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ORIGINAL PAPER

Aerobic MTBE biodegradation in the presence of BTEX by two consortia under batch and semi-batch conditions

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Abstract This study explores the effect of microbial consortium composition and reactor configuration on methyl *tert*-butyl ether (MTBE) biodegradation in the presence of benzene, toluene, ethylbenzene and *p*-xylenes(BTEX). MTBE biodegradation was monitored in the presence and absence of BTEX in duplicate batch reactors inoculated with distinct enrichment cultures: MTBE only (MO—originally enriched on MTBE) and/or MTBE BTEX (MB—originally enriched on MTBE and BTEX). The MO culture was also applied in a semi-batch reactor which received both MTBE and BTEX periodically in fresh medium after allowing cells to settle. The composition of the microbial consortia was explored using a combination of 16S rRNA gene cloning and quantitative polymerase chain reaction targeting the known MTBE-degrading strain PM1^T. MTBE biodegradation was completely inhibited by BTEX in the batch reactors inoculated with the MB culture, and severely retarded in those inoculated with the MO culture (0.18 ± 0.04 mg/L-day). In the semi-batch reactor, however, the MTBE biodegradation rate in the presence of BTEX was almost three times as high as in the batch reactors (0.48 ± 0.2 mg/L-day), but still slower than MTBE biodegradation in the absence of BTEX in the MO-inoculated batch reactors (1.47 ± 0.47 mg/L-day). A long lag phase in MTBE biodegradation was observed in batch reactors inoculated with the MB culture (20 days), but the ultimate rate was comparable to the MO culture (0.95 ± 0.44 mg/L-day). Analysis of the cultures revealed that strain PM1^T concentrations were lower in cultures that successfully biodegraded MTBE in the presence of BTEX. Also, other MTBE degraders, such as *Leptothrix* sp. and *Hydrogenophaga* sp. were found in these cultures. These results demonstrate that MTBE bioremediation in the presence of BTEX is feasible, and that culture composition and reactor configuration are key factors.

Keywords BTEX · *Hydrogenophaga* · MTBE · PM1 · Q-PCR · RFLP

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Introduction

Unintentional spills at refineries and gas stations from leakage of pipelines and underground storage tanks can lead to serious contamination of aquifers with gasoline constituents. Gasoline oxygenates such as methyl *tert*-butyl ether (MTBE) and ethanol are used in order to reduce the emission of carbon dioxide and ozone forming compounds. MTBE has been used since the late 1970s and became preferred over other gasoline oxygenates in the United States, replacing

lead as an anti-knocking compound (USEPA 2006). The U.S. Geological Survey found that MTBE was the second most common volatile compound present in urban aquifers in the United States (Squillace et al. 1999). MTBE has a high water solubility, low adsorption to soil and low biodegradation rate, and thus MTBE continues to be a prevalent groundwater contaminant (USEPA 2006). Even though the toxicity and carcinogenicity of MTBE has not been confirmed, the United States Environmental Protection Agency (U.S. EPA) has established a drinking water maximum contaminant level of 20 μg/L, since MTBE adds significant taste and odor (USEPA 2005).

Active plume remediation of gasoline impacted sites is a common treatment technique. Active remedial methods include groundwater pump-and-treat (air stripping and removal with granular activated carbon), vapor extraction, chemical oxidation and multiphase high-vacuum extraction (Deeb et al. 2003). However, the cost associated with the construction, maintenance and operation of these treatments diminishes their feasibility; therefore, bioremediation is a preferred treatment option.

Ever since Salanitro et al. (1994) demonstrated that MTBE was aerobically biodegradable, several efforts have been made to isolate MTBE degraders in pure culture. Aerobic MTBE-degrading bacteria that have been described to date, include: *Rhodococcus* sp. (Mo et al. 1997), *Mycobacterium austroafricanum* IFP 2012 (Francois et al. 2002), ENV 425 (Steffan et al. 1997), *Rubrivivax gelatinosus* strain PM1 (Bruns et al. 2001) and *Hydrogenophaga* sp. (Hatzinger et al. 2001). One common attribute among MTBE degraders is a low yield coefficient, with theoretical to experimental biomass yield ratios (δ_e) ranging from 2.6 to 5.8 (Salanitro et al. 1994; Hanson et al. 1999; Fortin et al. 2001; Hatzinger et al. 2001; Francois et al. 2002; Fayolle et al. 2003). Energetically, aerobic degradation is favorable over all other electron acceptors (Stocking et al. 2000; Fayolle et al. 2003; Finneran and Lovley 2001; Fortin et al. 2001).

The most well-known aerobic MTBE degrader, strain PM1^T, is capable of complete mineralization of MTBE and can also use benzene as a sole source of carbon and energy (Deeb et al. 2001). Because of its high MTBE biodegradation rate, this strain has been used as a standard to characterize MTBE aerobic biodegradation by other strains (Fortin et al. 2001). In addition, the identification of strain PM1^T in aerobic MTBE-degrading cultures or environmental samples has been used as a parameter to correlate the presence of MTBE degraders with MTBE biodegradation (Hristova et al. 2003).

