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In Situ Transformation of Deuterated Toluene and Xylene to Benzylsuccinic Acid Analogues in BTEX-Contaminated Aquifers

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Techniques for detecting and quantifying anaerobic transformations of benzene, toluene, ethylbenzene, and xylene (BTEX) are needed to assess the feasibility of using in situ bioremediation to treat BTEX-contaminated groundwater aquifers. Deuterated surrogates of toluene (toluene-d_8) and xylene (*o*-xylene-d_{10}) were injected into BTEX-contaminated aquifers during single-well push−pull tests to monitor for the in situ formation of deuterated benzylsuccinic acid (BSA-d_8) and *o*-methyl-BSA-d_{10}. Test solutions (250 L) containing toluene-d_8 (9−22 μM) and *o*-xylene-d_{10} (4−9 μM) along with a conservative bromide tracer (1.3 mM) and nitrate (4 mM) as an electron acceptor were injected into four wells at two sites. Detection of BSA-d_8 and *o*-methyl-BSA-d_{10} in groundwater samples collected from the same wells following injection unequivocally demonstrated anaerobic in situ toluene-d_8 and *o*-xylene-d_{10} transformation with calculated zero-order formation rates ranging from 1.0 to 7.4 nM/day. Concurrent utilization of co-injected nitrate was rapid in all tests at both sites, with zero-order rates ranging from 13 to 39 μM/h. The field tests conducted in this study represent the first reported use of deuterated aromatic hydrocarbons to detect and quantify anaerobic BTEX transformation product formation in the subsurface.

Introduction

Groundwater contamination by petroleum hydrocarbons, including benzene, toluene, ethylbenzene, and xylene isomers (BTEX) is widespread. Limitations and costs of conventional groundwater cleanup technologies (e.g., pump-and-treat) have made in situ bioremediation an attractive remediation alternative (*1*). Since many sites contaminated with these compounds are anaerobic, BTEX degradation under oxygen-limited conditions is of interest. Anaerobic BTEX degradation has been shown to occur under denitrifying, sulfate-reducing, iron-reducing, manganese-reducing, and methanogenic conditions (*2*, *3*). Benzylsuccinic acid (BSA) and methyl-BSA have been identified as products of the anaerobic metabolism of toluene and xylenes, respectively (*2*−*5*). The first intermediate of anaerobic toluene mineralization is BSA, and methyl-BSA can be formed as either an intermediate of anaerobic xylene mineralization or a dead-end product of anaerobic xylene cometabolism, depending on the bacterial culture involved (*2*, *3*, *5*−*7*). Few studies have confirmed the presence of these compounds within anaerobic BTEX-contaminated aquifers (*5*, *8*−*11*).

Techniques for detecting and quantifying anaerobic BTEX degradation are needed to assess the feasibility of using in situ bioremediation to treat BTEX-contaminated groundwater aquifers. However, the commonly used approach of monitoring temporal and spatial changes in concentrations of BTEX, electron acceptors, and reduction products (e.g., methane) is problematic for several reasons: (a) small changes in BTEX concentrations are difficult to quantify in the presence of high background concentrations, (b) concentrations of reduction products are typically low, and (c) BTEX concentration changes due to metabolism are often obscured by nonbiological processes such as advection, dispersion, sorption/desorption, and dissolution of non-aqueous-phase liquids.

Previously Beller et al. (*8*) and Reinhard et al. (*9*) used single-well "push−pull" tests to evaluate anaerobic BTEX metabolism in a BTEX-contaminated aquifer characterized by low background BTEX concentrations (12.9 μM). In a push−pull test, a prepared test solution containing the compounds of interest and a conservative tracer is injected ("pushed") into the saturated zone of an aquifer and then extracted ("pulled") from the same location (*12*, *13*). In the study by Beller et al. (*8*), the test solution was prepared by first extracting groundwater and passing it through granulated activated carbon filters and ion-exchange resin and then purging the groundwater with helium to remove background BTEX and residual O_2. A mineral salt solution was then added back along with known concentrations of BTEX, sulfate (to serve as an electron acceptor), and bromide as a conservative tracer. In addition, a BTEX-free "buffer" solution was injected prior to test solution injection in an attempt to isolate the injected test solution from the native groundwater because mixing with native groundwater would have confounded the interpretation of BTEX concentration data during the test (*8*).

