Bioremediation Journal
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400853

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To cite this Article Deeb, Rula A. andAlvarez-Cohen, Lisa(2000) 'Aerobic Biotransformation of Gasoline Aromatics in MultiComponent Mixtures', Bioremediation Journal, 4: 2, 171 — 179
To link to this Article: DOI: 10.1080/10889860091114211
URL: http://dx.doi.org/10.1080/10889860091114211

Aerobic Biotransformation of Gasoline Aromatics in MultiComponent Mixtures

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Abstract: The primary objective of this study was to evaluate the impact of substrate interactions on the biotransformation rates and mineralization potentials of gasoline monoaromatics and methyl *tert*-butyl ether (MTBE), compounds that commonly co-exist in groundwater contaminant plumes. A mixed culture was derived from gasoline-contaminated aquifer material using toluene as the enrichment substrate. Two pure cultures, *Rhodococcus* sp. RR1 and RR2, were isolated from the mixed culture. The three toluene-grown cultures were shown to biotransform all of the six BTEX compounds (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene), both individually and in mixtures, over a broad range of concentrations. The mixed culture was shown to degrade all of the BTEX compounds to $^{14}CO_2$, while the two isolates mineralized BTE(*m*-/*p*-)X, but biotransformed *o*-xylene without production of carbon dioxide. Studies to evaluate substrate interactions caused by the concurrent presence of multiple BTEX compounds during their biodegradation revealed a number of patterns ,including competitive inhibition and cometabolism. Ethylbenzene was shown to significantly inhibit BTX degradation in mixtures. MTBE was not biodegraded by any of the three toluene-grown cultures over a range of MTBE concentrations. Furthermore, the presence of MTBE at concentrations of 2 to 100 mg/L had no effect on BTEX biotransformation rates.

Introduction

Petroleum hydrocarbons are common groundwater contaminants. Of particular concern are gasoline aromatics and oxygenates, primarily benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene (BTEX) and methyl *tert*-butyl ether (MTBE). The toxicity of BTEX compounds has been well documented (Dean, 1985) and are relatively mobile in aqueous systems compared with aliphatic gasoline constituents. MTBE migrates in the subsurface at essentially the speed of groundwater due to its high solubility (ca. 50,000 mg/L), insignificant sorption to aquifer solids, and resistance to microbial degradation (Squillace et al., 1997).

Although the biochemistry of the aerobic degradation of individual BTEX compounds is fairly well understood (Smith, 1990), substrate interactions such as inhibition and competition can lead to uncertain BTEX biodegradation outcomes in contaminant mixtures (Oh et al., 1994). A few studies have been conducted to elucidate substrate interactions among monoaromatics in multicomponent liquids (Alvarez and Vogel, 1991; Chang et al., 1993; Oh et al., 1994); however, these studies did not include ethylbenzene and all three xylene isomers. Furthermore, most mixture studies have focused on BTEX biotransformation and not on mineralization to carbon dioxide. To date, no bacterial culture has been reported to mineralize all of the six BTEX components in a mixture. Accordingly, recent efforts in this area of research have been devoted to genetically constructing a bacterium that incorporates the requisite biological pathways for the mineralization of monoaromatic mixtures (Lee et al., 1994; 1995a; 1995b).

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1058-8337/00/$.50

Only a few microbial cultures have been reported to degrade MTBE under aerobic conditions (Garnier et al., 1999; Hanson et al., 1999; Hardison et al., 1997; Mo et al., 1997; Salanitro et al., 1994; Steffan et al., 1997). Field data indicate that gasoline plumes commonly contain MTBE together with BTEX compounds (Borden et al., 1997). High concentrations of MTBE can potentially impede BTEX biodegradation (Salanitro and Wisniewski, 1996). Thus, it is important to evaluate the effects of MTBE on BTEX biodegradation rates.

The objectives of this study are as follows: (1) to investigate the biodegradation and mineralization potentials of BTEX compounds by three microbial cultures, a consortium and two isolates derived from gasoline-contaminated aquifer material; (2) to characterize substrate interactions during the biodegradation of BTEX mixtures; and (3) to evaluate MTBE biodegradability and to quantify its impact on BTEX biotransformation rates.

