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Substrate Interactions of Benzene, Toluene, and *para*-Xylene during Microbial Degradation by Pure Cultures and Mixed Culture Aquifer Slurries

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Benzene, toluene, and *p*-xylene (BTX) were degraded by indigenous mixed cultures in sandy aquifer material and by two pure cultures isolated from the same site. Although BTX compounds have a similar chemical structure, the fate of individual BTX compounds differed when the compounds were fed to each pure culture and mixed culture aquifer slurries. The identification of substrate interactions aided the understanding of this behavior. Beneficial substrate interactions included enhanced degradation of benzene and *p*-xylene by the presence of toluene in *Pseudomonas* sp. strain CFS-215 incubations, as well as benzene-dependent degradation of toluene and *p*-xylene by *Arthrobacter* sp. strain HCB. Detrimental substrate interactions included retardation in benzene and toluene degradation by the presence of *p*-xylene in both aquifer slurries and *Pseudomonas* incubations. The catabolic diversity of microbes in the environment precludes generalizations about the capacity of individual BTX compounds to enhance or inhibit the degradation of other BTX compounds.

Release of petroleum hydrocarbons in the environment is a widespread occurrence. One particular concern is the contamination of drinking water sources by the toxic, water-soluble, and mobile petroleum components benzene, toluene, and xylene (BTX). Microbial degradation of these compounds in aquatic environments can serve as a significant attenuation mechanism (3, 5, 9, 12, 14, 17, 19, 20). However, in situ biodegradation of BTX compounds is not ubiquitous, and some BTX compounds have been reported to persist in the environment at levels exceeding regulatory thresholds even after the addition of nutrients and electron acceptors (3, 22). Several factors, such as pollutant concentration, active biomass concentration, temperature, pH, availability of inorganic nutrients and electron acceptors, and microbial adaptation, influence the rate and extent of biodegradation of BTX. Although these factors have been recognized, limited attention has been placed on the ability of individual BTX compounds to stimulate or inhibit the biodegradation of other BTX compounds.

The diversity of microbial degradative activity towards aromatic compounds has led to some apparently contradictory observations with respect to the degradation of BTX compounds in the presence of other cosubstrates. Benzene degradation by a mixed microbial population from an oil refinery settling pond was inhibited by phenol, a more easily degradable substrate (15). In other work, the presence of easily degradable substrates (e.g., amino acids) was found to enhance the degradation of monosubstituted phenols by a mixed microbial community from a mesotrophic reservoir (18). Degradation of polycyclic aromatic hydrocarbons in marine sediment slurries was enhanced by prior exposure to alternate polycyclic aromatic hydrocarbons and benzene (4). This was considered to be the result of initial selection, acclimation, and proliferation of microbes capable of degrading more than one polycyclic aromatic hydrocarbon. In a factorial experiment to study the degradation of benzene in the presence of other aromatic compounds, benzene degradation by mixed culture incubations was found to be stimulated by the presence of either toluene or *o*-xylene (1). However, an antagonistic effect was observed when both toluene and *o*-xylene were present. The degradation of toluene and *o*-xylene was not reported. Several researchers have reported that toluene is degraded more readily than benzene in aquifer systems (11, 19, 22). However, the opposite trend was observed in other field studies (6, 21). *o*-Xylene is usually reported to be the most recalcitrant of the BTX compounds (16, 19). Although pure cultures capable of growing on *o*-xylene have been isolated (2), partial degradation of *o*-xylene concomitant to toluene metabolism has been observed in several mixed cultures of denitrifying bacteria (7, 10).

Clearly, substrate interactions are important in understanding the behavior of BTX compounds in the environment and the similarity or differences in their biodegradation. The presence of a given BTX compound can theoretically stimulate the degradation of another BTX compound by inducing the required catabolic enzymes. Another beneficial substrate interaction would be a BTX compound (e.g., toluene) acting as a primary substrate and stimulating microbial growth, which could enhance cometabolism of another BTX compound (e.g., xylene). On the other hand, a BTX compound could inhibit the degradation of another by exerting toxicity, diauxy, catabolite repression, competitive inhibition for enzymes, or depletion of electron acceptors. The identification of operant interactions would contribute to our understanding of why a particular BTX compound may persist in a contaminated site while other BTX compounds are degraded. Substrate interactions during benzene, toluene, and *p*-xylene degradation by different microbial populations are the focus of this study. Specifically, two pure cultures, *Pseudomonas* sp. strain CFS-215 and *Arthrobacter* sp. strain HCB, and a mixed culture indigenous to a shallow sandy aquifer are compared.

