Environ. Sci. Technol. **2001**, *35,* 4235−4241

Biodegradation of Methyl *tert*-Butyl Ether under Various Substrate Conditions

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Five aerobic enrichments efficient at degrading methyl *tert*-butyl ether (MTBE) under different substrate conditions were developed in well-mixed reactors containing a polyethlene porous pot for biomass retention. The five substrate conditions were as follows: MTBE alone; MTBE and diethyl ether (DEE); MTBE and diisopropyl ether (DIPE); MTBE and ethanol (EtOH); and MTBE with benzene, toluene, ethylbenzene, and xylene (BTEX). All five cultures demonstrated greater than 99.9% removal of MTBE. Addition of alternative substrate was found to have no effect on the performance of the reactors. The bacterial communities of the reactors were monitored periodically by denaturing gradient gel electrophoresis (DGGE) to determine when homeostasis was achieved. Phylogenetic analysis of the excised DGGE bands was done in order to compare the bacterial community compositions of the reactors. All cultures were found to be mixed cultures, and each enrichment was shown to have a unique composition. A majority of the bands in all reactors represented a group of organisms belonging to the *Cytophaga-Flexibacter-Bacterioides* (C−F−B) Phylum of bacteria. This was also the only group found in all of the reactors. This study demonstrates that MTBE can be degraded effectively in bioreactors under several substrate conditions and gives insight into the microorganisms potentially involved in the process.

Introduction

The contamination of groundwater, surface water, and consequently drinking water with methyl *tert*-butyl ether (MTBE) has become an increasing problem over the past decade. MTBE has been used extensively as a fuel additive for the purpose of meeting the 1990 Clean Air Act Amendment's requirement that gasoline be at least 2% oxygen by weight. MTBE production in the United States was estimated at 6.2 billion kilograms in 1994 and 21 billion kilograms in 1995 (*1*), the second largest volume of production for any organic chemical. Because of gasoline spills, mainly due to leaking underground storage tanks, MTBE has become a prevalent contaminant in groundwater. The reason for such widespread contamination is that MTBE is a polar molecule, the most polar constituent of gasoline, and is therefore very water-soluble. In addition, MTBE resists biological degradation, compared to other gasoline components (*2*).

Recently, the U.S. Environmental Protection Agency (EPA) has recommended that the use of MTBE be phased out over the next several years, but the persistence of MTBE in the environment will remain to be a problem for some time. EPA is recommending an advisory of 20−40 μg/L for drinking water, which is at the low end of human taste and odor threshold. With such an advisory in place, it is urgent that effective treatment and remediation strategies be found. Bioremediation is one possible treatment strategy that could offer great promise in eradicating MTBE from groundwater if it can be developed into a cost-effective cleanup technology.

Early studies utilizing activated sludge and soil reported MTBE to be resistant to biodegradation (*3*). In a study at the Borden field site in Canada, reformulated gasoline containing MTBE was intentionally injected below the water table to determine its fate. After 16 months, the BTEX (benzene, toluene, ethylbenzene, and xylene) component of the gasoline had degraded significantly, but the mass of MTBE only declined slightly (*2*). Later in 1995/1996 when the aquifer was reevaluated, it was found that only 3% of the original MTBE mass remained (*4*), with loss attributed to biodegradation. This raises hope for the use of bioremediation in the field, but the slow rate indicates that there is need to optimize the MTBE biodegradation process.

In 1994, Salanitro et al. (*5*) isolated an aerobic culture (BC-1) capable of degrading MTBE from a refinery waste bioreactor. Biodegradation was confirmed with radiolabeled MTBE studies. More recently, Hanson et al. (*6*) isolated PM1, a fast growing MTBE degrader, from a compost biofilter. Limited anaerobic biodegradation but not mineralization of MTBE has also been reported (*7*−*9*). Yeh and Novak (*7*) found that the presence of rich organic matter in soil inhibits degradation. Competitive inhibition by a second organic compound has been demonstrated in other studies (*10*), while Deeb et al. (*11*) observed that BTEX directly interferes with MTBE degradation, citing enzymatic inhibition as the probable cause. A recent review by Prince (*12*) provides a useful summary of MTBE biodegradation research and implications for bioremediation.

Knowledge of the conditions under which MTBE can be biologically degraded is limited. In this study five aerobic cultures efficient at MTBE degradation were enriched in well-mixed porous-pot reactors, each under different substrate conditions. This was done in order to determine whether the presence of an alternative substrate, which is generally the case in the field, has an effect on MTBE degradation. The resident bacteria of the reactors were profiled with 16S rDNA targeted PCR-DGGE (DGGE) to identify and compare the dominant organisms with the addition of alternative substrates. DGGE has proven to be a powerful tool for monitoring and identifying microbial populations in several other studies (*13*, *14*).

Experimental Section

Substrate Conditions. The five cultures were enriched with the following substrates: (1) MTBE alone; (2) MTBE and diethyl ether (DEE); (3) MTBE and diisopropyl ether (DIPE); (4) MTBE and ethanol (EtOH); and (5) MTBE and benzene, toluene, ethylbenzene, and xylene (BTEX). Alternative substrates were chosen based on probability of co-occurring with MTBE in a gasoline spill or by chemical similarity to MTBE. The MTBE, DEE, DIPE, and ETOH reactors were all

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10.1021/es010572z CCC: $20.00
Published on Web 09/15/2001

initially seeded with the same material: 2 L of mixed liquor from the Metropolitan Sewer District (MSD), Cincinnati, OH, 600 mL of mixed liquor from Shell Development Co., Houston, TX (kindly provided by J. Salanitro), and 140 mL of aquifer material wash water from an MTBE contaminated site in Port Hueneme, CA. The BTEX reactor was seeded 100 days later with a mixture of 100 mL of each of the above acclimated cultures. All cultures were maintained on a total influent feed of 417 mg/L chemical oxygen demand (COD). For reactors in which more than one substrate was provided, half of the COD was provided by MTBE, and the other half by the alternative substrate(s).

