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Characterization of Methyl *tert*-Butyl Ether-Degrading Bacteria from a Gasoline-Contaminated Aquifer

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Abstract: Molecular microbial community analysis was combined with traditional cultivation strategies to investigate the presence of methyl *tert*-butyl ether (MTBE)-degrading bacteria in a gasoline-contaminated aquifer (Ronan, MT). A bacterial consortium, RS24, which is capable of complete mineralization of MTBE as a sole carbon and energy source was enriched from soil and aquifer materials taken from the contaminated site. The consortium was capable of degrading MTBE at rates up to 0.66 mg d^{-},^{1} with corresponding gross biomass yields of 0.25 ± 0.02 mg dry biomass (mg MTBE)$^{-1}$. Two MTBE-degrading isolates identified as *Pseudomonas* Ant9 and *Rhodococcus koreensis* were obtained from the consortium. However, both isolates required the presence of 2-propanol as a cosubstrate for MTBE degradation. Denaturing gradient gel electrophoresis (DGGE) of Polymerase Chain Reaction (PCR)-amplified 16S rDNA confirmed the presence of both isolates in the initial consortium and indicated their disappearance with transfer and subculturing. MTBE degradation and cell growth by the consortium was stimulated by the presence of spent culture medium, suggesting the production of a growth factor during MTBE degradation. These results indicate the presence of naturally occurring MTBE-degrading bacteria in a contaminated aquifer and suggest the potential for natural attenuation or enhanced aerobic oxidation.

Keywords: MTBE, hydrocarbon remediation, gasoline oxygenates, 16S rRNA, DGGE, molecular community analysis.

Introduction

Alkyl ethers such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) have been used as gasoline additives in the United States since the late 1970s to reduce vehicle emissions. MTBE, the most common fuel oxygenate, has been added at concentrations up to 15% (v/v) to more than 30% of all gasoline sold in the U.S. Due to the widespread use of MTBE in reformulated gasoline, there have been numerous reports of releases into the subsurface from point sources such as leaking underground storage tanks. In addition, non-point source contamination by motorized watercraft (Reuter et al., 1998), as well as urban air and rainwater (Grosjean et al., 1998; Pankow et al., 1997; Wilson, 1997), may also contribute significantly to detectable levels of MTBE in groundwater. A 1993 to 1994 survey conducted by the USGS identified MTBE as the second most common contaminant of urban aquifers in the U.S. (Squillace et al., 1996). Despite the work completed by the USGS in the early 1990s, only a few states were testing for MTBE prior to 1995. Most MTBE plumes were not characterized, because states

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had no action level for remediating the compound in groundwater or drinking water. Most states have only recently initiated routine sampling for MTBE and are now identifying MTBE plumes from petroleum releases at service stations.

The physical and chemical properties of MTBE contribute to its mobility in groundwater. In contrast to gasoline hydrocarbons, MTBE has a relatively low octanol-water partition coefficient (log K_{ow} = 0.94 to 1.16) and a high aqueous solubility (~ 50,000 mg/L at 25°C), both of which limit partitioning into soil organic matter (Mackay et al., 1993; Shaffer, 1997). While MTBE has a higher vapor pressure than benzene, its Henry's Law constant (0.022 at 25°C) is an order of magnitude lower than that of benzene. This results in minimal losses of MTBE from the aqueous phase to the atmosphere (Robbins et al., 1993). Due to significant partitioning of MTBE into the aqueous phase and limited sorption to soil, MTBE plumes in contaminated groundwater are generally longer and more stable than coexisting gasoline plumes (Landmeyer et al., 1998; Schirmer and Barker, 1998).

Traditional pump-and-treat technologies, such as air stripping, used to eliminate gasoline constituents from groundwater have not been as successful for MTBE removal. However, bioremediation remains a viable strategy for removal of MTBE from gasoline-contaminated aquifers. Recent studies suggest that biodegradation of MTBE and its degradation products of concern, *tert*-butyl alcohol (TBA) and *tert*-butyl formate (TBF), occur primarily under aerobic conditions (Barker et al., 1996; Borden et al., 1997; Bradley et al., 1999; Hanson et al., 1999; Park and Cowan, 1997a; Salanitro et al., 1994; Steffan et al., 1997). Hanson et al. (1999) recently reported a bacterial isolate (PM1) capable of utilizing MTBE as its sole carbon and energy source. In addition, cometabolism of MTBE may be a potentially important mechanism of degradation. Steffan et al. (1997) isolated several propane-oxidizing bacteria that could degrade MTBE, ETBE, and TAME after growth on propane or 2-propanol. MTBE degradation by *Pseudomonas aeruginosa* has been shown to occur in the presence of pentane (Garnier et al., 1999). Cometabolism of MTBE (in the presence of gaseous n-alkanes) by a filamentous fungus has also been reported (Hardison et al., 1997).

MTBE research is in its infancy and expanded knowledge of the fate and transport of MTBE in natural systems is essential as more risk-based approaches to contaminant remediation such as natural attenuation are evaluated. Therefore, the goals of this study were (1) to assay for the presence of MTBE-degrading bacteria at a contaminated aquifer as an initial step in assessing the potential for natural attenuation of MTBE and (2) to identify specific bacteria involved in MTBE degradation and their associated rates of biodegradation. In addition to traditional cultivation strategies, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments was used to determine the composition of MTBE-degrading consortia.

Materials And Methods

Chemicals

HPLC-grade MTBE and 2-propanol were purchased from Fisher Scientific (Bridgewater, NJ). Uniformly labeled [^{14}C]-MTBE (> 99% purity; 10.1 mCi mmol^{-1}) was synthesized by DuPont New England Nuclear Products (Boston, MA). TBA (99.6%) and TBF (99%) standards were obtained from J.T. Baker (Phillipsburg, NJ) and Aldrich Chemical Co. (Milwaukee, WI), respectively.

