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EFFECT OF ETHANOL AND METHYL-*TERT*-BUTYL ETHER ON MONOAROMATIC HYDROCARBON BIODEGRADATION: RESPONSE VARIABILITY FOR DIFFERENT AQUIFER MATERIALS UNDER VARIOUS ELECTRON-ACCEPTING CONDITIONS

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Abstract—Aquifer microcosms were used to determine how ethanol and methyl-*tert*-butyl ether (MtBE) affect monoaromatic hydrocarbon degradation under different electron-accepting conditions commonly found in contaminated sites experiencing natural attenuation. Response variability was investigated by using aquifer material from four sites with different exposure history. The lag phase prior to benzene, toluene, ethylbenzene, and xylenes (BTEX) and ethanol degradation was typically shorter in microcosms with previously contaminated aquifer material, although previous exposure did not always result in high degradation activity. Toluene was degraded in all aquifer materials and generally under a broader range of electron-accepting conditions compared to benzene, which was degraded only under aerobic conditions. The MtBE was not degraded within 100 d under any condition, and it did not affect BTEX or ethanol degradation patterns. Ethanol was often degraded before BTEX compounds and had a variable effect on BTEX degradation as a function of electron-accepting conditions and aquifer material source. An occasional enhancement of toluene degradation by ethanol occurred in denitrifying microcosms with unlimited nitrate; this may be attributable to the fortuitous growth of toluene-degrading bacteria during ethanol degradation. Nevertheless, experiments with flow-through aquifer columns showed that this beneficial effect could be eclipsed by an ethanol-driven depletion of electron acceptors, which significantly inhibited BTEX degradation and is probably the most important mechanism by which ethanol could hinder BTEX natural attenuation. A decrease in natural attenuation could increase the likelihood that BTEX compounds reach a receptor as well as the potential duration of exposure.

Keywords—Monoaromatic hydrocarbons   Oxygenates   Substrate interactions   Degradation kinetics   Natural attenuation

INTRODUCTION

A recent initiative to phase out MtBE as a gasoline oxygenate is likely to significantly increase the use of ethanol in gasoline to mitigate air pollution during combustion [1]. Because gasoline releases that contaminate the subsurface are likely to continue well into the future, a better understanding of how ethanol affects the natural attenuation of petroleum hydrocarbons is warranted. This is particularly important for the fate and transport of the benzene, toluene, ethylbenzene, and xylene (BTEX) compounds, which are the most toxic and soluble of the hydrocarbons in gasoline and the most important ones to consider for risk assessment and management purposes.

Past research on the possible groundwater impacts of ethanol in gasoline has focused primarily on cosolvent and biodegradation effects. The presence of ethanol at relatively high concentrations (>20%) facilitates the dissolution of BTEX from the fuel phase into groundwater [2]. This cosolvent effect can also decrease the extent to which BTEX adsorb onto aquifer material [3], which could decrease sorption-related retardation and enhance BTEX transport during bulk flow [4]. Such cosolvent effects, however, are concentration dependent. They could be important for neat ethanol releases over preexisting BTEX contamination at bulk terminals [5] but are unlikely to be significant at retail sites contaminated with gasohol (gasoline with 10% ethanol) [2].

Previous studies have also shown that the preferential degradation of ethanol by indigenous aquifer microorganisms and the accompanying depletion of oxygen could hinder BTEX biodegradation [6,7]. Inhibition of BTEX degradation is probably more influential than the cosolvency effect with respect to elongating BTEX plumes and increasing the region of influence of hydrocarbon contamination. However, little is known about how differences in site-specific conditions affect the extent to which ethanol could hinder BTEX biodegradation.

Several site-specific factors have not been studied within the context of substrate interactions between BTEX and gasoline oxygenates. Exposure history to BTEX, methyl-*tert*-butyl ether (MtBE), and ethanol in soil may be important to consider with the presumption that microbial communities with previous exposure to contaminants are better adapted to degradation. The terminal electron-accepting process is another potentially important factor that could affect the level of impact of ethanol on BTEX degradation kinetics. Furthermore, considerable interest exists in understanding how ethanol might affect the natural attenuation of preexisting MtBE contamination, which makes it desirable to incorporate MtBE in a response variability study.

This paper compares the effects of ethanol versus MtBE on BTEX degradation patterns. Response variability was ad-

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Table 1. Key constituent concentrations in groundwater at the sample collection point and characteristic of aquifer material used in this work; study sites located in California, USA, with the exception of Northwest Terminal, which is located in Oregon, USA

	Site			
Component	Travis Air Force Base	Tracy	Sacramento	Northwest Terminal
Benzene (ppb)	300	NA[a]	5,300	1,500
Toluene (ppb)	170	NA	35	6,090
Ethylbenzene (ppb)	4,700	NA	270	770
Xylenes (ppb)	3,100	NA	130	5,070
Ethanol (ppm)	ND[b]	NA	NA	16,100
Methyl-*tert*-butyl ether (ppb)	170	NA	2,700	ND
Nitrate (ppm)	ND	0.9	ND	ND
Sulfate (ppm)	27	19	13	310
Dissolved oxygen (ppm)	1–2	NA	1–2	ND
Aquifer material pH	6.9	7.3	7.4	5.7
Organic matter (%)	0.9	0.6	1.1	0.9
Cation exchange capacity (meq/100 g)	4.2	5.0	13.6	9.3
Texture	Loamy sand	Loamy sand	Silt loam	Sandy loam
Depth to groundwater (m)	2.6–2.9	7.6–9.1	~7.6	1.8
Hydraulic conductivity (cm/d)	35–691	1,218	674	1,063
Age of spill (years)	12	Not contaminated	>6	>20[c]

