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Byproducts of Anaerobic Alkylbenzene Metabolism Useful as Indicators of in Situ Bioremediation

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Based on the concordance of laboratory studies of anaerobic bacteria and field observations from an aquifer in Seal Beach, CA, a group of compounds including benzylsuccinic acid, benzylfumaric acid (or a closely related isomer), and the *o*-, *m*-, and *p*-methyl homologs of these compounds are proposed as biogeochemical indicators of in situ anaerobic alkylbenzene metabolism in gasoline-contaminated aquifers. Under the controlled conditions of the field study, a strong correspondence was observed between the disappearance of alkylbenzenes from groundwater over time and the appearance of associated metabolic byproducts. This correspondence was both qualitative (i.e., only products specific to the metabolism of toluene, *o*-xylene, and *m*-xylene were observed, and only these three hydrocarbons were depleted) and quantitative (i.e., metabolic byproduct concentrations tended to increase as the associated alkylbenzene concentrations decreased). These metabolites may prove useful for distinguishing biotic losses of BTEX (benzene, toluene, ethylbenzene, and xylenes) from abiotic losses when monitoring gasoline-contaminated, typically anaerobic, aquifers that are not being actively remediated or that are being remediated by stimulation of anaerobic bacteria.

Introduction

Leakage of gasoline from underground fuel storage tanks is a pervasive source of groundwater contamination in the United States. In 1986, the U.S. Environmental Protection Agency (EPA) estimated that 35% of the nation's underground motor fuel storage tanks were leaking (*1*); at that time, estimates of the total number of underground tanks approached 2 million (*2*). The leaking fuel from storage tanks has reached groundwater in approximately 40% of the reported cases nationwide (*2*). Surface spill accidents and landfill leachate intrusion are additional sources of groundwater contamination by gasoline, aviation fuel, and other refined petroleum derivatives. Benzene, toluene, ethylbenzene, and the three xylene isomers (known collectively as BTEX) are relatively water-soluble constituents of refined petroleum products (constituting over 50 wt % of the water-soluble fraction of unleaded gasoline; ref 3) that present human health concerns.

Bioremediation is one of a limited number of options for restoring fuel-contaminated aquifers. Toluene and other alkylbenzenes are readily degradable in aerobic surface water and soil systems (*4, 5*); however, in the subsurface environment, contamination by organic compounds often results in the complete consumption of available oxygen by indigenous microorganisms and the development of anaerobic conditions (e.g., ref 6). Considering the cost and technical difficulty associated with introducing oxygen into some aquifers, in situ bioremediation performed by indigenous, anaerobic bacteria merits serious consideration at some contaminated sites. Such processes have become more plausible in light of recent progress made in research of anaerobic BTEX degradation. For example, since 1989, 16 pure cultures capable of anaerobic toluene degradation have been reported, including 13 denitrifying cultures (*7–11*), two sulfate-reducing cultures (*12, 13*), and one ferric iron-reducing culture (*14, 15*); several fermentative-methanogenic consortia that degrade toluene have also been reported (*16–18*).

A major impediment to the acceptance of in situ bioremediation is the difficulty of demonstrating that decreases in the concentrations of BTEX in groundwater truly represent biological metabolism of these compounds rather than abiotic processes such as sorption, dilution, or volatilization. The difficulties of documenting in situ bioremediation have been discussed in detail elsewhere (*19, 20*). A recent National Research Council (NRC) report on in situ bioremediation (*19*) made general recommendations for demonstrating biodegradation using field measurements; among the variables listed in the NRC report were "byproducts of anaerobic activity" and "intermediary metabolite formation". Metabolites relevant specifically to BTEX degradation were not identified.

Regarding the NRC recommendations, we believe that ideal biogeochemical indicators for demonstrating in situ metabolism of BTEX would be metabolites with the following properties: (i) an unequivocal biochemical relationship to specific single-ring aromatic hydrocarbons, (ii) no commercial or industrial uses, and (iii) biological and chemical stability. A group of compounds that conforms partially to the above properties includes benzoic acid and alkylbenzoic (e.g., toluic) acids. Benzoic acid has been shown to be an intermediate of anaerobic toluene degradation (e.g., refs 10 and 21–24), and toluic acid isomers have been reported as metabolites of the corresponding xylene isomers under denitrifying conditions (*25*) and sulfate-reducing conditions (*13*). Furthermore, Cozzarelli and co-workers presented field evidence suggesting that benzoic acid and methylbenzoic acid isomers were formed from the corresponding alkylbenzenes in an oil-contaminated, anoxic aquifer located near Bemidji, MN (*26, 27*); similar findings were reported by Reinhard et al. (*28*) for studies of leachate plumes from two landfills in Ontario,