Site Characterization

The contaminated site is located at a fuel station east of U.S. Highway 93, 1 mile south of Ronan, MT, where an underground storage tank (removed in April 1994) released an estimated 40,000 L of gasoline over a 1-year period. The Montana Department of Environmental Quality established 20 monitoring wells at the site to identify plume boundaries, and 8 additional wells were installed in 1997 for free product removal. Figure 1 shows the free product and dissolved contaminant plumes at the site and Table 1 (MSE/HKM, unpublished data) presents selected well monitoring results of contaminant concentrations over a 3-year period. The maximum depth to groundwater is approximately 5 m at the point of release. The dominant lithology identified from bore holes was silt and fine sand with scattered clay lenses, typical of glacial lacustrine deposits common to the area. Remediation at the site has included a combination of passive recovery skimmers, air sparging systems coupled with soil vapor extraction and an interceptor trench.

Soil/Aquifer Materials

Soil and aquifer materials used as inocula for MTBE-degrading bacterial enrichments were collected from two bore holes drilled to a depth of 1 m below the groundwater surface. One bore hole was near the downgradient edge of the nonaqueous phase liquid (NAPL) gasoline plume at well M-12 and the second was adjacent to well M-19 near Spring Creek. Samples were retrieved from the vadose zone, at the groundwater interface, and below the groundwater surface, and were stored at 4°C for 1 week prior to inoculation of

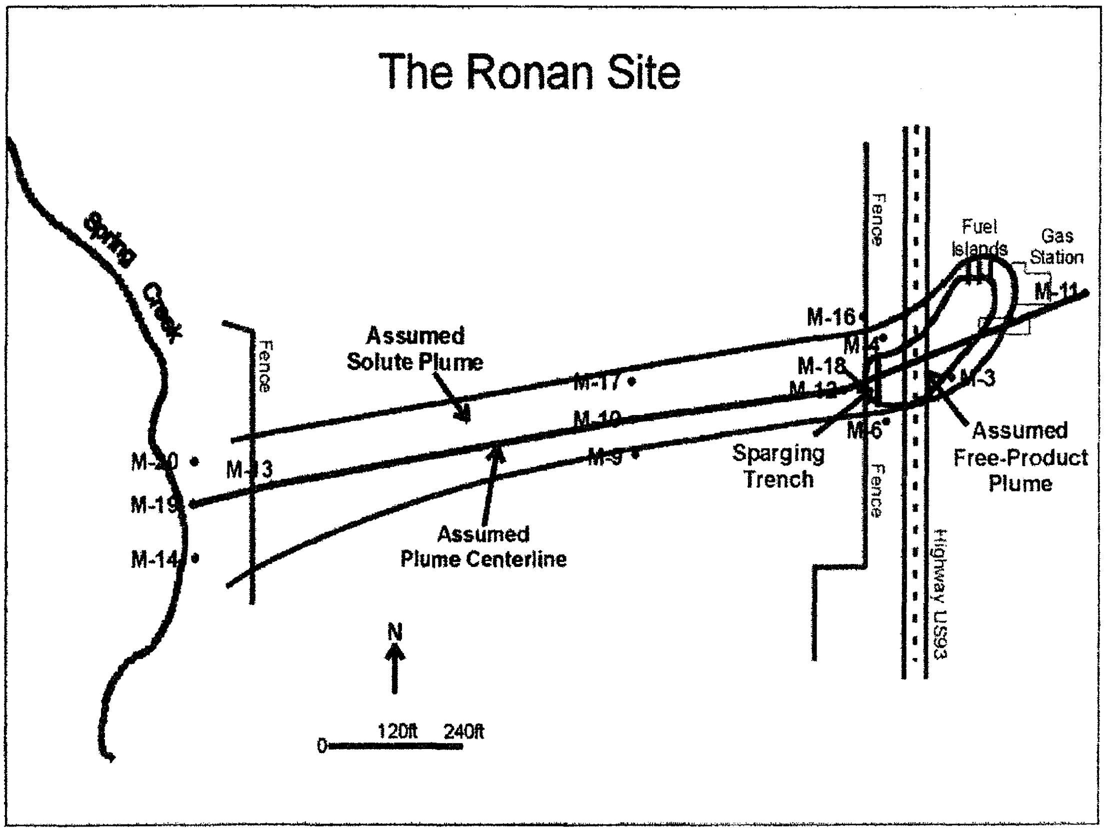

Figure 1. Schematic map of the MTBE-contaminated site in Ronan, MT.

Table 1. Site concentrations of total petroleum hydrocarbons (TPH) and

sampling date	well M-12		well M-10		well M-13	
	TPH*	MTBE*	TPH*	MTBE*	TPH*	MTBE*
5/96	101000	19800	1570	1460	nd	nd
12/96	94100	15800	3880	2900	100	221
12/97	98500	9540	8270	4570	201	204
11/98	68900	4950	9760	4650	50	57
6/99	21900	3500	5770	3580	58	72

* TPH and MTBE concentrations are given in μg/L

nd not determined

enrichments. A pristine agricultural soil (Arthur Post Research Station, Bozeman, MT) was used as inoculum in several control enrichments.

Enrichment Conditions

Degradation experiments were conducted in closed 125-mL Erlenmeyer flasks under aerobic conditions. The flasks contained 25 mL of a mineral medium (SSE) designed to simulate a typical soil solution (Angle et al., 1991) containing NH_4NO_3 (1.25 m*M*), $CaSO_4$ (2 m*M*), $MgCl_2$ (2 m*M*), KH_2PO_4 (10 μ*M*), KOH (1.25 m*M*), $FeCl_2$ (5 μ*M*), and supplemented with 100 μL L^{-1} of micronutrient solution (Skerman, 1967). Subsamples of the composite soil/aquifer material were used as inocula (1% w/v) for the MTBE-degrading microcosms. MTBE was added to enrichment flasks to a final concentration of 10 mg L^{-1} MTBE (~100,000 dpm $flask^{-1}$ [^{14}C]-MTBE), which was the approximate concentration of MTBE measured at well M-12 at the time of inoculum sampling. Radiolabeled $^{14}CO_2$ that had evolved from the enrichments was captured and used to track MTBE degradation. Solutions of 0.5 *M* NaOH (0.3 mL) were utilized as $^{14}CO_2$ traps and were placed in cups suspended from the stoppers. The NaOH was removed weekly and $^{14}CO_2$ was quantified using liquid scintillation analysis. Disappearance of MTBE from the aqueous phase was monitored by gas chromatography (GC). Treatments were tested in triplicate and compared with autoclaved controls that contained 250 mg L^{-1} $HgCl_2$.