The reactors (Autoclave Engineers, Erie, PA) were well-mixed and shared a common temperature control system maintaining the temperature at about 20 °C, to simulate groundwater conditions. Since other studies had reported that biomass yield is low when cultures are grown on MTBE (*5*), a polyethylene porous pot (Atlas Minerals & Chemicals, Mertztown, PA) was inserted into each reactor to retain high biomass levels. The pots consisted of 0.48 cm thick filter grade polyethylene (pore size = 20 μm), with an internal diameter of 19.1 cm and a height of 29.2 cm. Initially, a solids retention time (SRT) of 18 days was maintained by wasting intentionally from the reactor. Subsequently intentional wasting ceased and only took place during sampling of the reactors.

The combined influent flow rate was 2.37 L per day, with 80% of the total flow provided by a pH adjustment solution, and 20% of the flow provided by an acidified nutrient solution. MTBE was fed directly into the higher flow line with a Model 11 high precision syringe infusion pump (Harvard Apparatus, Inc., South Natick, MA) with a 2.5 mL fixed needle syringe (Hamilton Co., Reno, NV). The pH adjustment solution consisted of deionized water with an appropriate amount of 10 N sodium hydroxide added to maintain the pH between 7.4 and 8.0. The MTBE BTEX reactor was switched to a phosphate buffering system midway through the study for more precise pH control. The pH adjustment solutions also contained the alternative substrate if present, with the exception of BTEX, which was provided combined with MTBE in the syringe. The nutrient solution consisted of deionized water with essential salts and vitamins added to promote biological growth. Final nutrient concentrations inside the reactor were as follows: $(NH_4)_2SO_4$, 93 mg/L; $MgSO_4$, 69.6 mg/L; $CaCl_2 \cdot 2H_2O$, 22.5 mg/L; K_2HPO_4, 6.9 mg/L; $CuSO_4 \cdot H_2O$, 0.08 mg/L; $Na_2MoO_4 \cdot 2H_2O$, 0.15 mg/L; $MnSO_4 \cdot H_2O$, 0.13 mg/L; $ZnCl_2$, 0.23 mg/L; $CoCl_2 \cdot 6H_2O$, 0.42 mg/L; and $FeCl_2 \cdot 4H_2O$, 17.25 mg/L. Each reactor was fed from its own separate reservoir with a constant speed pump controlled with a programmable timer. The hydraulic residence time was 4.2 days with a total reactor volume of 9.95 L and an enrichment culture volume of 6 L.

Analysis. Effluent from the reactors was monitored on a weekly basis for the presence of MTBE or its degradation product *tert*-butyl alcohol (TBA), DEE, DIPE, EtOH, or BTEX using a Hewlett-Packard 5890 Series II gas chromatograph (GC) (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and 60/80 Carbopack B5% Carbowax 20 M glass column (Supelco, Bellefonte, PA). For analysis at low concentrations, a Tekmar Dohrmann (Cincinnati, OH) 3100 heated Sample Concentrator was used with a Hewlett-Packard 6890 Series Plus GC and a DB-1 (J&W Scientific, Folsom, CA) capillary column. The pH of all systems was measured daily using an Orion Model 720A pH meter (Orion Research Co., Boston, MA). Reactors were also monitored weekly for chemical oxygen demand (COD) using Hach (Loveland, CO) low range or ultralow range COD vials. Dissolved organic carbon (DOC) was measured using a Shimadzu TOC-5000 Analyzer (Shimadzu, Kyoto, Japan) as the difference between the total carbon and inorganic carbon or by using nonpurgeable organic carbon analysis when the effluent concentrations were below 0.1 mg/L.

Radiolabeled Study. To confirm mineralization of MTBE, radiolabeled ^{14}C-MTBE tracer studies were performed with each culture, as described in Knaebel and Vestal (*15*). One milliliter biomass samples were taken from each reactor and added to 7 mL of reactor effluent containing 40 mg/L of MTBE and spiked with ^{14}C-MTBE. All samples were done in quadruplicate, controls were killed with 2.72 g/L $HgCl_2$. Solutions were mixed in 30 mL capacity serum bottles equipped with a KOH trap for trapping $^{14}CO_2$ that evolved as a result of ^{14}C-MTBE mineralization. Killed controls also accounted for any radiolabeled MTBE that may have volatilized into the trap region. All bottles were sacrificed after 2 days. Absence of MTBE and TBA in the liquid phase was verified at this time by GC analysis. Bottles were sacrificed in their entirety and radioactivity was measured in four regions: the trap, homogenized unfiltered biomass suspension, filtered biomass suspension, and the 0.1 μm filter paper (Micron Separations, Inc., Westborough, MA) used to filter the suspension. Samples were acidified and mixed for 10 min immediately prior to sampling in order to drive any aqueous carbon dioxide onto the KOH trap. Radioactivity was determined using a Tri-Carb 2300TR Liquid Scintillation Counter (LSC) (Packard, Downers Grove, IL). Total radioactivity in each bottle was calculated as the sum of the counts per minute (cpm) measured in each of the four regions. The percent radioactivity of each region is reported relative to the total cpm for that sample.

Chemicals. The following chemicals were used as supplied: methyl *tert*-butyl ether (MTBE, 99%, Aldrich, Milwaukee, WI), *tert*-butyl alcohol (TBA, 99%, Aldrich), ethyl ether (99% Fisher, Fair Lawn, NJ), isopropyl ether (99%, Aldrich), ethanol (190 proof, Midwest Grain Products, Weston, MO), benzene (99%, Fisher), toluene (99%, Fisher), ethylbenzene (99%, Fisher) and *p*-xylene (99%, Fisher).

