tend to favor lighter isotopes and CSIA offers a way to measure isotopic

fractionation, it has particularly useful in anaerobic biodegradation studies and has potential to impact the future design of bioremediation and natural attenuation strategies. Chapter 5 of this dissertation reports on the use of CSIA in biodegradation studies involving MTBE and TBA.

**Isotope fractionation** – change of isotope ratios resulting from biological, chemical, or physical processes. Biological enzymatic processes proceed faster for molecules containing lighter isotopes, resulting in reaction products enriched in the lighter isotope while the remaining substrate is enriched in the heavier isotope.

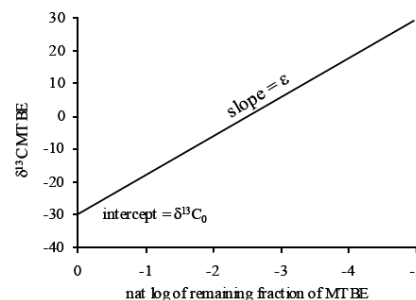
**Compound-specific isotope analysis** – permits stable isotope ratio determination for individual compounds within a complex environmental sample containing multiple compounds. Analyte separation is possible because of the GC component in the instrumentation used.

**Rayleigh fractionation model** – isotope fractionation in systems where there is a mass flux from reactant to product can be mathematically expressed for element X by the Rayleigh equation as follows:

$$\delta X_t = \delta X_{t=0} + \epsilon * \ln F$$

where:  $\delta X_t = \delta X$  of the contaminant at time t  
 $\delta X_{t=0} = \delta X$  of the contaminant at time 0  
 $\epsilon =$  isotopic enrichment factor  
 $F =$  ratio of contaminant concentrations at times t and t = 0

Example Rayleigh plot:



**Enrichment factor ( $\epsilon$ )** – shows the magnitude of isotopic fractionation.

Figure 1.1.2 – Equations and definitions used in CSIA analyses. Adapted from Kuder, T., et al. 2004. *Compound-specific isotope analysis of MTBE and TBA for bioremediation studies*. Proceedings of Petroleum Hydrocarbons and Organic Chemicals in Groundwater Conference, Baltimore, Md.

## 1.2 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique primarily used to determine the structure of organic compounds (31, 67). NMR can only be used to detect nuclei that have a nonzero magnetic property referred to as spin. Atoms with an even atomic number and even atomic mass, like  $^{12}\text{C}$  and  $^{16}\text{O}$ , have opposing paired spins that result in no overall spin. But, stable isotopes such as  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{19}\text{F}$  have an odd number of neutrons giving these atoms an overall spin (31, 67). When molecules are placed in a strong magnetic field and a radiofrequency that is tailored to the nuclei of interest is applied, the targeted spinning nuclei resonate at a frequency dependent on the atom's local chemical environment (63). These unique resonance frequencies are termed chemical shifts and are used to produce spectra that reflect the chemical structure of the molecule. This technique has been useful in microbial research focused on identifying intermediates in metabolic pathways. Sensitivity is problematic in these studies because they involve organic molecules which are not naturally  $^{13}\text{C}$  enriched and  $^1\text{H}$ -NMR cannot be used due to the high background signal resulting from water. For this reason, the signals often must be enhanced by the use of isotopically enriched substrates like  $^{13}\text{C}$ -glucose (129, 194). In addition to basic research, the application of NMR has been expanded to studies aimed at identifying metabolites produced during microbial biodegradation of environmental contaminants and characterizing the associated biodegradation pathways. For some compounds like fluorophenols, NMR analysis is particularly useful because it is enhanced by the natural presence

of  $^{19}\text{F}$ . Boersma, et al., have stated that “of all NMR-observable isotopes,  $^{19}\text{F}$  is the one perhaps most convenient for studies of biodegradation of environmental pollutants” (4-6). The  $^{19}\text{F}$  nucleus is 100% abundant (69), has a high intrinsic sensitivity and, more importantly, background noise signals are minimized because biological systems do not have substantial levels of endogenous fluorinated compounds (4-6). On the other hand, many polluting compounds, such as aromatic hydrocarbons, are organic molecules comprised of elements having low natural abundance stable isotopes. Here again, sensitivity can be a problem in NMR analysis, so biodegradation studies are often conducted with isotopically enriched compounds such as 1- $^{13}\text{C}$ -*n*-hexane (24),  $^{13}\text{C}$ -methyl bromide (170),  $^{13}\text{C}_5$ -MTBE (63), and  $^{15}\text{N}_3$ -2,4,6-trinitrotoluene (171).  $^{19}\text{F}$ -fluorinated analogs (63, 130) of environmental contaminants can also be used. However, these types of studies also have drawbacks. Most significantly, isotopically enriched compounds of this sort require synthesis in a specialized lab (52), and these processes can be prohibitively expensive. Nevertheless, NMR has and will continue to be an important technique for identifying degradation intermediates. Chapter 4 of this dissertation includes an NMR analysis which identified TBA and formate as the primary metabolites generated during MTBE biodegradation by the ammonia-oxidizing microorganism *Nitrosomonas europaea*.

### 1.3 Stable Isotope Probing

Understanding how microorganisms interact in complex environments like soil and groundwater is an important research interest for microbial ecologists. As Eugene Madsen has stated, “throughout evolutionary time, and each day in every habitat throughout the globe, microorganisms have been responsible for maintaining the biosphere. Despite the crucial part that they play in the cycling of nutrients in habitats such as soils, sediments, and waters, only rarely have the microorganisms actually responsible for key processes been identified” (103). Stable Isotope Probing (SIP) offers a way to begin identifying these organisms and characterizing the complex communities they form. It is being used to link specific environmental functions with a population of responsible organisms (133, 185, 188, 189). With this technique, environmental samples containing indigenous microbial populations are typically incubated with isotopically enriched growth substrates. Biologically relevant isotopes such as  $^{13}\text{C}$  or  $^{15}\text{N}$  are often used. Metabolically active organisms equipped with the enzymatic machinery needed to catabolize the substrate incorporate the stable isotopes into cellular biomolecules. The labeled biomolecules, such as phospholipid fatty acids, nucleic acids, and proteins can then be isolated and analyzed with various molecular techniques to characterize the population of organisms responsible for metabolism of the substrate. Phylogenetic resolution is largely dependent on the biomolecule used for analysis. SIP has been used extensively in laboratory microcosms but it has also been extended to field settings

for *in situ* analysis. This review will focus on the three most commonly employed SIP approaches: 1) phospholipid fatty acid analysis, 2) DNA-SIP, and 3) RNA-SIP.

### 1.3.1 Phospholipid Fatty Acid Analysis

The first stable isotope probing analysis was conducted with <sup>13</sup>C-labeling of lipid biomarkers (30). This approach was introduced in 1998 by Boschker, et al., in *Nature* (9). PLFA analysis is based on the separation of different lipid classes after they have been extracted from samples with various solvents (201). The individual fatty acids are separated, quantified, and identified with capillary gas chromatography and mass spectrometry (137). The types of fatty acids found are then linked to indicator organisms. A few examples, taken from a review provided by Zelles (201), are shown in Table 1.3.1.a.

**Table 1.3.1.a – Examples of fatty acid (FA) types and microbial indicators.** Taken from Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biology and Fertility of Soils* (29):111-129.

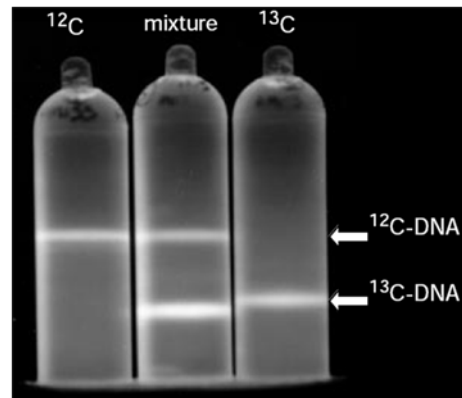
Type of FA	Indicator for
FAs containing cyclopropyl ring	Gram negative: <i>Rhodospirillum</i> , <i>Cromatium</i> / <i>Legionella</i> Gram positive: <i>Clostridium</i> , <i>Bifidobacterium</i>
Methyl branching on 10 <sup>th</sup> C atom	Actinomycetes, sulfate reducer
Ester-linked polyunsaturated FAs	Eukaryotes, cyanobacteria
Hydroxy substitution at position 3 nearest to carboxyl end	Gram negative except <i>Eikanella</i> , <i>Arthrobacter</i> , fungi

The information used to derive the microbial indicators largely comes from isolated pure cultures of microorganisms (201), so PLFA analysis is generally limited by the difficulties associated with reliably obtaining pure culture isolates from diverse and

complex environments. Other biomarkers such as nucleic acids offer the potential for improved phylogenetic resolution (30) without the need for isolated cultures.

### 1.3.2 DNA Stable Isotope Probing

In 2000, Radajewski, et al., published results of the first DNA-SIP investigation (132) in *Nature*. They first grew the methylophilic bacterium *Methylobacterium extorquens* in separate cultures each containing either  $^{12}\text{C}$ - or  $^{13}\text{C}$ -methanol as the sole source of carbon. Culture DNA was extracted and separated by CsCl density ultracentrifugation as shown in Figure 1.3.2 (132).



**Figure 1.3.2 – Equilibrium centrifugation of isotopically labeled DNA.** Shown are CsCl/ethidium bromide density gradients for pure fractions and a mixture of DNA extracted from *M. extorquens* AM155 culture utilizing either  $^{12}\text{C}$ - or  $^{13}\text{C}$ -methanol as the sole carbon source. Visualized with UV light. Photo taken from Radajewski, S., et al. 2000. Stable-isotope probing as a tool in microbial ecology. *Nature*. (403):646-649.

Ethidium bromide was added to the centrifugation tubes to aid in visualization of light ( $^{12}\text{C}$ ) and heavy ( $^{13}\text{C}$ ) DNA fractions, which could be removed with a sterile needle and syringe. It appeared that effective separation had been achieved. (Later,

Singleton et al. (150) improved this visual confirmation by adding an internal control to all sample tubes prior to centrifugation. They added  $^{12}\text{C}$ -DNA from *E. coli* to their sample tubes and then probed the  $^{13}\text{C}$ -DNA fractions for *E. coli* sequences with specially designed primers. With 25 cycle PCR reactions, they found little to no contamination in the  $^{13}\text{C}$ -DNA, suggesting that separation of heavy from light DNA in the CsCl gradients was effective.)

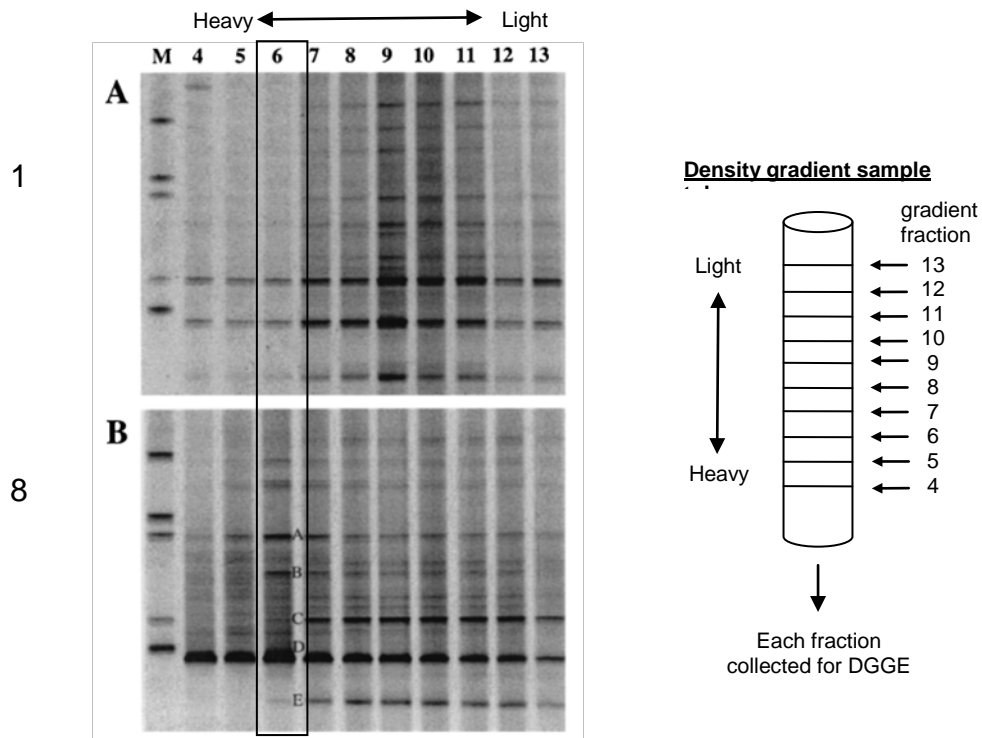
Radajewski, et al., then extended their method to experiment with an acidic forest soil (134). Soil samples were incubated with  $^{13}\text{C}$ -methanol as the sole carbon source. Methanol consumption was monitored by gas chromatography. Gradient separated  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA fractions were used to construct a clone library based on 16S rRNA genes. PCR analysis of the  $^{12}\text{C}$ -DNA fraction with primers targeting archaea, bacteria, and eukarya, revealed sequences representing all three domains. A similar analysis of the  $^{13}\text{C}$ -fraction produced only bacterial sequences, suggesting that only organisms from this domain were actively assimilating methanol in the soil microcosms. The majority of the cloned 16S rRNA sequences were novel sequences related to either *Beijerinckia indica* (49%) or *Rhodopseudomonas acidophila* (47%), two  $\alpha$ -Proteobacteria not generally associated with methanol utilization. The  $^{13}\text{C}$  fraction was also analyzed for methylotrophy-associated genes, like *mxoF* which encodes the  $\alpha$ -subunit of methanol dehydrogenase. The *mxoF* amino acid sequence analysis supported the 16S rRNA analysis which suggested that the samples represented new methylotrophic organisms belonging to the  $\alpha$ -Proteobacteria. This type of parallel gene analysis is one of the advantages of

using DNA-SIP. Later, however, it was recognized that the long incubation time (44 days) and multiple applications of methanol used in this initial experiment may have contributed to one of the primary criticisms associated with DNA-SIP and that is the potential for crossfeeding (30, 124, 133). Crossfeeding results when organisms do not directly assimilate the isotopically labeled substrate, but instead utilize a labeled metabolic intermediate generated by the primary consumer (30, 133). Avoiding crossfeeding often presents a challenge because researchers must balance this consideration with the need to retrieve an adequate amount of enriched DNA for analysis. One idea that has emerged to combat crossfeeding is the use of carrier-DNA (45). It is essentially an added source of isotopically enriched DNA that is not likely to be found in the samples being analyzed. Its use allows for shorter incubation times and improves sensitivity by aiding in the UV visualization of heavy DNA in the CsCl gradients.

### **1.3.3 RNA Stable Isotope Probing**

Another approach that reduces the likelihood of undetected cross-feeding is RNA-SIP. Cell copy numbers for 16S rRNA are higher than 16S rDNA (110) and RNA is produced independent of cellular replication. It is estimated to be labeled 6.5 times faster than DNA (107, 110) and therefore offers enhanced sensitivity and the potential for shorter incubation times. Manefield, et al., pioneered the first RNA-SIP (109). They first demonstrated that RNA gets labeled faster than DNA.  $^{13}\text{C}_6$ -phenol was applied to samples from a bioreactor treating groundwater heavily contaminated with phenolic compounds. Phenol concentration in samples was assessed over an

8 hour period, during which the  $^{13}\text{C}$  atom % for RNA increased 10-fold, while DNA increased 2-fold. In separate experiments, bioreactor samples were incubated with  $^{13}\text{C}_6$ -phenol and monitored for phenol consumption over 8 hours. RNA was extracted and subjected to centrifugation in a cesium trifluoroacetate density gradient, but efficient RNA separation into one heavy and one light gradient fraction (like that achieved with DNA-SIP) was not observed. The “presence of RNA in high-density gradient fractions does not necessarily mean it is isotopically enriched” (109). Therefore, multiple gradient fractions had to be analyzed by Isotope Ratio Mass Spectrometry (IRMS) to determine the gradient locations of isotopically enriched RNA. Once enrichment was confirmed for individual gradient fractions, RNA was amplified by RT-PCR and analyzed by DGGE. The DGGE profiles (shown in Figure 1.3.3) for the gradient fractions shift between the 1-hour and 8-hour sample points indicating the consumption of  $^{13}\text{C}_6$ -phenol and RNA  $^{13}\text{C}$ -enrichment. The emergence of bands A, B, and D in fraction 6, with  $^{13}\text{C}$  enrichment confirmed by IRMS, represents the primary phenol degraders in the bioreactor samples.



**Figure 1.3.3 – RNA-SIP DGGE profiles of community RNA present in fractions 4 to 13 in density gradients loaded with RNA extracted at 1 hour (Panel A) and 8 hours (Panel B) after  $^{13}\text{C}_6$ -phenol pulse.** Adapted from Manefield, et al. 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology*. (68)11:5367-5373.

### **1.3.4 SIP Applications in Biodegradation Research**

The three fundamental methods (PLFA analysis, DNA-SIP, and RNA-SIP) for stable isotope probing analysis have become widely applied in studies focused on biodegradation of environmental contaminants. Cleanup of such contaminants often requires massive pump and treat operations, but in some circumstances, especially where important natural resources such as lakes and rivers are not immediately threatened, natural attenuation can be an effective remediation option. Natural attenuation involves some abiotic processes like dilution, dispersion, and sorption, but the real effectiveness of this method is driven by indigenous microbial processes that transform and destroy the contaminants (139). Understanding the biodegradation dynamics *in situ* for such complex mixtures has been a challenge in bioremediation research. SIP offers one way of addressing this problem. Several SIP studies that demonstrate how SIP is being used in bioremediation research will be described and this section will conclude with a table that lists SIP studies which were not discussed in detail.

#### **1.3.4.a Biodegradation *In Situ***

Many environmental contaminants such as benzene and toluene are known to be biodegraded under aerobic conditions. However, environments where these compounds tend to persist contain anaerobic zones, which are difficult to study. So it is not surprising that early SIP biodegradation studies aimed at understanding natural attenuation focused on anaerobic conditions. In this work, the primary goal has been to demonstrate that *in situ* biodegradation was occurring.

For example, Geyer, et al., reported a PLFA-based SIP analysis that utilized Bio-Sep beads. Beads were amended with  $^{13}\text{C}$ -toluene or  $^{13}\text{C}$ -benzene and deployed in a monitoring well situated over an aquifer, which was heavily contaminated with benzene and toluene from a former hydrogenation plant. Microorganisms from the aquifer colonized the beads and consumption of benzene and toluene resulted in significant  $^{13}\text{C}$  enrichment ( $\delta^{13}\text{C}$  up to 13400‰) of microbial lipid fatty acids. The PLFA profiles indicated that benzene and toluene were assimilated by similar types of organisms, but a more detailed phylogenetic resolution was not provided. The fatty acid  $^{13}\text{C}$ -enrichments do however support the project aim of demonstrating microbial *in situ* benzene and toluene biodegradation.

Bio-Sep beads have also been used to demonstrate anaerobic biodegradation of fuel oxygenates MTBE and TBA in the field. Busch-Harris and colleagues utilized a PFLA-SIP approach (16) to investigate biodegradation at the site of a gasoline station with a history of underground storage tank leaks and above ground spills. Bio-Sep beads (referred to as biotraps) which were amended with either  $^{13}\text{C}$ -MTBE or  $^{13}\text{C}$ -TBA were deployed in several monitoring wells, and for both amendments,  $^{13}\text{C}$ -fatty acid enrichment was observed. Again, the fatty acid profiles did not provide detailed phylogenetic resolution, but the  $^{13}\text{C}$ -fatty acid enrichment provided evidence that anaerobic MTBE and TBA biodegradation was occurring.

#### **1.3.4.b Biodegradation in Microcosms**

SIP has also been used to study biodegradation in microcosms comprised of samples from contaminant-impacted environments. It has been used to help isolate

organisms with contaminant degrading activity, and when repeated isolation attempts have failed, it has been used to describe a unique commensal microbial relationship.

For example, Kasai, et al., used RNA-SIP to investigate  $^{13}\text{C}$ -benzene degradation under different electron accepting conditions (nitrate, sulfate, and oxygen) in microcosms of groundwater contaminated with aromatic hydrocarbons (81). Density gradient separated RNA fractions were analyzed by DGGE and for denitrifying conditions, a sequence with 100% homology to *Azoarcus evansii* was recovered. At the time, there were only two anaerobic benzene degraders in isolation and both are members of *Dechloromonas*. The SIP derived information was used to screen colonies from agar plates inoculated with groundwater samples. DGGE profiles for multiple plate colonies were compared to the SIP-derived *Azoarcus* and five isolate profiles were found to be identical to it. Two of the isolated *Azoarcus* strains degraded benzene under denitrifying conditions.

Kunapuli, et al., used DNA-SIP to investigate an iron-reducing enrichment culture maintained with benzene as the sole carbon and energy source (92). Multiple attempts to retrieve an isolate from the culture had failed. The culture was incubated with  $^{13}\text{C}$ -benzene, monitored for ferrous iron formation and  $^{13}\text{CO}_2$  evolution, and analyzed by DNA-SIP. Clone libraries were constructed for both the  $^{12}\text{C}$  and  $^{13}\text{C}$  DNA fractions. The majority (13/23) of the  $^{12}\text{C}$  clones were identified as *Actinobacteria*, but some belonged to *Desulfobulbaceae* (5/23) and *Clostridia* (4/23). The  $^{13}\text{C}$  clones were dominated by *Clostridia* (11/19), but a small number were also

found to be members of *Desulfobulbaceae* (3/19). Prior to these findings, anaerobic benzene degradation had only been linked to members of the family *Geobacteraceae*, so this study appears to be the first report of an association of *Clostridia* with benzene degradation under iron-reducing conditions. But while *Clostridia* incorporated the  $^{13}\text{C}$ -label from benzene, it is possible that these organisms were not the iron-reducers in the enrichment. A syntrophic model was proposed. Based on the observance of a high number of *Clostridia* in the  $^{13}\text{C}$  clones, it was hypothesized that these organisms were responsible for  $\text{H}_2$ -yielding benzene attack and degradation. Subsequent  $\text{H}_2$  oxidation by *Desulfobulbaceae* was thought to “pull the initial reaction towards completion” (92), in a manner that has been recognized for syntrophic microbial interactions under methanogenic conditions (92, 143). *Desulfobulbaceae* was further proposed to assimilate  $\text{CO}_2$  from the surrounding media (thereby explaining its relative lack of  $^{13}\text{C}$  labeling) and pass on part of the electrons to ferric iron. So in this use of SIP, the researchers developed evidence that not only linked *Clostridia* to anaerobic benzene degradation, but also described a potentially novel commensal relationship that clearly explains their repeated failures to isolate a microorganism from the enrichment culture.

#### **1.3.4.c Field-Based Studies of Biodegradation**

SIP has also been applied directly to contaminated field plots in an effort to describe microbial populations and processes occurring *in situ*. Unlike the CSIA-PLFA biodegradation studies that were aimed primarily at demonstrating that a

biological process was occurring, field based SIPs have focused more on describing the microbial populations and the relevant field degradation processes.

In 2003, Padmanabhan, et al., demonstrated an innovative method in which they combined field-based degradation assays with a DNA-SIP analysis (124). Four different  $^{13}\text{C}$ -substrates (glucose, phenol, naphthalene, and caffeine) were directly applied to soil plots in an experimental agricultural field station. Plots were covered with glass chambers and monitored for  $^{13}\text{CO}_2$  and  $^{12}\text{CO}_2$  evolution by GC/MS.  $^{13}\text{C}$ -DNA was successfully recovered from field soil and an SIP analysis showed that active organisms were consistent with the types of chemoorganotrophs expected in soil, water, and/or sewage habitats. Based on this demonstration, a similar field based study of  $^{13}\text{C}$ -naphthalene biodegradation was conducted.

Jeon, et al., investigated the naphthalene degrading population in soil contaminated with coal-tar waste (78). With DNA-SIP and cloning, the active populations of organisms with naphthalene degrading activity were found to be distinct from the majority of cultured organisms on which the understanding of naphthalene degradation is largely based. This information led to use of field relevant conditions (i.e.  $10^\circ\text{C}$  groundwater temperature) during attempts to isolate a microorganism capable of naphthalene degradation. The  $10^\circ\text{C}$  temperature turned out to be critical because at higher temperatures, the vapor pressure of naphthalene was toxic. An isolate with a 16S rRNA sequence matching one of the DNA-SIP clones was achieved. Further analysis of the naphthalene dioxygenase gene sequence in the new strain showed that it was distinctive from other known

sequences for the same gene, suggesting that this particular gene is important for naphthalene biodegradation in the environment.

Finally, Liou, et al., expanded the study of benzene biodegradation to “assess the influence of field-relevant parameters (native electron acceptors, concentration of benzene) upon the activity and identity of benzene biodegrading populations” in sediments from a coal-tar waste site (97). DNA-SIP and clone libraries were used to compare benzene-degrading microbial profiles from a field site receiving an uncontrolled benzene release with those of anaerobic microcosms comprised of site sediments. Microcosms were established for two different benzene concentrations (10 and 200 ppm) under aerobic, nitrate-, and sulfate-reducing conditions.  $^{13}\text{C}$ -DNA clone sequences for the aerobic microcosms were dominated (49%) by *Streptococcus*, while the anaerobic microcosms consistently produced  $^{13}\text{C}$ -DNA sequences belonging to *Pelomonas* and *Ralstonia* genera (10 ppm) or *Pseudomonas* and *Propionibacterium* (200 ppm).  $^{13}\text{C}$ -DNA clone sequences recovered for the field treatment showed that fully one-third of them clustered with *Pelomonas*. Recovery of *Pelomonas* sequences was surprising since denitrification activity had not been previously reported for cultivated strains of these organisms. Likewise, cultivated *Pseudomonas* spp. had not been associated with benzene oxidation coupled with nitrate reduction. But the high percentage of clones related to these two taxa suggested that organisms related to *Pelomonas* and *Pseudomonas* were responsible for anaerobic benzene oxidation in the microcosms. And the fact that a substantial number of clones from the field treatment was consistent with the

*Pelomonas* sequences observed for the 10 ppm anaerobic benzene microcosm led to the further interpretation of field biodegradation conditions as having low benzene concentrations and anaerobic benzene degradation activity.

#### **1.3.4.d Bioreactor Biodegradation and SIPs Using Multiple Substrates**

From 2005 to 2007, Singleton, et al., published a series of studies (149-151) in which they used DNA-SIP to evaluate naphthalene, phenanthrene, and pyrene biodegradation in a laboratory bioreactor treating polycyclic aromatic hydrocarbon (PAH) contaminated soil. In the initial study, they were able to associate naphthalene degradation with members of the genera *Pseudomonas* and *Ralstonia*, while members of the genus *Acidovorax* were linked to phenanthrene degradation (150). They followed up this study with a DNA-SIP focused on <sup>13</sup>C-pyrene degradation in the same bioreactor (151). The <sup>13</sup>C-DNA identified sequences for 3 groups of uncultivated pyrene degraders (designated PG1, PG2, PG3). qPCR primers, designed based on these sequences, were used in a separate time course experiment aimed at determining the relative abundance of each PG group in the bioreactor during an incubation with unlabeled pyrene. As pyrene was consumed, significant (2 to 4 orders of magnitude) copy number increases were observed for groups PG1 and PG3, each of which had a low starting abundance. In contrast, copy numbers for PG2, which had a highest starting abundance, did not increase significantly in response to overall pyrene consumption. Together, these data suggested that organisms belonging to groups PG1 and PG3 were the primary pyrene degraders in the bioreactor.

Singleton, et al., took this analysis further by combining DNA-SIP, qPCR, and DGGE to analyze the degradation of two PAHs (phenanthrene and pyrene) with various combinations of  $^{13}\text{C}$ -labeling (149). With the aim of analyzing growth substrate preferences within the degrading community, the compounds were added to the bioreactor either individually ( $^{13}\text{C}$ -labeled) or as a mixture in which one compound was  $^{13}\text{C}$ -labeled and the other was unlabeled. Their analysis method was based on the expectation that organisms capable of simultaneous growth on both a  $^{13}\text{C}$  carbon source and an unlabeled one would contain partially  $^{13}\text{C}$ -enriched DNA. With careful gradient fraction collection and analysis, they analyzed the full range of gradient fractions by qPCR and DGGE and measured 16S rRNA gene copy numbers for phenanthrene and pyrene degrading groups based on the primers designed from their previously identified sequences. The method was designed to detect carbon assimilation patterns by examining the distribution of group specific genes present in DNA after it has undergone the ultracentrifugation step in an SIP experiment. This analysis showed that PG1 organisms were capable of growth on both phenanthrene and pyrene, but in a mixed incubation these organisms exhibited a stronger response to pyrene as a growth substrate. PG2 organisms were also able to assimilate both substrates, but unlike PG1 organisms, PG2 organisms did not appear to prefer one substrate over the other as both substrates were utilized when they were presented as a mixture. PG3 organisms exhibited a strong preference for pyrene. Although this method is complex, it offers a way to characterize how different groups of organisms within complex microbial communities respond to

more than one SIP substrate. The use of multiple substrates in SIP experiments is particularly relevant to biodegradation studies because the associated environments are often impacted by multiple contaminants. Currently, there is limited understanding regarding the identity of metabolically active microorganisms in these habitats and there is even less understanding regarding growth substrate selection in these complex environments.

#### **1.3.4.e Biodegradation of Other Environmental Contaminants**

SIP studies have been applied to a growing list of environmental contaminants. As isotopically enriched compounds become more available, they are being used in much the same way as the studies described. A list of studies is provided in Table 1.3.4.e.

**Table 1.3.4.e – List of SIP Applications for Biodegradation of Other Environmental Contaminants**

Contaminant Type	Compound	Isotope	Methods	Conditions	Key Microorganisms or Genes Identified	Reference
Explosives	2,4,6-Trinitrotoluene (TNT)	<sup>15</sup> N <sup>13</sup> C	DNA-SIP Carrier DNA T-RFLP	Sulfate reducing	<i>Lysobacter</i>	(46)
	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	<sup>15</sup> N	DNA-SIP Clone library Functional gene analysis	Aerobic	<i>Actinobacteria</i> <i>α-Proteobacteria</i> <i>γ-Proteobacteria</i>  <i>xplA</i> -like genes	(140)
Pesticides	2,4-dichlorophenoxyacetic acid (2,4-D)	<sup>13</sup> C	DNA-SIP T-RFLP	Aerobic	<i>β-Proteobacteria</i>	(22)
	Pentachlorophenol (PCP)	<sup>13</sup> C	RNA-SIP DGGE HPLC	Aerobic	<i>Pseudomonas</i> <i>γ-Proteobacteria</i> <i>β-Proteobacteria</i> <i>Burkholderia</i> <i>Sphingomonas</i>  All strains associated with hydrocarbon degradation	(105)
	Methyl Bromide Methyl Chloride	<sup>13</sup> C	DNA-SIP CSIA Clone library	Aerobic	MeBr: <i>Burkholderia</i> MeCl: <i>Rhodobacter</i> , <i>Lysobacter</i> , <i>Nocardioideis</i>  Strains not previously associated with methyl halide degradation	(115)
	Methyl Chloride	<sup>13</sup> C	DNA-SIP Enrichments Clone library Functional gene analysis	Aerobic	Enrichments dominated by <i>Hyphomicrobium</i> , <i>Aminobacter</i> , <i>Mesorhizobium</i>  SIP indicated larger diversity based on <i>cmuA</i> gene sequences	(7)

**Table 1.3.4.e – continued**

Contaminant Type	Compound	Isotope	Methods	Conditions	Key Microorganisms or Genes Identified	Reference
Halogenated organics	Polychlorinated biphenyl (PCB)	<sup>13</sup> C	PLFA	Aerobic	<i>Burkholderia</i>	(172)
	Polychlorinated biphenyl (PCB)	<sup>13</sup> C	DNA-SIP Functional gene analysis	Aerobic	75 different genera including <i>Pseudonocardia</i> , <i>Kribella</i> , <i>Nocardiodes</i> , <i>Sphingomonas</i>  <i>Rhodococcus</i> not detected in SIP, but is abundant in enrichments  Functional gene array detected aromatic ring hydroxylating dioxygenases	(95)
Halogenated organics	Polychlorinated biphenyl (PCB)	<sup>13</sup> C	DNA-SIP T-RFLP Functional gene analysis	Aerobic	<i>Hydrogenophaga</i> <i>Paenibacillus</i> <i>Achromobacter</i> <i>Variovorax</i> <i>Methylovorus</i> <i>Methylophilus</i>  BphA deduced amino acid sequences nearly identical to that of <i>Pseudomonas alcaligenes</i> B-357	(174)
	Tetrachloroethene (PCE)	<sup>13</sup> C	RNA-SIP T-RFLP Clone library	Aerobic	<i>Chloroflexi</i>  Only distantly related to cultivated <i>Dehalococcoides</i> spp.	(84, 85)
BTEX intermediate	Benzoate	<sup>13</sup> C	DNA-SIP Carrier DNA T-RFLP Functional gene analysis	Denitrifying	Changes in only T-RFLP patterns reported	(45)
	Benzoic Acid	<sup>13</sup> C	DNA-SIP GC/MS T-RFLP Clone library	Aerobic, field based assay	Population shifts from <i>Pseudomonas</i> to <i>Burkholderia</i> on multiple additions of <sup>13</sup> C benzoic acid	(131)

**Table 1.3.4.e – continued**

Contaminant Type	Compound	Isotope	Methods	Conditions	Key Microorganisms or Genes Identified	Reference
Industrial wastes	Hexane	<sup>2</sup> H	PLFA FISH	Aerobic	<i>Gordonia</i>	(42)
	Phenol	<sup>13</sup> C	DNA-SIP GC/MS Clone library Carbon flow through analysis	Aerobic, field based assay	Enriched primary degraders: <i>Kocuria</i> , <i>Staphylococcus</i>  Crossfeeders: <i>Pseudomonas</i>	(29)
	Phenol	<sup>13</sup> C	DNA-SIP GC/MS Clone library HPLC Enrichment Fungi-targeted PCR	Aerobic, field based assay	<i>Trichosporon multisporum</i>	(28)
	Phenol	<sup>13</sup> C	RNA-SIP DGGE	Aerobic, activated sludge samples	<i>Acidovorax</i>	(108)

### 1.3.5 Stable Isotope Methods – Conclusions

Stable isotope methods have advanced our ability to study microbial activity in complex environments. The advantages offered by these techniques have clearly contributed to their rapid and expansive deployment in biodegradation studies. NMR offers a tool for analyzing reaction intermediates and understanding biodegradation pathways. CSIA and SIP have both offered ways to demonstrate *in situ* anaerobic biodegradation of important environmental contaminants such as benzene, toluene, MTBE, and TBA. And where microbial isolates capable of a particular metabolism have not been available for study, SIP analysis has directed efforts to successfully recover them from environmental samples. In other cases, SIP studies have suggested that obtaining microbial isolates for some processes, such as benzene degradation under iron-reducing conditions, may be less relevant to our understanding of contaminant biodegradation than exploring the syntrophy involved. SIP studies have also shown promise in direct field applications. Comparisons of physiological preferences demonstrated in lab microcosms with observed field parameters have led to increased understanding of field conditions. And finally, where crossfeeding was once considered a disadvantage for SIP, it is starting to be viewed differently. Recent studies have utilized multiple substrates and different labeling strategies to better detect carbon assimilation patterns across multiple organisms within a complex community.

## II – Fuel Oxygenates

### 2.0 Fuel Oxygenate Uses and Problems

Fuel oxygenates are oxygen-containing chemicals that are added to gasoline (65) for a variety of reasons. Initially, in the 1970s and 1980s, fuel oxygenates were used in gasoline blends as a replacement for tetraethyl lead, an anti-knocking agent which was neurotoxic (50, 66). More recently, oxygenates were used to promote efficient fuel combustion and reduce carbon monoxide and other harmful automobile emissions (50, 65). In 1990, the federal Clean Air Act (CAA) was amended so that reformulated gasoline (RFG), an existing gasoline blend designed to burn cleanly, was required to contain a minimum average of 2% (w/w) oxygen. The CAA amendments required use of 2% (w/w) oxygen RFG in areas of the U.S. with serious ozone pollution. And in areas where carbon monoxide levels were especially problematic, a 2.7% (w/w) oxygen RFG was required during winter months (27, 50). Although the CAA required the use of oxygenates, it did not specify which ones had to be used.

Methyl *tertiary*-butyl ether (MTBE) quickly became the most widely used fuel oxygenate in RFG for a variety of reasons. It blends well with gasoline, has a high octane level, and is easy for producers to distribute with existing networks (27). In addition, MTBE is synthesized in a chemical reaction involving inexpensive feedstocks methanol and isobutylene (27, 68). All combined, these factors resulted in favorable economics for gasoline refiners, so MTBE became the primary strategy for meeting RFG oxygen requirements. Production of MTBE increased rapidly and

was the second most produced organic chemical in the United States between 1993 and 1998 (178). By 1998, the EPA-appointed Blue Ribbon Panel on Oxygenates in Gasoline reported that over 85% of RFG contained MTBE (175). By 2000, total annual MTBE production in the U.S. exceeded 3 billion gallons (70). These intensive production levels made environmental MTBE releases almost inevitable. MTBE releases from underground storage tanks and pipelines, spills at industrial plants and refueling facilities, and accidental spills during transport are all referred to as “point source releases”. Non-point release sources include exhaust and evaporative emissions from vehicles, stormwater runoff, and atmospheric deposition (27).

Regardless of the release source, the presence of MTBE in the environment is particularly problematic for groundwater. MTBE has a low sorptive property ( $\text{Log } K_{ow} = 1.2$ ) and high solubility (50,000 mg/L), especially in comparison to other compounds that are found in high abundance in gasoline, such as benzene ( $\text{Log } K_{ow} = 2.0$ , solubility = 1,780 mg/L) and toluene ( $\text{Log } K_{ow} = 2.6$ ; solubility = 535 mg/L) (138). These properties give MTBE a tendency to be mobile in groundwater, where it has the potential to migrate long distances and impact drinking water supplies. While the potential toxicity of MTBE has been debated (25), it is clear that tiny amounts of it can make water undrinkable. MTBE is reported to impart an offensive, turpentine-like quality (37) to water and one study has shown that it could be detected in potable water based on odor at 15 ppb and taste at 40 ppb (197). In comparison, this same study reported an odor threshold for benzene and toluene as

190 and 960 ppb, respectively. In 1997, EPA issued a drinking water advisory that recommended keeping MTBE contamination levels in the range of 20 to 40 ppb for consumer acceptability (176).