[a] NA = not analyzed because sample was collected from a remote uncontaminated area, far from any potential source of organic contamination (e.g., no gas stations nearby).
[b] ND = not detected.
[c] A neat ethanol release occurred one year prior to sample collection.

dressed by considering four sites with different exposure histories under three electron-accepting conditions (aerobic, denitrifying, and sulfate reducing). A statistical analysis was conducted to evaluate the relative importance of the presence of MtBE or ethanol, the source of the aquifer material, and electron-accepting conditions on BTEX degradation kinetics. Aquifer columns were also used to study biodegradation and geochemical transitions in flow-through systems simulating BTEX natural attenuation in the presence or absence of ethanol.

MATERIALS AND METHODS

Aquifer microcosms were used to investigate substrate interactions between BTEX, ethanol, and MtBE. Microcosms were prepared using aquifer materials from four different sites in the United States with varying exposure history to BTEX, MtBE, and ethanol. Aquifer materials from the Travis Air Force Base (AFB) (Fairfield, CA, USA) and Sacramento (CA, USA) sites had been exposed to BTEX and MtBE contamination, whereas the Northwest Terminal in Tigard (OR, USA) had been exposed to BTEX and was subsequently impacted by a spill of neat ethanol. Material from the Tracy (CA, USA) site served as a control since it had no known previous exposure to BTEX, MtBE, or ethanol.

Sediments were collected from the saturated zone using a split-spoon sampler (Sacramento), a geoprobe direct push sampler (Travis AFB, Northwest Terminal), or a standard penetration test tool (Tracy). New sleeves or liners were used for sample collection, and care was taken to avoid exposing the aquifer material to oxygen. Specifically, the cores were immediately capped and shipped on ice and stored inside an anaerobic chamber (Coy Laboratory, Grass Lake, MI, USA) prior to use. The sediments near the ends of the core were discarded, and the remaining aquifer material was drained, homogenized, and equilibrated with the chamber's atmosphere for 2 d prior to transfer to the microcosms. Groundwater parameters at the sample collection points and aquifer material characteristics are summarized in Table 1.

Aquifer columns were used to study ethanol and BTEX migration and biodegradation in a flow-through system simulating natural attenuation. This experiment was performed to provide support for the microcosm results and was conducted with aquifer material from Travis AFB.

Microcosm study

For each site, aerobic microcosms were prepared with 20 g of drained aquifer material and 80 ml of aerated mineral medium in 250-ml amber glass bottles. The mineral medium was synthetic groundwater prepared as described by von Guten and Zobrist [8], except that $NaHCO_3$ was replaced by KH_2PO_4 (3.9 mM). In addition, NH_4Cl was substituted for $NaNO_3$ at 0.3 mM to provide a nitrogen source that could not be used as an electron acceptor. The medium contained (in mg/L of deionized water): KH_2PO_4 (531), K_2SO_4 (40), NH_4Cl (16), $MgCl_2{\cdot}6H_2O$ (12), $CaCl_2$ (6.7), $Ni(NO_3)_2{\cdot}6H_2O$ (0.002), $CuSO_4{\cdot}5H_2O$ (0.002), $ZnSO_4{\cdot}7H_2O$ (0.002), $CoSO_4{\cdot}7H_2O$ (0.002); $(NH_4)_6Mo_7O_{24}$ (0.001), and H_3BO_3 (0.0004).

Microcosms were prepared in triplicate and capped with Mini-nert valves (Alltech Associates, Deerfield, IL, USA). Air in the headspace was replaced with oxygen to facilitate the maintenance of aerobic conditions during the degradation assay. Three sets of aerobic microcosms were prepared: BTEX alone, BTEX plus ethanol, and BTEX plus MtBE. Abiotic controls were prepared with all compounds added and were poisoned with a commercial biocide (Kathon® cosmetic grade/in-can preservatives [CG/ICP], diluted 1:100; Supelco, Bellefonte, PA, USA). All microcosms were incubated in the dark under quiescent conditions at 28°C, and aqueous samples were collected periodically to determine changes in contaminant concentrations.

Anaerobic (denitrifying and sulfate-reducing) microcosms were prepared similarly and were incubated at 25 ± 3°C inside the anaerobic with an atmosphere composed of N_2 (80%), CO_2 (10%), and H_2 (10%). The mineral medium was the same as that used for the aerobic microcosms, except that it was buffered mainly with bicarbonate (7.4 mM). Phosphate buffer was also added (20 μM) to ensure that phosphorus was not limiting. The medium was autoclaved and purged for 2 h with N_2/CO_2

(80/20) to remove dissolved oxygen prior to transferring to the anaerobic chamber, where it was equilibrated with the chamber atmosphere for 3 d. The pH of the medium was 7.4 at that time. The medium was then amended with nitrate (5.3 mM) or sulfate (4 mM) prior to BTEX, ethanol, and/or MtBE addition. Initial concentrations were approximately 1 mg/L for each BTEX compound, 10 mg/L for MtBE, and 100 mg/L for ethanol, which are representative of sites contaminated with oxygenated gasoline. The Tracy and Northwest Terminal microcosms did not receive MtBE since these sites had no known previous exposure to MtBE. Anaerobic controls were also prepared by adding all tested compounds and electron acceptors and were poisoned with a commercial biocide (Kathon CG/ICP) at 10 ml/L. Note that repeated poisoning with the Kathon biocide was needed to maintain the sterility of the controls.