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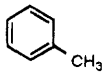
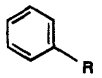
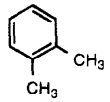
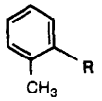
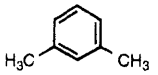
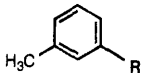
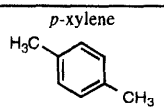
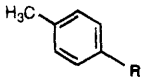
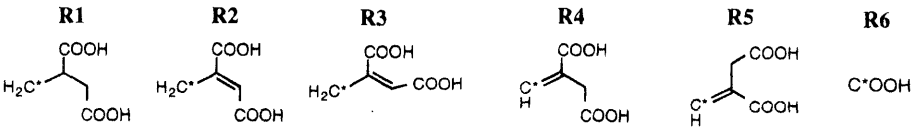
Substrate metabolized	Anaerobic metabolic by-products[a] Nucleus	R[b]	Reported observation of by-products
toluene 		R1 = benzylsuccinic acid R2 = benzylfumaric acid	Evans et al. (*30*); Beller et al. (*22*); Beller (*13*); Seyfried et al. (*25*)
o-xylene 		R1 = (2-methylbenzyl)succinic acid R2 = (2-methylbenzyl)fumaric acid	Evans et al. (*30*); Beller (*13*)
m-xylene 		R1 = (3-methylbenzyl)succinic acid R2 = (3-methylbenzyl)fumaric acid	Seyfried et al. (*25*) and this study
p-xylene 		R1 = (4-methylbenzyl)succinic acid R6 = *p*-toluic acid	Beller (*13*)

a Products found in spent medium of anaerobic laboratory cultures that were not metabolically inhibited.

b



where C* is a benzylic carbon atom.

FIGURE 1. Names and structures of reported byproducts of anaerobic alkylbenzene metabolism.

Canada. Nonetheless, benzoic acid is not an ideal biogeochemical indicator of anaerobic toluene degradation because it has widespread commercial use, is an intermediate of the anaerobic metabolism of a range of aromatic compounds (e.g., ref 29), and is not biologically stable. Regarding biological stability, benzoic acid is rarely observed as an intermediate of anaerobic toluene degradation in microbial lab studies unless the cultures are metabolically inhibited (*10, 22–25*). Toluic acids may be more biologically stable than benzoic acid but they are not well-studied in relation to anaerobic xylene metabolism.

In this paper, we provide evidence that benzylsuccinic acid and a group of related compounds (Figure 1) are valuable biogeochemical indicators of in situ anaerobic alkylbenzene metabolism in gasoline-contaminated aquifers. Benzylsuccinic acid and benzylfumaric acid were recently reported as metabolic dead-end products formed in conjunction with toluene mineralization under sulfate-reducing conditions (*13, 22*) and denitrifying conditions (*25, 30*). In addition, (2-methylbenzyl)succinic acid and (2-methylbenzyl)fumaric acid were reported as products from the anaerobic transformation of *o*-xylene (*13, 30*), (4-methylbenzyl)succinic acid was reported as a product of anaerobic *p*-xylene metabolism (*13*), and (3-methylbenzyl)-succinic acid and (3-methylbenzyl)fumaric acid were reported as products of anaerobic *m*-xylene metabolism (ref 23, citing unpublished data). As biogeochemical indicators, this group of compounds has the following advantageous properties: a specific biochemical relationship between the compounds and the alkylbenzenes from which they are produced, no commercial or industrial uses, and biological stability (they are dead-end metabolites under anaerobic conditions in the cultures studied thus far). Notably, these products have not been reported in the extensive studies of aerobic metabolism of alkylbenzenes, in contrast to benzoic acid and toluic acids (e.g., refs 5 and 31).

In addition to their potential use as biogeochemical indicators, these compounds may also be of interest because of human health concerns. For example, benzylsuccinic acid is a potent inhibitor of carboxypeptidase A, a pancreatic enzyme that catalyzes protein digestion (*32, 33*). Benzylfumaric acid also appears to be a strong inhibitor of this enzyme (*34*).

Experimental Section

Sample Collection and Analysis. The field site in Seal Beach, CA, and the sampling methods employed there are described elsewhere (*35*) and will not be detailed here. Briefly, this study of in situ biodegradation of BTEX involved a "slug test" with a single injection/extraction well and the controlled release of five single-ring aromatic compounds (BTEX, excluding *p*-xylene). The test slug consisted of approximately 840 L of Seal Beach groundwater that was (i) extracted from the aquifer; (ii) deionized; (iii) purged with helium to remove oxygen (to ≤0.2 mg/L) and volatile aromatic compounds (to ≤1 μg/L); (iv) amended with bromide (for use as a conservative tracer), mineral salts, and aromatic compounds at 2–3 μM (225–300 μg/L) per component; and (v) injected back into the same well to establish an aquifer test zone (*35*). Sulfate was the only electron acceptor supplied in this experiment; the test slug was amended with ca. 0.16 mM sulfate, which is approximately one-fifth of the typical background concentration. Sources of BTEX other than the injected test slug (e.g., desorption from aquifer material) were negligible during the experiment (35). The aquifer test zone was isolated from native groundwater by a geochemical "buffer" zone that had the composition of the test slug excluding BTEX and bromide. To monitor concentrations of analytes,

groundwater was pumped from the test zone at selected time intervals. Modeling of the hydraulics of the site and assessment of mass recovery using the slug test method at this site are presented elsewhere (*35*).

Groundwater samples were collected for BTEX analysis, semivolatile metabolite analysis, and bromide analysis. Samples analyzed for BTEX were collected by completely filling precleaned 40-mL glass vials, leaving no headspace, and were preserved upon collection by the addition of concentrated HCl. Samples for bromide were also collected in 40-mL vials but were not preserved with HCl. These samples were shipped to Stanford University on ice and were stored for less than 1 day at 4 °C before analysis. BTEX samples were analyzed by purge-and-trap gas chromatography (GC) with photoionization detection (*36*). Bromide samples were analyzed by ion chromatography with conductivity detection using methods described elsewhere (*36*).