The widespread nature of MTBE groundwater impacts was reported as part of an assessment of data from the U.S. Geological Survey's National Water Quality Assessment Program (NAWQA). Squillace, et al., evaluated NAWQA data collected between 1985 and 1995 (165) and their analysis summarizes the detection frequencies of 60 volatile organic compounds in ambient groundwater, which is defined as groundwater in areas with no known point sources of contamination prior to sampling. MTBE was among the four most frequently detected volatile organic compounds in urban settings, where concentration levels ranging from 0.2 ppb to 20,000 ppb had a detection frequency of 16.9%. In rural areas, MTBE had a detection frequency of 3.4% with concentration levels ranging from 0.2 ppb to over 100 ppb. Further, it was estimated that between 35 and 50 million people may rely on water from areas where the untreated ambient groundwater resource contains at least one volatile organic compound at a concentration of 0.2 ppb. So even in areas where there are no known point source releases of MTBE, the problem is widespread.

Of the MTBE release sources, underground storage tanks (UST) for gasoline have been recognized as being likely to pose the most serious threat to groundwater contamination (27). The Office of Underground Storage Tanks (OUST), within U.S. Environmental Protection Agency (U.S. EPA), administers a monitoring and

reporting program for USTs storing petroleum products and other designated hazardous substances. In the fiscal year (FY) 2009 report, there were ~611,500 federally regulated active USTs at ~223,000 sites across the country (177). It is not clear from the report how many of these USTs store petroleum products (potentially containing MTBE), but it is likely that they comprise the majority. Since the program inception, over 488,000 UST leaks have been reported and around 388,000 (~80%) cleanups have been completed. In the FY2009 report, EPA estimated that two-thirds of active USTs were fully complying with requirements to prevent and detect leaks. These measures are expensive. The UST program received over \$300 million of taxpayer funds in FY2009 to use for prevention, assessment, and leak cleanup (177). And there is still a backlog of over 100,000 UST sites awaiting cleanup.

Closely linked to environmental concerns about MTBE is TBA. TBA is well known as an intermediate in the aerobic and anaerobic biodegradation of MTBE. TBA is often detected along with MTBE in the environment, and in some instances, TBA concentrations can be higher than those for MTBE (193). While MTBE biodegradation is one potential source of TBA in the environment, it is not the only one. TBA has been used as an industrial solvent and a fuel oxygenate (albeit less extensively than MTBE), so industrial waste streams and leaking gasoline USTs could also lead to releases of TBA. Regardless of the source point, TBA poses its own set of environmental risks and problems that are separate from those of MTBE. First, TBA is a carcinogen at high doses (17). Second, it is fully miscible in water

and has a low sorptive property ( $\text{Log } K_{ow} = 0.35$ ), so its tendency to be mobile in groundwater outpaces that of MTBE. These combined characteristics make TBA a potentially greater threat to drinking water supplies than MTBE.

A variety of engineered methods for MTBE and TBA cleanup are available. Depending on site conditions, cleanup technologies such as soil vapor extraction, excavation, air sparging, and pump and treat options are often used (119, 122). All of these processes can be used in combination with natural microbial processes for maximum contaminant removal. Indeed, biodegradation is also an important part of cleanup strategies.

## **2.1 MTBE and TBA Anaerobic Biodegradation**

Because fuel oxygenates from leaking USTs and other sources tend to largely impact anoxic sediments, there has been considerable interest in understanding the anaerobic microbial processes that promote biodegradation in these environments. In 2001, Finneran and Lovley published calculations showing that anaerobic oxidation of MTBE is thermodynamically possible (35). Despite this, microbial isolates capable of growth on either MTBE or TBA as a sole source of carbon and energy under anaerobic conditions are currently not available. Biodegradation of both MTBE and TBA has been observed under anaerobic conditions (13, 35, 145), but MTBE degradation is typically very slow and likely to result in the undesirable accumulation of TBA (91, 145, 191). Anaerobic MTBE microcosm studies have been conducted under various redox conditions including denitrifying (10), sulfate reducing (118, 161, 187), iron reducing (35), and

methanogenic (192). Results have been conflicting. For example, Bradley, et al., found MTBE degradation under nitrate reducing conditions (11), but others only found MTBE degradation under sulfate reducing conditions (118, 161) or iron reducing conditions (35). In addition to the conflicting redox condition data, anaerobic MTBE biodegradation has been difficult to study because very long incubations have been required. For example, Somsamak, et al., incubated sediment samples under various enrichment conditions for 1,160 days before MTBE degradation was eventually observed under sulfate reducing conditions (161). Other incubations have required less, but still substantial, lags before MTBE transformation to TBA was observed. Wilson, et al., reported a small reduction in MTBE concentration under methanogenic conditions after 200 days, but reduction in MTBE from a starting concentration of 3,112 ppb to 40 ppb required at least 490 days (192). In another study, reduced MTBE concentrations were apparent under iron-reducing conditions after a 275 day lag (35). Apparently faster anaerobic MTBE degradation times have been observed under nitrate reducing conditions. Bradley, et al., reported a ~20% recovery of  $^{14}\text{C}$ -MTBE as  $^{14}\text{CO}_2$  after 77 days. (11).

More recent microcosm studies have focused less on redox conditions and more on characterizing the microbial populations involved in anaerobic MTBE biodegradation. Wei and Finneran recently reported on three anaerobic MTBE enrichment cultures that were derived from an MTBE-impacted aquifer (187). Clone libraries prepared for each culture indicated that the closest NCBI BLAST relative for

most of the sequences was an uncultured bacterium, suggesting that the MTBE-degrading microorganisms in the cultures are novel.

In 2008, researchers from the Häggblom lab analyzed a bacterial consortium from a decade long MTBE enrichment process (199) and suggested that anaerobic MTBE degradation is mediated by acetogenic bacteria. Several lines of evidence were presented. First, the MTBE-degrading cultures were shown to O-demethylate syringic acid, consistent with acetogenic bacteria which are known for cleaving the methyl group from methoxylated aromatic compounds. Second, the addition of syringic acid to cultures enhanced the rate of MTBE transformation to TBA. Third, MTBE degradation was observed when two different inhibitors were applied. Propyl iodide inhibits O-demethylation reactions in a light reversible manner by binding the corrinoid present in many methyl accepting proteins. When it was added to cultures incubated in the dark, MTBE degradation was inhibited. Cultures that were incubated in the light continued to exhibit MTBE degradation, suggesting that MTBE transformation to TBA involves a corrinoid mediated reaction like acetogenic O-demethylation. Rifampicin inhibits RNA polymerase in bacteria but not in archaea, so if MTBE degradation was mediated by methanogenic archaea, degradation in methanogenic cultures would be expected to continue in the presence of rifampicin. But when rifampicin was added to either methanogenic or sulfidogenic cultures, MTBE degradation was inhibited, suggesting that degradation is mediated by bacteria. A followup analysis of these cultures however did not confirm the involvement of acetogenic bacteria.

Most recently, the Häggblom group used a T-RFLP and cloning approach to characterize the microorganisms in their anaerobic MTBE degrading cultures (198). T-RFLP analysis showed that three T-RF profiles either persisted or increased in relative abundance over time. A clone library was constructed and 16S rRNA clone sequences matching the three primary T-RFLP profiles were compared to type strains listed in the Ribosomal Database Project II. The three most abundant profiles were related to type strains *Anaerococcus prevottii* (95% identity), *Geobacter metallireducens* (90% identity), and *Dehalococcoides ethenogenes* (79% identity). None of the sequences were closely related to known organisms capable of aryl-methyl ether O-demethylation. Based on this analysis, the organisms most likely responsible for MTBE degradation in the enrichment cultures could not be determined. “The continued presence of multiple T-RF peaks, even after several years with MTBE as the sole carbon source, suggests that interspecies interactions of a consortium may be required for appreciable anaerobic MTBE degradation to occur” (198).

The Häggblom group has also used CSIA to characterize MTBE degradation in methanogenic and sulfate reducing cultures (162, 163). TBA was again shown to accumulate and MTBE degradation required a lengthy lag period. However, <sup>13</sup>C isotopic enrichment was observed. Other studies have expanded the use of CSIA from enrichment cultures to field setting and they offer some compelling evidence for *in situ* anaerobic MTBE biodegradation (87, 91). Groundwater samples from a contaminated aquifer showed that MTBE concentrations along the contaminant

plume decreased by a factor of 40 while there was a concomitant increase in the  $^{13}\text{C}/^{12}\text{C}$  ratio of the remaining MTBE. As previously discussed, biological enzymes favor lighter isotopes, so the  $^{13}\text{C}$ -enrichment observed in the residual MTBE provided strong evidence for *in situ* anaerobic MTBE biodegradation.

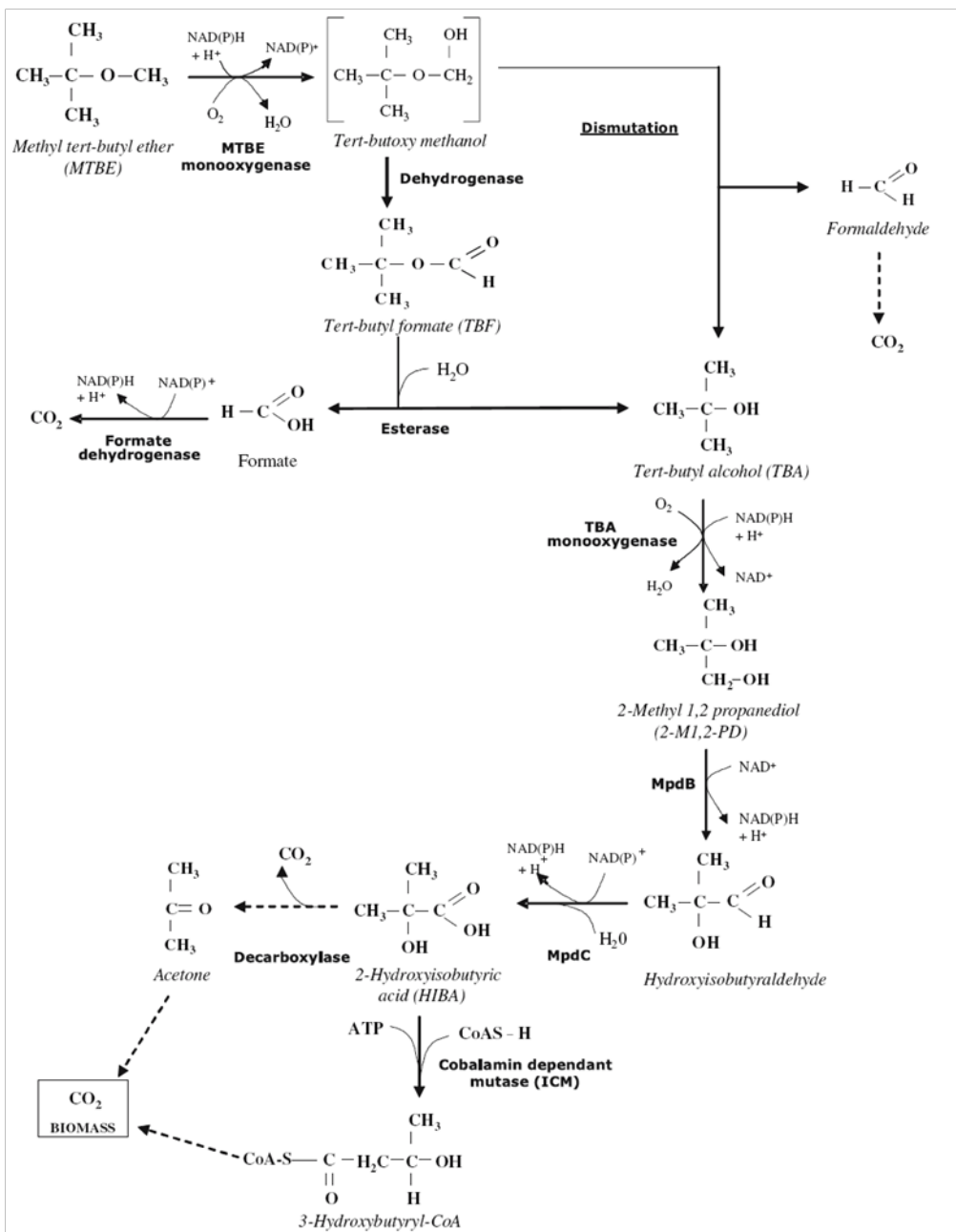
In summary, multiple studies using analysis tools such as CSIA, T-RFLP, and cloning have provided evidence for anaerobic MTBE biodegradation in enrichments, sediment microcosms, and field settings. Anaerobic TBA biodegradation has also been observed in sediment microcosms (13), but in general, it accumulates as a dead end reaction product when MTBE is anaerobically degraded (191). These processes are generally characterized by long incubation periods, which can be somewhat enhanced under methanogenic culture conditions by the addition of various methoxylated aromatic compounds such as syringic acid. But even with these enhanced analysis tools and improved information about anaerobic MTBE biodegradation, our understanding of this process and how specific microorganisms participate continues to be limited. A time-resolved SIP analysis may be an appropriate tool for gaining further insights about this process and the microorganisms involved.

## **2.2 Aerobic MTBE and TBA Biodegradation**

Even though multiple studies aimed at anaerobic MTBE biodegradation have been initiated in recent years, most of our understanding about MTBE and TBA biodegradation comes from the study of aerobic microorganisms. Aerobic MTBE degradation is generally thought to proceed according to the diagram shown in

Figure 2.2.a. Based on the assumption that a monooxygenase enzyme is involved in the initial reaction, the first MTBE degradation intermediate is thought to be an unstable hemiacetal, which has not been detected, but recently confirmed to exist (63). TBA, however, is a well recognized MTBE degradation intermediate and depending on the microorganisms involved, tertiary butyl formate (TBF) may also be detected.

MTBE and TBA biodegradation may be thought of in terms of two different types of microbial processes. First, MTBE and TBA can be aerobically biodegraded by microorganisms which use these compounds as a sole source of carbon and energy. This sort of direct MTBE and TBA metabolism has been described for growth of a limited number of microorganisms, most of which belong to the *β-Proteobacteria* (49, 56, 58, 94), but at least one is a *Mycobacterium* (41). The other aerobic biodegradation process with significant application regarding MTBE and TBA utilization spans a more diverse array of microorganisms and is often described as cometabolism. The idea of cometabolism was introduced by Leadbetter and Foster (93) when they used the term cooxidation to “describe the process in which a microorganism oxidizes a substance without being able to utilize the energy derived from this oxidation to support growth” (61). Later, the term cometabolism (61, 77) emerged. And although the term has been criticized (183), its use has persisted especially in the field of bioremediation where it is particularly relevant to the characterization of microbial processes such as aerobic



**Figure 2.2.a – Proposed Aerobic MTBE Biodegradation Pathways with Proposed Enzymes.** The unstable hemiacetal has not been detected, so its structure is enclosed by brackets. Recently, however, its existence was indirectly confirmed (63). Diagram taken from N. Lopes Ferreira, et al. 2006. Enzymes and genes involved in the aerobic biodegradation of methyl *tert*-butyl ether (MTBE). *Applied Microbiology and Biotechnology*. 72:252-262.

biodegradation of trichloroethylene and other chlorinated solvents (1, 2, 32, 33, 82, 147). Cometabolism is also relevant to the microbial biodegradation of MTBE. For the processes described in this dissertation, the term cometabolism follows the meaning provided by Smith, et al. (156). “In a cometabolic process, the fortuitous transformation of a non-growth-supporting substrate is catalyzed by an enzyme or cofactor that is expressed by the cell for an alternative, and often growth-related, purpose” (156). Aerobic MTBE and TBA biodegradation will be discussed in terms of the following: 1) microbial metabolism in which MTBE and TBA are utilized as sole sources of carbon and energy, and 2) microbial cometabolism in which MTBE is fortuitously transformed by microorganisms utilizing a different substrate for growth.

### **2.2.1 MTBE and TBA Aerobic Metabolism**

A number of sediment or sludge derived microcosms (12, 14, 36, 142) and enriched cultures consisting of bacterial consortia (39, 99, 135, 186, 200) which can utilize MTBE or TBA as the sole source of carbon have been reported. More detailed studies of these processes however involve microbial isolates. In 1997, Mo, et al., reported on three isolates identified by the API Rapid NFT test as belonging to genera *Methylobacterium*, *Rhodococcus*, and *Arthrobacter* (117). All three isolates were capable of slow growth on MTBE as a sole carbon source, but MTBE degradation was enhanced by the presence of yeast extract. And, in comparison to MTBE-degrading mixed cultures, these isolates showed less robust MTBE degradation activity. The low growth rates of the isolates on MTBE suggested that MTBE may be a poor growth substrate or that degradation intermediates may have

inhibitory or toxic effects on the cells. However, additional isolates have been reported.

*Hydrogenophaga flava* ENV735 is a hydrogen-oxidizing bacterium that has been reported to slowly utilize MTBE and TBA as growth substrates (58, 168). MTBE and TBA consumption are both enhanced when cultures are supplemented with yeast extract, but the presence of H<sub>2</sub> does not appear to have any stimulatory effects. Strain ENV735 does not degrade BTEX compounds, but MTBE degradation does not appear to be impacted by their presence. When ENV735 cells are incubated in the presence of 1-amino benzotriazole (ABT), a P450 monooxygenase enzyme inhibitor, MTBE degradation is inhibited, suggesting that P450s are utilized for MTBE degradation by strain ENV735. However, ABT did not inhibit TBA degradation by this strain, further suggesting that separate enzymes are utilized by ENV735 for MTBE and TBA degradation activity.

*Mycobacterium austroafricanum* IFP 2012 was isolated from activated sludge and has both MTBE and TBA degrading capabilities (40, 41), which are enhanced by the presence of yeast extract. Growth of IFP 2012 on MTBE and TBA was completely inhibited in the presence of acetylene, suggesting that these processes rely on a monooxygenase other than the P450 type suggested for ENV735. In addition, during MTBE degradation, IFP 2012 generates TBF, which was first reported as an intermediate in cometabolic MTBE degradation by *Graphium* sp. (57). But more importantly, studies with IFP 2012 have identified two putative dehydrogenase proteins, MpdB and MpdC, (101) that are likely involved near the

end of the proposed MTBE biodegradation pathway shown in Figure 2.2.a. The putative MpdB and MpdC sequences align closely with known sequences for choline dehydrogenase and betaine aldehyde dehydrogenase, respectively. And when DNA sequences for *mpdB* and *mpdC* were transferred into *Mycobacterium smegmatis* mc2 155, a strain with no MTBE or TBA degrading capacity, it was subsequently capable of transforming 2-M-1,2-PD to HIBA. And finally, TBA degradation in strain IFP 2012 has been linked to its expression of *alkB* after growth on propane, hexane, and hexadecane (102). This is unusual because MTBE degradation after growth on alkanes is typically associated with cometabolic degradation processes. In addition, *alkB* codes for alkane hydroxylase, which is an integral membrane non-heme iron protein initially identified (158, 179, 181) in non-TBA-degrading *Pseudomonas putida* Gpo1 (155).

Perhaps the best characterized microbial isolate with demonstrated capability for growth on either MTBE or TBA as a sole source of carbon and energy is *Methylibium petroleiphilum* PM1, a uniflagellated Gram negative rod belonging to the  $\beta$ -Proteobacteria and closely related to *Aquabacterium commune* DSM 11901 (96%) based on 16S rRNA gene sequence identity (121). Strain PM1 was isolated from a mixed culture derived from a compost biofilter and enriched on MTBE as the sole carbon source (15, 56, 121). It has also been reported to grow on a variety of other carbon substrates such as ethanol, pyruvate, acetate, butanol, methanol, toluene, benzene, phenol, ethylbenzene, and several dihydroxybenzoates (121). A whole genome analysis of strain PM1 showed that it has a circular ~4 Mb chromosome and

a ~600 kb megaplasmid (80). The chromosome was found to contain putative alcohol and aldehyde dehydrogenases but they are only 45% and 54% similar to sequences for MpdB and MpdC found in strain IFP 2012. The chromosome was also found to contain multiple genes that appear to code for monooxygenases, including those associated with benzene and toluene degradation. Also found was a putative propane monooxygenase pathway, with predicted proteins that are similar to those of *Gordonia* sp. strain TY-5, an *n*-alkane and propane oxidizer (88). This discovery was interesting because propane metabolism has been linked to cometabolic MTBE degradation in other organisms (62, 156, 167). However, plasmid curing by heat stress (173) demonstrated that the PM1 megaplasmid is essential for MTBE degradation, suggesting that the monooxygenase genes identified on the chromosome may not be important for this organism's metabolic capabilities toward MTBE.

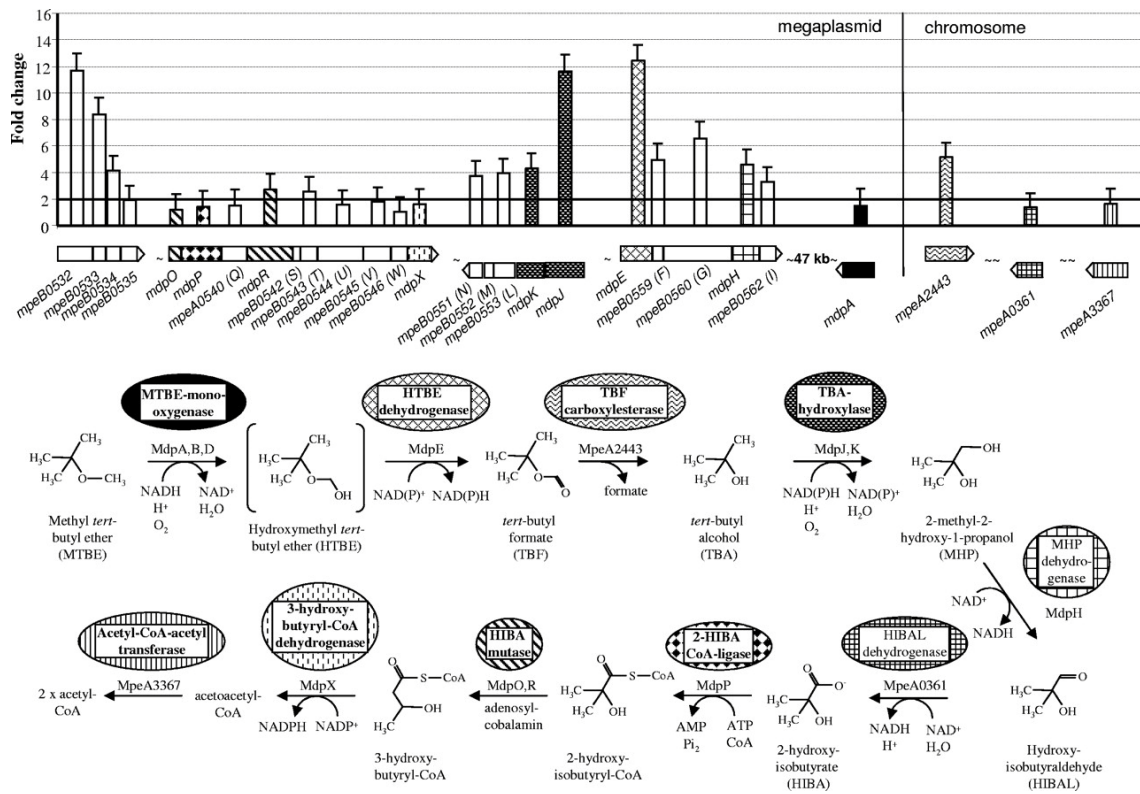
The PM1 megaplasmid contains a putative alkane monooxygenase that includes two rubredoxin genes and the gene *alkB*, which codes for the hydroxylase subunit. The putative AlkB sequence in PM1 is 66% similar to the alkane hydroxylase associated with MTBE cometabolism by *Pseudomonas putida* GPO1 (155) and 69% similar to that of *Alcanivorax borkumensis* AP1, an alkane degrading organism (180, 195). "The AlkB system in PM1 is likely involved in MTBE hydroxylation, based on its similarity to other AlkB proteins in organisms shown to be involved in MTBE degradation" (80). However, a cDNA microarray analysis in which the PM1 transcriptomes were compared after cell growth on MTBE and ethanol (64)

failed to show upregulation of *alkB* in MTBE grown cells. Genes for the corresponding rubredoxin (*mdpB*) and the rubredoxin reductase (*mdpD*) were not present in the microarray, so their responses could not be evaluated.

Differential expression was observed for other genes and key responses were summarized in a proposed PM1 degradation pathway as shown in Figure 2.2.1.c.

Upregulated responses to PM1 growth on MTBE were detected in genes *mdpE* (12-fold), *mpeA2443* (5.2-fold), *mdpJ* (11.7-fold), *mdpK* (4.3-fold), *mdpH* (4.6-fold), and *mpeB0532* through *mpeB0535* (~12-fold to ~2-fold). Gene *mdpE* encodes a putative dehydrogenase and was proposed to be involved in production of TBF. Gene *mpeA2443* is located on the chromosome and codes for a possible TBF esterase that may catalyze the conversion of TBF to TBA. Genes *mdpJ* and *mdpK* were reported as part of a new dioxygenase system that is involved in TBA oxidation. *mdpJ* codes for a protein reported to belong to group I of the phthalate dioxygenases as described by Parales and Resnick (125) and *mdpK* codes for an associated Fe-S reductase. Gene *mdpH* codes for a putative dehydrogenase that was proposed to be responsible for transformation of 2-M-1,2-PD to HIBA. Interestingly, genes for the PM1 dehydrogenases that were comparable to *MpdB* and *MpdC* in strain IFP 2012 were either neutral or downregulated when PM1 was grown on MTBE, suggesting that these genes are not involved in MTBE degradation by PM1.

And finally, a group of genes from *mpeB0532* to *mpeB0535* were at least two-fold upregulated, but they code for proteins with homology to no known proteins.



**Figure 2.2.1.c – Gene expression levels of the proposed MTBE degradation regulon and proposed MTBE degradation pathway in *M. petroleiphilum* PM1.** Error bars represent 1 standard deviation. Taken from Hristova, K. R., et al. 2007. Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to fuel oxygenates methyl *tert*-butyl ether and ethanol. *Applied and Environmental Microbiology*. 73(22): 7347-7357.

The predicted MdpA and its involvement in MTBE degradation by PM1 was further characterized with a PM1 mutant containing a disrupted version of *mdpA* (144). The PM1 mutant grew on TBA, but not MTBE, as the sole carbon source. Ethanol-grown resting cells of both the PM1 mutant and the wild type degraded TBA at similar rates, but only the wild type was able to degrade MTBE. These findings suggested the involvement of two enzymes in MTBE and TBA degradation, which was consistent with the transcriptome analysis that proposed two separate gene systems for MTBE (*mdpA*) and TBA (*mdpJ* and *mdpK*) degradation by PM1. Complementation failed to restore the PM1 mutant's ability to utilize MTBE as a growth substrate, but ethanol-grown resting cells of the complement strain and the wild type degraded MTBE and TBA at similar rates. These findings suggested that *mdpA* is essential for MTBE degradation by PM1. Methimazole inhibition assays further suggested MdpA is a flavin-dependent monooxygenase.

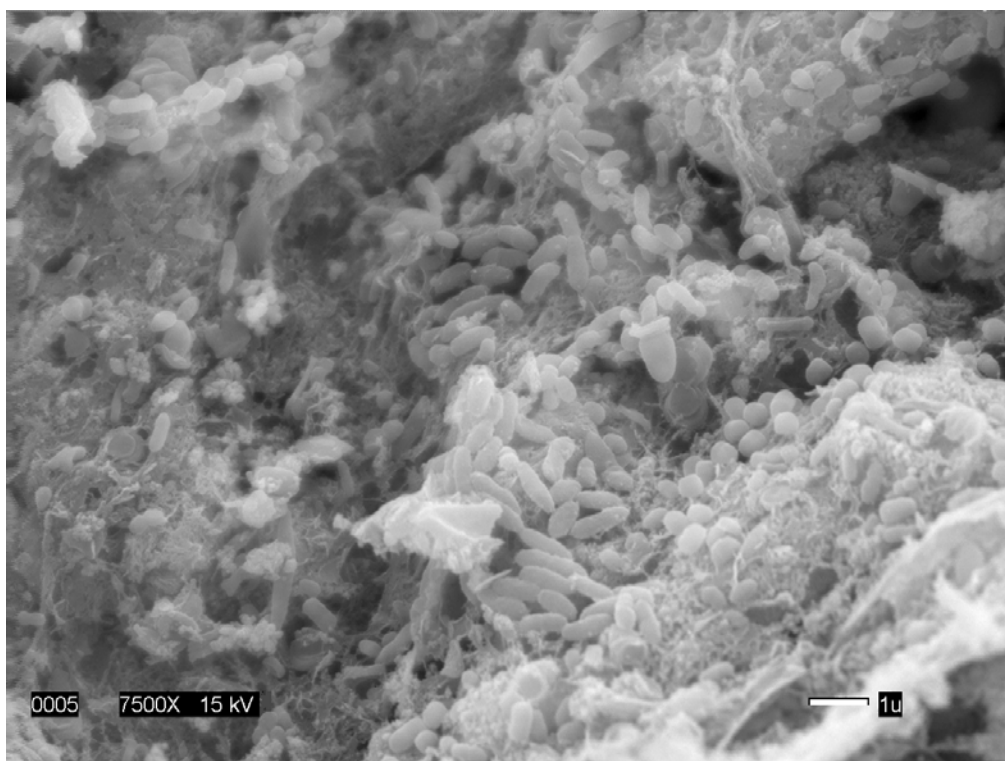
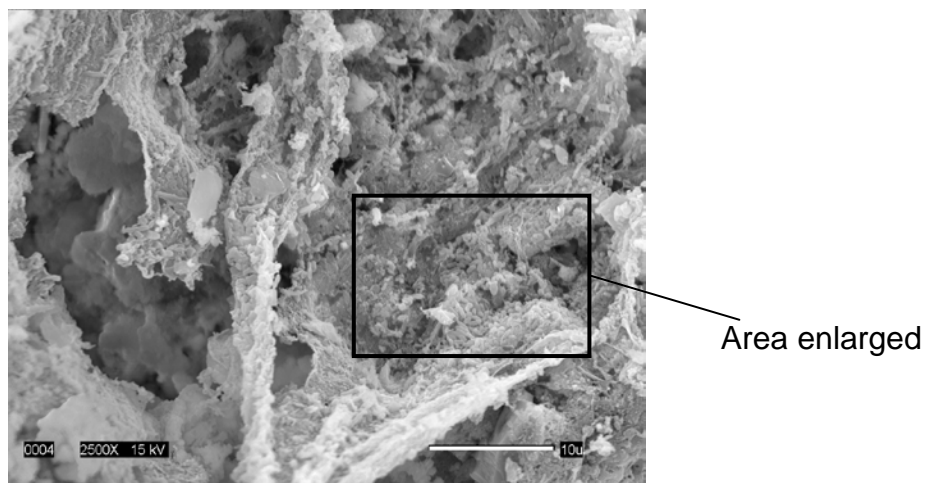
Studies of *Aquicola tertiaricarbonis* strains L108, L10, and CIP I-2052 (94) have also contributed to what is known about the proposed MTBE degradation pathway (Figure 2.2.a). Strain L108 was originally characterized by its ability to grow on MTBE, ETBE, and TAME, but faster growth rates have been observed for TBA, TAA, and 2-HIBA (120). Strains L10 (an L108 mutant) and CIP-I2052 grow on TBA but not on MTBE (127, 141). Cobalt was found to stimulate growth of these organisms on TBA and HIBA (141). And when cobalamin replaced cobalt in the growth media, doubling times were decreased from ~13 to ~5 hours. This result suggested that cobalamin synthesis is the rate limiting step for growth on TBA and

HIBA. Analysis via a 2-dimensional SDS-PAGE gel identified protein sequences corresponding to subunits (IcmA and IcmB) of a cobalamin-dependent mutase. Mutase enzymatic activity was demonstrated with extracts from HIBA-grown cells which produced 3-hydroxybutyrate from HIBA. Acetate grown cells did not have this activity. And interestingly, the PM1 genome was found to have ICM genes nearly identical to those found in L108, suggesting that HIBA is processed in the same way in these two strains.

MTBE and TBA degrading isolates have also been used in bioaugmentation trails that have informed our understanding of MTBE and TBA bioremediation in the field. For example, the *in situ* MTBE degrading capacity of strain PM1 was tested by inoculating laboratory cultivated cells into a field test plot at Port Hueneme Naval Base (154). Following a gasoline release from a gas station, a 1,500-m contaminant plume containing MTBE was found to impact a shallow aquifer. Test plots A and B were installed downgradient from the MTBE source. Both plots were sparged with O<sub>2</sub>, and after sparging, only Plot B was inoculated with PM1 cells. Groundwater was sampled throughout the test period and the rates of MTBE removal in the field trial were similar for both test plots. These results suggested that O<sub>2</sub> amendments alone may be an effective *in situ* biotreatment strategy for stimulating the MTBE degradation capacity of naturally occurring microorganisms.

Another study characterized the TBA-degrading microorganisms derived from an *ex situ* remediation system used to treat TBA-impacted groundwater (136). In such systems, contaminated groundwater is pumped through a bioreactor containing

granular activated carbon (GAC). Indigenous microorganisms colonize the GAC (forming bioGAC as shown in Figure 2.2.1.d) and remove groundwater contaminants. bioGAC was used in TBA enrichments to develop two mixed microbial cultures capable of growth on TBA as the sole carbon source. Culture growth on TBA was examined over a range of temperature and pH conditions. Resting cells of both cultures mineralized TBA most effectively at 37°C, but one tolerated a range of 4°C to 60°C. TBA was also mineralized over a pH range of 5.0 to 9.0, although the most effective degradation range was 8.0 to 9.0. These results demonstrated that TBA-degrading microorganisms can be recovered from GAC inoculated with naturally occurring microorganisms from TBA-impacted groundwater. And TBA-degrading capacity by such organisms may be retained under fluctuating temperature and pH ranges relevant to field conditions.



**Figure 2.2.1.d – bioGAC from a reactor treating TBA-impacted groundwater on Long Island, NY.** photos taken by Valerie Knowlton, SEM Center, North Carolina State University. An SIP analysis of this bioGAC is included in Chapter 2 of this dissertation.

### 2.2.2 MTBE Aerobic Cometabolism

Cometabolism is particularly relevant to MTBE biodegradation because MTBE frequently enters the environment as part of a gasoline mixture containing various *n*-alkanes, such as pentane, hexane, and octane (63). MTBE cometabolism has been reported for a variety of microorganisms that primarily utilize *n*-alkanes and branched alkanes as growth substrates (19, 47, 74-76, 98, 155-157, 167). And unlike most MTBE- and TBA-degrading isolates which cluster within the *β-Proteobacteria*, cometabolic MTBE-degrading organisms can be found in both bacterial and eukaryotic domains.

Interestingly, the first report of MTBE cometabolism did not involve a bacterial consortium or isolate, but rather a eukaryotic filamentous fungus, a *Graphium* sp. strain ATCC 58400 which is capable of growth on some gaseous *n*-alkanes as a sole carbon and energy source (23, 152). This organism cannot grow on MTBE but degrades it after growth on either propane (152, 153), *n*-butane (57), or diethyl ether (DEE) (57). The monooxygenase enzyme system which initiates growth on these substrates is also thought to be responsible for MTBE oxidation. Several lines of evidence support this idea. First, MTBE is readily oxidized by either propane, *n*-butane, or DEE-grown, but not dextrose-grown, mycelia suggesting that MTBE degradation activity is expressed only when a gaseous *n*-alkane or DEE is a growth substrate. Second, propane, *n*-butane, DEE, and MTBE oxidation are each inhibited by acetylene, which is known to inhibit fungal P450-type monooxygenase enzyme systems (23) as well as growth of *Graphium* sp. on gaseous *n*-alkanes.

Third, when *n*-butane-grown mycelia are exposed to either a mixture of *n*-butane and MTBE or MTBE alone, the MTBE degradation rate is slower for the mixture, suggesting that *n*-butane and MTBE compete for the same enzyme. And fourth, when *n*-butane grown mycelia were exposed to MTBE alone, the MTBE degradation rate became steadily slower but increased after the addition of *n*-butane. This MTBE degradation pattern is compatible with “the progressive exhaustion and subsequent replenishment of reductant required to support cytochrome P450-catalyzed oxidations” (57).

*Graphium* sp. was also the first organism reported to generate TBF during cometabolic MTBE degradation (57). In contrast to gaseous *n*-alkanes and MTBE, TBF is degraded in the absence of oxygen and in the presence of acetylene. This suggests that TBF degradation requires an enzyme system that is separate from the one responsible for propane and MTBE oxidation. And finally, TBA oxidation was not detected under any test conditions for this organism, suggesting that *Graphium* sp. have limited or no capacity to degrade TBA.

*Graphium* sp. is the only organism reported to generate TBF and not oxidize TBA during cometabolic MTBE degradation. Other organisms with cometabolic MTBE degrading activity are bacterial and have been characterized in two groups “based on the final extent of MTBE degradation and the intermediates generated during biodegradation” (153). The first group of organisms is represented by bacteria like *Pseudomonas mendocina* KR1 (157) and *Pseudomonas putida* GPo1 (155) which oxidize MTBE and generate TBA and formaldehyde (153). The fate of

formaldehyde is uncharacterized, but studies suggest that TBA is not oxidized further by these *n*-alkane-grown organisms (153, 155, 157). The second group is represented by propane-grown *Mycobacterium austroafricanum* JOB5 (formerly *vaccae*) (62, 63, 156) and *n*-butane-grown bacteria (98, 153) and is characterized by a cometabolic MTBE degradation process in which the first detectable degradation intermediate is TBF. Subsequent hydrolysis of TBF yields TBA (57, 153), which is further oxidized by the same monooxygenase enzyme that is responsible for oxidizing MTBE (153).

Perhaps the best characterized bacterium with demonstrated cometabolic MTBE-degrading capacity is *Mycobacterium austroafricanum* JOB5 (62, 63, 79, 156). Strain JOB5 was originally isolated from soil with enrichments containing isopentane as a sole source of carbon and energy (123). It is a versatile organism not only capable of growth on a wide variety of *n*-alkanes and isoalkanes (63, 123), but also known to cometabolically oxidize many organic pollutants after growth on propane (55, 104, 148, 182, 184). It is also known to oxidize MTBE and TBA after growth on a wide range of gasoline components including *n*-alkanes, isoalkanes, and BTEX compounds (63). The initial reactions during cometabolic oxidation of MTBE by propane-grown cells of JOB5 have been characterized and the proposed reaction pathway is shown in Figure 2.2.2.a.

In the first reaction, MTBE is oxidized by the same short chain alkane monooxygenase responsible for propane oxidation. Evidence supporting this proposal is consistent with previous *Graphium* sp. studies which indicated a



common enzyme system for *n*-alkane and MTBE oxidation. For example, propane grown cells responded to MTBE exposure with a high level of immediate MTBE oxidation, whereas casein-yeast extract-dextrose grown cells (in which alkane-oxidizing activity is not expressed), did not oxidize MTBE. In addition, propane was shown to inhibit MTBE oxidation in propane-grown cells, suggesting that the two substrates are competing for the same enzyme. And finally, acetylene completely inhibited both propane and MTBE oxidation by propane-grown cells of JOB5, further suggesting that one enzyme is common to both processes.

