Biodegradation **10:** 15–25, 1999.

Anaerobic biodegradation of BTEX and gasoline in various aquatic sediments

Craig D. Phelps & L.Y. Young*
*Rutgers, The State University of New Jersey, New Brunswick, NJ, USA (*author for correspondence)*

Accepted 16 September 1998

Key words: anaerobic, biodegradation, BTEX, gasoline, hydrocarbons

Abstract

We examined the extent of biodegradation of benzene, toluene, ethylbenzene and the three isomers of xylene (BTEX) as a mixture and from gasoline in four different sediments: the New York/New Jersey Harbor estuary (polluted); Tuckerton, N.J. (pristine); Onondaga Lake, N.Y. (polluted) and Blue Mtn. Lake, N.Y. (pristine). Enrichment cultures were established with each sediment using denitrifying, sulfidogenic, methanogenic and iron reducing media, as well as site water. BTEX loss, as measured by GC-FID, was extensive in the sediments which had a long history of pollution, with all compounds being utilized within 21–91 days in the most active cultures, and was very slight or non-existent in the pristine sediments. Also, the pattern of loss was different under the various reducing conditions within each sediment and between sediments. For example benzene loss was only observed in sulfidogenic cultures from the NY/NJ Harbor sediments while toluene was degraded under all redox conditions. The loss of BTEX was correlated to the reduction of the various electron acceptors. In cultures amended with gasoline the degradation was much slower and incomplete. These results show that the fate of the different BTEX components in anoxic sediments is dependent on the prevailing redox conditions as well as on the characteristics and pollution history of the sediment.

Introduction

The monoaromatic hydrocarbons: benzene, ethylbenzene, toluene and *o*-, *m*-, and *p*-xylene (BTEX) are constituents of petroleum and its products such as gasoline and diesel fuel. Because they have a relatively high water solubility, these chemicals readily travel from spills or leaking storage tanks into groundwater and from there into drinking water supplies. Benzene is of particular concern as a pollutant because of its carcinogenicity (Dean 1985). The others are toxic to varying degrees. BTEX also moves easily into sediments and other environments where oxygen is limited. In these anoxic environments, bacteria may use nitrate, iron, sulfate or carbonate as an electron acceptor while degrading organic compounds, but these ions cannot take the place of oxygen as a reactant in aromatic ring cleavage. Because of this, bacteria have had to evolve novel pathways for anaerobic ring cleavage (Harwood & Gibson 1997). The BTEX compounds are especially difficult to degrade because they lack an activating oxygen or nitrogen substituent group which would make oxidation of the ring more energetically feasible. Although biodegradation of these hydrocarbons has been conclusively shown to occur under several anaerobic conditions, information regarding the extent of this activity, or the conditions that encourage it is limited.

The capability of bacteria to anaerobically biodegrade BTEX has been clearly demonstrated. several pure strains of bacteria which mineralize toluene under denitrifying, sulfidogenic or iron-reducing conditions have been isolated (for a review see Frazer et al. 1995), as have denitrifyers which degrade ethylbenzene (Ball et al. 1996; Rabus & Widdel 1995) and *m*-xylene (Dolfing et al. 1990; Hess et al. 1997; Rabus & Widdel 1995). The mineralization of various isomers of xylene also occurs under denitrifying (Ball & Reinhard 1996 [*p*, *m*, *o*]; Bregnard et al. 1996 [*p*, *m*, *o*]; Evans et al. 1991 [*m*]; Haner et al. 1995

[*p*, *m*]; Hutchins et al. 1991 (*p*, *m*], 1991b (*p*, *m*], 1992 [*p*, *m*]; Kao & Borden 1997 [*m*]; Kuhn et al. 1985 [*p*, *m*], 1988 [*m*]; Zeyer et al. 1986 [*m*]) as well as sulfate-reducing (Ball & Reinhard 1996 [*p*, *m*, *o*]; Edwards et al. 1992 [*p*, *m*, *o*]; Haag et al. 1991 [*p*]; Rabus et al. 1996 [*m*, *o*]; Reinhard et al. 1997 [*p*, *m*, *o*]) and methanogenic (Edwards & Grbic-Galic 1994 [*o*]; Wilson et al. 1986 [*o*]) conditions. Recently benzene degradation has been demonstrated under iron-reducing (Kazumi et al. 1997; Lovley et al. 1994, 1996), sulfidogenic (Edwards & Grbic-Galic, 1992; Kazumi et al. 1997; Lovley et al. 1995; Phelps et al. 1996) and methanogenic conditions (Kazumi et al. 1997).

Although this activity has been clearly demonstrated, it has generally been found only in highly polluted areas. Most studies have focused on a single polluted site such as a heavily trafficked harbor or an aquifer which has a history of chronic spills. Few studies have examined the variability in biodegradative capacity between different sites under different reducing conditions. Kao & Borden (1997) looked at BTEX degradation in several aquifer sediments, both polluted and clean, in denitrifying microcosms. They found that some inocula resulted in loss of toluene, ethylbenzene, *m*-xylene and *o*-xylene, whereas others showed no or reduced activity. Variation within a site, depending on the terminal electron acceptor provided was studied by Ball & Reinhard (1996) and Reinhard et al. (1997) at a gasoline contaminated aquifer. BTEX loss was observed under denitrifying and sulfidogenic conditions. Differences were seen in both patterns and rates of degradation.