At gasoline impacted sites, the co-occurrence of MTBE and other organic compounds [such as benzene, toluene, ethylbenzene and xylenes (BTEX)] is a common scenario, especially at refineries. In order to successfully bioremediate these sites, the conditions under which MTBE and the other compounds can biodegrade simultaneously must be characterized. It was noted by Deeb et al. (2001) in batch reactors that the presence of ethylbenzene, *o*-xylene and *p*-xylene inhibited the biodegradation of MTBE by strain PM1^T, while toluene slightly inhibited the biodegradation of MTBE. Results such as these have discouraged attempts to bioremediate MTBE in the presence of BTEX or other contaminants. On the other hand, recent studies have demonstrated that under some conditions MTBE biodegradation can take place in the presence of other compounds such as BTEX in both pure and mixed cultures (Pruden et al. 2001; Sedran et al. 2002; Pruden and Suidan 2004). Thus, a better understanding of the conditions under which BTEX inhibits MTBE biodegradation is needed to advance bioremediation of these mixtures in the field.

Several laboratory experiments have been conducted under different conditions (i.e., various MTBE concentrations, batch or continuous flow tests), implementing different cultures (pure and mixed), and observing the effect of co-contaminants (Table 1). However, no study to date has explored the effect of the microbial consortium composition, nor has the effect of batch versus semi-batch conditions been directly compared. The main difference between the semi-batch reactor and the batch reactors in this study was that fresh medium was periodically provided after allowing the cells to settle in the semi-batch reactor. The objective of this study was to investigate the effect of BTEX on MTBE biodegradation on two levels: (1) the composition and overall diversity of the consortium and (2) the reactor configuration. Two distinct enrichment cultures were compared, MO (originally enriched on MTBE-only) and MB (originally enriched on MTBE and BTEX). The effect of batch versus semi-batch reactor configuration was also determined using the MO culture. Analyses of

Table 1 Summary of previous aerobic MTBE biodegradation studies: reactor and culture conditions

Reactor	Co-contaminants present	MTBE removal rate (mg/L/day)	Culture	Observations	Reference
Batch	*Tert-butyl* alcohol, butyl formate, isopropanol, acetone and pyruvate.	4.3–8.6	Pure cultures: *Methylobacterium* sp. *Rhodococcus* sp. and *Arthrobacter* sp.	Very slow growth, perhaps a result of metabolites. Other hydrocarbons decreased MTBE degradation rate. MTBE biodegradation by mixed cultures superior to that of pure cultures	Mo et al. 1997
Batch	BTEX	2.32–10.58	Strain UC1	BTEX had no effect on MTBE biodegradation	Pruden and Suidan 2004
Batch	BTEX	26	Enrichment culture	BTEX reduced TBA degradation rate	Sedran et al. 2002
Batch	BTEX	20–120	strain PM1^{T}	Inhibition of MTBE by some BTEX compounds (ethylbenzene and xylenes). Lag phase induced by benzene and toluene compounds	Deeb et al. 2001
Continuous flow porous-pot biomass concentrating	BTEX	5	Enrichment culture	Culture very sensitive to pH	Sedran et al. 2002
Continuous flow porous-pot biomass concentrating	Diethyl ether, diisopropyl ether, ethanol or BTEX	10	Enrichment culture	High biomass concentration was key to performance. Biodegradation of MTBE in the presence of BTEX was observed at a pH range of 7.4–7.6. A high microbial diversity was observed. Each condition studied had a unique microbial consortium composition	Pruden et al. 2001
Biotrickling filters	None	67	Enrichment culture (F-consortium)	The biodegradation rate of the F-consortium was similar to the biodegradation rate of strain PM1^{T}. pH did not affect biodegradation (range 4–8)	Fortin et al. 2001
Biotrickling filters	Ethanol, formate, pyruvate, lactate, acetate, hydroxylamine, ethyl ether	20–50	Enrichment culture (F-consortium)	MTBE was degraded after depletion of the co-contaminant added. The microbial consortium composition was not characterized	Fortin et al. 2001

the microbial consortium included: cloning, restriction fragment length polymorphism (RFLP) screening and sequencing of 16S rRNA genes and quantitative polymerase chain reaction (Q-PCR) quantification of the known MTBE-degrader, strain PM1^T. In addition to its application for addressing an important challenge for MTBE bioremediation, this study is unique in that it explores the relationship between complex microbial consortium dynamics to biodegradation performance.

Materials and methods

Reactor design and operation

Fifteen batch reactors (seven in duplicate and one uninoculated control) were used to compare the biodegradation of MTBE by two distinct enrichment cultures in the presence or absence of BTEX. The enrichment cultures were designated as the MTBE only culture (MO) and the MTBE and BTEX culture (MB), indicating the substrate conditions under which they were originally enriched. The cultures were obtained from two previously studied porous-pot biomass concentrating continuous flow reactors (Pruden et al. 2001; Sedran et al. 2002). Glycerol freezer stocks were generated upon sacrifice of the reactors in 2002 and maintained at −80°C until the time of this study. The MO and MB cultures were studied individually and mixed at a 1:1 ratio. A summary of the experimental conditions is presented in Table 2.

Batch experiments were carried out in 500 mL borosilicate glass bottles, into which 360 mL of mineral salt medium (MSM) and 40 mL 10× phosphate buffer solution (PBS) were initially aliquotted with a pH of 7.0. The MSM nitrogen source was ammonium and contained trace minerals, as described previously (Pruden et al. 2001). The bottles were sterilized in an autoclave, sealed with butyl rubber septa and continuously stirred at medium velocity with magnetic stir bars to ensure sufficient dissolved oxygen. The 100 mL headspace provided more than double the requirement for mineralization based on stoichiometry. Opening all bottles for 5 min at each sampling provided additional oxygen beyond the stoichiometric requirement, while control bottles accounted for any potential losses of MTBE or BTEX due to volatilization. The pH was measured periodically and consistently fell in the range of 7.0 ± 0.2 throughout the experiment.