In the second reaction, the unstable hemiacetal is oxidized to TBF by an alcohol dehydrogenase. This enzyme was proposed for at least two reasons. First, alcohol dehydrogenases are important in the alkane oxidation pathway and since JOB5 cells oxidizing MTBE were grown on propane, they were likely to express alcohol dehydrogenase activity. Second, hemiacetal oxidation reactions have been observed for methanol-grown yeasts, which utilize methyl formate synthase (an NAD<sup>+</sup>-reducing alcohol dehydrogenase) to generate methyl formate from the hemiacetal. It is this step in the pathway that distinguishes strain JOB5 from other cometabolic MTBE-degrading bacteria like *Pseudomonas mendocina* KR1, which does not generate TBF (157). The lack of TBF production is “likely a reflection of differences in catalytic capabilities of the alcohol dehydrogenases” in these two organisms (157).

The third reaction is an esterase-catalyzed hydrolysis of TBF to TBA. An esterase has been proposed here for several reasons. First, esterases are

common in mycobacteria. Second, TBF degradation was examined in the absence of MTBE, and propane-grown cells rapidly converted TBF to TBA. Propane-grown cells also converted TBF to TBA in the presence of acetylene suggesting this reaction does not involve a monooxygenase. Third, propane-grown cells were incubated with TBF and other *tert*-butyl esters (*tert*-butyl acetate and *tert*-butyl propionate) and TBA accumulation was observed for both esters. Fourth, the Tween 80 hydrolysis test (20, 128), which is often used to detect esterase activity in mycobacteria, was strongly positive for JOB5. And finally, heat stability has been associated with some mycobacterial esterases, and in a reaction containing heat treated (30 min, 95°C) TBF and propane-grown cells, TBF was consumed and TBA was generated suggesting the presence of a heat stable enzyme.

The final part of the proposed sequence in Figure 2.2.2.a shows the further oxidation of TBA by the same monooxygenase responsible for MTBE oxidation. Again, it was shown that both MTBE and TBA oxidation were inhibited by acetylene. And, other lines of evidence similar to those provided for MTBE oxidation were also provided for TBA. TBA oxidation products were not identified in this study, but as previously discussed for aerobic MTBE metabolism, others have suggested that 2-M-1,2-PD is the oxidation product (41) and 2-M-1,2-PD was detected in a subsequent JOB5 study (79). Overall, the reactions depicted in Figure 2.2.2.a are similar to those described for the cometabolic degradation of MTBE by *Graphium* sp.. Both organisms generate TBF, but only JOB5 has the ability to further oxidize TBA.

In a subsequent study of MTBE-oxidizing activity by JOB5, Johnson, et al., reported that MTBE oxidation occurs during growth of JOB5 on a wide range of non-alkane substrates (79). And, acetylene inhibited consumption of MTBE, one again implicating the involvement of a monooxygenase in MTBE oxidation. In addition, MTBE was shown to have an inductive effect on alkane monooxygenase. When glycerol-grown cells were exposed to MTBE, MTBE-oxidizing activity was not immediate, but was acquired over time, and was strongly inhibited by chloramphenicol and rifampin. These results suggested that MTBE oxidizing activity by these cells required both transcription and *de novo* protein synthesis, further suggesting that the presence of MTBE induced MTBE-oxidizing activity. To demonstrate that MTBE-oxidizing activity was not constitutively expressed, cells grown on valeric acid were used. If MTBE-oxidizing activity was constitutive, then a steadily increasing oxidation rate would be predicted with a corresponding increase in cell density. But this was not observed. Consumption of MTBE and production of TBF and TBA began only after residual valeric acid had been nearly depleted. This result suggests a catabolite repression effect, which also supports the idea of “MTBE-dependent gene induction” (79). These results are important because “organic acids can accumulate in gasoline impacted environments as a result of anaerobic degradation of gasoline components. If low concentrations of acids are present with MTBE in environments undergoing oxygenation, the physiological conditions for cometabolic degradation could be met”. (79)

In a more recent study with JOB5, the potential importance of cometabolic MTBE-degradation in gasoline impacted environments was further emphasized (63). House and Hyman examined the effects of individual gasoline hydrocarbons and BTEX compounds on growth of JOB5, as well as, cometabolism of MTBE and TBA by this organism. Hydrocarbons examined included *n*-alkanes (C<sub>5</sub> to C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>), isoalkanes (C<sub>5</sub> to C<sub>8</sub>), alicyclics (cyclopentane, methylcyclopentane), and BTEX compounds (benzene, toluene, ethylbenzene, *m*-, *o*-, and *p*-xylenes). All of the tested alkanes supported growth of JOB5, as well as, MTBE and TBA oxidation. And once again, acetylene inhibited MTBE and TBA oxidation by alkane-grown cells. Acetylene also inhibited JOB5 growth on C<sub>5-8</sub> *n*-alkanes and isoalkanes, but interestingly, acetylene did not affect growth on longer chain C<sub>10</sub>, C<sub>12</sub>, or C<sub>14</sub> *n*-alkanes. Based on these results, a model comprised of two enzyme systems was proposed. One was described as an MTBE- and TBA-oxidizing short chain alkane monooxygenase (SCAM) responsible for initiating oxidation and catabolism of shorter chain *n*-alkanes ( $\leq$  C<sub>9</sub>). Essentially, SCAM was the same MTBE- and TBA-oxidizing enzyme system found in propane-grown cells. A second system (LCAM), not involved in MTBE or TBA oxidation, was proposed for oxidation and catabolism of longer chain *n*-alkanes ( $\geq$  C<sub>10</sub>).

Experimental results suggested that SCAM was strongly expressed by strain JOB5 during growth on shorter chain *n*-alkanes with maximal MTBE oxidizing activity observed by cells grown on C<sub>5</sub> and C<sub>6</sub> *n*-alkanes. A similar trend was reported for isoalkanes. These observations are important because they suggest

that SCAM is maximally expressed after growth on two of the most abundant (138) alkanes (*n*-pentane and 2-methylbutane) found in gasoline. The tested alicyclics did not support growth and they inhibited MTBE and TBA oxidation by JOB5, suggesting a competitive interaction between these compounds at the SCAM active site. For the BTEX compounds, a broad range of effects was observed. Benzene and ethylbenzene did not support growth of JOB5, and both compounds inhibited MTBE and TBA oxidation by alkane-grown cells. Benzene, however, was cometabolically degraded by *n*-pentane grown cells. Toluene, *m*-, and *p*-xylenes did support growth of JOB5 and cells grown on these substrates also had MTBE and TBA oxidation activity. Interestingly, low concentrations of toluene (< 10µM) appeared to stimulate MTBE and TBA oxidation by alkane-grown JOB5, but high concentrations were inhibitory. Again, these findings are significant regarding the potential cometabolic MTBE-degrading activity in gasoline impacted environments. “The presence of high concentrations of *n*-alkanes in gasoline raises the possibility that cometabolic degradation of MTBE may occur in gas-impacted environments, if appropriate environmental conditions are met” (157).

In summary, cometabolic MTBE degradation systems have been described for a diverse range of microorganisms. *Graphium* sp. is a eukaryotic filamentous fungus which is the first reported cometabolic MTBE-degrading microorganism. TBF was first observed as an MTBE degradation intermediate in studies with this organism. The same monooxygenase is proposed to be responsible for both *n*-alkane and MTBE oxidation. TBF is formed prior to TBA, and TBA is not further

oxidized. A enzyme system separate from the MTBE degrading monooxygenase is proposed for TBF degradation.

Cometabolic MTBE-degrading systems have been described for two groups of bacteria. The first group is characterized by *Pseudomonas mendocina* KR1 and *Pseudomonas putida* GPo1 which oxidize MTBE and generate TBA and formaldehyde. The fate of formaldehyde is unknown but TBA is not further oxidized by these organisms. The second group is represented by organisms like *Mycobacterium austroafricanum* JOB5, which is characterized by the generation of TBF as the first detectable product of MTBE oxidation. Subsequent hydrolysis of TBF yields TBA, which is further oxidized by these organisms. MTBE and TBA oxidation processes share a common monooxygenase enzyme.

*Mycobacterium austroafricanum* JOB5 is the best characterized bacterium with a cometabolic MTBE-degrading system. It is known for a high level of MTBE oxidizing activity after growth on propane. Propane and MTBE are oxidized by the same short chain alkane monooxygenase. The unstable hemiacetal predicted to be generated by MTBE oxidation is transformed to TBF by an alcohol dehydrogenase. TBF is hydrolyzed to TBA in an esterase catalyzed reaction. TBA is further oxidized by the MTBE-oxidizing short chain alkane monooxygenase to 2-M-1,2-PD. Resting cells of strain JOB5 grown on various organic compounds, such as glycerol and valeric acid, can be induced by MTBE toward cometabolic MTBE-degradation activity. Strain JOB5 maintains cometabolic MTBE degradation activity over a wide

range of growth substrates including *n*-alkanes, isoalkanes, and some aromatics that are major constituents of gasoline.

## References

1. **Alvarez-Cohen, L., and G. E. Speitel.** 2001. Kinetics of aerobic cometabolism of chlorinated solvents. *Biodegradation* **12**:105-126.
2. **Arp, D. J., C. M. Yeager, and M. R. Hyman.** 2001. Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene. *Biodegradation* **12**:81-103.
3. **Aston, F. W.** 1935. Isotopes. *Nature* **135**:686-687.
4. **Boersma, M. G., T. Y. Dinarieva, W. J. Middelhoven, W. J. H. V. Berkel, J. Doran, J. Vervoort, and I. M. C. M. Rietjens.** 1998. <sup>19</sup>F Nuclear Magnetic Resonance as a tool to investigate microbial degradation of fluorophenols to fluorocatechols and fluoromuconates. *Applied and Environmental Microbiology* **64**:1256-1263.
5. **Boersma, M. G., I. P. Solyanikova, W. J. H. V. Berkel, J. Vervoort, L. A. Golovleva, and I. M. C. M. Rietjens.** 2001. <sup>19</sup>F NMR metabolomics for the elucidation of microbial degradation pathways of fluorophenols. *Journal of Industrial Microbiology & Biotechnology* **26**:22-34.
6. **Bondar, V. S., M. G. Boersma, E. L. Golovlev, J. Vervoort, W. J. H. V. Berkel, Z. I. Finkelstein, I. P. Solyanikova, L. A. Golovleva, and I. M. C. M. Rietjens.** 1998. <sup>19</sup>F NMR study on the biodegradation of fluorophenols by various *Rhodococcus* species. *Biodegradation* **9**:475-486.
7. **Borodina, E., M. J. Cox, I. R. McDonald, and J. C. Murrell.** 2005. Use of DNA-stable isotope probing and functional gene probes to investigate the diversity of methyl-chloride utilizing bacteria in soil. *Environmental Microbiology* **7**:1318-1328.
8. **Boschker, H. T. S., and J. J. Middelburg.** 2002. Minireview: Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiology Ecology* **40**:85-95.
9. **Boschker, H. T. S., S. C. Nold, P. Wellsbury, D. Bos, W. de Graaf, R. Pel, R. J. Parkes, and T. E. Cappenberg.** 1998. Direct linking of microbial populations to specific biogeochemical processes by <sup>13</sup>C-labelling of biomarkers. *Nature* **392**:801-805.
10. **Bradley, P. M., F. H. Chapelle, and J. E. Landmeyer.** 2001. Effect of redox conditions on MTBE Biodegradation in surface water sediments. *Environmental Science and Technology* **35**:4643-4647.

11. **Bradley, P. M., F. H. Chapelle, and J. E. Landmeyer.** 2001. Methyl *t*-butyl ether mineralization in surface-water sediment microcosms under denitrifying conditions. *Applied and Environmental Microbiology* **67**:1975-1978.
12. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 1999. Aerobic mineralization of MTBE and *tert*-butyl alcohol by stream-bed sediment microorganisms. *Environmental Science and Technology* **33**:1877-1879.
13. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 2002. TBA biodegradation in surface-water sediments under aerobic and anaerobic conditions. *Environmental Science & Technology* **36**:4087-4090.
14. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 2001. Widespread potential for microbial MTBE degradation in surface-water sediments. *Environmental Science & Technology* **35**:658-662.
15. **Bruns, M. A., J. R. Hanson, J. Mefford, and K. M. Scow.** 2001. Isolate PM1 populations are dominant and novel methyl *tert*-butyl ether-degrading bacteria in compost biofilter enrichments. *Environmental Microbiology* **3**:220-225.
16. **Busch-Harris, J., K. Sublette, k. P. Roberts, C. Landrum, A. D. Peacock, G. Davis, D. Ogles, W. E. Holmes, D. Harris, C. Ota, X. Yang, and A. Kolhatkar.** 2008. 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* **28**:47-62.
17. **Cirvello, J. D., A. Radovsky, J. E. Heath, D. R. Farnell, and C. Lindamood, III.** 1995. Toxicity and carcinogenicity of *t*-Butyl alcohol in rats and mice following chronic exposure in drinking water. *Toxicology and Industrial Health* **11**:151-165.
18. **Conrad, R.** 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. *Organic Geochemistry* **36**:739-752.
19. **Corcho, D., R. J. Watkinson, and D. N. Lerner.** 2000. Cometabolic degradation of MTBE by a cyclohexane-oxidizing bacteria, p. 183-189. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), *Bioremediation and phytoremediation of chlorinated and recalcitrant compounds*. Battelle Press, Columbus, OH.

20. **Cox, F. R., C. E. Slack, M. E. Cox, E. L. Pruden, and J. R. Martin.** 1978. Rapid Tween 80 hydrolysis test for mycobacteria. *Journal of Clinical Microbiology* **7**:104-105.
21. **Criss, R. E.** 1999. *Principles of Stable Isotope Distribution*. Oxford University Press, New York, NY.
22. **Cupples, A. M., and G. K. Sims.** 2007. Identification of in situ 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing. *Soil Biology & Biochemistry* **39**:232-238.
23. **Curry, S., L. Ciuffetti, and M. Hyman.** 1996. Inhibition of growth of a *Graphium* sp. on gaseous *n*-alkanes by gaseous *n*-alkynes and *n*-alkenes. *Applied and Environmental Microbiology* **62**:2198-2200.
24. **Davidova, I. A., L. M. Gieg, M. Nanny, K. G. Kropp, and J. M. Suflita.** 2005. Stable isotopic studies of *n*-alkane metabolism by a sulfate reducing bacterial enrichment culture. *Applied and Environmental Microbiology* **71**:8174-8182.
25. **Davis, J. M., and W. H. Farland.** 2001. The paradoxes of MTBE. *Toxicological Sciences* **61**:211-217.
26. **Dawson, T. E., and P. D. Brooks.** 2001. *Fundamentals of Stable Isotope Chemistry and Measurement*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
27. **Deeb, R. A., K.-H. Chu, T. Shih, S. Linder, I. Suffet, M. C. Kavanaugh, and L. Alvarez-Cohen.** 2003. MTBE and other oxygenates: environmental sources, analysis, occurrence, and treatment. *Environmental Engineering Science* **20**:433-447.
28. **DeRito, C. M., and E. L. Madsen.** 2008. Stable isotope probing reveals *Trichosporon* yeast to be active *in situ* in soil phenol metabolism. *The ISME Journal* **3**:477-485.
29. **DeRito, C. M., G. M. Pumphrey, and E. L. Madsen.** 2005. Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. *Applied and Environmental Microbiology* **71**:7858-7865.
30. **Dumont, M. G., and J. C. Murrell.** 2005. Stable isotope probing - linking microbial identity to function. *Nature Reviews Microbiology*:1-6.

31. **Edwards, J. C.** Principles of NMR. <http://www.process-nmr.com/nmr1.htm>, accessed 4/22/2010.
32. **Ely, R. L., K. J. Williamson, M. R. Hyman, and D. J. Arp.** 1999. Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects, and bacterial response. *Biotechnology and Bioengineering* **63**:756.
33. **Ely, R. L., K. J. Williamson, M. R. Hyman, and D. J. Arp.** 1997. Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects, and bacterial response. *Biotechnology and Bioengineering* **54**:520-534.
34. **Farquhar, G. D., J. R. Ehleringer, and K. T. Hubick.** 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**:503-537.
35. **Finneran, K. T., and D. R. Lovley.** 2001. Anaerobic degradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA). *Environmental Science and Technology* **35**:1785-1790.
36. **Fiorenza, S., and H. S. Rifai.** 2003. Review of MTBE biodegradation and bioremediation. *Bioremediation Journal* **7**:1-35.
37. **Fiorenza, S., M. P. Suarez, and H. S. Rifai.** 2002. MTBE in groundwater: status and remediation. *Journal of Environmental Engineering* **128**:773-781.
38. **Fischer, A., K. Theuerkorn, N. Stelzer, M. Gehre, M. Thullner, and H. H. Richnow.** 2007. Applicability of stable isotope fractionation analysis for the characterization of benzene biodegradation in a BTEX-contaminated aquifer. *Environmental Science and Technology* **41**:3689-3696.
39. **Fortin, N. Y., M. Morales, Y. Nakagawa, D. D. Focht, and M. A. Deshusses.** 2001. Methyl *tert*-butyl ether (MTBE) degradation by a microbial consortium. *Environmental Microbiology* **3**:407-416.
40. **François, A., L. Garnier, H. Mathis, F. Fayolle, and F. Monot.** 2003. Roles of *tert*-butyl formate, *tert*-butyl alcohol and acetone in the regulation of methyl *tert*-butyl ether degradation by *Mycobacterium austroafricanum* IFP 2012. *Applied Microbiology and Biotechnology* **62**:256-262.

41. **François, A., H. Mathis, D. Godefroy, P. Piveteau, F. Fayolle, and F. Monot.** 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Applied and Environmental Microbiology* **68**:2754-2762.
42. **Friedrich, M. M., and A. Lipski.** 2010. Characterization of hexane-degrading microorganisms in a biofilter by stable isotope-based fatty acid analysis, FISH and cultivation. *Environmental Biotechnology* **85**:1189-1199.
43. **Friedrich, M. W.** 2006. Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. *Current Opinion in Biotechnology* **17**:59-66.
44. **Fry, B.** 2006. *Stable Isotope Ecology*. Springer Science+Business Media, LLC, New York, NY.
45. **Gallagher, E., L. McGuinness, C. Phelps, L. Y. Young, and L. J. Kerkhof.** 2005. <sup>13</sup>C-carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Applied and Environmental Microbiology* **71**:5192-5196.
46. **Gallagher, E. M., L. Y. Young, L. M. McGuinness, and L. J. Kerkhof.** 2010. Detection of 2,4,6-trinitrotoluene-utilizing anaerobic bacteria by <sup>15</sup>N and <sup>13</sup>C incorporation. *Applied and Environmental Microbiology* **76**:1695-1698.
47. **Garnier, P. M., R. Auria, C. Augur, and S. Revah.** 1999. Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Applied Microbiology and Biotechnology* **51**:498-503.
48. **Gelwicks, J. T., J. B. Risatti, and J. M. Hayes.** 1989. Carbon isotope effects associated with autotrophic acetogenesis. *Organic Geochemistry* **14**:441-446.
49. **Golart, K. L.** 2007. *Physiology and Enzymology of Aerobic MTBE and TBA Biodegradation*. Ph.D. Thesis. North Carolina State University, Raleigh.
50. **Gomez, J., T. Brasil, and N. Chan.** 1998. An overview of the use of oxygenates in gasoline. California Air Resources Board, California Environmental Protection Agency.
51. **Gray, J. R., G. Lacrampe-Couloume, D. Gandhi, K. M. Scow, R. D. Wilson, D. M. Mackay, and B. S. Lollar.** 2002. Carbon and hydrogen isotopic fractionation during biodegradation of methyl *tert*-butyl ether. *Environmental Science and Technology* **36**:1931-1938.

52. **Grivet, J.-P., A.-M. Delort, and J.-C. Portais.** 2003. NMR and microbiology: from physiology to metabolomics. *Biochimie* **85**:823-840.
53. **Habicht, K. S., and D. E. Canfield.** 1997. Sulfur isotope fractionation during bacterial sulfate reduction in organic-rich sediments. *Geochimica et Cosmochimica Acta* **61**:5351-5361.
54. **Habicht, K. S., and D. E. Canfield.** 1996. Sulphur isotope fractionation in modern microbial mats and the evolution of the sulphur cycle. *Nature* **382**:342-343.
55. **Hamamura, N., C. Page, T. Long, L. Semprini, and D. J. Arp.** 1997. Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b. *Applied and Environmental Microbiology* **63**:3607-3613.
56. **Hanson, J. R., C. E. Ackerman, and K. M. Scow.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Applied and Environmental Microbiology* **65**:4788-4792.
57. **Hardison, L. K., S. S. Curry, L. M. Ciuffetti, and M. R. Hyman.** 1997. Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. *Applied and Environmental Microbiology* **63**:3059-3067.
58. **Hatzinger, P. B., K. McClay, S. Vainberg, M. Tugusheva, C. W. Condee, and R. J. Steffan.** 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. *Applied and Environmental Microbiology* **67**:5601-5607.
59. **Heider, J., A. M. Spormann, H. R. Beller, and F. Widdel.** 1999. Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiology Reviews* **22**:459-473.
60. **Holliger, C., S. Gaspard, G. Glod, C. Heijman, W. Schumacher, R. P. Schwarzenbach, and F. Vazquez.** 1997. Contaminated environments in the subsurface and bioremediation: organic contaminants. *FEMS Microbiology Reviews* **20**:517-523.
61. **Horvath, R. S.** 1972. Microbial co-metabolism and the degradation of organic compounds in nature. *Bacteriological Reviews* **36**:146-155.

62. **House, A., and M. Hyman.** published online December 10, 2009. Effects of gasoline components on MTBE and TBA cometabolism by *Mycobacterium austroafricanum* JOB5. Biodegradation.
63. **House, A. J.** 2009. Characterizing MTBE cometabolism and propane metabolism by *Mycobacterium austroafricanum* JOB5. Ph.D. Thesis. North Carolina State University, Raleigh.
64. **Hristova, K. R., R. Schmidt, A. Y. Chakicherla, T. C. Legler, J. Wu, P. S. Chain, K. M. Scow, and S. R. Kane.** 2007. Comparative transcriptome analysis of *Methylbium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. Applied and Environmental Microbiology **73**:7347-57.
65. **[http://toxics.usgs.gov/definitions/fuel\\_oxygenates.html](http://toxics.usgs.gov/definitions/fuel_oxygenates.html)** 2006, posting date. Fuel Oxygenates. Toxic Substances Hydrology Program. U.S. Geological Survey. [Online.]
66. **<http://www.atsdr.cdc.gov/tfacts13.pdf>** 2007, posting date. Lead. Agency for Toxic Substances and Disease Registry, U.S. Dept. of Health and Human Services. [Online.]
67. **<http://www.cem.msu.edu/~reusch/VirtualText/Spectrpy/nmr/nmr1.htm>**. accessed 4/22/2010. Organic Chemistry On Line: Nuclear Magnetic Resonance Spectroscopy.
68. **<http://www.epa.gov/mtbe/gas.htm>** 2008, posting date. MTBE in Fuels. U. S. Environmental Protection Agency, Washington, DC. [Online.]
69. **<http://www.nist.gov/physlab/data/comp.cfm>** 1994, posting date. Atomic Weights and Isotopic Compositions for All Elements. National Institute of Standards and Technology. [Online.]
70. **<http://www.thefreelibrary.com/Appendix>** 2000, posting date. Appendix D: EIA 819M Monthly Oxygenate Telephone Report.-a069652814. The Free Library. [Online.]
71. **Hunkeler, D., R. Aravena, and B. J. Butler.** 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: microcosm and field studies. Environmental Science and Technology **33**:2733-2738.

72. **Hunkeler, D., B. J. Butler, R. Aravena, and J. F. Barker.** 2001. Monitoring biodegradation of methyl *tert*-butyl ether (MTBE) using compound specific-isotope analysis. *Environmental Science and Technology* **2001**:676-681.
73. **Hunkeler, D., N. Chollet, X. Pittet, R. Aravena, J. A. Cherry, and B. L. Parker.** 2004. Effect of source variability and transport processes on carbon isotope ratios of TCE and PCE in two sandy aquifers. *Journal of Contaminant Hydrology* **74**:265-282.
74. **Hyman, M., P. Kwon, K. Williamson, and K. O'Reilly.** 1998. Cometabolism of MTBE by alkane-utilizing microorganisms, p. 321-326. *In* G. B. Wickramanayake and R. E. Hinchee (ed.), *Natural Attenuation: Chlorinated and Recalcitrant Compounds*. Battelle Press, Columbus, OH.
75. **Hyman, M., C. Smith, and K. O'Reilly.** 2001. Cometabolism of MTBE by an aromatic hydrocarbon-oxidizing bacterium, p. 145-152. *In* V. S. Magar, J. T. Gibbs, K. T. O'Reilly, M. R. Hyman, and A. Leeson (ed.), *Bioremediation of MTBE, alcohols, and ethers*. Battelle Press, Columbus, OH.
76. **Hyman, M., C. Taylor, and K. O'Reilly.** 2000. Cometabolic degradation of MTBE by *iso*-alkane-utilizing bacteria from gasoline-impacted soils, p. 149-155. *In* G. B. Wickramanayake, A. R. Gavaskar, and B. C. Alleman (ed.), *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Compounds*. Battelle Press, Columbus, OH.
77. **Jensen, H. L.** 1963. Carbon nutrition of some microorganisms decomposing halogen-substituted aliphatic acids. *Acta Agriculturae Scandinavica* **13**:404 - 412.
78. **Jeon, C. O., W. Park, P. Padmanabhan, C. DeRito, J. R. Snape, and E. L. Madsen.** 2003. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for *in situ* biodegradation in contaminated sediment. *Proceedings of the National Academy of Sciences* **100**:13591-13596.
79. **Johnson, E. L., C. A. Smith, K. T. O'Reilly, and M. R. Hyman.** 2004. Induction of methyl tertiary butyl ether (MTBE)-oxidizing activity in *Mycobacterium vaccae* JOB5 by MTBE. *Applied and Environmental Microbiology* **70**:1023-30.
80. **Kane, S. R., A. Y. Chakicherla, P. S. G. Chain, R. Schmidt, M. W. Shin, T. C. Legler, K. M. Scow, F. W. Larimer, S. M. Lucas, P. M. Richardson, and K. R. Hristova.** 2007. Whole-genome analysis of the methyl *tert*-butyl ether-

- degrading Beta-proteobacterium *Methylibium petroleiphilum* PM1. Journal of Bacteriology **189**:1931-1945.
81. **Kasai, Y., Y. Takahata, M. Manefield, and K. Watanabe.** 2006. RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Applied and Environmental Microbiology **72**:3586-3592.
  82. **Kim, Y., D. J. Arp, and L. Semprini.** 2000. Chlorinated solvent cometabolism by butane-grown mixed culture. Journal of Environmental Engineering **126**:934-942.
  83. **Kinnaman, F. S., D. I. Valentine, and S. C. Tyler.** 2007. Carbon and hydrogen isotope fractionation associated with the aerobic microbial oxidation of methane, ethane, propane, and butane. Geochimica et Cosmochimica Acta **71**:271-283.
  84. **Kittelmann, S., and M. W. Friedrich.** 2008. Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. Environmental Microbiology **10**:31-46.
  85. **Kittelmann, S., and M. W. Friedrich.** 2008. Novel uncultured *Chloroflexi* dechlorinate perchloroethene to *trans*-dichloroethene in tidal flat sediments. Environmental Microbiology **10**:1557-1570.
  86. **Kohl, D. H., and G. Shearer.** 1980. Isotopic fractionation associated with symbiotic N<sub>2</sub> fixation and uptake of NO<sub>3</sub><sup>-</sup> by plants. Plant Physiology **66**:51-56.
  87. **Kolhatkar, R., T. Kuder, P. Philp, J. Allen, and J. T. Wilson.** 2002. Use of compound-specific stable carbon isotope analyses to demonstrate anaerobic biodegradation of MTBE in groundwater at a gasoline release site. Environmental Science and Technology **36**:5139-5146.
  88. **Kotani, T., T. Yamamoto, H. Yurimoto, Y. Sakai, and N. Kato.** 2003. Propane monooxygenase and NAD<sup>+</sup>-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. Journal of Bacteriology **185**:7120-7128.
  89. **Krzycki, J. A., W. R. Kenealy, M. J. DeNiro, and J. G. Zeikus.** 1987. Stable carbon isotope fractionation by *Methanosarcina barkeri* during methanogenesis from acetate, methanol, or carbon dioxide-hydrogen. Applied and Environmental Microbiology **53**:2594-2599.

90. **Kuder, T., R. Kolhatkar, J. Wilson, P. Philp, and J. Allen.** 2004. Compound-specific isotope analysis of MTBE and TBA for bioremediation studies. Proceedings of Petroleum Hydrocarbons and Organic Chemicals in Ground Water: Prevention, Assessment, and Remediation Conference. Baltimore, Maryland, August 17-18, 2004.
91. **Kuder, T., J. T. Wilson, P. Kaiser, R. Kolhatkar, P. Philp, and J. Allen.** 2005. Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: microcosm and field evidence. *Environmental Science and Technology* **39**:213-220.
92. **Kunapuli, U., T. Lueders, and R. U. Meckenstock.** 2007. The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *The ISME Journal* **2007**:643-653.
93. **Leadbetter, E. R., and J. W. Foster.** 1959. Oxidation products formed from gaseous alkanes by the bacterium *Pseudomonas methanica*. *Archives of Biochemistry and Biophysics* **82**:491-492.
94. **Lechner, U., D. Brodkorb, R. Geyer, G. Hause, C. Hartig, G. Auling, F. Fayolle-Guichard, P. Piveteau, R. H. Muller, and T. Rohwerder.** 2007. *Aquincola tertiaricarbonis* gen. nov., sp. nov., a tertiary butyl moiety-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* **57**:1295-303.
95. **Leigh, M. B., V. H. Pellizari, O. Uhlik, R. Sutka, J. Rodrigues, N. E. Ostrom, J. Zhou, and J. M. Tiedje.** 2007. Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *The ISME Journal* **1**:134-148.
96. **Lichtfouse, E.** 2000. Compound specific isotope analysis. Application to archaeology, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. *Rapid Communications in Mass Spectrometry* **14**:1337-1344.
97. **Liou, J. S. C., C. M. DeRito, and E. L. Madsen.** 2008. Field-based and laboratory stable isotope probing surveys of the identities of both aerobic and anaerobic benzene-metabolizing microorganisms in freshwater sediment. *Environmental Microbiology* **10**:1964-1977.

98. **Liu, C. Y., G. E. Speitel, Jr., and G. Georgiou.** 2001. Kinetics of methyl *t*-butyl ether cometabolism at low concentrations by pure cultures of butane-degrading bacteria. *Applied and Environmental Microbiology* **67**:2197-2201.
99. **Liu, H., J. Yan, Q. Wang, U. Karlson, G. Zou, and Z. Yuan.** 2009. Biodegradation of methyl *tert*-butyl ether by enriched bacterial culture. *Current Microbiology* **59**:30-34.
100. **Lollar, B. S., G. F. Slater, J. Ahad, B. Sleep, J. Spivack, M. Brennan, and P. MacKenzie.** 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. *Organic Geochemistry* **30**:813-820.
101. **Lopes Ferreira, N., D. Labbé, F. Monot, F. Fayolle-Guichard, and C. W. Greer.** 2006. Genes involved in the methyl *tert*-butyl ether (MTBE) metabolic pathway of *Mycobacterium austroafricanum* IFP 2012. *Microbiology* **152**:1361-1374.
102. **Lopes Ferreira, N., H. Mathis, D. Labbé, F. Monot, C. Greer, and F. Fayolle-Guichard.** 2007. n-Alkane assimilation and *tert*-butyl alcohol (TBA) oxidation capacity in *Mycobacterium austroafricanum* strains. *Applied Microbiology and Biotechnology* **75**:909-919.
103. **Madsen, E. L.** 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Reviews Microbiology* **3**:439-446.
104. **Mahendra, S., and L. Alvarez-Cohen.** 2006. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. *Environmental Science & Technology* **40**:5435-5442.
105. **Mahmood, S., G. I. Paton, and J. I. Prosser.** 2005. Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environmental Microbiology* **7**:1349-1360.
106. **Mancini, S. A., A. C. Ulrich, G. Lacrampe-Couloume, B. Sleep, E. A. Edwards, and B. S. Lollar.** 2003. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. *Applied and Environmental Microbiology* **69**:191-198.
107. **Manefield, M., R. Griffiths, N. P. McNamara, D. Sleep, N. Ostle, and A. Whiteley.** 2007. Insights into the fate of a <sup>13</sup>C labelled phenol pulse for stable isotope (SIP) experiments. *Journal of Microbiological Methods* **69**:340-344.

108. **Manefield, M., R. I. Griffiths, M. B. Leigh, R. Fisher, and A. S. Whiteley.** 2005. Functional and compositional comparison of two activated sludge communities remediating coking effluent. *Environmental Microbiology* **7**:715-722.
109. **Manefield, M., A. S. Whiteley, R. I. Griffiths, and M. J. Bailey.** 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* **68**:5367-5373.
110. **Manefield, M., A. S. Whiteley, N. Ostle, P. Ineson, and M. J. Bailey.** 2002. Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. *Rapid Communications in Mass Spectrometry* **16**:2179-2183.
111. **McKelvie, J. R., M. R. Hyman, M. Elsner, C. Smith, D. M. Aslett, G. Lacrampe-Couloume, and B. S. Lollar.** 2009. Isotopic fractionation of methyl tert-butyl ether suggests different initial reaction mechanisms during aerobic biodegradation. *Environmental Science and Technology* **43**:2793-2799.
112. **Meckenstock, R. U., B. Morasch, C. Griebler, and H. H. Richnow.** 2004. Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. *Journal of Contaminant Hydrology* **75**:215-255.
113. **Meier-Augenstein, W.** 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. *Journal of Chromatography A* **842**:351-371.
114. **Meselson, M., and F. W. Stahl.** 1958. The replication of DNA in *Escherichia coli*. *Proceedings of the National Academy of Sciences* **44**:671-682.
115. **Miller, L. G., K. L. Warner, S. M. Baesman, R. S. Oremland, I. R. McDonald, S. Radajewski, and J. C. Murrell.** 2004. Degradation of methyl bromide and methyl chloride in soil microcosms: use of stable C isotope fractionation and stable isotope probing to identify reactions and the responsible microorganisms. *Geochimica et Cosmochimica Acta* **68**:3271-3283.
116. **Miltner, A., H.-H. Richnow, F.-D. Kopinke, and M. Kästner.** 2004. Assimilation of CO<sub>2</sub> by soil microorganisms and transformation into soil organic matter. *Organic Geochemistry* **35**:1015-1024.

117. **Mo, K., C. O. Lora, A. E. Wanken, M. Javanmardian, X. Yang, and C. F. Kulpa.** 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. *Applied Microbiology and Biotechnology* **47**:69-72.
118. **Mormille, M. R., S. Liu, and J. M. Suflita.** 1994. Anaerobic biodegradation of gasoline oxygenates: extrapolation of information to multiple sites and redox conditions. *Environmental Science and Technology* **28**:1727-1732.
119. **Moyer, E. E., and P. T. Kosteki (ed.).** 2003. *MTBE Remediation Handbook*. Amherst Scientific Publishers, Amherst, MA.
120. **Müller, R. H., T. Rohwerder, and H. Harms.** 2008. Degradation of fuel oxygenates and their main intermediates by *Aquicola tertiaricarbonis* L108. *Microbiology* **154**:1414-21.
121. **Nakatsu, C. H., K. Hristova, S. Hanada, X.-Y. Meng, J. R. Hanson, K. M. Scow, and Y. Kamagata.** 2006. *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the *Betaproteobacteria*. *International Journal of Systematic and Evolutionary Microbiology* **56**:983-989.
122. **Newell, C. J., J. A. Conner, and D. L. Rowen.** 2003. *Groundwater Remediation Strategies Tool*, Publication Number 4730. Regulatory Analysis & Scientific Affairs Department, American Petroleum Institute.
123. **Ooyama, J., and J. Foster.** 1965. Bacterial oxidation of cycloparaffinic hydrocarbons. *Antonie van Leeuwenhoek* **31**:45-65.
124. **Padmanabhan, P., S. Padmanabhan, C. DeRito, A. Gray, D. Gannon, J. R. Snape, C. S. Tsai, W. Park, C. Jeon, and E. L. Madsen.** 2003. Respiration of <sup>13</sup>C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of <sup>13</sup>C-labeled soil DNA. *Applied and Environmental Microbiology* **69**:1614-1622.
125. **Parales, R. E., and S. M. Resnick.** 2006. Aromatic Ring Hydroxylating Dioxygenases, p. 287-340. *In* J.-L. Ramos and R. C. Levesque (ed.), *Pseudomonas*. Springer Netherlands, The Netherlands.
126. **Peterson, B. J., and B. Fry.** 1987. Stable Isotopes in Ecosystem Studies. *Annual Review of Ecology and Systematics* **18**:293-320.
127. **Piveteau, P., F. Fayolle, J. P. Vandecasteele, and F. Monot.** 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a

- methylotrophic bacterial isolate. Applied Microbiology and Biotechnology **55**:369-373.
128. **Plou, F. J., M. Ferrer, O. M. Nuero, M. V. Calvo, M. Alcalde, F. Reyes, and A. Ballesteros.** 1998. Analysis of Tween 80 as an esterase/ lipase substrate for lipolytic activity assay. Biotechnology Techniques **12**:183-186.
  129. **Portais, J.-C., and A.-M. Delort.** 2002. Carbohydrate cycling in microorganisms: what can <sup>13</sup>C-NMR tell us? FEMS Microbiology Reviews **26**:375-402.
  130. **Prenafeta-Boldú, F. X., D. M. A. M. Luykx, J. Vervoort, and J. A. M. D. Bont.** 2001. Fungal metabolism of toluene: monitoring of fluorinated analogs by <sup>19</sup>F Nuclear Magnetic Resonance Spectroscopy. Applied and Environmental Microbiology **67**:1030-1034.
  131. **Pumphrey, G. M., and E. L. Madsen.** 2008. Field-based stable isotope probing reveals the identities of benzoic acid-metabolizing microorganisms and their *in situ* growth in agricultural soil. Applied and Environmental Microbiology **74**:4111-4118.
  132. **Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell.** 2000. Stable-isotope probing as a tool in microbial ecology. Nature **403**:646-649.
  133. **Radajewski, S., I. R. McDonald, and J. C. Murrell.** 2003. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. Current Opinion in Biotechnology **14**:296-302.
  134. **Radajewski, S., G. Webster, D. S. Reay, S. A. Morris, P. Ineson, D. B. Nedwell, J. I. Prosser, and J. C. Murrell.** 2002. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. Microbiology **148**:2331-2342.
  135. **Raynal, M., and A. Pruden.** 2008. Aerobic MTBE biodegradation in the presence of BTEX by two consortia under batch and semi-batch conditions. Biodegradation **19**:269-82.
  136. **Reinauer, K., Y. Zhang, X. Yang, and K. Finneran.** 2008. Aerobic biodegradation of *tert*-butyl alcohol (TBA) by psychro- and thermo-tolerant cultures derived from granular activated carbon (GAC). Biodegradation **19**:259-268.