Benzene mineralization, in particular, has proven to be an elusive activity. Many researchers have reported that benzene was recalcitrant when sulfate was present (Ball & Reinhard 1996; Edwards et al. 1992; Langenhoff et al. 1996; Reinhard et al. 1997) but others have demonstrated biodegradation under these same conditions (Edwards & Grbic-Galic, 1992; Kazumi et al. 1997; Lovley et al. 1995; Phelps et al. 1996). We were interested in finding out if there was a difference in the degradation of BTEX in enrichment cultures established under identical conditions using different sediments as inocula.

It was our goal to determine if differences between contaminated and pristine environments would have an effect on the degradation of BTEX under different anaerobic conditions. We aim to comprehensively compare sediments from these environments in microcosms using identical protocols, media and incubation conditions in order to demonstrate the effects of the different inocula and electron acceptors.

We established enrichment cultures using sediment from four different sites, under five different reducing conditions in order to examine the effect of sediment characteristics and electron acceptors on BTEX biodegradation. The four sites were chosen because of their different histories of contamination and sediment characteristics. Two marine sites, one polluted (Arthur Kill) (AK) and the other pristine (Tuckerton) (TKT) were compared to two freshwater sites, also polluted (Onondaga Lake) (OL) and pristine (Blue Mtn. Lake) (BML). The Arthur Kill is part of the New York/New Jersey Harbor Estuary and is characterized by a long history of industrial development and pollution. Petroleum and other hazardous chemicals are frequently released into its waters (Brosnan & O'Shea 1993; Gunster et al. 1993; Holliday & Klein 1993). Similarly, Onondaga Lake, N.Y. has been the subject of industrial inputs, including mercury, petroleum, PCB's and other chlorinated compounds since the late 1800's (Effler & Harnett 1996; Perkins & Romanowicz 1996). In contrast, the other sites are both considered to be free from gross anthropogenic impacts. The Tuckerton site is an unpolluted site (Gary Taghon, personal communication), located on the continental shelf off the coast of an undeveloped part of southern New Jersey. Blue Mtn. Lake is an oligotrophic lake located within the boundaries of the Adirondack Park (New York), and has low annual inputs of nutrients or other pollutants (Martin & Hyde 1995).

Materials and methods

Sediment characterization

Inocula for the enrichment cultures were collected from four different sites. All samples were taken from the top 20 cm of sediment. At the two polluted sites (AK and OL), this material was highly anaerobic, in the others (BML and TKT) the samples quickly became anoxic during storage. AK sediment was collected in ~5 m of water using a coring device. OL was sampled using a Ponar dredge at a depth of ~19 m. Surface sediments at the other sites (BML and TKT) were collected with simple scoops. Water from the site of collection was also sampled at each location. All sediments and site water were stored in sealed jars at 4 °C until needed. In addition to the pollution history,

these sites also differed in the amount of organic matter and nutrients present in their sediments (Table 1). Sediment characterization was performed by the Soil testing Laboratory – New Jersey Agricultural Experiment Station using standard soil testing methods. The concentration of several ions in the site collected water were analyzed using ion chromatography.

Enrichment conditions

All enrichment cultures were set up as a 10% (vol./vol.) sediment slurry in defined mineral media, or in water collected at the site. The defined medium contained the following salts (in grams per liter): KCl, 1.30; KH_2PO_4, 0.20; NaCl, 1.17; NH_4Cl, 0.50; $CaCl_2 \cdot 2H_2O$, 0.10; $MgCl_2 \cdot 6H_2O$, 0.41; and $NaHCO_3$, 2.52 as well as 0.1 ml trace salts, 1 ml vitamins and resazurin as a redox indicator. The trace metal supplement was composed of the following (in grams per liter): H_3BO_3, 0.062; $MnCl \cdot 4H_2O$, 0.098; $FeCl_2 \cdot 4H_2O$ 1.49; $CoCl_2 \cdot 6H_2O$, 0.119; $NiCl_2 \cdot 6H_2O$, 0.237; $CuCl_2$, 0.134; and $ZnCl_2$, 0.068. The vitamin supplement contained (in milligrams per liter): biotin, 20; folic acid, 20; pyridoxine HCl, 100; riboflavin, 50; thiamin, 50; nicotinic acid, 50; pantothenic acid, 50; cyanocobalamin, 1; p-aminobenzoic acid, 50; and thiotic acid, 50. The medium used for the AK and TKT enrichments contained 23 g NaCl and 1.0 g $MgCl_2 \cdot 6H_2O$ per liter to approximate the salinity of sea water. In addition the methanogenic medium contained 0.4 g/l of $FeCl_2 \cdot 4H_2O$ and 1.0 ml of 2.1 M $Na_2S \cdot 9H_2O$ as a reducing agent. The iron-reducing medium contained a slurry of amorphous FeOOH (∼200 mM Fe(III)) formed by the reaction of $FeCl_3$ with NaOH. Denitrifying cultures were amended with 3.03 g/l of KNO_3 (30 mM), and 2.84 g/l of Na_2SO_4 (20 mM) was added to the sulfidogenic medium along with 0.7 ml of 2.1M $Na_2S \cdot 9H_2O$.