The removal of a compound from viable microcosms (with concomitant electron acceptor utilization) but not from sterile controls was taken as evidence of biodegradation. Some anaerobic microcosms (including controls) exhibited considerable BTEX losses, possibly due to volatilization exacerbated by intensive sampling over time. In such cases, the ratio of toluene to benzene was considered as an additional criterion to determine if degradation had occurred. The presumption was that this ratio would decrease significantly as a result of biodegradation (e.g., by 50% or more) because benzene is relatively recalcitrant, whereas toluene is commonly reported to degrade under anaerobic conditions [6,9]. For cases where biodegradation was unequivocally established, biodegradation patterns were characterized for each compound by determining lag periods and first-order degradation rate coefficients. The lag period, which reflects the acclimation phase, was determined as the time during which contaminant concentrations remained constant or did not decrease relative to sterile controls. The first-order rate coefficient (k) was determined by fitting an exponential decay model ($C = C_o e^{-kt}$) to concentration (C) versus time (t) data obtained after the lag period. This value was then corrected for volatile losses by subtracting the k value obtained for the controls.

Analysis of variance was used to determine if differences in lag phase and rate coefficients for each BTEX compound were statistically significant for different treatments and to determine the relative importance of different factors (presence of ethanol or MtBE, aquifer material source, and electron-accepting conditions). This analysis was conducted using Minitab software version 13.1 (Minitab, State College, PA, USA). Statistical significance was set at the 95% level of confidence.

Aquifer column study

Three columns (30 cm long, 2.5 cm in diameter) were used to further investigate natural attenuation of BTEX and ethanol and their potential interactive effects. Emphasis was placed on obtaining concentration profiles along the length of the columns to investigate geochemical transitions and spatial variations in removal efficiency. The columns were equipped with six sampling ports (at 3, 6, 10, 15, 20, and 25 cm from the inlet) and packed with aquifer material from Travis AFB. One column was amended with BTEX alone (benzene 5.2 mg/L, toluene 4.1 mg/L, ethylbenzene 2.3 mg/L, m+p-xylenes 2.4 mg/L, o-xylene 2.5 mg/L) to provide a baseline for the effect of ethanol on BTEX attenuation. Another column was amended with BTEX plus ethanol (100 mg/L). The third column was a sterile control to distinguish biodegradation from potential abiotic losses. This column was poisoned with Kathon biocide (1.5 mg/L) and amended with BTEX plus ethanol. Each column was fed continuously in an up-flow mode at 3 ml/h using both a peristaltic pump (Masterflex Model 7519-15, Barnant, Barrington, IL, USA) to supply the mineral medium and a syringe pump (Harvard Apparatus Model 22, South Natick, MA, USA) to supply the volatile organic compounds (BTEX and ethanol). The ratio of the peristaltic to syringe pump rates was set at 20:1. The total flow rate was 3 ml/h (superficial velocity of 0.61 cm/h), and approximately 2 d were required to displace one pore volume. The mineral medium was the same used for the anaerobic microcosms, except that it was air saturated and amended with nitrate (0.2 mM) and sulfate (0.4 mM) as potential electron acceptors. The $NaHCO_3$ buffer concentration was 2.4 mM.

Analytical methods

Aqueous samples (1 ml) were collected from microcosms or column sampling ports using gastight syringes and analyzed for BTEX, MtBE, and ethanol using a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP 19395A headspace autosampler and flame-ionization and photoionization detectors in series. Separation was achieved using a J&W Scientific DB-WAX column (Agilent Technologies, Palo Alto, CA, USA) at 35°C. Detection limits were approximately 0.02 mg/L for each BTEX compound, 0.01 mg/L for MtBE, and 0.15 mg/L for ethanol.

Nitrate and sulfate were analyzed using a Dionex 4500 ion chromatograph with an AS4A column (Dionex, Sunnyvale, CA, USA) for separation followed by chemical suppression and conductivity detection. The samples were passed through a 0.20-μm filter prior to ion chromatographic analysis.

The oxidation-reduction potential was monitored in aerobic microcosms (to verify that oxygen was not depleted) and along the length of aquifer columns (to characterize geochemical transitions) using a microelectrode MI-16/800 (Microelectrodes, Bedford, NH, USA) connected to a 16-702 Flow-Through pH reference and to the pH meter (Beckman Instruments, Fullerton, CA, USA).

RESULTS AND DISCUSSION

Ethanol was rapidly degraded in all aquifer materials, and electron-accepting conditions were tested, with half-lives ranging from about 3 to 7 d (Table 2). Previous exposure to ethanol at the Northwest Terminal site did not result in higher degradation activity. In general, ethanol was degraded fastest under denitrifying conditions, and the rates under aerobic conditions were similar. The rate coefficients determined for ethanol degradation (0.1–2/d) are markedly faster than that reported for a field site where ethanol was used as a cosolvent for extraction of free-phase chlorinated solvents (0.33/year) [10]. It is unclear whether this discrepancy is due to toxicity of the high ethanol concentrations at this site (>10,000 mg/L) or to more favorable conditions for biodegradation provided for the microcosms (e.g., higher temperature and nutrient addition). The effect of ethanol on BTEX degradation patterns is discussed in the following for different aquifer materials and electron-accepting conditions.