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MATERIALS AND METHODS

Experimental design. Serum bottles (120 ml) with Teflon-coated silicone septa and aluminum crimp caps were used for incubations. Two types were prepared: (i) mixed cultures, in which BTX biodegradation was carried out by the indigenous microbial consortia of a sandy aquifer from Kalkaska, Mich., and (ii) pure culture incubations, in which either of two BTX-degrading microbes isolated from the aquifer material were used. The aquifer material was collected aseptically with a split-spoon sampler and plastic coring tubes. Aquifer slurries were prepared by adding 16 g of drained aquifer material (10-ml bulk volume) and 50 ml of mineral medium into 120-ml serum bottles. *Pseudomonas* sp. CFS-215, isolated with toluene by R. H. Olsen, Department of Immunology and Microbiology, The University of Michigan, and *Arthrobacter* sp. HCB, isolated with benzene by Henry Corseuil, Department of Civil and Environmental Engineering, The University of Michigan, were used in this study. Both pure cultures were grown on Casamino Acids (1 g/liter) without any BTX compounds until A_{298} stabilized ca. 1.8 in order to have sufficient biomass for relatively rapid biodegradation studies. Fifty milliliters of the pure cultures was transferred to 120-ml serum bottles. Cells used in pure culture experiments were initially in a stationary phase. Pure culture cell concentrations (ca. 460 mg/liter as total suspended solids) were large enough that they did not undergo substantial relative increases as a result of substrate degradation during the experiment. This was not necessarily the case with microbes in aquifer slurries.

Biodegradation of all seven possible combinations of benzene, toluene, and *p*-xylene were tested in duplicate. These seven combinations were (i) benzene alone, (ii) toluene alone, (iii) *p*-xylene alone, (iv) benzene with toluene, (v) benzene with *p*-xylene, (vi) toluene with *p*-xylene, and (vii) benzene with toluene and *p*-xylene. BTX compounds were injected as pure stock with a 10-μl syringe. Oxygen gas (10 ml at 1 atm [101.29 kPa]) was also injected into the headspace (60 ml) in order to ensure an oxygen supply in excess of the stoichiometric requirements for mineralization of BTX. The serum bottles were sealed with Teflon-lined caps and aluminum crimps, inverted, and incubated at 25°C in the dark while mixing on an orbital shaker (100 rpm). Sterile controls were prepared by autoclaving replicates for 30 min at 120°C. Sodium azide (2,000 mg/liter) was also added to the controls to aid in inhibiting potential microbial activity due to microbial contamination of the controls during sampling. The initial concentration of each BTX compound was approximately 50 mg/liter, and no additional carbon source was added. Biodegradation was assessed by comparing the disappearance of BTX in samples and controls over time. Sterile controls containing benzene, toluene, and *p*-xylene were prepared for each batch set to discern volatilization and adsorption losses. Abiotic BTX losses were consistently less than 5% during the first 3 weeks of incubation. Two additional bottles were prepared with *Pseudomonas* sp. CFS-215. Both were fed with benzene (50 mg/liter) and relatively low concentrations of toluene (1 and 0.1 mg/liter, respectively) as cosubstrates.

Media. Basal mineral medium was prepared to provide essential inorganic nutrients and vitamins for microbial growth. The medium contained the following (in milligrams per liter of Milli-Q water): NH_4Cl (100), KH_2PO_4 (700), K_2HPO_4 (1,000), NaCl (10), $CaCl_2$ (5), $MgCl_2$ (10), $CuCl_2 \cdot 2H_2O$ (0.0392), $ZnCl_2$ (0.1363), $NiCl_2$ (0.013), $FeCl_2 \cdot 4H2O$ (0.7016), $AlCl_3$ (0.1106), $MnCl_2 \cdot 4H_2O$ (0.2807), $CoCl_2 \cdot 6H_2O$ (0.0382), $Na_2MoO_4 \cdot 2H_2O$ (0.0254), H_3BO_3 (0.0382), Na_2SO_4 (0.1420), biotin (0.02), folic acid (0.02), pyridoxine HCl (0.1), riboflavin (0.05), thiamine (0.05), nicotinic acid (0.05), pantothenic acid (0.05), vitamin B_{12} (0.001), and *p*-aminobenzoic acid (0.05).