Samples for semivolatile metabolite analysis were collected by completely filling solvent-cleaned 1-L amber glass bottles and were preserved upon collection by adding concentrated HCl (final pH ≤ 1). Samples were shipped on ice and were stored at 4 °C until extraction. Liquid–liquid extraction was performed with 1-L groundwater samples using 2-L separatory funnels. Samples were spiked with 4-fluorobenzoic acid (0.1 μM final concentration) as a surrogate standard to assess recovery and were then extracted three times with high-purity diethyl ether (B & J Brand, ethanol-preserved, Baxter Healthcare Corporation, Muskegon, MI). The combined ether extract was rotary evaporated to 2–5 mL, dried with precleaned sodium sulfate, derivatized with ethereal diazomethane to convert carboxylic acids to methyl esters (*37*), exchanged into high-purity dichloromethane (Ultra Resi-Analyzed, distilled-in-glass; J.T. Baker, Inc., Phillipsburg, NJ) using a gentle stream of high-purity nitrogen at room temperature, spiked with chrysene-d_{12} as an internal standard, and analyzed by gas chromatography/mass spectrometry (GC/MS). GC/MS analyses were performed with an HP Model 5890A GC (Hewlett-Packard Company, Palo Alto, CA) with a DB-5 fused silica capillary column (30 m length by 0.32 mm i.d.; 0.25 μm film thickness; J & W Scientific, Folsom, CA) coupled to an HP 5970 Series MSD; data analysis was performed with HP G1034C software designed for the MS ChemStation. The GC oven was programmed from 45 (held 2 min) to 110 °C at 8 °C/min and then from 110 to 250 °C at 4 °C/min (held 5 min); the injection port temperature was 275 °C, and the transfer line temperature was 280 °C. The column inlet pressure was maintained at 10 kPa.

Internal standard quantification was used for all compounds analyzed by GC/MS. The benzylsuccinic acid (dimethyl ester) response factor was used to estimate concentrations of all metabolic products for which authentic standards were not available (see next section). The tropylium ion (*m/z* 91) was used as the quantification ion for establishing the dimethyl benzylsuccinate response factor. Characteristic ions (e.g., the methyl tropylium ion, *m/z* 105, for methylbenzylsuccinates) were used for other compounds quantified with the benzylsuccinate response factor. This quantification technique, which yielded semiquantitative data for compounds without authentic standards, was sufficient for the method of data analysis used in this study; data analysis for metabolic byproducts focuses on concentration trends rather than on absolute concentrations. Detection limits for semivolatile metabolites were on the order of 1 nM. Method blanks contained none of the metabolic byproducts reported in this paper. Surrogate recoveries in groundwater samples averaged approximately 95% and were ≥70% in all samples. The recovery of benzylsuccinic acid from a spiked blank was approximately 89%.

Standards for Metabolic Byproducts of Alkylbenzene Metabolism. Of the metabolic byproducts shown in Figure 1, only benzylsuccinic acid (Sigma Chemical Company, St. Louis, MO) and toluic acids (Aldrich Chemical Company, Milwaukee, WI; purchased as methyl esters) were commercially available. However, for the purposes of this study, byproducts formed as a result of BTEX metabolism by anaerobic bacteria under controlled laboratory conditions served well as qualitative standards. Thus, identification of compounds found in Seal Beach groundwater was based principally on comparisons of the GC retention times and mass spectra of compounds found in groundwater extracts to those of metabolic byproducts produced by two bacterial cultures: (i) strain PRTOL1, a sulfate-reducing bacterium that is being studied in our laboratory (*13*), and (ii) *Pseudomonas* sp. strain T, a denitrifying bacterium isolated by Zeyer and co-workers (*7, 11*). Strain PRTOL1, which was isolated from contaminated subsurface soil from an aviation fuel storage facility near the Patuxent River (Maryland), forms metabolic byproducts from toluene and *o*- and *p*-xylene under anaerobic conditions (*13*). Strain T grows on toluene and *m*-xylene under denitrifying conditions and also forms metabolic byproducts (*25*). Although these particular bacterial species may not be present at the Seal Beach site, their byproducts are not species-specific, as similar or identical products have been reported for other anaerobic cultures that metabolize toluene and other alkylbenzenes (see Introduction).

Regarding previous studies that reported benzylfumaric acid (toluene + R2 in Figure 1) and methylbenzylfumaric acids (Figure 1), it is noteworthy that authentic standards were not available for compound confirmation. Although benzylmaleic acid (toluene + R3 in Figure 1) synthesized in one study had an identical HPLC retention time and very similar mass spectrum to the toluene metabolite reported as benzylfumaric acid (*30*), benzylfumaric acid itself was not synthesized. A recent study in which benzylfumaric acid and three structurally similar isomers were synthesized [i.e., benzylmaleic acid, (*Z*)-phenylitaconic acid (toluene + R4, Figure 1), (*E*)-phenylitaconic acid (toluene + R5, Figure 1)] indicated that the four isomers could be distinguished by their GC retention times but not by their mass spectra (*38*). Thus, all four isomers would have to be available to make definitive identifications of such metabolic byproducts. For the purpose of this study, which is to propose biogeochemical indicators of anaerobic alkylbenzene metabolism, the exact identity of the compounds is less important than the demonstration that compounds found in the field are identical to or extremely similar to (i.e., with identical GC retention times and mass spectra as) those found in controlled laboratory studies of anaerobic alkylbenzene metabolism. For the sake of consistency and brevity, we will retain the names of compounds previously reported as benzylfumaric acid or methylbenzylfumaric acid with the understanding that the isomers represented by substituting R3, R4, or R5 for R2 (Figure 1) may actually apply.

