

Report for 2005NJ86B: Microbial degradation of MTBE in anaerobic environments

Publications

- Conference Proceedings:
 - Youngster, L., P. Somsamak, L. Kerkhoff, and MM Haggblom. 2006. Characterization of anaerobic MTBE-degrading bacterial communities. Abstract Q-081. American Society for Microbiology 106th General Meeting, Orlando, FL, May 20-25, 2006.
 - Youngster, L., P. Somsamak, L. Kerkhoff, and MM Haggblom. 2006. Characterization of anaerobic MTBE-degrading bacterial communities. International Symposium on Environmental Biotechnology, Leipzig, Germany, July 9-13, 2006.

Report Follows

Problem and Research Objectives

Methyl *tert*-butyl ether is a synthetic chemical, primarily used as a fuel additive to reduce emissions (NJDEP, 2001). It is also a common environmental contaminant, introduced into water by spills and leaks during production, transportation, and storage. MTBE has a strong turpentine-like taste and smell and can only be tolerated in drinking water at low levels. MTBE is also a skin and respiratory irritant and can be carcinogenic in rats and mice (Werner, 2001). The USEPA issued a 1996 recommended limit of 20-35 ppb in drinking water (NJDEP, 2001). Many states have adopted lower thresholds of 13-14 ppb (Stefan, 2000, Ayotte, 2005). Municipal water supplies have been closed due to MTBE contamination and a USGS survey found MTBE to be the second most common aquifer contaminant in urban United States areas (Squillace, 1996).

Aquifer contamination with MTBE is widespread and travels quickly. For most contaminated groundwater, the most financially realistic treatment option is natural attenuation (Bradley, 2001). Physical and chemical properties of MTBE make environmental contamination a challenging problem. Most treatment plans for handling gasoline spills are optimized for removing BTEX components (benzene, toluene, ethylbenzene, or *o*-, *m*-, *p*-xylene) and are not very effective for MTBE removal (USEPA, 2004). Relative to other gasoline components, MTBE has a higher vapor pressure, higher solubility, and low Henry's constant (USEPA, 2004). Together these properties mean that when MTBE is spilled it is likely to dissolve in water and migrate quickly throughout the water system without being hindered by volatilization or adherence to soil. MTBE is also less prone to biodegradation. The tertiary carbon structure and stable, unreactive ether bond increase its resistance (Stocking, 2000). MTBE was initially thought to be entirely unsusceptible to microbial attack. Now there have been several reports of MTBE biodegradation by both aerobic and anaerobic cultures (Bradley, 2001, Pruden, 2001, Hristova, 2003, Somsamak, 2001). The aerobic cultures have been investigated and several organisms have been identified as able to biodegrade MTBE however, there is little information about anaerobic MTBE-biodegradation.

If we want to rely on natural attenuation for most MTBE removal, it is important that we know whether or not biodegradation is occurring in contaminated aquifers. We need to be able to measure the natural attenuation rate in the environment and to determine which metabolites are being formed. Since many fuel-contaminated aquifers have large anoxic zones (Mormile, 1996), it is important that we find out more about anaerobic MTBE biodegradation. In the Häggblom lab, anaerobic MTBE-degrading microcosms have been established using inocula from various sites (Somsamak, 2001, Youngster, 2004). Enrichments were initially established in 1996 with polluted estuarine sediment and since then, these cultures have been successfully transferred into fresh medium with enrichment of MTBE-degrading populations. They have retained MTBE degradation activity when tested with fresh sediments from different sites, different microbial populations, and different terminal electron accepting processes (Somsamak, 2005). These are the first, and very likely the only stable MTBE-utilizing anaerobic enrichment cultures available for more detailed microbial analysis. For my thesis research, I intend to identify the microbes in these communities, to develop methods for determining the rate of biodegradation in anaerobic environments, and to find out how to optimize conditions for such biodegradation to occur.

Methodology

Funded by the NJWRRI fellowship, complementary molecular tools are now being used to identify organisms in the MTBE-degrading cultures. Metagenomic techniques are valuable for analyzing the physiology and genetics of uncultured organisms from environmental samples. Comparative community analysis has been started by terminally labeled restriction fragment length polymorphism (T-RFLP) analysis. From several consortiums, 16S rRNA genes have been amplified and fluorescently labeled using PCR with a 5' labeled 6-FAM 27F primer and an unlabeled 1525R primer (Figure 2). The amplified DNA was digested with restriction enzymes and the fragments were separated on an ABI sequence analyzer. Chromatograms are produced indicating the fragment sizes their relative abundance.