All reactors were inoculated from the same enrichment culture freezer stocks, which allowed direct day by day comparison of the resulting consortia dynamics in different experiments. Enrichment cultures stored at −80°C were warmed initially overnight at −20°C and then placed on ice to melt

Table 2 Experimental conditions for the batch reactors

Reactor ID	Culture	Initial MTBE concentration (mg/L)	Initial BTEX concentration (mg/L)	Days operated
MO MTBE a	MO	10	0	51
MO MTBE b	MO	10	0	51
MO MBTEX a	MO	10	20	51
MO MBTEX b	MO	10	20	51
MOMB MTBE a	MO + MB	10	0	51
MOMB MTBE b	MO + MB	10	0	51
MOMB MBTEX a	MO + MB	10	20	51
MOMB MBTEX b	MO + MB	10	20	51
MB MTBE a	MB	10	0	93
MB MTBE b	MB	10	0	93
MB MBTEX a	MB	10	20	51
MB MBTEX b	MB	10	20	51
Killed control	None	10	20	93

slowly. The cells were washed three times with MSM by vortexing, centrifuging and pouring off the supernatant to remove the glycerol used to store the cells. The washed cells were diluted in MSM and 5 mL were used to inoculate each batch reactor, for an initial average concentration of 1×10^3 cells/mL. MTBE and BTEX were dissolved in the mixture by adding the appropriate volume with a gastight syringe (Hamilton Company, Reno, NV, USA). A target initial MTBE (Acros, Morris Plane, NJ, USA) aqueous concentration of 10 mg/L and either 0 or 20 mg/L (5 mg/L each) of BTEX (Fisher, Fair Lawn, NJ, USA) compounds were spiked in the reactors. When biodegradation was observed, the batch reactors were re-spiked periodically with MTBE and/or BTEX. A killed control dosed with 0.1 g of sodium azide (Fisher, Fair Lawn, NJ, USA) was also monitored. The batch reactors were maintained at room temperature ($\sim$25°C), with a total experiment time of 93 days.

A semi-batch reactor was also studied. A 4-L borosilicate glass cylindrical reactor (3,200 mL aqueous phase, 800 mL head-space), closed to the atmosphere and continuously stirred with a magnetic stir bar and stir plate, was sterilized with MSM and PBS, inoculated with the MO culture, and spiked with MTBE and BTEX as described for the batch reactors. The target concentrations of MTBE and BTEX were 10 and 20 mg/L, respectively, as applied in the batch reactors containing BTEX. Instead of spiking the compounds directly when necessary, fresh MSM and PBS containing MTBE and BTEX were provided. Cells were allowed to settle for 1 h before removing the spent medium and adding the fresh medium. The performance of the semi-batch reactors was monitored over a period of 105 days. All reactors were named as summarized in Table 2.

Monitoring MTBE and BTEX

Aqueous samples were taken periodically from each reactor and analyzed within 1 week. The stir plates were stopped and the cells were allowed to settle for 1 h before 1 mL of the supernatant was sampled at about 2 cm below the water surface. In a 25-mL borosilicate glass sampling vial with a Teflon cap, the 1-mL sample was diluted into 23 mL of deionized water with 1 mL of 0.1 M HCl for sample preservation.

Five milliliters of this mixture were used to measure the concentration of MTBE and BTEX compounds. The concentration of these compounds was determined using a Tekmar-Dohrman (Cincinnati, OH, USA) heated purge and trap 2016 and a Hewlett Packard (Rolling Meadows, IL, USA) 5890 Series II Gas Chromatograph with a capillary column and flame-ionization detector. Eight point calibration curves were generated with concentrations varying from 4 to 2,000 μg/L, with R^2 ranging from 98.95% for *p*-xylene to 99.96% for benzene. Because the R^2 value of the calibration curve for *tert*-butyl alcohol (TBA), an MTBE intermediate, was 87.29%, it was concluded that the TBA concentration could not be accurately quantified, so its presence or absence in each sample was reported instead. The biodegradation rates for each reactor were calculated from the linear portion of each degradation curve (neglecting lag phase) assuming that the transformations of MTBE to TBA or CO_2 were zero order. Most studies described in a recent review assumed zero order kinetics (Fortin et al. 2001) and its use was further justified based on the relatively high MTBE to cell ratio of this study.

Characterization of the microbial consortia

Two-milliliter samples were collected from the batch reactors for microbial analysis just before the stir plates were turned off and cells settled for MTBE and BTEX sampling. Samples were stored at −80°C and were later selected for further analysis based on their relationship to three key MTBE biodegradation time points during the second observed degradation event: onset (just before MTBE biodegradation commenced), middle (when MTBE was at $\sim$5 mg/L) and end (just before spiking back to 10 mg/L). Because the MO MBTEX batch reactor biodegraded MTBE at a very slow rate, it was further analyzed on days when the MO MBTEX semi-batch reactor was sampled for comparative purposes.

DNA extraction

The MoBio UltraClean Soil DNA kit (Solana Beach, CA, USA) was used for the extraction of microbial DNA according to the manufacturer's protocol. The only modification made to the kit procedure was that the bead-beating time was reduced from 10 to 4 min, in order to reduce shearing of DNA.

Cloning of 16S rRNA genes

Cloning of 16S rRNA genes was performed to estimate the diversity of the cultures and to identify the dominant microorganisms present in the following samples: MO and MB freezer stocks, one of the MO MBTEX batch reactors and the semi-batch reactor. This provided a means to compare a consortium efficient at biodegrading MTBE in the presence of BTEX, with a BTEX-inhibited consortium, both of which resulted from the same inoculum.