137. **Ringelberg, D. B., S. Sutton, and D. C. White.** 1997. Biomass, bioactivity and biodiversity: microbial ecology of the deep subsurface: analysis of ester-linked phospholipid fatty acids. *FEMS Microbiology Reviews* **20**:371-377.
138. **Riser-Roberts, E.** 1998. Remediation of petroleum-contaminated soils: biological, physical, and chemical processes. CRC Press, Boca Raton.
139. **Rittmann, B. E.** 2004. Monitored Natural Attenuation of MTBE. *In* E. E. Moyer and P. T. Kosteki (ed.), *MTBE Remediation Handbook*. Kluwer Academic Publishers, Amherst, MA.
140. **Roh, H., C.-P. Yu, M. E. Fuller, and K.-H. Chu.** 2009. Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading microorganisms via <sup>15</sup>N-stable isotope probing. *Environmental Science and Technology* **43**:2505-2511.
141. **Rohwerder, T., U. Breuer, D. Benndorf, U. Lechner, and R. H. Müller.** 2006. The alkyl *tert*-butyl ether intermediate 2-hydroxyisobutyrate is degraded via a novel cobalamin-dependent mutase pathway. *Applied and Environmental Microbiology* **72**:4128-4135.
142. **Salanitro, J. P., L. A. Diaz, M. P. Williams, and H. L. Wisniewski.** 1994. Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Applied and Environmental Microbiology* **60**:2593-2596.
143. **Schink, B.** 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* **61**:262-280.
144. **Schmidt, R., V. Battaglia, K. Scow, S. Kane, and K. R. Hristova.** 2008. Involvement of a novel enzyme, MdpA, in methyl *tert*-butyl ether degradation in *Methylibium petroleiphilum* PM1. *Applied and Environmental Microbiology* **74**:6631-8.
145. **Schmidt, T. C., M. Shirmer, H. Weiß, and S. B. Haderlein.** 2004. Microbial degradation of methyl *tert*-butyl ether and *tert*-butyl alcohol in the subsurface. *Journal of Contaminant Hydrology* **70**:173-203.
146. **Schmidt, T. C., L. Zwank, M. Elsner, M. Berg, R. U. Meckenstock, and S. B. Haderlein.** 2004. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. *Analytical and Bioanalytical Chemistry* **378**:283-300.

147. **Semprini, L.** 1997. Strategies for the aerobic co-metabolism of chlorinated solvents. *Current Opinion in Biotechnology* **8**:296-308.
148. **Sharp, J. O., T. K. Wood, and L. Alvarez-Cohen.** 2005. Aerobic biodegradation of *n*-nitrosodimethylamine (NDMA) by axenic bacterial strains. *Biotechnology and Bioengineering* **89**:608-618.
149. **Singleton, D. R., M. Hunt, S. N. Powell, R. Frontera-Suau, and M. D. Aitken.** 2007. Stable-isotope probing with multiple growth substrates to determine substrate specificity of uncultivated bacteria. *Journal of Microbiological Methods* **69**:180-187.
150. **Singleton, D. R., S. N. Powell, R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2005. Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. *Applied and Environmental Microbiology* **71**:1202-1209.
151. **Singleton, D. R., R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2006. Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. *Environmental Microbiology* **8**:1736-1745.
152. **Skinner, K., L. Cuiffetti, and M. Hyman.** 2009. Metabolism and cometabolism of cyclic ethers by a filamentous fungus, a *Graphium* sp. *Applied and Environmental Microbiology* **75**:5514-5522.
153. **Skinner, K. M., A. Martinez-Prado, M. R. Hyman, K. J. Williamson, and L. M. Ciuffetti.** 2008. Pathway, inhibition and regulation of methyl *tertiary* butyl ether oxidation in a filamentous fungus, *Graphium* sp. *Applied Microbiology & Biotechnology* **77**:1359-1365.
154. **Smith, A. E., K. Hristova, I. Wood, D. M. Mackay, E. Lory, D. Lorenzana, and K. M. Scow.** 2005. Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl *tertiary* butyl ether (MTBE). *Environmental Health Perspectives* **113**:317-322.
155. **Smith, C. A., and M. R. Hyman.** 2004. Oxidation of methyl *tert*-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and *n*-octane-grown *Pseudomonas putida* GPo1. *Applied and Environmental Microbiology* **70**:4544-4550.

156. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Applied and Environmental Microbiology* **69**:796-804.
157. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Cometabolism of methyl *tertiary* butyl ether and gaseous n-alkanes by *Pseudomonas mendocina* KR-1 grown on C<sub>5</sub> to C<sub>8</sub> n-alkanes. *Applied and Environmental Microbiology* **69**:7385-94.
158. **Smits, T., B. Witholt, and J. van Beilen.** 2003. Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*. *Antonie van Leeuwenhoek* **84**:193-200.
159. **Soddy, F.** 1913. Intra-atomic charge (Letter to the Editor). *Nature* **92**:399-400.
160. **Soddy, F.** 1922. The origins of the conceptions of isotopes, Nobel Lecture.
161. **Somsamak, P., R. M. Cowan, and M. M. Häggblom.** 2001. Anaerobic biotransformation of fuel oxygenates under sulfate-reducing conditions. *FEMS Microbiology Ecology* **37**:259-264.
162. **Somsamak, P., H. H. Richnow, and M. M. Häggblom.** 2006. Carbon isotope fractionation during anaerobic degradation of methyl *tert*-butyl ether under sulfate-reducing and methanogenic conditions. *Applied and Environmental Microbiology* **72**:1157-1163.
163. **Somsamak, P., H. H. Richnow, and M. M. Häggblom.** 2005. Carbon isotopic fractionation during anaerobic biotransformation of methyl *tert*-butyl ether and *tert*-amyl methyl ether. *Environmental Science and Technology* **39**:103-109.
164. **Spormann, A. M., and F. Widdel.** 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **11**:85-105.
165. **Squillace, P. J., M. J. Moran, W. W. Lapham, C. V. Price, R. M. Clawges, and J. S. Zogorski.** 1999. Volatile organic compounds in untreated ambient groundwater of the United States, 1985-1995. *Environmental Science & Technology* **33**:4176-4187.

166. **Steele, K. W., P. M. Bonish, R. M. Daniel, and G. W. O'Hara.** 1983. Effect of rhizobial strain and host plant on nitrogen isotopic fractionation in legumes. *Plant Physiology* **72**:1001-1004.
167. **Steffan, R. J., K. McClay, S. Vainberg, C. W. Condee, and D. Zhang.** 1997. 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* **63**:4216-4222.
168. **Steffan, R. J., S. Vainberg, C. W. Condee, K. McClay, and P. Hatzinger.** 2000. Biotreatment of MTBE with a new bacterial isolate. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), *Bioremediation and phytoremediation of chlorinated and recalcitrant compounds*. Battelle Press, Columbus, OH.
169. **Steinbach, A., R. Seifert, E. Annweiler, and W. Michaelis.** 2004. Hydrogen and carbon isotope fractionation during anaerobic biodegradation of aromatic hydrocarbons - a field study. *Environmental Science and Technology* **38**:609-616.
170. **Tao, T., and G. E. Maciel.** 2002. Interaction of methyl bromide with soil. *Environmental Science and Technology* **36**:603-607.
171. **Thiele, S., E. Fernandes, and J.-M. Bollag.** 2002. Enzymatic transformation and binding of labeled 2,4,6-Trinitrotoluene to humic substances during an anaerobic/aerobic incubation. *Journal of Environmental Quality* **31**:437-444.
172. **Tillmann, S., C. Strompl, K. N. Timmis, and W.-R. Abraham.** 2005. Stable isotope probing reveals the dominant role of Burkholderia species in aerobic biodegradation of PCBs. *FEMS Microbiology Ecology* **52**:207-217.
173. **Trevors, J. T.** 1986. Plasmid curing in bacteria. *FEMS Microbiology Letters* **32**:149-157.
174. **Uhlik, O., K. Jecna, M. Mackova, C. Vlack, M. Hroudova, K. Demnerova, V. Paces, and T. Macek.** 2009. Biphenyl-metabolizing bacteria in the rhizosphere of horseradish and bulk soil contaminated by polychlorinated biphenyls as revealed by stable isotope probing. *Applied and Environmental Microbiology* **75**:6471-6477.
175. **USEPA.** 1999. Blue Ribbon Panel on Oxygenates in Gasoline. Achieving clean air and clean water, EPA420-R-99-021, Washington, DC.

176. **USEPA.** 1997. EPA-822-F-97-009 Drinking water advisory: consumer acceptability advice and health effects analysis on methyl tertiary-butyl ether (MtBE). United States Environmental Protection Agency, Washington, D.C.
177. **USEPA.** 2010. FY2009 Annual Report on the Underground Storage Tank Program, EPA-510-R-10-001, Washington, D.C.
178. **USEPA.** 1998. Use and distribution of MTBE and ethanol, MTBE fact sheet #3, EPA 510-F-97-016. Office of Underground Storage Tanks, Washington, D.C.
179. **van Beilen, J. B., and E. G. Funhoff.** 2005. Expanding the alkane oxygenase toolbox: new enzymes and applications. *Current Opinion in Biotechnology* **16**:308-314.
180. **van Beilen, J. B., M. M. Marín, T. H. M. Smits, M. Röthlisberger, A. G. Franchini, B. Witholt, and F. Rojo.** 2004. Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. *Environmental Microbiology* **6**:264-273.
181. **van Beilen, J. B., S. Panke, S. Lucchini, A. G. Franchini, M. Rothlisberger, and B. Witholt.** 2001. Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiology* **147**:1621-1630.
182. **Vanderberg, L., J. Perry, and P. Unkefer.** 1995. Catabolism of 2,4,6-trinitrotoluene by *Mycobacterium vaccae*. *Applied Microbiology and Biotechnology* **43**:937-945.
183. **Wackett, L. P.** 1996. Co-metabolism: is the emperor wearing any clothes? *Current Opinion in Biotechnology* **7**:321-325.
184. **Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson.** 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. *Applied and Environmental Microbiology* **55**:2960-2964.
185. **Wagner, M.** 2004. Deciphering functions of uncultured microorganisms. *ASM News* **70**:63-70.
186. **Wang, X., and M. A. Deshusses.** 2007. Biotreatment of groundwater contaminated with MTBE: interaction of common environmental co-contaminants. *Biodegradation* **18**:37-50.

187. **Wei, N., and K. T. Finneran.** 2009. Microbial community analyses of three distinct, liquid cultures that degrade methyl tert-butyl ether using anaerobic metabolism. *Biodegradation* **20**:695-707.
188. **Wellington, E. M. H., A. Berry, and M. Krsek.** 2003. Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. *Current Opinion in Microbiology* **6**.
189. **Whiteley, A. S., M. Manefield, and T. Leuders.** 2006. Unlocking the 'microbial black box' using RNA-based stable isotope probing technologies. *Current Opinion in Biotechnology* **17**:67-71.
190. **Wieser, M. E.** 2006. Atomic Weights of the Elements 2005 (IUPAC Technical Report). *Pure and Applied Chemistry* **78**:2051-2066.
191. **Wilson, J. T., C. Adair, P. M. Kaiser, and R. Kolhatkar.** 2005. Anaerobic biodegradation of MTBE at a gasoline spill site. *Ground Water Monitoring and Remediation* **25**:103-115.
192. **Wilson, J. T., J. S. Cho, and B. H. Wilson.** 2000. *Natural Attenuation of MTBE in the subsurface under methanogenic conditions*. EPA/600/R-00/006. Environmental Protection Agency; Office of Research and Development; Washington, DC.
193. **Wilson, J. T., R. Kolhatkar, T. Kuder, P. Philp, and S. J. Daugherty.** 2005. Stable isotope analysis of MTBE to evaluate the source of TBA in ground water. *Ground Water Monitoring & Remediation* **25**:108-116.
194. **Xavier, K. B., M. S. D. Costa, and H. Santos.** 2000. Demonstration of a novel glycolytic pathway in the hyperthermophilic archaeon *Thermococcus zilligii* by <sup>13</sup>C-labeling experiments and Nuclear Magnetic Resonance analysis. *Journal of Bacteriology* **182**:4632-4636.
195. **Yakimov, M. M., P. N. Golyshin, S. Lang, E. R. B. Moore, W.-R. Abraham, H. Lnsdorf, and K. N. Timmis.** 1998. *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *International Journal of Systematic and Evolutionary Microbiology* **48**:339-348.
196. **Yoneyama, T., K. Fujita, T. Yoshida, T. Matsumoto, I. Kambayashi, and J. Yazaki.** 1986. Variation in natural abundance of <sup>15</sup>N among plant parts and in

- $^{15}\text{N}/^{14}\text{N}$  fractionation during  $\text{N}_2$  fixation in the legume-rhizobia symbiotic system. *Plant and Cell Physiology* **27**:791-799.
197. **Young, W. F., H. Horth, R. Crane, T. Ogden, and M. Arnott.** 1996. Taste and odour threshold concentrations of potential potable water contaminants. *Water Research* **30**:331-340.
198. **Youngster, L. K. G., L. J. Kerkhof, and M. M. Häggblom.** 2010. Community characterization of anaerobic methyl *tert*-butyl ether (MTBE)-degrading enrichment cultures. *FEMS Microbiology Ecology* **72**:279-288.
199. **Youngster, L. K. G., P. Somsamak, and M. M. Häggblom.** 2008. Effects of co-substrates and inhibitors on the anaerobic O-demethylation of methyl *tert*-butyl ether (MTBE). *Applied Microbiology and Biotechnology* **80**:1113-1120.
200. **Zaitsev, G. M., J. S. Uotila, and M. M. Häggblom.** 2007. Biodegradation of methyl *tert*-butyl ether by cold-adapted mixed and pure bacterial cultures. *Applied Microbiology and Biotechnology* **74**:1092-102.
201. **Zelles, L.** 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biology and Fertility of Soils* **29**:111-129.
202. **Zhang, C., and G. N. Bennett.** 2005. Biodegradation of xenobiotics by anaerobic bacteria. *Applied Microbiology and Biotechnology* **67**:600-618.
203. **Zwank, L., M. Berg, T. C. Schmidt, and S. B. Haderlein.** 2003. Compound-specific carbon isotope analysis of volatile organic compounds in the low-microgram per liter range. *Analytical Chemistry* **75**:5575-5583.

## CHAPTER 2

### Identification of *Tertiary* Butyl Alcohol (TBA)-Utilizing Organisms in BioGAC Reactors Using <sup>13</sup>C-DNA Stable Isotope Probing

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*Methylibium*

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## ABSTRACT

*Tertiary*-butyl alcohol (TBA) is an industrial solvent and a product of the biodegradation of gasoline oxygenates including methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE). Biodegradation of MTBE and ETBE can lead to TBA accumulation in the environment particularly in groundwater. One clean up option for TBA contaminated groundwater involves the use of bioreactors containing granulated activated carbon (GAC) which has been self-inoculated with indigenous organisms. Identification of these organisms is important for understanding conditions necessary to support and sustain TBA biodegradation activity in bioreactors. In this study, we have used  $^{13}\text{C}$ -DNA-SIP to characterize TBA-utilizing organisms in self-inoculated BioGAC reactors used to treat TBA-contaminated ground water at sites in New York and California.  $^{13}\text{C}$ -DNA recovered from our BioGAC incubations was also analyzed for the presence of genes associated with TBA biodegradation. Using  $^{13}\text{C}$ -DNA SIP, we identified novel TBA-utilizing organisms from self-inoculated BioGAC reactors and detected genes that have been associated with TBA biodegradation in pure cultures of TBA-oxidizing organisms. These findings have potential implications for operation of bioreactors intended to treat TBA impacted groundwater and evaluation of field sites regarding the potential for TBA degrading activity.

## INTRODUCTION

*Tertiary*-butyl alcohol (TBA) is widely used as an industrial solvent (5). However, environmental contamination by this compound occurs primarily because it is a product of the biodegradation of gasoline oxygenates including methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE). Anaerobic biodegradation of MTBE can generate TBA and as anaerobic conditions are frequently encountered at gasoline spill sites, TBA concentrations can often exceed those of MTBE (33). Accumulation of TBA in ground water sources of drinking water is of concern as this compound is potentially more toxic than its parent, MTBE (4).

Although TBA is generally resistant to biodegradation under anaerobic conditions (41), aerobic TBA biodegradation has been demonstrated in microcosms derived from stream bed sediments (2), surface water sediments (3) and various cultures (7-9, 11, 12, 15, 21, 26, 30, 38). Several pure cultures of TBA-utilizing bacteria have also been isolated and characterized and these include, among others, *Methylibium petroleiphilum* PM1 (11), *Aquicola tertiaricarbonis* L108 (22), and *Hydrogenophaga flava* ENV 735 (38). Aerobic TBA biodegradation also occurs through a cometabolic process in which some alkane-oxidizing bacteria fortuitously convert TBA to products such as 2-methyl-1,2-propanediol and 2-hydroxyisobutyric acid (37).

Removal of TBA from contaminated ground water is particularly problematic as this compound is fully miscible with water. Commonly used water purification

approaches involving sorption of trace organics by granulated activated carbon (GAC) are typically inefficient due to the limited capacity of these matrixes for TBA. Treatment of TBA contamination by conventional GAC is therefore expensive due to the need for frequent carbon replacement. However, many microorganisms can colonize GAC beds and these materials can then be used as effective biological water treatment systems if the environmental and physiological requirements of the attached microorganisms can be met and maintained.

Beyers, et al., described a GAC-containing packed bed reactor for the aerobic treatment of water contaminated with alkyl ethers and TBA (1). This reactor received oxygen and nutrients to support and sustain microbial activity. Operational versions of these systems have since become known as BioGAC reactors. To decrease reactor start time times some BioGAC reactors have been bioaugmented with commercially available MTBE- and TBA-degrading cultures (39). In other instances the reactors have been allowed to self-inoculate using indigenous organisms present in ground water extracted from MTBE and TBA-contaminated aquifers. The commercial cultures used to bioaugment BioGAC reactors have not been extensively characterized but in the case of the BioRemedy product the cultures were derived from mixed culture BC-1 originally described by Salanitro, et al. (30). *Rhodococcus aetheriovorans* is a significant component of this culture (10). The only previous microbiological study of organisms in a self-inoculated BioGAC reactor described two enrichment cultures (KR1 and YZ1) that both contained *Hydrogenophaga* strains (27).

Identification of organisms in self-inoculated BioGAC reactors is important as it can provide insights into the range of naturally occurring organisms that can degrade MTBE and TBA and the conditions necessary to support the activities of these organisms in attached cultures. Identification of these organisms can also potentially provide approaches to predict whether treatment of contaminated ground water sources could rely on self-inoculated BioGAC systems or whether bioaugmentation would be required to overcome the lack of native organisms with appropriate metabolic capabilities. Last but not least, identification of organisms in self-inoculated reactors can also provide supporting evidence for the possibility of ongoing MTBE and TBA biodegradation within the aquifers from which contaminated ground water is extracted and treated by the BioGAC systems. In this study, we have used  $^{13}\text{C}$ -DNA-SIP to characterize TBA-utilizing organisms in self-inoculated BioGAC reactors used to treat TBA-contaminated ground water at sites in New York and California. The BioGAC sample obtained from California was the same reactor previously studied by Reinauer et al (27). In addition to identifying the TBA-metabolizing organisms, we were also interested to investigate whether these organisms possessed genes that have been implicated in TBA-oxidizing activity in pure cultures of TBA-metabolizing strains. The results of this study demonstrate  $^{13}\text{C}$ -DNA SIP enabled us to identify several novel TBA-utilizing organisms from self-inoculated BioGAC reactors. Several genes that have been associated with TBA biodegradation in pure cultures of TBA-oxidizing organisms were also detected

in BioGAC samples. These findings have potential implications for decisions regarding bioaugmentation and self inoculation of reactors intended to treat TBA impacted groundwater.

## MATERIALS AND METHODS

**Materials.**  $^{13}\text{C}_4$ -TBA (99 atom %  $^{13}\text{C}$ ) and  $^{13}\text{C}_6$ -glucose (99 atom %  $^{13}\text{C}$ ) were obtained from Isotec (Sigma Aldrich, Isotec, Miamisburg, OH). CsCl was obtained from J.T. Baker Ultrapure Bioreagents. Denaturing gradient gels were made with OmniPur® Acrylamide/Bis (37.5:1) and OmniPur® formamide obtained from EMD Chemicals Inc. (Gibbstown, NJ), and molecular biology grade urea obtained from Eastman Kodak Company (Rochester, NY). PCR primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Compressed gases ( $\text{H}_2$ ,  $\text{N}_2$ , air) used for gas chromatography were obtained from local industrial vendors. BioGAC samples were obtained from two self-inoculated reactors. The reactors situated at sites in Hampton Bays, Long Island (NY) and Fountain Valley (CA).

**Microcosms.** Microcosms were assembled with BioGAC (5 g wet weight) added to sterile glass serum vials (160 ml) using flame-sterilized spatulas. Mineral salts medium (25 ml) (42) was added and the vials were sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton, Millville, NJ). Sterile control microcosms were constructed in the same manner except the vials were autoclaved (25 min at  $121^\circ\text{C}$ ) three times on three separate days. When required, TBA ( $^{12}\text{C}$  or  $^{13}\text{C}$ ) (120  $\mu\text{moles}$ ) was added to microcosms as a neat compound using heat-treated ( $350^\circ\text{C}$  for 30 s) glass microsyringes. When required, TBA was replenished in the microcosms by opening the microcosms in a sterile laminar flow hood. After allowing re-aeration of the gas phase for 5 min, the vials were resealed and TBA added as described

previously. All microcosms were incubated in the dark at 25°C on a rotary shaker (150 rpm).

**Cell cultures.** Cultures of *Methylobium petroleiphilum* PM1 (PM1) (ATCC BAA-1232) and *Escherichia coli* BW545 were grown in glass vials (160 ml) sealed with butyl rubber stoppers and aluminum crimp seals and incubated on a rotary shaker (150 rpm) at either 30°C (PM1) or 37°C (*E. coli*). PM1 was grown in mineral salts (25 ml) (42) with TBA (130 µmole) as the sole carbon source. *E. coli* was grown in M9 minimal media (31) (20 ml) containing either <sup>12</sup>C-glucose or <sup>13</sup>C<sub>6</sub>-glucose. Cells were harvested by centrifugation (10,000 x g, 10 min) and DNA was isolated from the pellet using the Ultraclean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA).

**Analytical methods.** Consumption of TBA consumption in all microcosms was monitored by gas chromatography (GC). Aqueous phase samples (2 µl) were taken directly from each microcosm using a heat-treated (350°C for 30 s) microsyringe and injected into a gas chromatograph (Shimadzu model GC-8A) (Kyoto, Japan) fitted with a flame ionization detector. The chromatograph was fitted with stainless steel column (0.3 by 183 cm) filled with Porapak Q (60/80 mesh) (Waters Associates, Framingham, Mass.). The column was used at a temperature of 160°C while the injection port and detector temperatures were 200°C and 220°C respectively. Nitrogen was used as carrier gas and the chromatograph was interfaced with a Hewlett-Packard (Palo-Alto, CA) HP3395 integrator for data collection. The aqueous

concentration of TBA was determined from a 10 point calibration plot ( $r^2 \geq 0.997$ ) developed by adding known amounts of TBA to sterile mineral salts.

**DNA isolation.** The contents of each microcosm were stored at  $-20^\circ\text{C}$  in sterile 50 ml conical tubes until processing. Samples were thawed at  $50^\circ\text{C}$  and then centrifuged ( $10,000 \times g$  for 10 min). DNA was isolated from each BioGAC pellet with the PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions.

**CsCl density gradient ultracentrifugation and DNA extraction.**  $^{12}\text{C}$ -DNA was separated from  $^{13}\text{C}$ -DNA by CsCl density gradient ultracentrifugation. CsCl gradients were assembled in polyallomer quick-seal ultracentrifugation tubes (11.5 ml) (Sorvall, Kendro Laboratory Products, Newtown, CT). Each tube contained TE buffer pH 8, the sample DNA solution ( $\sim 30 \mu\text{g}$  total DNA),  $200 \mu\text{l}$  of ethidium bromide (1%), and CsCl ( $1 \text{ g ml}^{-1}$ ). The tubes were ultracentrifuged in a Beckman L8-55 using a Sorvall T-1270 rotor ( $140,000 \times g$  for 69 hours at  $20^\circ\text{C}$ ). Separated DNA fractions were visualized with UV light and carefully removed from each tube using a sterile 20 gauge needle and a 1 ml syringe (24). DNA was isolated from each fraction by *n*-butanol extraction (5 times) and ethanol precipitation. The DNA pellet was resuspended in  $\frac{1}{2}\text{X}$  TE buffer pH 8 and visualized on a 1% agarose gel stained with ethidium bromide (1%).  $^{12}\text{C}$ -DNA (100 ng) from *E. coli* was also added to each gradient as a control.  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA fractions were analyzed for the presence of *E. coli* DNA as previously described (29, 34) in PCR reactions using both 25-cycle and 40-cycle DNA amplifications. In 25-cycle reactions, *E. coli* was not detected in

any fractions, but in 40-cycle PCR reactions, *E. coli* could be strongly detected in all  $^{12}\text{C}$  fractions and occasionally in  $^{13}\text{C}$  fractions. To avoid amplification of traces of  $^{12}\text{C}$  DNA in  $^{13}\text{C}$  DNA fractions, subsequent molecular analyses were conducted with 25-cycle reactions.

**Molecular analyses.** Purified  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA samples from CsCl gradients were used as templates in PCR reactions. For denaturing gradient gel electrophoresis (DGGE), PCR was performed using purified DNA (3 ng to 5 ng), primers 341GC forward (23) and 907 reverse (16) (0.5  $\mu\text{M}$  each), molecular grade PCR water, and an illustra PuReTaq Ready-To-Go™ PCR Bead (GE Healthcare Life Sciences) in a final volume of 25  $\mu\text{l}$ . The hot start PCR consisted of a touchdown protocol as follows: denature at 94°C for 5 min; 10 cycles of denature at 94°C for 45 s, anneal at 65°C (decreasing by 1°C/cycle after the initial cycle to 55°C) for 45 s, and extend at 72°C for 90 s; 25 cycles of denature at 94°C, anneal at 55°C, and extend at 72°C for 90 s; and a final extension at 72°C for 7 min. Separation of PCR products was performed by DGGE using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). DGGE gels were made according to manufacturer's instructions for 6% polyacrylamide and a 30%-55% denaturing gradient. Gels were electrophoresed for 14 h at 70V and 60°C in TAE running buffer. Gels were post-stained for 25 min on a rotary shaker (30 rpm) in TAE running buffer containing ethidium bromide (1%). Gels were destained for 20 min under static conditions in TAE running buffer. DGGE gel bands were visualized with a FOTO/Prep™ UV transilluminator (Fotodyne Inc., Hartland, WI) and excised using

sterile blades and forceps. Excised bands were transferred to sterile 1.5 ml tubes and washed for 15 min with 300  $\mu$ l of 1/2X TE buffer pH 8. Wash buffer was removed and replaced with 50  $\mu$ l fresh 1/2X TE buffer pH 8. Tubes were stored overnight (4°C) after which the gel slices were discarded. Eluted DNA was stored at -20°C. PCR was performed as previously described using extracted DNA (2  $\mu$ l) as template. PCR products were purified using the QIAquick nucleotide purification kit (Qiagen, Germantown, MD), and sent to Eurofins MWG Operon (Huntsville, AL) for sequencing.

**Phylogenetic Analyses.** PCR sequences of partial 16S rRNA genes were analyzed and phylogenetic trees were constructed with algorithms available in the Ribosomal Database Project (RDP) Release 10 (6, 40).

**Gene level analysis.** Primers (synthesized by Eurofins MWG Operon, Huntsville, AL) were designed using Primer3 (28) and GenBank sequences for *Methylibium petroleiphilum* PM1 genes Mpe\_B0532, Mpe\_B0554, Mpe\_B0555, and Mpe\_B0561. Primer sequences, including the GC clamp sequence which was added to the 5' end of all forward primers, are shown in Table 1. As previously described, purified <sup>13</sup>C-DNA from BioGAC microcosms and total DNA from PM1 cultures were used in PCR reactions. PCR products were separated on a DGGE gel (6% polyacrylamide with a 40% to 70% denaturing gradient) electrophoresed for 11.5 h in TAE running buffer. Bands were extracted from the DGGE gel and sequenced as previously described. Sample sequences were aligned with the corresponding GenBank PM1

gene sequence using Clustal version 2.0.8 (17) and translated to amino acid sequences based on the PM1 reading frame.

## RESULTS

**Biodegradation of TBA in Microcosms.** When TBA (120  $\mu$ moles) was added to sterilized microcosms containing BioGAC material there was an immediate and dramatic (5-fold) decrease in the aqueous concentration of TBA (Figure 1). However, after this initial decrease, the concentration of TBA remained close to constant of the remainder of the incubation (18 d). When TBA was added to sterile microcosms that contained mineral salts medium but no BioGAC material, the immediate decrease in TBA concentration described above was not observed. However, the aqueous concentration of TBA also remained close to constant throughout the remainder of the incubation. Based on these observations, the immediate decrease in TBA concentration that occurred in the presence of BioGAC was attributed to sorption of TBA by the GAC materials. In active microcosms that same initial drop in TBA concentration was also observed. However, unlike the sterile control microcosms, all of the initial TBA was subsequently consumed over the following 12 d in the microcosm containing NY BioGAC and within 9 d in the microcosm containing CA BioGAC. In the case of NY BioGAC, a separate microcosm showed that subsequent cycles of re-aeration and addition of  $^{13}\text{C}_4$ -TBA resulted in more rapid rates of TBA consumption. In each case newly added TBA was consumed within 24 to 48 h.

**Sequence and Phylogenetic Analyses.** After the single-pulse  $^{13}\text{C}_4$ -TBA incubations described in Figure 1 were completed, total DNA was extracted and  $^{12}\text{C}$ -

and  $^{13}\text{C}$ -DNA were separated using CsCl density gradient centrifugation. The 16S rRNA genes present in each separated DNA fraction were PCR amplified and analyzed by DGGE (Figure 2). Individual DGGE gel bands were excised, PCR-amplified, and sequenced as described in the Methods section. Searches in the Ribosomal Database Project Release 10 showed that multiple DGGE-resolved 16S rRNA sequences derived from  $^{13}\text{C}$ -DNA fractions were related to  $\beta$ -*proteobacteria* (Table 2), while the single  $^{12}\text{C}$  sequence recovered was related to an  $\alpha$ -*Proteobacteria* in the genus *Thermomonas*. Comparison of  $^{13}\text{C}$  sequences from the NY and CA microcosms showed both similarities and differences in the microbial diversity of the TBA-oxidizing populations. Sequences related to *Cupriavidus necator* and *Mitsuaria chitosanitabida* were obtained from both the NY and CA BioGAC microcosms. Differences in the two populations were highlighted by sequences related to *Polaromonas aquatica* and *Rhodoferrax ferrireducens* (NY only) and *Hydrogenophaga pseudoflava* and *Methylibium petroleiphilum* PM1 (CA only). The *Hydrogenophaga* sequence was similar only at the genus level to the closest BLAST sequence match (accession no. AM403226) for a microbial isolate (27) derived from the same CA bioreactor used for our SIP BioGAC sample analysis. A phylogenetic tree (Figure 3) of the recovered DNA sequences from  $^{13}\text{C}$ -enriched DNA indicates these sequences cluster tightly within the *Burkholderiales* order of the  $\beta$ -*proteobacteria*, along with other well known TBA-metabolizing organisms such as *Methylibium petroleiphilum* PM1 (11), *Hydrogenophaga flava* ENV735 (38), and *Aquincola tertiaricarbonis* L108 (22). In contrast, organisms associated with

cometabolic MTBE biodegradation, such as *Mycobacterium austroafricanum* JOB5 (35) and *Pseudomonas mendocina* KR1 (36), are more widely dispersed throughout the *Actinobacteria* and  $\gamma$ -*proteobacteria* respectively.

**Crossfeeding Assessment.** One concern with all forms of stable isotope probing is that these approaches can detect organisms that are not primarily responsible for metabolism of the isotopically-labeled test substrate but have assimilated labeled metabolites excreted by primary degraders. To address this issue of cross-feeding, DGGE profiles of the partial 16S rRNA sequences derived from the  $^{13}\text{C}$ -DNA from NY BioGAC samples receiving either one or multiple pulses of  $^{13}\text{C}_4$ -TBA were compared (Figure 4). The most sequence diversity as detected by a larger number of DGGE bands was observed with the single addition of  $^{13}\text{C}_4$ -TBA. Five distinct DNA bands were detected for the microcosm that received only one  $^{13}\text{C}_4$ -TBA addition whereas only three bands were detected for the microcosm that received five  $^{13}\text{C}_4$ -TBA additions.

**Gene level analysis.** Genomic DNA from pure cultures of *M. petroleiphilum* PM1 and  $^{13}\text{C}$ -DNA obtained from both NY and CA BioGAC that received a single  $^{13}\text{C}_4$ -TBA addition were further analyzed for the presence of specific genes that have been associated with TBA biodegradation in *M. petroleiphilum* PM1 (13). PCR reactions targeting four PM1 genes (Mpe\_B0532, Mpe\_B0541, Mpe\_B0555, and Mpe\_B0561) were conducted on all three DNA samples, as described in the Methods section. The products of these amplification reactions were further analyzed by DGGE (Figure 5). All visible bands were extracted from the DGGE gel,

purified, sequenced, and aligned, as described in the Methods section. In all cases, the DGGE analysis resulted in a single clear band for each PM1 gene and all DGGE-recovered PM1 sequences were identical to the partial PM1 gene sequences available in GenBank. The differences in band migration for amplification products generated from  $^{13}\text{C}$ -DNA from our microcosm studies and strain PM1 suggested that the DNA sequences of these products were different than the sequences for the products obtained from strain PM1. However, in most cases, the deduced amino acid sequences encoded by these differently migrating DNA fragments were highly similar (Table 2). For example, for gene Mpe\_B0555 there were 14 nucleotide differences between sequences amplified from  $^{13}\text{C}$ -DNA obtained from our microcosms as compared to the PM1 sequence. However, only two of these DNA sequence changes encode amino acid substitutions. In contrast, for gene Mpe\_B0541, 52 nucleotide differences were detected in DNA sequences from  $^{13}\text{C}$ -DNA obtained from our microcosms compared to the corresponding gene sequence for strain PM1. In this instance 13 of the 52 differences encode for amino acid substitutions.

## DISCUSSION

The results of this study demonstrate that  $^{13}\text{C}$ -DNA SIP using  $^{13}\text{C}_4$ -TBA enabled us to identify several novel TBA-utilizing organisms from self-inoculated BioGAC reactors. The 16S rRNA gene sequences recovered from NY BioGAC microcosms incubated with a single pulse of  $^{13}\text{C}$ -TBA indicated that at least five different sequences representing five potentially different microorganisms were recovered. The DNA sequences of partial 16S rRNA genes showed these organisms all belonged to class *Betaproteobacteria*, order *Burkholderiales* and included representatives from the genera *Polaromonas*, *Cupriavidus*, *Rhodoferax*, and *Mitsuaria*. *Polaromonas* strains have previously been shown to be important members of surface-attached microbial communities in GAC-based water purification systems (20). *Cupriavidus* strains are well known for their metabolic versatility (18, 25). However, as far as we are aware, no members of the four genera identified from our studies of NY BioGAC have previously been shown to grow on either TBA or MTBE.

For the CA BioGAC we found that all four of the 16S rRNA sequences recovered from the DGGE analysis were also all related to organisms belonging to the *Betaproteobacteria*. However, the organisms represented by these sequences were different from those obtained from the NY BioGAC and included representatives of the genera *Methylibium*, *Hydrogenophaga*, *Mitsuaria*, and *Cupriavidus*. Among these genera, only two out of four are related to organisms that

have previously been shown to metabolize TBA. The best-characterized aerobic MTBE- and TBA-utilizing organism is *M. petroleiphilum* PM1. This organism was originally isolated from a peat moss biofilter in California and similar strains have been found at several sites in that state (14). The *Hydrogenophaga* sequence we obtained was not identical to the closest BLAST sequence match (accession no. AM403226) for a microbial isolate obtained from the only other previous study of aerobic TBA-metabolizing bacteria in BioGAC systems (27) . The organism identified by Reinauer et al. was isolated from samples of the same BioGAC reactor used in our present SIP-based study. This result illustrates that SIP has the potential to identify a wide diversity of metabolically active microorganisms that is not currently possible with enrichment culture techniques. Even so, SIP may also under represent the diversity of active metabolizers in a community, as evidenced by the decrease in the number of different 16S rRNA sequences recovered when the number of <sup>13</sup>C-TBA pulses was increased from one to five.

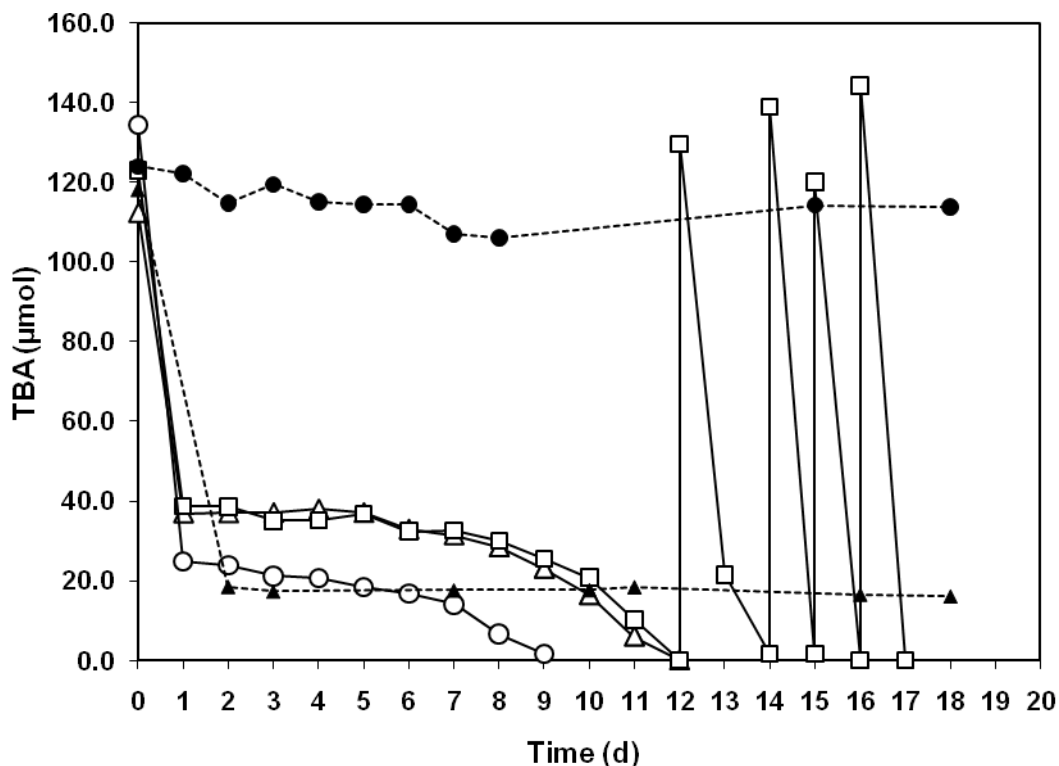
One concern with SIP-based approaches is that use of labeled substrates can often be used at concentrations that may not be environmentally relevant (19) and that this can cause shifts in the microbial population so that detected organisms are no longer representative of the targeted natural community. The BioGAC samples used in our analyses came from NY- and CA-based reactors treating groundwater with TBA levels as high as 10 and 350 ppm, respectively. As we have also shown in this study, the sorptive properties of the GAC can cause substantial decreases in aqueous phase TBA concentrations and presumably equally large increases in the

concentration of organics experienced by surface-attached bacteria. We also focused on results obtained when BioGAC samples were exposed to a single addition of  $^{13}\text{C}_4$ -TBA and also demonstrated that for NY BioGAC, multiple TBA exposures decreased rather than increased the microbial diversity detected in our samples. We therefore conclude that the aqueous TBA concentration in our microcosms (~300 ppm) is comparable, albeit at the high end, to concentrations of TBA these reactor samples may have experienced during field operation. Additionally, we can conclude that the microorganisms identified by the recovered 16S rRNA sequences are representative of the primary TBA-metabolizers in the BioGAC microcosms rather than organisms that have assimilated  $^{13}\text{C}$  label through crossfeeding processes.