Recently, less logistically complex push−pull tests were used to monitor the in situ reductive dechlorination of trichlorofluoroethene (TCFE) under anaerobic conditions in a trichloroethene (TCE)-contaminated aquifer at a former chemical manufacturing plant in the San Francisco Bay area. The Hageman et al. study (*14*) was unique in that it utilized TCFE as a fluorinated "surrogate" for TCE in the push−pull tests. The fluorine atom of TCFE was retained during reductive dechlorination (*15*), which allowed for the unambiguous detection and quantification of TCFE transformation products in the presence of high background TCE concentrations. The successful application of the push−pull test with a fluorinated surrogate of TCE to detect reductive dechlorination (*14*) indicated that it was possible to use an analogous approach to detect and measure anaerobic BTEX transformations.

In this study, we evaluated the approach of injecting deuterated toluene and *o*-xylene into BTEX-contaminated groundwater aquifers and following the subsequent formation of their deuterated transformation products. The distinct advantage of this approach is that the injected deuterated toluene and xylene as well as their deuterated transformation products can be unambiguously identified and quantified in the presence of background BTEX, BSA, and methyl-BSA. Deuterium-labeled compounds previously were used in

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laboratory studies to investigate the mechanistic aspects of the first steps of anaerobic toluene and xylene degradation (*7*, *16*, *17*). The use of deuterated surrogates makes field tests logistically simpler and more cost-effective since there is no need to purge background BTEX when test solutions are prepared. This approach of using deuterated surrogates is also more widely applicable because the uniqueness of the deuterated surrogates and their transformation products means that the tests can be executed in wells with high background BTEX contamination. Among isotopically labeled compounds, deuterated surrogates are preferred because they are less expensive than ^{13}C-labeled chemicals and are not radioactive.

The objective of this study was to monitor the formation of deuterated benzylsuccinic acid (BSA-d_8) and *o*-methylbenzylsuccinic acid (*o*-methyl-BSA-d_{10}) resulting from the injection of deuterated toluene-d_8 and *o*-xylene-d_{10}, respectively, using single-well push−pull tests. Test solutions containing toluene-d_8 or *o*-xylene-d_{10} along with a conservative tracer (bromide) and electron acceptor (nitrate) were injected into two BTEX-contaminated aquifers with background BTEX concentrations ranging from 0.7 to 193 μM. Nitrate was selected as the electron acceptor in order to obtain higher potential rates of toluene and *o*-xylene transformation as compared to sulfate. At the Northwest Terminal site, no information on background nitrate concentrations upgradient of the contaminated zone was available. At the Kansas City site, nitrate concentrations upgradient of the BTEX-contaminated site ranged from 4.8 to 21 μM. The experiments were not designed to provide information on intrinsic transformation rates but rather as a demonstration of the use of deuterated compounds to monitor in situ anaerobic BTEX transformations. The detection of the deuterated transformation products provided direct evidence for anaerobic transformation of the injected deuterated parent compounds. Moreover, it was possible to quantify the rate of formation of the deuterated transformation products BSA-d_8 and *o*-methyl-BSA-d_{10}.

Experimental Section

Reagents and Standards. Toluene-d_8 (99.95 atom % D) and *o*-xylene-d_{10} (>99 atom % D) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Potassium bromide (Fisher, Fair Lawn, NJ) was used as a conservative tracer (*18*). A BTEX standard and an internal standard (4-bromofluorobenzene, 4-BFB) for volatiles analysis were purchased from Chem Service (West Chester, PA). Methylene chloride (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA).

