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Effect of benzene, toluene, ethylbenzene, and *p*-xylene (BTEX) mixture on biodegradation of methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) by pure culture UC1

Amy Pruden[1,2] & Makram Suidan[1,*]
[1]*Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, OH 45221, USA;* [2]*Present address: Department of Civil Engineering, Colorado State University, Fort Collins, CO 80523-1372, USA (*author for correspondence: e-mail: makram.suidan@uc.edu)*

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Abstract

The effect of a BTEX mixture on the biodegradation of methyl *tert*-butyl ether (MTBE) and its degradation intermediate, *tert*-butyl alcohol (TBA) was investigated in the pure bacterial culture UC1, which has been identified to be a strain of the known MTBE-degrader PM1 based on greater than 99% 16S rDNA similarity. Several degradation studies were carried out on UC1 at three initial concentration levels of MTBE or TBA: 6–7; 15–17; and 40–45 mg/l, both with and without BTEX present cumulatively at about half of the MTBE or TBA molar mass in the system. The BTEX mixture was observed not to affect either the rate or the degradation lag period of MTBE or TBA degradation, except that the TBA degradation rate actually increased when BTEX was present initially in the highest concentration studies. When serving as the sole substrate, the MTBE degradation rate ranged from 48 ± 1.2 to 200 ± 7.0 mg_{MTBE}/g_{dw} h, and the TBA degradation rate from 140 ± 18 to 530 ± 70 mg_{TBA}/g_{dw} h. When present with BTEX, MTBE and TBA rates ranged from 46 ± 2.2 to 210 ± 14 and 170 ± 28 to 780 ± 43 mg_{TBA}/g_{dw} h, respectively. In studies where varying concentrations of TBA were present with 5 mg/l MTBE, both compounds were degraded simultaneously with no obvious preference for either substrate. In the highest concentration study of TBA with 5 mg/l MTBE, BTEX was also observed to increase the ultimate rate of TBA degradation. In addition to exploring the affect of BTEX, this study also provides general insight into the metabolism of MTBE and TBA by pure culture UC1.

Introduction

Methyl *tert*-butyl ether (MTBE) is a fuel additive which has gained increasing attention over the past decade because of its widespread contamination in the environment as a result of leaking underground fuel storage tanks. *Tert*-butyl alcohol (TBA) is a degradation intermediate of MTBE and is also applied as a fuel additive. MTBE is considered to be a possible human carcinogen and TBA is considered to be a probable human carcinogen by the US Environmental Protection Agency. One especially critical issue to MTBE and TBA remediation in the environment is the effect of benzene, toluene, ethylbenzene and xylenes (BTEX) on their biodegradation. The health hazards of these compounds are well-known, and because they are also major constituents of gasoline the co-contamination of MTBE, TBA and BTEX is commonplace. While it is a widespread conception that BTEX inhibits MTBE biodegradation, the effect of BTEX on MTBE and TBA degradation has not been thoroughly researched. This is especially true with respect to the effect of BTEX mixtures.

To date, Deeb et al. (2001) have presented the most detailed study of substrate interactions

between MTBE and BTEX compounds. In this study, the effect of individual BTEX compounds on MTBE biodegradation was investigated in the pure culture PM1. Most studies were carried out with 20 mg/l of MTBE with 5–20 mg/l of the individual BTEX compounds. It was found that 20 mg/l of ethylbenzene or xylenes completely inhibited MTBE degradation, while concentrations as low as 5 mg/l of ethylbenzene or toluene noticeably slowed the MTBE degradation rate by strain PM1. While benzene did not completely inhibit MTBE degradation, it did noticeably slow degradation when present initially at 20 mg/l. Only after benzene was completely degraded did MTBE degradation rates increase back to those observed initially. However, in another recent study carried out with a mixed-culture containing PM1-related microorganisms, no effect of BTEX mixtures on MTBE degradation rates or lag periods was found, but a slight decrease in the rate of TBA degradation was observed (Sedran et al. 2002). This study consisted of both a batch and a continuous-flow reactor component. The maximum concentration of BTEX tested in the batch study was an initial concentration of about 10 mg/l of each of the four compounds together with 40 mg/l MTBE or TBA. In the continuous-flow reactor, which demonstrated more than 99.99% removal of MTBE, TBA, and BTEX, the influent BTEX was about 17 mg/l for each of the four compounds with 75 mg/l MTBE, but their actual concentration inside the reactor was only about 1 μg/l. Therefore, there may be a concentration and/or mixture effect of BTEX compounds present at or below concentrations of 20 mg/l. Mixtures of BTEX at these lower concentrations and their effects on MTBE and TBA are highly relevant to environmental remediation. Thus, there is a significant need to better understand the effect of low concentrations of BTEX mixtures on MTBE and TBA degradation.

This study examines the effect of a BTEX mixture on both MTBE and TBA degradation in an MTBE-degrading pure culture, UC1. This culture was isolated from a porous pot reactor receiving MTBE as the sole carbon substrate (Wilson et al. 2002). UC1 can be considered to be a strain of PM1 (Hanson et al. 1999), considering that the similarity of the 16S rDNA gene sequence was found to be greater than 99%.

Materials and methods

Isolation of pure cultures

Two pure cultures (UC1 and UC2) were isolated from a porous pot reactor receiving MTBE as the sole carbon substrate (Wilson et al. 2002) on day 856 of operation (1.0 day hydraulic retention time). Another pure culture (UC3) was isolated from the granular activated carbon of a fluidized-bed reactor (Pruden et al. 2003). The fluidized-bed reactor also received MTBE as the sole carbon source at the time of isolation and was seeded solely from the above porous-pot reactor. The porous-pot reactor was originally seeded with a mixture of culture provided by J. Salanitro (Shell Technology Center, Houston, TX), rinse water from MTBE contaminated soil collected from Port Hueneme, CA, and activated sludge from the Cincinnati Municipal Sewer District. The three pure cultures were kindly isolated from the reactor samples with the help of Dr Robert Steffan of Envirogen, Inc. (Lawrenceville, NJ). In order to isolate individual strains, reactor samples were diluted and plated onto R2A media. Individual colonies were then further purified by streaking them onto R2A plates. Purified strains were subsequently tested for MTBE degradative capability in aqueous media. UC1 and UC2 both formed light yellow colonies on R2A plates, and produced a sticky exopolymeric substance, as described by Hanson et al. (1999) for PM1, which caused the cells to flocculate in liquid media and made the culture unsuitable for turbidity measurements as an indicator of growth. UC3 formed small white colonies on R2A plates. Partial sequences ($\sim$550 bp) of the 16S rDNA gene were obtained for all three pure cultures, and were submitted to GenBank with the following accession numbers: AY185812, AY185813, and AY185814. Both UC1 and UC2 showed high similarity (99% and 98%, respectively) to the known MTBE degrader, PM1, of the Burkholderiales order of the β-Proteobacteria. UC3 showed 97% similarity to the genus *Mycobacterium*. UC3 exhibited very slow growth on MTBE, and a steady build-up of TBA. UC1 was chosen for further studies as a representative of the two fast-growing PM1 strains.

