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Use of Benzoate to Establish Reactive Buffer Zones for Enhanced Attenuation of BTX Migration: Aquifer Column Experiments

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Flow-through aquifer columns were used to evaluate the efficacy of using benzoate as a biostimulatory substrate to enhance the aerobic biodegradation of benzene, toluene, and *o*-xylene (BTX), fed continuously at low concentrations (about 0.2 mg/L each). When used as a cosubstrate, benzoate addition (1 mg/L) enhanced BTX degradation kinetics and attenuated BTX breakthrough relative to acetate-amended (2 mg/L) or unamended control columns. The benzoate-amended column also experienced an increase in predominance of pseudomonad species capable of degrading BTX. The feasibility of injecting benzoate to enhance the growth of BTX degraders and establish a buffer zone downgradient of a BTX plume was also investigated. Using pristine aquifer material without previous exposure to BTX, aquifer columns were fed benzoate (2 mg/L), acetate (4 mg/L), or mineral medium without supplemental substrates during a 2-day acclimation stage. All columns were subsequently fed BTX alone, and their breakthrough was monitored. Previous exposure to benzoate, but not to acetate, shortened the acclimation period to BTX degradation and enhanced the short-term bioattenuation potential of the indigenous consortium. This suggests that benzoate could potentially be used to establish and sustain *in situ* reactive zones to attenuate BTX migration and protect downgradient groundwater resources.

INTRODUCTION

Benzene, toluene, and the isomers of xylene (BTX) are ubiquitous priority pollutants commonly associated with petroleum product releases from leaking underground storage tanks. Without appropriate cleanup measures, BTX often persist in subsoils and endanger groundwater resources and public health. Bioremediation, in conjunction with free product recovery, is one of the most cost-effective approaches to clean up BTX-contaminated aquifers (*1*). However, while all BTX compounds are biodegradable, there are several factors that can limit the success of BTX bioremediation. Factors that affect the rate and extent of BTX degradation include pollutant concentration, active biomass concentration, temperature, pH, presence of other substrates or toxicants, availability of nutrients and electron acceptors, mass transfer limitations, and microbial adaptation. These factors have been recognized in various attempts to optimize clean-up operations. Yet, limited attention has been given to the exploitation of favorable substrate interactions to enhance *in situ* BTX biodegradation.

BTX bioremediation projects often focus on overcoming limitations to natural degradative processes associated with the insufficient supply of inorganic nutrients and electron acceptors. However, other limitations associated with the presence and expression of appropriate microbial catabolic capacities may also hinder the effectiveness of bioremediation. Thus, while subsurface addition of oxygen or nitrate has proven sufficient to remove BTX below detection levels (*2*−*5*), it has been only marginally effective at some sites (*6*−*8*). Sometimes, the concentration of a target BTX compound fails to decrease below a threshold level even after years of continuous addition of nutrients and electron acceptors (*9*). This phenomenon has also been observed for many other xenobiotic and natural substrates under various experimental conditions (*10*−*15*).

Residual concentrations of carcinogenic compounds such as benzene could exceed applicable clean-up standards and remain a threat to public health. Possible reasons for residual BTX concentrations include mass transfer and diffusion limitations (*16*), the requirement for a minimum substrate concentration to satisfy the maintenance energy demand and sustain a sufficient concentration of BTX degraders (*17*) and the existence of a threshold substrate concentration below which induction of the necessary catabolic enzymes does not occur (*18, 19*). Therefore, overcoming limitations associated with the presence and expression of appropriate catabolic capacities might be required in some BTX bioremediation projects. Hypothetically, this might be accomplished by the addition of supplemental substrates that increase the concentration of desirable phenotypes without repressing the required catabolic enzymes.

Biostimulation through substrate addition is commonly practiced to support cometabolic biodegradation processes (*20*−*22*). Addition of stimulatory substrates to enhance bacterial growth and metabolic activity has also been used in bioaugmentation experiments involving both environmental clean-up (*23*) and agricultural applications (*24*). This approach, however, has not yet been used to enhance BTX bioremediation because BTX are often present in hydrocarbon plumes at sufficiently high concentrations to induce and sustain their degradation. In addition, there are concerns about potential diauxic effects and exacerbating the oxygen demand when additional substrates are added. Nevertheless, controlled addition of stimulatory substrates to groundwater contaminated with traces of BTX could help achieve lower residual BTX concentrations.