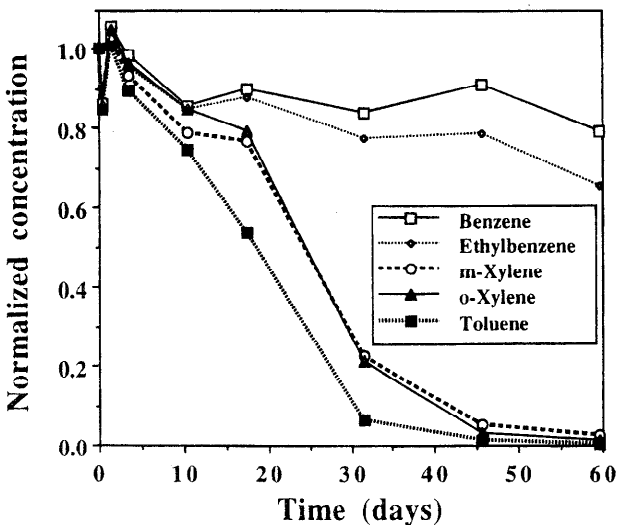


FIGURE 2. Normalized concentrations of BTEX in Seal Beach groundwater versus time. Concentrations are normalized to bromide and to the initial concentrations of the compounds themselves.

Results and Discussion

Depletion of Single-Ring Aromatic Hydrocarbons from Groundwater. Three of the five single-ring aromatic compounds added to Seal Beach groundwater were depleted during the study. Concentrations of toluene and *o*- and *m*-xylene decreased by >95% by day 60, whereas benzene and ethylbenzene concentrations did not decrease markedly over the duration of the experiment (Figure 2). Concentrations in Figure 2 and all others plotted in this paper were normalized to bromide concentration to correct for changes resulting from dilution and dispersion of the injected test slug.

Qualitative Relationship between Metabolic Byproducts and BTEX. Extraction and GC/MS analysis of Seal Beach groundwater samples collected at the sampling times shown in Figure 2 revealed the eventual appearance of benzylsuccinic acid and two methylbenzylsuccinic acid isomers. The presence of benzylsuccinic acid was confirmed in Seal Beach groundwater based on GC retention time and mass spectral comparisons of sample peaks to an authentic derivatized standard. Standards were not commercially available for the methyl homologs of benzylsuccinic acid shown in Figure 1. However, byproducts of *o*- and *p*-xylene metabolism by sulfate-reducing strain PRTOL1 (*13*) and of *m*-xylene metabolism by denitrifying strain T served as qualitative standards. These byproducts are believed to be methylbenzylsuccinate isomers because (i) they were produced by organisms known to make benzylsuccinic acid from toluene and (ii) they have mass spectral fragmentation patterns analogous to benzylsuccinate but with masses of major fragment ions that are 14 amu (one CH_2 unit) higher (*30*). Figure 3A is the mass spectrum of a derivatized metabolic byproduct of *o*-xylene metabolism by strain PRTOL1 tentatively identified as (2-methylbenzyl)succinic acid (*13*), and Figure 3B is the spectrum of a derivatized compound found in Seal Beach groundwater; these compounds had the same retention time, as demonstrated by co-injection on GC/MS, and thus are concluded to be identical. A compound with a similar mass spectral fragmentation pattern but different GC retention time is represented in Figure 3C. Figure 3C is the mass spectrum of a derivatized metabolic byproduct of *m*-xylene metabolism by strain T tentatively identified as