Materials and Methods

Cultures and Cell Growth

A mixed microbial culture was derived using soil from a gasoline-contaminated aquifer located at Lawrence Livermore National Laboratory, Livermore, CA (Deeb and Alvarez-Cohen, 1999). The culture was enriched using toluene as the sole carbon and energy source and was grown in a continuous flow reactor at 35°C (Deeb and Alvarez-Cohen, 1999). This temperature was experimentally shown to enhance microbial activity and to optimize BTEX degradation rates by the culture (Deeb and Alvarez-Cohen, 1994a,b; 1999). Two bacterial strains were isolated from the mixed culture using toluene as the sole source of carbon and energy. Preliminary identification of these isolates by Microbial ID, Inc. (Newark, DE) using Fatty Acid Methyl Ester (FAME) analysis revealed that one of the cultures was most closely related to *Rhodococcus rhodochrous*, while the other was most closely related to *Gordona bronchialis*, formerly classified as *Rhodococcus bronchialis*. 16S rRNA gene sequencing indicated that both isolates are members of the *Rhodococcus* genus. In this study, the two isolates are referred to as *Rhodococcus* sp. RR1 and RR2.

Rhodococcus sp. RR1 and RR2 were grown on toluene in gas-tight Teflon®-sealed 250-mL glass bottles. Cells were harvested during the exponential growth phase by centrifugation and resuspended in Berkeley mineral salts media (BMSM) (Deeb and Alvarez-Cohen, 1999). A 1 to 2 mL sample of the stock cell suspension was used to inoculate experimental bottles leading to culture densities in the bottles of 35 to 250 mg/L. For the mixed culture, the cell inocula consisted of a 10 to 20 mL sample from the continuous flow reactor leading to culture densities in the bottles of 140 to 310 mg/L. The culture samples from the continuous flow reactor were purged with nitrogen at a flow rate of 4 L/min for 5 min to remove residual toluene. Culture densities in each of the experimental bottles were measured in duplicate as the difference between sample weights after drying at 105°C for 8 h followed by combustion at 550°C for 30 min.

Experimental and Analytical Procedures

Single and multiple substrate biotransformation studies were conducted in 250-mL clear glass bottles containing 100 mL of BMSM and six glass beads (diameter = 5 mm) to promote mixing. The bottles were sealed with Teflon®-lined Mininert® valves (Alltech Co., Deerfield, IL) and incubated at 35°C with shaking at 150 rpm. Disappearance of aromatic compounds was monitored by headspace analysis using a gas chromatograph equipped with a flame ionization detector as described previously (Deeb and Alvarez-Cohen, 1999).

Mineralization studies were conducted in bottles like those described above using ^{14}C-labeled BTEX compounds. Sterile controls containing BTEX compounds, but no cells, were used to monitor abiotic losses of aromatic compounds. To evaluate the mineralization of a specific component within a BTEX mixture, only one radiolabeled chemical was added to the six component mixture. Following culture inoculation, the bottles were incubated at 35°C with shaking at 150 rpm for 48 to 60 h after which the experiment was terminated with the addition of 0.3 mL of 6.15 N NaOH. The procedure for ^{14}C analysis was modified from Alvarez-Cohen and McCarty (1991). Three different measurements were performed for each bottle: one sample was removed from the reactor, added to 10 mL of ScintVerse scintillation cocktail (Fisher Scientific Co., Fair Lawn, NJ) in a 20 mL glass scintillation vial, and assayed for radioactivity; a second sample was purged with N_2 (800 mL/min for 3 min), and added to 10 mL of scintillation cocktail prior to analysis; finally, a third sample was acidified to pH 2, purged with N_2, and added to 10 mL of scintillation cocktail prior to analysis. The ^{14}C content in each sample was measured using a Tri-Carb liquid scintillation analyzer (Model 1900-TR, Packard, Deerfield, IL). Some of the automated features of this system

included direct background subtractions and self-normalization and calibration. The counting efficiency of the liquid scintillation analyzer was automatically determined for each sample and the detected counts were converted to disintegrations per minute (dpm) to correct for quenching effects. Values for the counting efficiency in each case exceeded 96%.