16S rDNA PCR-DGGE. Denaturing gradient gel electrophoresis (DGGE) analysis using polymerase chain reaction amplified 16S rDNA was done on the cultures in all the reactors beginning in April 1999 every other month and then on a monthly basis beginning in September 1999. Cultures were monitored by DGGE until banding pattern remained consistent for at least 2 consecutive months, indicating that cultures had become stable.

DNA extractions were done in duplicate on each reactor using a FastDNA and a FastPrep sample homogenizer kit provided by Bio101 (Vista, CA). Polymerase Chain reaction (PCR) was used to amplify a 193 base pair portion of the V3 region of the 16S rDNA. Primers 534R (*E. coli* numbering system) and 341F (containing a GC clamp) were used as described in Muyzer et al. (*16*) and synthesized by Stratagene, Inc. (La Jolla, CA). This primer combination was chosen based on the superior ability of the resulting fragments to be resolved on a DGGE gel in addition to their higher sensitivity when compared to other "universal" PCR-DGGE primers (*17*). While such a short target sequence does not result in the most robust phylogenetic information, it is advantageous in that shorter target sequences are less likely to result in the formation of undesirable chimeras (*18*). The actual length of the sequence data generated was in the range of 150−160 bp because of gaps in the highly variableV3 region. The V3 region was chosen because of this variability and resulting resolving power in phylogenetic analysis. PCR reactions were done with 1.25 units of Expand Hi Fidelity DNA polymerase (Roche, Indianapolis, IN), and 10 pmol of each of the primers in a total volume of 25 μL. Negative PCR controls were monitored with every round of PCR to ensure that the background solution was not contaminated. Temperature cycling was done with a Robocycler PCR block (Stratagene) with the following regime: 93 °C-2 min (denaturing), 35 cycles of 92

°C-1 min, 55 °C-1 min, 68 °C-45 s, followed by 72 °C-2 min (extension).

DGGE employed a D-Code 16/16 cm acrylamide gel system (BioRad, Hercules, CA) maintained at a constant temperature of 60 °C in 6 L of 0.5 × TAE buffer (20 mM tris-acetate, 0.5 mM EDTA, pH 8.0). The total volume of the gel was approximately 30 mL. Gradients were formed between 15% and 55% denaturant (100% denaturant defined as 7 M urea plus 40% v/v formamide), and the gels were run at 35 V for 20 h. Gels were stained in purified water containing ethidium bromide at 0.5 mg/L and destained once in 0.5 × TAE. Images were documented using a GelDoc 2000 and Quantity One software (BioRad).

The central 1 mm^2 portions of the bands of interest were excised with razor blades and soaked in 36 μL of purified water for 3 days at −20 °C as described in Kowalchuck et al. (*19*). One microliter of this was used as template for PCR reaction, as described above. PCR products were purified using a GeneClean Spin Kit (Bio101). DNA sequence analysis was done using the reverse primer, described above. Sequencing was done by Davis Sequencing (Davis, CA) using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Foster City, CA).

Phylogenetic Analysis. Sequences were compared to the GenBank database by use of the BLAST facility of the National Center for Biotechnology Information (NCBI) and to the Ribosomal Database Project (RDP) using the "Similarity Rank" tool (*20*). All sequences were screened for chimeras using the "Check Chimera" tool also available from the RDP. Sequences were initially aligned with each other and with the closest RDP matches using ClustalX (*21*), followed by manual adjustment. Phylogenetic trees were constructed using maximum likelihood analysis, maximum parsimony, and neighbor joining analyses as implemented by Paup Star version 8b using default parameters (Sinauer Associates, Inc. Sunderland, MA). For the neighbor joining analysis, the Jukes-Cantor correction factor was implemented to account for the short sequence information. Bootstrap values were determined using 100 replicates. Sequences were submitted to GenBank and assigned accession numbers AYO40741-AYO40759 and AYO44871-AYO44875.

Results and Discussion

All cultures exhibited greater than 99.9% removal of MTBE on a consistent basis, regardless of substrate conditions. Effluent concentrations of MTBE were in the range of 0.5−5 μg/L in all reactors once they had attained steady-state. This is well below the EPA advisory level of 20−40 μg/L. Comparable removal of alternative substrate was also achieved. TBA was consistently measured at levels below 10 μg/L. See Figure 1 for MTBE removal plots. Schroeder et al. (*22*) also found that, after a short adaptation period, MTBE and toluene could be removed simultaneously from a compost biofilter.

Mineralization of MTBE, TBA, or other substrate by all cultures was confirmed based on carbon balance of the systems and radiolabeled ^{14}C-MTBE fate studies (Figure 2). ^{14}C-MTBE was found to be both mineralized to $^{14}CO_2$ as well as incorporated into the biomass of the microorganisms. The removal rates of all reactors, considering that MTBE in the effluent is negligible, is equal to the feed rate of MTBE to the reactor, which was 4.2 mmol/day for the MTBE only reactor, 2.3 mmol/day for the BTEX reactor, and 2.1 mmol/day for the remaining reactors.