Analytical procedure. A 500-μl gastight syringe was used to withdraw aqueous samples (0.5 ml) from each incubation. Samples were transferred to 5-ml vials and capped with Teflon-coated septa prior to automated headspace analysis. BTX compounds were analyzed in a Hewlett-Packard 5890 gas chromatograph equipped with a Hewlett-Packard 19395A automatic headspace sampler and a flame ionization detector. A relative standard deviation of ±10%, including inconsistencies in sample collection and analysis, was obtained for BTX measurements. The limit of detection of this procedure was approximately 0.01 ppm. A biological oxygen monitor (YSI 530) equipped with a microchamber and an oxygen microprobe was used to determine whether dissolved oxygen had become limiting.

BTX concentrations were monitored over time in order to compare lag periods and biodegradation rates for different cosubstrate combinations. The lag period was determined as the time during which BTX concentrations remained relatively constant. This was followed by an abrupt decrease in BTX concentrations relative to sterile controls. Pseudo zero-order rates were estimated as the ratio of BTX removed (corrected for sterile controls) to the corresponding time after the lag period.

RESULTS

Aquifer material slurries. Benzene, toluene, and *p*-xylene were degraded concurrently to concentrations below the limit of detection (0.01 ppm) after 8, 15, and 43 days, respectively. An acclimation period of 6 days for benzene and toluene and 10 days for *p*-xylene was observed before appreciable biodegradation occurred. The pseudo zero-order biodegradation rates were about 25, 5, and 2 mg/liter/day for benzene, toluene, and *p*-xylene, respectively (Table 1).

No difference was observed in benzene degradation between the sediment slurries that contained benzene alone and those that contained benzene and toluene (Table 1). In both cases, a lag period of 2 days was observed and benzene disappeared within the following 2 days (25 mg/liter/day for both conditions). The lag period for benzene degradation was 4 days when *p*-xylene was present, and then benzene disappeared within the following 2 days, although the rate was relatively similar (24 mg/liter/day). The lag increased further to 6 days when the three BTX compounds were present, but benzene still disappeared within 2 days of the onset of biodegradation. Therefore, the presence of *p*-xylene significantly increased the lag period for benzene degradation, although the rate of benzene degradation was not affected.

Toluene degradation was similar in aquifer material that contained toluene alone and in that containing toluene and benzene (Table 1). In both cases, a lag period of 2 days was observed and toluene disappeared at a rate of 23 mg/liter/day. The lag period for toluene degradation increased to 7 days when *p*-xylene was present, and toluene also degraded at a slower rate (11 mg/liter/day). The lag period was 6 days when both benzene and *p*-xylene were present, and toluene disappeared at an even slower rate (5 mg/liter/day). Therefore, cosubstrate combinations containing *p*-xylene both increased the lag period and decreased the rate of toluene degradation.

TABLE 1. Lag periods and pseudo zero-order biodegradation rates for benzene (B), toluene (T), and *para*-xylene (X)

Compound[a]	Aquifer material		*Pseudomonas* sp. CFS-215		*Arthrobacter* sp. HCB	
	Lag period (days)	Pseudo zero-order biodegradation rate (mg/liter day)[b]	Lag period (days)	Pseudo zero-order biodegradation rate (mg/liter/day)	Lag period (days)	Pseudo zero-order biodegradation rate (mg/liter/day)
B	2	25	6	7	2	52
B (with T)	2	25	1	36	2	24
B (with X)	4	24	ND[c]	ND	2	25
B (with T + X)	6	25	5	10	4	45
T	2	23	1	48	ND	ND
T (with B)	2	23	1	36	3	20, 0[d]
T (with X)	7	11	4	23	ND	ND
T (with B + X)	6	5	5	13	5	20, 0[d]
X	22	5	ND	ND	ND	ND
X (with B)	22	2	ND	ND	8	3, 0[d]
X (with T)	10	6, 1[e]	5	12, 0.3[e]	ND	ND
X (with B + T)	10	2, 1[e]	5	3, 0[e]	ND	ND