The carbon dioxide fraction was calculated as the difference between the alkaline/purged and the acidified/purged samples. The volatile fraction was calculated from the difference between the alkaline/purged and alkaline samples. The non-volatile fraction was calculated from the acidified/purged sample.

The production of $^{14}CO_2$ was confirmed on several occasions using a modification of the barium carbonate assay (Rapkin, 1962; Vogel, 1988). Ten mL were removed from the alkaline reactor by syringe and were centrifuged at maximum speed for 20 min using an MSE GT-2 centrifuge (VWR Scientific, West Chester, PA). After the cell pellet was discarded, 0.5 to 2 g of $Ba(NO_3)_2$ were added to the supernatant in a vial and mixed at 150 rpm for 30 min. The particulate fraction consisting of the precipitated $Ba^{14}CO_3$ was captured on a 0.2 μm membrane filter using vacuum filtration. The filter was washed twice with deionized water and placed in a scintillation vial containing 10 mL of scintillation cocktail to be assayed for radioactivity. The barium carbonate assay confirmed $^{14}CO_2$ production, but an attempt to quantify carbon dioxide using this assay yielded $^{14}CO_2$ values that were 64 to 78% of the values measured using the previous method. Between 22 and 36% of the radiolabel was lost during the filtering process due to the difficulty in fully removing the particulate fraction from the reaction vial or to partial solids dissolution when the filter was washed.

Before and after adding each of the ^{14}C-BTEX compounds to the reactors, an identical volume of each of the radiolabeled aromatic compounds was directly spiked into 10 mL of scintillation cocktail using a high-precision syringe. This was then analyzed for ^{14}C content in order to quantify the total radiolabel added to the reactors for estimating radiolabel recovery at the end of each experiment. In all experiments, the total recovery of the radiolabel was greater than 92%.

Sterile controls containing radiolabeled BTEX, but no cells were used to monitor abiotic losses of volatile compounds from the sealed bottles. Experiments were either repeated several times to validate results, or were performed using duplicate or triplicate bottles. The experimental error was calculated as the range of duplicate samples or as the standard deviation of triplicate samples.

Chemicals

Benzene (>99% ACS reagent) was from Mallinckrodt, Inc., Paris, KY. Toluene (99.8% ACS reagent), ethylbenzene (99.9% certified grade), and *p*-xylene (99.8% certified grade) were from Fisher Scientific Co., Fair Lawn, NJ. *o*-Xylene (spectro grade) was from J. T. Baker, Inc., Phillipsburg, NJ. *m*-Xylene (spectro grade), *n*-propylbenzene (no grade listed), and 1,2,4-trimethylbenzene (no grade listed) were from Eastman Kodak, Rochester, NY.

All of the radiolabeled compounds were from Sigma Chemical Company, St. Louis, MO. Benzene-UL-^{14}C (19.3 mCi/mmol) and toluene-ring-UL-^{14}C (6.2 mCi/mmol) were available commercially. Ethylbenzene-ring-UL-^{14}C (1.06 mCi/mmol), *o*-xylene-ring-UL-^{14}C (0.32 mCi/mmol), *m*-xylene-ring-UL-^{14}C (0.06 mCi/mmol), and *p*-xylene-ring-UL-^{14}C (0.15 mCi/mmol) were custom made. Chemical and radiochemical purities exceeded 98% in all cases as determined by the manufacturer using gas and liquid chromatography. Chemical purities were further confirmed in our laboratory using gas chromatography.

All other chemicals were of the highest purity commercially available and were obtained from standard sources.