Due to potential loss of vapor phase MTBE during sampling of the base traps, subsequent kinetic degradation experiments with one consortium were performed in 120-mL serum bottles with sealed Teflon®-coated septa. MTBE was introduced into 40 mL of SSE medium at concentrations ranging from 10 to 70 mg L^{-1} and 2 mL samples were aseptically removed for GC analysis. At each sampling time, 3 mL of sterile air was injected into the serum bottle headspace to maintain aerobic conditions.

In studies with spent culture medium amendment, duplicate MTBE-degrading cultures of RS24 were centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was decanted and saved, and the cell pellets were resuspended in 3 mL of 0.85% phosphate buffered saline. Aliquots (1 mL) from each cell suspension were used as inocula for duplicate cultures with 40 mL spent growth medium. Two mL aliquots were used as inocula for duplicate cultures with 40 mL of fresh SSE medium. The four new cultures were amended with MTBE to a final concentration of 32 ± 6 mg L^{-1}.

Gas Chromatography

GC analysis of 2-propanol, MTBE, TBA, and TBF was conducted using a Hewlett-Packard 5890 Series II gas chromatograph with a FID detector. A Porapak PS 80/100 mesh packed column (2 mm I.D. × 1.22 m glass; Supelco, Bellefonte, PA) was used with a He carrier gas flow rate of 18 mL min^{-1} at 100°C and a head pressure of 28 psi. The injector temperature was set at 190°C, and the detector temperature was 250°C. The initial oven temperature was set at 100°C for 4 min then ramped at 10°C min^{-1} to a final temperature of 200°C. The 2-propanol, TBA, MTBE, and TBF peaks showed retention times of approximately 2.7, 4.5, 6.3, 8.3 min, respectively.

Bacterial Cell Counts

0.5-mL samples of MTBE-degrading enrichments were placed above a 0.2-μm, 25-mm-diameter black polycarbonate membrane (Poretics Products, Livermore, CA) clamped in a filter chimney assembly and vacuum manifold apparatus. A 100-μL aliquot of 10 mg L^{-1} DAPI was added, cells were stained for 2 min and then rinsed 2 to 3 times with dH_2O to remove any unbound stain. Direct counts of DAPI-stained cells were conducted using a Nikon Eclipse E800 epifluorescence microscope with a mercury lamp UV source. The filter block used had an excitation bandwidth of 340 to 380 nm and an emission bandwidth of 435 to 495 nm. Total cell counts were based on a calibrated ocular grid, calculated total membrane area, and an average of 20 enumerated grid fields.

Isolation of MTBE-Degrading Bacteria

Samples from an MTBE-degrading consortium were spread on R2A agar plates (Difco Corp., Detroit, MI) and Noble agar plates amended with SSE. Noble agar plates were incubated in sealed metal cans containing MTBE vapors produced by volatilization from MTBE-saturated filter paper. Half of the Noble agar plates also received 50 μL of 20 mg L^{-1} 2-propanol applied to the plate surface prior to inoculation with bacteria. Single colonies fom both types of plates were purified through repeated transfer under identical cultivation conditions. Isolated colonies were selected by differences in colony color and morphology and used to inoculate 120-mL serum bottles containing 40 mL of SSE amended with 10 mg L^{-1} MTBE. 2-Propanol (20 mg L^{-1}) was added as a cosubstrate in selected treatments. Gas chromatography was used to monitor MTBE disappearance and accumulation of degradation products.

Nucleic Acid Extraction, PCR, DGGE, and Sequence Analysis

Cell lysis and DNA extraction protocols were based on those of Moré et al. (1994). Briefly, cells were lysed by mechanical disruption at 6.5 m s^{-1} for 45 sec using a beadbeater (Savant Instruments, Qbiogene, Carlsbad, CA). The crude cell lysates were precipitated with 12 *M* ammonium acetate, and the DNA in the resulting supernatant was precipitated with isopropanol. PCR conditions and primers were identical to those previously described (Ferris et al., 1996). These primers amplify a region of the 16S rRNA gene from nucleotide positions 1070 to 1392 (based on *E. coli*). These have been shown to be highly conserved for numerous bacterial lineages, including the proteobacterial, Gram-positive, cyanobacterial, green nonsulfur, and *Cytophagales-Flavobacterium-Bacteriodes* lineages. DGGE conditions were similar to those used by Ferris et al. (1996), with a 6 to 11% concentration gradient of acrylamide used in addition to a 45 to 70% urea/formamide gradient (Cremonesi et al., 1997). DGGE bands were purified for sequence analysis by touching individual bands with a 10-μL pipet tip and immersing the tip into previously prepared PCR reaction mixtures. Band purity was confirmed by analyzing the reamplified bands on a DGGE gel compared with the parent community. Sequencing of isolate DNA and DGGE bands was performed on an ABI Prism 310 Genetic Analyzer using a 47-cm capillary (PE Applied Biosystems, Foster City, CA) and Prism BigDye terminator cycle sequencing ready reaction kits (PE Applied Biosystems, Foster City, CA).

Results

Soil-Derived Enrichments

A series of enrichment cultures amended with 10 mg L^{-1} MTBE was established to assay the presence of MTBE-degrading bacteria at the gasoline-contaminated site. The treatment matrix consisted of three inoculum types (M-12, M-19, and pristine) and two concentrations of inorganic nutrients (1× and 0.1 × SSE). In addition, a set of 0.1x SSE-treated enrichments was amended with 2-propanol. After an initial lag time of approximately 15 days, one flask (RS24) inoculated with soil from well M-12 and amended with 0.1× SSE and 2-propanol displayed significant MTBE degradation compared with sterile controls and other treatments (Figure 2). $^{14}CO_2$ analyses from this enrichment indicated a maximum of 33% MTBE mineralization to CO_2 after 35 days. All other treatments displayed only minimal $^{14}CO_2$ evolution over the 35-day trial relative to the sterile controls.

