



WATER RESOURCES RESEARCH GRANT PROPOSAL

Title: FEASIBILITY OF USING BIOAUGMENTATION WITH BACTERIAL STRAIN PM 1 FOR BIOREMEDIATION OF MTBE-CONTAMINATED VADOSE AND GROUNDWATER ENVIRONMENTS

Research Category: Category III Water Quality: wastewater treatment and reclamation processes

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Executive Summary

The fuel additive, methyl tertiary-butyl ether (MTBE), has become a widespread environmental contaminant in the past decade. Since MTBE was introduced to gasoline as an additive in 1988, its production has increased to 17 billion pounds per year and currently comprises up to 15% of some reformulated gasoline (Kirshner, 1995). This increased usage coupled with high incidences of leaking underground storage tanks has led to MTBE contamination of groundwater, soils and sediments. There is little evidence that extensive intrinsic remediation is occurring at MTBE contaminated sites. Thus it is important to explore the potential of using active bioremediation, a potentially promising technology for inexpensive treatment of MTBE contaminated groundwater. Many challenges must be overcome before bioremediation of MTBE can be successfully implemented at the field scale. Challenges include the identification and culturing of an MTBE-degrading inoculant, the engineering constraints associated with in situ remediation, and insurance of inoculant survival and activity in contaminated environments. Our laboratory has recently isolated a bacterial culture, Strain PM 1, which is capable of using MTBE as its sole carbon and energy source at relatively rapid rates. In addition, a preliminary study indicates that the organism can also degrade MTBE when inoculated into a soil microcosm.

The objectives of this study are to measure the potential for and rates of biodegradation of MTBE in vadose and groundwater materials inoculated with Strain PM 1. We will also measure the survival of Strain PM 1 when it is inoculated into environmental samples, both contaminated and not contaminated with MTBE, and bioreactors. In order to more fully optimize conditions for use of Strain PM 1 as an inoculant we will continue to characterize the physiology of the bacterial strain with regard to nutrient and growth factor requirements, MTBE kinetic parameters, MTBE concentration range, maintenance

of MTBE degrading activity, and starvation survival. Finally we will characterize the metabolic pathway of MTBE degradation by Strain PM 1.

Predicted results and benefits of this study will be information about the feasibility of using Strain PM 1 in bioremediation of MTBE-contaminated subsurface environments and in bioreactors. This study will also increase our basic understanding of the physiology and metabolic activity of Strain PM 1 that can, in turn, be used to optimize bioremediation technologies involving Strain PM 1.

Key words: MTBE, bioremediation, biodegradation, microorganisms, groundwater treatment, vadose, organic pollutants, inoculation, groundwater contamination.

Nature, Scope and Objectives

The fuel additive, methyl tertiary-butyl ether (MTBE), has become a widespread environmental contaminant in the past decade. Since MTBE was introduced to gasoline as an additive in 1988, its production has increased to 17 billion pounds per year and currently comprises up to 15% of some reformulated gasoline (Cooney, 1997). This increased usage coupled with high incidences of leaking underground storage tanks has led to MTBE contamination of groundwater, soils and sediments. In addition recreational watercraft operation has resulted in contamination of surface waters, most notably the Santa Monica Reservoir in which concentrations of MTBE as high as 10 ug per ml have been measured. Once MTBE enters the environment, it is considered to be relatively recalcitrant to degradation. MTBE is very water-soluble (48 g/L), thus it is highly mobile in both ground and surface waters and its transport resembles that of conservative tracers used to predict groundwater flow. The pollutant is also moderately volatile (unitless Henry's law constant 0.04) which can lead to redistribution and further contamination of the vadose zone, surface soils and sediments.

A recent study of shallow groundwater conducted by the U.S. Geological Survey's National Water Quality Assessment program found MTBE contamination in 79% of wells tested in urban Denver (Squillace et al. 1996). A 1996 reformulated gasoline spill in Santa Monica, CA resulted in the closure of 80% of the city's drinking water supply (Cooney, 1997). Incidences such as these have led to increasing concerns regarding both the acute and chronic toxicity of MTBE. The Environmental Protection Agency (EPA) lists MTBE as a possible carcinogen, but toxicity limits are a subject of current debate. The compound can be detected by both taste and odor at concentrations as low as 35 ug/L, thus the EPA has recommended keeping MTBE concentrations in drinking water below a 20-40 ug/L nuisance limit.