The performance of all reactors greatly improved upon cessation of intentional biomass wasting. This demonstrates that biomass retention is a critical factor for effective MTBE removal. This was true under all substrate conditions, indicating that addition of alternative substrate did not sufficiently improve the yield of MTBE degrading organisms. Since performance was so unstable at the relatively long SRT of 18 days, removal of the pot, which would result in a SRT of 4.2 days, would likely lead to complete breakdown of reactor function. The pH of the systems was also an important parameter, especially for the MTBE and BTEX reactor. Performance of this reactor rapidly deteriorated outside a pH range of 7.4−7.6 (data not shown), whereas all other reactors could tolerate a range of 7.3−8.0. Conversion of the MTBE and BTEX reactor to a phosphate buffer allowed for more precise control of pH, and performance greatly improved. Phosphate was determined not to be the limiting factor considering that performance still deteriorated outside the established pH range, even with the application of phosphate buffer.

Figure 3 shows an example of how the cultures were monitored by DGGE until a stable banding pattern was achieved. Figure 4 compares the equilibrium gel banding patterns of the reactors. The multiple DGGE bands present at equilibrium demonstrated that all cultures are mixed cultures. Phylogenetic analysis further revealed that most of the sequenced DGGE bands belonged to the *Cytophaga-Flexibacter-Bacterioides* Phylum (C−F−B Phylum) of bacteria suggesting that all communities had significant populations of C−F−B. Figure 5 shows a phylogenetic tree of all of the organisms from the reactors found to be members of the C−F−B Phylum. DGGE bands identified in this study are shown in bold. The presence of some members of the α-Proteobacteria was found in the MTBE and EtOH chemostat and one member in the MTBE and DIPE chemostat. One organism belonging to the Nitrospina division of bacteria was detected in the MTBE only chemostat (DGGE band MTBE 1 ACC# AYO44871). Because some bands comigrate on top of each other, it was not possible to successfully sequence all of the bands. The arrows in Figure 4 indicate the bands that were successfully sequenced. Table 1 indicates the number of bands observed in each culture, the number sequenced, the number of C−F−B organisms identified, and the number of chimeras found.

No bands that were analyzed showed greater than 90% similarity with any known or cultured strain of bacteria, except DGGE band EtOH 7 ACC# AYO44873 from the MTBE and EtOH reactor which was 93% similar to *Hyphomicrobium vulgare*. This is a member of the *Rhizobium Agrobacterium* group of α-*Proteobacteria*. DGGE band MTBE 1 did show 90% similarity with an unnamed environmental clone (AzC021) identified in Kuske et al. (*23*) and assigned to the subdivision *Nitrospina*. An organism belonging to the α-Proteobacteria found in the MTBE and DIPE reactor (DGGE band DIPE 4 ACC# AYO44875) showed high similarity with the *Sphingomonas* group (RDP 83%). This indicates that most of the other recovered bands represent previously undescribed organisms. Though the majority of bands from all reactors were associated with the same kinds of organisms, namely C−F−B and α-Proteobacteria, only two organisms among the reactors showed 100% similarity to each other. These two organisms were from the MTBE and BTEX reactor and from the MTBE and DIPE reactor (DGGE band BTEX 1 and DGGE band DIPE 3) and fall within the C−F−B group (Figure 4).

It is probable that some of the C−F−B organisms identified in the MTBE only reactor are degrading MTBE, considering that MTBE was the only carbon substrate supplied to this reactor and that five out of six bands were determined to belong to C−F−B. Also, C−F−B organisms were the only ones identified in the BTEX and DEE reactor and were represented in the EtOH and DIPE reactor as well. This suggests that organisms belonging to C−F−B group may be the principle MTBE degraders. To our knowledge, this is the first report of the potential involvement of members of the C−F−B Phylum in MTBE biodegradation. Other reports have sited *Methylobacterium*, *Rhodococcus*, *Arthrobacter*, and