The sediment slurries were dispensed in 50 ml aliquots into 60 ml serum bottles under an atmosphere of 70% N_2/30% CO_2 (methanogenic and sulfidogenic) or Argon (denitrifying, iron-reducing and site water). Bottles were sealed with Teflon-coated, butyl rubber stoppers (Emsco, Philadelphia, PA) and crimp sealed. All enrichments were made in triplicate with duplicate sterile controls. The sterile controls were autoclaved three times on three consecutive days before initiation of the experiment. In addition, duplicate background controls were prepared in the same manner as the experimental cultures except that no substrate was added. These controls were used to account for electron acceptor loss due to metabolism of endogenous carbon in the sediment inoculum. The cultures were incubated at 30 °C in the dark without shaking. Strict anaerobic procedures were followed at all times.

Each of the cultures, except background controls, was amended with a mixture of benzene, toluene, ethylbenzene, *o*-, *m*, and *p*-xylene (BTEX) (Aldrich Chemical Co., Milwaukee, WI) each at a concentration of ∼100 μM. BTEX was added neat using a microliter syringe. The BTEX compounds were the only carbon and energy source added to the enrichments.

Enrichments used to compare the degradation of BTEX to gasoline were set up in the same way using site water as the medium and Arthur Kill sediment as the inoculum in a 10% (vol./vol.) slurry under an Argon headspace. The BTEX fed cultures received ∼100 μM of each compound, the gasoline cultures were fed 30 μl of commercial gasoline.

Analytical procedures

BTEX loss was monitored by withdrawing a 1 ml sample of the slurry and extracting with 0.4 ml of pentane containing 100 μM of fluorobenzene as an internal standard. The extract was analyzed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Avondale, PA) and a 30 m × 0.32 mm DBWax capillary column (J&W Scientific, Folsom, CA).

Nitrate and sulfate were measured on a Dionex model 100 ion chromatograph (Sunnyvale, CA) equipped with an IonPac AS9-SC column and a conductivity detector. The eluent was 2 mM Na_2CO_3, 0.75 mM $NaHCO_3$ at a flow rate of 2 ml min^{-1}. Ions were quantitated by comparison with external standards using a Chrome-Jet integrator (Spectra-Physics, San Jose, CA). The production of methane was checked by withdrawing a 0.25 ml sample of the headspace gas with a gas-tight syringe (Precision Sampling, Baton Rouge, La.) which had been flushed with Argon, and analyzing it on a model 1200 gas partitioner (Fisher Scientific, Pittsburgh, Pa.).

Results

BTEX degradation

The rate and pattern of BTEX degradation from our enrichment cultures showed distinct differences between the sediments used as the inoculum and the

Table 1. Characterization of sediments

Sediment	% O.M.	pH	% TKN	Nutrients (ppm) P	K	Mg	Ca	Texture (%) Sand	Silt	Clay
Arthur Kill	14.6	7.6	0.54	0.1	84	151	248	49	46	5
Tuckerton	0.2	8.7	0.01	1.4	10	28	14	100	0	
Onondaga	14.6	7.8	0.51	0.1	12	28	445		ND*	
Blue Mtn.	1.0	6.9	0.08	1.5	1	4	66	94	4	2

Site Water

Site	mM NO_3^-	$SO_4^=$	Cl^-
Arthur Kill	0.05	8.2	164
Tuckerton	u.d.	17.0	245
Onondaga	0.06	0.97	9.8
Blue Mtn.	0.03	0.07	1.0

* Sample was estimated to be less than 50% Sand and greater than 50% silt.
Analyses were performed by the Soil Testing Laboratory – New Jersey Agricultural Experiment Station.

electron acceptors provided. The results are summarized in Table 2. Degradation did not occur in any of the cultures from the BML sediment, and only one compound (toluene) was degraded in the TKT cultures (site water) (data not shown). Both of these sites are characterized by a lack of pollution history, low organic matter content and relatively low nutrient concentrations (Table 1). In contrast, BTEX loss was extensive in cultures established from the heavily polluted, high organic matter, Arthur Kill and Onondaga Lake sites.

Activity was especially rapid in the nitrate amended cultures of both polluted sites (AK and OL). Toluene, *m*-xylene and ethylbenzene were all lost, with no apparent lag, in both sets of enrichments (Figure 1A). However, no subsequent loss of the other compounds was observed in the AK cultures, and only *p*-xylene was lost (after a very long lag period) in the OL cultures. The pattern of degradation was the same in both sediments. Toluene, and *m*-xylene in OL, was lost within the initial 21 days of incubation. These were followed closely by ethylbenzene. There was also a small (∼20–40%) decrease in the concentration of *o*-xylene during the first three weeks, possibly due to cometabolism.

In the sulfate amended enrichments, degradation of BTEX began more slowly, but in the case of AK, proceeded to completion for all six of the substrates (Figure 1B). The same pattern was seen in the site water cultures (Figure 1C) which were actively sulfidogenic. Toluene was degraded first, followed by *o*- and *m*-xylene, then *p*-xylene and ethylbenzene, and finally benzene. In the sulfate-reducing OL enrichments (Figure 1B), only toluene and *m*-xylene were degraded during the course of the experiment.

As expected, the only methanogenic cultures which were active at degrading BTEX were those derived from freshwater (OL) sediment (Figure 1D), this includes the site water enrichment. toluene and ethylbenzene were lost during the 161 day incubation. When these same cultures were checked after 371 days, *o*-xylene loss was also seen (data not shown).

Iron-reducing cultures (Figure 1E) demonstrated the ability to degrade toluene (AK and OL) and *p*-xylene (AK) or *m*-xylene and *o*-xylene (OL). There was also some loss of ethylbenzene, *m*-xylene and *o*-xylene in the AK enrichment but the results were inconsistent.