The 16S rRNA genes were polymerase chain reaction (PCR) amplified with the primers 8F and 1492R (Weisburg et al. 1991). The PCR mix consisted of 2.5 µl of 10× buffer, 5 µl of 5× buffer, 0.5 µl of dNTP (10 mM), 0.25 µl of formamide,1 µM of each primer (5 µm) and 0.35 µl of Taq polymerase (Eppendorf, Wesbury, NY, USA) for each 25 µl reaction. The thermocycler program consisted of an initial denaturing step for 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C. A final extension step was conducted for 10 min at 68°C. The resulting PCR products were cloned using the TOPO TA Cloning Kit® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Resulting clones were screened for the presence of an insert by PCR using vector-specific M13F and M13R primers. The amplified inserts were digested with the restriction enzyme *Msp*I (Promega, Madison, WI, USA) to identify unique inserts by RFLP. The species richness (number of operational taxonomic units present in each reactor) was estimated using Analytic Rarefaction 1.3 developed by Steven Holland (UGA Stratigraphy Lab, Athens, GA, USA) to determine the average value within 95 and 99% confidence limits. Unique inserts were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced by the Macromolecular Resources facility at Colorado State University (Fort Collins, CO, USA) using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Quantification of strain PM1^T by real-time quantitative PCR

Hristova et al. (2001) recently reported a TaqMan Q-PCR protocol for quantifying the known MTBE-degrading strain PM1^T using primers 963F and 1076R and the corresponding TaqMan probe PM1. An adaptation of this Q-PCR protocol was used to measure the concentrations of strain PM1^T in the enrichment cultures used as inocula, in all of the batch reactors and in the semi-batch reactor. Twenty-four microliters of PCR master mix was mixed with 1 µl of DNA extract. The master mix consisted of 2.5 µl 10× buffer, 5 µl 5× buffer, 0.5 µl dNTP (10 mM), 0.25 µl of each primer (10 µM), 0.375 µl TaqMan probe PM1 (10 mM), 0.35 µl Taq polymerase (Eppendorf, Wesbuty, NY, USA), and 1.5 µl Mg^{2+}. All Q-PCR reactions were performed in duplicate using a Cepheid Smart Cycler (Sunnyvale, CA, USA). The amplification program consisted of 2 min at 95°C, followed by 50 cycles at 95°C for 15 s, 53°C for 60 s and 72°C for 20 s. The relative concentrations of total bacteria were determined by quantifying total 16S rRNA genes using the TaqMan Q-PCR protocol described by Suzuki et al. (2000). The reaction conditions were the same as described above, except that primers 1369F (10 µM) and 1492R (10 µM) and the TaqMan probe 16S (10 µM) were used. All Q-PCR reactions were carried out in duplicate.

A generalized least-square mean procedure was conducted to determine if the difference in strain PM1^T proportions observed for each reactor at the three different times studied was significant. SAS 9.1 software was used to conduct the procedure and determine the *p* values (SAS Institute Inc., Cary, NC, USA).

Sequence analysis and assignment to functional groups

Clones that represented more than 6% of the total in each library were selected for DNA sequencing. The BLAST tool of the National Institute of Health database was used to search and identify the highest matches for each clone insert. Sequences were also analyzed using the Ribosomal Database Project Sequence Match Tool to aid in phylogenetically classifying the microorganisms represented by clone inserts. A literature review was performed to further characterize the microorganisms corresponding to the highest matches.