Travis AFB microcosms

These microcosms were prepared with aquifer material with a history of BTEX and MtBE contamination and exhibited a relatively high BTEX degradation activity compared to the material from the other study sites. The MtBE, however, was

Table 2. Ethanol degradation patterns in the presence of monoaromatic hydrocarbons for microcosms from the four study sites, California and Oregon, USA

Site	Aerobic		Denitrifying		Sulfate reducing	
	Lag phase (d)	*k* (per day)	Lag phase (d)	*k* (per day)	Lag phase (d)	*k* (per day)
Travis Air Force Base[a]	0	0.5	0	1.3	0	0.1
Tracy[b]	2	1.0	1	0.8	6	0.8
Sacramento[c]	0	0.5	0	2.0	23	0.4
Northwest Terminal[d]	2	0.6	0	0.7	0	0.8

[a] Initial concentration (mg/L): benzene 1.8 ± 0.1, toluene 1.5 ± 0.2, ethylbenzene 0.4 ± 0.1, *o*-xylene 0.5 ± 0.7, *m*+*p*-xylenes 0.5 ± 0.1, ethanol 55 ± 4.
[b] Initial concentration (mg/L): benzene 2.4 ± 0.5, toluene 1.5 ± 0.2, ethylbenzene 0.5 ± 0.1, *o*-xylene 0.6 ± 0.1, *m*+*p*-xylenes 0.7 ± 0.1, ethanol 75 ± 11.
[c] Initial concentration (mg/L): benzene 2.3 ± 0.2, toluene 1.6 ± 0.2, ethylbenzene 0.5 ± 0.1, *o*-xylene 0.7 ± 0.1, *m*+*p*-xylenes 0.7 ± 0.1, ethanol 72 ± 3.
[d] Initial concentration (mg/L): benzene 2.4 ± 0.2, toluene 1.5 ± 0.1, ethylbenzene 0.5 ± 0.1, *o*-xylene 0.7 ± 0.1, *m*+*p*-xylenes 0.6 ± 0.1, ethanol 70 ± 13.

not degraded in this experiment, and its presence did not significantly affect BTEX or ethanol degradation patterns (data not shown).

Ethanol significantly inhibited aerobic benzene degradation (Fig. 1), even though oxygen was available in excess and was not depleted during ethanol degradation. Interestingly, some BTEX compounds were degraded faster than ethanol. For example, the *k* value for ethanol was 0.5/d (Table 2) versus 2.9/d for ethylbenzene (Table 3). This is contrary to previous reports that ethanol degrades preferentially and BTEX degradation does not begin until most of the ethanol is removed [6].

No anaerobic benzene degradation was observed in two months of incubation. Toluene was the only hydrocarbon degraded under all electron-accepting conditions and substrate combinations tested in the Travis microcosms, and its degradation appeared to support the cometabolism of *p*+*m*-xylenes under denitrifying conditions (xylene consumption coincided with that of toluene and subsided after toluene was removed). This trend was observed in both the presence and the absence of ethanol (Fig. 2). Cometabolism of xylene by toluene degraders appears to be a common substrate interaction under denitrifying conditions [11–14].

Ethanol (60–80 mg/L) was always the first compound degraded under anaerobic conditions, and its presence significantly inhibited *o*-xylene degradation in sulfate-reducing microcosms; *o*-xylene was degraded only in microcosms amended with BTEX alone or with MtBE (Table 3). Logistic constraints precluded the determination of whether a longer incubation time would be required to observe *o*-xylene degradation in microcosms with ethanol.

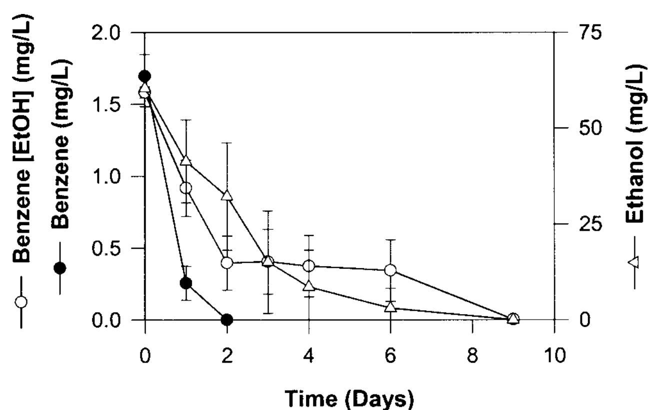

Fig. 1. Inhibition of benzene degradation by ethanol in aerobic microcosms from Travis Air Force Base (CA, USA). Data points represent the average of three replicate microcosms, and error bars depict one standard deviation.

Similar to microcosms from the other study sites, BTEX and ethanol degradation generally coincided with the utilization of the appropriate electron acceptor, as illustrated for nitrate removal (Fig. 2). However, no electron balances were calculated in this study because the electron acceptor demand from the added compounds was overshadowed by the higher and more variable background demand of the sediments.