Construction of phylogenetic tree

A phylogenetic tree was constructed with the three sequences obtained in this study and compared with known MTBE-degrading microorganisms (utilizing MTBE as a carbon and energy source). Sequences of known MTBE-degraders and related microorganisms were obtained from GenBank at the National Institute of Health database: http://www.ncbi.nlm.nih.gov/. Sequences were aligned with each other using ClustalX (Thompson et al. 1997). Trees were constructed using maximum likelihood analysis with 100 bootstrap replicates, as implemented by PAUP* version b10 (Sinaur Associates, Sunderland, MA). Taxonomic classification utilized in this study is according to the 2003 release of the Ribosomal Database Project (http://rdp.cme.msu.edu/html/analyses_preview. html).

Experimental design

All cultures were grown on an initial concentration of 50 mg/l of MTBE and were utilized for the experiments when this concentration reached about 1 mg/l. Inoculum was taken directly from these bottles for the degradation experiments. Degradation studies took place in three series: (1) MTBE with and without BTEX, (2) TBA with and without BTEX, and (3) TBA with 5 mg/l MTBE, with and without BTEX. Three MTBE and TBA initial concentration levels were studied: 6–7, 15–17, and 40–45 mg/l. The reason that three ranges of concentrations rather than precise initial concentrations were targeted is because the scintillation vials were not standardized for total volume, and thus there was some slight variation in head space which had some effect on the final aqueous concentration. An additional source of variation in the initial concentrations was possible volatile losses during filter sterilization of the concentrated MTBE and TBA solutions prior to addition.

The target aqueous concentration for the BTEX compounds was one-half of the molar MTBE or TBA concentration (the four BTEX compounds present cumulatively in the aqueous phase at half the concentration of MTBE or TBA). 'BTEX' consisted of: benzene; toluene; ethylbenzene; and *p*-xylene mixed at equimolar concentrations. Attaining target BTEX compound concentrations was somewhat problematic, presumably because of their higher Henry's value, and also because any errors in the addition of the four compounds (each with different Henry's value) were additive. In order to account for the relatively higher Henry's constant and consequent losses during filter sterilization of the BTEX solutions, BTEX was actually provided at 10% greater than the target concentration. However, despite this adjustment, the initial concentration of BTEX still showed some degree of variability in the studies. The BTEX concentrations actually ranged from 0.3 to 0.6 times the molar concentration of MTBE or TBA. All of the actual measured values of BTEX and all other compounds were plotted and used in calculations. The effect of *m*-xylene or *o*-xylene was not investigated in this study.

Experiments were carried out in 30 ml (approx.) borosilicate glass scintillation vials sealed with Teflon septa, and maintained inverted (to preserve septa moisture) on a rotary shaker at 105 rpm. A salt solution containing NH_4^+-N nitrogen and trace minerals (Pruden et al. 2001) was first aliquotted into the 30 ml vials and sterilized in an autoclave with the caps and the septa. MTBE, TBA and BTEX were dissolved in a separate portion of the same salt solution, filter sterilized and added to the sterilized vials using aseptic techniques in a laminar flow hood. Each series was inoculated from the same bottle, in order to maintain approximately the same cell density throughout the series. Vortexing was required to disperse the cells in the medium prior to inoculating the vials. Average initial inoculum density for the three series of experiments as determined by triplicate plate-counting on R2A medium was: $7.5 \times 10^6 \pm 4.1 \times 10^6$ CFU/ml (MTBE), $4.2 \times 10^6 \pm 2.1 \times 10^6$ CFU/ml (TBA), and $2.3 \times 10^6 \pm 0.98 \times 10^6$ CFU/ml (TBA with low MTBE concentrations). Triplicate bottles were prepared for all studies. Controls were autoclaved and uninoculated. The total initial volume of the solutions was 10 ml.

Monitoring cell growth

In order to monitor cell growth, 100 μl of the batch cultures was removed at each sampling event through the septa of the scintillation vials. The vials were vortexed vigorously (about 20 s total in pulses of 5 s) prior to sampling in order to resuspend microbes and break-up any flocs present.

One dilution series was carried out for each of the triplicate samples in sterile saline (0.85% NaCl) to within the range of 10^{-2} to 10^{-6} and plated onto R2A plates as described in Deeb et al. (2001). The dilutions were vortexed for about 3 s prior to plating. Plate counting was deemed to be the most reliable means of monitoring changes in UC1 population density with time, considering that the mass was too small for accurate dry weight or protein measurements (only 100 μl available at each sampling event). Also, R2A medium was chosen because MTBE is volatile and it was thus infeasible to make agar plates with MTBE as the carbon substrate.

Cells required about 1 week of incubation at room temperature on R2A in order to form visible colonies. Plates yielding greater than 300 cfu, or less than 20 cfu were discarded as statistically unreliable. Dilution series which yielded two statistically reliable plates were found to vary in the number of cfus by a log order, which demonstrated that plate counting was a reliable means of monitoring cell growth in this culture. Monitoring cultures with plate counts also served as a control to verify the purity of the cultures. All experimental vials were observed to maintain purity throughout the studies.

Measurement of compounds

Degradation was monitored by aseptically removing 100 μl of the solution through the septa, diluting it in a 40 ml purge and trap autosampler vial filled with distilled and nitrogen purged water, acidifying it, and sealing it with a Teflon septum. MTBE, TBA, and BTEX concentrations were determined using a Tekmar–Dohrman (Cincinnati, OH) 3100 heated purge and trap (Type 'K' trap, Supelco, Bellefontaine, PA) linked to an Agilent (Palo Alto, CA) Gas Chromatograph with a DB-1 capillary column (J&W Scientific, Folsom, CA) and flame-ionization detector (FID). Ten point calibration curves were prepared for all standards in the range of 0.05–300 μg/l, with an acceptable R^2 value of 99.99% or better. Standards were checked with each set of samples run, with an acceptable error rate of ±5%. MTBE, TBA, benzene, toluene, ethylbenzene, and *p*-xylene were ACS grade and were obtained from Fisher Scientific (Fairlawn, NJ).

Calculation of degradation rates

Degradation rates were determined by plotting concentration versus time of each of the triplicate experiments in Sigma Plot 8.0 (SPSS, Inc., Chicago, IL) and determining the slope of the linear portion of each curve with the linear regression tool. Rates were normalized to the initial grams of dry weight by converting the average initial CFUs present to dry weight. This was necessary in order to provide a means of comparison between the three series of degradation studies which had slightly differing inoculum densities, and also for comparison with other studies in the literature. For consistency, the value chosen for conversion of CFU to gram dry weight throughout this study was 2.8×10^{-13} g dry wt/cell which is considered average for gram negative bacteria (Neidhardt et al. 1990). Obviously, an experimentally determined dry weight conversion specific to UC1 would have been ideal. However, attempts to grow UC1 to sufficient densities for dry weight determination were unsuccessful. Furthermore, there were concerns that the densities needed for accurate dry weight measurements were irrelevant to the much lower densities present in the degradation studies, and would incorporate a significant amount of error from build-up of inactive biomass. Therefore, the implementation of a standard value used consistently throughout the study appears to be the most appropriate. Standard deviations of the triplicates of all rates were determined using Excel 2002 (Microsoft, Corp.).