In situ bioremediation is a potentially promising technology for decreasing environmental levels of MTBE). The use of microorganisms for remediation of MTBE is potentially less costly than other methods including soil vapor phase extraction, air stripping, and adsorption to activated carbon (NRC, 1993). The moderate volatility of MTBE makes air stripping techniques energy-intensive, and MTBE's weak sorption potential means that methods using activated carbon would require large amounts of this material to be

effective. In contrast, if it were possible to develop *in situ* and *ex situ* treatment methods using MTBE-degrading bacteria, these approaches could prove less costly than physical and chemical methods. In fact, research at UC Davis indicate that *ex situ* bioremediation methods are indeed possible to develop (Eweis et al., 1997, 1998).

Our laboratory has isolated a bacterial culture, Strain PM 1, which is capable of using MTBE as its sole carbon and energy source. Our research thus far has involved the preliminary characterization of Strain PM 1. This organism is a Gram-negative rod most closely related to organisms of the genus *Sphingomonas* by 16S rDNA analysis. Strain PM 1 can mineralize ^{14}C -MTBE to $^{14}\text{CO}_2$, and growth yields on this substrate are low (0.235). In addition we have found this strain can degrade MTBE when inoculated into soil microcosms. Thus, Strain PM 1 has a high potential for use in the remediation of MTBE-contaminated environments. The overall goals of the project proposed here are to further characterize this organism and test its efficacy as an inoculant into subsurface samples.

The objectives of the proposed study are the following:

1. Measure the potential for and rates of biodegradation of MTBE in vadose, and groundwater materials (contaminated and non-contaminated) inoculated with Strain PM 1.
2. Measure the survival of Strain PM 1 when inoculated into environmental samples and bioreactors.
3. Characterize the physiology of Strain PM 1 with regard to nutrient and growth factor requirements, MTBE kinetic parameters, MTBE concentration range, maintenance of MTBE degrading activity, and starvation survival.
4. Determine the metabolic pathway of MTBE degradation by Strain PM 1.

Methods, Procedures and Facilities

Environmental samples

Samples will be collected from various MTBE-contaminated and uncontaminated locations in California. We will utilize vadose zone and soil samples with organic matter contents ranging from 0.5% to 9%. Most of these samples are already collected and have been well-characterized in previous research projects. Groundwater and vadose zone samples from within and outside of the MTBE plume will be obtained from the Port Hueneme Naval Base (contact person: Fred Goetz, Port Hueneme) and from two contaminated gasoline stations in northern California (contacts: David Hull, Cambria Environmental Technology and Ed Schroeder, Dept. of Civil and Environmental Engineering, UCD).

We will also provide technical support in microbial community characterization to engineers in the Dept. of Civil and Environmental Engineering at UC Davis. MTBE treatment studies are ongoing in biofilters, sand columns, sequencing batch reactors, and a biological GAC treatment system. All of these systems originally were inoculated with the original mixed culture of MTBE degraders from which Strain PM1 was isolated. Using DNA fingerprinting methods, we will evaluate the presence and importance of Strain PM1 in these different waste treatment environments and also look for the potential of other microorganisms being important members of these MTBE-degrading consortia.

Routine growth and maintenance of Strain PM 1

Cultures will be grown in mineral salts medium (MSM) with MTBE as the sole carbon and energy source. Cultures will be incubated in 250-ml bottles sealed with teflon-lined Mini-Nert caps at 25° C in the dark on an orbital shaker. Inocula for bioaugmentation experiments will be grown on rich growth media (e.g. tripticase soy broth) in the presence of MTBE if experiments demonstrate that MTBE-degradation activity is not lost under these conditions. After growth, cultures will be centrifuged, the cell pellet washed twice and resuspended, and then used in inoculation. Inoculation densities will be determined by protein analysis.

Measurement of MTBE biodegradation potential in environmental samples

To examine the ability of strain PM1 to degrade MTBE in environmental samples, we will perform a series of inoculation experiments. Vadose zone and groundwater core samples will be inoculated with MTBE-grown Strain PM 1, and MTBE degradation will be measured by gas chromatography of 50 ul headspace samples using a Shimadzu GC-14A equipped with a photonization detector. Vadose samples of varying organic matter contents will be used to examine the effect of different carbon concentrations on MTBE biodegradation by Strain PM 1. The effect of inoculum density on MTBE degradation will also be determined. Rates of MTBE degradation will be calculated based on time course analysis of MTBE disappearance from the microcosms. All experiments will be performed in triplicate and inoculated samples will be compared to sterile controls.