[a] Initial concentration was approximately 50 mg/liter for each compound.
[b] Rates were estimated as the ratio of BTX removed (corrected for sterile controls) to the corresponding time after the lag period. All values were derived from duplicate incubations. Rates are linear regressions of at least four points and are accurate to within 10%.
[c] ND, not determined because degradation was not observed within 3 weeks.
[d] Rates while benzene was present and after benzene had been degraded, respectively.
[e] Rates while toluene was present and after toluene had been degraded, respectively.

The lag phase was considerably longer (22 days) for *p*-xylene degradation than for benzene or toluene, even when benzene was present. However, the lag for *p*-xylene degradation was reduced to 10 days in the two serum bottles where toluene was present. The observed *p*-xylene degradation rate was fastest while toluene was being degraded (6 mg/liter/day) but slowed down after toluene was degraded (1 mg/liter/day) (Table 1). This phenomenon was reproduced by respiking toluene.

In summary, all BTX compounds were degraded within 6 weeks to concentrations below 0.01 mg/liter, the presence of *p*-xylene increased the lag period for benzene and toluene degradation, and the degradation of *p*-xylene was fastest when toluene was concomitantly being degraded.

CFS-215 incubations. When present concurrently, benzene, toluene, and *p*-xylene all had a lag period of 5 days, followed by degradation rates of 10, 13, and 3 mg/liter/day, respectively, while toluene was present. After toluene was degraded, benzene continued to be degraded, but *p*-xylene did not.

Benzene degradation by CFS-215 was enhanced by the presence of toluene (Fig. 1). When toluene was fed as the sole cosubstrate (50 mg/liter), the lag period was reduced (from 6 days to 1 day) and the rate was increased (from 7 to 36 mg/liter/day) (Table 1). The presence of relatively low initial concentrations of toluene (e.g., 0.1 and 1 mg/liter) also enhanced benzene degradation. Benzene was degraded after a 3-day lag for both toluene concentrations at a rate of 9 mg/liter/day (not shown in Table 1). The presence of *p*-xylene, on the other hand, inhibited benzene biodegradation. No benzene or *p*-xylene degradation occurred in incubations containing only these two compounds. However, when both toluene and *p*-xylene were present with benzene, the enhancement of toluene outweighed the inhibition by *p*-xylene. Therefore, benzene underwent faster degradation when all three BTX compounds were present (10 mg/liter/day) than when benzene was present alone (7 mg/liter/day).

Serum bottles fed toluene alone or toluene with benzene degraded toluene after a lag period of only 1 day at rates of 48 and 36 mg/liter/day, respectively. Bottles fed toluene and *p*-xylene showed an increased lag period (4 days) and decreased toluene degradation rate (23 mg/liter/day). The lag period increased to 5 days when both benzene and xylene were present, and the rate of toluene degradation decreased to 13 mg/liter/day.

p-Xylene biodegradation by CFS-215 was relatively slow. When *p*-xylene was present alone, no degradation was observed in the first 3 weeks, and concentrations on the order of 2 mg/liter persisted after 9 weeks. *p*-Xylene degradation was accelerated while toluene was being degraded (12 mg/liter/day) but slowed down once toluene had disappeared (0.3 mg/liter/day). This phenomenon was reproduced by respiking toluene. The incubations fed toluene had degraded