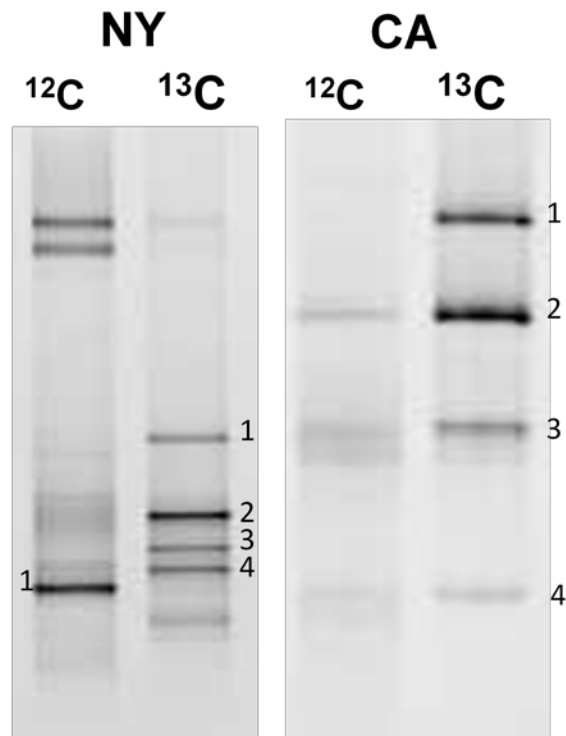
And finally our gene level analysis targeted four genes that have been associated with TBA biodegradation in pure cultures of *M. petroleiphilum* PM1. Genes Mpe\_B0532, Mpe\_B0541, Mpe\_B0555, Mpe\_B0561 were selected for our study based on the results of a comparative transcriptome analysis which indicated they were differentially expressed (3- to 12-fold upregulated) when PM1 was grown on MTBE versus ethanol (13). These genes were all detected in our NY and CA BioGAC samples, suggesting that they are widespread in environments favorable for TBA biodegradation. Additionally, the deduced amino acid sequences for genes Mpe\_B0555, Mpe\_B0532, and Mpe\_B0561 detected in our BioGAC samples were highly similar to the corresponding sequences for PM1, further suggesting that the predicted proteins are well conserved. Our findings are particularly relevant for

Mpe\_B0555 which codes for phthalate dioxygenase, a protein recently detected during a proteomic analysis of *Aquicola tertiaricarbonis L108* after growth on TBA (32). Detection of these genes, specifically Mpe\_B0555, may indicate a site's potential for TBA degrading activity and therefore could inform decisions regarding self inoculation or bioaugmentation of BioGAC reactors intended to treat TBA-impacted ground water.

## FIGURES AND TABLES



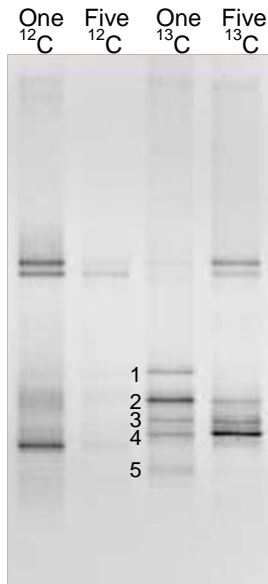
**Figure 1: Biodegradation of TBA in microcosms.** The Figure shows the time course for TBA consumption in sterile (closed symbols) and active (open symbols) microcosms constructed using BioGAC samples obtained from both Hampton Bays (NY) and Fountain Valley (CA). The microcosms were constructed as described in the Methods section. The symbols correspond to the following treatments; (●) mineral salts alone, (▲) mineral salts + NY BioGAC, (△) mineral salts + NY BioGAC + a single <sup>13</sup>C<sub>4</sub>-TBA addition, (□) mineral salts + NY BioGAC + five sequential additions of <sup>13</sup>C<sub>4</sub>-TBA and (○) mineral salts + CA BioGAC + a single <sup>13</sup>C<sub>4</sub>-TBA addition.



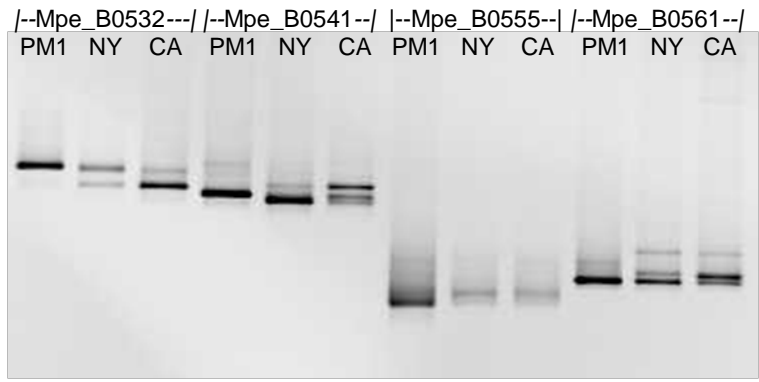
**Figure 2: DGGE analysis of partial 16S rRNA genes PCR-amplified from  $^{12}\text{C}$  and  $^{13}\text{C}$ -DNA fractions from microcosms conducted with BioGAC samples from New York (NY) and California (CA). Numbered bands were extracted, sequenced, and compared to type strain 16S rRNA sequences available in the Ribosomal Database Project. Type strains with closest matching 16S rRNA sequences are as follows: *Thermomonas fusca* (NY  $^{12}\text{C}$  Band 1); *Cupriavidus necator* (NY  $^{13}\text{C}$  Band 1); *Polaromonas aquatica* (NY  $^{13}\text{C}$  Band 2); *Rhodoferrax ferrireducens* (NY  $^{13}\text{C}$  Band 3); *Mitsuaria chitosanitabida* (NY  $^{13}\text{C}$  Band 4); *Cupriavidus necator* (CA  $^{13}\text{C}$  Band 1); *Hydrogenophaga pseudoflava* (CA  $^{13}\text{C}$  Band 2); *Mitsuaria chitosanitabida* (CA  $^{13}\text{C}$  Band 3); *Methylibium petroleiphilum* PM1 (CA  $^{13}\text{C}$  Band 4).**



**Figure 3: Phylogenetic tree of partial 16S rRNA gene sequences obtained from DGGE analysis described in Figure 2. <sup>13</sup>C-DNA sample sequences cluster in the *Burkholderiales*.**



**Figure 4: Effect of TBA additions on microbial diversity in NY BioGAC samples.** The figure shows different <sup>13</sup>C-TBA pulsing strategies were applied to the NY BioGAC microcosms. In one, the microcosm received a single pulse of <sup>13</sup>C-TBA and in the other, the microcosm received five total pulses of <sup>13</sup>C-TBA. DGGE banding patterns are shown here for the two pulsing strategies.



**FIGURE 5. PM1 genes analysis.** Selected genes associated with TBA biodegradation in *M. petroleiphilum* PM1 were detected in BioGAC samples. Sequence heterogeneity was evaluated by DGGE.

**Table 1 – Primer sequences for gene level analysis**

<b>Gene</b>	<b>Primer Sequences forward (top) and reverse (bottom)</b>
Mpe_B0532	5'–GACAACCTGCGCATCATCTA–3' 5'–GTGCCTCTTCCTCGTAGTCG–3'
Mpe_B0541	5'–ACGCGAAGATCATGAAGGAG–3' 5'–GTTGTCGTACGGGTGGATCT–3'
Mpe_B0555	5'–CGAGCGACACATGTACACAC–3' 5'–ACGAAAACCAGATCGACCAC–3'
Mpe_B0561	5'–CAGCAGGTTGATGTCGTTGT–3' 5'–TTCGATGACCTTCTGGAACC–3'
GC clamp sequence	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC-3'

**Table 2 - Heterogeneity analysis for 4 putative TBA biodegradation genes found in DGGE separated DNA sequences from NY and CA BioGAC samples**

Gene ID	PM1 gene length (bp)	Sample sequence overlap of PM1 sequence (bp)	% gene coverage	Number of nucleotide differences in overlapping region (% similarity to PM1 sequence)	Number of nucleotide differences coding for AA substitutions	AA changes
Mpe_B0532	1248	417	33	10 (97.6)	3	Val → Leu Pro → Ser Isol → Met
Mpe_B0541	1689	462	26	52 (88.7)	13	
Mpe_B0555	1413	493	35	14 (97.1)	2	Tryp → Ser Val → Leu
Mpe_B0561	894	423	47	19 (95.5)	4	Pro → Ala Lys → Glut Acid Isol → Val Val → Isol

## REFERENCES

1. **Beyers, D. L., C. L. Meyer, P. T. Sun, and J. P. Salanitro.** 2001. Method and apparatus for biodegradation of alkyl ethers and tertiary butyl alcohol. . US Patent # 6458276.
2. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 1999. Aerobic mineralization of MTBE and *tert*-butyl alcohol by stream-bed sediment microorganisms. *Environmental Science and Technology* **33**:1877-1879.
3. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 2002. TBA biodegradation in surface-water sediments under aerobic and anaerobic conditions. *Environmental Science & Technology* **36**:4087-4090.
4. **Cirvello, J. D., A. Radovsky, J. E. Heath, D. R. Farnell, and C. Lindamood, III.** 1995. Toxicity and carcinogenicity of t-Butyl alcohol in rats and mice following chronic exposure in drinking water. *Toxicology and Industrial Health* **11**:151-165.
5. **Clark, J. J. J.** 2002. *tert*-butyl alcohol: chemical properties, production and use, fate and transport, toxicology, and detection in groundwater and regulatory standards, p. 92-106. *In* A. F. Diaz and D. L. Drogos (ed.), *Oxygenates in Gasoline: Environmental Aspects*, vol. Chapter 7. American Chemical Society, Washington.
6. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje.** 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35**.
7. **Deeb, R. A., K.-H. Chu, T. Shih, S. Linder, I. Suffet, M. C. Kavanaugh, and L. Alvarez-Cohen.** 2003. MTBE and other oxygenates: environmental sources, analysis, occurrence, and treatment. *Environmental Engineering Science* **20**:433-447.
8. **Fortin, N. Y., M. Morales, Y. Nakagawa, D. D. Focht, and M. A. Deshusses.** 2001. Methyl *tert*-butyl ether (MTBE) degradation by a microbial consortium. *Environmental Microbiology* **3**:407-416.
9. **François, A., H. Mathis, D. Godefroy, P. Piveteau, F. Fayolle, and F. Monot.** 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Applied and Environmental Microbiology* **68**:2754-2762.

10. **Goodfellow, M., A. L. Jones, L. A. Maldonado, and J. Salanitro.** 2004. *Rhodococcus aetherivorans* sp. nov., A new species that contains methyl *t*-butyl ether-degrading Actinomycetes. *Systematic and Applied Microbiology* **27**:61-65.
11. **Hanson, J. R., C. E. Ackerman, and K. M. Scow.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Applied and Environmental Microbiology* **65**:4788-4792.
12. **Hatzinger, P. B., K. McClay, S. Vainberg, M. Tugusheva, C. W. Condee, and R. J. Steffan.** 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. *Applied and Environmental Microbiology* **67**:5601-5607.
13. **Hristova, K. R., R. Schmidt, A. Y. Chakicherla, T. C. Legler, J. Wu, P. S. Chain, K. M. Scow, and S. R. Kane.** 2007. Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. *Applied and Environmental Microbiology* **73**:7347-57.
14. **Kane, S. R., H. R. Beller, T. C. Legler, C. J. Koester, H. C. Pinkart, R. U. Halden, and A. M. Happel.** 2001. Aerobic biodegradation of methyl *tert*-butyl ether by aquifer bacteria from leaking underground storage tank sites. *Applied and Environmental Microbiology* **67**:5824-5829.
15. **Kharoune, M., L. Kharoune, J. M. Lebeault, and A. Pauss.** 2001. Isolation and characterization of two aerobic bacterial strains that completely degrade ethyl *tert*-butyl ether (ETBE). *Applied Microbiology and Biotechnology* **55**:348-353.
16. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academies of Science* **82**:6955-6959.
17. **Larkin, M. A., G. Blackshields, N. P. Brown, C. R. McGettigan, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. ClustalW and ClustalX version 2. *Bioinformatics* **23**:2947-2948.
18. **Lykidis, A., D. Pérez-Pantoja, T. Ledger, K. Mavromatis, I. J. Anderson, N. n. Ivanova, S. D. Hooper, A. Lapidus, S. Lucas, B. González, and N. C. Kyrpides.** 2010. The complete multipartite genome sequence of *Cupriavidus necator* JMP134, a versatile pollutant degrader. *PLoS ONE* **5**:e9729.

19. **Madsen, E. L.** 2006. The use of stable isotope probing techniques in bioreactor and field studies on bioremediation. *Current Opinion in Biotechnology* **17**:92-97.
20. **Magic-Knezev, A., B. Wullings, and D. V. d. Kooij.** 2009. *Polaromonas* and *Hydrogenophaga* species are the predominant bacteria cultured from granular activated carbon filters in water treatment. *Journal of Applied Microbiology* **107**:1457-1467.
21. **Mo, K., C. O. Lora, A. E. Wanken, M. Javanmardian, X. Yang, and C. F. Kulpa.** 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. *Applied Microbiology and Biotechnology* **47**:69-72.
22. **Müller, R. H., T. Rohwerder, and H. Harms.** 2008. Degradation of fuel oxygenates and their main intermediates by *Aquicola tertiaricarbonis* L108. *Microbiology* **154**:1414-21.
23. **Muyzer, G., E. C. D. Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**:695-700.
24. **Neufeld, J. D., J. Vohra, M. G. Dumont, T. Lueders, M. Manefield, M. W. Friedrich, and J. C. Murrell.** 2007. DNA stable-isotope probing. *Nature Protocols* **2**:860-866.
25. **Pérez-Pantoja, D., R. D. I. Iglesia, D. H. Pieper, and B. González.** 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. *FEMS Microbiology Reviews* **32**:736-794.
26. **Piveteau, P., F. Fayolle, J. P. Vandecasteele, and F. Monot.** 2001. Biodegradation of *tert*-butyl alcohol and related xenobiotics by a methylotrophic bacterial isolate. *Applied Microbiology and Biotechnology* **55**:369-373.
27. **Reinauer, K., Y. Zhang, X. Yang, and K. Finneran.** 2008. Aerobic biodegradation of *tert*-butyl alcohol (TBA) by psychro- and thermo-tolerant cultures derived from granular activated carbon (GAC). *Biodegradation* **19**:259-268.

28. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Humana Press, Totowa, NJ.
29. **Sabat, G., P. Rose, W. J. Hickey, and J. M. Harkin.** 2000. Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. *Applied and Environmental Microbiology* **66**:844-9.
30. **Salanitro, J. P., L. A. Diaz, M. P. Williams, and H. L. Wisniewski.** 1994. Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Applied and Environmental Microbiology* **60**:2593-2596.
31. **Sambrook, J., and D. W. Russell.** 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.
32. **Schäfer, F., U. Breuer, D. Benndorf, M. v. Bergen, H. Harms, and R. H. Müller.** 2007. Growth of *Aquicola tertiarycarbonis* L108 on *tert*-butyl alcohol leads to the induction of a phthalate dioxygenase-related protein and its associated oxidoreductase subunit. *Engineering in Life Sciences* **7**:512-519.
33. **Schmidt, T. C., L. Zwank, M. Elsner, M. Berg, R. U. Meckenstock, and S. B. Haderlein.** 2004. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. *Analytical and Bioanalytical Chemistry* **378**:283-300.
34. **Singleton, D. R., S. N. Powell, R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2005. Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. *Applied and Environmental Microbiology* **71**:1202-1209.
35. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Applied and Environmental Microbiology* **69**:796-804.
36. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Cometabolism of methyl *tertiary* butyl ether and gaseous *n*-alkanes by *Pseudomonas mendocina* KR-1 grown on C<sub>5</sub> to C<sub>8</sub> *n*-alkanes. *Applied and Environmental Microbiology* **69**:7385-94.
37. **Steffan, R. J., K. McClay, S. Vainberg, C. W. Condee, and D. Zhang.** 1997. 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* **63**:4216-4222.
38. **Steffan, R. J., S. Vainberg, C. W. Condee, K. McClay, and P. Hatzinger.** 2000. Biotreatment of MTBE with a new bacterial isolate. *In* G. B. Wickramanayake, A. R. Gavaskar, B. C. Alleman, and V. S. Magar (ed.), *Bioremediation and phytoremediation of chlorinated and recalcitrant compounds*. Battelle Press, Columbus, OH.
  39. **Sun, P. T., D. Walsh, C. Meyer, and D. Pickle.** 2003. The treatment of MTBE-contaminated groundwater in bioaugmented granular activated carbon beds - a case history. *Proceedings of the Water Environment Federation* **14**:450-463.
  40. **Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole.** 2007. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**:5261-5267.
  41. **Wilson, J. T., C. Adair, P. M. Kaiser, and R. Kolhatkar.** 2005. Anaerobic biodegradation of MTBE at a gasoline spill site. *Ground Water Monitoring and Remediation* **25**:103-115.
  42. **Yeager, C. M., P. J. Bottomley, D. J. Arp, and M. R. Hyman.** 1999. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. *Applied and Environmental Microbiology* **65**:632-639.

## CHAPTER 3

### **A DNA-Stable Isotope Probing Analysis of Aerobic Methyl *tertiary* Butyl Ether Degradation via Cometabolism and Commensalism**

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## ABSTRACT

Methyl *tertiary* butyl ether (MTBE) is a pervasive ground water pollutant that can be biodegraded both metabolically and cometabolically by aerobic bacteria. *Tertiary* butyl alcohol (TBA) is a central metabolite in both of these processes. We have used  $^{13}\text{C}$ -DNA-stable isotope probing to investigate the potential for complete MTBE biodegradation initiated by propane-dependent cometabolism in soil microcosms. Studies using  $^{12}\text{C}$ -labeled substrates demonstrated MTBE was not biodegraded over 56 d without propane while rapid MTBE biodegradation to TBA occurred in the presence of propane. Further degradation of TBA also occurred. Microcosms exposed to various mixtures of  $^{13}\text{C}$ - and  $^{12}\text{C}$ -labeled propane, MTBE and TBA followed by DGGE analyses of 16S rRNA genes in  $^{13}\text{C}$ -enriched DNA led to the identification of propane-utilizing strains similar to *Dechloromonas hortensis* and *Rhodococcus wratislaviensis*. Strains closely related to *Cupriavidus necator* and *Methylibium petroleiphilum* PM1 were identified as TBA-utilizing organisms. Our results indicate a commensalistic interaction involving cometabolic oxidation of MTBE to TBA by propane-oxidizing bacteria and concurrent TBA-utilization by non-propane-utilizing strains led to complete degradation of MTBE. Our results have been interpreted in terms of their impacts on our understanding of the role of different types of alkane- MTBE- and TBA-utilizing organisms in the aerobic biodegradation of MTBE.

## INTRODUCTION

Methyl *tertiary*-butyl ether (MTBE) is a branched alkyl ether that was widely used in the United States as a gasoline oxygenate and octane enhancer from the early 1980's until the mid 2000's (10). Because of its extensive use during this period, MTBE became widely distributed in the environment. Ground water resources were, and remain, particularly impacted by MTBE derived from gasoline leaked from underground fuel storage tanks (41).

Anaerobic biodegradation of MTBE can occur under various electron-accepting conditions (5, 6, 11, 39, 44) and *tertiary* butyl alcohol (TBA) often accumulates as a stable product in these processes. Aerobic MTBE biodegradation also involves TBA as a key metabolite. A limited number of bacteria typified by *Methylibium petroleiphilum* PM1 can use MTBE as a sole source of carbon and energy for growth (15). Strain PM1 grows on both MTBE and TBA although TBA does not typically accumulate during MTBE oxidation (31). A diverse range of microorganisms can also cometabolically oxidize MTBE after growth on alkanes (14, 22, 37, 38, 40). Organisms such as *Pseudomonas mendocina* KR1 oxidize MTBE to TBA and formaldehyde through the activity of an *n*-alkane-inducible alkane hydroxylase (38). These organisms do not further oxidize TBA and, other than the possible further metabolism of formaldehyde, derive no apparent metabolic benefit from MTBE oxidation. In contrast, organisms such as *Mycobacterium austroafricanum* (*vaccae*) JOB5 cometabolically oxidize MTBE to *tertiary* butyl formate (TBF), which is then biotically and abiotically hydrolyzed to TBA (37). These

organisms also further oxidize TBA to 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate (12, 37). As these metabolites are also thought to be generated by MTBE-metabolizing organisms such as strain PM1 (16), the inability of strain JOB5 to grow on MTBE may reflect a kinetic rather than metabolic limitation.

Historically, MTBE has been viewed as slowly biodegradable in the environment. However, studies of other similarly poorly biodegradable gasoline components such as cyclohexane have shown mineralization can occur through a combination of cometabolism and commensalism (3, 19). For example, propane-grown strain JOB5 can oxidize cyclohexane to cyclohexanone although this strain does not grow on either cyclic compound. However, mineralization of  $^{14}\text{C}$ -labeled cyclohexane to  $^{14}\text{CO}_2$  can be achieved when strain JOB5 is grown on propane with *Xanthobacter* CY6, a strain that grows on cyclohexanone but not on either propane or cyclohexane (3). In the present study, we have explored the possibility that alkane-dependent cometabolism of MTBE to TBA can also be used to initiate the complete degradation of MTBE through commensal interactions between microorganisms. Unlike the studies described above that used  $^{14}\text{C}$ -labeled substrates and defined cocultures to demonstrate cyclohexane mineralization, we have used  $^{13}\text{C}$ -DNA stable isotope probing (SIP) to investigate MTBE biodegradation in undefined microbial communities in soil systems. One concern with all SIP-based approaches is that cross-feeding can occur when secondary organisms subsequently assimilate isotopically-labeled metabolites excreted by organisms that initially degrade the isotopically-labeled test compound. In this study

we have specifically exploited this effect to identify organisms capable of metabolizing TBA generated from an initial alkane-dependent cometabolic biodegradation of MTBE.

## MATERIALS AND METHODS

**Materials.**  $^{13}\text{C}_4$ -TBA (99 atom %  $^{13}\text{C}$ ),  $^{13}\text{C}_5$ -MTBE (99 atom %  $^{13}\text{C}$ ), and  $^{13}\text{C}_3$ -propane (99 atom %  $^{13}\text{C}$ ) were obtained from Isotec (Sigma-Aldrich, Isotec, Miamisburg, OH) CsCl was obtained from J.T. Baker Ultrapure Bioreagents. Denaturing gradient gels were made with OmniPur<sup>®</sup> Acrylamide/Bis (37.5:1) and OmniPur<sup>®</sup> formamide obtained from EMD Chemicals Inc. (Gibbstown, NJ), and molecular biology grade urea obtained from Eastman Kodak Company (Rochester, NY). Compressed gases ( $\text{H}_2$ ,  $\text{N}_2$ , air) used for gas chromatography were obtained from local industrial vendors.

**Microcosms.** Soil microcosms were constructed using a sandy soil obtained from a coastal farm in North Carolina. As far as we are aware, this soil had no previous exposure to gasoline. Soil samples (25 g) were added to sterile glass serum vials (160 ml) using flame-sterilized spatulas. The soil was mixed with mineral salts medium (20 ml) (47) and the microcosms were sealed with Teflon-lined Mininert valves (Alltech Associates, Inc., Deerfield, Ill). Control microcosms were constructed in the same manner and were autoclaved (25 min at  $121^\circ\text{C}$ ) four times on four separate, nonconsecutive days. After each autoclaving cycle a soil sample (~0.5 g) was mixed with sterile water (1 ml) and a sample (10  $\mu\text{l}$ ) was then plated on Difco<sup>™</sup> Plate Count Agar (Becton, Dickinson and Company, Sparks MD). This medium contained (in g/L) dextrose (1.0), pancreatic digest of casein (5.0) and yeast extract (2.5). No growth was observed on any of the plates over 30 d. When required, propane ( $^{12}\text{C}$  or  $^{13}\text{C}$ ) (220  $\mu\text{moles}$ ) was added to the sealed microcosms using sterile

disposable plastic syringes fitted with sterile disposable 0.1  $\mu\text{m}$  Acrodisc filters (Millipore, Ireland).  $^{12}\text{C}$ - or  $^{13}\text{C}$ -labeled MTBE and TBA were added to microcosms as neat compounds using heat-treated ( $350^{\circ}\text{C}$  for 30 s) glass microsyringes. When required, organic substrates were replenished in the microcosms by opening the microcosms in a sterile laminar flow hood. After allowing for re-aeration of the gas phase for 5 min, the vials were resealed and organic substrates were added as described above. All microcosms were incubated in the dark at  $30^{\circ}\text{C}$  on a rotary shaker (150 rpm).

**Analytical methods.** Substrate consumption and product accumulation in all microcosms was monitored using gas chromatography (GC). To quantify propane consumption, gas phase samples (10  $\mu\text{l}$ ) were taken from each microcosm using a UV- and heat-treated gastight Hamilton syringe. Samples were injected into a Shimadzu GC-14A gas chromatograph (Kyoto, Japan) fitted with a flame ionization detector and a DB-MTBE capillary column (30 m by 0.45 mm (internal diameter), 2.55  $\mu\text{m}$  film; J and W Scientific, Folsom, CA). The column, injector, and detector temperatures were  $35^{\circ}\text{C}$ ,  $200^{\circ}\text{C}$ , and  $220^{\circ}\text{C}$ , respectively. To quantify MTBE and TBA, aqueous samples (2  $\mu\text{l}$ ) were injected using separate UV- and heat-sterilized Hamilton syringes into another GC-14A gas chromatograph fitted with a flame ionization detector and a stainless steel column (0.3 x 180 cm) packed with Porapak Q 80-100 mesh (Waters Associates, Framingham, Mass.). The column, injector, and detector temperatures were  $160^{\circ}\text{C}$ ,  $200^{\circ}\text{C}$ , and  $220^{\circ}\text{C}$ , respectively. In all case  $\text{N}_2$  was used as carrier gas and the GCs were interfaced with HP3395 integrators

(Hewlett Packard, Palo Alto, CA) for data collection. The concentrations of MTBE, TBA and propane were determined from 6 point calibration plots ( $r^2 \geq 0.997$ ) developed by adding known amounts of each compound to sterilized microcosms.

**DNA isolation.** The contents of each microcosm were stored at  $-20^\circ\text{C}$  in sterile 50 ml conical tubes until processing. Samples were thawed at  $50^\circ\text{C}$  and then centrifuged ( $2,500 \times g$  for 30 min). DNA was isolated from each soil pellet with the PowerMax™ Soil DNA isolation kit (MoBio Laboratories, Inc, Carlsbad, CA.) following manufacturer's instructions except cell lysis was carried out using a FastPrep-24 cell disrupter (MP Biomedicals, Solon, OH). DNA was further purified using the PowerClean™ DNA clean-up kit (MoBio Laboratories, Inc., Carlsbad, CA) following manufacturer's instructions.

**CsCl density gradient ultracentrifugation and DNA extraction.**  $^{12}\text{C}$ -DNA was separated from  $^{13}\text{C}$ -DNA by CsCl density gradient ultracentrifugation. CsCl gradients were assembled in polyallomer quick-seal ultracentrifugation tubes (Sorvall, 11.5 ml). Each tube contained TE buffer pH 8, the sample DNA solution (approximately  $7 \mu\text{g}$  total DNA),  $200 \mu\text{l}$  of ethidium bromide (1%), and CsCl at a final concentration of  $1 \text{ g ml}^{-1}$ . The tubes were centrifuged in a Beckman L8-55 ultracentrifuge using a Sorvall T-1270 rotor ( $140,000 \times g$  for 69 hours at  $20^\circ\text{C}$ ). Separated DNA fractions were visualized with UV light and carefully removed from each tube using a sterile 20 gauge needle and a 1 ml syringe. DNA was isolated from each fraction by *n*-butanol extraction ( $\geq 5$  times) and ethanol precipitation. The DNA pellet was resuspended in  $\frac{1}{2}\text{X}$  TE buffer pH 8 and visualized on a 1% agarose

gel stained with ethidium bromide (1%).  $^{12}\text{C}$ - DNA (100 ng) from *Campylobacter jejuni* was also added to each gradient as a control.  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA fractions were analyzed with *C. jejuni* specific primers in PCR reactions using both 25-cycle and 40-cycle DNA amplifications. In 25-cycle reactions, *C. jejuni* was not detected in any DNA fractions, but in 40-cycle PCR reactions, *C. jejuni* could be strongly detected in all  $^{12}\text{C}$  fractions and slightly detected in  $^{13}\text{C}$  fractions. To avoid amplification of traces of  $^{12}\text{C}$  DNA in  $^{13}\text{C}$  DNA fractions, subsequent molecular analyses were conducted with 25-cycle reactions.

**Molecular analyses.** Purified  $^{12}\text{C}$ - and  $^{13}\text{C}$ -DNA samples from CsCl gradients were used as templates in PCR reactions. For denaturing gradient gel electrophoresis (DGGE) PCR was performed using purified DNA (5 to 10 ng), primers 341GC forward and 907 reverse (0.5  $\mu\text{M}$  each), molecular grade PCR water, and an illustra PuReTaq Ready-To-Go™ PCR Bead (GE Healthcare Life Sciences) in a final volume of 25  $\mu\text{l}$ . The hot start PCR consisted of a touchdown protocol as follows: denature at  $94^{\circ}\text{C}$  for 5 min; 10 cycles of denature at  $94^{\circ}\text{C}$  for 45 s, anneal at  $65^{\circ}\text{C}$  (decreasing by  $1^{\circ}\text{C}/\text{cycle}$  after the initial cycle to  $55^{\circ}\text{C}$ ) for 45 s, and extend at  $72^{\circ}\text{C}$  for 90 s; 25 cycles of denature at  $94^{\circ}\text{C}$ , anneal at  $55^{\circ}\text{C}$ , and extend at  $72^{\circ}\text{C}$  for 90 s; and a final extension at  $72^{\circ}\text{C}$  for 7 min. Separation of PCR products was performed by DGGE using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). DGGE gels (6% polyacrylamide and a 30%-55% denaturing gradient) were made according to manufacturer's instructions. Gels were electrophoresed for 14 h (70V at  $60^{\circ}\text{C}$ ) in TAE running buffer. Gels were post-

stained for 25 min on a rotary shaker (30 rpm) in TAE running buffer containing ethidium bromide (1%). Gels were destained for 20 min under static conditions in TAE running buffer. DGGE gel bands were visualized with a FOTO/Prep™ UV transilluminator (Fotodyne Inc., Hartland, WI) and excised using sterile blades and forceps. Excised bands were transferred to sterile 1.5 ml tubes and washed with 1/2X TE buffer (300 µl) for 15 min. Wash buffer was removed and replaced with fresh 1/2X TE buffer (50 µl). The tubes were stored overnight (4°C) after which the gel slices were discarded. Eluted DNA was stored at -20°C. PCR was performed as described earlier using extracted DNA (2 µl) as template. PCR products were purified using the QIAquick nucleotide purification kit (Qiagen, Germantown, MD), and sent to Eurofins MWG Operon (Huntsville, AL) for sequencing.

**Phylogenetic Analyses.** Searches of 16S rRNA DNA sequences in the NCBI Genbank database were conducted using the BLAST algorithm. DNA sequences were also searched against 16S rRNA sequences for Type strain bacterial isolates available in the Ribosomal Database Project (RDP) Release 10 .

## RESULTS AND DISCUSSION

We initially examined the time course of biodegradation of unlabeled propane, MTBE and TBA in soil microcosms. When propane was added alone it was fully consumed within 11 d after a slight lag phase (Figure 1A). The microcosm was then re-aerated and MTBE was added. No consumption of MTBE occurred over the following 28 d and there was no detectable TBA production. After 28 d, propane was re-added to this microcosm. Immediately after this, both propane and MTBE were rapidly consumed. The newly added propane was more rapidly consumed than the initial propane addition. Although MTBE was also rapidly consumed following the second addition of propane, the rate of MTBE consumption decreased abruptly once the newly added propane had been fully consumed. The lack of MTBE consumption during the period between the two propane additions, the rapid onset of MTBE consumption immediately following the second addition of propane, and the subsequent decline in the rate of MTBE consumption following exhaustion of the second propane addition all suggest MTBE consumption was a propane-dependent process.

During MTBE consumption (Figure 1A), TBA also accumulated in the microcosm but TBF and other recognized MTBE-derived metabolites such as 2-methyl-1,2-propanediol were not detected. The maximal amount of TBA (14  $\mu$ moles) detected was observed 3 d after the onset of MTBE consumption when  $\sim$ 30  $\mu$ moles of MTBE had been consumed. The TBA was subsequently consumed and this

activity continued even after the propane had been fully consumed and MTBE consumption had ceased.

Propane-independent consumption of TBA was also characterized in two other microcosms. One microcosm was exposed to several cycles of simultaneous additions of both propane and MTBE (Figure 1B). During the first cycle, propane was again rapidly consumed within 11d. Consumption of MTBE also occurred and again effectively ceased once propane had been fully consumed. Accumulation of TBA during the first cycle reached a maximum after 11 d and then subsequently decreased to undetectable levels over the following 8 d. A similar consumption of both propane and MTBE occurred in the following two cycles of re-aeration and substrate re-addition. During the second cycle, propane was more rapidly consumed than during the first cycle, again suggesting a population of propane-utilizing organisms developed during the first cycle of additions. During the second cycle, MTBE was also again consumed and this again led to the accumulation of TBA. The TBA that accumulated was more rapidly consumed during the second cycle than the first, suggesting that like propane, the presence of TBA had also stimulated growth of a population of TBA-utilizing organisms. Further evidence of this was apparent during the third cycle of additions. During this cycle both propane and MTBE were rapidly consumed but only low levels of TBA (~2  $\mu$ moles) accumulated even though ~40  $\mu$ moles of MTBE were consumed while propane was present in the microcosm.

We also examined the fate of TBA in the absence of either MTBE or propane (Figure 1C). The TBA was not consumed over the initial 10 d of the incubation. However, this lag phase was followed by the onset and complete consumption of TBA over the following 8 d. Subsequent cycles of re-aeration and re-additions of TBA to the same microcosm resulted in progressively faster rates of TBA consumption and the disappearance of the initial lag phase. In a final microcosm, no consumption of MTBE was observed over 56 d in the absence of propane (Figure 1D). When propane was subsequently added to this microcosm there was a lag phase of ~10 d, after which both propane and MTBE were rapidly consumed. The consumption of MTBE also led to the accumulation of TBA, which was consumed within ~13 d. Collectively the results shown in Figure 1 indicate MTBE biodegradation in this soil system is a propane-dependent cometabolic process. Our results also suggest TBA was a substantial metabolite of MTBE degradation and that a separate population of propane-independent TBA-utilizing organisms was stimulated by cometabolic TBA production. The combined activities of these two populations acting in concert then allowed the otherwise recalcitrant MTBE to be degraded through a commensalistic interaction between these groups of organisms.

To identify the organisms involved in this proposed interaction we repeated some of the experiments described above using highly enriched  $^{13}\text{C}$ -labeled substrates. In duplicate autoclaved microcosms, no consumption of  $^{13}\text{C}_3$ -propane,  $^{13}\text{C}_5$ -MTBE or  $^{13}\text{C}_4$ -TBA occurred over 25 d (Figure 2A). In active microcosms we observed substantially similar time courses of substrate consumption and product

accumulation to those described in Figure 1. For example, complete consumption of  $^{13}\text{C}_3$ -propane occurred within 12-15 d in microcosms containing either  $^{13}\text{C}_3$ -propane alone or unlabelled propane plus  $^{13}\text{C}_5$ -MTBE (Figure 2B). While we observed no consumption of  $^{13}\text{C}_5$ -MTBE when it was added alone (Figure 2B), ~20  $\mu\text{moles}$  of  $^{13}\text{C}_5$ -MTBE was consumed in the presence of propane and ~15  $\mu\text{moles}$  TBA accumulated during this reaction (Figure 2C). Further consumption of  $^{13}\text{C}_5$ -MTBE did not occur once propane had been fully consumed while the TBA generated from MTBE oxidation continued to be consumed until only low levels (~2  $\mu\text{moles}$ ) were detected at the end of the incubation (23 d). When  $^{13}\text{C}_4$ -TBA was added in the absence of either propane or MTBE, there was a lag phase of ~10 d before consumption of TBA was detected (Figure 2D). Complete consumption of the TBA was achieved after 16 d. The presence of  $^{12}\text{C}$ -propane had little or no effect on the time course of TBA consumption (Figure 2D).