A 16S rRNA clonal library is being constructed. To do this, DNA has been extracted from the samples. 16S rRNA genes have been amplified by PCR using standard eubacterial primers 27F and 1525R. DNA fragments are currently being cloned into a plasmid vector. The ligated vector/insert plasmids will be transformed into high transforming efficiency *Escherichia coli* DH5, then plated onto a selective media plate. Colonies will be screened by plasmid extraction and digestion with restriction enzyme to identify unique clones.

I am also studying carbon isotope fractionation patterns in our MTBE degrading cultures. Natural attenuation rates of MTBE cannot be determined based on concentration measurements. MTBE's high solubility in water means that a decrease in concentration could be due to dispersal throughout the water system rather than degradation. If contamination occurs from multiple, sometimes unknown sources, concentration may be remaining stable or actually increasing despite the occurrence of degradation. Stable carbon isotope fractionation has been used to monitor in situ degradation of several environmental contaminants. Due to the greater stability of bonds involving ^{13}C versus ^{12}C , MTBE containing the lighter isotope is degraded preferentially, leading to an increased $^{13}\text{C}:^{12}\text{C}$ ratio in the remaining MTBE.

Principal Findings and Significance

So far, DNA has been extracted from several different enrichment consortia (Figure 1) and community analysis by T-RFLP has been conducted using this DNA (Figure 2).

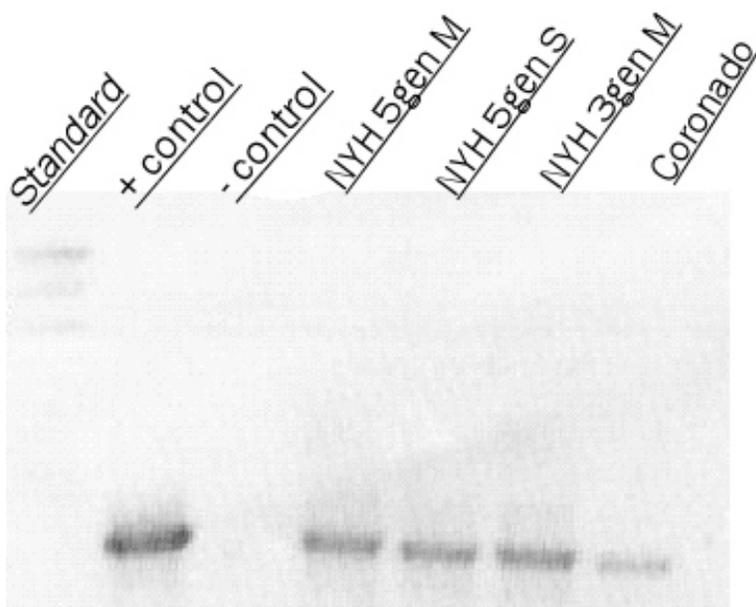


Figure 1. 16S rRNA gene PCR product

PCR amplification of 16S rRNA genes from community DNA extracts with 27 Forward fluorescent and 1525 Reverse primers

This initial analysis of the dilute cultures gives us several useful pieces of information. The T-RFLP fingerprints show reduced diversity in the communities as a result of the enrichment process. Therefore, identification of these organisms will provide information about how MTBE-degradation occurs.

The profiles obtained from sediments from New York Harbor, Coronado Cay, and Cheesequake Park are all strikingly different in composition and diversity. Further analysis of these cultures and identification of the microbes may reveal multiple organisms that are capable of anaerobic MTBE-biodegradation. Even within the 3rd generation samples which are both from New York Harbor there are differences between communities. The two 5th generation New York Harbor samples, however, are less diverse and extremely similar to each other, indicating that methanogenic and sulfidogenic conditions are selecting for the same population from this sediment.

This project is ongoing. T-RFLP analysis of additional enrichment cultures is in progress. This data will be compared to current data to determine the effects of substrate variation and cultural conditions on the community. As mentioned above, a clonal library is in the process of being constructed. Unique clones will then be sequenced. At this point a computer program will be used to align the sequences from unique clones and construct phylogenetic trees. Gene databases will be searched for the closest matches to the sequences obtained from clones. The genes identified may elucidate the community physiology and phylogeny. T-RFLP analysis of clonal libraries will also be done to compare the representation seen in the T-RFLP fingerprint to the clones that are obtained