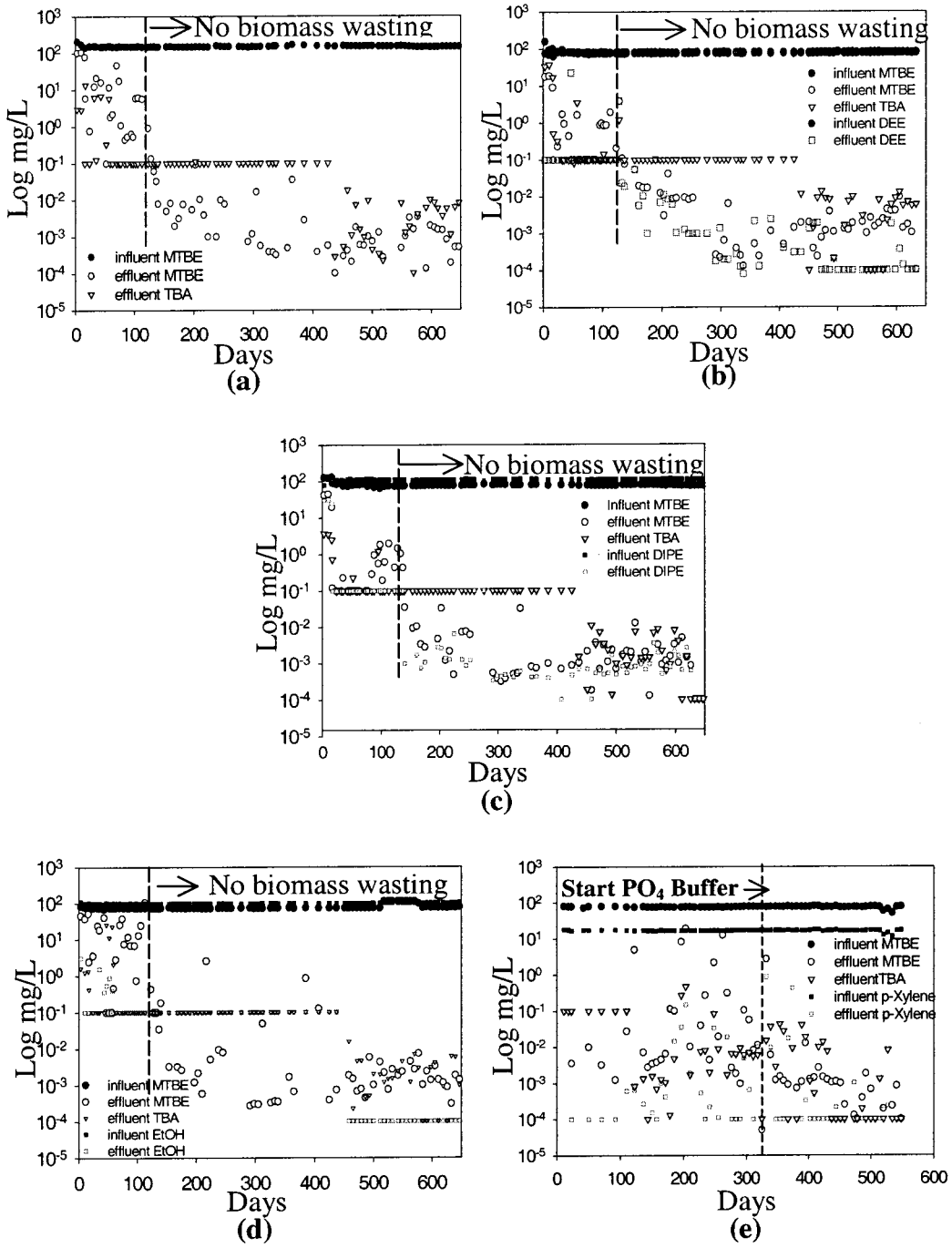

FIGURE 1. Porous-pot chemostat performance plots for (a) MTBE only, (b) MTBE and diethyl ether (DEE), (c) MTBE and diisopropyl ether (DIPE), (d) MTBE and ethanol (EtOH), and (e) MTBE and benzene, toluene, ethylbenzene, and *p*-xylene (BTEX). Method detection limit of *tert*-butyl alcohol (TBA) shifted from 0.1 mg/L to 0.1 μg/L midway through the study. For simplicity, only *p*-xylene is plotted of the four BTEX compounds

β-Proteobacteria as MTBE degraders in pure culture (*10*, *6*).

The C−F−B group are well-known for their abilities to degrade complex compounds, such as proteins, chitin, pectin, agar, starch, and cellulose (*24*). Also, they are known to be dominant in nearly all activated sludge (*25*, *26*), which was originally used to seed the cultures. This group has also been implicated in the degradation of polycyclic aromatic hydrocarbons (*27*), phenol (*28*, *29*), and various other substituted aromatic ring compounds (*30*, *31*). It is therefore not surprising that they would be involved in the degradation of MTBE, a xenobiotic which contains a tertiary carbon group and an ether bond, both of which resist biological degradation. The ability of C−F−B to degrade substituted aromatics may also explain why only these organisms were found in the MTBE and BTEX reactor.

C−F−B organisms are abundant in a variety of organically rich habitats and in both soil and water. They are prevalent in biofilms and bacterial flocs. One study using rRNA in situ hybridization probes showed that Flavobacteria-Cytophaga are the most numerically dominant of any organism in eutrophic river water but are much less represented in oligotrophic river water (*32*). Their presence in groundwater, which is one of the most oligotrophic environments, is not well documented. Some studies have cited these organisms being present in contaminated aquifers, such as one in Finland highly contaminated with chlorophenol (*28*) and

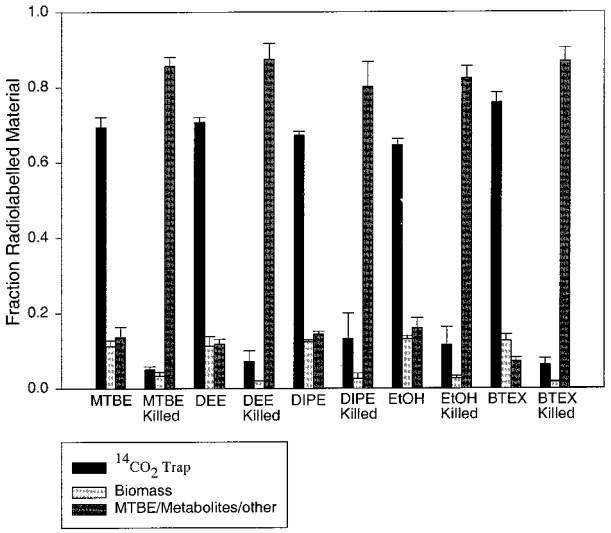

FIGURE 2. Radiolabeled ^{14}C-MTBE fate study. Size of bar indicates fraction of radioactivity found in each of three regions: 0.5 M KOH^{14}-CO_2 trap, biomass, and the filtrate containing any remaining ^{14}C-MTBE or intermediates. Error bars indicate standard deviation of quadruplicate samples.

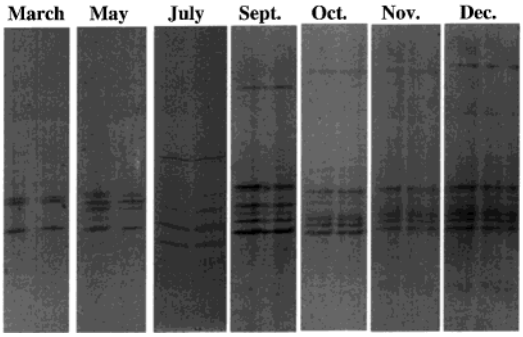

FIGURE 3. DGGE monitoring of MTBE only reactor until stable banding pattern was observed, indicating that equilibrium was achieved.