Measurement of Strain PM1 survival in environmental samples

DNA-based methods will be used to follow the survival of Strain PM 1 in environmental samples. Our laboratory has expertise in the extraction and analysis of nucleic acids from complex environmental matrices such as the vadose zone and surface soils. These methods are based on direct lysis procedures, adapted from Malik et al. (1994) and Zhou et al. (1996), that involve enzyme-treatment/freeze-thaw and high-salt/heat-treatment of

the samples to release microbial DNA. Subsequent steps to purify DNA from cellular debris and environmental contaminants include chloroform extraction, precipitation of DNA with ethanol, and agarose gel electrophoresis of crude DNA extracts. Portions of the gel containing DNA are excised, residual agarose is removed and the sample is desalted and concentrated by centrifugal filtration. DNA will be quantified by measuring its absorbance at 260 nm. DNA purity is checked by measuring absorbances at 230 and 280 nm, which indicate contamination with humic acids and proteins, respectively. The resultant DNA is generally of sufficient purity for polymerase chain reaction (PCR) amplification and restriction enzyme digestion. Groundwater samples are expected to have fewer humic acid contaminants than vadose and soil samples, thus they may warrant fewer purification steps.

Once environmental DNA samples are obtained, two PCR-based approaches will be used to produce fingerprints of the microbial communities. In each case, purified DNA will be amplified with nonrandom oligonucleotide primers complementary to bacterial 16S ribosomal RNA (rRNA) genes (Pace et al., 1985). In the first approach, small subunit ribosomal RNA (srRNA) genes in the bacterial community DNA will be amplified to yield a mixture of PCR products reflecting the taxonomic composition of the community. The primers used will generate small PCR products (<600 bp) that will be applied to polyacrylamide gels containing urea and/or formamide for thermal gradient gel electrophoresis (TGGE) (Riesner et al., 1991). TGGE separates similarly sized PCR products based on their basepair composition, thus DNA bands of differing G+C content will migrate to different positions in the gel. The location and intensities of DNA bands appearing in a gel lane will provide a fingerprint reflecting the types and numbers of ribosomal genes found in the DNA from the environmental sample (Heuer and Smalla, 1997). A TGGE system has been constructed in our laboratory from a vertical electrophoresis apparatus fitted with an aluminum block, against which glass plates containing polyacrylamide gel can be clamped. The upper portion of the aluminum block contains an internal channel through which water is pumped using a circulating water bath. The bottom portion of the block contains an electrical heating strip connected to a temperature controller. The temperatures of the water bath (for the cooled upper block) and the controller unit (for the hotter lower block) can be adjusted to provide a linear temperature gradient across the length of the gel. Gels will be stained either with ethidium bromide or with more sensitive silver reagents (Mitchell et al., 1994) to visualize the bands.

The second DNA-based approach for community fingerprinting involves analysis of the intergenic transcribed spacer (ITS) region of the microbial chromosome. In this technique, primers specific for the highly conserved regions at the 3' end of the 16S rDNA gene and the 5' start of the 23S rDNA gene are used for PCR amplification of the intervening less conserved region. This method has been used successfully to distinguish closely related bacterial strains, and thus provides a high level of discrimination for use in tracking specific bacterial populations in environmental samples. To generate ITS patterns, purified DNA from mixed and pure cultures will be amplified by PCR using the primers 1406f (5'-TGYACACACCGCCCGT-3') and 115r (5'-GGGTTBCCCCATTCRG-3') (Borneman and Triplett, 1997). The 1406f primer is complementary to positions 1391-1406 of the *E. coli* small subunit rRNA gene, and the

115r primer corresponds to positions 115-130 of the *E. coli* large subunit rRNA gene. These primers will amplify DNA fragments ranging from 140 to 1500 bases in length, depending on the ITS regions found within bacterial DNAs. PCR products will then be applied to wells in 5% polyacrylamide gels, and electrophoresed. Gels will be stained with 0.01% SYBR Green I stain illuminated with UV light, and photographed through a yellow filter with a charge-coupled-device camera.

Images for TGGE and ITS gels will be recorded using a charge-coupled-device camera with the BP-M1/722 TWAIN digital imaging kit (Bioimage, Ann Arbor, MI) and evaluated with Photofinish and GPTools image analysis software. For both TGGE and ITS analyses, comparison of the band(s) obtained from Strain PM 1 with those from inoculated environmental samples will allow us to measure the survival of this organism. Statistical analysis to measure the relatedness of DNA fingerprints will be performed using cluster analysis.