After the incubations described in Figure 2 were completed, total DNA in individual microcosms was extracted and  $^{13}\text{C}$ - and  $^{12}\text{C}$ -DNA were separated using CsCl density gradient centrifugation. The 16S rRNA genes present in each purified  $^{13}\text{C}$ -enriched DNA sample were PCR amplified and analyzed by DGGE (Figure 3). This analysis revealed that a limited number of bands were obtained for each treatment and that banding patterns were highly reproducible for replicate microcosms receiving the same substrate additions. Based on comigration of bands, the DGGE analysis suggests at least three different organisms represented by bands A+B, 1+2 and 3+4 (lanes  $^{13}\text{P}_1$  and  $^{13}\text{P}_2$ ) consumed  $^{13}\text{C}_3$ -propane when it was

added as a sole substrate. Similarly, at least three organisms, represented by bands E-J, 5-10 and 11-16 metabolized TBA. These bands were detected irrespective of whether the TBA was generated from propane-dependent  $^{13}\text{C}_5$ -MTBE cometabolism (lanes  $^{13}\text{M}^{12}\text{P}_1$  and  $^{13}\text{M}^{12}\text{P}_2$ ), or was exogenous  $^{13}\text{C}_4$ -TBA added in the absence of either MTBE or propane (lanes  $^{13}\text{T}_1$  and  $^{13}\text{T}_2$ ). This suggests these organisms only derived  $^{13}\text{C}$  from TBA and not from the products of the methoxy group of MTBE such as formaldehyde or formate. As the same banding pattern was also observed for microcosms incubated with  $^{13}\text{C}_4$ -TBA plus  $^{12}\text{C}$ -propane (lanes  $^{13}\text{T}^{12}\text{P}_1$  and  $^{13}\text{T}^{12}\text{P}_2$ ), propane had no discernable effect on the diversity of TBA-metabolizing organisms in these microcosms.

To verify the comigrating bands were derived from the same organisms, and to relate phylogeny to physiology, individual bands were extracted, reamplified by PCR and their nucleotide sequence was determined. Chimera checks through the RDP database and reviews of chromatograms were used to eliminate unreliable sequences. Acceptable DGGE-derived sequences were compared with sequences available through the BLAST feature of the GenBank database, as well as the Ribosomal Database Project (RDP), Release 10. The results of this analysis confirm that all comigrating bands between 1-20 were derived from highly similar DNA sequences (Table 1). Many of the organisms identified from the Genbank database were highly similar ( $\geq 97\%$ ) to several undefined organisms. An equivalent search restricted to physiologically characterized type strains in the RDP database also revealed high levels of similarity ( $\geq 95.2\%$ ) to several organisms with physiological

traits compatible with the cometabolic and commensalistic process proposed for these microcosms.

Based on the search results from the RDP database (Table 1), the organisms identified from purified  $^{13}\text{C}$ -DNA from microcosms containing only  $^{13}\text{C}_3$ -propane were highly similar ( $\leq 95\%$  and  $\geq 99.5\%$ ) to *Dechloromonas hortensis* MA-1 (bands 1+2), and *Rhodococcus wratislaviensis* (bands 3+4), respectively. No usable sequences were obtained for the presumed single organism represented by the comigrating bands A+B. *D. hortensis* strain MA-1 is a perchlorate-reducing, facultative anaerobe (46). We could find no reports describing *n*-alkane oxidation by *Dechloromonas* strains, but strain RCB can degrade monoaromatics under both aerobic and anaerobic conditions (8). In contrast, propane oxidation has been reported for various *Rhodococci* (1, 20, 32). A particularly relevant isolate, *R. wratislaviensis* IFP 2016, oxidizes MTBE to TBA in the presence of *n*-alkanes (2) although it does not degrade MTBE when incubated with this compound alone. Oxidation of MTBE appears to be a cometabolic process as TBA is not degraded by this strain, even in the presence of hydrocarbons.

What is known about the physiology of *R. wratislaviensis* IFP 2016 is compatible with the activities expected of organisms responsible for generating TBA as a terminal metabolite in the presence of propane and MTBE. However, other evidence suggests this organism may not be an important component of the MTBE-oxidizing microflora in this system. For example, in separate microcosms we compared the effects of the presence and absence of MTBE on the diversity of

organisms that utilize propane. A DGGE and subsequent DNA sequence analysis of 16S rRNA genes amplified from  $^{13}\text{C}$ -DNA from a single microcosm incubated with  $^{13}\text{C}_3$ -propane alone (lane  $^{13}\text{P}_3$ ) revealed the same banding pattern and 16S rDNA sequences observed for the duplicate microcosms incubated under identical conditions (lanes  $^{13}\text{P}_1$  and  $^{13}\text{P}_2$ ) (Table 1). When a comparable analysis of purified  $^{13}\text{C}$ -DNA was conducted for a single microcosm incubated with  $^{13}\text{C}_3$ -propane plus  $^{12}\text{C}$ -MTBE (lane  $^{13}\text{P}^{12}\text{M}$ ), a band that comigrated with bands 3+4 and 20 was not detected. The absence of this band, attributed to *R. wratislaviensis*, suggests presence of MTBE selected against propane-dependent growth of this organism and this effect might be attributable to inhibitory effects of formaldehyde generated as a co-product of MTBE oxidation. The production of formaldehyde rather than formate as a major  $\text{C}_1$  metabolite of MTBE oxidation is certainly supported by our observation that TBF did not accumulate in our microcosms. Formaldehyde production has also previously been implicated as a factor controlling the composition of other MTBE-degrading microbial communities (48). Irrespective of the underlying mechanism, our results do suggest that the unidentified organism represented by bands A through D and a *Dechloromonas* strain (bands 1+2 and 18+19) are more likely to be responsible for oxidizing MTBE to TBA than the *R. wratislaviensis* strain we identified.

A second effect of MTBE on the diversity of propane-utilizing organisms was the appearance of a novel band (band 17) amplified from  $^{13}\text{C}$ -DNA from a microcosm incubated with  $^{13}\text{C}_3$ -propane plus  $^{12}\text{C}$ -MTBE (Figure 3; lane  $^{13}\text{P}^{12}\text{M}_3$ ).

The DNA sequence of this band was >95% similar to the type strain of *Ensifer adhaerens* (ATCC 33212) (Table 1). *E. adhaerens* is often found in aerobic soils and is a non-obligate bacterial predator (7). We could find no reports of propane-associated growth by members of the genus *Ensifer*. In light of this organism's well-documented predatory activities, it is likely that its DNA became <sup>13</sup>C-enriched by predation of organisms that utilized <sup>13</sup>C-propane as a growth substrate.

Based on type strains in the RDP database, the TBA-utilizing organisms identified in this study were highly similar (≥98.6%) to *Cupriavidus necator* strain N-1 (bands 5-10) and *M. petroleiphilum* PM1 (bands 11-16) (Table 1). A third consistently observed organism represented by bands E through J was not identified. Like *E. adhaerens*, *C. necator* has been described as a non-obligate bacterial predator (24) and DNA from this organism may also have become <sup>13</sup>C-enriched through predation. However, this seems unlikely as DNA sequences attributed to this strain were only recovered from microcosms that contained <sup>13</sup>C<sub>4</sub>-TBA. Some well-characterized *C. necator* isolates such as strain JMP134 can metabolize numerous organics and its genome encodes more than 70 predicted oxygenase enzymes (23, 28). However, to date no *Cupriavidus* strains have been shown to metabolize TBA.

In contrast, the type strain *M. petroleiphilum* PM1 is well known for its MTBE-oxidizing capabilities (15). The genome of the type strain includes a ~600kb megaplasmid (16) that encodes a phthalate dioxygenase-like enzyme implicated in TBA oxidation (17, 30) and a flavin-dependent monooxygenase (*mpdA*), linked to

oxidation of MTBE, but not TBA (31). In our microcosms MTBE was not biodegraded unless propane was also present. If the PM-1-like organism we have detected has the same metabolic capabilities as the type strain, we might have expected to observe MTBE biodegradation in the absence of propane. However, as this was not observed, the PM1-like organism detected may lack genes encoding the putative oxygenase enzyme required to oxidize MTBE to TBA. Notably, the type strain of *M. petroleiphilum* PM1 also possesses a chromosomal gene that encodes a putative propane monooxygenase (PrMO) (16). The ability of the type strain to oxidize propane has not been described and our <sup>13</sup>C-DNA-SIP approach provided no evidence that the PM1-like organism we have identified utilized propane in this study.

Previously DNA- and RNA-SIP-based studies have investigated biodegradation of several important pollutants including explosives (13), pesticides (9), polychlorinated biphenyls (42, 43), polyaromatic hydrocarbons (34, 35) and aromatics (18, 21, 45). In some of these SIP studies cross-feeding has been exploited to follow carbon flow through complex systems (4, 27, 29). <sup>13</sup>C-DNA-SIP studies using combinations of both labeled and unlabeled substrates has also been used to determine the substrate preferences of organisms grown on mixed substrates (33). While these studies all touch on aspects of SIP approaches used in the work reported here, as far as we are aware, the present study is the first to make use of <sup>13</sup>C-DNA-SIP to characterize the metabolic interactions involved in the cometabolic degradation of an environmental pollutant.

In this study we focused on use of propane as this substrate has previously been well-characterized as a substrate that supports MTBE cometabolism [12,14]. This gas is also available as a highly  $^{13}\text{C}$ -enriched, universally- $^{13}\text{C}$ -labeled form suitable for DNA-SIP studies. However, in addition to propane, many gasoline-range alkanes also support MTBE cometabolism to TBA (38, 40). Our findings presented here may help explain other previous studies by Morales et al. (25, 26) examining MTBE mineralization by mixed cultures exposed to longer chain *n*-alkanes. These studies have shown mixed microbial communities can substantially mineralize ( $\leq 92\%$ ) MTBE, TBA and other ether oxygenates and *tertiary* alcohols (25) in the presence of *n*-pentane and *n*-alkanes up to *n*-heptane. The reaction time course in these mixed cultures is very similar to that reported here and involves an initial rapid degradation of MTBE to TBA that occurs in the presence of *n*-pentane, followed by further oxidation of TBA to  $\text{CO}_2$  after *n*-pentane has been depleted. It has been claimed that *Pseudomonas* strains isolated from these mixed cultures can fully mineralize MTBE to  $\text{CO}_2$  in pure culture although no data has been presented to verify this. Based on our findings presented here, an alternative explanation is that these mixed cultures, which were maintained on mixtures of *n*-pentane and MTBE, consist of both *n*-alkane-utilizing *Pseudomonas* strains that cometabolically oxidize MTBE to TBA and a separate group of TBA-metabolizing organisms. This interpretation is further supported by the fact that *Pseudomonas* strains grown on gasoline range *n*-alkanes ( $\text{C}_5\text{-C}_8$ ) oxidize MTBE to TBA through the activity of non-heme iron alkane hydroxylases while these strains further oxidize TBA (36, 38).

If our interpretation of our own results and those presented by Morales *et al.* (25, 26) is correct, it suggests MTBE mineralization based on aerobic cometabolism and commensalism can be supported by a wide range of *n*-alkanes, including compounds like *n*-pentane that are abundant in gasoline. This further suggests that an *n*-alkane-dependent biodegradation process based on cometabolism and commensalism could also occur in oxygenated environments generated by remediation approaches such as air sparging and bioventing. These techniques are often used to treat source areas at gasoline spill sites where high concentrations of MTBE can occur, along with the gasoline range *n*-alkanes that could support MTBE cometabolism to TBA by ubiquitous *n*-alkane-oxidizing bacteria.

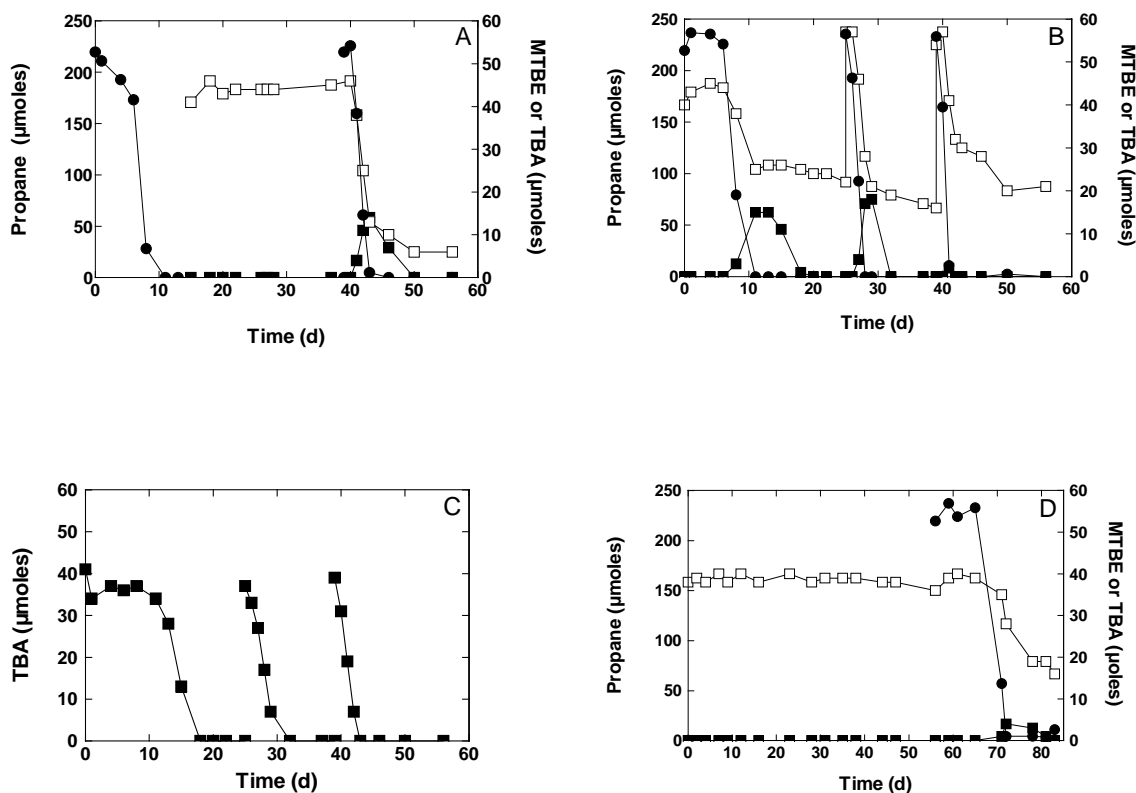
Another aspect of this study that relates to potential MTBE remediation processes is our observation that repeated cycles of TBA production stimulated growth and activity of a TBA-metabolizing microbial population such that the amount of TBA accumulation from MTBE cometabolism progressively decreased. Our results (Figure 1C) suggest that with further or continuous exposure to mixtures of alkanes and MTBE, the rate of TBA production could eventually be matched by an equivalent rate of TBA metabolism. Without apparent TBA production, MTBE biodegradation would then have all the appearances of a metabolic rather than cometabolic process. If, as we have also shown, the microbial community responsible for these activities contained PM1-like organisms, this could be taken to suggest that PM1-like organisms were solely responsible for metabolizing MTBE in these environments. Our results again provide an alternative explanation and

suggest that rather than initiating MTBE oxidation, growth and activity of PM-1-like organisms in aerobic environments could be fully dependent on an initial cometabolic degradation of MTBE to TBA by *n*-alkane-oxidizing bacteria. This alternative interpretation highlights a potential important role for *n*-alkanes in MTBE oxidation that could have important implications for the design, operation, and monitoring of aerobic treatment processes for MTBE.

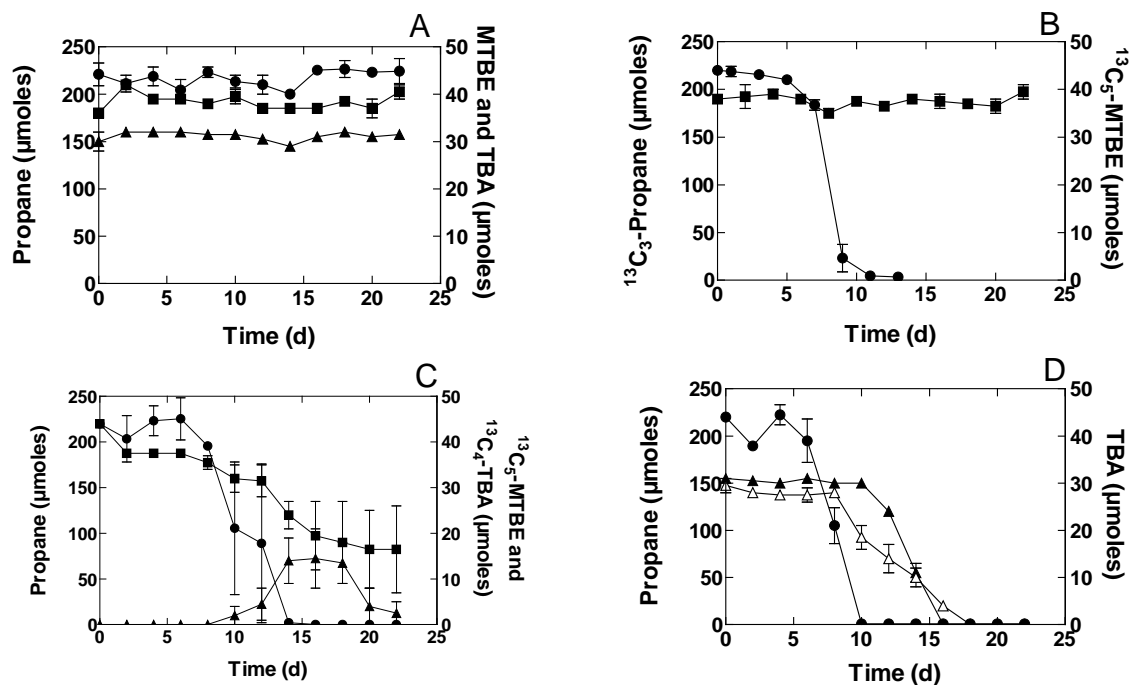
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**BRIEF:** <sup>13</sup>C-DNA-SIP has been used to demonstrate MTBE can be mineralized in soil microcosms through a combination of propane-dependent cometabolism and commensalism.

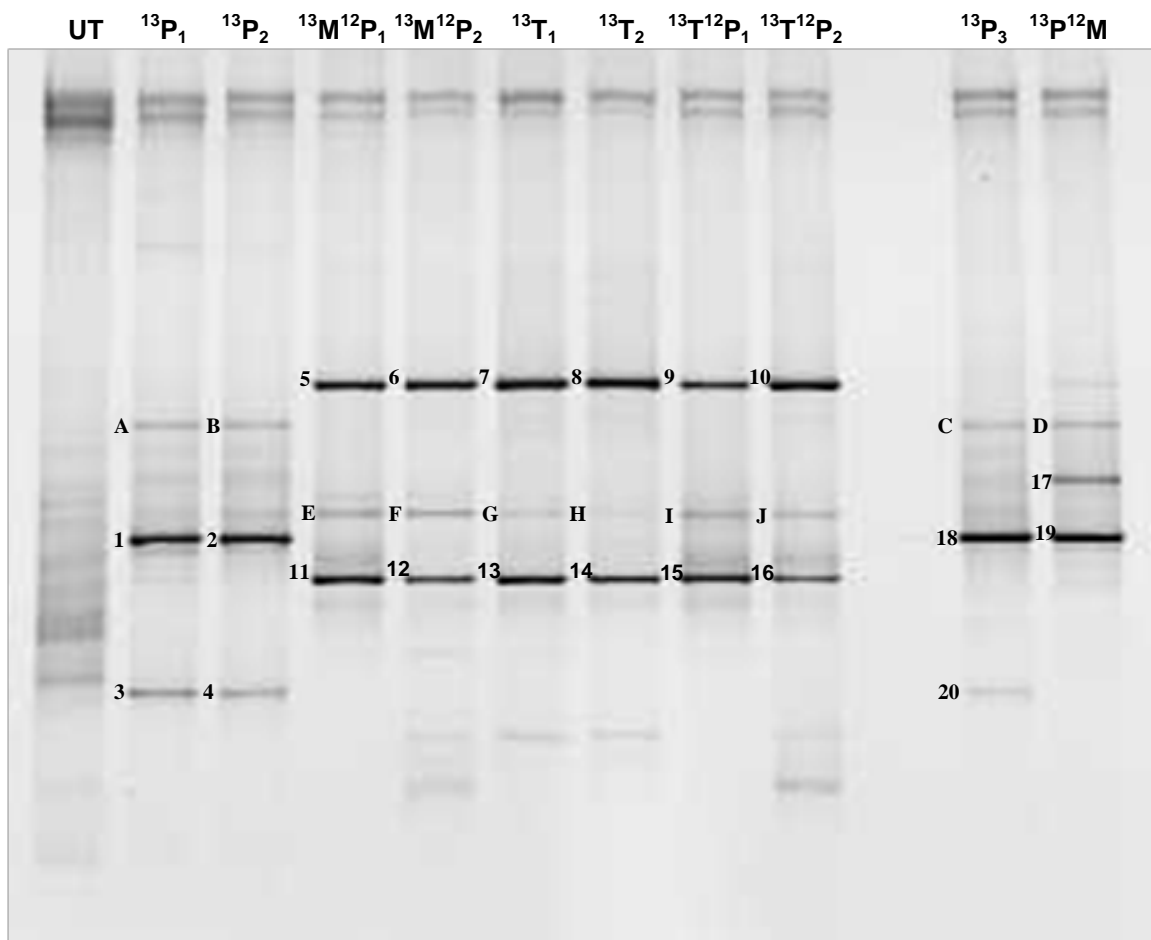
## FIGURES AND TABLES



**FIGURE 1. Time course for substrate consumption and product accumulation in soil microcosms treated with  $^{12}\text{C}$  compounds.** The Figure shows the time course for (●) propane consumption, (□) MTBE consumption, and (■) both TBA production and consumption for four individual soil microcosms. The microcosms were constructed as described in the Methods section and the additions to the individual microcosms were as follows: **Panel A.** At  $t = 0$  propane (220  $\mu\text{moles}$ ) was added. At  $t = 15$  d the microcosm was re-aerated and MTBE (42  $\mu\text{moles}$ ) was added. At  $t = 41$  d propane (220  $\mu\text{moles}$ ) was added. **Panel B.** At  $t = 0$  d propane (220  $\mu\text{moles}$ ) and MTBE (42  $\mu\text{moles}$ ) were added. At  $t = 28$  d the microcosm was re-aerated and propane (220  $\mu\text{moles}$ ) and additional MTBE (42  $\mu\text{moles}$ ) were added. At  $t = 41$  d the microcosm was re-aerated and propane (220  $\mu\text{moles}$ ) and additional MTBE (42  $\mu\text{moles}$ ) were added. **Panel C:** At  $t = 0$  d TBA (30  $\mu\text{moles}$ ) was added. At  $t = 25$  d the microcosm was re-aerated and TBA (30  $\mu\text{moles}$ ) was added. At  $t = 39$  d, the microcosm was re-aerated and TBA (30  $\mu\text{moles}$ ) was added. **Panel D.** At  $t = 0$  d MTBE (42  $\mu\text{moles}$ ) was added. At  $t = 56$  d propane (220  $\mu\text{moles}$ ) was added.



**FIGURE 2. Time course for substrate consumption and product accumulation in soil microcosms treated with  $^{12}\text{C}$ - and  $^{13}\text{C}$ -compounds. Panel A** shows the time course for consumption of unlabeled ( $^{12}\text{C}$ ) (●) propane, (■) MTBE, and (▲) TBA in autoclaved soil microcosms. **Panel B** shows the time course for substrate consumption in microcosms treated with (●)  $^{13}\text{C}_3$ -propane alone and (■)  $^{13}\text{C}_5$ -MTBE alone. **Panel C:** shows the time course for substrate consumption for microcosms treated with a mixture of (●)  $^{13}\text{C}_3$ -propane plus and (■)  $^{13}\text{C}_5$ -MTBE. The panel also shows the time course of production and consumption of (▲)  $^{13}\text{C}_4$ -TBA. **Panel D** shows the time course for substrate consumption for microcosms treated with (▲)  $^{13}\text{C}_4$ -TBA alone or a mixture of (●)  $^{12}\text{C}_3$ -propane plus (△)  $^{13}\text{C}_4$ -TBA. In all cases the data presented are the means of two replicate soil microcosms. The error bars represent the range of detected values for each substrate or product.



**FIGURE 3. DGGE analysis of CsCl-density gradient purified  $^{13}\text{C}$ -DNA from soil microcosms.** The Figure shows the analysis of 16S rRNA gene amplification products derived from the CsCl-density gradient purified  $^{13}\text{C}$ -DNA fraction of total DNA extracted from duplicate microcosms exposed to the following substrates: (lanes  $^{13}\text{P}_1$  &  $^{13}\text{P}_2$ )  $^{13}\text{C}$ -propane only (220  $\mu\text{moles}$ ); (lanes  $^{13}\text{M}^{12}\text{P}_1$  &  $^{13}\text{M}^{12}\text{P}_2$ )  $^{13}\text{C}_5$ -MTBE (40  $\mu\text{moles}$ ) plus  $^{12}\text{C}$ -propane (220  $\mu\text{moles}$ ); (lanes  $^{13}\text{T}_1$  &  $^{13}\text{T}_2$ )  $^{13}\text{C}_4$ -TBA alone (30  $\mu\text{moles}$ ); (lanes  $^{13}\text{T}^{12}\text{P}_1$  &  $^{13}\text{T}^{12}\text{P}_2$ )  $^{13}\text{C}_4$ -TBA (30  $\mu\text{moles}$ ) plus  $^{12}\text{C}$ -propane (220  $\mu\text{moles}$ ). The most right hand lanes show amplification products obtained from purified  $^{13}\text{C}$ -DNA obtained from single microcosms treated with  $^{13}\text{C}_3$ -propane alone (220  $\mu\text{moles}$ ) (lane  $^{13}\text{P}_3$ ) or  $^{13}\text{C}_3$ -propane plus  $^{12}\text{C}$ -MTBE (lane  $^{13}\text{P}^{12}\text{M}$ ). The most left hand lane (lane UT) shows amplification products from  $^{12}\text{C}$ -DNA directly extracted from a single untreated microcosm without prior CsCl density gradient centrifugation. Labeled bands were extracted for analysis. Bands A through J did not return useable sequences. Sequence matches for Bands 1 through 20 are shown in Table 1.

**Table 1 – DGGE sequences compared to GenBank and RDP**

<b>Band</b>	<b>Genbank</b>	<b>Similarity</b>	<b>RDP</b>	<b>Similarity</b>
1+2	Uncultured bacterium (GU454876.1)	97-98%	<i>Dechloromonas hortensis</i> MA-1 (AY277621)	95.2-95.6%
3+4	<i>Rhodococcus</i> sp. MIDE17 (GU3000457.1)	98-99%	<i>Rhodococcus wratislaviensis</i> (Z37138)	99.6-99.8%
5-10	<i>Cupriavidus</i> sp. Csa-24 (GU338038.1)	98-100%	<i>Cupriavidus necator</i> N-1 (AF191737)	99.8-100%
11-16	<i>Methylibium/Aquabacterium</i>	98-100%	<i>Methylibium petroleiphilum</i> PM1 (AF176594).	98.6-99.3%
17	Arsenite-oxidizing bacterium SDB 1 (FJ774943.2)	92%	<i>Ensifer adhaerens</i> (AM181733).	95.4%
	<i>Ensifer</i> sp. M_9 (GQ495034.1)	92%		
18+19	Uncultured bacterium clone (GU454876.1)	98%	<i>Dechloromonas hortensis</i> MA-1 (AY277621)	95.1 – 95.7%
20	<i>Rhodococcus</i> sp. MIDE17	97%	<i>Rhodococcus wratislaviensis</i> (Z37138)	99.1%

## REFERENCES

1. **Ashraf, W., A. Mihdir, and J. C. Murrell.** 1994. Bacterial oxidation of propane. *FEMS Microbiology Letters* **122**:1-6.
2. **Auffret, M., D. Labbé, G. Thouand, C. W. Greer, and F. Fayolle-Guichard.** 2009. Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. *Appl Environ Microbiol* **75**:7774-7782.
3. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. *Journal of General Microbiology* **82**:163-169.
4. **Bombach, P., A. Chatzinotas, T. R. Neu, M. Kastner, T. Leuders, and C. Vogt.** 2009. Enrichment and characterization of a sulfate-reducing toluene-degrading microbial consortium by combining *in situ* microcosms and stable isotope probing techniques. *FEMS Microbiology Ecology* **71**:237-246.
5. **Bradley, P. M., F. H. Chapelle, and J. E. Landmeyer.** 2001. Effect of redox conditions on MTBE biodegradation in surface water sediments. *Environ Sci Technol* **35**:4643-7.
6. **Bradley, P. M., F. H. Chapelle, and J. E. Landmeyer.** 2001. Methyl *t*-butyl ether mineralization in surface-water sediment microcosms under denitrifying conditions. *Appl Environ Microbiol* **67**:1975-1978.
7. **Casida Jr., L. E.** 1982. *Ensifer adhaerens* gen. nov., sp. nov; a bacterial predator of bacteria in soil. *International Journal of systematic Bacteriology* **32**:339-345.
8. **Chakraborty, R., S. M. O'Connor, E. Chan, and J. D. Coates.** 2005. Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Appl Environ Microbiol* **71**:8649-8655.
9. **Cupples, A. M., and G. K. Sims.** 2007. Identification of *in situ* 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing. *Soil Biology & Biochemistry* **39**:232-238.
10. **Deeb, R. A., K.-H. Chu, T. Shih, S. Linder, I. M. Suffet, M. C. Kavanaugh, and L. Alvarez-Cohen.** 2003. MTBE and Other Oxygenates: Environmental

Sources, Analysis, Occurrence, and Treatment. Environmental Engineering Science **20**:433-447.

11. **Finneran, K. T., and D. R. Lovley.** 2001. Anaerobic Degradation of Methyl *tert*-Butyl Ether (MTBE) and *tert*-butyl alcohol (TBA). Environmental Science and Technology **35**:1785-1790.
12. **François, A., H. Mathis, D. Godefroy, P. Piveteau, F. Fayolle, and F. Monot.** 2002. Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. Applied and Environmental Microbiology **68**:2754-2762.
13. **Gallagher, E. M., L. Y. Young, L. M. McGuinness, and L. J. Kerkhof.** 2010. Detecting 2,4,6-Trinitotoluene utilizing, anaerobic bacteria by <sup>15</sup>N and <sup>13</sup>C incorporation. Applied and Environmental Microbiology: AEM.02274-09.
14. **Garnier, P. M., R. Auria, C. Augur, and S. Revah.** 1999. Cometabolic degradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. Appl Microbiol Biotechnol **51**:498-503.
15. **Hanson, J. R., C. E. Ackerman, and K. M. Scow.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. Applied and Environmental Microbiology **65**:4788-4792.
16. **Hristova, K. R., R. Schmidt, A. Y. Chakicherla, T. C. Legler, J. Wu, P. S. Chain, K. M. Scow, and S. R. Kane.** 2007. Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. Appl Environ Microbiol **73**:7347-7357.
17. **Kane, S. R., A. Y. Chakicherla, P. S. G. Chain, R. Schmidt, M. W. Shin, T. C. Legler, K. M. Scow, F. W. Larimer, S. M. Lucas, P. M. Richardson, and K. R. Hristova.** 2007. Whole-Genome Analysis of the Methyl *tert*-Butyl Ether-Degrading Beta-Proteobacterium *Methylibium petroleiphilum* PM1. Journal of Bacteriology **189**:1931-1945.
18. **Kasai, Y., Y. Takahata, M. Manefield, and K. Watanabe.** 2006. RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Applied and Environmental Microbiology **72**:3586-3592.
19. **Klerk, H. D., and A. C. V. D. Linden.** 1974. Bacterial degradation of cyclohexane Participation of a co-oxidation reaction. Antonie van Leeuwenhoek **40**:7-15.

20. **Kulikova, A. K., and A. M. Bezborodov.** 2001. Assimilation of propane and characterization of propane monooxygenase from *Rhodococcus erythropolis* 3/89. Applied Biochemistry and Microbiology **37**:164-167.
21. **Liou, J. S. C., C. M. DeRito, and E. L. Madsen.** 2008. Field-based and laboratory stable isotope probing surveys of the identities of both aerobic and anaerobic benzene-metabolizing microorganisms in freshwater sediment. Environmental Microbiology **10**:1964-1977.
22. **Liu, C. Y., J. Gerald E. Speitel, and G. Georgiou.** 2001. Kinetics of Methyl *t*-Butyl Ether Cometabolism at Low Concentrations by Pure Cultures of Butane-Degrading Bacteria. Appl Environ Microbiol **67**:2197-2201.
23. **Lykidis, A., D. Pérez-Pantoja, T. Ledger, K. Mavromatis, I. J. Anderson, N. n. Ivanova, S. D. Hooper, A. Lapidus, S. Lucas, B. González, and N. C. Kyrpides.** 2010. The complete multipartite genome sequence of *Cupriavidus necator* JMP134, a versatile pollutant degrader. PLoS ONE **5**:e9729.
24. **Makkar, N. S., and J. L. E. Casida.** 1987. *Cupriavidus necator* gen. nov., sp. nov.; a nonobligate bacterial predator of bacteria in soil. International Journal of systematic Bacteriology **37**:323-326.
25. **Morales, M., V. Nava, E. Velásquez, E. Razo-Flores, and S. Revah.** 2009. Mineralization of methyl *tert*-butyl ether and other gasoline oxygenates by Pseudomonads using short *n*-alkanes as growth source. Biodegradation **20**:271-280.
26. **Morales, M., E. Velázquez, J. Jan, S. Revah, U. González, and E. Razo-Flores.** 2004. Methyl *tert*-butyl ether biodegradation by microbial consortia obtained from soil samples of gasoline-polluted sites in Mexico. Biotechnology Letters **26**:269-275.
27. **Moreno, A. M., C. Matz, S. Kjelleberg, and M. Manefield.** 2010. Identification of ciliate grazers of autotrophic bacteria in ammonia-oxidizing activated sludge by RNA stable isotope probing. Applied and Environmental Microbiology **76**:2203-2211.
28. **Pérez-Pantoja, D., R. D. I. Iglesia, D. H. Pieper, and B. González.** 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. FEMS Microbiology Reviews **32**:736-794.

29. **Rangel-Castro, J. I., K. Killham, N. Ostle, G. W. Nicol, I. C. Anderson, C. M. Scrimgeour, P. Ineson, A. Meharg, and J. I. Prosser.** 2005. Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. *Environmental Microbiology* **7**:828-838.
30. **Schäfer, F., U. Breuer, D. Benndorf, M. v. Bergen, H. Harms, and R. H. Müller.** 2007. Growth of *Aquincola tertiaricarbonis* L108 on *tert*-butyl alcohol leads to the induction of a phthalate dioxygenase-related protein and its associated oxidoreductase subunit. *Engineering in Life Sciences* **7**:512-519.
31. **Schmidt, R., V. Battaglia, K. Scow, S. Kane, and K. R. Hristova.** 2008. Involvement of a novel enzyme, MdpA, in methyl *tert*-butyl ether degradation in *Methylibium petroleiphilum* PM1. *Appl Environ Microbiol* **74**:6631-6638.
32. **Sharp, J. O., C. M. Sales, J. C. LeBlanc, J. Liu, T. K. Wood, L. D. Eltis, W. W. Mohn, and L. Alvarez-Cohen.** 2007. An inducible propane monooxygenase is responsible for *N*-Nitrosodimethylamine degradation by *Rhodococcus* sp. strain RHA1. *Appl Environ Microbiol* **73**:6930-6938.
33. **Singleton, D. R., M. Hunt, S. N. Powell, R. Frontera-Suau, and M. D. Aitken.** 2007. Stable-isotope probing with multiple growth substrates to determine substrate specificity of uncultivated bacteria. *Journal of Microbiological Methods* **69**:180-187.
34. **Singleton, D. R., S. N. Powell, R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2005. Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. *Applied and Environmental Microbiology* **71**:1202-1209.
35. **Singleton, D. R., R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken.** 2006. Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. *Environmental Microbiology* **8**:1736-1745.
36. **Smith, C. A., and M. R. Hyman.** 2010. Oxidation of gasoline oxygenates by closely related non-haem-iron hydroxylases in *Pseudomonas mendocina* KR1 and other *n*-octane utilizing *Pseudomonas* strains. *Environmental Microbiology Reports* **2**:426-432.
37. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Appl Environ Microbiol* **69**:796-804.

38. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Cometabolism of Methyl *tertiary* Butyl Ether and Gaseous *n*-Alkanes by *Pseudomonas mendocina* KR-1 Grown on C<sub>5</sub> to C<sub>8</sub> *n*-Alkanes. *Appl Environ Microbiol* **69**:7385-7394.
39. **Somsamak, P., R. M. Cowan, and M. M. Haggblom.** 2001. Anaerobic biotransformation of fuel oxygenates under sulfate-reducing conditions. *FEMS Microbiology Ecology* **37**:259-264.
40. **Steffan, R. J., K. McClay, S. Vainberg, C. W. Condee, and D. Zhang.** 1997. Biodegradation of the Gasoline Oxygenates Methyl *tert*-Butyl Ether, Ethyl *tert*-Butyl Ether and *tert*-Amyl Methyl Ether by Propane-Oxidizing Bacteria. *Appl Environ Microbiol* **63**:4216-4222.
41. **Stocking, A. J., R. A. Deeb, A. E. Flores, W. Stringfellow, J. Talley, R. Brownell, and M. C. Kavanaugh.** 2000. Bioremediation of MTBE: a review from a practical perspective. *Biodegradation* **11**:187-201.
42. **Tillmann, S., C. Strompl, K. N. Timmis, and W.-R. Abraham.** 2005. Stable isotope probing reveals the dominant role of Burkholderia species in aerobic biodegradation of PCBs. *FEMS Microbiology Ecology* **52**:207-217.
43. **Uhlik, O., K. Jecna, M. Mackova, C. Vacek, M. Hroudova, K. Demnerova, V. Paces, and T. Macek.** 2009. Biphenyl-metabolizing bacteria in the rhizosphere of horseradish and bulk soil contaminated by polychlorinated biphenyls as revealed by stable isotope probing. *Applied and Environmental Microbiology* **75**:6471-6477.
44. **Wei, N., and K. T. Finneran.** 2008. Microbial community analyses of three distinct, liquid cultures that degrade methyl *tert*-butyl ether using anaerobic metabolism. *Biodegradation* **20**:695-707.
45. **Winderl, C., H. Penning, F. v. Netzer, R. U. Meckenstock, and T. Lueders.** 2010. DNA-SIP identifies sulfate-reducing Clostridia as important toluene degraders in tar-oil-contaminated aquifer sediment. *The ISME Journal*.
46. **Wolterink, A., S. Kim, M. Muusse, I. S. Kim, P. J. M. Roholl, C. G. v. Ginkel, A. J. M. Stams, and S. W. M. Kengen.** 2005. *Dechloromonas hortensis* sp. nov. and strain ASK-1, two novel (per)chlorate-reducing bacteria, and taxonomic description of strain GR-1. *International Journal of Systematic and Evolutionary Microbiology* **55**:2063-2068.

47. **Yeager, C. M., P. J. Bottomley, D. J. Arp, and M. R. Hyman.** 1999. Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. *Appl Environ Microbiol* **65**:632-9.
48. **Zaitsev, G., J. Uotila, and M. Häggblom.** 2007. Biodegradation of methyl *tert*-butyl ether by cold-adapted mixed and pure bacterial cultures. *Applied Microbiology and Biotechnology* **74**:1092-1102.