Results and Discussion

Biotransformation of BTEX Compounds

A microbial consortium and two *Rhodococcus* strains derived from gasoline-contaminated aquifer material completely biotransformed each of the BTEX compounds, individually and in mixtures, over a broad range of concentrations (up to 80 mg/L for the majority of BTEX compounds). Representative results depicting BTEX biotransformation patterns by the mixed culture in single- and in multiple-substrate studies are shown in Figure 1. In addition, the mixed culture was capable of transforming other gasoline monoaromatics, including *n*-propylbenzene and 1,2,4-trimethylbenzene in mixtures with BTEX (Figure 1B). The mixed culture also transformed the BTEX compounds in a gasoline solution (16 to 160 μL of gasoline in 150 mL of liquid) (data not shown).

Substrate Interactions in BTEX Mixtures

In view of the fact that the bioremediation of gasoline-contaminated sites requires the microbial degradation of complex waste mixtures, it is important to understand the potential enhancement or inhibition caused

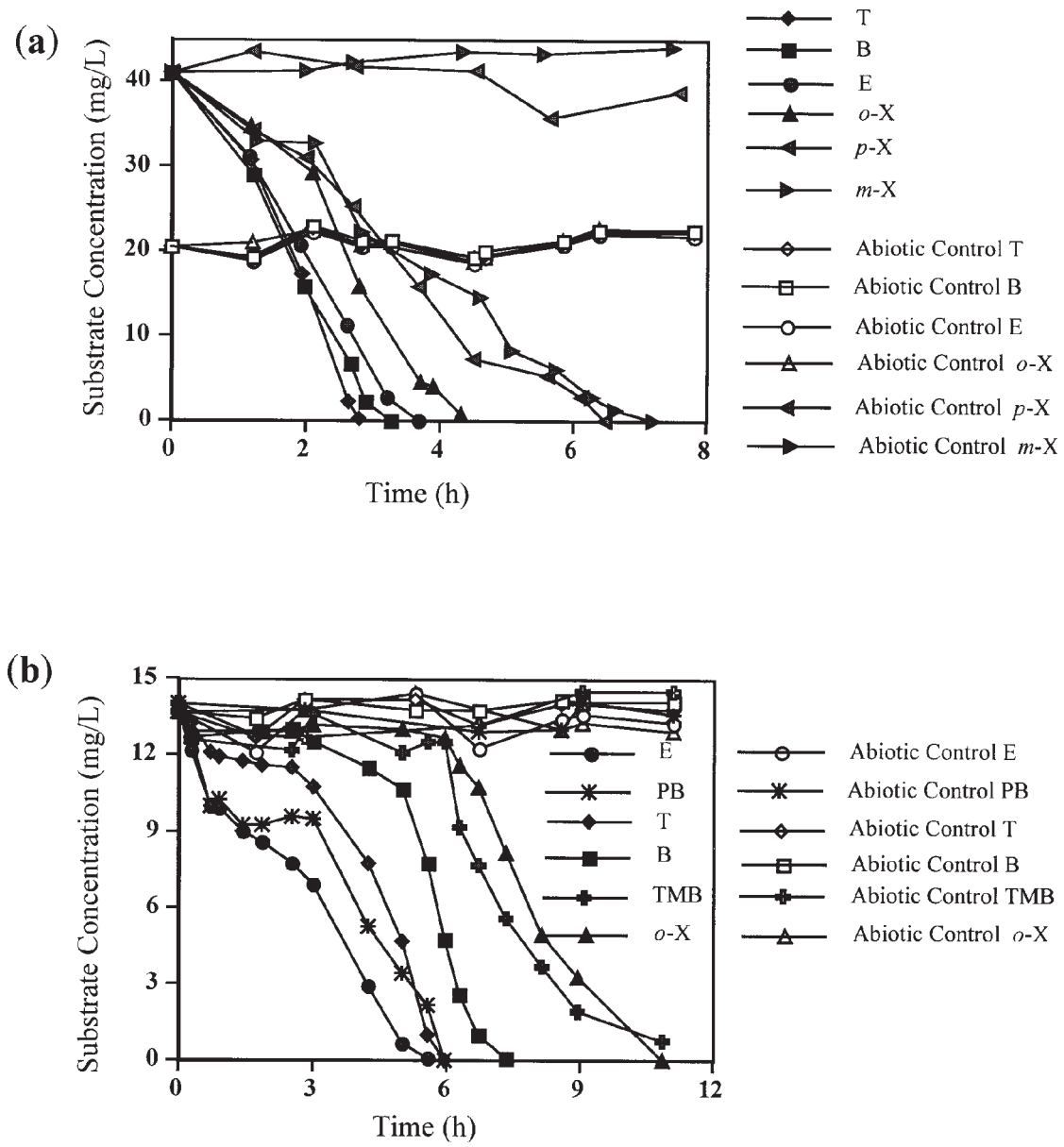

Figure 1. (A) Biotransformation of benzene (B), toluene (T), ethylbenzene (E), *o*-xylene (*o*-X), *m*-xylene (*m*-X) and *p*-xylene (*p*-X) individually by a mixed culture (culture density = 270 mg/L); (B) biotransformation of a mixture of benzene (B), toluene (T), ethylbenzene (E), *o*-xylene (*o*-X), *m*-xylene (*m*-X) and *p*-xylene (*p*-X), *n*-propylbenzene (*n*-PB) and 1,2,4-trimethylbenzene (TMB) by a mixed culture (culture density = 220 mg/L).

by the concurrent presence of multiple BTEX compounds. In this study, one of the major research objectives was to identify both negative (e.g., inhibition) and positive (e.g., cometabolism) substrate interactions in comprehensive BTEX mixtures.

Both negative and positive substrate interactions were observed during the biotransformation of BTEX mixtures by the three cultures used in this study. During the biotransformation of binary mixtures of BTEX compounds by the mixed culture, the presence of ethylbenzene was shown to have a distinct inhibitory effect on benzene, toluene, and xylene transformation rates. On the other hand, the presence of other compounds in binary mixtures with ethylbenzene had negligible effects on ethylbenzene transformation (Deeb and