Calculation of degradation lags

A degradation lag was defined as the amount of time elapsed before reaching the linear portion of the degradation curve. In order to obtain a numerical estimate of the lag, a horizontal line was extrapolated from the initial concentration point on the *Y*-axis of the degradation plots to the linear regression line of the linear portion of the curve. The point of intersection was determined with respect to time in order to find the degradation lag period. The lag period of each of the triplicate degradation experiments was determined, and the standard deviation was calculated using Excel.

All data presented in this study was plotted using Sigma Plot 8.0.

Results

MTBE, TBA and BTEX degradation

MTBE degradation plots, with and without BTEX are presented along with the resulting growth in Figure 1. Although the culture had no prior exposure to BTEX, the compounds were observed to degrade readily in all experiments. Intermediate TBA was not detected in any of the MTBE degradation studies. The detection limit of TBA after the required dilutions for bringing MTBE within the upper limit of instrument detection was 10–20 μg/l. Degradation curves appeared very similar at all concentration levels, regardless of BTEX being present. Growth corresponding to degradation was also observed in the cultures at all concentration levels. BTEX did not appear to have a positive or negative effect on the growth of the cultures.

Similarly, TBA degradation, presented in Figure 2, was not negatively impacted by BTEX.

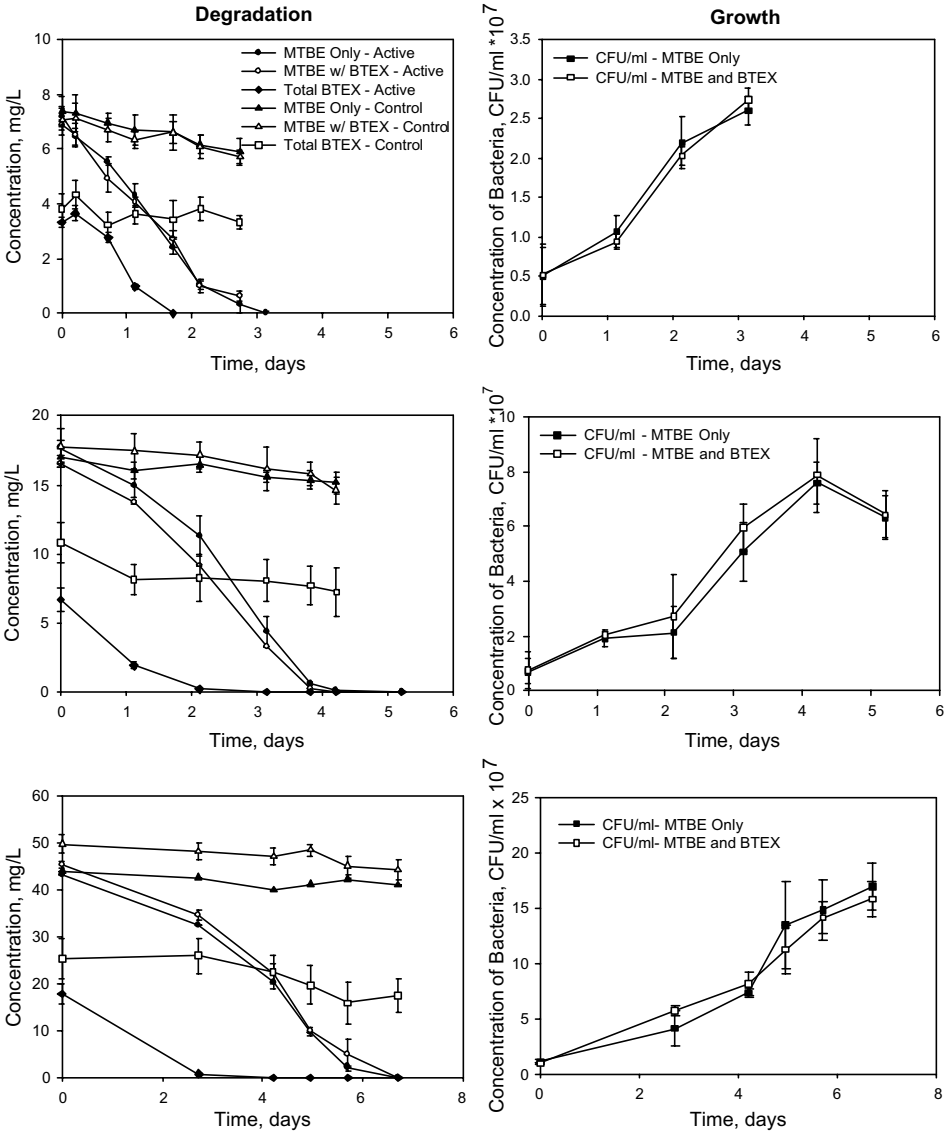

Figure 1. Plots of MTBE degradation and growth at three initial concentration levels: 7, 17 mg/l, and 44–45 mg/l, with and without BTEX present. TBA was not observed to be present as an intermediate.