Electron acceptor loss

The concentrations of nitrate and sulfate were measured in the denitrifying and sulfidogenic (including AK site water) cultures at the beginning and end of the incubation. In those cultures where BTEX degradation had taken place, the amount of electron acceptor loss in fed cultures above that in un-fed backgrounds was compared to the amount of loss that would be expected if all the BTEX were mineralized to CO_2. The results are shown in Table 3. Values ranged from 73–75% of expected for denitrifying cultures, and 88–186% for sulfidogenic. Because of the small amount of substrate utilized (from 6–21 μmol) and the large background

Table 2. Summary of BTEX degradation in different sediments

	Benzene	Toluene	Ethylbenzene	*p*-Xylene	*m*-Xylene	*o*-Xylene
Arthur Kill						
NO_3^-	–	++++++	+++++	–	++++++	–
$SO_4^=$	+	++++++	++	++	+++	++++
CO_3^-	–	–	–	–	–	–
Fe(III)	–	++++	+	++	+/–	+/–
Site water	+++	++++++	+++	+++	+++	++++
Tuckerton						
NO_3^-	–	–	–	–	–	–
$SO_4^=$	–	–	–	–	–	–
CO_3^-	–	–	–	–	–	–
Fe(III)	–	–	–	–	–	–
Site water	–	++++	–	–	–	–
Onondaga						
NO_3^-	–	++++++	++++++	+	++++++	–
$SO_4^=$	–	++++	–	–	++	–
CO_3^-	–	++++	+	–	–	–
Fe(III)	–	++++++	–	–	+++	++
Site water	–	++	–	–	–	–
Blue Mtn.						
NO_3^- [b]	–	–	–	–	–	–
$SO_4^=$ [b]	–	–	–	–	–	–
CO_3^-	–	–	–	–	–	–
Fe(III)	–	–	–	–	–	–
Site water	–	–	–	–	–	–

++++++, loss within 21 days (avg. is less than 10% sterile controls).
+++++, loss within 35 days.
++++, loss within 63 days.
+++, loss within 91 days.
++, loss within 119 days.
+, loss within 161 days.
+/– loss in only 1 replicate after 161 days.
[b] Only 2 replicates available.

loss in some cultures, it is difficult to obtain good estimates for these values. They are, however, in general agreement with the hypothesis that the BTEX is being mineralized rather than lost by some other process.

The presence of methane in the headspace of the methanogenic enrichments, including OL site water (data not shown), indicated that methanogenesis was the terminal electron accepting process in these cultures. Iron reduction was not measured.

Effects of gasoline

When commercial gasoline was used as the substrate, degradation of BTEX was much slower and less complete than when the BTEX was added alone (Figure 2). BTEX fed cultures showed complete loss of all six compounds in less than 150 days. In contrast, gasoline fed cultures showed only partial loss of toluene and the three xylenes, and no loss of benzene or ethylbenzene after nearly 300 days.

In order to determine which components of gasoline were causing the inhibitory effect we established enrichments containing toluene alone, BTEX, toluene plus a mixture of C5-C11 alkanes, or gasoline, and following the degradation of the toluene over time. The concentration of toluene in each was approximately equal. Results are shown in Figure 3. Complete loss of toluene was observed within 28 days in the toluene alone cultures, 42 days in BTEX cultures and 70 days in toluene plus alkanes. Degradation was not signifi-