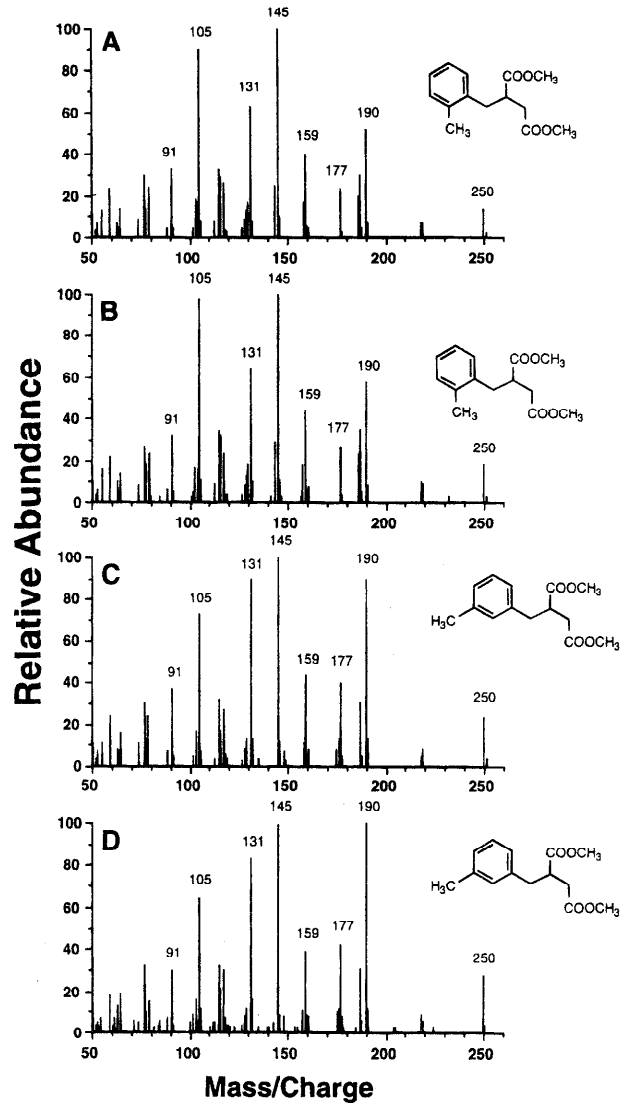


FIGURE 3. Mass spectra of (A) a derivatized metabolite, tentatively identified as (2-methylbenzyl)succinic acid (see text), resulting from *o*-xylene metabolism by strain PRTOL1; (B) a compound found in a derivatized Seal Beach extract that co-eluted with the compound represented by A; (C) a derivatized metabolite, tentatively identified as (3-methylbenzyl)succinic acid (see text), resulting from *m*-xylene metabolism by strain T; and (D) a compound found in a derivatized Seal Beach extract that co-eluted with the compound represented by C. Retention times relative to benzylsuccinic acid (dimethyl ester): (A) 1.100 and (C) 1.094 at the GC conditions described in the text.

(3-methylbenzyl)succinic acid, and Figure 3D is the spectrum of a derivatized compound found in Seal Beach groundwater; these compounds had the same GC retention time and are concluded to be identical. A qualitative standard for (4-methylbenzyl)succinic acid was available from *p*-xylene metabolism by strain PRTOL1 (*13*); however, this compound was not observed in Seal Beach groundwater. In summary, the appearance of benzylsuccinic acid and (2-methylbenzyl)- and (3-methylbenzyl)succinic acid in Seal Beach groundwater is consistent with the disappearance of toluene and *o*- and *m*-xylene from the groundwater over time; the absence of (4-methylbenzyl)-succinic acid from the groundwater is consistent with the absence of *p*-xylene from the system throughout the experiment.

Extraction and GC/MS analysis of Seal Beach groundwater samples also revealed the eventual appearance of

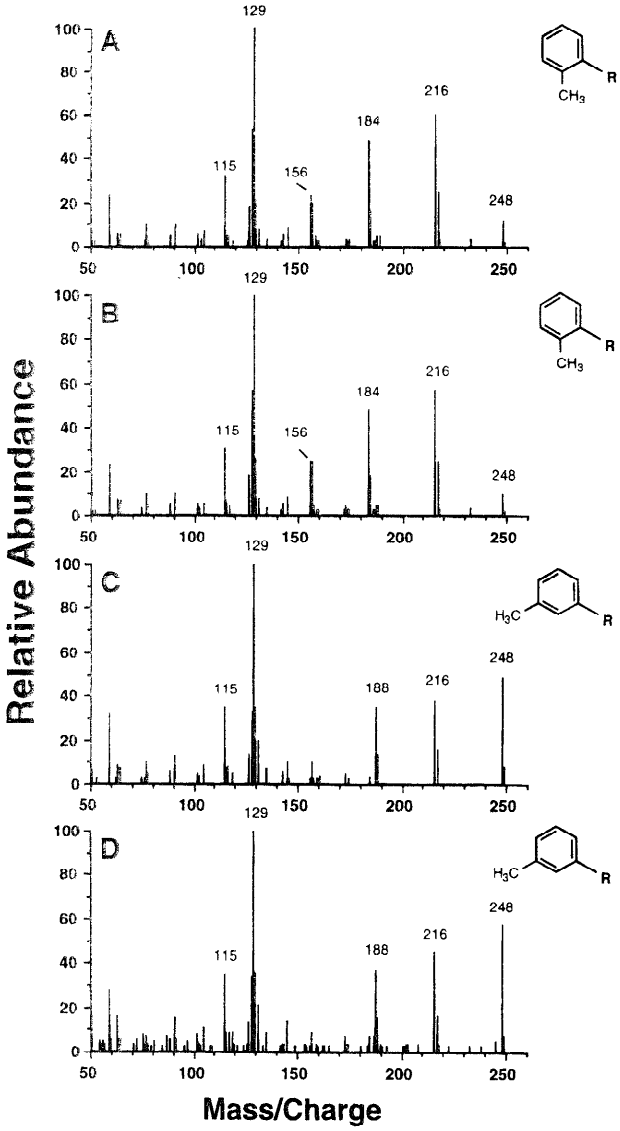


FIGURE 4. Mass spectra of (A) a derivatized metabolite, tentatively identified as (2-methylbenzyl)fumaric acid (see text), resulting from *o*-xylene metabolism by strain PRTOL1; (B) a compound found in a derivatized Seal Beach extract that co-eluted with the compound represented by A; (C) a derivatized metabolite, tentatively identified as (3-methylbenzyl)fumaric acid, resulting from *m*-xylene metabolism by strain T; and (D) a compound found in a derivatized Seal Beach extract that co-eluted with the compound represented by C. In all cases, R = R2, R3, R4, or R5 (see Figure 1) as dimethyl esters. Retention times relative to benzylsuccinic acid (dimethyl ester): (A) 1.137 and (C) 1.193 at the GC conditions described in the text.