Alvarez-Cohen, 1999). An earlier attempt to characterize ethylbenzene inhibition effects for the mixed culture using Lineweaver-Burk kinetic models revealed a mixed inhibition pattern encompassing both competitive and noncompetitive inhibition (Deeb and Alvarez-Cohen, 1999). These results were not surprising considering that multiple species with a range of

BTEX degradation pathways may be present within the mixed culture.

In studies similar to the ones performed with the mixed culture, ethylbenzene also was the most potent inhibitor of benzene, toluene, and xylene degradation by the two pure cultures in bi-substrate mixtures. Representative data for one of the isolates, *Rhodococcus* sp. RR1, is shown in Figure 2. Ethylbenzene inhibition was more distinctive with the isolates than with the parent mixed culture. In fact, transformation of other aromatic compounds in the presence of ethylbenzene did not commence until most of the ethylbenzene was removed. As a result, we were unable to obtain initial BTX biotransformation rates in the presence of ethylbenzene over a range of ethylbenzene concentrations. This prevented us from generating Lineweaver-Burk plots, and therefore from quantifying the observed inhibition effects using conventional methods. Results, however, were useful in clarifying the mechanism of ethylbenzene inhibition. For example, the transformation rate of ethylbenzene in a mixture with toluene by toluene-grown cells was similar to that of toluene when it was present alone. Furthermore, ethylbenzene transformation proceeded without a lag (Figure 2), which suggests that the pathway responsible for toluene degradation may also be responsible for ethylbenzene metabolism by this culture. The observation that the transformation of toluene by the toluene-grown cells did not start until all of the ethylbenzene was removed suggests that the enzymes responsible for the degradation of both toluene and ethylbenzene might have a much higher affinity for ethylbenzene than toluene. This in turn suggests that ethylbenzene might be competitively inhibiting toluene degradation. Recent biochemical studies performed in an effort to elucidate BTEX biodegradation pathways by the two pure cultures confirmed that both toluene and ethylbenzene are degraded by the same metabolic pathway (Deeb et al., 1999).