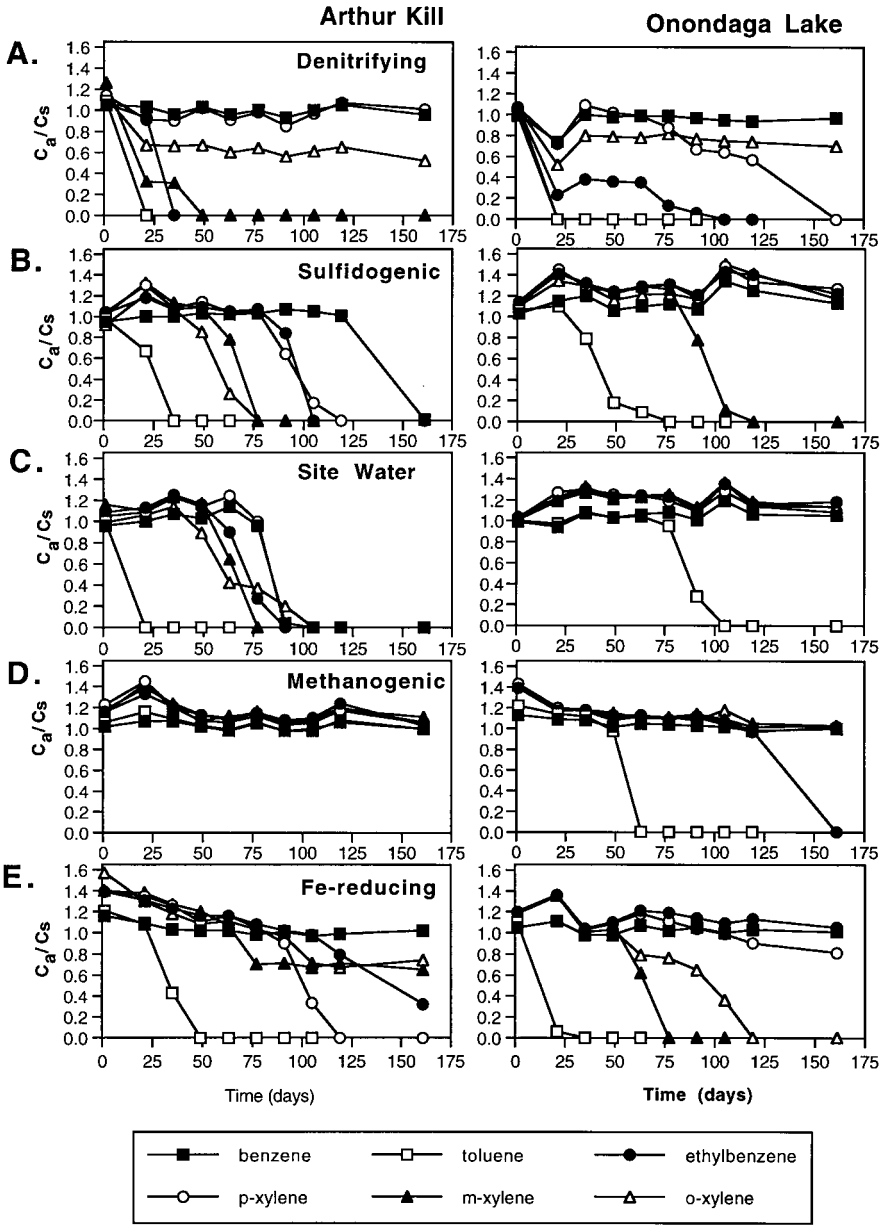

Figure 1. Loss of BTEX from anaerobic enrichment cultures. Each culture was fed a mixture of BTEX at a concentration of ~100 μM for each component. All data points are the average of triplicate bottles and are expressed as the ratio of the concentration in the active cultures to the concentration in the sterile controls (C_a/C_s).

cant over the course of the experiment for the gasoline cultures.

Discussion

Although previous research has demonstrated that individual BTEX compounds are biodegradable under anaerobic conditions, few investigators have studied the differences between sites, or the effect that different electron acceptors have on degradation. This study is significant because we have tested the activity from several sites in a side-by-side comparison using the full range of BTEX components as substrates. Enrichment conditions were identical except

Table 3. Utilization of electron acceptors

Culture	mM Nitrate loss Active	bkgd.	Net	BTEX loss (mM)	NO_3 loss expected	% of expected
Denitrifying						
Arthur Kill	8.90	6.75	2.15	0.156	1.23	175 ± 35
Onondaga	13.05	11.05	2.00	0.341	2.73	73 ± 20
Sulfidogenic						
Culture	mM Sulfate loss Active	bkgd.	Net	BTEX loss (mM)	SO_4 loss expected	% of expected
Arthur Kill	5.20	3.05	2.15	0.249	1.16	186 ± 12
Arthur Kill (site water)	6.00	3.90	2.10	0.425	1.97	107 ± 19
Onondaga	5.30	4.80	0.50	0.119	0.57	88 ± 0

The amount of nitrate or sulfate reduced in the active cultures was compared to the amount expected if all BTEX lost was mineralized to CO_2. The expected amounts of nitrate or sulfate loss were calculated from the theoretical stoichiometric balance for mineralization of each substrate and the amount of the substrate lost. For example, toluene under denitrifying conditions:

$$C_7H_8 + 7.2NO_3^- + 7.2H^+ \rightarrow 7CO_2 + 3.6N_2 + 7.6H_2O$$

or under sulfidogenic conditions:

$$C_7H_8 + 4.5SO_4^= + 9H^+ \rightarrow 7CO_2 + 4.5H_2S + 4H_2O.$$

These individual values were then summed to find the expected nitrate or sulfate loss, which was compared to the actual net loss in the active cultures.

for the inoculum and the electron acceptor provided. This allows us to draw conclusions about the nature of the microbial populations present and the potential for bioremediation.

We have shown that there is a great deal of variability in the fate of BTEX in anaerobic sediments, with the rate and extent of loss depending on the inoculum used and the terminal electron acceptor available. While some enrichments resulted in complete loss of all six compounds (e.g. AK-sulfidogenic), others showed no activity at all (e.g. BML – all conditions). Within each sediment, the activity was affected by which electron acceptor was provided. Marine cultures had robust activity under sulfidogenic conditions, while only freshwater cultures were effective under methanogenic. Both sets responded well to nitrate addition, with rapid degradation of some compounds. Furthermore, the degradation of BTEX was severely limited by the presence of some, as yet unidentified, compound or compounds in gasoline. This information has duplications for the type of remediation strategy to be used on BTEX spills in a specific area depending on the type of material spilled, the pollution history, and the sediment characteristics.

Hydrocarbon utilization was extensive in cultures from the organic rich, polluted sites (AK and OL), and very slight or lacking in the low carbon, pristine sites (TKT and BML). Toluene in particular was quickly degraded in cultures from both of the polluted sites, under all reducing conditions tested (with the exception of methanogenic cultures derived from AK) (Table 1). Only one example of toluene degradation was seen in one of the unpolluted sediments (TKT- site water), however, and none of the other aromatics was lost. The rapid utilization of toluene under all reducing conditions is consistent with many reports in the literature, including Ball & Reinhard (1996), Barbaro et al (1992), Reinhard et al (1997) and especially Langenhoff et al (1996) who showed that in columns filled with mixed, polluted sediments, toluene degradation took place under denitrifying, sulfidogenic, methanogenic, iron-reducing, and manganese-reducing conditions. There were no organic-rich, unpolluted sediments or organic-poor, polluted sediments included in this study, so it is not possible to distinguish between the effects of these two factors.