Biostimulation through substrate addition may be even more valuable to support a new technological area quickly developing in the remediation field: *in situ* reactive zones. This novel remediation approach is based on the creation of a subsurface zone where migrating contaminants are intercepted and immobilized or degraded (*25*). This is different from reactive walls and funnel and gate systems where the groundwater flow pattern is also controlled. *In situ* reactive zones allow groundwater to continue to flow naturally and are particularly attractive in that they conserve energy and water and, through long-term low operating and maintenance costs, have the potential to be considerably less costly than conventional clean-up methods (*26*). To this end, injecting a nontoxic stimulatory substrate downgradient of a BTX plume might be a cost effective approach to enhance the growth and viability of BTX degraders before the arrival of

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the plume. This would attenuate BTX migration and protect downgradient groundwater resources.

Benzoate, a common food preserver, may be a suitable substrate to achieve this end. It is a relatively inexpensive, harmless aromatic compound that has been previously used in "analogue enrichment" schemes to enhance biodegradation of the aromatic herbicide, 2,3,6-trichlorobenzoate (*27*). Benzoate is also an intermediate in the *tol* pathway and it can induce related enzymes involved in the degradation of toluene and *m*- and *p*-xylenes (*28*). In addition, the anionic nature of benzoate would minimize its retardation and facilitate its distribution when injected into an aquifer. Albeit, the feasibility of adding benzoate to establish and sustain a reactive zone would depend mainly on its ability to acclimate the indigenous consortium and enhance the growth and viability of BTX degraders, even in the absence of BTX.

In this work, benzoate was evaluated as a stimulatory cosubstrate to enhance BTX degradation by indigenous aquifer microorganisms exposed to trace BTX concentrations. The potential efficacy of injecting benzoate to enhance the viability of BTX degraders and establish a reactive buffer zone downgradient of a BTX plume is also discussed.

MATERIALS AND METHODS

Experimental Design. Flow-through aquifer columns were used to mimic *in situ* conditions of microbial exposure to BTX and supplemental substrates (e.g., plug flow with dispersion). Two different exposure scenarios were studied to evaluate the effect of adding supplemental substrate on BTX breakthrough concentrations and microbial population shifts.

The first experiment evaluated the feasibility of using benzoate as a stimulatory cosubstrate to enhance BTX degradation by indigenous aquifer microorganisms exposed to trace BTX concentrations. In this experiment, four columns were continuously fed benzene (193 ± 16 μg/L), toluene (183 ± 18 μg/L), and *o*-xylene (192 ± 15 μg/L). The first (treatment) column was supplemented with benzoate (1 ± 0.1 mg/L) as a stimulatory substrate. The second column was supplemented with acetate (2 ± 0.1 mg/L) as a control substrate to investigate whether any common substrate could enhance biodegradation of trace BTX concentrations. Acetate, which is widely used as primary substrate to sustain a variety of biological treatment processes (e.g., denitrification and reductive dechlorination), was fed at an equal concentration as benzoate in terms of electron donor equivalents (i.e., equal chemical oxygen demand, [COD]). The third column was a no-treatment control and received no supplemental substrates to control for any stimulatory effect of the mineral medium. The fourth column was poisoned with mercuric chloride (200 mg/L) to discern BTX biodegradation from potential volatilization losses. Benzoate or acetate were added at low concentrations (each at about 2 mg/L as COD) to avoid exacerbating the oxygen demand and to preclude potential diauxic effects which are common at high substrate concentrations (*29*).

The second experiment investigated the feasibility of injecting benzoate downgradient of a BTX plume to establish a reactive buffer zone for attenuating BTX migration and protecting downgradient groundwater resources. Similar to the first experiment, the columns were packed with aquifer material of no known previous BTX exposure. During a 2-day acclimation stage, the treatment column was fed benzoate (2 ± 0.2 mg/L), the substrate-control column was fed acetate at an equivalent COD concentration (4 ± 0.2 mg/L), and the no-treatment control column was fed mineral medium alone. Benzoate and acetate were subsequently removed from the feeds, and all columns were fed benzene, toluene, and *o*-xylene at about 150 μg/L each. In both experiments, effluent BTX concentrations were monitored to compare the peak concentrations breaking through and the time required for effluent concentrations to drop below detection levels. To further evaluate the effect of different treatments on BTX degradation, mass loss rates were calculated for each BTX compound using the concentration breakthrough data, as follows:

TABLE 1. Hydraulic Characteristics of Flow-Through Aquifer Columns

parameter	value
length, *L* (cm)	15
i.d. (cm)	1
porosity (fraction of total volume)	0.37
avg pore velocity, *v* (cm/h)	6.8
hydraulic residence time (h)	2.18
dispersion coefficient, *D* (cm^2/h)	0.29
pecklet number = *vL*/*D*	352
retardation factors (relative to Br^-)	
benzene	2.1
toluene	4.2
o-Xylene	8.5

$$\dot{M} = Q(C_{\text{control}} - C_{\text{viable}}) \quad (1)$$

where $\dot{M}$ = BTX biodegradation rate (mass/time) within a given viable column, Q = flow rate applied to the aquifer column, C_{control} = effluent BTX concentration from the sterile column, and C_{viable} = effluent BTX concentration from the viable column.