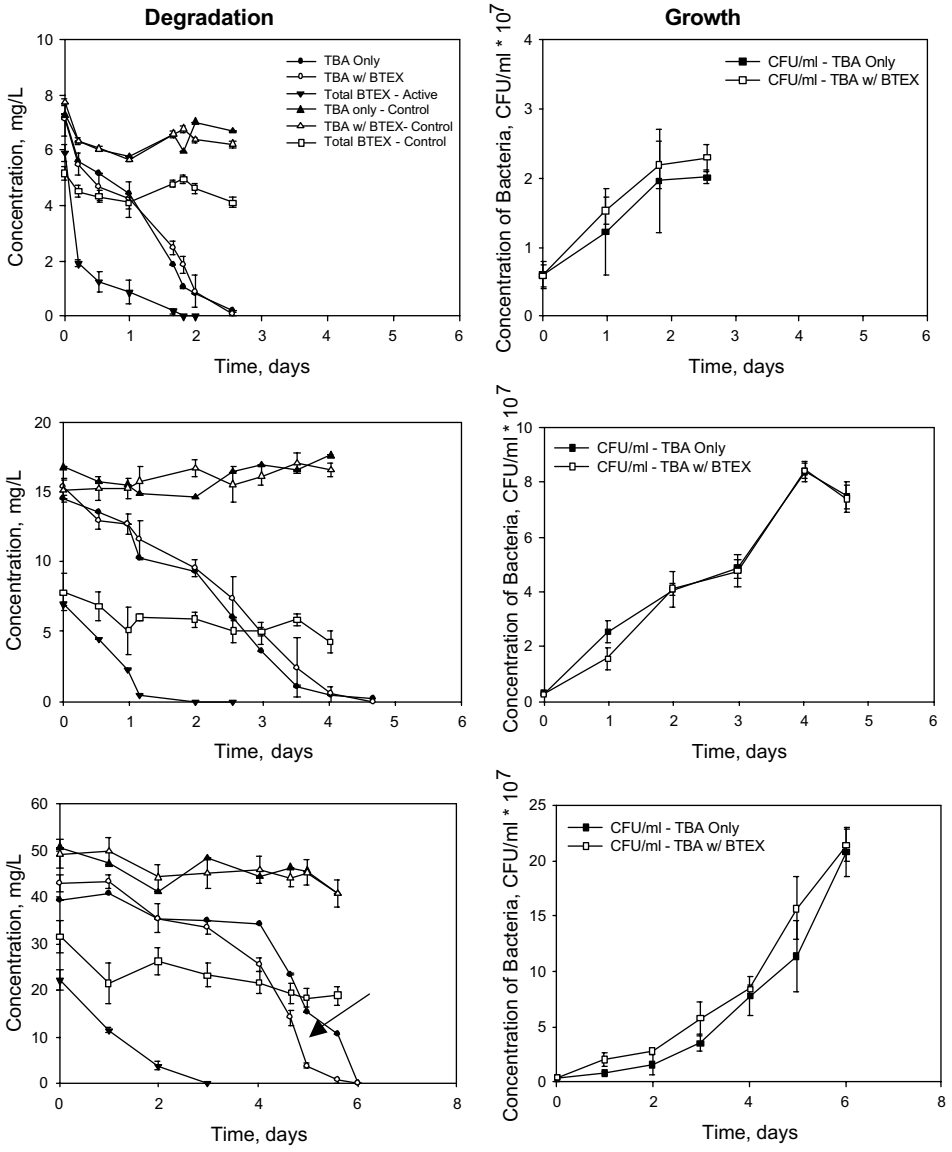

Figure 2. Plots of TBA degradation and growth at three initial concentration levels: 7, 15, and 40–42 mg/l, with and without BTEX. Arrow indicates evidence of faster ultimate TBA degradation rate when BTEX present initially.

Plots of growth are also presented in Figure 2, and indicate that UC1 is capable of utilizing TBA as a growth substrate. The growth pattern was similar to the pattern observed with MTBE as the growth substrate, and BTEX did not positively or negatively affect the growth observed.

In Figure 3, degradation studies with the same three initial concentration levels of TBA and BTEX are plotted, but with low concentrations of MTBE (5 mg/l) present in all studies. In all of these studies, MTBE and TBA were degraded simultaneously, with no noticeable preference for either substrate. Growth was also observed when TBA was present with MTBE, and the net growth in the two higher concentration studies was within one standard deviation of the growth of TBA without MTBE, as determined by $CFU/ml_{final} - CFU/ml_{initial}$. In the lowest concentration study, the relative amount of MTBE present was significant enough to noticeably affect the measured growth, and the growth observed for TBA with and without MTBE present was

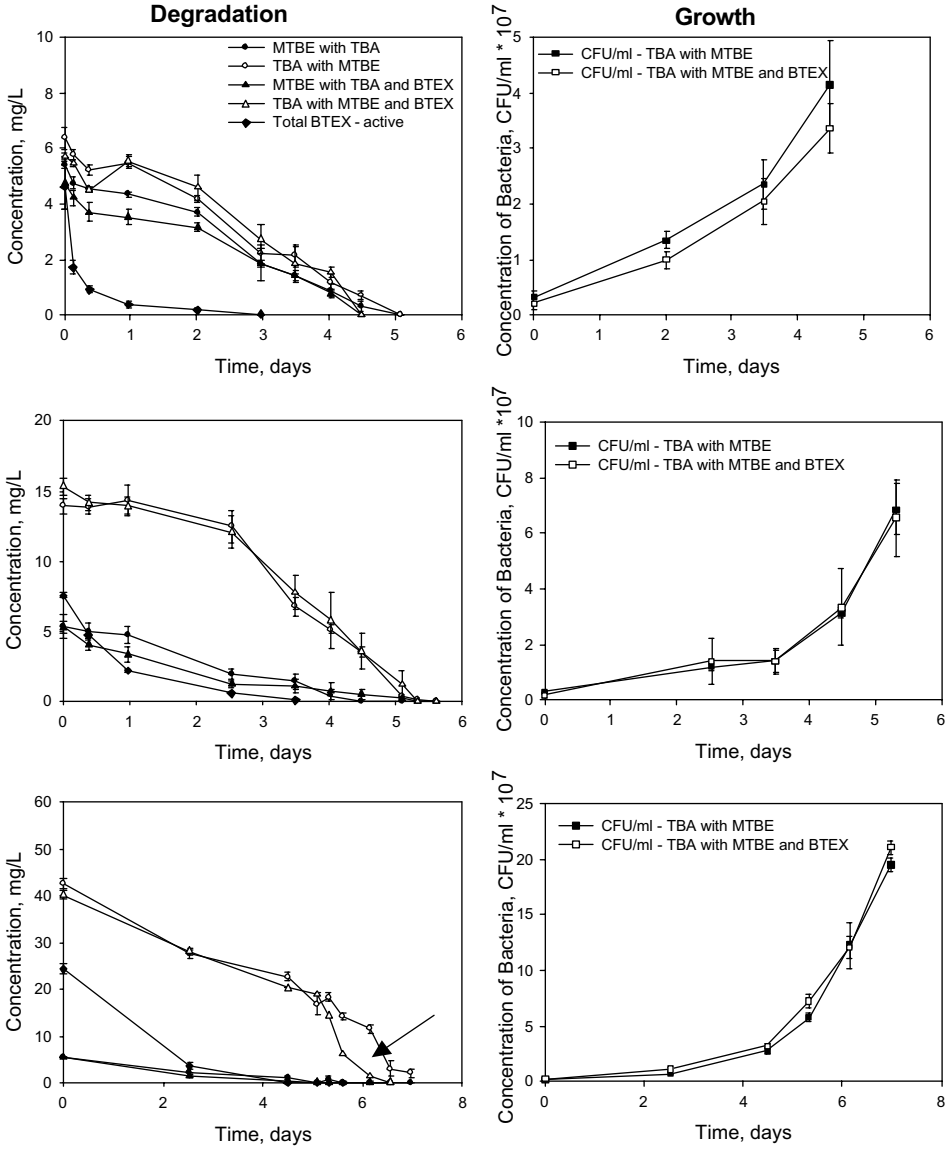

Figure 3. Plots of TBA degradation and growth (with MTBE initially present at 5 mg/l in all studies) at three initial concentration levels: 6, 15, and 40–42 mg/l, with and without BTEX provided at half the molar concentration. For brevity, controls not plotted. Arrow indicates evidence of faster ultimate TBA degradation rate when BTEX present initially.

$3.0 \times 10^7 \pm 6.1 \times 10^6$ and $1.4 \times 10^7 \pm 2.6 \times 10^6$ CFU/ml, respectively. BTEX was not observed to have a significant effect on growth.