Tracy microcosms

These microcosms were prepared with uncontaminated aquifer material and exhibited lower BTEX degradation activity than the Travis AFB microcosms but higher activity than previously contaminated material from the Sacramento and Northwest Terminal sites. All BTEX compounds were degraded in aerobic Tracy microcosms (Table 4), which reflects the ubiquitous nature of aerobic BTEX degraders. Ethanol was degraded earlier (within one week) than all BTEX compounds in aerobic microcosms, and its presence significantly decreased the rate of BTEX degradation.

Ethanol was rapidly degraded (within two weeks) in all anaerobic microcosms (Table 2). However, no anaerobic BTEX degradation was observed except for toluene in denitrifying microcosms (Table 4).

Sacramento microcosms

This aquifer material had a history of BTEX and MtBE contamination. Nevertheless, it exhibited a relatively narrow range of BTEX degradation. Benzene and *o*-xylene were not degraded in aerobic microcosms within the two-week incubation period. Furthermore, no anaerobic BTEX degradation was observed except for toluene under denitrifying conditions (Table 5).

Ethanol had an inhibitory effect on BTEX degradation; no BTEX compound was degraded in triplicate microcosms with ethanol. On the other hand, ethanol enhanced toluene degradation in denitrifying microcosms, and no toluene degradation was observed in replicate microcosms without ethanol (Table 5).

The observed enhancement of toluene degradation by ethanol in denitrifying microcosms represents a caveat against

Table 3. Monoaromatic hydrocarbon degradation patterns in Travis Air Force Base (CA, USA) microcosms

Compound[a]	Aerobic Lag phase (d)	Aerobic *k* (per day)	Denitrifying Lag phase (d)	Denitrifying *k* (per day)	Sulfate reducing Lag phase (d)	Sulfate reducing *k* (per day)
Benzene						
With BTEX[b]	0	3.0	ND[c]	ND	ND	ND
With BTEX+EtOH[d]	0	0.5	ND	ND	ND	ND
With BTEX+MtBE	0	3.0	ND	ND	ND	ND
Toluene						
With BTEX	0	0.8	2	0.4	6	0.3
With BTEX+EtOH	0	0.9	0	0.2	3	0.3
With BTEX+MtBE	0	0.7	2	0.5	6	0.2
Ethylbenzene						
With BTEX	0	2.9	2	0.3	ND	ND
With BTEX+EtOH	0	4.9	3	0.1[e]	ND	ND
With BTEX+MtBE	0	4.0	2	0.2	ND	ND
m+*p*-Xylenes						
With BTEX	0	0.9	2	0.1[e]	6	0.1
With BTEX+EtOH	0	0.3	0	0.1[e]	3	0.1[e]
With BTEX+MtBE	0	0.8	2	0.1[e]	9	0.1
o-Xylene						
With BTEX	0	1.2	3	0.1[e]	27	0.2
With BTEX+EtOH	6	1.5	3	0.1[e]	ND	ND
With BTEX+MtBE	0	1.4	3	0.1[e]	27	0.1

[a] Initial concentration (mg/L): benzene 1.8 ± 0.1, toluene 1.5 ± 0.2, ethylbenzene 0.4 ± 0.1, *o*-xylene 0.5 ± 0.7, *m*+*p*-xylenes 0.5 ± 0.1, ethanol 55 ± 4, methyl-*tert*-butyl ether (MtBE) 6.2 ± 0.5.
[b] BTEX = monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene).
[c] ND = no significant removal relative to sterile control was observed within the 59-d incubation period.
[d] EtOH = ethanol.
[e] Degradation ceased after toluene was removed.

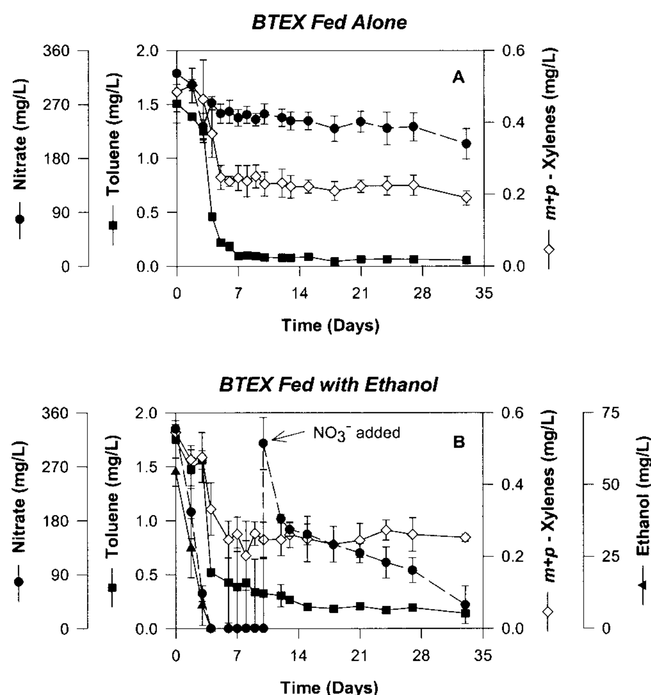

Fig. 2. Toluene-dependent degradation of *m*+*p*-xylenes in denitrifying microcosms from Travis Air Force Base (CA, USA). Data points represent the average of three replicate microcosms, and error bars depict one standard deviation; BTEX = benzene, toluene, ethylbenzene, xylenes.