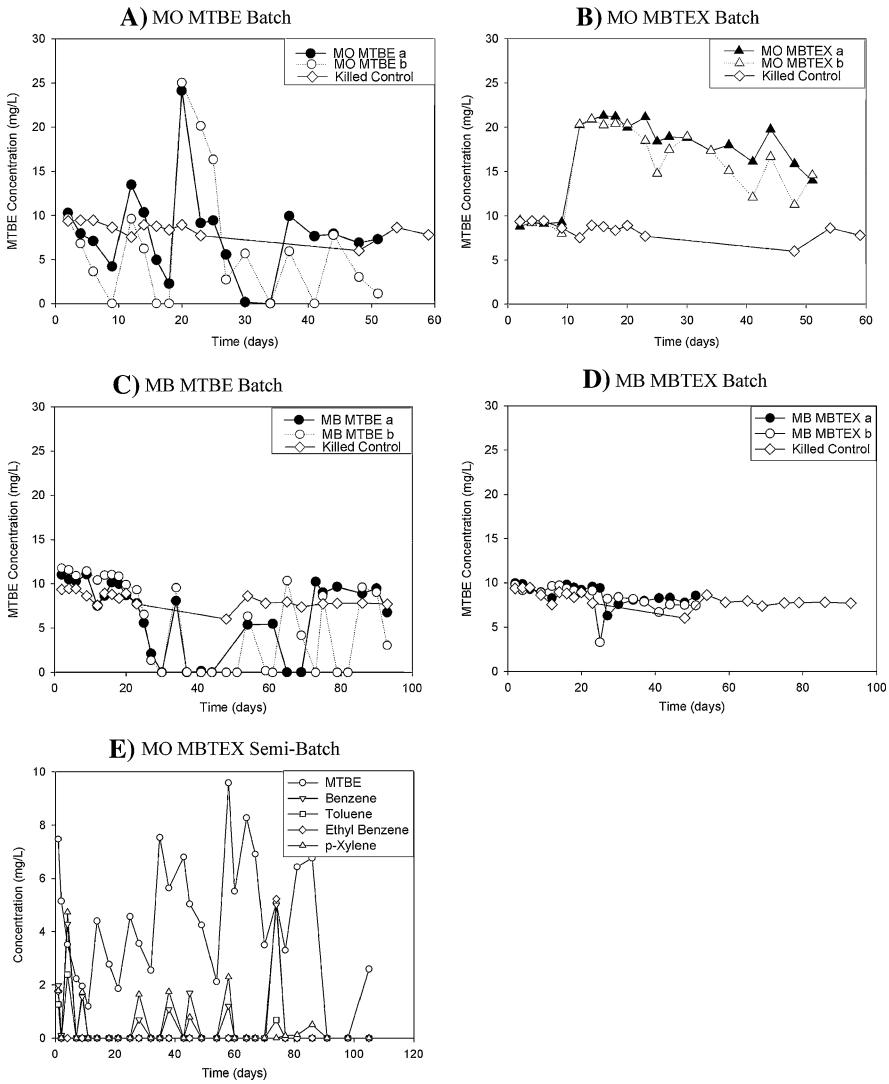

Fig. 1 Biodegradation of MTBE in batch reactors: (**A**) MO culture in the absence of BTEX; (**B**) MO culture in the presence of BTEX; (**C**) MB culture in the absence of BTEX; (**D**) MB culture in the presence of BTEX and (**E**) biodegradation of MTBE and BTEX by the MO culture in the semi-batch reactor. MTBE and BTEX were spiked in periodically at target concentrations 10 and 20 mg/L, respectively, except for Day 12 in the MO MBTEX reactor and Day 20 in the MO MTBE reactor, when MTBE was spiked to $\sim$20 mg/L

Results and discussion

Reactor effect

To determine the reactor configuration effect on MTBE biodegradation in the presence of BTEX, concentrations of MTBE and BTEX in the batch and semi-batch reactors were monitored with time (Fig. 1). It was observed that MTBE biodegraded readily in the presence of BTEX in the semi-batch reactor, but not in most of the batch reactors, even when inoculated with the same MO culture. However, the rate of MTBE biodegradation in the MO MBTEX semi-batch reactor (0.48 ± 0.2 mg/L-day) was about 40% the rate of MTBE biodegradation in the MO MTBE batch reactor (1.47 ± 0.47 mg/L-day); though it was more than three times faster than the MTBE biodegradation rate in the MO MBTEX batch reactor (0.18 ± 0.04 mg/L-day). BTEX compounds were readily biodegraded in all reactors. Based on these results, it is hypothesized that the accumulation of byproducts from the biodegradation of BTEX inhibits biodegradation of MTBE in batch reactors, whereas the semi-batch conditions significantly dilute these compounds. BTEX itself may be responsible for some level of inhibition, but in the semi-batch reactor MTBE biodegradation preceded even with continued additions of BTEX. Inhibition of MTBE

biodegradation in the presence of BTEX due to potential build-up of byproducts has been suggested by others (Deeb et al. 2001; Sedran et al. 2002). If MTBE biodegradation pathways differ between the two reactor configurations, then MTBE intermediates could be an alternative cause of inhibition in the batch reactors; however, this kind of inhibition has not been reported previously. TBA was noted to be consistently below detection as a metabolite in all batch reactors and in the semi-batch reactor (data not shown). Thus MTBE biodegradation progressed beyond TBA in all reactors. No abiotic losses were observed in the killed control; therefore removal of MTBE by the cultures could be attributed to biodegradation.

Culture effect

Overall effect on performance

The effect of the culture on performance was determined by monitoring MTBE and BTEX biodegradation in batch reactors inoculated with either the MO or MB culture, or a combination of both. The MB culture appeared to be inferior to the MO culture in terms of two main performance indicators: (1) MTBE biodegradation by the MB culture was completely inhibited in the presence of BTEX (Fig. 1D) even when mixed with the MO culture (data not shown), whereas the MO culture showed some MTBE biodegradation in the presence of BTEX (Fig. 1B); (2) the lag period for MTBE biodegradation by the MB culture in the absence of BTEX was very long (20 days), whereas there was no apparent lag phase for the MO culture (Fig. 1A, C). However, discounting the lag period, the actual rate of biodegradation in the absence of BTEX was comparable between the MO and MB cultures (1.47 ± 0.47 mg/L-day versus 0.95 ± 0.44 mg/L-day, respectively). MTBE biodegradation by both cultures was inhibited in the presence of BTEX; however, the MO-inoculated reactors showed less inhibition than the MB-inoculated reactors.

The concentration of MTBE spiked into the MO MTBE and MO MBTEX reactors on Day 22 and Day 10, respectively, was increased to ~20 mg/L. It was observed that the cells in the MO MTBE reactor were not shocked by this change, and that they were able to biodegrade the ~20 mg/L with no apparent rate change. However, the MO MBTEX batch reactor still showed inhibited MTBE biodegradation in the presence of BTEX.

It is interesting to note that the MB culture failed to degrade MTBE in the presence of BTEX, even though this culture was originally collected from a reactor that was simultaneously degrading MTBE and BTEX and had been observed to degrade these compounds simultaneously in batch reactors (Sedran et al. 2002). Thus, storage in the freezer had an apparent effect on the capabilities of the culture. However, this effect did not negatively impact the main purpose of this study, which was to compare the performance of cultures with different compositions. This observation does suggest some caution in using freezer stocks for bioaugmentation purposes.