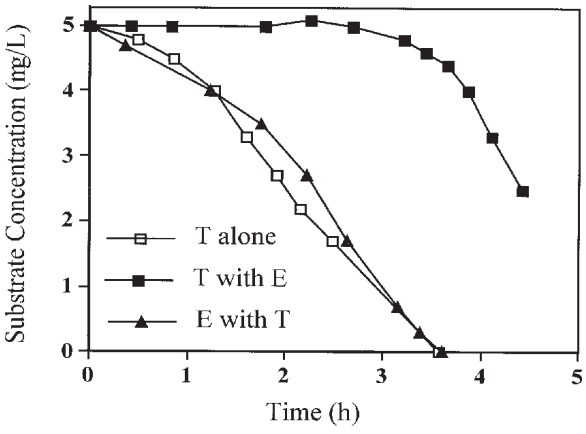

Figure 2. Toluene (T) and ethylbenzene (E) biotransformation alone or in a binary mixture by *Rhodococcus* sp. RR1 (culture density = 35 mg/L).

In addition to toluene, all three cultures utilized benzene and ethylbenzene as growth substrates. Although the cultures were able to degrade the three xylenes, it was not clear whether the xylenes were effectively utilized as primary carbon sources. The cultures degraded only limited amounts of the xylenes and marked decreases in cell densities took place after repeated exposure to xylene, in particular to the *ortho* and *meta* isomers.

In the case of the mixed culture, the failure to degrade continual additions of the xylenes in the absence of another substrate suggests that the xylenes might be cometabolically degraded. Cometabolic degradation of the xylenes in this study is consistent with previous reports of xylene cometabolism in mixtures of aromatic compounds (Alvarez and Vogel, 1991; Chang et al., 1993; Oh et al., 1994).

Mineralization of BTEX Compounds

BTEX mineralization was investigated at a range of hydrocarbon concentrations using radiolabeled compounds. The mixed culture mineralized all six BTEX compounds to some extent, both individually and in mixtures, within experimental time frames of 48 to 60 h. In Table 1, the fractions of the added radiolabeled BTEX compounds recovered as $^{14}CO_2$ are reported. Unlike the mixed culture, the two isolates were able to mineralize only five of the six BTEX components. Although the isolates were able to biotransform *o*-xylene (data not shown), they were unable to mineralize it to carbon dioxide.

As illustrated in the previous section, substrate interactions during BTEX degradation by the three cultures sometimes had a negative effect on the disappearance rates of the individual BTEX compounds. In particular, ethylbenzene inhibited BTX degradation by all three cultures. However, whether the presence of ethylbenzene in a mixture of aromatic compounds would negatively impact eventual BTX mineralization potential was not clear. With both the mixed culture and the two *Rhodococcus* strains, although the number of BTEX compounds mineralized was similar in the single- and multiple-substrate experiments, the final fractions of carbon dioxide produced varied somewhat between the two conditions (Table 1). Only the mineralization of ethylbenzene and *o*-xylene by the mixed culture appeared to be negatively affected by the presence of mixtures. Therefore, it does not seem that ethylbenzene caused an overall decrease in BTX mineralization capacity, or that the concurrent presence of other aromatic components arrested the mineralization

Table 1. Fraction (%) of the added ^{14}C-BTEX recovered as $^{14}CO_2$ when the aromatic compounds were exposed (i) individually and (ii) in six-component mixtures to the mixed culture and *Rhodococcus* sp. RR1 and RR2 for a period of 48 to 60 h

(i)	Benzene	Toluene	Ethylbenzene	*o*-Xylene	*m*-Xylene	*p*-Xylene
Mixed culture[a,b]	92 ± 2	92 ± 6	91 ± 5	89 ± 1	35 ± 5	22 ± 3
RR1[a,c]	90 ± 3	59 ± 1	89 ± 5	0 ± 1	41 ± 0	17 ± 1
RR2[a,c]	85 ± 6	57 ± 3	91 ± 5	4 ± 0	38 ± 6	15 ± 2

[a]Initial BTEX concentration = 20 mg/L.
[b]Initial culture density = 140 mg dry wt/L.
[a]Initial culture density = 240 mg dry wt/L.