MTBE Degradation by Consortium RS24

The MTBE-degrading enrichment (RS24) was transferred (10% v/v) and enriched further with 14 mg L^{-1} MTBE and 20 mg L^{-1} 2-propanol. After 7 days GC analysis revealed the disappearance of 2-propanol and the accumulation of two metabolites identified by mass spectrometry as TBA and TBF. TBA and TBF were still present at day 14 and disappeared by day 21. Over the 21-day period, the MTBE concentration decreased to 12 mg L^{-1} and continued to decrease steadily at a rate of about 0.1 mg L^{-1} d^{-1}. Following the complete disappearance of MTBE after 146 days, another transfer (10% v/v) was made and the enrichment was amended with 6 mg L^{-1} MTBE without the addition of 2-propanol. An extended lag time (~18 days) was again observed before significant degradation occurred (Figure 3). However, with subsequent addition of MTBE on days 40, 47, and 53, the onset of degradation became increasingly rapid. As amendments of MTBE increased from 10.3 mg L^{-1} to 14.7 mg L^{-1} to 66.4 mg L^{-1}, no significant decrease in degradation rates were observed. Beginning on day 47, MTBE disappearance was monitored daily and MTBE degradation rates of 0.15 mg d^{-1} and 0.66 mg d^{-1} were calculated from days 47 to 53 and days 53 to 57, respectively. In contrast to the previous enrichments in the presence of 2-propanol, no metabolites were observed at any point in the degradation. Figure 4 shows increasing bacterial cell density during MTBE degradation in duplicate transfer cultures (10% v/v) of RS24, indicating that microbial growth was coupled to MTBE degradation. A gross estimate of biomass yield was calculated based on maximum MTBE utilization rates represented in the graphs. Assuming an approximate dry mass of 2×10^{-10} mg $cell^{-1}$, a yield of 0.25 ± 0.02 mg dry biomass $(mg\ MTBE)^{-1}$ was observed.

Growth Factor Experiments

A significant lag phase was repeatedly observed after transfer of active MTBE-degrading cultures of RS24 to fresh medium. However, when active cultures were spiked with MTBE, no lag in degradation was observed (Figure 3). To determine if a growth-related factor in the medium may be involved in the metabolism of MTBE by enrichment RS24, degradation experiments were conducted with fresh SSE and spent culture medium (i.e., supernatant from active MTBE-degrading cultures). Figure 5A shows MTBE degradation with corresponding increases in cell density for the cultures with spent growth medium. Despite a twofold higher initial cell density, the cultures with

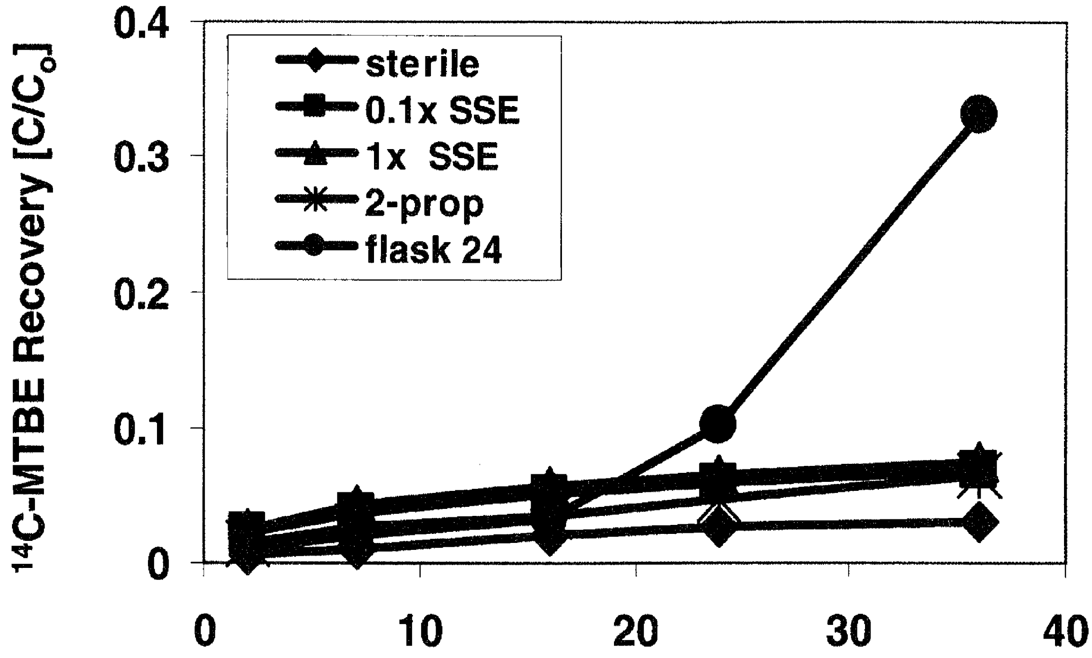

Figure 2. Recovery of $^{14}CO_2$ from ^{14}C-MTBE amended soil enrichments. Treatments labeled 2-Prop were amended with 20 mg L^{-1} 2-propanol.