MTBE, TBA, and BTEX degradation rates

Rates were determined from the regression line of the linear portions of the degradation curves plotted in Figures 1–3. Figure 4 provides a plot of the MTBE and TBA degradation rates observed with respect to initial concentration. The range of MTBE degradation rates for 7–44 mg/l of MTBE without BTEX was: 48 ± 1.2–200 ± 7.0 mg_{MTBE}/g_{dw} h, and for 7–45 mg/l MTBE with BTEX was: 46 ± 2.2–210 ± 14 mg_{MTBE}/g_{dw} h. The range of TBA degradation rates for 7–40 mg/l TBA without BTEX was: 140 ± 18–530 ± 70 mg_{TBA}/g_{dw} h, and for 7–42 mg/l TBA with BTEX was: 170 ± 28–780 ± 43 mg_{TBA}/g_{dw} h. It is clear from the rates obtained that

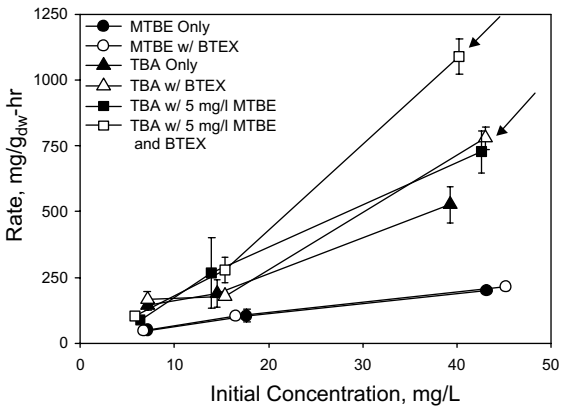

Figure 4. Plots of rate versus Initial concentration for MTBE and TBA. Error bars indicate standard deviation of triplicate samples. Arrows indicate evidence of faster TBA degradation rate when BTEX present initially at the highest concentration.

BTEX did not slow the rate of either MTBE or TBA degradation. In fact, at the highest initial TBA concentration the TBA degradation rate was noticeably higher when BTEX was present initially (Figure 4).

The same effect of BTEX was observed in the TBA studies with 5 mg/l MTBE: the rate of TBA degradation in the 40–42 mg/l experiment was significantly higher when BTEX was present, while BTEX had no noticeable effect at the two lower concentrations (Figure 4). In comparison with the TBA studies without MTBE, in the 40–42 mg/l range the TBA rates were higher with 5 mg/l MTBE present (530 ± 70 versus 730 ± 79 mg_{TBA}/g_{dw} h). In the 6–7 mg/l studies, however, the TBA rate was slightly slower when MTBE was present (88 ± 6 versus 140 ± 18 mg_{TBA}/g_{dw} h), and in the 15–17 mg/l studies the rates were within one standard deviation of each other (Figure 4). The 5 mg/l MTBE rates with varying TBA concentrations were all found to be within one standard deviation of each other, and ranged from 75 ± 14 to 103 ± 45 mg_{MTBE}/g_{dw} h, with an average and standard deviation of all 18 experiments of: 85 ± 48 mg_{MTBE}/g_{dw} h. There was no clear trend observed with respect to the concentration of TBA present and the rate of MTBE degradation observed. The degradation rates calculated for the 7 mg/l MTBE studies (series 1) were also noted to be within the standard deviation of the 5 mg/l MTBE rates in the third series, though on average the rates of 5 mg/l MTBE with TBA were about double.

Finally, BTEX degradation rates, determined as the total disappearance of all four BTEX compounds, were similar for all experiments with respect to concentration, and were within the range of 28 ± 14–520 ± 14 mg_{BTEX}/g_{dw} h for the concentration ranges of the three series of studies.

Degradation lag periods

As described above, a degradation lag was defined as the amount of time required before linear degradation commenced. The lag times were determined from Figures 1–3. It should be noted that a lag period may be reported which is shorter than the time of the initial sampling point by the method the lags were calculated. A plot of the effect of BTEX on the degradation lag period of MTBE and TBA is presented in Figure 5. The lag periods were found to increase linearly with initial concentration in all studies. Degradation lag periods were within one standard deviation of each other at each concentration whether or not BTEX was present (Figure 5). Therefore, BTEX did not noticeably affect the degradation lag of either MTBE or TBA.

Because this study primarily focused on the effect of BTEX on MTBE and TBA degradation, rather than BTEX degradation itself, there were too few data points in some of the studies to quantify the BTEX degradation lag. Clearly any BTEX lags if present did not extend beyond the

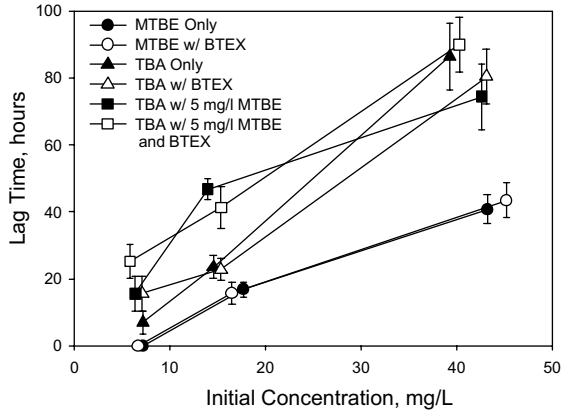

Figure 5. Plots of degradation lag observed versus Initial concentration (MTBE or TBA). Error bars indicate standard deviation of triplicate samples.

first few hours in any of the studies (Figures 1–3). Previous studies have shown that benzene and toluene lags in PM1 are in the order of about 2–3 h (Deeb et al. 2001), which might not have been detectable in this study. In the 7 mg/l MTBE study, it appears there might have been a lag in BTEX degradation of about 5 h (Figure 1). In most of the BTEX studies, however, the linear portion of the degradation curve extended through the initial concentration point, indicating an estimated lag time of zero.

Growth of UC1

Growth of UC1 was observed in all degradation studies, as shown in Figures 1–3. Figure 6 presents a plot of the observed growth as mg dry weight versus mg MTBE or TBA consumed. Mass of BTEX was not considered in this plot, and TBA growth with 5 mg/l MTBE present is plotted, but not included in the regression analysis in order to avoid introduction of error. Ninety-five percent confidence intervals on the linear regression line estimating the yield of MTBE and TBA did not overlap, indicating a difference in the yield between the two compounds. From this plot it is also apparent that the presence of BTEX did not enhance nor diminish the amount of growth observed, and both MTBE and TBA values with and without BTEX could be plotted on the same linear regression line. Also, values for growth on TBA with 5 mg/l MTBE present in the two higher concentration studies are within one standard deviation of the values obtained without MTBE present, while some difference is noted in the lowest concentration study, where the relative concentrations of MTBE and TBA were similar.