Aquifer Columns. Sterilized glass columns (15 cm long, 1 cm i.d.) (Kontes) were packed with pristine, low organic carbon (0.1%) sandy aquifer material, as described by Siegrist and McCarty (*30*). Columns were continuously fed mineral medium amended with BTX and/or supplemental substrates, as appropriate, in an upflow mode at a constant flow rate of 2 mL/h. Harvard syringe pumps with 100 mL gas-tight syringes (SGE) were used for this purpose. A 0.2 μm Teflon Acrodisc syringe filter (Gelman Sciences) was placed at the syringe tip to protect the feed against possible bacterial contamination. All tubing and fittings in the flow train were Teflon or Teflon-lined. The influent tubing was approximately 9.5 cm long and 1/16 mm i.d. The effluent tubing was approximately 36 cm long and 1/8 mm i.d. The end of the effluent tubing was adapted for sampling with a flangeless ferrule and nut arrangement, a 1/4−28 adapter male luer lock fitting, and a thin (30-gage) disposable syringe needle (Becton Dickinson). Influent and effluent samples were collected and analyzed regularly for BTX, acetate, and/or benzoate. BTX samples (0.5 mL) were collected in 10 mL GC vials (Kimble) that had been previously closed with Teflon-lined septa and aluminum crimps (The West Company). Benzoate and acetate samples (0.8 mL) were passed through 0.2 μm syringe filters and collected in 1 mL HPLC vials.

Three-way valves were placed influent and effluent to the columns in order to redirect flow for on-line dissolved oxygen (DO) readings. All columns were operated aerobically. Influent DO concentrations were about 8 mg/L while effluent DO concentrations never dropped below 3 mg/L.

The hydraulic characteristics of the columns were determined from bromide tracer studies. Mineral medium spiked with 200 mg/L of sodium bromide was fed at 2 mL/h and sampled every 15 min. The breakthrough curve for bromide was used to calculate the average porosity, pore velocity, dispersion coefficient, and retardation factors for benzene, toluene, and *o*-xylene (Table 1). These parameters were estimated by fitting tracer breakthrough concentrations to a solution of the advective-dispersive transport equation (*31*). BTX breakthrough data from a sterile column were used to estimate the retardation factors. The aquifer columns

had a plug flow with dispersion hydraulic regime representative of aquifers, as indicated by the magnitude of the Pecklet number (Pe = 352) (*32*).

Dominant (Culturable) Species Identification. A qualitative assessment of microbial population shifts was conducted by spread plating dilutions of aquifer material collected from the inlet of aquifer columns at the end of the first experiment. Dilutions were plated onto tryptic soy agar, and dominant colonies which differed in regard to color, texture, margins and concavity were isolated, Gram-stained, and identified based on their substrate utilization patterns using a Biolog bacterial identification system. The dominant isolates were also tested for BTX degradation capacity. In these biodegradability assays, sterilized mineral medium was put into 250 mL serum bottles, spiked with BTX (10 mg/L each), and inoculated with a given isolate prior to sealing with Mininert caps. Bottles were incubated for two weeks at 20 °C. Sterile controls were also run to differentiate biodegradation from potential volatilization losses.

Instrumental Analysis. BTX were analyzed with a Hewlett-Packard 5890 Series II GC equipped with a Hewlett-Packard 19395A headspace autosampler and flame-ionization and photoionization detectors in series. Separation was achieved using a J&W Scientific DB-WAX column. Benzoate was analyzed with a Gilson HPLC equipped with a UV−vis detector set at 221 nm. Separation was achieved with an Alltech econosphere Lc-18 column. Acetate was analyzed with a Hewlett Packard 5890 GC equipped with a flame-ionization detector. Separation was achieved with a Supelco 1%SP-1000 60/80 Carbopack B column. A YSI 5300 biological oxygen monitor, equipped with an Instech microchamber and an oxygen microprobe (Instech Instruments), was used to sample the columns effluent to verify that aerobic conditions prevailed in the columns. Detection limits were about 2 μg/L for each BTX compound and 0.1 mg/L for acetate, benzoate, and dissolved oxygen.