(ii)	Benzene	Toluene	Ethylbenzene	*o*-Xylene	*m*-Xylene	*p*-Xylene
Mixed culture[d,e]	92 ± 2	93 ± 6	77 ± 5	49 ± 13	34 ± 3	37 ± 6
RR1[f,g]	94 ± 0	81 ± 2	92 ± 4	4 ± 1	46 ± 0	28 ± 0
RR2[f,h]	97 ± 16	83 ± 1	99 ± 3	8 ± 1	58 ± 1	29 ± 2

[d]Initial concentration of each aromatic compound = 14 mg/L.
[e]Initial culture density = 310 mg dry wt/L.

of any individual BTEX compound. On the other hand, the mineralization of *p*-xylene by each culture, *m*-xylene by RR2, and toluene by both isolates were all increased in the presence of mixtures, suggesting a positive substrate interaction. Conflicting results were observed in previously reported mixture studies. For example, substrate interactions during the biodegradation of BT(*p*-)X mixtures by mixed and pure cultures were shown to lead to the incomplete metabolism of *p*-xylene and a strong inhibition of benzene biodegradation (Oh et al., 1994; Oh and Bartha, 1997). Therefore, caution should be exercised when making generalizations about substrate interactions in pollutant mixtures.

The broad mineralization capacity exhibited by the mixed culture in this study is not surprising and may be attributed either to the presence of different microbial species with a number of metabolic pathways or to interspecies interactions. The mineralization of BTE(*m*-/*p*-)X by the pure cultures is a unique result and merits further study. Preliminary results from biochemical studies with these cultures suggest that BTEX mineralization by the two *Rhodococcus* strains is taking place via a TOD-like pathway involving dioxygenase attack on the aromatic ring leading to the formation of the corresponding catechols that are then cleaved by either catechol-1,2- or 2,3-dioxygenase (Deeb et al., 1999).

Finally, comparison of the mineralization potentials of the mixed and pure cultures revealed that the mixed culture is slightly more effective than the pure cultures in mineralizing BTEX mixtures. The two isolates in this study exhibited a biodegradation pattern that was a subset of that exhibited by the mixed culture. Mixed cultures therefore may be more effective than pure cultures in biotreatment systems because interspecies interactions may be necessary for the complete biodegradation of multicomponent hydrocarbon mixtures.

Biodegradability of MTBE and Its Effect on BTEX Biotransformation Rates

MTBE was not degraded by the three cultures over a broad range of MTBE concentrations. Furthermore, the presence of MTBE at concentrations comparable to those reportedly detected in groundwater at gasoline-contaminated sites had no negative effect on BTEX biotransformation rates (Figure 3). Even at high concentrations (up to 100 mg/L), the presence of MTBE in bi-substrate mixtures with each of the BTEX compounds had no discernible effect on BTEX biodegradation by the mixed culture (data not shown). The results are in agreement with previous reports and suggest that the presence of MTBE does not affect the