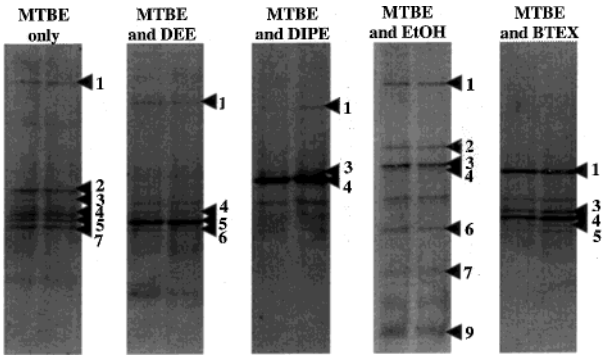

FIGURE 4. Equilibrium DGGE bands of five aerobic MTBE degrading cultures enriched under different substrate conditions. Substrate conditions are indicated above each gel. The arrows identify bands that were successfully excised and sequenced.

another in Korea receiving livestock waste (*33*). C−F−B organisms were found to originate from the animal waste and were not present in a nearby uncontaminated aquifer. This suggests that ex situ "pump and treat" using biomass concentrated within a biomass retaining reactor might be the most effective means of treating MTBE-laden groundwater. This may be an especially attractive option considering the extremely high biomass concentrations that were required in this study which will not naturally exist in an aquifer.

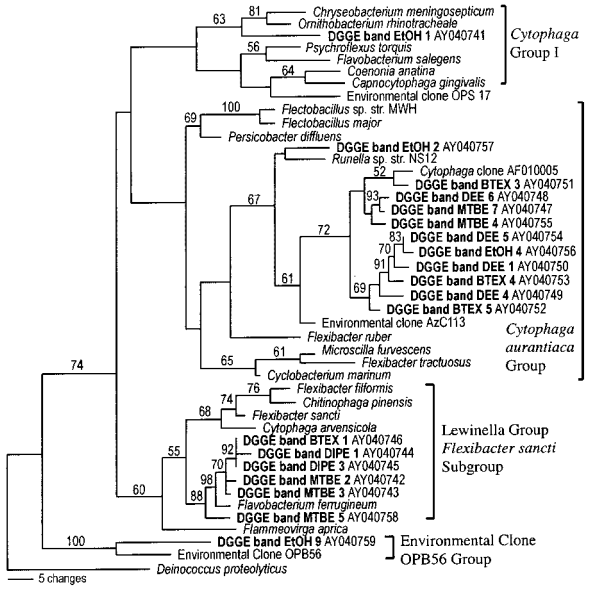

FIGURE 5. Phylogenetic analysis of DGGE bands falling within the *Cytophaga-Flexibacter-Bacterioides* (C−F−B) Phylum based on neighbor-joining analysis with Jukes-Cantor correction factor. Sequences obtained from DGGE bands are marked in bold labeled by their respective enrichment substrates and band numbers from Figure 4. GenBank accession numbers are provided for organisms that have not yet been classified. Brackets indicate major C−F−B groups and subgroups; *Flammeovirga aprica* and *Persicobacter diffluens* belong to the *Persicobacter* group, and Environmental clone OPS 17 belongs to the *Thermonema lapsum* group. *Deinococcus Proteolyticus* is the outgroup.

TABLE 1. Analysis of DGGE Bands

reactor	no. of bands	bands sequenced	bands identified as C−F−B	chimera
MTBE only	7	7	5	1
MTBE and DEE	6	4	4	0
MTBE and DIPE	6	4	2	1
MTBE and EtOH	9	7	4	2
MTBE and BTEX	6	4	4	0

One DGGE band from the MTBE only reactor (DGGE band MTBE 1) most closely matched (RDP 90% similarity) an uncultured group of the *Nitrospina* subdivision. This is an almost entirely uncultured division widespread in distribution but about which little is known (*23*). Involvement in MTBE biodegradation, however, should not be entirely ruled out.

The α-Proteobacteria, being present only in reactors with dual substrates, were most likely responsible for the degradation of the additional substrate, rather than MTBE. One organism from the MTBE and DIPE reactor showed high association with members of the genus *Sphingomonas*. *Sphingomonas* are well documented as degraders of complex compounds, and some are even able to degrade pentachlorophenol (PCP), a very toxic and recalcitrant xenobiotic (*34*). A *Sphingomona* (sp. PM1) capable of MTBE degradation was isolated by Hanson et al. (*6*) but not subjected to further study because of its inferior growth rate. Unlike Flavobacteria-Cytophaga, *Sphingomonas* are often detected in samples from the subsurface (*35*, *36*). *Hyphomicrobium vulgare*, the only species that was found to have greater than 90% similarity with any of MTBE reactor organisms, is a facultative methylotroph known to degrade alcohols (*37*). This may explain its residence in the ethanol reactor.

All five cultures achieved long-term stability as a mixed consortia. Considering this, as well as the excellent MTBE

removal performance of these cultures, mixed cultures may be more suitable for treatment options than pure cultures. In addition, as was observed seen in the initial seeding and subsequent disappearance of several of the organisms in the MTBE and BTEX culture (data not shown), not all organisms are appropriate for every substrate condition. Each substrate condition fostered the development of a community of bacteria that was distinct from all others. Because there were no replicate reactors at each substrate condition, it cannot be said for sure whether identical communities would always develop when the same substrate condition is provided.

Considering that all cultures were mixed cultures, it is possible that each organism is carrying out a different step in the degradation pathways of MTBE, TBA, DIPE, DEE, or EtOH or that some are carrying out the same steps in a cooperative manner. *Flavobacterium-Cytophaga* and α-Proteobacteria in particular have been shown to work cooperatively for the complete degradation of complex organic matter (*38*). It is important to note that none of the cultures applied in this study require a cometabolic mechanism for MTBE degradation, considering that the radiolabeled study confirmed MTBE mineralization of all culture in the absence of any other carbon substrate.