Mineral Medium and Chemicals. Basal mineral medium was prepared using the recipe of Alvarez and Vogel (*33*) to provide essential inorganic nutrients and vitamins for microbial growth and to buffer aqueous solutions at pH 7. Mineral medium chemicals, mercuric chloride, sodium benzoate, and sodium acetate were purchased from Fisher Scientific. HPLC grade benzene was obtained from Mallinckrodt Chemical Works, toluene was purchased from the Fisher Scientific, and *o*-xylene was obtained from Aldrich Chemical Company. Tryptic Soy Broth and Bacto-agar were purchased from Difco Laboratories.

RESULTS

Benzoate as a Stimulatory Cosubstrate. The removal of BTX, benzoate, and acetate in biologically active but not in sterile columns provided evidence of biodegradation. Benzoate and acetate were completely utilized within the columns and were not detected in the effluents. Benzoate addition enhanced aerobic BTX degradation and attenuated the breakthrough of benzene (Figure 1A), toluene (Figure 1B), and *o*-xylene (Figure 1C) relative to acetate-amended and unamended control columns. Both the maximum BTX concentrations that broke through and the time required for the effluent BTX concentration to drop below detection levels were reduced in the benzoate-amended column. The benzoate-fed column exhibited peak effluent concentrations (expressed as a percentage of the corresponding influent concentrations) of 85% for benzene, 64% for toluene, and 45% for *o*-xylene. The respective peak BTX concentrations breaking through the acetate-amended (91, 89, and 83%) and unamended (92, 95, and 94%) columns were higher. In addition, BTX compounds were not detected after 2 days in the effluent of the benzoate-fed column, while it took longer than 8 days for the acetate-fed and the unamended control columns to remove BTX below detection levels. The observed improvement in short-term BTX degradation capacity in the benzoate-fed column was reproduced in three additional trials.

Benzoate as Preparatory Substrate to Enhance Acclimation to BTX Degradation. Similar to the previous experiment, preacclimation with benzoate had a pronounced beneficial effect on subsequent degradation of benzene (Figure 2A), toluene (Figure 2B), and *o*-xylene (Figure 2C) relative to acetate-acclimated or unamended control columns. The benzoate-acclimated column exhibited peak effluent concentrations (as a percentage of the influent concentrations) of 72% for benzene, 31% for toluene, and 42% for *o*-xylene. The respective peak BTX concentrations breaking through the acetate-amended (75, 35, and 49%) and unamended (80, 40, and 63%) columns were higher. In addition, BTX compounds were not detected after 2.5 days in the effluent of the benzoate-acclimated column, while effluent BTX concentrations had not begun to decline at this time in the acetate-amended or unamended control columns.

Microbial Population Shifts. Inlet samples from the columns used in the first experiment were qualitatively analyzed for changes in dominant (culturable) heterotrophic species. Sample dilutions were spread onto Tryptic Soy Agar plates, and the dominant colonies were identified using Biolog. Compared to initial conditions, different treatments caused different shifts in genotypic dominance. The dominant culturable heterotrophs in the unexposed aquifer material, in order of relative abundance, were identified as *Xanthomonas oryzae, Deleya marina,* and *Pseudomonas putida* B1. Two of these species were also predominant in the sample from the column fed BTX plus acetate, which exhibited the following order of dominant species: *X. oryzae, Acidovorax avenae SS catetleya, Pseudomonas flourescens,* and *D. marina.* Predominant species in the sample from the column fed BTX alone were identified as *P. putida* B1, *X. oryzae, P. putida* A1, and *Burkholderia* (formerly *Pseudomonas*) *cepacia.* The sample from the column fed BTX plus benzoate exhibited a much greater dominance of *P. putida* A1 and *B. cepacia.* Two of the four isolates from the column fed BTX plus acetate (i.e., *D. marina* and *Acidovorax avenae* SSP. *catetleya*) failed to degrade BTX in further characterization studies. All other column isolates (i.e., *P. putida, P. fluorescens, B. cepacia, and X. oryzae*) were pseudomonads that exhibited BTX degradation capabilities.