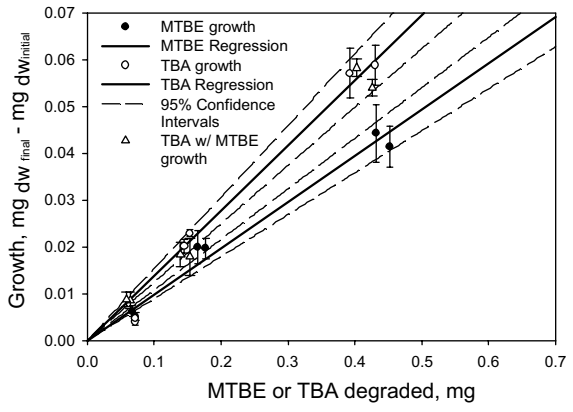

Figure 6. Linear regression of growth (mg dry weight) versus mass (mg) of MTBE or TBA utilized. Dashed lines indicate 95% confidence intervals on the regression line estimates. TBA growth with 5 mg/l MTBE present (triangles) is also plotted, but not included in the regression analysis.

Phylogenetics

Figure 7 presents a phylogenetic tree of the three MTBE-degrading pure cultures (UC1, UC2, UC3) isolated in this study compared with other MTBE-degrading microorganisms utilizing MTBE as a carbon and energy source reported in the literature. The bootstrap values indicate the percentage of tree replicates (100 total) which clustered together the groups indicated by the node. For example, the gram negative bacteria were grouped together 100 times out of 100, while UC1 was found to be more closely related to UC2 58 times out of 100 (and thus more closely related to PM1 42 times out of 100). All three of these strains; PM1, UC1 and UC2 showed greater than 98% similarity to each other, indicating that they are variations of the same strain.

Discussion

MTBE and TBA degradation by pure culture UC1

Both MTBE and TBA were degraded by UC1, which utilized each as a growth substrate. UC1 thus contributes to the growing list of MTBE degrading pure cultures which have been isolated to date. UC1 showed 99% 16S rDNA sequence homology to the known MTBE degrader PM1, which is sufficient to identify it as a strain of PM1. Rates of MTBE degradation by UC1 in this study were very similar to what had previously been estimated for PM1. For a concentration range of 5, 50, and 500 mg/l the rates of MTBE degradation by PM1 estimated were: 0.07, 1.17, and 3.56 μg/ml h, for an inoculum density of 2×10^6 cells/ml (Hanson et al. 1999). In this study, for a range of concentrations of 7, 17 and 44 mg/l, the rates were: 0.101, 0.218, and 0.415 μg/ml h. The inoculum density in this study was just slightly higher ($7.48 \times 10^6 \pm 4.09 \times 10^6$ CFU/ml). Recently it has been reported that PM1-related

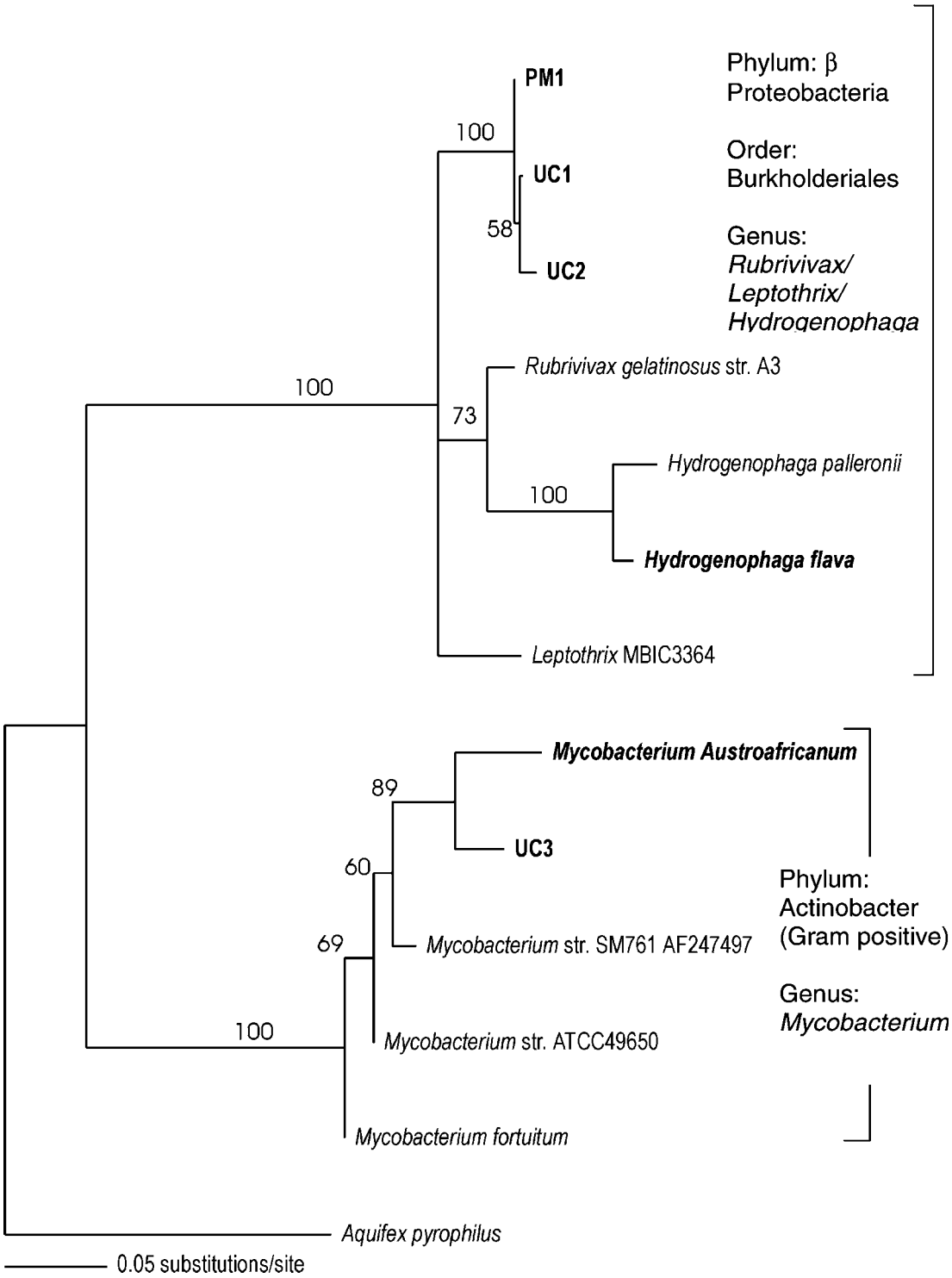

Figure 7. Phylogenetic analysis of pure cultures isolated in this study (UC1, UC2, UC3) compared with three known pure cultures (bold): PM1, *Hydrogenophaga flava*, and *Mycobacterium austroafricanum*. Tree was constructed by maximum likelihood analysis with 100 bootstrap replicates, reported for nodes greater than 50. *Aquifex pyrophilus* is the outgroup.

microorganisms are widespread in MTBE-contaminated environments (Hristova et al. 2003). PM1-related microorganisms have also been commonly found in MTBE-degrading bioreactors (Pruden et al. 2003; Sedran et al. 2002).