Table 4. Monoaromatic hydrocarbon degradation patterns in Tracy (CA, USA) microcosms

Compound[a]	Aerobic Lag phase (d)	Aerobic *k* (per day)	Denitrifying Lag phase (d)	Denitrifying *k* (per day)
Benzene				
With BTEX[b]	7	1.5	ND[c]	ND
With BTEX+EtOH[d]	3	0.3	ND	ND
Toluene				
With BTEX	7	1.5	29	0.1
With BTEX+EtOH	3	0.3	21	0.1
Ethylbenzene				
With BTEX	7	1.3	ND	ND
With BTEX+EtOH	3	0.2	ND	ND
m+*p*-Xylenes				
With BTEX	7	1.1	ND	ND
With BTEX+EtOH	3	0.1	ND	ND
o-Xylene				
With BTEX	7	0.7	ND	ND
With BTEX+EtOH	5	0.1	ND	ND

[a] Initial concentration (mg/L): benzene 2.4 ± 0.5, toluene 1.5 ± 0.2, ethylbenzene 0.5 ± 0.1, *o*-xylene 0.6 ± 0.1, *m*+*p*-xylenes 0.7 ± 0.1, ethanol 75 ± 11.
[b] BTEX = monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene).
[c] ND = no significant removal relative to sterile control was observed within the 75-d incubation period. Data for all BTEX compounds under sulfate-reducing conditions were ND.
[d] EtOH = ethanol.

Table 5. Toluene degradation patterns in Sacramento (CA, USA) microcosms

Compound[a]	Aerobic		Denitrifying	
	Lag phase (d)	*k* (per day)	Lag phase (d)	*k* (per day)
Toluene				
With BTEX[b]	0	0.1	ND	ND
With BTEX+EtOH[c]	ND[d]	ND	0	0.1
With BTEX+MtBE	0	0.1	ND	ND
Ethylbenzene				
With BTEX	0	0.2	ND	ND
With BTEX+EtOH	ND	ND	ND	ND
With BTEX+MtBE	0	01	ND	ND
m+*p*-Xylenes				
With BTEX	0	0.6	ND	ND
With BTEX+EtOH	ND	ND	ND	ND
With BTEX+MtBE	0	0.3	ND	ND

[a] Initial concentration (mg/L): benzene 2.3 ± 0.2, toluene 1.6 ± 0.2, ethylbenzene 0.5 ± 1.0, *o*-xylene 0.7 ± 0.1, *m*+*p*-xylenes 0.7 ± 0.1, ethanol 72 ± 3, methyl-*tert*-butyl ether (MtBE) 6.8 ± 0.6.
[b] BTEX = monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene).
[c] EtOH = ethanol.
[d] ND = no significant removal relative to sterile control was observed within the 59-d incubation period. Data for benzene and *o*-xylene under all electron-accepting conditions and data for all BTEX compounds under sulfate-reducing conditions were ND.

generalizations about the overall effect of fuel additives on BTEX degradation. Specifically, if electron acceptors are not limiting, ethanol could exert both positive and negative effects. The overall effect would depend both on exposure conditions and on the initial microbial community structure.

The MtBE was not degraded under any condition tested, and its presence did not significantly affect the previously described BTEX and ethanol degradation patterns (data not shown).

Northwest Terminal microcosms

These microcosms were prepared with aquifer material that had experienced BTEX contamination and a subsequent spill of neat ethanol [5]. Aerobic BTEX degradation activity was relatively low (with *k* values ranging from 0.1–0.4/d), and no BTEX compound was completely removed within two weeks of incubation. Furthermore, none of the xylene isomers was degraded in these aerobic microcosms (Table 6). Although ethanol was degraded preferentially over BTEX compounds, its presence did not significantly inhibit aerobic BTEX degradation compared to replicates without ethanol.

Ethanol was readily degraded (within one week) under all anaerobic electron acceptor conditions tested. Similar to microcosms from the Sacramento site, ethanol had a stimulatory effect on toluene degradation under denitrifying conditions, and no toluene degradation was observed in microcosms without ethanol within 50 d (Fig. 3).

Toluene was also degraded in sulfate-reducing microcosms, and its consumption coincided with that of *m*+*p*-xylenes (data not shown). Cometabolism of xylenes with toluene as primary substrate has also been reported under sulfate-reducing conditions in studies with pure bacterial cultures [15].

General trends and statistical analysis of microcosm data

Analysis of variance showed that, if electron acceptors are not limiting, site-specific factors such as aquifer material properties and electron-accepting conditions have a more significant overall effect on BTEX degradation than the presence of oxygenates (Table 7). Apparently, the type of aquifer material (including geochemical characteristics and exposure history)

Table 6. Monoaromatic hydrocarbon and ethanol degradation in Northwest Terminal (OR, USA) microcosms

Compound[a]	Aerobic		Denitrifying		Sulfate reducing	
	Lag phase (d)	*k* (per day)	Lag phase (d)	*k* (per day)	Lag phase (d)	*k* (per day)
Benzene						
With BTEX[b]	8	0.2	ND[c]	ND	ND	ND
With BTEX+EtOH[d]	11	0.1	ND	ND	ND	ND
Toluene						
With BTEX	4	0.1	ND	ND	17	0.2
With BTEX+EtOH	10	0.1	13	0.4	27	0.3
Ethylbenzene						
With BTEX	5	0.2	ND	ND	ND	ND
With BTEX+EtOH	10	0.4	ND	ND	ND	ND
m+*p*-Xylenes						
With BTEX	ND	ND	ND	ND	17	0.1
With BTEX+EtOH	ND	ND	ND	ND	27	0.1

[a] Initial concentration (mg/L): benzene 2.4 ± 0.2, toluene 1.5 ± 0.1, ethylbenzene 0.5 ± 0.1, *o*-xylene 0.7 ± 0.1, *m*+*p*-xylenes 0.6 ± 0.1, ethanol 70 ± 13.
[b] BTEX = monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene).
[c] ND = no significant removal relative to sterile control was observed within the 50-d incubation period. Data for *o*-xylene under all electron-accepting conditions were ND.
[d] EtOH = ethanol.