Differences in substrate utilization due to the available electron acceptor were evident in both the AK and OL enrichments. The most rapid loss of any compounds occurred when nitrate was added as the terminal electron acceptor, but the loss was most extensive

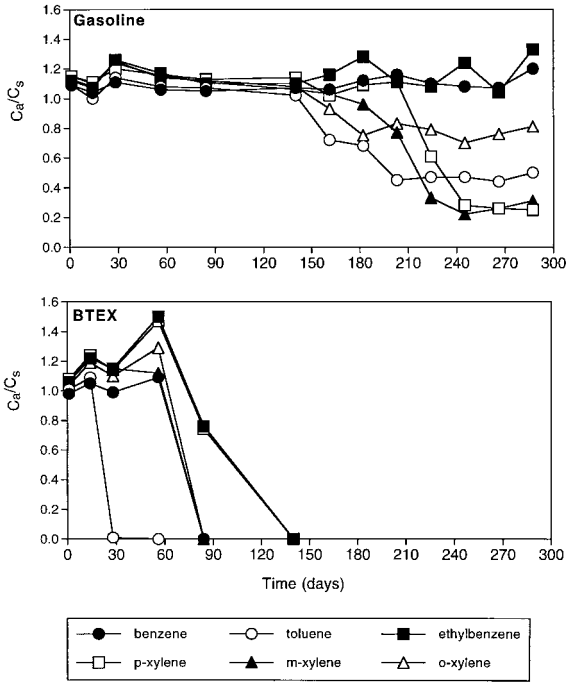

Figure 2. Comparison of BTEX degradation in enrichment cultures containing gasoline or BTEX alone. Cultures were fed either 30 μl of gasoline (for a final toluene concentration of 100–150 μM) or a mixture of BTEX at a concentration of −100 μM for each component. All data points are the average of triplicate bottles and are expressed as the ratio of the concentration in the active cultures to the concentration in the sterile controls (C_a/C_s).

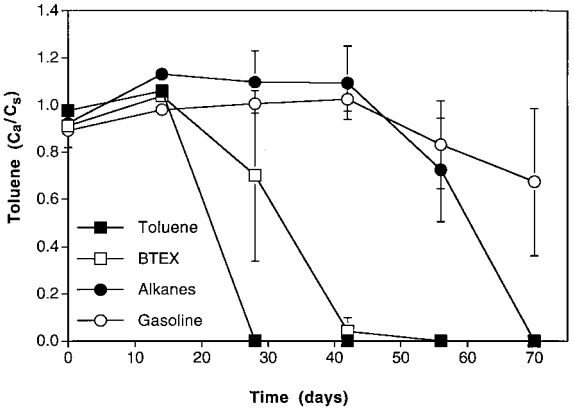

Figure 3. Degradation of toluene in cultures amended with toluene alone, BTEX, a mixture of C_5–C_{11} alkanes, or gasoline. Error bars represent ±1 standard deviation.

when sulfate was added. In the AK enrichments, only toluene, *m*-xylene and ethylbenzene were degraded when nitrate was provided. In contrast, when ferric iron was added, *m*-xylene was not used but *p*-xylene was, and when sulfate was present, all six compounds including benzene were lost. Similarly, differences were seen in the OL cultures. Toluene, ethylbenzene, *m*-xylene and, after a long lag, *p*-xylene disappeared under denitrifying conditions, only toluene and *m*-xylene were utilized under sulfidogenic conditions. Iron-reducing activity was similar to denitrifying, with the exception of *o*-xylene being degraded instead of *p*-xylene. These differences are in contrast to the results of Reinhard et al. (1997), who found that at one site all of the BTEX compounds, with the exception of benzene, were degraded under both denitrifying and sulfidogenic conditions. The degradation of toluene, ethylbenzene, *m*-xylene and sometimes *p*-xylene, without loss of benzene or *o*-xylene, in denitrifying enrichments has been reported numerous times however (Hutchins et al. 1991, 1991b; Rabus & Widdel 1995).

Interestingly, but not surprisingly, toluene was the first compound to be lost in all cultures. toluene was followed by ethylbenzene and *m*-xylene in denitrifying cultures and *o*- and *m*-xylene in sulfidogenic cultures (AK). Benzene was the last compound utilized. Edwards et al. (1992) also found toluene and *m*-xylene to be the preferred substrates when BTEX was fed to a sulfate-reducing enrichment. They found, in contrast to our results however, that *o*-xylene was consumed after *p*-xylene.

In denitrifying cultures from both AK and OL there is some evidence that *o*-xylene is being cometabolized during toluene degradation. The concentration of *o*-xylene decreased approximately 10% relative to the sterile controls during the first three weeks of incubation (when toluene degradation was occurring), but did not change afterwards. In sub-cultures of the AK enrichment, the addition of more toluene resulted in a further decrease in *o*-xylene concentrations (data not shown). This type of cometabolism was observed by Evans et al. (1991) in enrichment cultures and, later, in a pure culture of strain T1 (Evans et al. 1991b). The transformation of *o*-xylene in T1 was tied to the production of 2-methyl-benzylsuccinate by the enzymes responsible for toluene metabolism (Evans et al. 1992). Apparent cometabolism has since been reported in other studies of denitrifying systems (Ball & Reinhard 1996; Kao & Borden 1997), and the by-products have been isolated from contaminated

groundwater, where sulfate was the terminal electron acceptor (Beller et al. 1995). Though cometabolism cannot be completely excluded from these consortia, *o*-xylene metabolism began only after toluene was exhausted, suggesting that it was not the dominant mechanism of degradation.