Composition of the microbial consortia

For a summary of the putative functions of the microorganisms detected in the cultures and the reactors by cloning and the corresponding phylogenetic identities see Table 3. It was observed that the majority of the microorganisms belonged to the α-, β- and γ-Proteobacteria classes, which contain several oil-degrading bacteria (Hristova et al. 2001; Feris et al. 2004). The most frequently detected class was β-Proteobacteria, to which most currently known MTBE-degraders belong (Bruns et al. 2001; Hatzinger et al. 2001). Many of the microorganisms identified in the reactors did not have any obvious function related to MTBE or BTEX biodegradation (Table 3). While cloning is dependent on PCR, which is known to exhibit biases, this approach was considered to be suitable for comparative purposes, especially with respect to the same culture(s) under different conditions, which would be expected to normalize any potential biases. Also, the PCR cycle number was kept low (25 cycles) to reduce bias as much as possible.

The relative proportions of detected microorganisms is shown in Fig. 2. This semi-quantitative comparison is based on frequency of occurrence in the clone libraries according to RFLP patterns. Observation of the initial cultures' compositions revealed two different strains of known MTBE-degraders in the MO culture: PM1^T and *Leptothrix* sp. However, only one known MTBE-degrader, strain PM1^T, was detected in the MB culture. This suggests

Table 3 Summary of highest matches and characteristics of sequenced clones

Highest match (score)[a,b,c]	Class	Function/association	Reactor/Culture	Reference
MTBE biodegradation				
Strain PM1: AF176594 (0.96)	β-Proteobacteria	MTBE-degradation	MO and MB cultures	Bruns et al. 2001
Leptothrix sp. MBIC3364: Y8; AB015048 (0.90)	β-Proteobacteria	MTBE-degradation	MO culture	Hamada et al. 2003
Hydrogenophaga sp. PG-10: AY566583 (0.95)	β-Proteobacteria	MTBE-degradation	Semi-batch	Hatzinger et al. 2001
BTEX/petroleum biodegradation				
Bacteroidetes: AY211072 (0.95)	Bacteroidetes	Oil-degrading bacterial consortium	MO culture	Feris et al. 2004
Uncultured γ-proteobacterium: AF529326 (0.95)	γ-Proteobacteria	Oil-degrading cyanobacterial consortium	Semi-batch	Sanchez et al. 2005
Rhodobacter sp. TCRI 14: AB017799 (0.94)	α-Proteobacteria	Petroleum-degrading photosynthetic bacteria	MB culture	Hristova et al. 2001
Ralstonia sp. DUT_AHX: DQ40908 (0.95)	β-Proteobacteria	*o*-Xylene-degradation	Batch	Lee and Sun Bok 2002
Comamonas sp. *AV1A*: AF434169 (0.96)	β-Proteobacteria	mcl-PHA synthesizing bacteria, TCE-degradation	Batch	Ciesielski et al. 2004; Fennell et al. 2001
Pietermaritzburg bacterium Y14-2: AF312216 (0.94)	γ-Proteobacteria	BTX-catabolizing consortium	Batch	Ralebitso et al. 2001
Rhodobacter sp.: AF494542 (0.93)	β-Proteobacteria	Benzo[*a*]pyrene-mineralizing consortium	Batch	Thomas et al. 1996
Alcaligenes sp.: DQ366091 (0.95)	β-Proteobacteria	Oil-degrading bacterial consortium	Batch and semi-batch	Krooneman et al. 1996
Unknown relevance				
Denitrifying β-Proteobacterium: U51102 (0.94)	β-Proteobacteria	Denitrifying Fe-II oxidization	MO and MB cultures	Straub et al. 1996
Uncultured bacterium PHOS-HE31: AF314430 (0.97)	Sphingobacteria	Aerobic phosphorus-removal	Semi-batch	Dabert et al. 2001
Uncultured bacterium: AF268998 (0.716)	Genera Incertae Sedis	Putative uncultured phylum	Semi-batch	Bond et al. 1995
Uncultured α-proteobacterium: AB245350 (0.97)	α-Proteobacteria	Unknown	Semi-batch	Lee et al. 2006
Uncultured Flavobacteriales: AY509269 (0.94)	Flavobacteria	Unknown	Semi-batch	Eiler and Bertilsson 2004

[a] National Center for Biotechnology Information Blast similarity score http://www.ncbi.nlm.nih.gov/BLAST/

[b] Based on 800–1,100 bp of 16S rRNA gene sequence information

[c] GenBank accession numbers provided following name (eight characters)

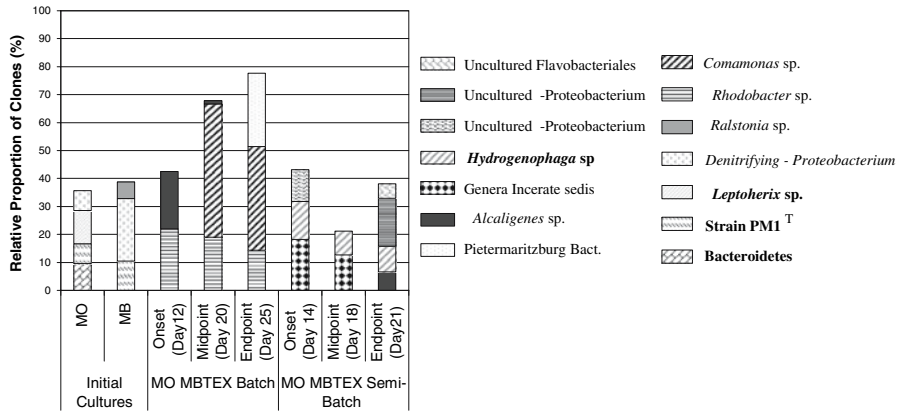

Fig. 2 Identification of the dominant microorganisms present in the initial cultures (before inoculation), the MO MBTEX batch reactor and the MO MBTEX semi-batch reactor based on cloning, RFLP screening and sequencing of most common clones (≥6% total library). Three time points were investigated based on the second observed biodegradation event: onset, midpoint and endpoint. Known MTBE-degraders are shown in *bold* in the legend. Further characterization of the identified strains is presented in Table 3