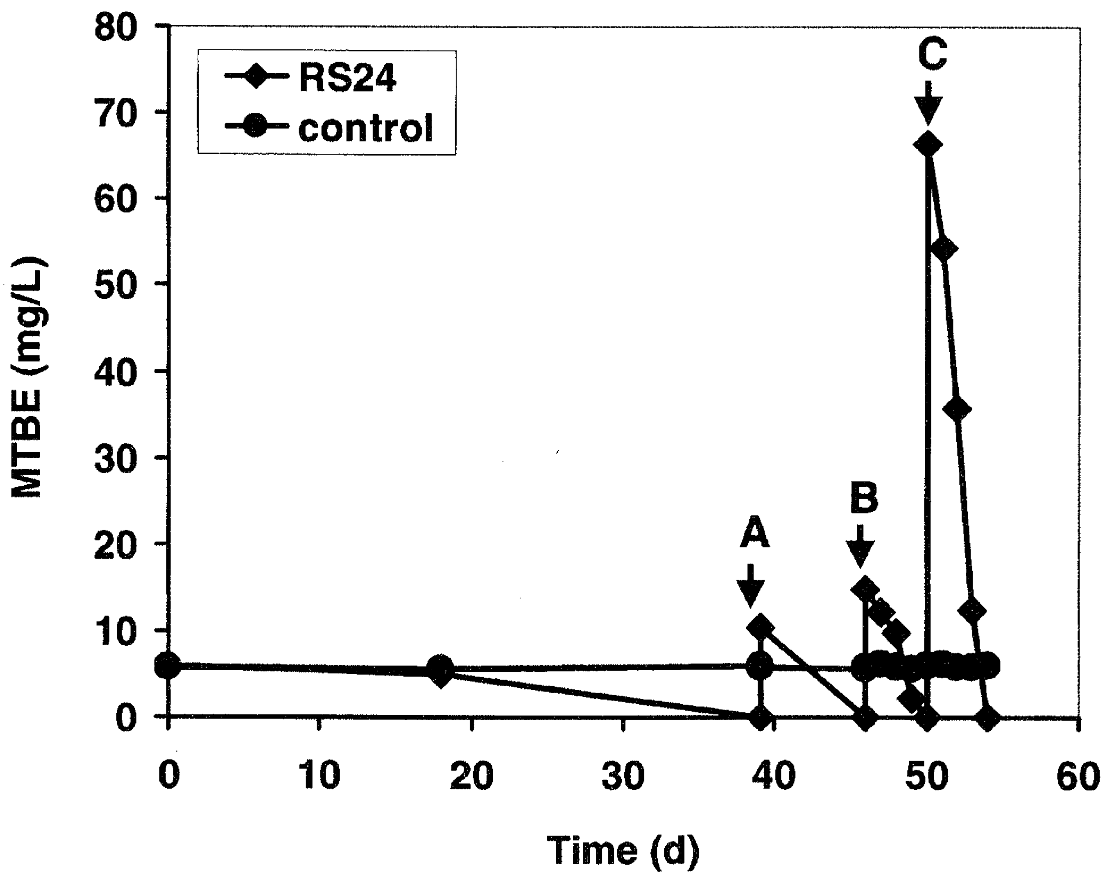

Figure 3. Degradation of increasing concentrations of MTBE by enrichment culture RS24. Letters correspond to MTBE additions of A, 10.3 mg L^{-1}; B, 14.7 mg L^{-1}; and C, 66.4 mg L^{-1}. Treatment labeled 01.x and 1x inorganic nutrient solution and flask 24 is the culture from which the consortium RS24 was derived.

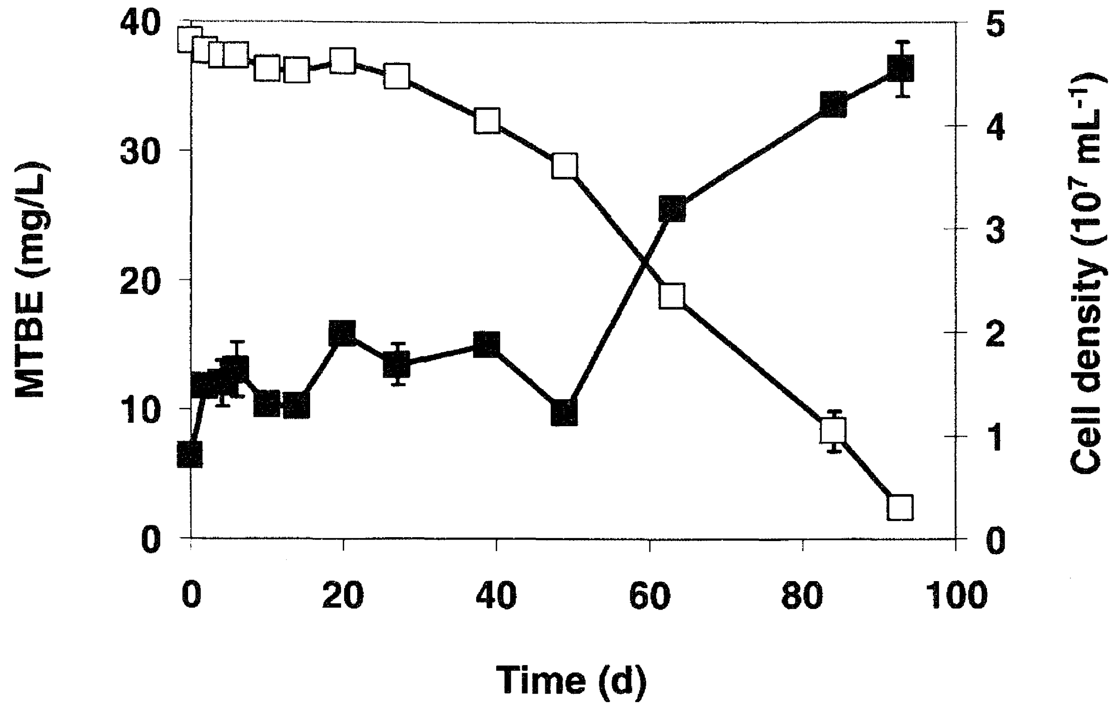

Figure 4. Degradation of MTBE with corresponding increases in cell density with enrichment culture RS24. MTBE disappearance (□) and bacterial cell density (■) were monitored over time. Error bars represent standard deviation from the mean of duplicate cultures.

fresh SSE showed reduced MTBE degradation and cell growth over the 60-day trial period (Figure 5B).

Isolation of MTBE-Degrading Bacteria

Thirteen MTBE-degrading isolates were cultivated from enrichment RS24. For each isolate MTBE degradation kinetics were determined in the presence and absence of 2-propanol. The degradation of MTBE was more rapid and complete in the presence of 2-propanol for all isolates tested. Because many isolates had similar colony morphologies and MTBE degradation kinetics, nucleotide sequence analysis of a 340-bp region of the 16S rRNA gene was performed to identify unique isolates. Of the nine isolates characterized, four isolates showed 100% sequence similarity to *Pseudomonas* sp. Ant9 and five isolates were 100% similar to *Rhodococcus koreensis*. Figure 6 shows degradation kinetics of MTBE in the presence and absence of 2-propanol for representative isolates identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*. Both isolates had similar MTBE degradation rates in the presence of 2-propanol as a cosubstrate and displayed minimal MTBE degradation in the absence of 2-propanol. GC analysis of 20 mg L^{-1} 2-propanol treatments revealed an initial decrease in 2-propanol concentration prior to the onset of MTBE disappearance, indicating a probable cometabolic induction of MTBE degradation. Accumulation of TBA and TBF was observed during MTBE degradation with both isolates in the presence of 2-propanol.