## CHAPTER 4

### Oxidation of Methyl *tertiary* Butyl Ether (MTBE) by *Nitrosomonas europaea*

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## ABSTRACT

*Nitrosomonas europaea* is an aerobic lithoautotrophic bacterium that obtains all of its energy for growth from oxidizing ammonia ( $\text{NH}_3$ ) to nitrite ( $\text{NO}_2^-$ ). This bacterium can also cometabolically oxidize a wide range of organics including chlorinated solvents, aromatics and ethers. In this study we have characterized the biodegradation of the gasoline oxygenate, methyl *tertiary* butyl ether (MTBE) by this organism. Kinetic studies indicate MTBE is a weak uncompetitive inhibitor of ammonia oxidation ( $K_i = \sim 27\text{mM}$ ). In the presence of ammonia, resting cells immediately oxidized MTBE to *tertiary* butyl alcohol (TBA). *Tertiary* butyl formate (TBF) was not produced during MTBE oxidation and neither TBA nor TBF were consumed by this organism. The MTBE-dependent production of TBA was inhibited by acetylene and allylthiourea, two specific inhibitors of ammonia monooxygenase. An analysis of  $^{13}\text{C}_5$ -MTBE oxidation products using  $^{13}\text{C}$ -NMR indicated no other multicarbon products other than TBA were generated during MTBE oxidation although trace amounts of formate were detected. Short-term preexposure of cells to formaldehyde (5 mM) also had little or no inhibitory effect on  $\text{NH}_3$ - or  $\text{NH}_2\text{OH}$ -dependent nitrite-generating activity. The results of this study suggest *N. europaea* is unlikely to contribute significantly to MTBE biodegradation in the environment.

## INTRODUCTION

*Nitrosomonas europaea* is an aerobic lithoautotrophic bacterium that obtains energy for growth from the oxidation of ammonia ( $\text{NH}_3$ ) to nitrite ( $\text{NO}_2^-$ ). Ammonia oxidation is initiated by ammonia monooxygenase (AMO) that generates hydroxylamine ( $\text{NH}_2\text{OH}$ ) as an immediate product. Further oxidation of hydroxylamine to nitrite is catalyzed by the multiheme enzyme, hydroxylamine oxidoreductase (HAO). Reductant generated from hydroxylamine oxidation is returned to AMO to support further activity of this enzyme (3, 45). In addition to oxidizing ammonia, *N. europaea* can also oxidize a wide range of organics including  $\text{C}_1$  to  $\text{C}_8$  *n*-alkanes (21, 24),  $\text{C}_2$ - $\text{C}_4$  alkenes (21, 24), aromatics (5, 23, 29, 32), halogenated hydrocarbons (2, 28, 33, 42), and ethers such as dimethyl ether (22). Evidence for the role of AMO in these reactions is often based on detection of monooxygenase-compatible oxidation products, selective inhibition by Cu-selective metal binding compounds such as allylthiourea (4, 25) or inactivation by acetylene, a mechanism-based inactivator, (20, 26). Products of these organic transformation reactions usually do not undergo further substantial oxidation and accumulate extracellularly.

In this study we have examined the ability of *N. europaea* to oxidize methyl tertiary butyl ether (MTBE). Until recently, this compound was widely used in gasoline sold in the United States (41). Initially, MTBE was used as an octane enhancer to compensate for the loss of octane rating following elimination of alkyl lead compounds from gasoline. Subsequently, MTBE was used as an oxygenate to increase fuel combustion efficiency and decrease automobile exhaust emissions (7).

The extensive use of MTBE in the United States has led to widespread contamination of ground water resources as a result of leaking underground gasoline storage tanks(38). Although MTBE is no longer added to gasoline sold in the United States, this compound remains an important ground water contaminant (40) in this country and is still widely used elsewhere in the world.

Although MTBE biodegrades anaerobically(13), this compound is more rapidly biodegraded under aerobic conditions. In some instances MTBE biodegradation is catalyzed by slow-growing, MTBE-metabolizing bacteria (14, 16, 30). In other instances MTBE is cometabolically oxidized by alkane-oxidizing microorganisms (10, 15, 19, 35, 36, 39). In all MTBE-oxidizing aerobes it is thought that MTBE is first oxidized by a monooxygenase enzyme to yield an unstable hemiacetal (36). This transient intermediate then abiotically decomposes to yield formaldehyde and *tertiary* butyl alcohol (TBA). Growth of MTBE-metabolizing organisms is supported by the further mineralization of TBA and potentially formaldehyde. During MTBE cometabolism, further oxidation of TBA often does not occur (37) although some *Mycobacterium* strains can further slowly cometabolically oxidize this product TBA (36). These organisms are further distinguished by the fact that they also generate *tertiary* butyl formate (TBF) as an initial product of MTBE oxidation (36). This ester is thought to be generated by an alcohol dehydrogenase-catalyzed oxidation of the MTBE-derived hemiacetal. Subsequent hydrolysis of TBF generates both TBA and formate as products.

The potential for MTBE oxidation by ammonia-oxidizing bacteria (AOB) typified by *N. europaea* has not been previously reported although nitrifying activity has been noted in several studies of mixed aerobic MTBE-degrading consortia (9, 34). In one consortium (9), a nitrifying-denitrifying subculture was reported to oxidize MTBE although this process was not further characterized. In the present study we have characterized the ability of pure cultures of *N. europaea* to oxidize MTBE. Our results indicate this bacterium slowly oxidizes MTBE to TBA through the activity of AMO. The kinetic features of this process have been characterized and the potential toxic effects of MTBE-derived metabolites have been examined. Our results are discussed in terms of their impact on our understanding of MTBE biodegradation processes and the possible role of AOB in MTBE biodegradation in natural and engineered environments.

## MATERIALS AND METHODS

**Materials:** *Nitrosomonas europaea* (ATCC 19178) was obtained from the American Type Culture Collection (Manassas, Va.). Universally  $^{13}\text{C}$ -labeled  $^{13}\text{C}_5$ -MTBE (99% purity), was obtained from ISOTEC (Miamisburg, Ohio). Allylthiourea (98%), calcium carbide (for acetylene generation ~80%), hydrazine sulfate (99.999% purity), hydroxylamine hydrochloride (99.9999% purity), MTBE (99.8% purity), TBA (>99.3% purity), TBF (99% purity), formaldehyde (~37% [wt/vol] solution in water), and deuterium oxide ( $^2\text{H}_2\text{O}$ ) (99.9% purity) were all obtained from Sigma-Aldrich (Milwaukee, Wis.). All other chemicals were of reagent grade. Compressed gases ( $\text{H}_2$ ,  $\text{N}_2$ , and air) used for gas chromatography (GC) were obtained from local industrial vendors.

**Cell growth and harvesting.** Cells were grown in the dark in 1-liter cultures in 2-liter flasks incubated in a rotary shaker (150 rpm) maintained at 30°C. Cells were grown in media and harvested by centrifugation 3 days after inoculation, as previously described (20). Harvested cells were washed in phosphate buffer [PB] (50 mM  $\text{NaH}_2\text{PO}_4$  plus 2mM  $\text{MgCl}_2$  pH 7.8), re-sedimented, and suspended in fresh PB at a final concentration of ~10 mg total protein  $\text{ml}^{-1}$ . Cells were stored in the dark at 4°C and used within 1 hour of harvesting.

**Incubation conditions:** Unless otherwise noted, all incubations were conducted in glass serum vials (15 ml) sealed with butyl rubber stoppers and crimp seals (Wheaton Scientific, Millville, NJ). The reactions contained PB (~900  $\mu\text{l}$ ) and substrates and inhibitors added using glass microsyringes (Hamilton Company,

Reno, NV) as either neat liquids (MTBE), gases (acetylene), or freshly prepared aqueous stock solutions (allylthiourea, formaldehyde,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_2\text{OH}\cdot\text{HCl}$ ,  $\text{N}_2\text{H}_4\cdot\text{SO}_4$ , MTBE, TBA). Before use, the reaction vials were incubated in the dark at  $30^\circ\text{C}$  in a shaking water bath (150 rpm) to allow equilibration of the organic reactants between the gas and liquid phases. The reactions were initiated by the addition of cells (30-100  $\mu\text{l}$ ;  $\sim 0.3$ -1 mg total protein) to give a final reaction volume of 1 ml. The reaction vials were then returned to the water bath. For short-term kinetic studies (Figure 1), nitrite accumulation was determined using samples (10  $\mu\text{l}$ ) of the reaction medium that were removed immediately after the addition of cells ( $t = 0$  min) and every 5 min thereafter throughout the 15 min reaction period. In other experiments (Figures 2, 3, and 4) the concentrations of nitrite and organic reactants and products were determined in aqueous samples (10  $\mu\text{l}$  and 2  $\mu\text{l}$ , respectively) withdrawn from the reaction vials at the times indicated in the respective Figure legends.

For experiments examining the potential toxic effects of formaldehyde on nitrite-producing activity (Figure 5), cells ( $\sim 300$   $\mu\text{g}$  total protein) were incubated in sealed glass serum vials in PB containing either 0, 1, or 5 mM formaldehyde in a total reaction volume of 1 ml. The reaction vials were incubated in the dark in at  $30^\circ\text{C}$  in a shaking water bath (150 rpm). After 15 min, the reaction medium was transferred to 1.5 ml centrifuge tubes and cells were sedimented by centrifugation using an Eppendorf model 5415C microcentrifuge (14,000 rpm, 5 min). The resulting supernatant was removed and the cells were resuspended in PB (1 ml), re-sedimented by centrifugation, and finally resuspended in fresh PB (30  $\mu\text{l}$ ). The cells

were then added to reaction vials containing PB (~900  $\mu$ l), and either  $\text{NH}_4^+$  or  $\text{NH}_2\text{OH}$  (final concentration 2 mM) to give a total reaction volume of 1 ml. The reaction vials were then returned to the water bath. For the determination of nitrite, samples (10  $\mu$ l) were removed immediately ( $t = 0$  min) and every 5 min thereafter throughout the 15 min reaction period.

For experiments using  $^{13}\text{C}_5$ -MTBE, reactions were conducted in glass serum vials (25 ml) sealed with butyl rubber stoppers and crimp seals. The reaction medium contained PB, cells (~10 mg total protein, MTBE (200  $\mu$ moles) and  $(\text{NH}_4)_2\text{SO}_4$  (10 mM) in a total reaction volume of 4 ml. The reaction vials were incubated in the dark at  $30^\circ\text{C}$  in a shaking water bath (150 rpm).

**Gas chromatography.** In all experiments, the concentrations of MTBE and TBA were determined by gas chromatography (GC). At the indicated times, aqueous samples (2  $\mu$ l) were taken from sealed reaction vials using glass microsyringes (Hamilton Company, Reno, NV) and were immediately injected into a Shimadzu GC-14A (Kyoto, Japan) fitted with a flame ionization detector and a stainless steel column (6' x 18") packed with Porapak Q 80-100 mesh (Waters Associates, Framingham, Mass.). The column, injector, and detector temperatures were  $160^\circ\text{C}$ ,  $200^\circ\text{C}$ , and  $220^\circ\text{C}$ , respectively. The GC was interfaced with an HP3395 integrator (Hewlett Packard, Palo Alto, CA) for data collection. Concentrations for MTBE and TBA were determined using standard curves generated by adding known amounts of each compound to stoppered reaction vials (10 ml) containing PB (1 ml) without cells. The standard curves were linear with an  $r^2$  value  $\geq 0.99$ .

**NMR Spectroscopy.** Products formed during  $^{13}\text{C}_5$ -MTBE-oxidation were identified using  $^{13}\text{C}$ -NMR. For NMR analysis, samples (~600  $\mu\text{l}$ ) of the reaction medium were removed and centrifuged using an Eppendorf model 5415C microcentrifuge (14,000 rpm, 5 min) to remove whole cells. A portion (510  $\mu\text{l}$ ) of the supernatant was transferred to a 5 mm NMR tube.  $^2\text{H}_2\text{O}$  (75  $\mu\text{l}$ ) was added to the sample, followed by methanol (2  $\mu\text{l}$  neat) as an internal reference ( $\delta = 49.15$  ppm relative to tetramethylsilane). The spectrometer was locked on  $^2\text{H}_2\text{O}$  and proton decoupled  $^{13}\text{C}$ -NMR spectra were collected using a Varian Mercury 300 spectrometer operated at 75 MHz with a spectral width of 18.8 kHz and a pulse width of 8.7  $\mu\text{s}$  (corresponding to a flip angle of  $43.5^\circ$ ), with no delay between pulses. A total of 1024 scans, with 68K data points in the time domain, were collected for Fourier transformation.

**Nitrite and protein assays.** Nitrite was determined colorimetrically, as previously described (12). Protein concentration was determined with the Biuret assay (11) after solubilization of cell protein in aqueous 3 N NaOH (30 min at  $60^\circ\text{C}$ ) and sedimentation of insoluble material in an Eppendorf model 5415C microcentrifuge (14,000 rpm, 5 min). Bovine serum albumin was used as a standard.

## RESULTS

Our initial experiments used short-term incubations (15 min) to characterize the effect of varying MTBE concentrations on the initial rates of ammonia-dependent nitrite production. The data were fitted to a hyperbolic single substrate-binding model and good fits were obtained in all cases ( $r^2 \geq 0.975$ ). The inhibition pattern indicated MTBE appeared to act as an uncompetitive inhibitor (Figure 1A and B) as the  $K_s$  values for  $\text{NH}_4^+$  decreased from 0.9 mM to 0.30 mM with increases in MTBE concentration from 0 to 45 mM. The  $V_{\max}$  values for  $\text{NH}_4^+$ -dependent nitrite production also decreased from 116 to 13 nmoles  $\text{min}^{-1}$  mg total protein $^{-1}$  over the same range of MTBE concentrations.  $K_i$  values for MTBE derived from double reciprocal plots (Figure 1 B) were estimated using the following equation  $1/K_{m(\text{app})} = (1 + [\text{MTBE}]/K_i)/K_m$  and ranged from 22 to 31 mM (mean = 27.6 mM).

In experiments involving longer incubations (30 min), ~50 mM MTBE was required to inhibit the rate of ammonia-dependent nitrite production by 50% when concentrated cell suspensions were incubated with 10 mM  $\text{NH}_4^+$  and increasing concentrations of MTBE (Figure 2). An analysis of the reaction media by GC revealed that TBA accumulated during these reactions. With lower initial MTBE concentrations (<50 mM), the amount of TBA generated steadily increased with increases in MTBE concentration. The maximal amount of TBA (~350 nmoles) was generated in the presence of 50 mM MTBE. At the highest concentration of MTBE tested (~110 mM), nitrite production was inhibited by >90% relative to reactions conducted in the absence of MTBE. The presence of acetylene (1% v/v) fully (<98%)

inhibited both ammonia-dependent nitrite and TBA production. In separate short-term experiments (1 h), TBA (20 nmoles) was also generated when cells were incubated with MTBE (50 mM) and either the HAO substrates hydrazine or hydroxylamine (5 mM). In these reactions TBA accumulation was fully inhibited (<95%) by allylthiourea (100  $\mu$ M) (data not shown).

While accumulation of TBA was detected during the reactions described in Figure 2, TBF was not detected as a product of MTBE oxidation. Hydrolysis of TBF to TBA can occur both biotically or abiotically. Ammonia oxidation is also an acidogenic reaction that can result in substantial decreases in pH, even in buffered media. To test whether biotic or abiotic processes could have led to rapid hydrolysis of TBF in our reactions, cells were incubated with TBF (500 nmoles) in PB adjusted to pH values of 6.0, pH 7.0 and 8.0. No consumption of TBF or accumulation of TBA was observed over 1 h. Likewise, no consumption of TBA was observed over the same period when cells were incubated with TBA (200 nmoles) either in the presence or absence of  $\text{NH}_4^+$  (10 mM) (Figure 3).

Together the results shown in Figures 2 and 3 suggest MTBE is oxidized directly to TBA and that TBA is not further oxidized by *N. europaea*. To verify this we also used  $^{13}\text{C}$ -NMR to analyze the reaction medium when cells were incubated with  $^{13}\text{C}_5$ -MTBE (50 mM) and 20 mM  $\text{NH}_4^+$ . The time course of this reaction showed that consumption of MTBE was minimal (1%) over 240 min while the stoichiometric accumulation of nitrite indicated that complete consumption of  $\text{NH}_4^+$  occurred within ~120 min (Figure 4A). Accumulation of TBA occurred concurrently with nitrite

production and effectively ceased after ~120 min when complete consumption of the added  $\text{NH}_4^+$  had occurred (Figure 4A). The  $^{13}\text{C}$ -NMR spectrum of the reaction medium after 240 min (Figure 4B) confirmed accumulation of TBA but no other multicarbon products derived from MTBE were detected. The  $^{13}\text{C}$ -NMR spectrum also did not provide any evidence for accumulation of formaldehyde but a small signal attributed to formate was detected.

Despite the lack of evidence for formaldehyde accumulation during MTBE oxidation, formaldehyde is associated with the direct pathway of TBA production from MTBE. Formaldehyde is also a reactive and potentially toxic metabolite that could impact the kinetics and sustainability of MTBE oxidation by *N. europaea*. To investigate the effects of formaldehyde on *N. europaea*, cells were initially exposed to formaldehyde, washed and then assayed for ammonia- and hydroxylamine-dependent nitrite-generation activities, as described in Materials and Methods. ANOVA analyses with significance levels = 0.05 showed that neither ammonia- ( $p = 0.127$ ) nor hydroxylamine-dependent nitrite production ( $p = 0.315$ ) were affected by preexposure to formaldehyde, even at concentrations as high as 5 mM (Figure 5).

## DISCUSSION

Several lines of evidence presented in this study indicate that like many other organics, MTBE is a substrate for AMO in *N. europaea*. First, MTBE inhibited ammonia-dependent nitrite production in a concentration-dependent manner. Second, a monooxygenase-compatible product, TBA, was generated in the presence of MTBE. Third, like ammonia oxidation itself, production of TBA from MTBE was strongly inhibited by both acetylene and allylthiourea, two selective inhibitors of AMO. Several features of these lines of evidence are further discussed in the following sections.

Several previous studies have characterized the effects of organic co-substrates on the oxidation of ammonia ( $\text{NH}_3$ ) by *N. europaea*. Some co-substrates such as methane (25) and ethylene (24) exhibit a classical competitive interaction *versus* ammonia. These effects have been interpreted in terms of a mutually exclusive binding of these substrates to the active site of AMO. Like MTBE, other co-substrates such as benzene exhibit non-competitive inhibition of ammonia oxidation (23). Many other co-substrates still exhibit non-competitive behavior when hydrazine is added to alleviate the effects of reductant limitation caused by co-substrate oxidation (28). The consistency with which non-competitive inhibition patterns can be observed with organic AMO substrates has led to a two-site model for active site of AMO (28).

A similar profile of the effects of MTBE on ammonia-dependent nitrite production and concurrent TBA production shown in Figure 2 has been observed in

several previous studies (22, 33). This profile has been interpreted in terms of the effects of co-substrate binding and oxidation on the reductant supply to AMO when *N. europaea* is incubated with both ammonia and a co-substrate. For example, at low co-substrate concentrations the co-substrate has limited access to AMO and the effects on the rate of ammonia oxidation are equally limited. As the co-substrate concentration is increased, an increased proportion of the electrons derived from concurrent ammonia oxidation are used in non-reductant-regenerating co-substrate oxidation reactions. The overall rate of AMO catalyzed reactions therefore decreases. At even higher co-substrate concentrations the co-substrate can effectively fully prevent ammonia binding and oxidation by AMO. The overall level of electron flow is then decreased until only low levels of AMO activity are supported by endogenous reductant sources.

In the absence of any toxic effects, the extent to which a co-substrate impacts electron flow in *N. europaea* is dictated primarily by the potency of the co-substrate as an inhibitor of ammonia oxidation. An estimate of the  $K_i$  value for MTBE as an inhibitor of ammonia oxidation can be derived from our results (Figure 2) that showed ~50 mM MTBE was required to inhibit nitrite production from ammonium (10 mM  $\text{NH}_4^+$ ) by 50%. Using these values, the equation  $K_{i(\text{MTBE})} = K_i^{\text{app}}(\text{MTBE}) / (1 + [\text{NH}_4^+]/K_{s(\text{NH}_4^+)})$  and previously determined  $K_s$  values for  $\text{NH}_4^+$  of ~0.9 mM at pH 7.8 (Figure 1), the  $K_i$  for MTBE can be estimated to be ~4.2 mM (~370 ppm). Notably, the  $K_i$  value for a competitive inhibitor is equivalent to the  $K_s$  value for this compound as a substrate (25). Recognizing MTBE appears to act as a non-competitive rather

than competitive inhibitor, this estimated  $K_s$  value for MTBE is as much as 2-fold higher than the  $K_s$  values previously determined for methane (2-3.2 mM) (25, 28). Based on this comparison, MTBE is one of the poorest substrates yet characterized for *N. europaea*. The fact that MTBE is such a poor substrate is further supported by our observation that less than 1% of the MTBE added to both short term (30 min) or longer (240 min) incubations was oxidized to TBA (Figures 2, 4A, and 4B).

Our experiments demonstrated that both acetylene and allylthiourea inhibited production of TBA from MTBE and this suggests MTBE is oxidized by AMO. Our results obtained from analyses using both GC (Figures 2, 4A) and  $^{13}\text{C}$ -NMR (Figure 4B) also indicate TBA is the sole multicarbon metabolite generated by MTBE oxidation. This further suggests MTBE is simply dealkylated by AMO and that TBA is not further oxidized by AMO or other enzymes. As we did not observe production of TBF during MTBE oxidation and TBF was not transformed by this organism, our results imply TBA is generated directly from the chemical decomposition of an initial hemiacetal generated by oxidation of the methoxy carbon of MTBE by AMO, rather than *via* TBF. The fact that our  $^{13}\text{C}$ -NMR analysis detected formate but not formaldehyde accumulation is still compatible with a TBF-independent pathway of MTBE oxidation as previous studies have shown *N. europaea* can consume formaldehyde (22, 43) and that formate is a product of this process (43). Evidence from studies conducted with  $^{14}\text{CH}_4$  also suggest some AOB including *N. europaea* can mineralize methane to  $\text{CO}_2$  (27). If the pathway of methane oxidation in AOB

overlaps the pathway in methanotrophic bacteria (1), the formate we detected during  $^{13}\text{C}_5$ -MTBE oxidation may also have been further oxidized to  $\text{CO}_2$ .

Our results also demonstrated that short-term exposure of cells to formaldehyde had no detectable effect on initial rates of ammonia- and hydroxylamine-dependent nitrite-generating activity. This observation suggests that this immediate product of MTBE oxidation is unlikely to contribute to the inhibitory effects of MTBE we observed on ammonia oxidation. However, formaldoxime can be generated spontaneously through a direct chemical reaction between even low micromolar concentrations of formaldehyde and hydroxylamine (43). Formaldoxime is also many fold more potent than formaldehyde as an inhibitor of ammonia oxidation in *N. europaea*. The effects of formaldoxime appear to be directed primarily at HAO (43), the enzyme responsible for oxidizing hydroxylamine and generating the electrons required for AMO activity. It may be that the non-competitive effects of MTBE on ammonia oxidation we have observed could be a reflection of inhibitory effects of low levels of formaldoxime generated by concurrent oxidation of ammonia to hydroxylamine and MTBE to TBA and formaldehyde.

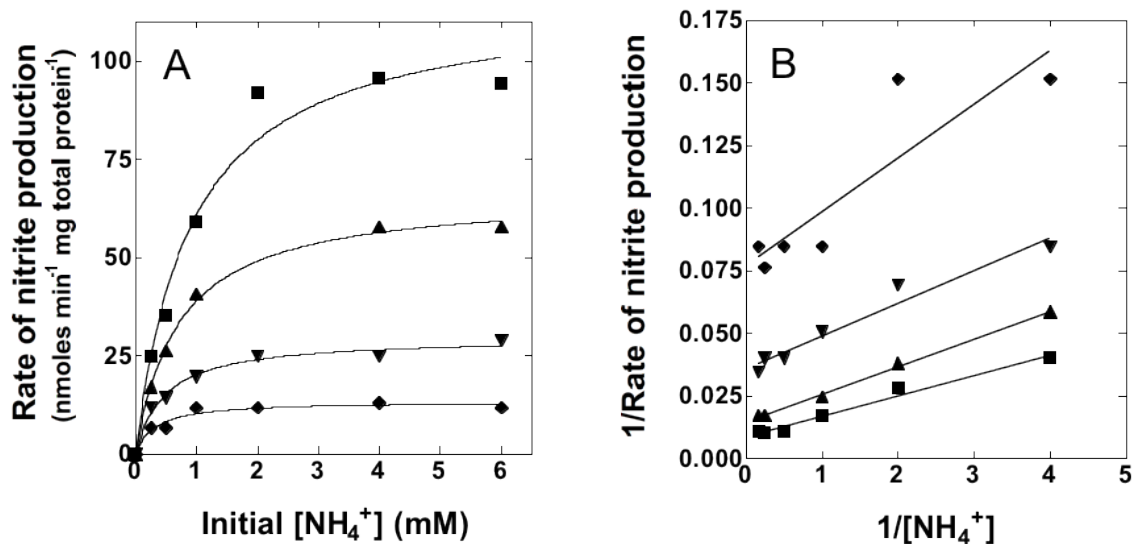
*N. europaea* and other related AOB are widely distributed in the environment and the potential for MTBE oxidation by these organisms could therefore be equally broad. However, based on the low affinity of AMO for MTBE we have characterized in this study it seems unlikely that these organisms would contribute significantly to MTBE biodegradation in most environments. The kinetic features of MTBE oxidation

by *N. europaea* may also explain why this reaction has not been observed by methanotrophic bacteria expressing either particulate (pMMO) or soluble (sMMO) methane monooxygenases (17). Structurally, AMO is closely related to pMMO (18) although the co-substrate range of AMO is comparable to sMMO (6). Our results presented here suggest that future studies with methanotrophs using high MTBE concentrations might reveal slow and partial MTBE oxidation by these organisms.

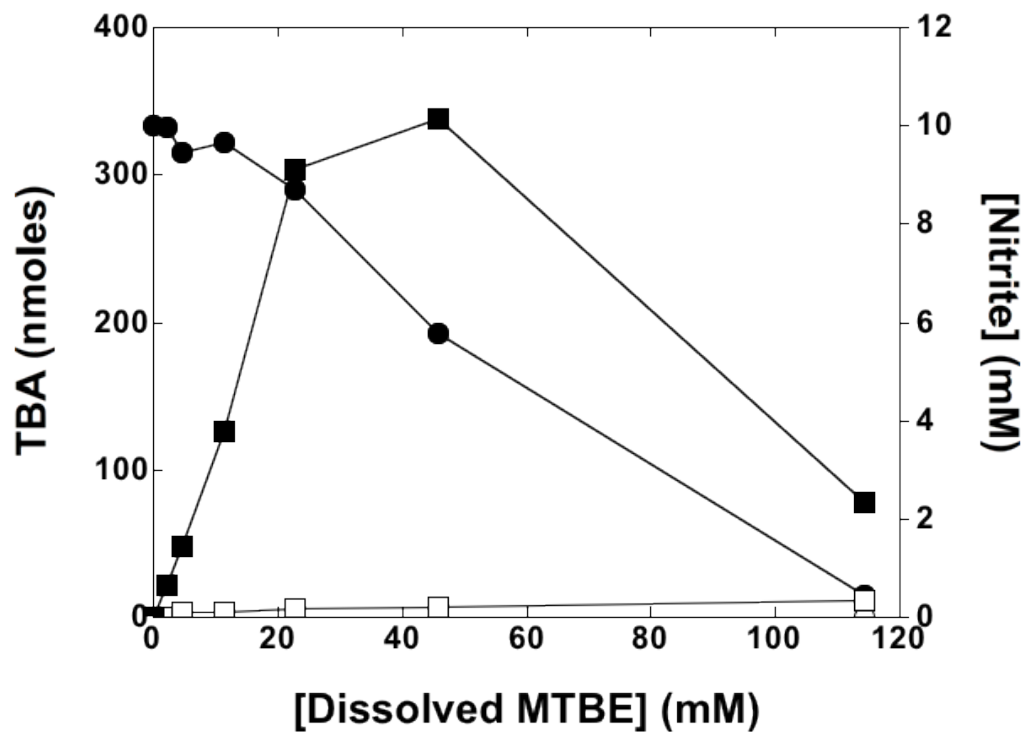
While AOB like *N. europaea* may not contribute significantly to MTBE biodegradation in the environments where MTBE concentrations are low, these organisms could potentially contribute to MTBE biodegradation in environments that contain high ammonia and MTBE concentrations. For example, an extensive field study at the Leuna site in Germany identified ammonium as an important co-contaminant with MTBE (31). The possibility that AOB could oxidize MTBE at this site was not addressed. However, ammonia oxidation was suggested to be a competitive oxygen-consuming process that could decrease the rate and extent of aerobic MTBE degradation. It is also possible that AOB could contribute to MTBE oxidation in packed bed reactors that combine sorption and biological activity. In these reactors sorption of target organics into support materials such as activated carbon can result in surface-attached organisms being exposed to high concentrations of organics. In the case of AOB like *N. europaea*, this effect might be sufficient to overcome the kinetic limitations of MTBE oxidation we have characterized. However, in a recent study (44), it was again suggested that slow-growing AOB in these types of reactors could inhibit the growth and activities of

even slower growing MTBE-metabolizing bacteria through competition for limiting oxygen. As it has also been reported that MTBE-metabolizing consortia are also highly sensitive to nitrite produced during concurrent ammonia oxidation by AOB (8). Overall, it therefore seems likely that the activities of AOB are more likely to be detrimental rather than beneficial in aerobic biotreatment processes directed at MTBE.

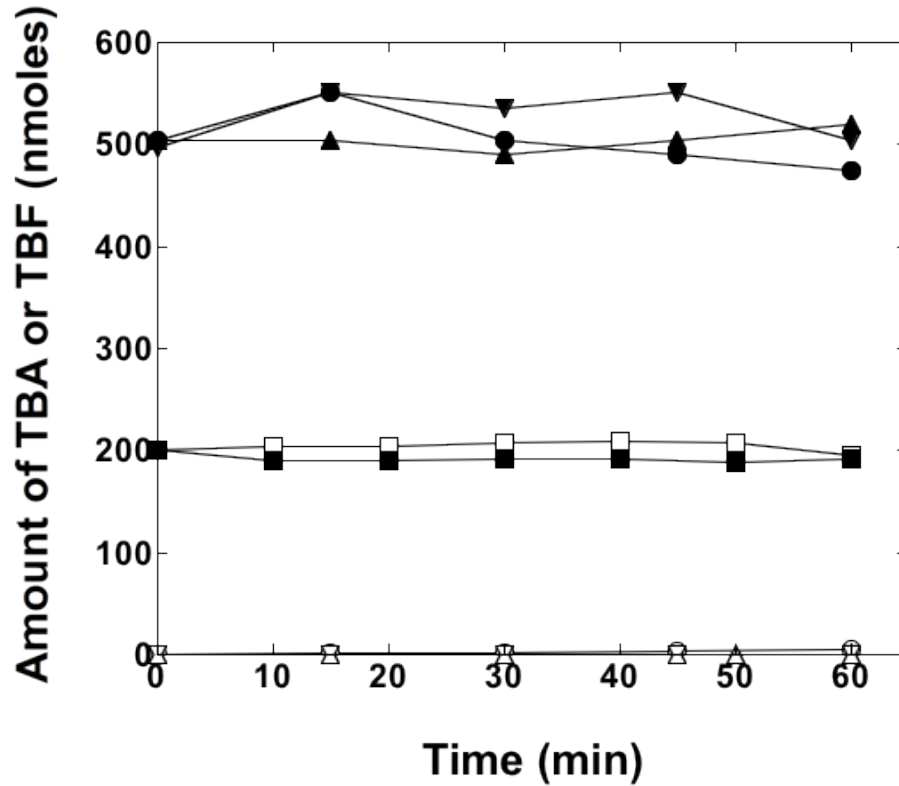
## FIGURES



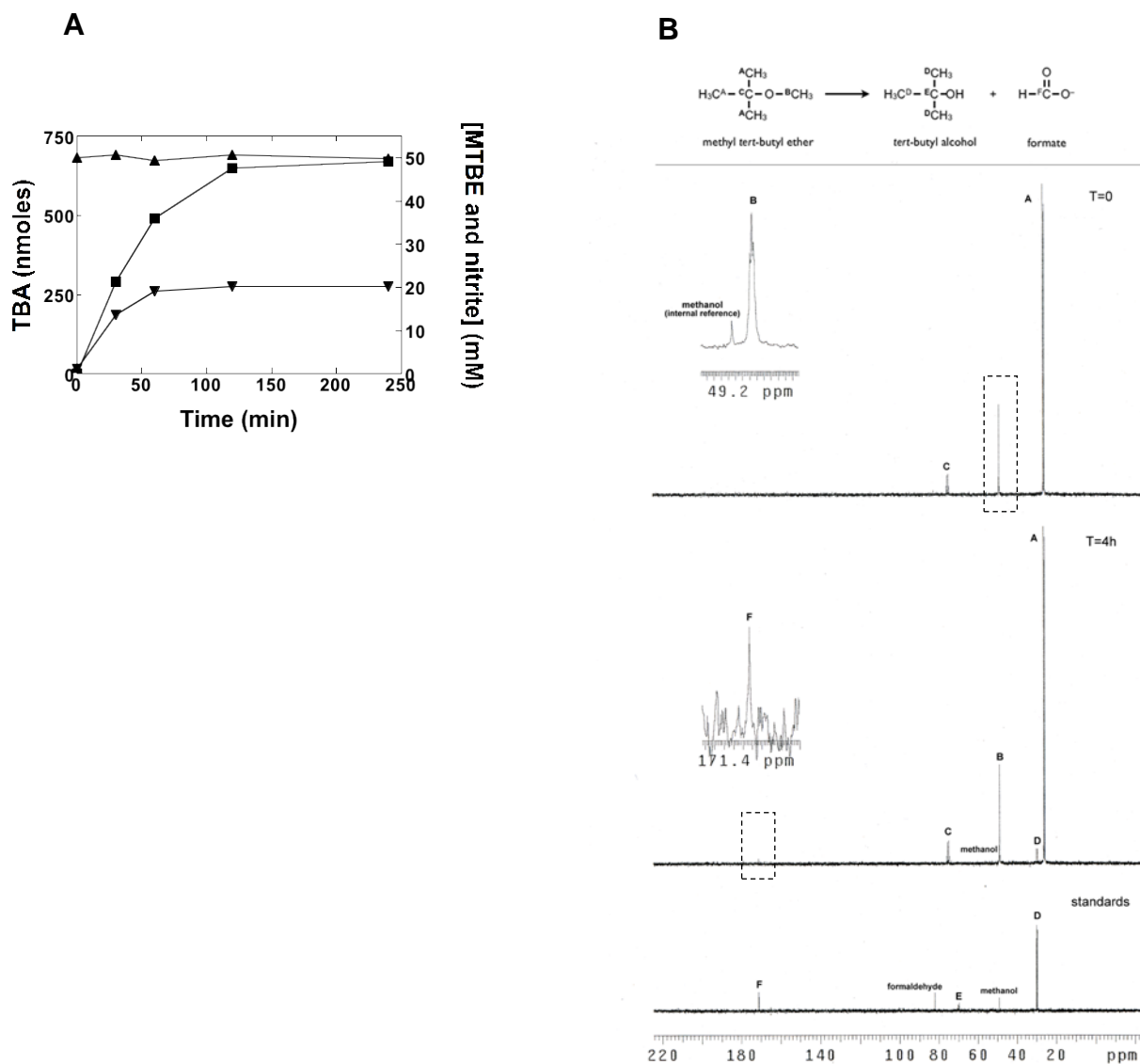
**Figure 1: Effect of MTBE on  $\text{NH}_4^+$ -dependent nitrite production by *N. europaea*.** Panel A shows the effects of (■) 0 mM, (●) 10 mM, (▼) 25 mM and (◆) 50 mM dissolved MTBE on the rates of  $\text{NH}_4^+$ -dependent nitrite production for *N. europaea* (~0.3 mg total protein) in short term (15 min) incubations conducted as described in the Methods section. Panel B shows a double reciprocal plot of the data presented in Panel A.



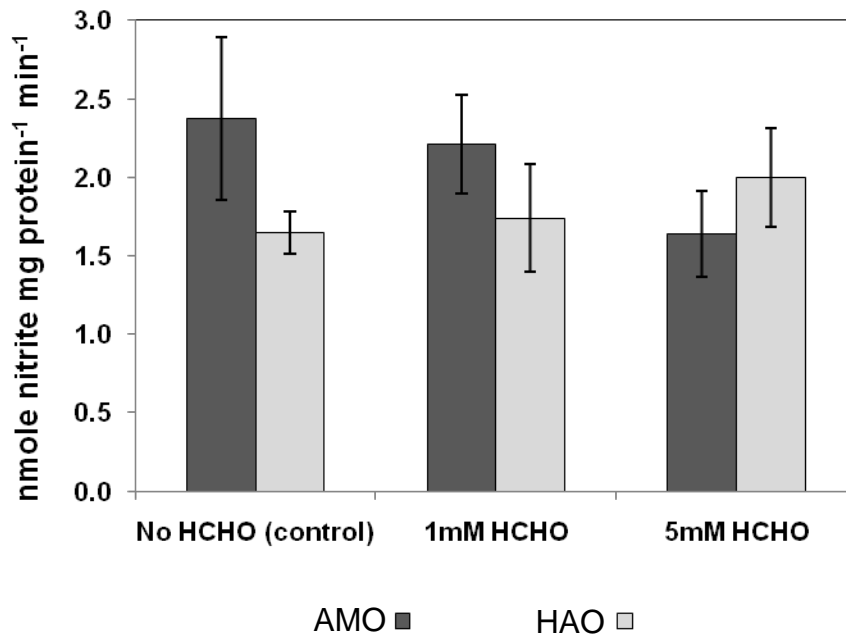
**Figure 2: Effect acetylene on TBA production from MTBE and  $\text{NH}_4^+$ -dependent nitrite production by *N. europaea*.** The Figure shows the effects of increasing dissolved concentrations of MTBE on (●,○)  $\text{NH}_4^+$ -dependent nitrite production and (■,□) TBA production for cells of *N. europaea* (~1 mg total protein) incubated with 10 mM  $\text{NH}_4^+$  in the absence (solid symbols) and presence (open symbols) of acetylene (1% gas phase).



**Figure 3: Consumption of TBF and TBA by *N. europaea*.** The Figure shows the time course for consumption of TBF (closed symbols) and production of TBA (open symbols) for cells of *N. europaea* (~1 mg total protein) incubated with TBF in PB adjusted to initial pH values of (▼,▽) 6.0, (▲,△) 7.0 and (●,○) 8.0. The Figure also shows the time course for consumption of TBA for cells of *N. europaea* (~1 mg total protein) incubated in the (■) presence and (□) absence of 10 mM NH<sub>4</sub><sup>+</sup>.