that initial diversity among MTBE-degraders may play a role in the ability to biodegrade MTBE in the presence of BTEX. Interestingly, the reactor conditions significantly altered the composition of the initial consortia in the inocula, though some populations may also have died during freezer storage. In the case of the MO MBTEX batch reactor, RFLP detected no known MTBE-degraders, which corresponds well to the inhibited MTBE biodegradation observed in this reactor. Furthermore, a BTEX-degrader, Pietermaritzburg bacterium, was found that was not detected in the initial culture (Table 3 and Fig. 2). In the MO MBTEX semi-batch reactor, which was actively biodegrading MTBE, a very different consortium was characterized. Neither strain PM1^T nor *Leptothrix* sp. were found in the semi-batch reactor by RFLP, however, another MTBE-degrader, *Hydrogenophaga* sp., was identified. This microorganism has been studied in other experiments and was characterized as having a slow MTBE biodegradation rate; however, the effect of BTEX has not been reported (Hatzinger et al. 2001). Overall, these results demonstrated that the microbial consortium composition varied between a reactor actively biodegrading MTBE in the presence of BTEX, and a BTEX-inhibited reactor, even when originally inoculated with the same culture. Therefore, reactor configuration had a major influence on the microbial consortium that developed and its ultimate performance.

Relative diversity

The results of cloning and RFLP screening of 16S rRNA genes are presented in Fig. 3. Comparing the number of phylotypes present in the initial cultures revealed that the estimated species richness was higher for the MO culture than for the MB culture (28.5 ± 1 versus 27.8 ± 1, respectively), but that the difference was not significant. However, two different patterns emerged in examining the diversity of the MO BTEX batch and semi-batch reactors with time (Fig. 3). In the batch reactors, where MTBE biodegradation was inhibited, the microbial diversity decreased with time (species richness = 32.8 ± 1, 18.9 ± 0.7 and 13.8 ± 1, respectively). This may have been a result of further enrichment of MTBE degraders, though this is unlikely considering that MTBE biodegradation was poor. Thus, failure of the populations to adapt to the reactor conditions is the more likely scenario. In the semi-batch reactor, species richness increased from Day 14 (21.7 ± 1.1) to Day 18 (45.5 ± 1.2), but then decreased to a moderate value by Day 21 (31.8 ± 1). Notably, the final microbial diversity was higher than the initial microbial diversity in the semi-batch reactor, and about equivalent to the initial diversity in the batch reactor. These results suggest that a diverse microbial consortium may play a role in MTBE biodegradation in the presence of BTEX.

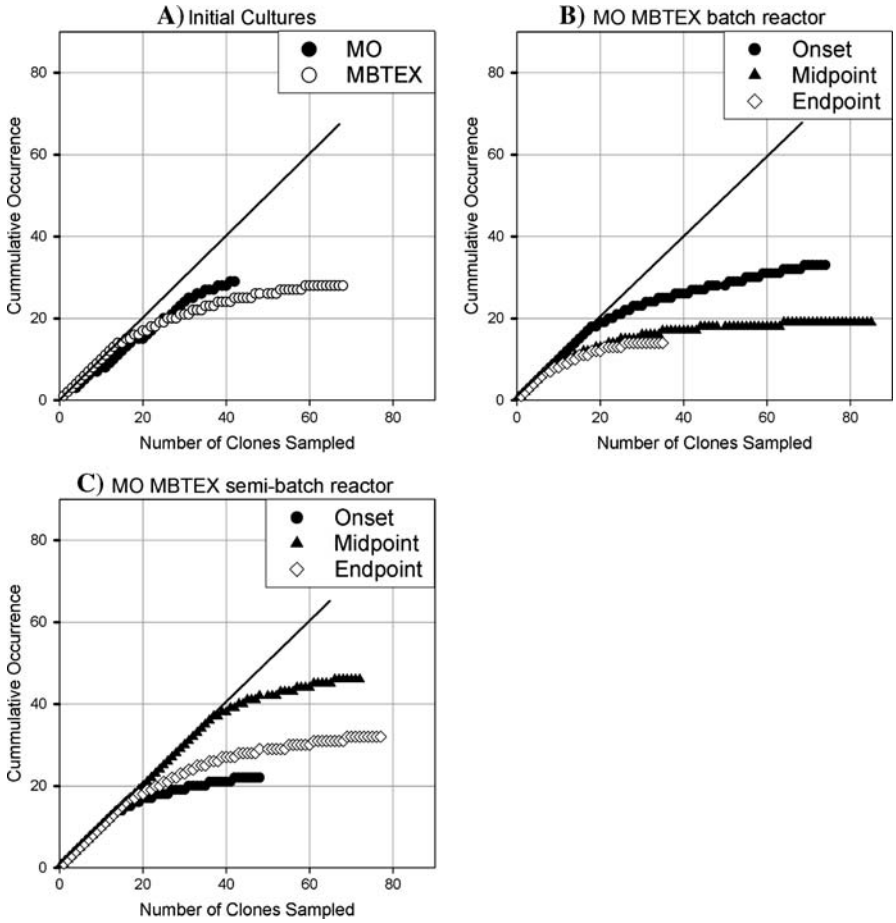

Fig. 3 Microbial diversity estimated by rarefraction curves generated from cloning and restriction fragment length polymorphism of 16S rRNA genes using *Msp*I restriction enzyme: (**A**) Initial cultures enriched on MTBE only (MO) or MTBE and BTEX (MB); (**B**) Batch reactor inoculated with the MO culture and fed MTBE and BTEX (MO MBTEX) and (**C**) Semi-batch reactor inoculated with the MO culture and fed MTBE and BTEX (MO MBTEX semi-batch reactor). The three sampling days for the reactors corresponded to the second observed biodegradation event: onset, midpoint and endpoint. The *solid lines* represent the hypothetical case that all sampled clones are unique (maximum possible diversity)