Effect of BTEX on MTBE and TBA degradation

A significant outcome of this study is the demonstration that the BTEX mixture did not inhibit MTBE or TBA degradation at the concentrations studied. This result is somewhat unexpected in light of previous research on MTBE and BTEX substrate interactions. First of all, BTEX, in this case X being *p*-xylene, did not inhibit degradation of MTBE, nor did it induce a lag in MTBE degradation, as was expected based on the studies by Deeb et al. (2001) on PM1. There may be several reasons for these differences, one being that the concentrations of BTEX were individually about equal to or lower than those in the previous study. The maximum initial total BTEX concentration in this study was 30 mg/l, with each of the four BTEX compounds being in the range of 6–10 mg/l. In the study done with PM1, the initial concentration of ethylbenzene or xylene which completely inhibited MTBE degradation was 20 mg/l, while 20 mg/l of benzene, 5 mg/l of ethybenzene, or 5 mg/l of toluene demonstrated some inhibition. However, although an initial concentration of 20 mg/l of benzene showed inhibition, this inhibition continued until the benzene was degraded to completion. Another possibility is that mixtures of BTEX do not have an additive inhibitory effect. Results from Deeb et al. suggested that the BTEX compounds are degraded via the same aromatic degradation pathway, but that benzene, followed by toluene, are the most efficient inducers of this pathway. Perhaps when BTEX compounds are present in mixtures, this pathway is more efficiently induced, and minimizes the inhibiting effect of ethylbenzene and xylene. Another possibility is that the UC1 strain of PM1 is not as sensitive to BTEX toxicity.

The results of this study with respect to the effect of BTEX are supported by observations in several mixed culture studies. For example, Sedran et al. (2002) showed that a mixed culture containing PM1-like microorganisms could efficiently degrade MTBE or TBA together with BTEX in both batch and continuous-flow studies. Also an MTBE-degrading fluidized-bed reactor, which was seeded with the same culture from which UC1 was isolated, degraded BTEX spontaneously with no prior acclimation period (Pruden et al. 2003). Furthermore, the same observation was recently made in a membrane bioreactor which had also been acclimated to MTBE as the sole carbon substrate (unpublished data), and which also contains PM1-like microorganisms (Morrison et al. 2001). Previously these observations had been attributed to the effect of having a mixed culture (Sedran et al. 2002), but results of this study indicate that this is not necessarily the case. An important conclusion of this study therefore is that BTEX mixtures and MTBE can be biodegraded simultaneously, which could help with their bioremediation in the field.

One particularly interesting finding in this study was TBA was degraded at a faster rate when BTEX was present, especially at the highest concentration. This observation is also supported by results recently reported by Sedran et al. (2002) which suggest that the presence of BTEX in the feed enhanced the overall capacity of a culture to degrade TBA in a continuous-flow system. One possibility is that BTEX enzymes are also able to degrade TBA. Exploring the exact mechanism of BTEX-enhanced TBA degradation would be an interesting topic of further research and could reveal further points of optimization in MTBE/BTEX remediation.

MTBE and TBA metabolism of UC1

In UC1 it appears that TBA degradation is faster than MTBE degradation. This is supported by three major observations: (1) In the study with 7 mg/l TBA and 5 mg/l MTBE together, the TBA rate was significantly faster in each of the triplicate studies than the MTBE rate (though the average of the three shows some overlap in error: 88 ± 6 versus 77 ± 9 mg_{MTBE}/g_{dw} h); (2) TBA rates from the second and third series of studies normalized to the initial cell mass were all significantly higher than the corresponding MTBE rates in the first series of studies (Figure 4) (this was also true if the rates were not normalized to cell mass, and the TBA studies both had a slightly lower inoculum density than the MTBE studies); (3) there was not any detectable build-up of TBA as an intermediate in any of the MTBE experiments (Figure 1). Sev-

eral studies have found varied results concerning the rates of MTBE versus TBA degradation and microbial preference for these substrates. For example, other pure culture studies reported thus far also have shown that some organisms metabolize MTBE faster than TBA, such as *Hydrogenophaga flava* (Hatzinger et al. 2001), and others TBA faster than MTBE, such as *Mycobacterium austroaficanum* (François et al. 2002). In mixed-culture studies, some have also found MTBE to degrade faster (Wilson et al. 2002) while others have found that TBA degrades faster (Sedran et al. 2002). Thus it appears that diversity exists among various cultures and their relative rates of MTBE and TBA degradation.

With respect to the enzymes responsible for MTBE and TBA degradation, some important observations can be made. (1) In the 7 mg/l TBA/MTBE study (Figure 3–top panel) both MTBE and TBA were degraded simultaneously with no clear preference for either substrate. (2) Average 5 mg/l MTBE rates with TBA at all concentrations were within one standard deviation of 7 mg/l MTBE rates from the first series without TBA, though on average were about double (85 ± 48 versus 48 ± 12 mg_{MTBE}/g_{dw} h). (3) TBA rates with MTBE were comparable to rates without MTBE in the 15–17 mg/l studies, but were almost half the TBA rates without MTBE in the 6–7 mg/l studies (88 ± 6 versus 140 ± 18 mg_{TBA}/g_{dw} h). (4) In the 40–45 mg/l studies, the TBA rates with MTBE initially present were significantly higher than TBA rates without MTBE (Figure 4). One possible explanation for these results is that two separate enzymes are primarily responsible for MTBE and TBA degradation, but that the MTBE enzyme is secondarily able to degrade TBA. This would explain why the two compounds were degraded simultaneously, and why TBA rates were elevated in the 45 mg/l studies after the 5 mg/l MTBE was degraded. MTBE enzymes, after consuming all of the MTBE, were able to contribute to the degradation of TBA. If the same enzyme is primarily responsible for both TBA and MTBE degradation, then these results are difficult to explain. In this case, it be expected that increasing ratios of TBA:MTBE would slow the MTBE rate, assuming that they are competing for the same active site. Instead, it was observed that the rate of degradation of 5 mg/l of MTBE remained consistent throughout the TBA/MTBE studies and were within one standard deviation of each other. These MTBE degradation rates were also within one standard deviation of the 7 mg/l MTBE rates observed in the first series of studies, though actually slightly higher on average. If the increased average rate of MTBE degradation in the presence of TBA is a real phenomenon, this could be due to the TBA helping boost the microbial populations. The fact that 6–7 mg/l TBA rates were lower on average when MTBE was present is likely due to the processing of intermediate TBA from MTBE degradation. When considering the TBA intermediate, the net amount of TBA degraded almost doubles, which would account for the observed rate of degradation decreasing by almost half. Considering all of the above, it seems that degradation of MTBE and TBA by two separate enzymes with secondary degradation of TBA by the MTBE enzyme presents the most plausible explanation for the results observed.