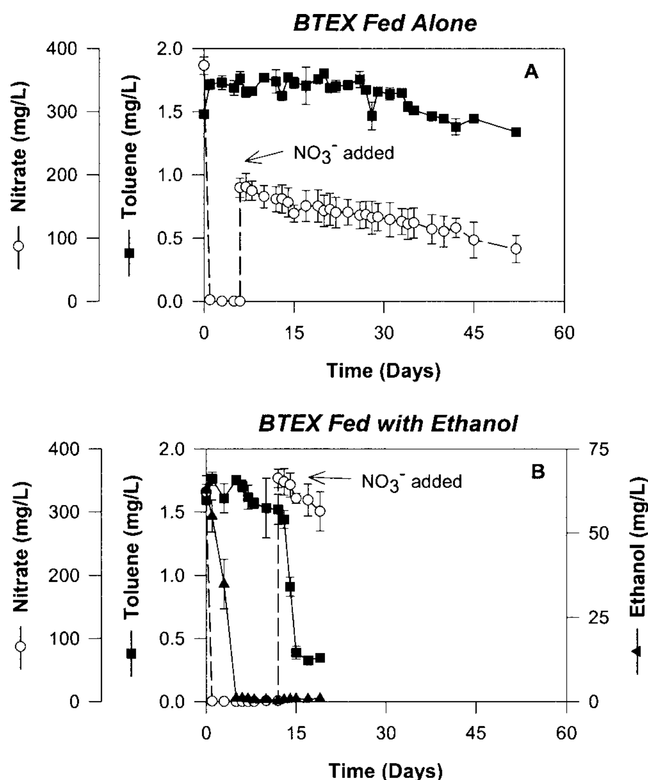

Fig. 3. Enhanced toluene degradation by ethanol in denitrifying microcosms from the Northwest Terminal (Tigard, OR, USA). The bottom panel shows little toluene removal after nitrate was depleted during ethanol degradation and fast removal when nitrate was respiked. Error bars depict one standard deviation from the mean of three microcosms; BTEX = benzene, toluene, ethylbenzene, xylenes.

influences the microbial community structure and the initial concentration of desirable phenotypes, which in turn has a significant effect on whether BTEX degrade readily.

The electron-accepting condition also had a significant overall effect on BTEX degradation (Table 7), with faster degradation generally corresponding to electron acceptors with higher reduction potential ($O_2 > NO_3^- > SO_4^{2-}$) (Tables 3 to 6). Toluene was the most frequently degraded hydrocarbon, and it was consumed under all electron-accepting conditions

Table 7. Analysis of variance of selected factors potentially influencing the lag period and the rate coefficient for monoaromatic hydrocarbon degradation[a]

	Attained level of significance (p value)	
Factor	For lag period	For k
Site	<0.001*	<0.001*
Electron-accepting condition[b]	<0.001*	<0.001*
BTEX[c] compound	<0.001*	0.255
Presence of ethanol	0.519	0.140
Presence of MtBE[d]	0.989	0.862

[a] Sites in California and Oregon, USA: Travis Air Force Base (CA), Tracy (CA), Sacramento (CA), Northwest Terminal (OR), and Tigard (OR).
[b] Electron-accepting condition = aerobic, denitrifying, and sulfate reducing.
[c] BTEX = monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene).
[d] MtBE = methyl-*tert*-butyl ether.
* Significant effect ($p < 0.05$).

tested. On the other hand, benzene (which is the most toxic of the BTEX compounds) was not degraded anaerobically under any condition tested. This corroborates previous studies that benzene is the most recalcitrant of the BTEX compounds under anaerobic conditions [9,16] and suggests that oxygen depletion during ethanol biodegradation is likely to hinder the biodegradation of benzene to a greater extent than that of other BTEX compounds.

Under the conditions tested (electron acceptors supplied in excess), ethanol had no statistically significant effect on BTEX degradation (Table 7). The lack of a clear overall effect is exemplified in microcosms from the Sacramento site, where ethanol hindered aerobic toluene degradation while it enhanced it under denitrifying conditions (Table 4). Enhancement of toluene degradation by ethanol could be attributable to the fortuitous growth of BTEX-degrading bacteria during ethanol degradation.

All microcosms were prepared with electron acceptors in excess of their stoichiometric requirement for the mineralization of the added BTEX and ethanol. However, the high electron acceptor demand exerted by ethanol exacerbated by the background demand of the aquifer material caused the depletion of nitrate and sulfate in microcosms from the Travis AFB, Sacramento, and Northwest Terminal sites. This caused toluene degradation to stop until more electron acceptors were added, as depicted for denitrifying microcosms from the Northwest Terminal (Fig. 3). This shows that ethanol can exert a significant electron acceptor demand compared to the other soluble components in gasoline and that electron acceptor depletion during ethanol degradation could be a very important mechanism by which ethanol could hinder BTEX natural attenuation.