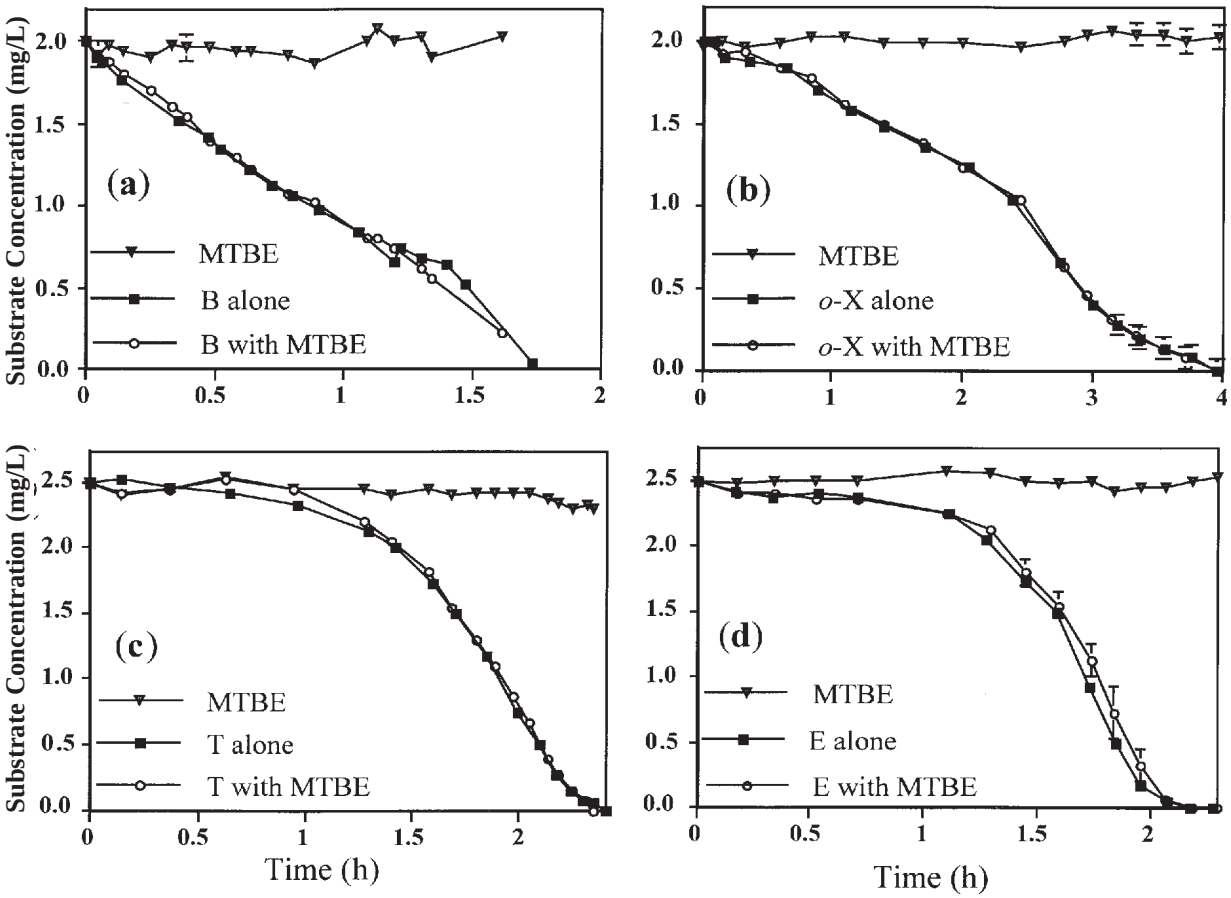

Figure 3. Biotransformation of benzene (B), toluene (T), ethylbenzene (E), *o*-xylene (*o*-X), *m*-xylene (*m*-X), and *p*-xylene (*p*-X) in mixtures with methyl *tert*-butyl ether (MTBE) by (a,b) a mixed culture (culture density = 176 mg/L), (c) *Rhodococcus* sp. RR1 (culture density = 94 mg/L) and (d) *Rhodococcus* sp. RR2 (culture density = 188 mg/L).

cell viability and activity of non-MTBE-degrading cultures. For example, Jensen and Arvin (1990) showed that MTBE up to concentrations of 40 mg/L had no effect on BTEX degradation in batch experiments using aquifer material. Weak inhibition of BTEX biodegradation was observed in the presence of MTBE at a concentration of 200 mg/L.

The broader implications of these results may be important for predicting whether the presence of MTBE in groundwater plumes would impede BTEX bioattenuation in the field. The effect of MTBE on the biodegradation of BTEX compounds by an MTBE-grown pure culture is currently being characterized in collaboration with researchers at the University of California at Davis.

Acknowledgments

The authors gratefully acknowledge Dr. Jim Spain for reviewing this paper. This work was supported by graduate student fellowships from the National Student Foundation and the U.S. Environmental Protection Agency (U914983-01-2) and by a research grant from the National Institutes of Environmental Health Sciences (P42-ES04705-09).

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