While no study has yet provided precise identification of the enzymes involved with MTBE or TBA degradation, other experiments with pure culture have provided some insight. An enzyme inhibition study done with *Hydrogenophaga flava* ENV735 suggested that separate enzymes are responsible for MTBE and TBA metabolism (Hatzinger et al. 2001). Evidence for this was also supported by lack of stoichiometric conversion of MTBE to TBA which had previously been observed in co-metabolizing cultures (Steffan et al. 1997). Both MTBE and TBA were degraded simultaneously, but TBA rates were slower than MTBE rates in *H. flava*. In contrast, studies reported with *Mycobacterium autroafricanum* IFP 2012 demonstrate the involvement of very similar and possibly identical monooxygenase enzyme systems responsible for both MTBE and TBA metabolism. In general, monooxygenase enzymes appear to be the prime candidate for MTBE and TBA degradation. Thus, while no study has precisely characterized the enzymes involved with MTBE and TBA degradation, it is evident that similarities and differences exist with respect to the metabolic diversity of MTBE degraders.

Growth of UC1 on MTBE and TBA

Monitoring of bacterial growth during these degradation studies clearly indicated that UC1 is capable of degradation and growth on both

MTBE and TBA. Plotting the observed growth against milligrams substrate degraded in Figure 6 allows for an estimate of the observed yield (Y_{obs}). Growth observed in the TBA studies with 5 mg/l MTBE present is also plotted in Figure 6, but not included in the regression analysis in order to avoid introduction of error from the low levels of MTBE present. From Figure 6, Y_{obs} for MTBE is 0.098 mg cells/mg MTBE, and Y_{obs} for TBA is 0.14 mg cells/mg TBA. These values are comparable with those reported in the literature (Fortin et al. 2001; Hanson et al. 1999; Morrison et al. 2001; Salanitro et al. 1994; Wilson et al. 2002).

In examining the yield of MTBE and TBA in this study, two observations are readily apparent: (1) Both the MTBE and TBA yields are low, and (2) the TBA yield is slightly higher than the MTBE yield. With respect to the first point, low yields are a common factor in MTBE biodegradation studies (Fortin et al. 2001; Hanson et al. 1999; Morrison et al. 2001; Salanitro et al. 1994; Wilson et al. 2002). The ultimate reason for this low yield has been subject to significant debate. This issue is explored in-depth in a recent study by Fortin et al. (2001). One of the first explanations for the low yield of MTBE suggested was that MTBE may be an 'uncoupler' of oxidative phosphorylation (Salanitro 1995). Uncouplers significantly reduce the observed yield from that theoretically expected by rendering the cytoplasmic membrane permeable to protons, thus diminishing the proton motive force and its energetic potential for ATP synthesis and resulting cell growth. However, if MTBE is an uncoupler, then lower yields should be observed when MTBE is added to cultures growing on an alternative substrate, which is not generally observed (Fortin et al. 2001). In this study, the yields were about the same for the TBA experiments with and without MTBE (Figure 6). Also, no effect was seen in the degradation behavior of TBA when MTBE was present (except that the TBA rate was actually higher in the 45 mg/l TBA/MTBE study). Other possible causes of the low yield of MTBE proposed by Fortin et al. include both high energy requirements for catabolism (i.e. NADH demand) and/or for anabolism, but this could not be confirmed. Thus, though the observation that MTBE has a lower than expected yield has been established, the exact causes remain disputed.

The observation that the yield of TBA is slightly higher than MTBE is also in agreement with other studies and has interesting implications (Fortin et al. 2001; François et al. 2002). Based on available electrons, MTBE (30e−) should theoretically have a higher yield than TBA (26e−) (Fortin et al. 2001). It is widely accepted that the cause for discrepancy is that the MTBE to TBA conversion step is catalyzed by a monooxygenase enzyme, and thus requires highly energetic input of NADH. Fortin et al. attempted to stimulate the degradation of MTBE through the addition of reducing equivalents in order to support the validity of this theory, but they were not successful. TBA itself also has a low yield, which may be the result of either of the possible reasons explored above, though this has yet to be validated. Repeating the experiments described by Fortin et al. with a pure culture may provide better insight into the cause of the discrepancy in yield between TBA and MTBE.

The reason(s) that BTEX failed to stimulate growth in either the MTBE/BTEX or TBA/BTEX experiments are not exactly clear. From studies carried out with PM1 (Deeb et al. 2001) it was demonstrated that benzene and toluene can each serve as sole growth substrates. Therefore, it would have been expected that BTEX would have affected growth, and higher yields would have been observed when BTEX was present. Instead, growth observed in experiments with BTEX was about the same as growth without BTEX (Figure 6). One possibility considering that benzene is a known growth uncoupler (Lee 1999) is that any growth stimulated by BTEX was dissipated proportionally by uncoupling. However, it seems unlikely that such an effect could be exactly proportional, such that no difference in growth with or without BTEX was observed for any of the concentration levels. Further experiments would be required, such as studying BTEX and individual BTEX compounds without MTBE or TBA, in order to determine why BTEX did not stimulate growth in these experiments.

Phylogenetics of UC1 and other MTBE-degrading bacteria

Only three MTBE-mineralizing pure cultures using MTBE as the sole carbon and energy substrate have been reported to date. These include: PM1 of the Burkholderiales order of the β-Proteobacteria (Hanson et al. 1999); *Hydrogenophaga flava* ENV735 (Hatzinger et al. 2001), a hydrogen-oxi-

dizing bacterium also belonging to the β-Proteobacteria; and *Mycobacterium austroafricanum* IFP 2012, a gram positive bacterium belonging to the Actinobacteria phylum (François et al. 2002). UC1 shows 99% similarity to the known MTBE degrader PM1 of the β-Proteobacteria and based on this similarity is considered to be a strain of PM1. The morphology is also identical to PM1, in terms of the light yellow color and the sticky exopolymeric substances which it produces.

The phylogenetic tree presented in Figure 7 includes the sequences obtained from UC1, as well as an additional PM1-related pure culture (UC2) which was isolated from the same reactor, and UC3, which was isolated from the same seed culture. UC3, belonging to the genus *Mycobacterium*, represents a phylogenetically distinct MTBE degrader from the one in this study. The phylogenetics of the MTBE-degrading bacteria to date seems to indicate that they fall within either of two groups, the Burkholderiales order of the β-Proteobacteria, or the genus *Mycobacterium* of the Actinobacter phylum. As indications are that MTBE is not a very energetically favorable substrate, many of the bacterial lineages may have developed similar strategies in degrading MTBE. In pure culture studies such as these, it is hoped that general trends concerning limitations as well as diverse metabolic capabilities may be identified and eventually exploited for the purposes of improving our current technologies in the application of bioremediation to cleaning the environment.

Conclusions

This study investigated the effect of BTEX on MTBE and TBA biodegradation by pure culture UC1. It was found that BTEX mixtures did not to have a negative impact on MTBE or TBA degradation, either in terms of the degradation rate or the degradation lag time. At higher concentrations, the presence of BTEX actually increased the TBA degradation rate. In addition, insight into the general metabolism of UC1 was gained. This study suggests that TBA is degraded simultaneously with MTBE and at a slightly faster rate and higher cell yield than MTBE. Overall results of this research are promising for the development of remediation strategies involving simultaneous biodegradation of MTBE, TBA and BTEX mixtures.

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