Methyl-*tert*-butyl ether has been reported to degrade under both aerobic and anaerobic conditions [17–22]. However, the ubiquity of such biodegradation capabilities has not been established, and no convincing evidence exists that MtBE biodegradation occurs rapidly in the field under natural conditions [23]. This notion is supported by the lack of MtBE degradation in this study under any condition tested within up to 100 d of incubation (data not shown), which is consistent with the apparent lack of MtBE degradation observed at the Sacramento site (M. Peterson, ETIC Engineering, personal communication).

The presence of MtBE did not significantly affect BTEX degradation patterns (Table 7), which corroborates previous findings that MtBE had a negligible effect on BTEX degradation by non-MtBE-degrading cultures [24,25]. The recalcitrance of MtBE precluded the assessment of how ethanol might affect the natural attenuation (if any) of preexisting MtBE contamination. Nevertheless, MtBE is even more recalcitrant under anaerobic conditions, which suggests that competition for oxygen by ethanol-degrading bacteria would also hinder MtBE biodegradation.

Natural attenuation profiles along aquifer columns

No significant decreases in BTEX or ethanol concentrations (<10%) were observed in the sterile control column (data not shown), indicating that volatile losses were relatively minor. The BTEX were rapidly degraded within the first 10 cm of the column inlet when added without ethanol (Fig. 4A). Oxidation-reduction potential measurements (+150 to +200 mV) and the lack of significant sulfate and nitrate consumption (Fig. 4B) suggest that BTEX were degraded mainly under aerobic

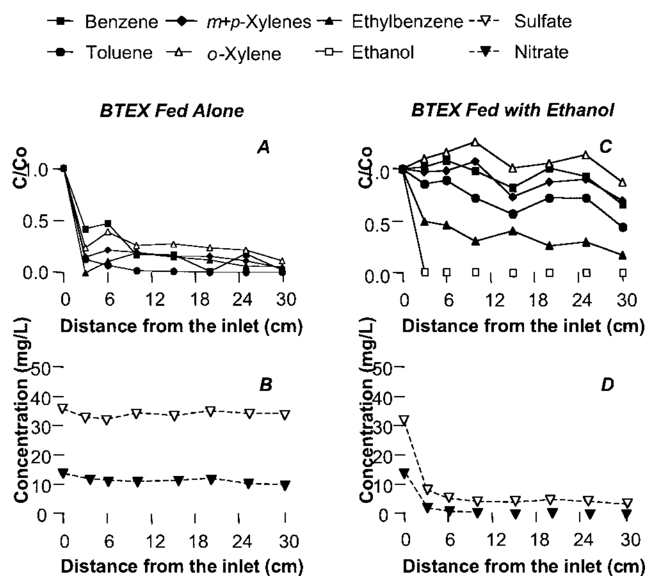

Fig. 4. (**A**) Normalized hydrocarbon concentrations in column fed benzene, toluene, ethylbenzene, and xylenes (BTEX) alone. (**B**) Nitrate and sulfate concentrations in column fed BTEX alone. (**C**) Normalized hydrocarbon and ethanol concentrations in column fed BTEX with ethanol. (**D**) Nitrate and sulfate concentrations in column fed BTEX with ethanol.

conditions. Note that the mineral medium contained ammonium as a nitrogen source.

The degradation of BTEX was significantly inhibited by ethanol, which was preferentially utilized within 3 cm of the column inlet (Fig. 4C). The high oxygen demand exerted by ethanol rapidly created reducing conditions near the column inlet (−29 mV) and contributed to the depletion of the added electron acceptors, nitrate and sulfate (Fig. 4D). As a result, little BTEX degradation occurred in this column. This observation corroborates microcosm results that underscored the dependence of hydrocarbon degradation on electron acceptor availability (Fig. 3). In more general terms, it illustrates that the high ethanol concentrations expected at a gasohol spill (>1,000 mg/L near the source [2]) are likely to contribute significantly to the depletion of electron acceptors and nutrients that could otherwise be available for BTEX degradation.

SUMMARY AND CONCLUSIONS

This study investigated potential effects of ethanol and MtBE on the natural attenuation of BTEX compounds under different conditions commonly encountered in contaminated sites. The data indicate that ethanol is likely to be preferentially utilized relative to the BTEX compounds under both aerobic and anaerobic conditions. Therefore, whereas ethanol is unlikely to persist for extended periods of time at gasohol-contaminated sites, its presence may prevent the bacterial population capable of degrading BTEX from fully expressing its catabolic potential. If electron acceptors are available in excess, ethanol may exhibit a variable effect. While ethanol is more likely to hinder aerobic BTEX metabolism, it may occasionally enhance anaerobic alkylbenzene degradation, possibly due to additional growth of BTEX-degrading bacteria during ethanol degradation. Nevertheless, if electron acceptors are limiting (as is likely to be the case in gasohol plumes), their depletion during ethanol degradation will likely exacerbate the negative effect of ethanol. The magnitude of this effect was shown to be variable, depending on the characteristics of the aquifer material, microbial consortium, and electron-accepting conditions. Yet a decrease in the extent of aerobic BTEX degradation is particularly important for the fate of benzene, which is the most toxic of the BTEXs and degrades very slowly, if at all, under anaerobic conditions. Results also corroborated the recalcitrance of MtBE and showed that its presence is unlikely to affect BTEX or ethanol degradation.

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