Characterization of PM 1 physiology and MTBE biodegradation kinetics

Further elucidation of the general physiology of Strain PM 1 will include determination of nutrient and growth factor requirements, measurement of kinetic parameters for MTBE biodegradation, determination of the effective MTBE concentration range, investigation of the maintenance of MTBE-degrading activity, and resistance to and survival during starvation conditions. As Strain PM 1 flocculates in liquid culture, it will not be possible to standardize inoculum sizes based on turbidity. Instead we will employ measurement of total cellular protein to ensure inoculum consistency.

For total protein analysis cells will be harvested by centrifugation, washed, and resuspended in 500 μ l 0.85% NaCl. Cell lysis was accomplished by adding $_$ volume of acid-washed glass beads and vortexing the samples three times for one minute bursts. Total protein will be measured in 50 μ l subsamples using the Micro Protein Determination kit (Sigma Diagnostics, St. Louis, MO) according to manufacturer's instructions.

Nutrient and growth factor requirements will be investigated using standard vitamin, amino acid and yeast extract mixes. Mineral salts medium (MSM) with MTBE present as the sole carbon and energy source will be amended with the nutrient mixtures alone and in combination. MTBE degradation rates and Strain PM 1 cellular yields will be compared to determine which nutrient additions provide the most suitable conditions for cell growth and activity.

The kinetics of MTBE biodegradation will be measured by time course analysis of MTBE degradation at varying pollutant concentrations. MTBE disappearance will be measured by GC analysis and/or carbon dioxide evolution from 14 C-labeled MTBE, and replicate bottles will be sacrificed at different time points for measurement of cell biomass. Based on biomass and degradation rate measurements, other kinetic parameters will be determined by nonlinear curve fitting using the Monod equation. Maximum and minimum MTBE concentrations degradable by Strain PM 1 will be measured by growing

the organism on selected pollutant concentrations ranging from the MTBE solubility limit in water (48 g/L) to levels commonly found in groundwater cores (100 ug/L). We will monitor signs of MTBE toxicity to the organism as well as measure the lowest threshold MTBE concentration required for induction of MTBE degradation by Strain PM 1.

Strain PM 1's ability to maintain MTBE degradation will be measured by analyzing MTBE disappearance after the organism has been grown on other carbon sources (e.g., complex media, toluene, alkanes), followed by transfer to MTBE-containing media. The length of the lag period, an indication of the time required for induction of the appropriate metabolic pathway, will be measured. Starvation survival will be analyzed by removing MTBE from the growth media for varying periods of time. The cells will then be placed in MTBE-containing MSM and the lag period and rates of MTBE degradation will be measured. In order to distinguish between starvation and loss of metabolic activity, we will also measure viable cell numbers using both microscopy with dyes specific for living cells and also using plate counts on specific medium.

Determination of metabolic pathway for MTBE degradation

The MTBE degradation pathway employed by Strain PM 1 will be initially determined by analysis of MTBE breakdown products identified in the culture supernatant. Both formaldehyde and tertiary-butyl alcohol, putative MTBE-degradation products, are detectable by gas chromatography and high performance liquid chromatography, and we will employ these methods for analysis of pathway intermediates. In addition, formaldehyde concentrations can be measured colorimetrically by the addition of chromotropic acid and concentrated sulfuric to culture supernatants.

Preliminary enzymological studies will focus on determination of the presence or absence of a cytochrome P-450 system in Strain PM 1. These enzyme systems are involved in the monooxygenation of a variety of organic pollutants and enzymes with homology to P-450s have been previously identified in *Sphingomonads* (Armengaud and Timmis, 1998). We will measure the effect of addition of the cytochrome P-450 inhibitors 1-aminobenzotriazole and carbon monoxide on MTBE degradation by the organism (Ortiz and Mathews, 1981). If this enzyme system is involved in MTBE biodegradation by Strain PM 1, we should be able to measure significant decreases in pollutant disappearance in the presence of the inhibitors.

If cytochrome P-450 appears to be involved, we will do further enzyme characterization studies. An advantage of studying cytochrome P-450 enzyme systems is the high level of sequence conservation among members of the enzyme family. Based on amino acid homology it is possible to design degenerate PCR primers that we will use to amplify putative cytochrome P-450 gene(s) from Strain PM 1. Once we have obtained the PCR fragments we will determine their DNA and putative amino acid sequences and compare them with previously published P-450 sequences. As cytochrome P-450 systems are obligately aerobic, knowledge of whether these enzymes are involved in MTBE-degradation by Strain PM 1 will provide more complete information for optimization of bioreactor and *in situ* remediation technologies.