benzylfumaric acid and two methylbenzylfumaric acid isomers. Benzylfumaric acid was tentatively identified in Seal Beach groundwater based on GC retention time and mass spectral comparisons of sample peaks to a metabolic product of anaerobic toluene degradation by strain PRTOL1 (data not shown). In addition, a derivatized compound found in Seal Beach groundwater (represented by Figure 4B) had the same mass spectrum and GC retention time as a derivatized byproduct of *o*-xylene metabolism by strain PRTOL1 (Figure 4A). A very similar spectrum was reported by Evans et al. (*30*) for a derivatized product of *o*-xylene metabolism by a denitrifying bacterium; the product was reported as (2-methylbenzyl)fumaric acid (dimethyl ester). A related compound, tentatively identified as (3-methylbenzyl)fumaric acid, is represented in Figure 4C. Figure 4C is the spectrum of a derivatized product of *m*-xylene metabolism by strain T, and Figure 4D is the spectrum of a derivatized compound found in Seal Beach groundwater; the compounds represented by Figure 4C,D had the same GC retention time and are concluded to be identical. The spectra shown in Figure 4C,D are similar to, but distinct from, the spectra shown in Figure 4A,B. The greatest differences are the relatively low abundances of *m/z* 156, 157, and 184 and the relatively high abundances of *m/z* 188 and 248 in Figure 4C,D. Overall, the presence of benzylfumaric, (2-methylbenzyl)fumaric, and (3-methylbenzyl)fumaric acid is consistent with the disappearance of toluene and *o*- and *m*-xylene from Seal Beach groundwater over time.

Quantitative Relationship between Metabolic Byproducts and BTEX. Trends in concentration data provide additional evidence that benzylsuccinic acid, benzylfumaric acid, and the related compounds found in Seal Beach groundwater were indeed products of in situ alkylbenzene metabolism. None of the compounds reported as metabolic byproducts in this study were detectable in the initial Seal Beach groundwater sample. In general, as alkylbenzene concentrations decreased with time in Seal Beach groundwater, metabolic byproduct concentrations increased until the alkylbenzenes were depleted. For example, the correspondence between alkylbenzene disappearance and the appearance of benzylsuccinic acid homologs is depicted in Figure 5. The concentrations of toluene, *o*-xylene, and *m*-xylene and of the associated metabolic byproducts are normalized to bromide and to the maximum concentration of each compound itself in Figure 5. Analogous data for presumed benzylfumaric acid homologs are shown in Figure 6. Figure 6C presents data for *m*-toluic acid in addition to *m*-xylene and (3-methylbenzyl)fumaric acid. In general, similar concentration trends were apparent for all observed byproducts (e.g., all byproduct concentrations maximized on day 32; Figures 5 and 6).

An interesting trend observed for the concentrations of all metabolic byproducts is a decrease after the concentration maximum, suggesting a loss of these compounds beyond simple dilution, which was corrected for by bromide normalization. If this decrease represents biodegradation of these products, this is in contrast to laboratory observations of pure and mixed cultures in which these compounds were accumulating dead-end products (*22, 30*). Although degradability would detract somewhat from the use of these compounds as biogeochemical indicators, it must be noted that this study entailed the one-time release of low concentrations of BTEX, whereas a contaminated aquifer would be expected to contain ongoing sources of BTEX to groundwater (e.g., from a nonaqueous gasoline phase or desorption from solids). In such situations, a continuing steady-state concentration of metabolic byproducts is more likely than in this study.

As discussed previously, determination of absolute concentrations of metabolic byproducts other than benzylsuccinic acid and *m*-toluic acid was not possible; however, estimations of concentrations were made using benzylsuccinic acid as a standard for analogous compounds. As an approximation, benzylsuccinic acid or benzylfumaric acid analogs at their maximum concentrations constituted <10 wt % (<5 mol %) of the consumed quantities (ca. 225–300 μg/L or 2–3 μM) of the corresponding aromatic hydrocarbons. The actual yields of the byproducts would be higher than indicated by their maximum concentrations if, as suggested by Figures 5 and 6, the byproducts were

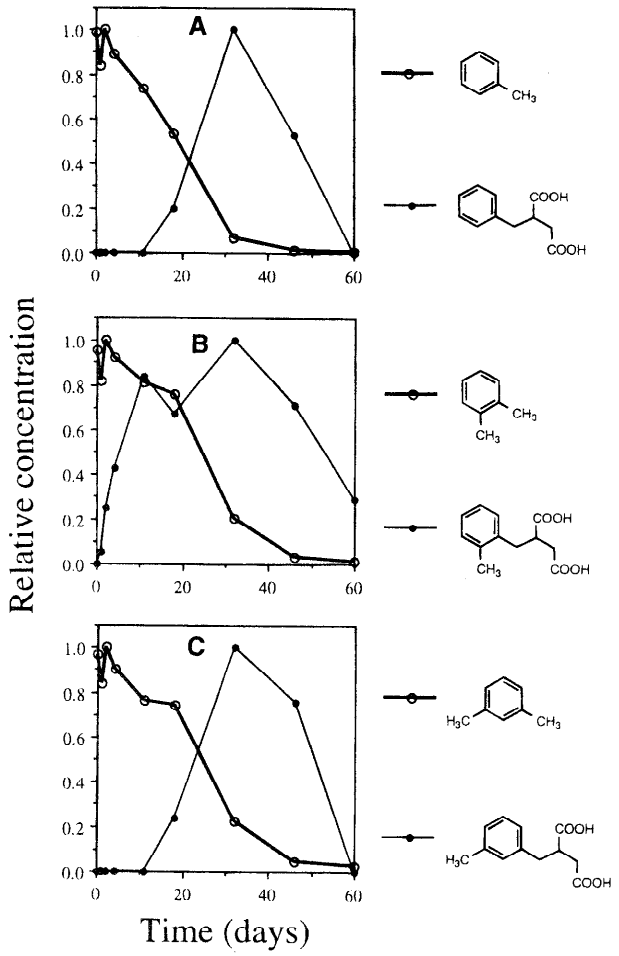


FIGURE 5. Normalized concentrations of alkylbenzenes and corresponding benzylsuccinic acid homologs in Seal Beach groundwater versus time: (A) toluene and benzylsuccinic acid, (B) *o*-xylene and (2-methylbenzyl)succinic acid, and (C) *m*-xylene and (3-methylbenzyl)-succinic acid. All concentrations are normalized to bromide and to the maximum concentrations of the compounds themselves.