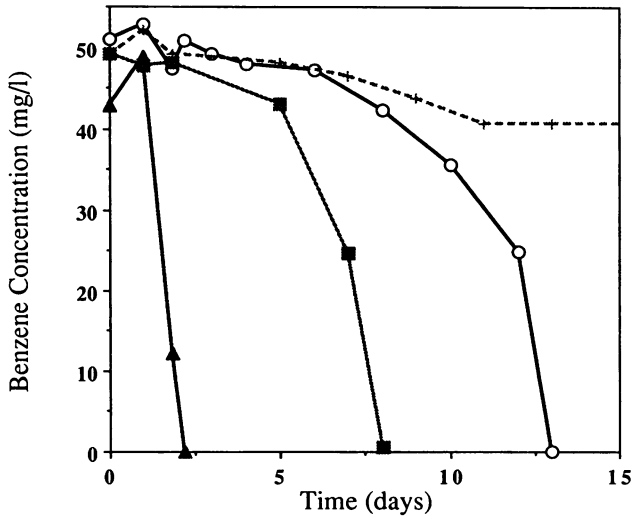

FIG. 1. Effect of toluene on benzene degradation by *Pseudomonas* sp. CFS-215. Symbols: ○, benzene alone; ▲, benzene with toluene (50 mg/liter); ■, benzene with toluene (0.1 mg/liter); –+–, sterile control.

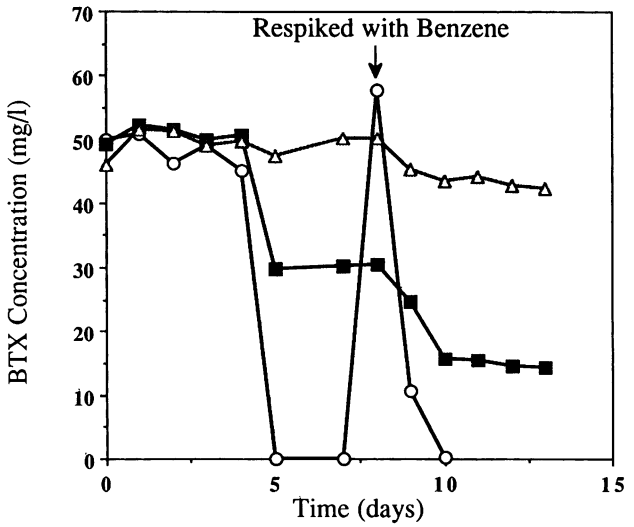

FIG. 2. Concurrent degradation of BTX compounds by *Arthrobacter* sp. HCB. Sterile controls (not shown) exhibited BTX loses on the order of 2% over the depicted time course. Symbols: ○, benzene; ■, toluene; △, *p*-xylene.

about 25% of the initial *p*-xylene concentration within the first 2 weeks of incubation, and no detectable levels of *p*-xylene remained after 6 weeks. Therefore, the presence of toluene enhanced *p*-xylene degradation by CFS-215.

In summary, all BTX compounds were degraded by *Pseudomonas* sp. CFS-215, traces of toluene enhanced the degradation of benzene, *p*-xylene degradation was dependent (i.e., enhanced) on toluene degradation, and the presence of *p*-xylene increased the lag period for benzene and toluene degradation.

HCB incubations. When the three BTX compounds were present concurrently, benzene and toluene were degraded after a 4- and 5-day lag period, respectively. *p*-Xylene was not significantly degraded. Benzene and toluene were degraded at relatively high rates (45 and 20 mg/liter/day, respectively). Toluene was only degraded while benzene was being degraded. This phenomenon was reproduced by respiking benzene and observing further toluene degradation (Fig. 2).

The lag period for benzene degradation (2 days) was not affected by the presence of either toluene or *p*-xylene alone. However, when toluene and *p*-xylene were present concurrently the lag increased to 4 days. Benzene degradation was relatively rapid in all of the tested cosubstrate combinations, ranging from 24 to 52 mg/liter/day (Table 1). Degradation was most rapid when benzene was present alone.

Toluene was not degraded by HCB in the absence of benzene. *p*-Xylene was not degraded in the HCB batches containing *p*-xylene alone or *p*-xylene and toluene. However, incubations containing *p*-xylene and benzene showed a slow but measurable decrease in *p*-xylene relative to the controls (Table 1). Therefore, the presence of benzene enhanced the degradation of both toluene and *p*-xylene.

DISCUSSION

Although the aquifer material used in this study had no detectable levels of BTX, the sample was collected from an aquifer that had an overall BTX contamination of 200 μg of total BTX per liter (5, 13). Therefore, the consortium present in the slurries had probably been previously exposed to some BTX compounds.