It is significant that benzene degradation was only observed in cultures from one site (AK), and only under sulfidogenic conditions. Benzene degradation has previously been shown to occur with sulfate as the terminal electron acceptor in four separate studies, one of which used the Arthur Kill as its sediment source (Kazumi et al. 1997). The other reports also involved highly polluted source material (Edwards & Grbic-Galic, 1992; Lovley et al. 1995) including an area exposed to hydrothermally produced hydrocarbons over a geological time scale (Phelps et al. 1996). Other researchers have noted a lack of degradation in similar sediments under the same conditions (Ball & Reinhard, 1996; Beller et al. 1992; Edwards et al. 1992; Rabus et al. 1996; Reinhard et al. 1997). The lack of degradation is sometimes ascribed to the preferential utilization of other carbon compounds (Edwards & Grbic-Galic, 1992), an observation which is consistent with, but not proven by the observation that benzene was the last compound to be degraded in our experiments (see Figure 1B). Complete inhibition of benzene utilization did not, however, occur in our enrichments as it may have in other reports. It appears that the ability to anaerobically degrade benzene is not widespread throughout the environment, but can only be found in certain sites with the right combination of pollution history and environment.

The mineralization of benzene under iron-reducing (Kazumi et al. 1997; Lovley et al. 1994; Lovley et al. 1996) and methanogenic (Grbic-Galic & Vogel, 1987; Kazumi et al. 1997; Wilson et al. 1986) conditions has also been reported, but was not observed in this study. The concentrations of benzene used in our enrichments was much higher than in the previous iron-reducing reports, and the lag times in the methanogenic experiments was often longer than the length of our experiment, this may explain our lack of success.

The relationship between the amount of electron acceptor lost and the amount of BTEX degraded during the course of the experiment (Table 3) supports the hypothesis that these aromatic compounds are being mineralized to carbon dioxide and water. The agreement between expected and predicted values was best in the AK-site water enrichment (107%), where the largest amount of BTEX (0.425 mM) was lost in a relatively short time (∼100 days). Ball & Reinhard (1996) observed an excess of from 2.4–6.6 times the amount of electron acceptor usage over what would be required to mineralize the added BTEX in their microcosms. This effect was accounted for in our study by subtracting out the amount of nitrate and sulfate reduced in un-fed controls. The values that we obtained in these background controls (2.0–8.4 times) is in good agreement with that they reported. The amount of background utilization was especially high in the denitrifying cultures, and this made it difficult to obtain good estimates of the actual stoichiometry.

Degradation of BTEX was much slower and incomplete in cultures amended with gasoline than when BTEX alone was added. Other researchers have noticed similar results when feeding gasoline (Barbaro et al. 1992; Hutchins et al. 1991b). There are many possible explanations for this effect. Although the concentrations of BTEX were approximately the same, the total amount of hydrocarbons in the gasoline fed cultures was much higher. This may have led to a general toxic solvent effect on the whole microbial community leading to a longer lag time, or these other hydrocarbons may have been degraded preferentially over the aromatics. It is also possible that some other component or additive in the gasoline exerted a selective toxicity on the aromatic degraders. Because there was no decrease in mineralization of the background organic matter (as indicated by electron acceptor loss) in gasoline amended cultures as compared to un-fed controls (data not shown), it seems likely that the inhibitory effect is not directed at the entire microbial community. The results of an experiment where various components of gasoline were added to cultures along with toluene (Figure 3), however, show slower rates of degradation in the presence of alkanes and BTEX but that the inhibition of toluene degradation can not be explained entirely by the presence of either. There may be a synergistic effect between the different components or some other, untested, compound may be causing the lag. More work needs to be done to determine the mechanism behind these observations.

We have shown that the fate of BTEX in anaerobic sediments is dependent on characteristics of the site, including its pollution history and the terminal electron acceptor available. Furthermore, the degradation of BTEX may be severely limited by the form (alone or as a component of gasoline) in which it is introduced to the site. This information leads to the conclusion that bioremediation strategies must take

into account the previous history and other characteristics of an area, as well as the type of material spilled, if they hope to take advantage of native microbial communities.

Acknowledgments

This research was supported in part by a grant from DARPA (grant No. N00014-92-J-88).

We are grateful to the following people for assistance in collecting sediments: Dr. Steven Effler of the Upstate Freshwater Institute (Onondaga Lake), Dr. Lee Kerkhof of the IMCS at Rutgers and Rosemarie Petrecca of MAB-NURC (Tuckerton) and Beau Ranheim and the crew of the HSV Osprey of the NYC-DEP (Arthur Kill). Also to Dr. Michael Martin for providing a copy of the limnological report on Blue Mtn. Lake, and especially to Monica Tukiendorf for assistance in the lab.