Molecular Characterization of MTBE-Degrading Consortia

Figure 7 illustrates DGGE profiles of enrichment RS24 and two successive subcultures taken from this consortium. Sequence analysis of purified DGGE bands revealed a phylogenetically diverse group of bacteria present in the MTBE-degrading consortium that was comprised of members of Gram-positive, proteobacterial and *Cytophaga-Flavobacterium-Bacteroides* lineages. A decrease in the total number of bands in the community profiles was observed with successive subculturing of enrichment RS24. DGGE bands identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis* showed 100% sequence similarity to the two cultivated MTBE-degrading isolates identified above. Interestingly, the *Rhodococcus koreensis* band disappeared completely by the second subculture, while the band corresponding to *Pseudomonas* sp. Ant9 decreased in intensity with subculturing. However, due to bias inherent in the PCR, differences in DGGE band intensity may not reflect population abundance in the consortium. The band corresponding to *Rhodoferax fermentans* was present in the original consortium and appeared stable in both subcul-

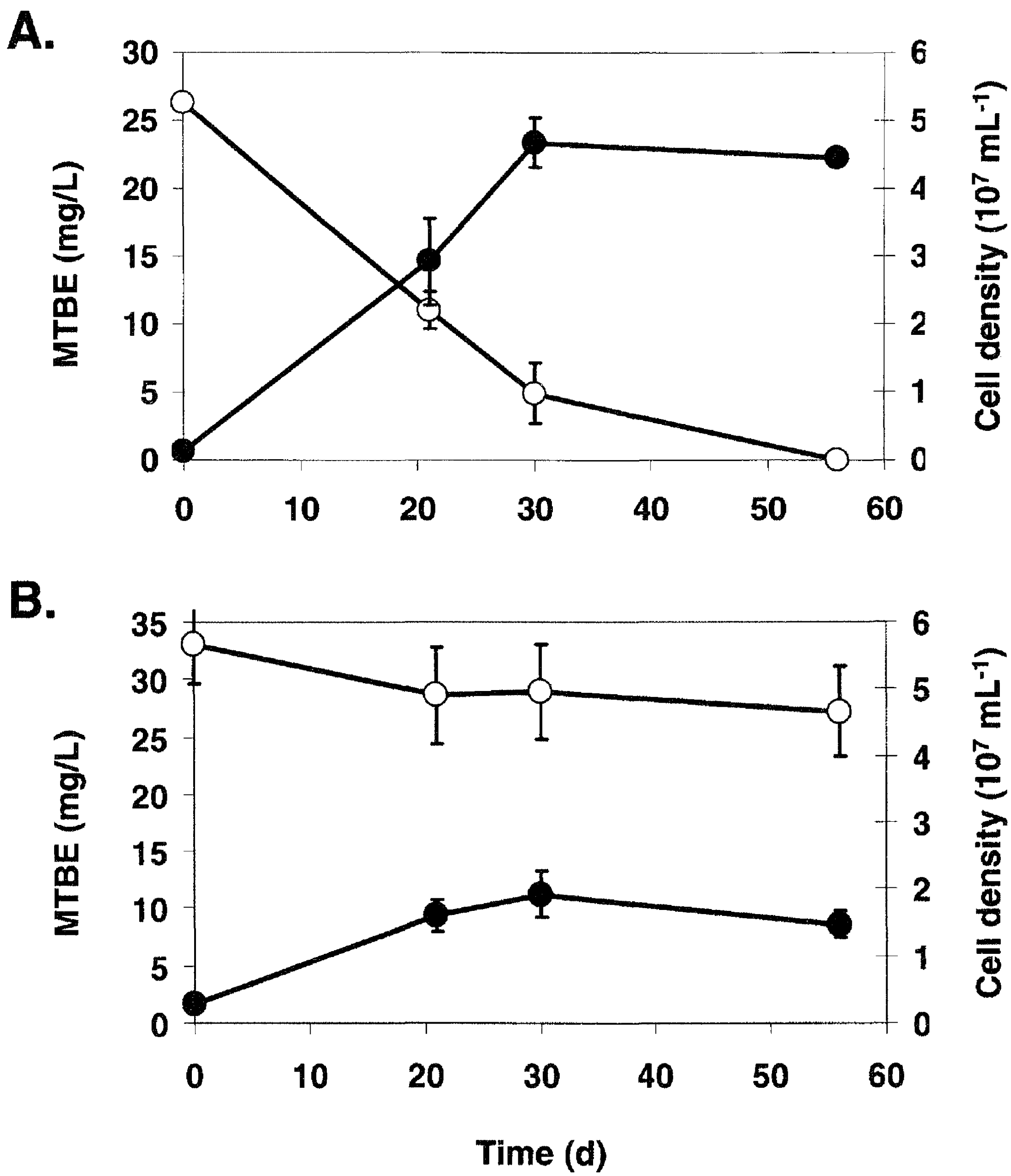

Figure 5. Degradation of MTBE by enrichment culture RS24 in the presence of (A) spent and (B) fresh culture medium. MTBE concentration (open symbols) and bacterial cell density (closed symbols) were monitored over time. Error bars represent standard deviation from the mean of duplicate cultures.