The presence of *p*-xylene in the mixed culture slurries increased the lag period for the degradation of both benzene and toluene. The inhibitory effect on acclimation of benzene degraders was increased when both toluene and *p*-xylene were present. Since the initial total BTX concentration was relatively high in this case (150 mg/liter), toxicity was probably responsible for this phenomenon. This conclusion is supported by unpublished kinetics studies with the same aquifer material in which total benzene-plus-toluene concentrations in excess of 150 mg/liter decreased the maximum specific substrate utilization rate of benzene.

Cometabolism of *p*-xylene by toluene degraders was probably an important interaction observed in the mixed culture slurries. This phenomenon is suggested by the rapid disappearance of *p*-xylene while toluene was being degraded and by the subsidence of *p*-xylene degradation rate after toluene had been degraded.

p-Xylene was degraded in the slurries when it was present alone. However, this degradation occurred after a relatively long lag period of 3 weeks. This could be explained by two untested hypotheses. One hypothesis is that there was initially a relatively small number of xylene degraders in the aquifer material and that these microbes were relatively slow growers. This explanation, however is not very attractive because the slurries were aerobic, and aerobes grow relatively fast. Three weeks would have certainly been sufficient time to grow significant numbers of *p*-xylene degraders. Another hypothesis is that microbes which metabolize benzene and toluene can also degrade *p*-xylene but are subject to some *p*-xylene toxicity at 50 mg of *p*-xylene per liter. The circumstantial evidence of the increase in the lag period of benzene and toluene degradation under the two combinations that included *p*-xylene supports this hypothesis.

CFS-215 was isolated with toluene as the sole carbon and energy source. As expected, this bacterium degraded toluene preferentially over benzene and *p*-xylene. The presence of toluene enhanced the degradation of the other two BTX compounds. The increase in the rate of benzene degradation as a result of the presence of toluene might be explained by one of two mechanisms. The first is that CFS-215 grows better on toluene, and that the presence of this compound aids the proliferation of the bacteria, leading to a greater number of microbes capable of attacking benzene. However, benzene degradation was substantially accelerated even when only traces of toluene (i.e., 0.1 mg/liter) were initially present (Fig. 1). This initial amount of toluene was not sufficient to increase the number of bacteria significantly since the starting microbial concentration was on the order of 460 mg/liter (as total suspended solids). Therefore, the enhancement of benzene degradation by CFS-215 must be explained by the second mechanism: toluene enhanced microbial activity by affecting enzyme induction. This suggests that CFS-215 might utilize the same enzyme to degrade both benzene and toluene. The oxidation of benzene catalyzed by toluene dioxygenase with a broad substrate specificity has previously been observed (8). The fact that the rate of toluene degradation was slightly reduced from 48 to 36 mg/liter/day when benzene was present (i.e., competitive inhibition) supports this hypothesis.

The accelerated degradation of *p*-xylene while toluene was being degraded suggests that CFS-215 was also degrading *p*-xylene. The fact that *p*-xylene was also degraded when present alone after a long acclimation (i.e., greater than 3

weeks) suggests that *p*-xylene was not as effective as toluene in inducing the required catabolic enzymes.

HCB was isolated with benzene as the sole carbon source from the same aquifer material used in the mixed culture slurries. This bacterium preferred benzene over toluene and *p*-xylene as expected. Surprisingly, HCB could not degrade either toluene or *p*-xylene unless benzene was present. This was evident by the fact that toluene and *p*-xylene degradation stopped when benzene had been degraded. Yet, toluene and *p*-xylene biodegradation resumed upon respiking benzene (Fig. 2). This suggests that cometabolism of toluene and *p*-xylene by HCB degrading benzene was responsible for this beneficial substrate interaction.

Conclusions. Although BTX compounds have similar chemical structures, different organisms can metabolize certain BTX compounds but not others (e.g., HCB). In these cases, the presence of the primary BTX substrate could be beneficial in the cometabolism of other BTX compounds. Some microbes, however, possess the capability to degrade all three compounds, benzene, toluene, and *p*-xylene (e.g., CFS-215), but some compounds are more efficient enzyme inducers (e.g., toluene).

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