Related Research

Unlike other gasoline components including benzene, toluene and various alkanes, there are few reports of microorganisms in either pure or mixed cultures capable of biodegrading MTBE. Salanitro et al. were the first to report bacterial degradation of MTBE (Salanitro et al., 1994). In their study, a mixed microbial consortium was found to degrade 120 ppm MTBE at a rate of 34 mg/g cells per hour. Although the metabolic intermediate tert-butyl alcohol (TBA) were observed, the analysis was complicated by the presence of more than one bacterial species in the degrading culture. This initial account was followed by research showing pure bacterial cultures capable of using MTBE as a sole carbon and energy source. Mo et al. described three strains (an *Arthrobacter*, a *Rhodococcus*, and a *Methanobacterium*) that degraded up to 29% of the initial 200 ppm MTBE in up to 2 weeks (Mo et al., 1997). One hundred percent removal of MTBE by these cultures, however, was not shown. Others have reported that propane-enriched environmental isolates are capable of mineralizing MTBE, but these organisms cannot grow on MTBE without prior induction by another compound (Steffan et al., 1997). Finally there is one report of a strain of the fungal species *Graphium* capable of cometabolizing MTBE. In this case, cells achieved rates of pollutant removal of 0.6 nmol MTBE/mg cells per hour when incubated in the presence of n-butane (Hardison et al., 1997).

There have also been a few microcosm studies on MTBE biodegradation in environmental samples. Yeh and Novak examined the anaerobic biodegradation of MTBE in soil microcosms and found that MTBE was only degraded in soils containing lower amounts of organic matter (Yeh and Novak, 1994). Mormille et al. found anaerobic degradation of MTBE in one replicate of a fuel-contaminated river sediment, although a 152-day acclimation period preceded the onset of degradation (Mormille et al., 1994). In addition to soil and sediment studies, there is indirect evidence for degradation of MTBE in groundwater. Schrimmer and Barker (1998) compared model predictions of MTBE concentrations, in the absence of biodegradation, with actual MTBE field concentrations in the Borden aquifer. They suggested that over a period of eight years natural attenuation was responsible for up to 97% loss of the initial concentration of 3 mg MTBE/L. Laboratory confirmation of biodegradation in aquifer material, however, has not yet been demonstrated.

MTBE research in our laboratory thus far has involved isolation and preliminary characterization of an organism that is capable of growth with MTBE as its sole carbon and energy source. We have demonstrated that this organism, Strain PM 1, mineralizes ^{14}C -MTBE to $^{14}\text{CO}_2$ and can degrade MTBE when inoculated into microcosms containing nonsterile Yolo silt loam soil. We are also collaborating with researchers at the University of California at Berkeley to investigate the effect of BTEX compounds on MTBE biodegradation by Strain PM 1 in liquid culture. Members of the University of California Department of Civil and Environmental Engineering currently operate a variety of bioreactors (vapor and liquid phase systems) which were originally inoculated with a microbial consortium containing Strain PM 1 (Eweis et al., 1997, 1998). These bioreactors are all actively degrading MTBE, and we have begun preliminary work on

fingerprinting microbial communities in one of these systems to determine whether Strain PM 1 is present in significant abundance. Finally, for what it's worth, we have recently received considerable attention from the news media (Associated Press wire, San Jose Mercury News, Orange County Register, Davis Enterprise, Sacramento Bee, Channels 10, 3, 13 in Sacramento, Channel KGO (ABC) in San Francisco--October 8,9, 1998). We believe this attention underscores the strong public concern about MTBE pollution and interest in exploring bioremediation as a treatment alternative.

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Investigator's Overall Research

I am a soil microbial ecologist in the Dept. of Land, Air and Water Resources. My lab is interested in the biodegradation of organic pollutants by indigenous microbial communities (intrinsic remediation), how to describe the kinetics of biodegradation, and factors controlling rates of degradation in surface and subsurface soils and groundwater. Over the past decade, we have conducted research on the biodegradation of toluene and trichloroethylene, methylene chloride and chloroform, PAHs, phenol, and PCBs. We have also put considerable effort into developing approaches for characterizing microbial community composition and linking this information to microbial process rates (e.g., for biodegradation). The isolation of Strain PM 1 opens up a new area of research for which we are well prepared. We are interested in the larger questions of the feasibility of using bacterial inoculants in in situ bioremediation and determining conditions when intrinsic remediation does and does not work.

Our research on MTBE biodegradation so far has been supported only by seed funding (< \$6,000). In writing this grant, we are seeking sufficient funds to support the more indepth studies that are needed to determine the feasibility of using this promising microorganism in bioremediation of MTBE.