Quantitative response of strain PM1^T

Concentrations of the known MTBE-degrader, strain PM1^T, normalized to total Bacteria were measured by Q-PCR (Fig. 4). The presence of strain PM1^T in environmental samples has been used as a parameter to demonstrate the presence of MTBE-degraders and thus the likelihood of MTBE biodegradation (Hristova et al. 2003). No changes in total Bacterial concentration, as determined by number of 16S rRNA genes, were detectable in the reactors with time (data not shown). Interestingly, the proportion of strain PM1^T decreased initially in all of the reactors with respect to the original inocula. In the batch reactors inoculated with the MO culture only, the proportion of strain PM1^T remained constant with time. However, in the MB-inoculated batch reactors in the absence of BTEX, a significant increase in strain PM1^T was observed by the time of the MTBE biodegradation endpoint ($p < 0.0001$). Though it appears that strain PM1^T in the semi-batch reactor increased and then decreased to the initial level, this change was not statistically significant ($p \sim 0.98$).

The fact that strain PM1^T was present in all of the reactors, even when MTBE biodegradation was inhibited by BTEX, indicates that strain PM1^T may not be an optimal indicator of MTBE biodegradation potential when BTEX is present. Deeb et al. (2001) noted that MTBE-biodegradation by this strain can be inhibited in the presence of BTEX. The authors attributed this to preference of the MTBE-degrading enzyme for BTEX over MTBE. It is also of interest that concentrations of strain PM1^T were the lowest in the semi-batch reactor, which was most effective at biodegrading MTBE in the presence of BTEX. Strain PM1^T was also below the RFLP/cloning 6% detection limit as applied in this study, which is in agreement with the more sensitive Q-PCR results. These

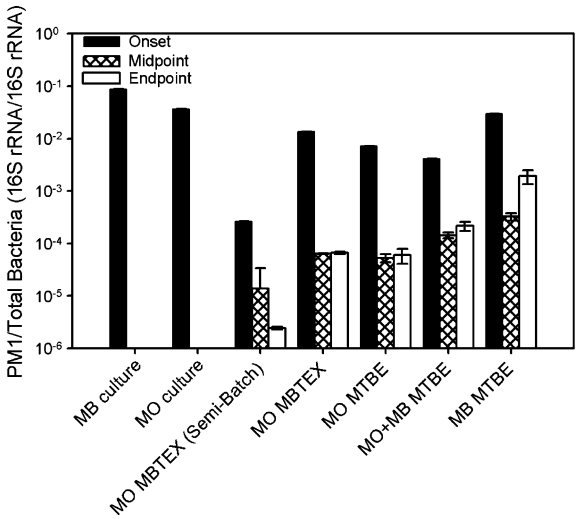

Fig. 4 Quantitative polymerase chain reaction (Q-PCR) analysis of initial (MO and MB), semi-batch reactor (MO MBTEX) and the batch reactors (MO MBTEX, MO MTBE, MO + MB MTBE, MB MTBE) cultures at three experimental time points during the second observed biodegradation event: onset, midpoint and endpoint, except for the MO MBTEX batch experiment, which showed inhibited MTBE degradation and was instead sampled on Day 12, 20 and 25, corresponding approximately to the MO MBTEX semi-batch reactor. *Error bars* represent the standard error of two replicate Q-PCR measurements

observations suggest that other MTBE-degrading microorganisms identified in the semi-batch reactor by cloning, such as *Hydrogenophaga* sp., may play a key role in biodegrading MTBE in the presence of BTEX.

Implications for MTBE treatment in the presence of BTEX

A major driver of this study was inconsistent reports of the ability to biodegrade MTBE in the presence of BTEX, which is often a critical factor in bioremediation considering the likelihood of co-contamination of these compounds. This study suggests that MTBE bioremediation in the presence of BTEX is feasible, and that overall success may be enhanced by several factors. First, it is important to recognize that the characteristics of the microbial consortium do matter. MTBE-degraders besides strain PM1^T may be key for overcoming BTEX inhibition of MTBE biodegradation. The consortia that were most successful in biodegrading MTBE in the presence of BTEX actually had the lowest numbers of strain PM1^T, but contained *Hydrogenophaga* sp. There was some evidence that higher diversity, as represented by estimated species richness, was associated with successful MTBE biodegradation in the presence of BTEX. Reactor configuration is also important for controlling the composition and performance of the microbial consortium. Previous studies characterizing BTEX inhibition of MTBE biodegradation have been carried out in batch reactors (Deeb et al. 2001). However, this study demonstrates that a semi-batch reactor provides better performance, probably because BTEX biodegradation intermediates can be flushed out. In other studies using continuous-flow reactors, comparable biodegradation rates of MTBE were observed in the presence and absence of BTEX (Pruden et al. 2001; Sedran et al. 2002; Pruden and Suidan 2004). Continuous-flow conditions are also more realistic for most remediation scenarios in the field.

In the broader sense, the results of this study also support further research into the possibility of designing microbial communities to achieve desired bioremediation performance. This approach has recently been suggested by Curtis and Sloan (2006). Implementation of appropriate molecular biological tools and modeling techniques makes this more feasible.

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