Discussion

Bioremediation is widely recognized as a potentially cost-effective but underutilized alternative to destroy toxic, hazardous, or other unwanted compounds at contaminated sites. Although considerable progress has been made toward understanding and managing the hydrogeochemical factors that influence the rate and extent of BTX biodegradation, potential limitations associated with the insufficient presence and expression of indigenous catabolic capacities are often overlooked. This work suggests that benzoate could potentially serve as a stimulatory substrate to overcome such limitations and enhance the capabilities of natural degradative processes. Specifically, aquifer column experiments show that using benzoate either as a cosubstrate with trace BTX concentrations (Figure 1) or as a preparatory substrate to acclimate pristine aquifer material (Figure 2) could reduce the time required for the indigenous consortium to adapt to BTX and attenuate their migration. This is evident in the rapid decline in effluent BTX concentrations that followed the initial BTX breakthrough in benzoate-amended columns. Such breakthrough patterns are characteristic of increased biodegradation rates as a result of enhanced microbial adaptation and proliferation of desirable phenotypes (*34*).

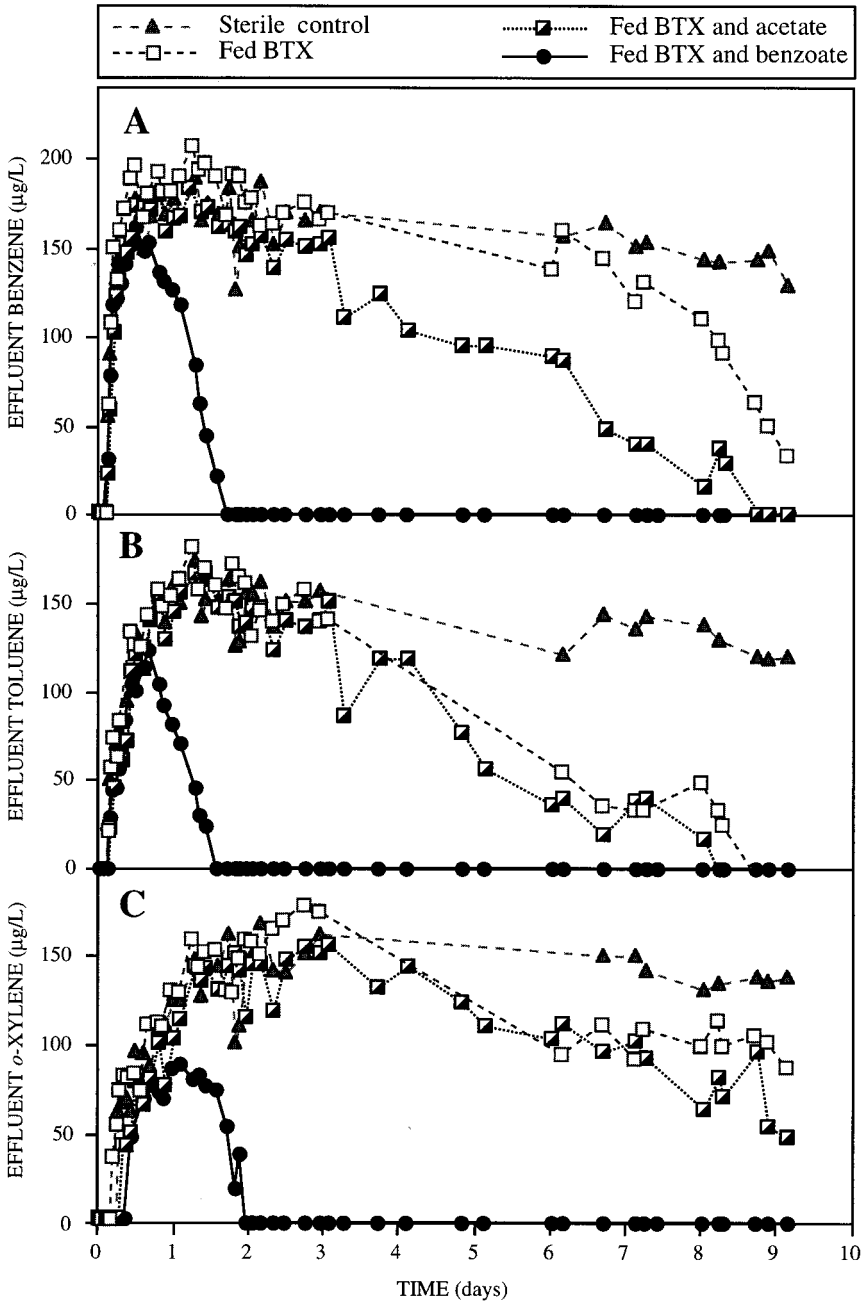

FIGURE 1. Breakthrough curves for benzene (A); toluene (B); and *o*-xylene (C) from columns fed different cosubstrates. All three BTX compounds were fed concurrently at about 190 μg/L each. The feed to two viable columns was supplemented with either acetate (2 mg/L) or benzoate (1 mg/L).