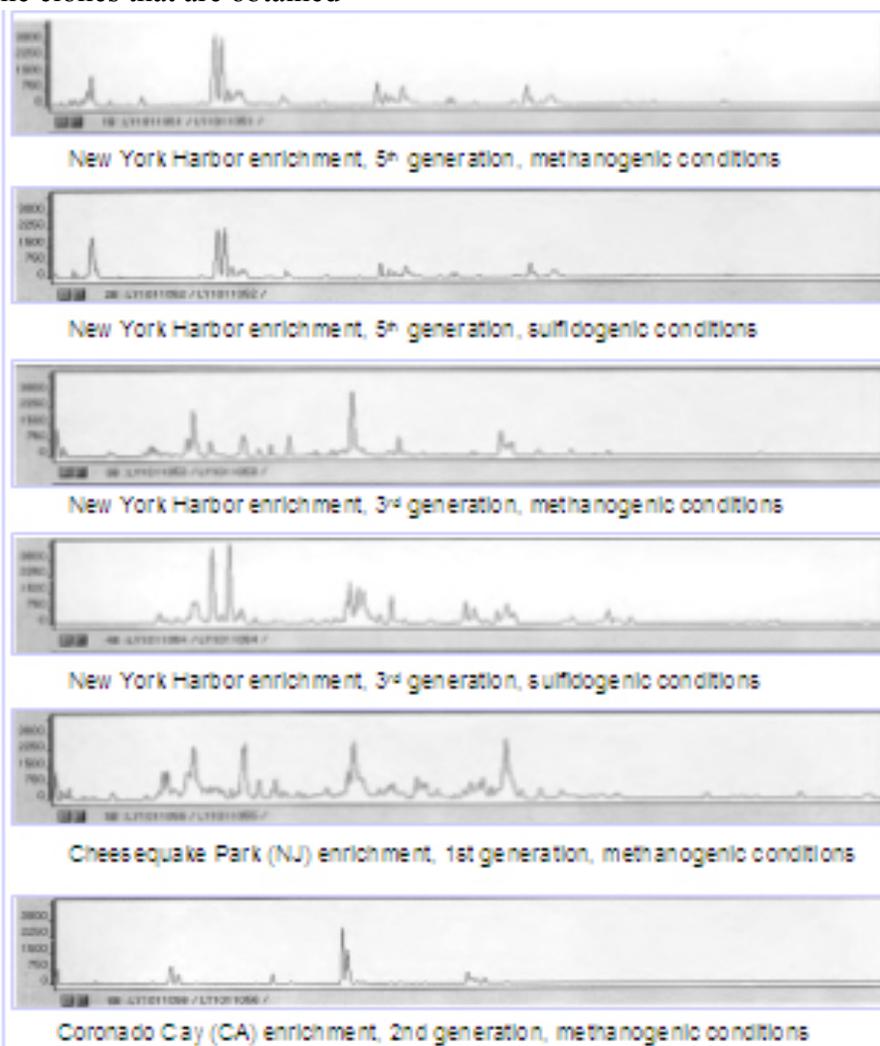


Figure 2.

**Terminal
Restriction
Fragment Length
Polymorphism
analysis of various
enrichment
communities**

Other plans include RNA extraction from enrichment cultures and subsequent analysis to determine which members of the population are actively growing, cultural isolation experiments, and eventually constructing anaerobic MTBE-degrading microcosms consisting of identified organisms

Characterization of MTBE-degrading anaerobic communities will be an important step toward assessing how to enhance MTBE biodegradation in the environment and to encourage complete mineralization of MTBE. It will also be useful for developing methods to monitor *in situ* biodegradation and thus determine whether or not remediation of polluted environments by natural attenuation is a viable option.

Studies done by Piyapawn Somsamak have demonstrated that the carbon isotopic fractionation during anaerobic biodegradation of MTBE is greater than that observed in aerobic culture (Somsamak, 2005). In July of 2006 I am going to be using Gas Chromatography-Isotope Ratio Mass Spectrometry to analyze the stable carbon isotope fractionation that occurs in sulfidogenic and methanogenic MTBE-degrading microcosms. Variations of culture conditions are currently being tested, including cultures grown in the presence of substrates which are likely to be present in fuel contaminated aquifers, such as ethanol and benzene. Other conditions that are being tested are amendments with syringate, a methoxylated aromatic carbon which may enhance the rate of MTBE degradation by enhancing acetogen growth. The effects of additional chemicals on the isotope fractionation that occurs during MTBE-degradation will help develop this assay as a tool for monitoring contaminated areas. We have also acquired an aerobic strain of MTBE-degrading bacteria from Finland. This strain is being tested for the effects of temperature on the growth rate, for degradation of tert amyl methyl ether (TAME), and the carbon and hydrogen isotope fractionation rates will be studied for all of these conditions.

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