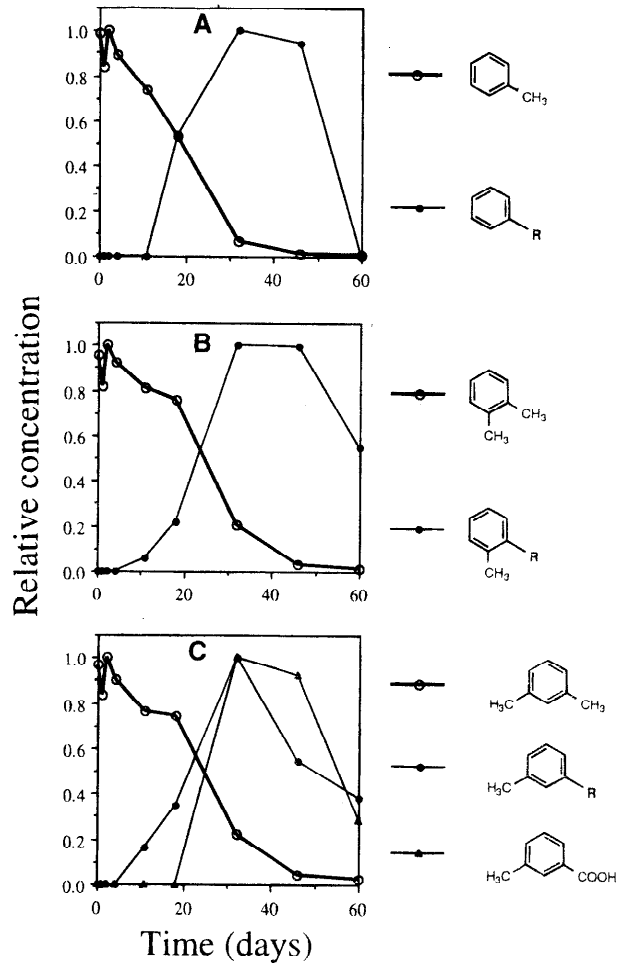


FIGURE 6. Normalized concentrations of alkylbenzenes and corresponding benzylfumaric acid homologs (or closely related compounds) in Seal Beach groundwater versus time. In all cases, R = R2, R3, R4, or R5 (see Figure 1). *m*-Toluic acid is also included in C. All concentrations are normalized to bromide and to the maximum concentrations of the compounds themselves.

being degraded or otherwise removed from the groundwater. Based on total ion chromatogram areas, the 2-methyl (ortho-) isomers of benzylsuccinic and benzylfumaric acid were clearly present at the greatest concentration of all the metabolic byproducts detected (data not shown).

In summary, in this field study of in situ biodegradation of BTEX under anaerobic conditions, a strong correspondence was observed between the disappearance of alkylbenzenes from groundwater and the appearance of associated metabolic byproducts, such as benzylsuccinic acid, benzylfumaric acid (or a closely related isomer), and the methyl homologs of these compounds. These observations support the use of such byproducts as biogeochemical indicators of anaerobic BTEX degradation in gasoline-contaminated aquifers. Confirmation of the widespread applicability of the observed metabolic byproducts as biogeochemical indicators will require (i) an assessment of their presence in other gasoline-contaminated aquifers that have become anaerobic and (ii) a better understanding of their biodegradability by mixed bacterial cultures under anaerobic conditions.

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Literature Cited

(1) *Underground motor fuel storage tanks: a national survey*; U.S. Environmental Protection Agency: Washington, DC, 1986; NTIS PB 86-216512.

(2) *Underground storage tanks: Hearing before the Subcommittee on Energy and Agriculture of the Committee on Small Business*, November 18, 1987, U.S. House of Representatives; U.S. Government Printing Office: Washington, DC, 1988.

(3) Coleman, W. E.; Munch, J. W.; Streicher, R. P.; Ringhand, H. P.; Kopfler, F. C. *Arch. Environ. Contam. Toxicol.* **1984**, *13*, 171–178.

(4) Ridgway, H. F.; Safarik, J.; Phipps, D.; Carl, P.; Clark, D. *Appl. Environ. Microbiol.* **1990**, *56*, 3565–3575.