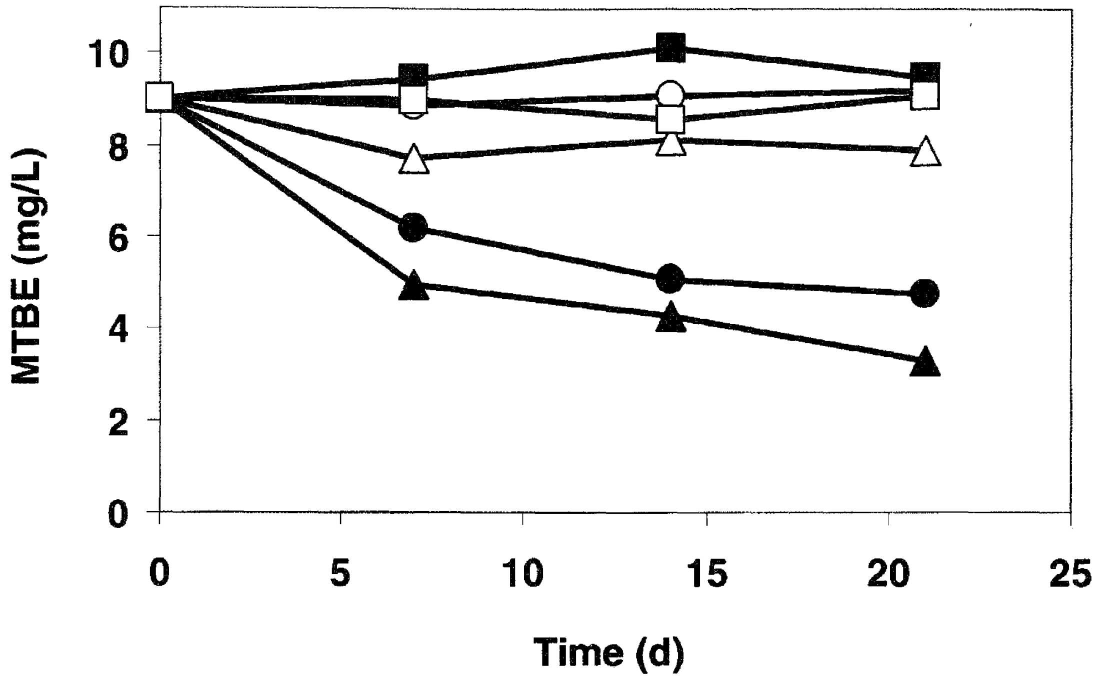

Figure 6. MTBE degradation by bacterial isolates in the presence and absence of 2-propanol as a cosubstrate. Symbols: *Pseudomonas* sp. Ant9 with (●) and without (○) 2-propanol; *Rhodococcus koreensis* with (▲) and without (△) 2-propanol; sterile controls with (■) and without (□) 2-propanol.

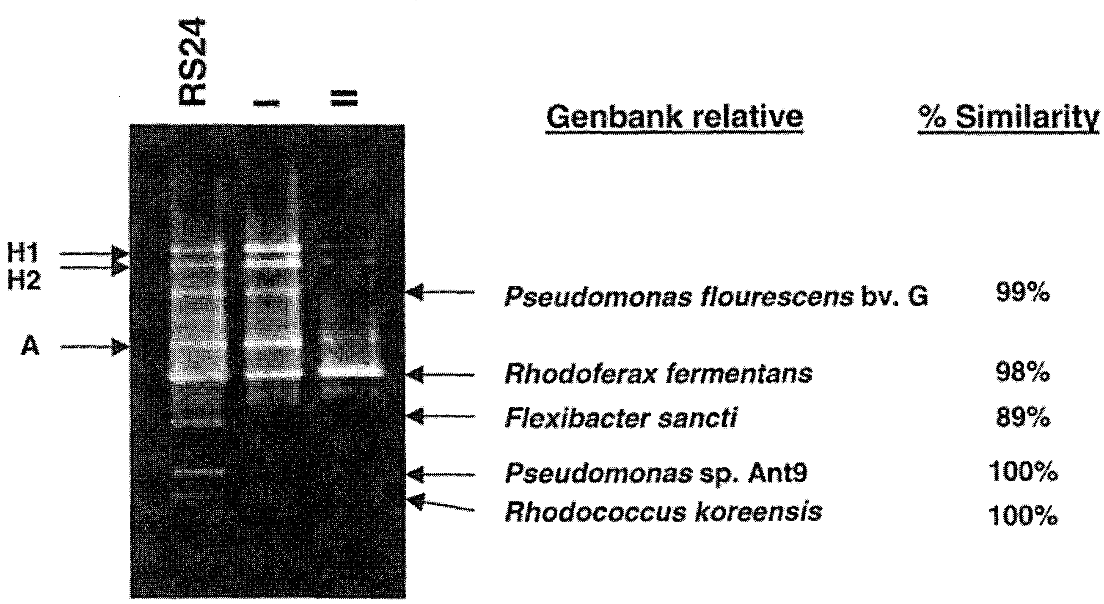

Figure 7. DGGE profiles of RS24 and two subcultures taken from RS24 at different times. Genbank accession numbers for the sequences above are *Pseudomonas flourescens* bv. G (AF228366), *Rhodoferax fermentans* (D16212), *Flexibacter sancti* (M62795), *Pseudomonas* sp. Ant9 (AF184219) and *Rhodococcus koreensis* (AF134343).

tures. After numerous attempts, band A could not be sufficiently purified for sequence analysis. Bands H1 and H2 are believed to be heteroduplex molecules formed during the PCR reaction, resulting from single strands of DNA from different parent molecules that combine to form a double-stranded product (Ferris et al., 1996). Purification and reamplification of bands H1 and H2 always yielded band A and the band corresponding to *Rhodoferax fermentans*, suggesting these are the parent molecules that form the heteroduplexes. Because heteroduplexes are often formed from very similar parent molecules, this indicates that the organism contributing band A may be a close relative of *Rhodoferax fermentans*.

Discussion

Initial screening of the gasoline/MTBE-contaminated soils yielded a single enrichment capable of degrading MTBE in the presence of 2-propanol. Maintenance and transfer of this enrichment resulted in a bacterial consortium (RS24) that could degrade MTBE at rates of 0.66 mg d^{-1} in the absence of 2-propanol as a cosubstrate. In this consortium, cell growth coupled to MTBE degradation as the sole carbon source, as well as the lack of metabolite accumulation, suggested that the bacterial consortium derived energy from the mineralization of MTBE. Calculated gross biomass yields for enrichment RS24 (~0.25 mg dry biomass (mg $MTBE)^{-1}$) were comparable to the estimated biomass yield of 0.18 mg cells (mg $MTBE)^{-1}$ reported by Hanson et al. (1999) and to the estimated biomass yields of 0.21 to 0.28 mg dry biomass (mg $MTBE)^{-1}$ reported by Salanitro et al. (1994). Bacterial strain PM1 isolated by Hanson et al. (1999) was identified by 16S rRNA gene sequence analysis as a member of the Beta subgroup of Proteobacteria. In our study, sequence analysis of a purified DGGE band identified *Rhodoferax fermentans*, also a member of the Beta subgroup of proteobacteria, as an important member of the consortium RS24 due to its stability over subculturing.