Breakthrough data were also used to calculate BTX mass loss rates attributable to biodegradation (Figure 3). This analysis confirms that degradation rates increased with time for all BTX compounds, and that the rates increased faster when benzoate was added.

Increased degradation rates in the benzoate-amended column reduced the time required to fully attenuate BTX migration from about 9 to 2 days (Figure 1), representing a relative reduction in "breakthrough" time of 70%. Although a net reduction in "breakthrough" time of 1 week is not particularly impressive, biostimulation tends to yield greater benefits in the field where the need to overcome limitations to natural degradative processes is greater. Indeed, BTX degradation rates are generally orders of magnitude slower *in situ* than in the lab (*35*), and a similar relative increase in degradation rates in the field would be conducive to a more dramatic reduction in the duration of clean-up operations. For example, if benzoate increased BTX degradation rates and reduced the half-life of benzene by 70%, from a typical value of 100 to 30 days, the time required to degrade 1 mg/L to the drinking water standard of 5 μg/L would decrease by 1.5 years.

Enhanced BTX degradation in the benzoate-amended columns was attributed to microbial population changes. Pseudomonad species that are capable of degrading BTX and growing on benzoate (i.e., *P. putida* and *B. cepacia*) (*36*–*39*) became dominant in these columns. In contrast, dominant (culturable) species in unexposed aquifer material or in the columns fed BTX plus acetate were not all BTX degraders. *P. putida* and (to a lesser extent) *B. cepacia* were also dominant in the column-fed BTX alone, but not in the column-fed BTX plus acetate. Regardless of the reasons why they predominated in benzoate-amended but not in acetate-amended columns, these pseudomonads are *r*-strategists that