(5) Gibson, D. T.; Subramanian, V. In *Microbial degradation of organic compounds*; Gibson, D. T., Ed.; Marcel Dekker, Inc.: New York, 1984; pp 181–252.
(6) Lee, M. D.; Thomas, J. M.; Borden, R. C.; Bedient, P. B.; Wilson, J. T.; Ward, C. H. *CRC Crit. Rev. Environ. Control* **1988**, *18*, 29–89.
(7) Dolfing, J.; Zeyer, J.; Binder-Eicher, P.; Schwarzenbach, R. P. *Arch. Microbiol.* **1990**, *154*, 336–341.
(8) Evans, P. J.; Mang, D. T.; Kim, K. S.; Young, L. Y. *Appl. Environ. Microbiol.* **1991**, *57*, 1139–1145.
(9) Fries, M. R.; Zhou, J.; Chee-Sanford, J.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1994**, *60*, 2802–2810.
(10) Schocher, R. J.; Seyfried, B.; Vazquez, F.; Zeyer, J. *Arch. Microbiol.* **1991**, *157*, 7–12.
(11) Zeyer, J.; Eicher, P.; Dolfing, J.; Schwarzenbach, R. P. In *Biotechnology and Biodegradation*; Kamely, D., Chakrabarty, A., Omenn, G. S., Eds.; Portfolio Publishing Company: The Woodlands, TX, 1990; Vol. 4, pp 33–40.
(12) Rabus, R.; Nordhaus, R.; Ludwig, W.; Widdel, F. *Appl. Environ. Microbiol.* **1993**, *59*, 1444–1451.
(13) Beller, H. R. Ph.D. Thesis, Stanford University, 1995.
(14) Lovley, D. R.; Baedecker, M. J.; Lonergan, D. J.; Cozzarelli, I. M.; Phillips, E. J. P.; Siegel, D. I. *Nature (London)* **1989**, *339*, 297–300.
(15) Lovley, D. R.; Lonergan, D. J. *Appl. Environ. Microbiol.* **1990**, *56*, 1858–1864.
(16) Vogel, T. M.; Grbić-Galić, D. *Appl. Environ. Microbiol.* **1986**, *52*, 200–202.
(17) Grbić-Galić, D.; Vogel, T. M. *Appl. Environ. Microbiol.* **1987**, *53*, 254–260.
(18) Edwards, E. A.; Grbić-Galić, D. *Appl. Environ. Microbiol.* **1994**, *60*, 313–322.
(19) National Research Council. *In situ bioremediation: when does it work?*; National Academy Press: Washington, DC, 1993.
(20) Madsen, E. L. *Environ. Sci. Technol.* **1991**, *25*, 1662–1673.
(21) Altenschmidt, U.; Fuchs, G. *Arch. Microbiol.* **1991**, *156*, 152–158.
(22) Beller, H. R.; Reinhard, M.; Grbić-Galić, D. *Appl. Environ. Microbiol.* **1992**, *58*, 3192–3195.
(23) Frazer, A. C.; Ling, W.; Young, L. Y. *Appl. Environ. Microbiol.* **1993**, *59*, 3157–3160.
(24) Kuhn, E. P.; Zeyer, J.; Eicher, P.; Schwarzenbach, R. P. *Appl. Environ. Microbiol.* **1988**, *54*, 490–496.
(25) Seyfried, B.; Glod, G.; Schocher, R.; Tschech, A.; Zeyer, J. *Appl. Environ. Microbiol.* **1994**, *60*, 4047–4052.
(26) Cozzarelli, I. M.; Eganhouse, R. P.; Baedecker, M. J. *Environ. Geol. Water Sci.* **1990**, *16*, 135–141.
(27) Cozzarelli, I. M.; Baedecker, M. J.; Eganhouse, R. P.; Goerlitz, D. F. *Geochim. Cosmochim. Acta* **1994**, *58*, 863–877.
(28) Reinhard, M.; Goodman, N. L.; Barker, J. F. *Environ. Sci. Technol.* **1984**, *18*, 953–961.
(29) Dangel, W.; Brackmann, R.; Lack, A.; Mohamed, M.; Koch, J.; Oswald, B.; Seyfried, B.; Tschech, A.; Fuchs, G. *Arch. Microbiol.* **1991**, *155*, 256–262.
(30) Evans, P. J.; Ling, W.; Goldschmidt, B.; Ritter, E. R.; Young, L. Y. *Appl. Environ. Microbiol.* **1992**, *58*, 496–501.
(31) Smith, M. R. *Biodegradation* **1990**, *1*, 191–206.
(32) Byers, L. D.; Wolfenden, R. *J. Biol. Chem.* **1972**, *247*, 606–608.
(33) Byers, L. D.; Wolfenden, R. *Biochemistry* **1973**, *12*, 2070–2078.
(34) Okuyama, A.; Ohuchi, S.; Tanaka, T.; Naganawa, H.; Aoyagi, T.; Umezawa, H. *Biochem. Int.* **1986**, *12*, 361–366.
(35) Reinhard, M.; Shang, S.; Kitanidis, P. K.; Orwin, E.; Hopkins, G. D. *Measurement of in situ BTEX biotransformation rates in an anaerobic gasoline plume under different electron-accepting conditions*; Technical Report WRC-6; Western Region Hazardous Substance Research Center: Stanford, CA, 1995.
(36) Ball, H. A.; Reinhard, M.; Orwin, E.; Ridgway, H. F.; Phipps, D. *Factors influencing the anaerobic biodegradation of gasoline hydrocarbons by groundwater bacteria at Seal Beach, CA*; Technical Report WRC-5; Western Region Hazardous Substance Research Center: Stanford, CA, 1993.
(37) Fales, H. M.; Jaouni, T. M.; Babashak, J. F. *Anal. Chem.* **1973**, *45*, 2302–2303.
(38) Frost, J. Michigan State University, personal communication, 1995.

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