DGGE is a powerful tool in terms of its sensitivity in detecting organisms that culturing cannot. Muyzer et al. (*16*) showed that dilution of the template to extinction did not affect the patterns of major bands, and Brüggemann et al. (*39*) confirmed that it can readily detect organisms that comprise as low as 1−2% of the total population. Nonetheless, DGGE is often subject to criticism because of the biases inherent in extraction, purification, and PCR techniques (*40*). Limitations of DGGE are reviewed in Muyzer and Smalla (*41*). A recent study comparing DGGE to quantitative microscopy techniques, however, demonstrated that the two techniques revealed nearly identical microbial communities, with DGGE having somewhat more sensitive detection (*42*). Henckel et al. (*43*) further demonstrated that the major DGGE bands represent major components of the community. Though DGGE can be adapted as a quantitative tool (*39*, *44*), this study applied a simplified approach of recognizing that identified bands represent at least 1% of the total community.

This study was purposefully executed as a mixed culture study rather than as a pure culture study. A mixed culture is a more real-world scenario for both microorganisms native to an aquifer as well as those applied in ex situ bioreactors. Specifically, DGGE is an advantageous tool for this particular kind of study. While future studies may employ culture based methods, it is important to keep in mind that most organisms have not been isolated as pure cultures. This is because many are difficult or even impossible culture due to very specific ecological requirements. This could cause the principle degraders to be grossly overlooked, especially if they are working in cooperation with each other. These results are important especially considering that all reactors equilibrated as mixed cultures.

In summary, MTBE can be successfully and consistently degraded and mineralized in high biomass aerobic reactors regardless of the presence or absence of an alternative substrate. In all cases, whether MTBE is the only carbon source or not, mixed communities developed within the reactors. This indicates that mixed cultures may be most suitable for bioremediation or water treatment efforts. The prevalent group of organisms associated with the biodegradation of MTBE whether MTBE was the sole carbon and energy source was a group of organisms associated with the *Cytophaga-Flexibacter-Bacterioides* group. These organisms are not known to be dominant in pristine groundwater but are present in some contaminated groundwaters. This will be important to consider when deciding whether to treat MTBE contamination of subsurface waters in situ or ex situ. In addition, the adeptness of C−F−B for forming flocs and biofilms will be an added advantage in both attached growth and suspended growth processes for MTBE removal.

Acknowledgments

The authors are grateful to Dr. John Stephen and to Dr. Brian Kinkle for their consultation in DGGE and phylogenetic analysis. Thanks also goes to Mr. James Baltzer, a work-study student, for his assistance in care of the MTBE degrading cultures. This research was made possible under Contract No. 68-C7-0057 from the U.S. Environmental Protection Agency. The findings and conclusions expressed in this paper are solely those of the authors and do not necessarily reflect the views of the Agency.

Literature Cited

(1) *Interagency Assessment of Oxygenated Fuels*; Committee on Environment and Natural Resources, National Science and Technology Council (NSTC): 1997.

(2) Hubbard, C. E.; Barker, J. F.; O'Hannesin, S. F.; Vandegriendt, M.; Gillham R. *Transport and fate of dissolved methanol, methyl-tertiary-butyl-ether, and monoaromatic hydrocarbons in a shallow sand aquifer*; American Petroleum Institute, Health & Environmental Sciences Department: Publication 4601, Washington, DC, 1994; p 226.

(3) Jensen, H. M.; Arvin, E. Solubility and degradability of the gasoline additive MTBE, and gasoline compounds in water. In *Contaminated soil 90;* Arendt, F., Hinsenveld, M., van den Brink, W. J., Eds.; Kluwer Academic Publishers: Dorderecht, The Netherlands, 1990; pp 445−448.

(4) Schirmer, M.; Butler, B. J.; Barker, C. D.; Church, C. D.; Schirmer, K. *Phys. Chem. Earth* **1999**, *24*, 557−560.

(5) Salanitro, J. P.; Diaz L. A.; Williams, M. P.; Wisniewski, H. L. *Appl. Environ. Microbiol.* **1994**, *60*, 2593−2596.

(6) Hanson, J. R.; Ackerman, C. E.; Scow, K. M. *Appl. Environ. Microbiol.* **1999**, *65*, 4788−4792.

(7) Yeh, C. K.; Novak, J. T. *Water Environ Res.* **1994**, *66*, 744−752.

(8) Suflita, J. M.; Mormile, M. R. *Environ. Sci. Technol.* **1993**, *27*, 976−978.

(9) Mormile, M. R.; Liu, S., Suflita, J. M. *Environ. Sci. Technol.* **1994**, *28*, 1727−1732.

(10) Mo, K.; Lora, C. O.; Wanken, A. E.; Javanmardian, M.; Yang, X.; Kulpa, C. F. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 69−72.

(11) Deeb, R. A.; HU, H.-Y.; Hanson, J. R.; Scow, K. M.; Alvarez-Cohen, L. *Environ. Sci. Technol.* **2001**, *35*, 312−317.

(12) Prince, R. C. *Crit. Rev. Microbiol.* **2000**, *26*, 163−178.

(13) Macnaughton, S. J.; Stephen, J. R.; Venosa, A. D.; Davis, G. A.; Chang, Y.-J.; White, D. C. *Appl. Environ. Microbiol.* **1999**, *65*, 3566−3574.

(14) Stephen, J. R.; Chang, Y.-J.; Macnaughton, S. J.; Whitaker, S. L.; Hicks, C. L.; Leung, K. T.; Flemming, C. A.; White, D. C. *Environ. Toxicol. Chem.* **1999**, *18*, 1118−1123.