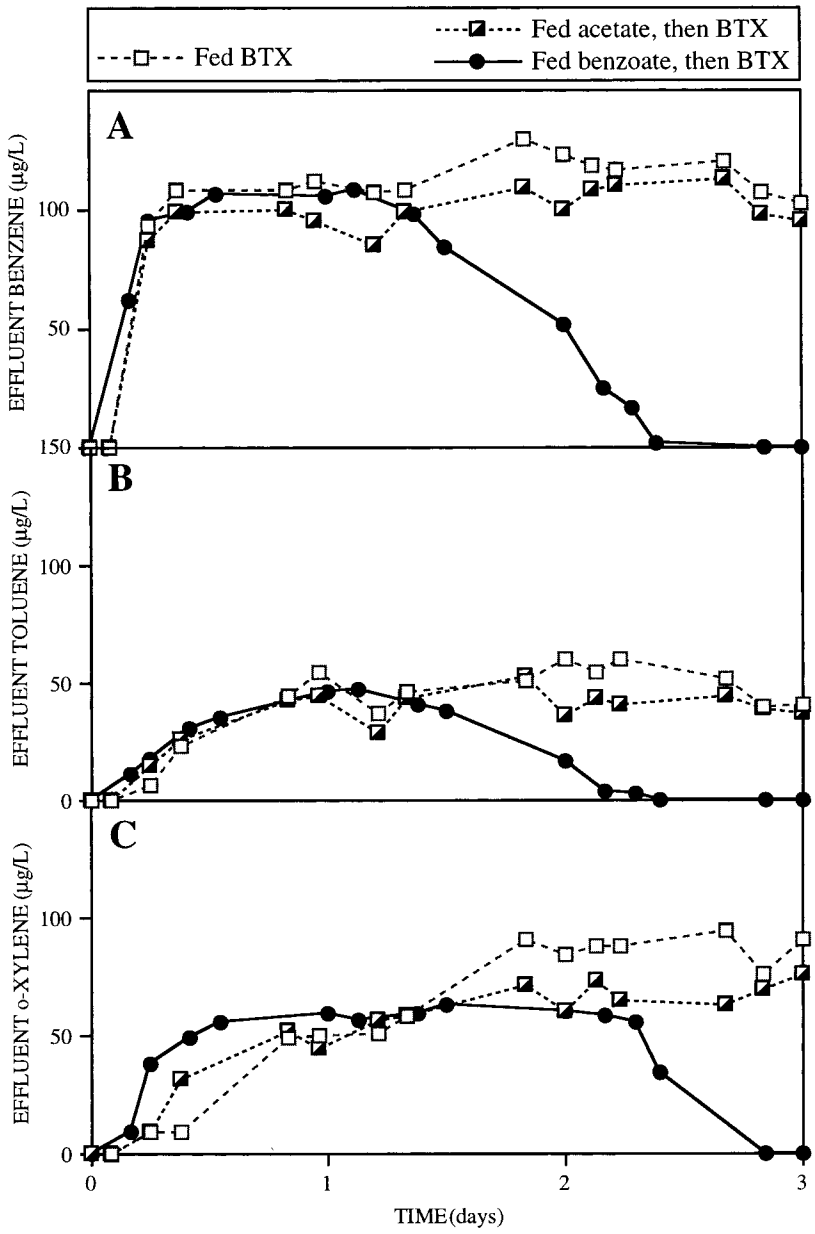

FIGURE 2. Breakthrough curves for benzene (A); toluene (B); and *o*-xylene (C) from columns previously acclimated to different substrates. Columns were initially fed benzoate (2 mg/L), acetate (4 mg/L), or basal medium alone for 2 days. Benzoate and acetate were subsequently removed from the feeds, and all columns were fed BTX at about 150 µg/L each.

exhibit relatively fast degradation rates (*40*) and their presence seems to be common and beneficial in BTX contaminated sites (*41*).

Although BTX should be the best substrates to select for BTX degraders, these compounds are not always present at sufficient concentrations to sustain a viable microbial population, particularly in pristine (oligotrophic) zones downgradient of BTX plumes. Benzoate is a nontoxic substrate that can serve as additional carbon and energy source to enhance the viability of BTX degraders, as indicated by the enhanced attenuation of BTX migration (Figures 1 and 2), by a faster increase in BTX degradation rates (Figure 3), and by the beneficial changes observed in dominant (culturable) isolates. Therefore, biostimulation with benzoate might fill a niche in pollution control associated with enhancing BTX degradation when rates are limited by low concentrations and/or activity of BTX degraders.

Other frequently cited adaptation mechanisms, such as gene transfer and mutation and enzyme induction, are unlikely to have played a significant role in the observed enhancement of BTX degradation. Gene transfer and mutation usually requires longer time than was allowed (*42*). Regarding enzyme induction, benzoate induces the TOL plasmid (*28*) which is commonly found in the dominant species isolated from benzoate-amended columns (i.e., *P. putida*). However, the TOL enzymes cannot degrade benzene or *o*-xylene. Thus, the enhanced degradation of these compounds cannot be attributed to enhanced induction of the TOL plasmid. *P. putida* is also known to express toluene dioxygenase (TDO), which can oxidize all BTX compounds (*36*). However, benzoate does not induce TDO (*43*), which suggests that the increase in BTX degradation activity in benzoate-amended columns was not due to enhanced catabolic enzyme induction. Other studies have found that common substrates can enhance xenobiotic degradation due to general stimulation of microbial metabolism rather than specific operon activation (*18, 44*). Yet, acetate addition did not have the beneficial effect that benzoate addition did