**Figure 4:**  $^{13}\text{C}$ -NMR analysis of products generated from  $^{13}\text{C}_5$ -MTBE by *N. europaea*. Cells of *N. europaea* (~10 mg total protein) were incubated with 20 mM  $\text{NH}_4^+$  and 50 mM dissolved  $^{13}\text{C}_5$ -MTBE, as described in the Methods section. **Panel A** shows the time course of (■) MTBE consumption (◆)  $\text{NH}_4^+$ -dependent nitrite and (●) TBA production. **Panel B** shows the  $^{13}\text{C}$ -NMR spectra for samples of the reaction taken at  $t = 0$  min (upper) and  $t = 4$  h (middle). A reference spectrum for a mixture of unlabeled TBA, formaldehyde and formate (5 mM each) is also shown (lower). The pathway at the top of the panel shows the assignment of individual detected resonances to the products detected in the experimental system. The upper inset shows the resolution of the methoxy carbon of MTBE from methanol that was added as an internal reference. The lower inset shows an expanded section of the spectrum indicating the detection of trace amounts of formate. (NMR data by Alan House.)



**Figure 5: Effect of exposure of *N. europaea* to formaldehyde.** Cells of *N. europaea* were exposed to formaldehyde and assayed for residual nitrite-generating activities, as described in the methods section. The Figure shows the effects of exposure to 0, 1 and 5 mM formaldehyde on the residual (dark bars)  $\text{NH}_4^+$ -dependent and (light bars)  $\text{NH}_2\text{OH}$ -dependent nitrite-generating activity. The data presented are the mean and range for three separate determinations made using three different cell cultures.

## REFERENCES

1. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, London.
2. **Arciero, D., T. Vannelli, M. Logan, and A. B. Hopper.** 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochemical and Biophysical Research Communications **159**:640-643.
3. **Arp, D., L. Sayavedra-Soto, and N. Hommes.** 2002. Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. Archives of Microbiology **178**:250-255.
4. **Bedard, C., and R. Knowles.** 1989. Physiology, biochemistry, and specific inhibitors of CH<sub>4</sub>, NH<sub>4</sub><sup>+</sup>, and CO oxidation by methanotrophs and nitrifiers. Microbiology and Molecular Biology Reviews **53**:68-84.
5. **Chang, S. W., M. R. Hyman, and K. J. Williamson.** 2002. Cooxidation of naphthalene and other polycyclic aromatic hydrocarbons by the nitrifying bacterium, *Nitrosomonas europaea*. Biodegradation **13**:373-381.
6. **Colby, J., D. I. Stirling, and H. Dalton.** 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochemical Journal **165**:395-402.
7. **Deeb, R. A., K.-H. Chu, T. Shih, S. Linder, I. Suffet, M. C. Kavanaugh, and L. Alvarez-Cohen.** 2003. MTBE and other oxygenates: environmental sources, analysis, occurrence, and treatment. Environmental Engineering Science **20**:433-447.
8. **Deshusses, M. A., M. Morales, and S. Revah.** 2000. Microcosm and column studies on the biodegradation of methyl *tert*-butyl ether (MTBE) in soil-water systems. Paper 795. Proceedings of the Annual Meeting and Exhibition of the Air and Waste Management Association, Salt Lake City, Utah, June 18-22, 2000.
9. **Fortin, N. Y., M. Morales, Y. Nakagawa, D. D. Focht, and M. A. Deshusses.** 2001. Methyl *tert*-butyl ether (MTBE) degradation by a microbial consortium. Environmental Microbiology **3**:407-416.

10. **Garnier, P. M., R. Auria, C. Augur, and S. Revah.** 1999. Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Applied Microbiology and Biotechnology* **51**:498-503.
11. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry* **177**:751-766.
12. **Hageman, R. H., and D. P. Hucklesby.** 1971. Nitrate reductase from higher plants. *Methods in Enzymology* **23**:491-503.
13. **Hägglom, M. M., L. K. G. Youngster, P. Somsamak, H. H. Richnow, S. S. Allen I. Laskin, and M. G. Geoffrey.** 2007. Anaerobic biodegradation of methyl *tert*-butyl ether (MTBE) and related fuel oxygenates. *Advances in Applied Microbiology* **62**:1-20.
14. **Hanson, J. R., C. E. Ackerman, and K. M. Scow.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Applied and Environmental Microbiology* **65**:4788-4792.
15. **Hardison, L. K., S. S. Curry, L. M. Ciuffetti, and M. R. Hyman.** 1997. Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. *Applied and Environmental Microbiology* **63**:3059-3067.
16. **Hatzinger, P. B., K. McClay, S. Vainberg, M. Tugusheva, C. W. Condee, and R. J. Steffan.** 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. *Applied and Environmental Microbiology* **67**:5601-5607.
17. **Hesselsoe, M., S. Boysen, N. Iversen, L. Jørgensen, J. Murrell, I. McDonald, S. Radajewski, H. Thestrup, and P. Roslev.** 2005. Degradation of organic pollutants by methane grown microbial consortia. *Biodegradation* **16**:435-448.
18. **Holmes, A. J., A. Costello, M. E. Lidstrom, and J. C. Murrell.** 1995. Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiology Letters* **132**:203-208.
19. **House, A., and M. Hyman.** 2010. Effects of gasoline components on MTBE and TBA cometabolism by *Mycobacterium austroafricanum* JOB5. *Biodegradation* **21**:525-541.

20. **Hyman, M. R., and D. J. Arp.** 1992.  $^{14}\text{C}_2\text{H}_2$ - and  $^{14}\text{CO}_2$ -labeling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. *Journal of Biological Chemistry* **267**:1534-1545.
21. **Hyman, M. R., I. B. Murton, and D. J. Arp.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. *Applied and Environmental Microbiology* **54**:3187-3190.
22. **Hyman, M. R., C. L. Page, and D. J. Arp.** 1994. Oxidation of methyl fluoride and dimethyl ether by ammonia monooxygenase in *Nitrosomonas europaea*. *Applied and Environmental Microbiology* **60**:3033-3035.
23. **Hyman, M. R., A. W. Sansome-Smith, J. H. Shears, and P. M. Wood.** 1985. A kinetic study of benzene oxidation to phenol by whole cells of *Nitrosomonas europaea* and evidence for the further oxidation of phenol to hydroquinone. *Archives of Microbiology* **143**:302-306.
24. **Hyman, M. R., and P. M. Wood.** 1984. Ethylene oxidation by *Nitrosomonas europaea*. *Archives of Microbiology* **137**:155-158.
25. **Hyman, M. R., and P. M. Wood.** 1983. Methane oxidation by *Nitrosomonas europaea*. *Biochemical Journal* **212**:31-37.
26. **Hyman, M. R., and P. M. Wood.** 1985. Suicidal inactivation and labelling of ammonia mono-oxygenase by acetylene. *Biochemical Journal* **227**:719-725.
27. **Jones, R. D., and R. Y. Morita.** 1983. Methane Oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europaea*. *Applied and Environmental Microbiology* **45**:401-410.
28. **Keener, W. K., and D. J. Arp.** 1993. Kinetic studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. *Applied and Environmental Microbiology* **59**:2501-2510.
29. **Keener, W. K., and D. J. Arp.** 1994. Transformations of aromatic compounds by *Nitrosomonas europaea*. *Applied and Environmental Microbiology* **60**:1914-1920.
30. **Lechner, U., D. Brodkorb, R. Geyer, G. Hause, C. Hartig, G. Auling, F. Fayolle-Guichard, P. Piveteau, R. H. Muller, and T. Rohwerder.** 2007. *Aquincola tertiaricarbonis* gen. nov., sp. nov., a tertiary butyl moiety-

- degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* **57**:1295-303.
31. **Martienssen, M., H. Fabritius, S. Kukla, G. U. Balcke, E. Hasselwander, and M. Schirmer.** 2006. Determination of naturally occurring MTBE biodegradation by analysing metabolites and biodegradation by-products. *Journal of Contaminant Hydrology* **87**:37-53.
  32. **Radniecki, T. S., M. E. Dolan, and L. Semprini.** 2008. Physiological and transcriptional responses of *Nitrosomonas europaea* to toluene and benzene inhibition. *Environmental Science & Technology* **42**:4093-4098.
  33. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. *Applied and Environmental Microbiology* **57**:2986-2994.
  34. **Salanitro, J. P., L. A. Diaz, M. P. Williams, and H. L. Wisniewski.** 1994. Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Applied and Environmental Microbiology* **60**:2593-2596.
  35. **Smith, C. A., and M. R. Hyman.** 2004. Oxidation of methyl *tert*-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and n-octane-grown *Pseudomonas putida* GPo1. *Applied and Environmental Microbiology* **70**:4544-4550.
  36. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. *Applied and Environmental Microbiology* **69**:796-804.
  37. **Smith, C. A., K. T. O'Reilly, and M. R. Hyman.** 2003. Cometabolism of methyl *tertiary* butyl ether and gaseous n-alkanes by *Pseudomonas mendocina* KR-1 grown on C<sub>5</sub> to C<sub>8</sub> n-alkanes. *Applied and Environmental Microbiology* **69**:7385-94.
  38. **Squillace, P. J., M. J. Moran, W. W. Lapham, C. V. Price, R. M. Clawges, and J. S. Zogorski.** 1999. Volatile organic compounds in untreated ambient groundwater of the United States, 1985-1995. *Environmental Science & Technology* **33**:4176-4187.
  39. **Steffan, R. J., K. McClay, S. Vainberg, C. W. Condee, and D. Zhang.** 1997. 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* **63**:4216-4222.
40. **USEPA.** 2010. FY2009 Annual Report on the Underground Storage Tank Program, EPA-510-R-10-001, Washington, D.C.
  41. **USEPA.** 2007. State Actions Banning MTBE, EPA420-B-07-013.
  42. **Vannelli, T., M. Logan, D. M. Arciero, and A. B. Hooper.** 1990. Degradation of halogenated aliphatic compounds by the ammonia- oxidizing bacterium *Nitrosomonas europaea*. *Applied and Environmental Microbiology* **56**:1169-1171.
  43. **Voysey, P. A., and P. M. Wood.** 1987. Methanol and formaldehyde oxidation by an autotrophic nitrifying bacterium. *Journal of General Microbiology* **133**:283-290.
  44. **Waul, C., E. Arvin, and J. E. Schmidt.** 2008. Model description and kinetic parameter analysis of MTBE biodegradation in a packed bed reactor. *Water Research* **42**:3122-3134.
  45. **Wood, P. M.** 1986. Nitrification as a bacterial energy source, p. 39 - 62. *In* J. I. Prosser (ed.), *Nitrification*. Society for General Microbiology (IRL Press), Washington, D.C.

## CHAPTER 5

### Compound Specific Isotope Analysis (CSIA) of Fuel Oxygenate

#### Biodegradation

The work in this chapter describes the author's contribution to two manuscripts as follows:

- I) Acid hydrolysis control reactions were prepared for the work described in "Isotopic Fractionation of Methyl *tert*-Butyl Ether Suggests Different Initial Reaction Mechanisms during Aerobic Biodegradation" published in *Environmental Science and Technology*, 2009, (43): 2793 – 2799. The authors of this manuscript are Jennifer R. McKelvie, Michael R. Hyman, Martin Elsner, Christy Smith, Denise M. Aslett, Georges Lacrampe-Couloume, and Barbara Sherwood Lollar.
- II) Culture growth, sample collection, and gas chromatography analysis were contributions to "Isolation and characterization of *tertiary* butyl alcohol-metabolizing strains *Aquicola* sp. S1B1 and *Hydrogenophaga* sp. G2B2." to be submitted for publication in *Applied and Environmental Microbiology*. The authors of this manuscript will be Kimberly L. Golart, L. Denise Aslett, Jennifer R. McKelvie, and Michael R. Hyman.

## ABSTRACT

Compound specific isotope analysis (CSIA) has been used in field studies to provide evidence for biodegradation of environmental contaminants such as benzene, tetrachloroethylene, and the fuel oxygenate methyl *tertiary*-butyl ether (MTBE). In this work, we used CSIA to compare the  $^{13}\text{C}$  and  $^2\text{D}$  enrichments associated with MTBE oxidation by different biological cultures. The strain used in this analysis was *Pseudonocardia tetrahydrofuranoxydans* K1, an actinomycete that grows on tetrahydrofuran (THF) and cometabolically oxidizes MTBE to TBA, but does not further utilize TBA. Results from this analysis indicate that strain K1, in comparison to other MTBE-oxidizing organisms, appears to use a mechanistically different enzyme to catalyze the oxidation of MTBE and other ether oxygenates. To further evaluate this reaction mechanism, we compared the carbon isotopic values ( $\delta^{13}\text{C}$ ) of parent fuel oxygenates MTBE, ethyl *tertiary*-butyl ether (ETBE), and *tertiary*-amyl methyl ether (TAME) and their daughter products *tertiary*-butyl alcohol (TBA) and *tertiary*-amyl alcohol (TAA). Results of this analysis further suggested that the *tertiary*-butyl and *tertiary*-amyl carbons are not involved in the initial degradation reactions for the parent compounds. We also used two TBA-oxidizing organisms *Aquicola* strain S1B1 and *Hydrogenophaga* strain G2B2, to determine the  $^{13}\text{C}$  enrichment associated with tertiary alcohol metabolism. This data begins to address the need for CSIA benchmarks that can be used for identifying and monitoring natural attenuation of TBA in the field.

## INTRODUCTION

Compound specific isotope analysis (CSIA) has been used in field studies to provide evidence for biodegradation of environmental contaminants such as benzene (2, 9), tetrachloroethylene (4), and the fuel oxygenate MTBE (5, 8). These studies have often been aimed at demonstrating an overall decrease in contaminant concentration while at the time detecting  $^{13}\text{C}$  enrichment within the remaining contaminant fraction. Because biological enzymes prefer lighter isotopes, these types of observations helped implicate biological processes (as opposed to abiotic processes such as dispersion and sorption) in contaminant removal in the field.  $^{13}\text{C}$  enrichment benchmarks have been provided by studies with cultures of contaminant degrading organisms. In the case of MTBE, these studies detect  $^{13}\text{C}$  enrichment based on removal of the methoxy carbon and for this reason, measured  $^{13}\text{C}$  enrichments ( $\epsilon_c$ ) have been found to be similar across different types of samples. To help distinguish potentially unique reaction mechanisms utilized by organisms in these samples,  $^2\text{D}$  enrichments which are created when C–H bonds are broken are also being measured.

In this work, we used CSIA to compare the  $^{13}\text{C}$  and  $^2\text{D}$  enrichments associated with MTBE oxidation by different biological cultures. Abiotic acid hydrolysis was used in control reactions (10). The benchmark strain used in this analysis was *Pseudonocardia tetrahydrofuranoxydans* K1, an actinomycete which grows on tetrahydrofuran (THF) (6, 7) and cometabolically oxidizes MTBE to TBA, but does not further utilize TBA. The oxidation of THF is initiated by tetrahydrofuran

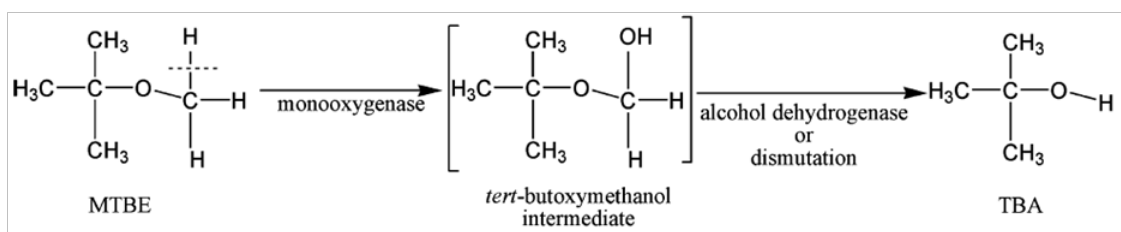
monooxygenase (11, 12), which is also implicated in MTBE oxidation to TBA. This enzyme oxidizes THF and generates 2-hydroxytetrahydrofuran from a C–H adjacent to an ether bond (11). A similar reaction with MTBE would generate the unstable hemiacetal as shown in Figure 5.1. Results from this analysis indicate that while  $^{13}\text{C}$  enrichments were similar across sample types,  $^2\text{D}$  enrichments suggest that strain K1, in comparison to other MTBE-oxidizing organisms such as *Methylibium petroleiphilum* PM1, uses a different mechanism for the initial reaction of MTBE degradation.

To provide further evidence that the *tert*-butyl group does not participate in the initial reaction of MTBE degradation, we also evaluated the isotopic composition of TBA. If carbons on the *tertiary*-butyl group of MTBE are involved in the initial reaction, we would expect to see potentially large differences in the isotopic compositions of parent MTBE and daughter TBA. To evaluate this question, we compared the carbon isotopic values ( $\delta^{13}\text{C}$ ) of parent fuel oxygenates with those of daughter products using not only MTBE but other ether oxygenates, ethyl *tertiary*-butyl ether (ETBE) and *tertiary*-amyl methyl ether (TAME). Biological reactions were set up for strain K1 and acid hydrolysis was used as an abiotic comparison. Results of this analysis were consistent with monooxygenase-catalyzed oxidation for all three oxygenates and further suggested that the *tert*-butyl and *tert*-amyl carbons are not involved in the initial degradation reactions.

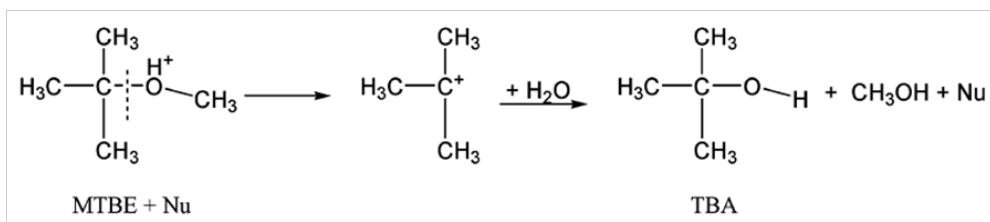
We have also applied CSIA in the study of intermediate alcohols generated during biodegradation of fuel oxygenates MTBE and TAME. The need for CSIA

data associated with TBA oxidation has recently been highlighted (13) and organisms isolated by our lab are particularly suited for such a data analysis. TBA-oxidizing organisms, *Aquicola* strain S1B1 and *Hydrogenophaga* strain G2B2, were isolated from a BioGAC reactor treating TBA-contaminated groundwater (3). Strain S1B1 is phylogenetically related to *M. petroleiphilum* PM1, but is physiologically distinct in terms of growth substrate ranges. PM1 utilizes either MTBE or TBA as a sole source of carbon and energy, but S1B1 cannot grow on MTBE. And while S1B1 is capable of growth on *tertiary*-amyl alcohol (TAA), PM1 is not. Using TBA-oxidizing organisms *Aquicola* strain S1B1 and *Hydrogenophaga* strain G2B2, <sup>13</sup>C enrichment data was collected. This data begins to address the need for CSIA benchmarks that can be used for identifying and monitoring natural attenuation of TBA in the field.

**A Oxidation of a C-H bond on the methoxy group:**



**B Hydrolytic reaction:**



**Figure 5.1 – Possible Mechanisms for Degradation of MTBE to TBA – Oxidation vs. Hydrolysis.** Dashed lines represent bond breakage. Panel A) MTBE is oxidized by strain K1 in a monooxygenase-catalyzed reaction. Panel B) Acid hydrolysis and  $\text{S}_{\text{N}}1$  enzymatic reactions generate isotopic fractionation patterns distinct from oxidation reactions. Diagrams taken from McKelvie, J.R., et al. 2009. Isotopic fractionation of methyl tert-butyl ether suggests different initial reaction mechanisms during aerobic biodegradation. *Environmental Science and Technology*. 43: 2793-2799.

## MATERIALS AND METHODS

**Acid Hydrolysis of Fuel Oxygenates.** Acid hydrolysis of MTBE, ETBE, and TAME was conducted in glass serum vials (15 ml) crimp sealed with Teflon-coated butyl rubber stoppers. Samples of each neat ether (7 – 11  $\mu$ l) were added to buffer (1 ml, 50 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  adjusted to pH 1.0 with 12 N HCl) and incubated in a shaking water bath (150 rpm) at 40°C. Ether hydrolysis was measured over time using gas chromatography. Once the hydrolysis was complete (98.4% reacted), samples were added to 40 ml VOA vials containing trisodium phosphate (1 %w/v) for a total volume of 40 ml and a 10 mg/L final concentration of TBA or TAA.

**Gas Chromatography (GC).** MTBE, TBA, and TAA concentrations were determined by direct aqueous injection of 2  $\mu$ l into a Shimadzu GC-8A Gas Chromatograph (Kyoto, Japan) fitted with a flame ionization detector and a stainless steel column (0.3 x 183 cm) filled with Porapak Q (60-80 mesh) (Waters Associates, Framingham, MA). GC analysis was conducted using a column temperature of 160°C isothermal, an injector port temperature of 200°C and a detector temperature of 220°C. The GC was interfaced to Hewlett Packard HP3395 (Palo Alto, CA) integrator for data collection.

**Mineral Salts Media.** *Aquinicola* sp. S1B1 was grown in mineral salts media comprised of (per L): Solution (10 ml) A, vitamin solution (1 ml), and Solution B (989 ml) *Hydrogenophaga* sp. G2B2 was grown in mineral salts media comprised of (per L): Solution A (ml) and Solution B (990 ml). Solution A contained the following (per L): ferric ammonium citrate (0.5g);  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (1.0 g). The vitamin solution was

stored at 4°C and contained the following (per L): biotin (20 mg); pantothenic acid (20 mg); thiamine-HCl (20 mg) and pyridoxine-HCl (20 mg). Solution B contained the following (per L): Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (6.74 g), KH<sub>2</sub>PO<sub>4</sub> (1.5 g) NH<sub>4</sub>Cl (1.0 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g) and trace element solution (1 ml). The trace element solution contained the following (per L): ZnSO<sub>4</sub>·7H<sub>2</sub>O (100 mg), MnCl<sub>2</sub>·4H<sub>2</sub>O (30 mg), H<sub>3</sub>BO<sub>3</sub> (300 mg) CoCl<sub>2</sub>·6H<sub>2</sub>O (200 mg) CuCl<sub>2</sub>·2H<sub>2</sub>O (10 mg) NiCl<sub>2</sub>·6H<sub>2</sub>O (20 mg) and Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (30 mg). All media was sterilized by autoclaving at 121°C for 30 min.

**Growth of S1B1 and G2B2 cells.** Approximately 500 µl of cells from existing cultures were inoculated into each of six 160 ml glass vials containing 25 ml mineral salts and 12.5 µl either neat TBA or TAA. Vials were incubated in the dark at room temperature (~24°C) on a rotary shaker (150 rpm). After growth for 7 d, cultures were combined and cells were pelleted by centrifugation (6,000 x g, 15 min), resuspended, and washed in phosphate buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, pH 7). Cells were resedimented by centrifugation (10,000 x g, 5 min) and resuspended in 5 ml mineral salts media. Cells were added to glass culture bottles (500 ml) containing either neat TBA or TAA (520 µl) and mineral salts in a final volume of 100 ml. Duplicate cultures were prepared for each compound. The optical density (OD<sub>600</sub>) of each culture was measured by removing 100 µl and mixing it with 900 µl mineral salts. Beginning ODs for cultures were as follows: G2B2 (0.093, 0.121); S1B1 + TBA (0.152, 0.141); S1B1 + TAA (0.141, 0.167). Bottles were sealed with screw caps fitted with butyl rubber inserts (Wheaton Scientific, Millville, NJ). Cultures were

incubated at room temperature, in the dark, on a rotary shaker (150 rpm). Two samples were extracted directly from culture bottles with a heat sterilized syringe (Hamilton Syringe Company, Reno, NV) immediately after cell addition and every 24 hours thereafter for analysis as follows: a) 2  $\mu$ l samples were used for GC analysis to monitor TBA and TAA biodegradation, b) appropriate volumes (calculated to provide a final TBA concentration of 10 mg/L) were extracted from cultures, filtered to remove biomass, and added to 40 ml VOA vials overfilled with trisodium phosphate (1% w/v). Biomass was filtered out of the samples by sedimentation using an Eppendorf model 5415C microcentrifuge (14,000 rpm, 5 min). VOA vials were stored at 4°C prior to overnight shipment (with ice packs) to Drs. Jennifer R. McKelvie and Barbara Sherwood Lollar (University of Toronto, Canada) for analysis by GC/C/IRMS.

## RESULTS AND DISCUSSION

CSIA was used to investigate the  $^{13}\text{C}$  and  $^2\text{D}$  enrichments exhibited during the oxidation of MTBE by strain K1 and several other cultures with aerobic MTBE-oxidizing activity. The  $^{13}\text{C}$  enrichment ( $\epsilon_{\text{C}}$ ) for the oxidation of MTBE for strain K1 is similar to that observed for other organisms as shown in Table 5.1. In contrast, the  $^2\text{D}$  enrichments ( $\epsilon_{\text{H}}$ ) are consistently much larger than those described for other aerobes and are close to the theoretical maximum previously determined for the abiotic permanganate oxidation of MTBE (1). This result suggests that the initial reaction in MTBE degradation by strain K1 is consistent with an oxidative, monooxygenase-catalyzed reaction in which a C–H bond on the methoxy group is cleaved. The lower enrichments ( $\epsilon_{\text{H}}$ ) observed for strains PM1 and R8 further suggest the enzyme systems responsible for MTBE degradation in these organisms

**Table 5.1 – CSIA Analysis of MTBE Biodegradation by Selected Aerobic MTBE Degrading Cultures**

Culture	$\epsilon_{\text{C}} \pm 95\% \text{ CI}$	$\epsilon_{\text{H}} \pm 95\% \text{ CI}$
<u>Abiotic reactions:</u>		
Oxidation by permanganate	NA	$-109 \pm 9\text{‰}$
Acid Hydrolysis	$-4.9 \pm 0.6 \text{‰}$	$-55 \pm 7\text{‰}$
<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	$-2.3 \pm 0.2 \text{‰}$	$-100 \pm 10 \text{‰}$
<i>Methylibium petroleiphilum</i> PM1	$-2.2 \pm 0.1 \text{‰}$	$-35 \pm 2 \text{‰}$
<i>Methylibium</i> R8	$-2.4 \pm 0.1 \text{‰}$	$-42 \pm 4 \text{‰}$
<i>Rhodococcus ruber</i> IFP2001	$-0.3 \pm 0.1 \text{‰}$	No enrichment

Data from McKelvie, J. R., et al. 2009. Isotopic fractionation of methyl tert-butyl ether suggests different initial reaction mechanisms during aerobic biodegradation. *Environmental Science and Technology*. 43: 2793-2799.

may involve hydrolysis rather than oxidation of MTBE.

The carbon isotopic values ( $\delta^{13}\text{C}$ ) of parent fuel oxygenates MTBE, ETBE, and TAME and daughter products TBA and TAA were also compared for strain K1 and abiotic acid hydrolysis as shown in Table 5.2. These results show that the values for the parent oxygenates are similar to those of daughter products TBA and TAA, suggesting that the *tertiary*-butyl or *tertiary*-amyl groups do not participate in the initial reaction of fuel ether degradation. These results are also consistent with an oxidative mechanism for biodegradation of not only MTBE, but also ETBE and TAME, by strain K1.

**Table 5.2 – Carbon Isotopic Values ( $\delta^{13}\text{C}$ ) of Parent Fuel Oxygenates and Daughter Products (TBA and TAA) Produced during Biodegradation by *Pseudonocardia* K1 and Abiotic Acid Hydrolysis**

Fuel Oxygenate	Daughter Product	Degradation method	Initial $\delta^{13}\text{C}$ (‰) of Parent	$\delta^{13}\text{C}$ (‰) of daughter product	% Reacted
MTBE	TBA	Biodegradation	-30.7	-28.2	98.7
		Acid hydrolysis	-30.9	-29.9	
ETBE	TBA	Biodegradation	-22.3	-28.4	99.2
		Acid hydrolysis	-24.7	-30.3	
TAME	TAA	Biodegradation	-23.8	-22.4	99.9
		Acid hydrolysis	-23.8	-19.8	

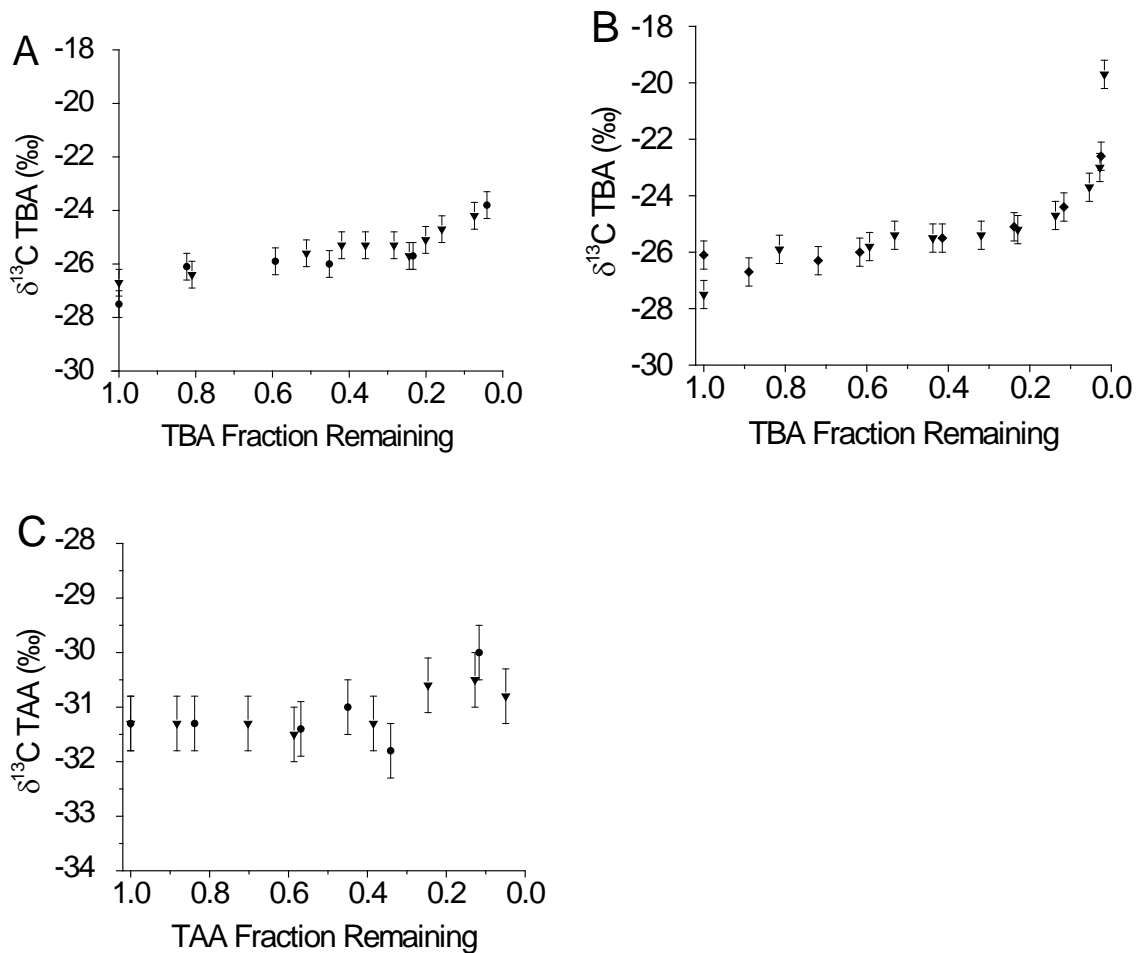
Data from McKelvie, J. R., et al. 2009. Isotopic fractionation of methyl tert-butyl ether suggests different initial reaction mechanisms during aerobic biodegradation. *Environmental Science and Technology*. 43: 2793-2799.

In addition to CSIA studies with *Pseudonocardia* strain K1, CSIA studies of the  $^{13}\text{C}$  enrichments associated with TBA and TAA oxidation by strains S1B1 and G2B2 were also conducted. The  $^{13}\text{C}$  enrichments associated with TBA and TAA oxidation were small or non-detectable, as shown in Table 5.3 and Figure 5.2. These results indicate further studies examining  $^2\text{D}$  enrichment will be required to determine if CSIA-based analyses can be useful for quantifying TBA degradation in field samples.

**Table 5.3 – Isotopic Enrichment Factors for TBA and TAA.**

Cell culture	Growth Substrate	Initial $\delta^{13}\text{C}$ (‰) ± standard deviation	Enrichment Factor (‰) ± standard deviation
G2B2	TBA	$-27.1 \pm 0.6$	$-1.2 \pm 0.2$ , $r^2 = 0.72$
S1B1	TBA	$-26.8 \pm 1.0$	$-1.3 \pm 0.2$ , $r^2 = 0.87$
S1B1	TAA	$-31.3 \pm 0.1$	ND*

\* An isotopic enrichment factor could not be calculated. The fit to the Rayleigh model was poor ( $r^2 = 0.42$ ). Data provided by Jennifer R. McKelvie.



**Figure 5.2 - Carbon isotopic values versus the fraction of parent compound remaining during aerobic biodegradation of TBA and TAA.** Panel A) TBA by G2B2; Panel B) TBA by S1B1; and Panel C) TAA by S1B1. Vertical error bars represent the total uncertainty (accuracy and reproducibility) of  $\pm 0.5\%$  for  $\delta^{13}\text{C}$  values. Data and graphs provided by Jennifer R. McKelvie.

## REFERENCES

1. **Elsner, M., J. McKelvie, G. Lacrampe Couloume, and B. Sherwood Lollar.** 2007. Insight into methyl *tert*-butyl ether (MTBE) stable isotope fractionation from abiotic reference experiments. *Environmental Science & Technology* **41**:5693-5700.
2. **Fischer, A., K. Theuerkorn, N. Stelzer, M. Gehre, M. Thullner, and H. H. Richnow.** 2007. Applicability of stable isotope fractionation analysis for the characterization of benzene biodegradation in a BTEX-contaminated aquifer. *Environmental Science and Technology* **41**:3689-3696.
3. **Golart, K. L.** 2007. Physiology and Enzymology of Aerobic MTBE and TBA Biodegradation. Ph.D. Thesis. North Carolina State University, Raleigh.
4. **Hunkeler, D., R. Aravena, and B. J. Butler.** 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: microcosm and field studies. *Environmental Science and Technology* **33**:2733-2738.
5. **Hunkeler, D., B. J. Butler, R. Aravena, and J. F. Barker.** 2001. Monitoring biodegradation of methyl *tert*-butyl ether (MTBE) using compound specific-isotope analysis. *Environmental Science and Technology* **2001**:676-681.
6. **Kampfer, P., U. Kohlweyer, B. Thiemer, and J. R. Andreesen.** 2006. *Pseudonocardia tetrahydrofuranoxydans* sp. nov. *International Journal of Systematic and Evolutionary Biology* **56**:1535-1538.
7. **Kohlweyer, U., B. Thiemer, T. Schröder, and J. R. Andreesen.** 2000. Tetrahydrofuran degradation by a newly isolated culture of *Pseudonocardia* sp. strain K1. *FEMS Microbiology Letters* **186**:301-306.
8. **Kolhatkar, R., T. Kuder, P. Philp, J. Allen, and J. T. Wilson.** 2002. Use of compound-specific stable carbon isotope analyses to demonstrate anaerobic biodegradation of MTBE in groundwater at a gasoline release site. *Environmental Science and Technology* **36**:5139-5146.
9. **Mancini, S. A., A. C. Ulrich, G. Lacrampe-Couloume, B. Sleep, E. A. Edwards, and B. S. Lollar.** 2003. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. *Applied and Environmental Microbiology* **69**:191-198.
10. **O'Reilly, K. T., M. E. Moir, C. D. Taylor, C. A. Smith, and M. R. Hyman.** 2001. Hydrolysis of *tert*-butyl methyl ether (MTBE) in dilute aqueous acid. *Environmental Science & Technology* **35**:3954-3961.

11. **Thierner, B., J. R. Andreesen, and T. Schröder.** 2003. Cloning and characterization of a gene cluster involved in tetrahydrofuran degradation in *Pseudonocardia* sp. strain K1. *Archives of Microbiology* **179**:266-277.
12. **Thierner, B., J. R. Andreesen, and T. Schröder.** 2001. The NADH-dependent reductase of a putative multicomponent tetrahydrofuran mono-oxygenase contains a covalently bound FAD. *European Journal of Biochemistry* **268**:3774-3782.
13. **Wilson, J. T., and C. Adair.** 2007. Monitored natural attenuation of *tertiary* butyl alcohol (TBA) in ground water at gasoline spill sites. EPA/600/R-07/100. U.S. Environmental Protection Agency Washington, D.C.

## CONCLUDING REMARKS

In this work, multiple stable isotope methods were used in novel approaches to investigate fuel oxygenate biodegradation.

Stable isotope probing was used to identify microbial populations associated with the aerobic biodegradation of fuel oxygenates with metabolic as well as cometabolic processes. DNA-SIP was used to identify tertiary *butyl* alcohol (TBA) degrading bacteria in reactor systems designed to treat contaminated groundwater. Identifying these organisms and detecting the presence of tested genes, especially Mpe\_B0555, have the potential to predict a site's compatibility with TBA biodegradation. This information may also inform decisions regarding bioreactor self inoculation vs. bioaugmentation. Detection of Mpe\_B0555 is particularly relevant because it was recently detected during a proteomic analysis of *Aquicola* strain L108 after growth on TBA. And finally, the presence of these organisms and genes may indicate ongoing biodegradation in TBA-impacted aquifers.

DNA-SIP was also used in a novel application to characterize oxygenate biodegradation by a cometabolic and commensal process. Although propane was used in our study to initiate MTBE cometabolism, alkane-dependent MTBE degradation to TBA and alkane-independent consumption of TBA occur with gasoline-relevant C<sub>5</sub> to C<sub>8</sub> *n*-alkanes. A prior study has attributed these activities to a single *Pseudomonas* strain in a mixed culture maintained on alkane + MTBE. Our results however suggest cometabolism and commensalism are more likely. And

finally, detection of PM1-like organisms has previously led to the assumption that MTBE is degraded through direct metabolism. Our results suggest that the activity of PM-1 like organisms is entirely dependent on an initial alkane-dependent oxidation of MTBE to TBA.

Nuclear magnetic resonance was also used to detect and characterize key metabolites generated during MTBE cometabolic biodegradation by an ammonia-oxidizing bacterium common to soil environments.

Compound specific isotope analysis was also used to evaluate MTBE biodegradation and results suggested that microorganisms utilize multiple initial reaction mechanisms for MTBE biodegradation.

The research described in this dissertation contributes to the knowledge of fuel oxygenate biodegradation with the use of novel investigative approaches that improve our understanding of the diversity of microorganisms and processes relevant to the environmental fates of these compounds.