(15) Knaebel, D. B.; Vestal, J. R. *J. Microbiol. Methods* **1988**, *7*, 309−317.

(16) Muyzer, G.; de Waal, E. C.; Uitterlinden, A. G. *Appl. Environ. Microbiol.* **1993**, *59*, 695−700.

(17) Chang, Y.-J.; Stephen, J. R.; Richter, A. P.; Venosa, A. D.; Brüggemann, J.; Macnaughton, S. J.; Kowalchuk, G. A.; Haines, J. R.; Kline, E.; White, D. C. *J. Microbiol. Methods* **2000**, *40*, 19−31.

(18) Wang, G. C.-Y; Wang, Y. *Microbiology* **1996**, *142*, 1107−1114.

(19) Kowalchuk, G. A.; Gerards, S.; Woldendorp, J. W. *Appl. Environ. Microbiol.* **1997**, *63*, 3858−3865.

(20) Maidak, B. L.; Cole, J. R.; Lilburn, T. G.; Parker Jr, C. T.; Saxman, P. R.; Farris R. J.; Garrity, G. M.; Olsen G. J.; Schmidt T. M.; Tiedje J. M. *Nucleic Acids Res.* **2001**, *29*, 173−174.

(21) Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F.; Higgins, D. G. *Nucl. Acid Res.* **1997**, *24*, 4876−4882.

(22) Schroeder, E. D.; Eweis, J. B.; Chang, D. P. Y.; Veir, J. K. *Water, Air, Soil Pollut.* **2000**, *123*, 133−146.

(23) Kuske, C. R.; Barns, S. M.; Busch, J. D. *Appl. Environ. Microbiol.* **1997**, *63*, 3614−3621.

(24) Reichenbach, H. The order *Cytophagales*. In *The Prokaryotes*; Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. H., Eds.; Springer: New York, 1992; pp 3631−3675.

(25) Manz, W.; Amann R.; Wolfgang, L.; Vancanneyt, M.; Schleifer, K. H. *Microbiology* **1996**, *142*, 1097−1106.
(26) Pike, L. F. Aerobic bacteria. In *Ecological Aspects of Used Water Treatment*; Curds, C. R., Hawkes, H. A., Eds.; Academic Press: London, 1975; Vol. 1, pp 1−63.
(27) Maue, G.; Dott, W.; Kampfer, P. *ACTA Biotechnologica* **1994**, *14*, 337−345.
(28) Männistö, M. K.; Tiirola, M. A.; Salkinoja-Salonen, M. S.; Kulomaa, M. S.; Puhakka, J. A. *Arch. Microbiol.* **1999**, *171*, 189−197.
(29) Whiteley, A. S.; Bailey, M. J. *Appl. Environ. Microbiol.* **2000**, *66*, 2400−2407.
(30) Kampfer, P.; Steiof, M.; Becker, P. M.; Dott, W. *Microb. Ecol.* **1993**, *26*, 161−188.
(31) Langenhoff, A. A. M.; Nijenhuis, I.; Tan, N. C. G.; Briglia, M.; Zehnder, A. J. B.; Schraa, G. *FEMS Microbiol. Ecol.* **1997**, *24*, 113−125.
(32) Kenzaka, T.; Yamaguchi, N.; Tani, K.; Nasu, M. *Microbiology-UK.* **1998**, *144*, 2085−2093.
(33) Cho, J.; Kim, S. *Appl. Environ. Microbiol.* **2000**, *66*, 956−965.
(34) Ederer, M. M.; Crawford, R. L.; Herwig, R. P.; Orser, C. S. *Mol. Ecol.* **1997**, *6*, 39−49.
(35) Balkwill, D. L.; Drake, G. R.; Reeves, R. H.; Fredrickson, J. K.; White, D. C.; Ringelberg, D. B.; Chandler, D. P.; Romine, M. F.; Kennedy, D. W.; Spadoni, C. M. *Int. J. Syst. Bacteriol.* **1997**, *47*, 191−201.
(36) Fredrickson, J. K.; Balkwill, D. L.; Romine, M. F.; Shi, T. *J. Indust. Microbiol. Biotechnol.* **1999**, *23*, 273−283.
(37) Nikitin, D. I.; Slabova, O. I.; Pitryuk, I. A.; Sorokin, V. V.; Oranskaya, M. S. *Microbiology* **1998**, *67*, 54−60.
(38) Cottrell, M. T.; Kirchman, D. L. *Appl. Environ. Microbiol.* **2000**, *66*, 1692−1697.
(39) Brüggemann, J.; Stephen, J. R.; Chang, Y.-J.; Macnaughton, S. J.; Kowalchuck, G. A.; Kline, E.; White, D. C. *J. Microbiol. Methods* **2000**, *40*, 111−123.
(40) Suzuki, M. T.; Giovannoni, S. J. *Appl. Environ. Microbiol.* **1996**, *62*, 625−630.
(41) Muyzer, G.; Smalla, K. *Antonie Van Leeuwenhoek* **1998**, *73*, 127−141.
(42) Casamayor, E. O.; Schafer, H.; Baneras, L.; Pedros-Alio, C.; Muyzer, G. *Appl. Environ. Microbiol.* **2000**, *66*, 499−508.
(43) Henckel, T.; Friedrich, M.; Conrad, R. *Appl. Environ. Microbiol.* **1999**, *65*, 1980−1999.
(44) Felske, A.; Akkermans, A. D.; De Vos, W. M. *Appl. Environ. Microbiol.* **1998**, *64*, 4581−4587.

Received for review January 24, 2001. Revised manuscript received July 31, 2001. Accepted August 6, 2001.

ES010572Z