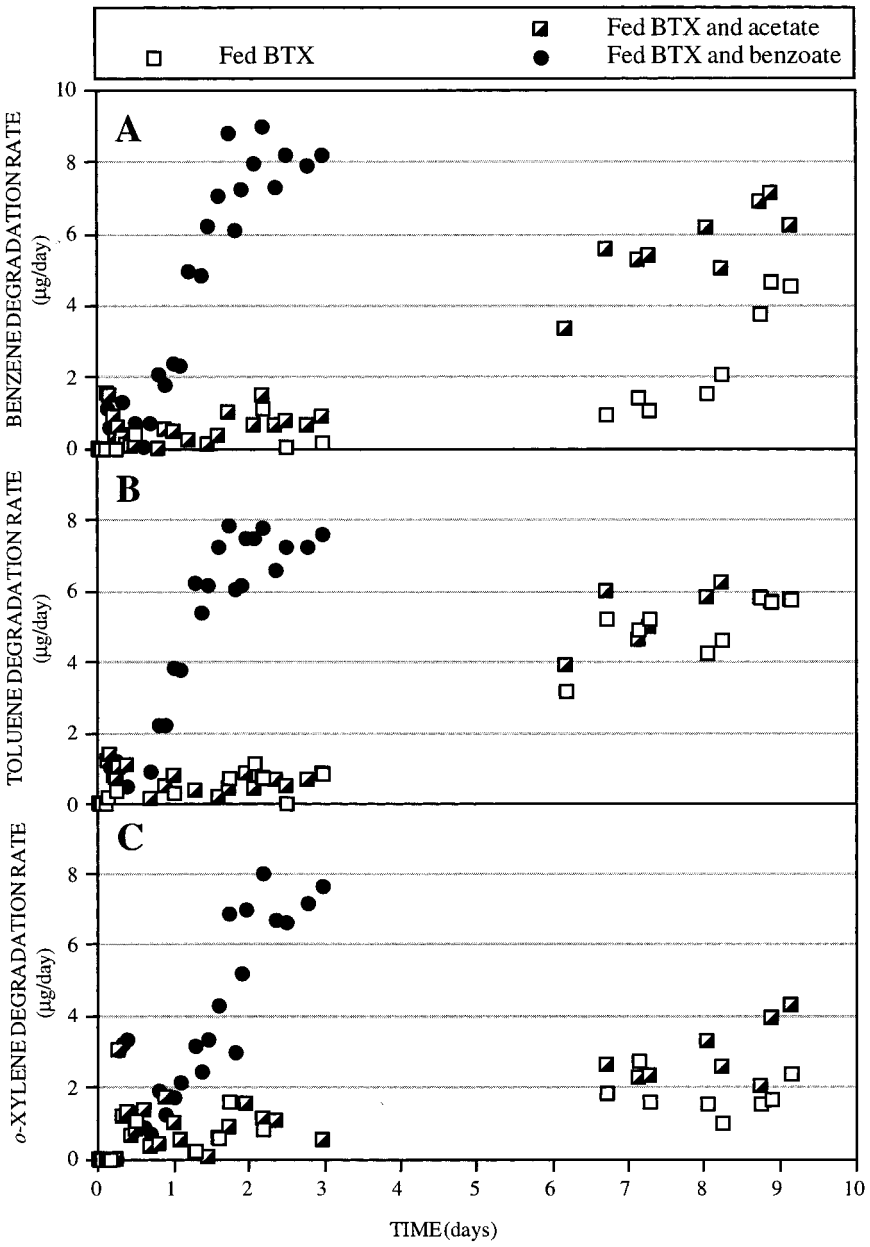

FIGURE 3. Mass loss rates attributable to biodegradation for benzene (A); toluene (B); and *o*-xylene (C) in columns fed different cosubstrates. Rates were calculated using equation 1 with breaktrhough data depicted in Figure 1.

(Figures 1 and 2), indicating that not just any common supplemental substrate can stimulate BTX degradation.

Although benzoate addition enhanced BTX degradation kinetics in this study, it should be kept in mind that *in situ* biodegradation rates may reflect a number of processes other than intracellular metabolism, including solubilization or desorption of target contaminant, liquid phase transport to cell surface, microbial adaptation (e.g., enzyme induction and other metabolic shifts), diffusion of electron acceptors, and contaminant transport through cytoplasmatic membrane. The conditions under which biochemical reactions or mass transport processes are rate limiting are site specific, and benzoate addition would only be beneficial when rates are limited by biochemical processes (e.g., low concentration and/or activity of BTX degraders). Nevertheless, such conditions seem to be common in groundwater contaminated with trace BTX concentrations, or in pristine (oligotrophic) zones downgradient of BTX plumes, as suggested by a study of five pristine groundwaters that found only 1−10% of the total bacteria present to be metabolically active (*45*). In such cases, long lag periods preceding contaminant degradation often reflect the time required for specific degraders to grow to a critical concentration capable of exerting measurable degradation rates (*46*−*48*). Because benzoate addition could serve as growth substrate for BTX degraders, its addition could shorten the adaptation period and thus enhance the short-term biodegradation potential of unacclimated indigenous consortia. This would be particularly important for rapid degradation of contaminants that are briefly exposed to indigenous microorganisms before further transport into pristine groundwater. Consequently, injecting benzoate downgradient of a BTX plume to establish and sustain an *in situ* reactive (buffer) zone might develop into a practical additional tool for pollution control.

In summary, this work shows that small amounts of benzoate (1−2 mg/L) can enhance the growth of desirable genotypes and potentially increase BTX degradation activity, even in the absence of BTX. Thus, investigating the feasibility

of adding benzoate to establish and sustain a reactive buffer zone downgradient of a BTX plume could be a fruitful avenue of field research.

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