While no metabolites were detected during degradation of MTBE by enrichment RS24 in the absence of 2-propanol, the accumulation of both TBA and TBF was consistently observed when RS24 was grown with 2-propanol. Both were also found in the presence of 10 mg L^{-1} benzene and 20 mg L^{-1} 2-propanol (data not shown). In addition, both TBA and TBF were observed repeatedly during MTBE degradation by both isolates, *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*, in the presence of 2-propanol. The presence of these organisms in the community profile of RS24 and their subsequent disappearance with subculturing suggests that these two isolates may be limited to cometabolic MTBE degradation. Mo et al. (1997) reported a reduction in MTBE degradation by a *Rhodococcus* sp. in the presence of butyl formate, *t*-butanol, and several other simple organic compounds. These results are reinforced by results from our study, showing an accumulation of TBA and TBF by *Rhodococcus koreensis* and *Pseudomonas* sp. Ant9. This suggests an inhibitory effect caused by accumulation of these metabolites.

The production of TBA and TBF support an MTBE degradation pathway such as that proposed by Hardison et al. (1997) in which the enzyme, cytochrome P-450, oxidizes MTBE to *tert*-butoxymethanol, which then may be converted to TBF via an alcohol dehydrogenase. The TBF may then undergo hydrolysis to TBA. This latter hydrolysis step was also proposed by Church et al. (1997). However, they suggested that the initial formation of TBF may be due to abiotic chemical oxidation by atmospheric oxygen. Our results do not support chemical oxidation as a major MTBE degradation step, as no disappearance of MTBE or accumulation of TBA or TBF was observed in our sterile controls in the presence or absence of 2-propanol.

As the lag phase in MTBE degradation by RS24 transfer cultures grown in spent supernatant vs. fresh SSE medium was reduced, this suggests the production of a factor in the medium that stimulates MTBE degradation. The mechanism governing this regulation may involve either a repressor-regulated transcriptional system or activator-dependent regulation. In the first case, a constitutively expressed repressor protein would prevent transcription of genes involved in MTBE degradation. Presumably, production or activation of an inducer protein could be initiated by the presence of MTBE or an effector protein. The production of the inducer over time would increase its concentration and affect more binding to and release of the repressor protein, thus allowing transcription of genes responsible for MTBE degradation. This type of mechanism has already been proposed for other genes involved in contaminant biodegradation. Leisinger et al. (1994) constructed a model for dichloromethane (DCM) degradation in *Methylobacterium* sp. In this model, production of the protein DcmA, which is responsible for DCM dehalogenase production, is normally repressed by binding of the regulatory protein DcmR to the *dcmA* promoter. Derepression (i.e., activation) of *dcmA* occurs by putative substrate-induced release of DcmR. In addition, Bertoni et al. (1997) showed that the XylR protein has both repressor and activator roles in the control of toluene biodegradation by *Pseudomonas putida*. Undoubtedly, some of these regulatory mechanisms are strictly intracellular in nature. However, we speculate that extracellular release of regulatory proteins acting as autoinducers such as those already identified in other types of quorum sensing and cell-cell communication would confer a selective advantage. For example, Visick and Ruby (1999) suggest that quorum sensing may prevent unnecessary gene induction, allow a rapid sensing of changes in the environment, and serve to coordinate consortial metabolism among bacterial species. Such concentration-dependent phenomena could result in observations of MTBE degradation similar to those documented in this study.

While several MTBE-degrading bacterial species were cultivated from the Ronan aquifer, MTBE biodegradation in the aquifer is difficult to assess in relation to other processes of natural attenuation. However, low dissolved oxygen (DO) measurements and low temperatures (4 to 12°C) suggest that biodegradation in the aquifer is relatively slow. DO measurements from the first to the fourth quarters of 1999 were consistently less than 0.5 mg L^{-1} for MW-10. Over this time period for the same monitoring well, MTBE measurements first increased over an order of magnitude to 3580 $\mu g\ L^{-1}$ and then gradually decreased again to < 300 $\mu g\ L^{-1}$. Likewise, total petroleum hydrocarbon (TPH) measurements increased almost two orders of magnitude to 10,900 $\mu g\ L^{-1}$ and then gradually decreased to < 900 $\mu g\ L^{-1}$. Background DO levels obtained from well MW-11 upgradient of the contaminant plume, as well as from well MW-6 and MW-16 (adjacent to MW-12 but outside the contaminant plume), were consistently between 4 to 6 mg L^{-1}. This indicates oxygen utilization coupled to the degradation of gasoline hydrocarbons inside the plume. Other studies have reported that low concentrations of DO and low temperatures severely slowed rates of MTBE degradation rate (Park and Cowan, 1997b) and injection of pure oxygen into an impacted aquifer enhanced MTBE degradation (Carter et al., 1997). Because measured DO levels indicate that oxygen may be limiting biodegradation at this site, we recommend that any applied treatment technology should investigate oxygen addition.

This study demonstrates that bacteria capable of degrading MTBE were present in the gasoline-contaminated aquifer near Ronan, Montana. To our knowledge, this is only the second study of a gasoline-contaminated site in which indigenous MTBE-degrading bacteria have been reported (Bradley et al., 1999). Other studies targeting the identification of native bacterial populations with the capacity to degrade MTBE in natural environments will help to establish the ubiquity of MTBE-degrading organisms. They will also allow better prediction of the efficacy of biodegradation as a means to remediate MTBE-contaminated aquifers. In addition, further laboratory studies can be designed to elucidate factors that influence MTBE degradation, such as co-substrates or the growth factor proposed in this study. Thus, they may provide insight into more efficient methods of stimulating MTBE degradation by naturally occurring bacteria.

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