References

Ball HA, Johnson HA, Reinhard M & Spormann AM (1996) Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178: 5755–5761

Ball HA & Reinhard M (1996) Monoaromatic hydrocarbon transformation under anaerobic conditions at Seal Beach, California: laboratory studies. Environ. Tosicol. Chem. 15: 114–122

Barbaro JR, Barker JF, Lemon LA & Mayfield CI (1992) Biotransformation of BTEX under anaerobic, denitrifying conditions: field and laboratory observations. J. Contam. Hydrol. 11: 245–272

Beller HR, Ding W-H & Reinhard M (1995) Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. Environ. Sci. Technol. 29: 2864–2870

Beller HR, Reinhard M & Grbic-Galic D (1992) Metabolic byproducts of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 58: 3192–3195

Bregnard TP, Höhener P, Häner A & Zeyer J (1996) Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. Environ. Toxicol. Chem. 15: 299–307

Brosnan TM & O'Shea ML (1993) New York Harbor water quality survey. New York City Department of Environmental Protection

Dean BJ (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. 145: 153–181

Dolfing J, Zeyer J, Binder-Eicher P & Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154: 336–341

Edwards EA & Grbic-Galic D (1992) Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. 58: 2663–2666

Edwards EA & Grbic-Galic D (1994) Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60: 313–322

Edwards EA, Wills LE, Reinhard M & Grbic-Galic D (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl. Environ. Micro. 58: 794–800

Effler SW & Hamett G (1996) Background. In: Effler SW (Ed) Limnological and Engineering Analysis of a Polluted Urban Lake. Prelude to Environmental Management of Onondaga Lake, New York (pp. 1–31). Springer-Verlag, New York

Evans PJ, Mang DT & Young LY (1991) Degradation of toluene and m-xylene and transformation of o-xyiene by denitrifying enrichment cultures. Appl. Environ. Microbiol. 57:450–454

Evans PJ, Ling W, Goldschmidt B, Ritter ER & Young LY (1992) Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. 58: 496–501

Evans PJ, Mang DT, Kim KS & Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57: 1139–1145

Frazer AC, Coschigano PW & Young LY (1995) Toluene metabolism under anaerobic conditions: a review. Anaerobe 1: 293–303

Grbic-Galic D & Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. 53: 254–260

Grunster DG, Bonnevie NL, Gillis CA & Wenning RJ (1993) Assessment of chemical loadings to Newark Bay, New Jersey from petroleum and hazardous chemical accidents occurring from 1986 to 1991. Ecotoxicology and Environmental Safety 25: 202–213

Haag F, Reinhard M & McCarty PL (1991) Degradation of toluene and p-xylene in anaerobic microcosms: evidence for sulfate as a terminal electron acceptor. Environ. Toxicol. Chem. 10: 1379–1389

Haner A, Hohener P & Zeyer J (1995) Degradation of p-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61: 3185–3188

Harwood CS & Gibson J (1997) Shedding light on anaerobic benzene ring degradation: a process unique to prokaryotes? J. Bacteriol. 179: 301–309

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P & Zeyer J (1997) In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63: 2136–2141

Holliday SE & Klein III CJ (1993) Surficial sediments in Mid-Atlantic estuaries. US Department of Commerce, NOAA

Hutchins SR, Downs WC, Wilson JT, Smith GB, Kovacs DA, Fine DD, Douglass RH & Hendrix DJ (1991) Effect of nitrate addition on biorestoration of fuel-contaminated aquifer: field demonstration. Ground Water 29: 571–580

Hutchins SR, Moolenaar SW & Rhodes DE (1992) Column studies on BTEX biodegradation under microaerophilic and denitrifying conditions. J. Hazardous Mater. 32: 195–214

Hutchins SR, Sewell GW, Kovacs DA & Smith GA (1991) Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. 25: 68–76

Kao C-M & Borden RC (1997) Site-specific variability in BTEX biodegradation under denitrifying conditions. Ground Water 35: 305–311

Kazumi J, Caldwell ME, Suflita JM, Lovley DR & Young LY (1997) Anaerobic degradation of benzene in diverse anoxic environments. Environ. Sci. Technol. 31: 813–818

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB & Schwarzenbach RP (1985) Microbial Transformations of Substi-

tuted Benzenes during Infiltration of River Water to Groundwater: Laboratory Column Studies. Environ. Sci. Technol. 19(10): 961–968

Kuhn EP, Zeyer J, Eicher P & Schwarzenbach RP (1988) Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. Appl. Environ. Microbiol. 54: 490–496

Langenhoff AAM, Zehnder JB & Schraa G (1996) Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. Biodegradation 7: 267–274

Lovley DR, Coates JD, Woodward JC & Phillips EJP (1995) Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. 61: 953–958

Lovley DR, Woodward JC & Chapelle FH (1994) Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. Nature 370: 128–131

Lovley DR, Woodward JC & Chapelle FH (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl. Environ. Microbiol. 62: 288–291

Martin MR & Hyde JB (1995) The limnological condition of Blue Mountain Lake: final report of the 1994 monitoring program. The Adirondack Aquatic Institute. Paul Smiths, New York

Phelps CD, Kazumi J & Young LY (1996) Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction. FEMS Microbiol. Lett. 145: 433–437

Perkins MG & Romanowicz EA (1996) Hydrogeologic Setting. In: Effler SW (Ed) Limnological and Engineering Analysis of a Polluted Urban Lake. Prelude to Environmental Management of Onondaga Lake, New York (pp 32–96). Springer-Verlag, New York

Rabus R, Fukui M, Wilkes H & Widdel F (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. 62: 3605–3613

Rabus R & Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163: 96–103

Reinhard M, Shang S, Kitanidis PK, Orwin E, Hopkins GD & Lebron CA (1997) In situ BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. Environ. Sci. Technol. 31: 28–36

Wilson BH, Smith GB & Rees JF (1986) Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. 20: 997–1002

Zeyer J, Kuhn EP & Schwarzenbach RP (1986) Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. Appl. Environ. Microbiol. 52: 944–947