Benzylsuccinic acid (99% purity) was purchased from Sigma Chemical (St. Louis, MO). Qualitative standards of deuterated benzylsuccinic acid (BSA-d_8) and deuterated (2-methylbenzyl)succinic acid (*o*-methyl-BSA-d_{10}) were produced enzymatically with partially purified benzylsuccinate synthase, as described by Beller and Spormann (*19*). Standards of 2-chlorolepidine (2Cl, 2-chloro-4-methylquinoline; 99% purity) and 4-(trifluoromethyl)hydrocinnamic acid (4TFM, 95% purity) were purchased from Aldrich Chemical (Milwaukee, WI) and were used as the internal and surrogate standards, respectively. Standards of BSA, 2Cl, and 4TFM were prepared in acetonitrile at 1 mg/mL. Acetone, methanol, and acetonitrile (HPLC grade) were purchased from Fisher Scientific, and ethyl acetate (HPLC grade) was obtained from Mallinckrodt (Paris, Kentucky).

Field Sites. Experiments were performed at two field sites. The first site is a bulk fuel terminal (referred to as the Northwest Terminal) located near Portland, OR (*20*). The unconfined aquifer consists of ~5 m of medium dense- to fine-grained sand and silty sand overlaying clayey silt with sand interbedded with silty clays and clays. The water table is ~2−3 m below land surface, and groundwater flow is toward the east with an estimated velocity of 100 m/yr (*20*). Total BTEX concentrations in wells used at the site were 71.5 and 193 μM (Table 1). The wells tested for this study were also impacted by a neat ethanol release that occurred in 1999 (*20*). Anoxic conditions exist at this site, and dissolved oxygen, nitrate, and sulfate concentrations are near or below detection limits while the dissolved methane concentration was 600 μM (Table 1). Tests were conducted in 5-cm-inner diameter PVC monitoring wells with 3-m screened intervals starting at 1.6 m below land surface. Aerobic respiration and denitrification rates (Table 1) at the Northwest Terminal were measured as part of a separate study (*12*) using push−pull tests conducted 2 months prior to the experiments described in this study. Denitrification rates of 4 and 16 μM/h in the absence of exogenous organic substrates (e.g., BTEX) were measured in wells CR12 and CR15. Background BSA concentrations were below detection (<0.001 μM), but methyl-BSA concentrations of 1.0 and 0.05 μM were detected by liquid chromatography/tandem mass spectrometry (*21*) in wells CR12 and CR15, respectively, which provided evidence for intrinsic anaerobic xylene transformation at this site (Table 1). In a previous study, concentrations of methyl-BSA detected in three wells at this site, including CR12 and CR15, ranged from 0.01 to 0.9 μM (*21*).

TABLE 1. Characterization of Groundwater in Wells Used in Field Push−Pull Tests at Two BTEX-Contaminated Sites[a]

	Northwest Terminal		Kansas City site	
	CR15	CR12	105s	106s
benzene (μM)	170	1.9	54	0.26
toluene (μM)	1	30	3.5	nd
ethylbenzene (μM)	4.7	5.7	3.6	0.30
xylene (μM)	17	34	3.6	0.12
total BTEX (μM)	193	71.5	64.6	0.68
ethanol (μM)	<(0.04)	1.0	n/a	n/a
dissolved oxygen (μM)	nd	16	20	57
nitrate (μM)	nd	nd	nd	nd
sulfate (μM)	<(10. 4)	<(10.4)	0.08	0.07
methane (μM)	690	630	1100	894
aerobic respiration (μM/h)	43	84	n/a	n/a
denitrification (μM/h)	16	4	n/a	n/a
BSA (μM)	nd	nd	n/a	n/a
methyl-BSA (μM)	0.05	1.0	n/a	n/a

[a] nd, not detected; n/a, not analyzed. "Less than" sign indicates detection but below the quantitation limit given in parentheses.

The second field site (the Kansas City site) is a former petroleum refinery near Kansas City, KS. The unconfined aquifer consists of ~3 m of fine sand with clayey silt or silt overlying sand (*22*). The water table is 7-9 m below land surface, and groundwater flow is toward the east with an estimated velocity of 0.05 m/day. Dissolved oxygen, sulfate, and methane were detected at this site (Table 1). Total BTEX concentrations in the wells tested at the site were 0.68 and 64.4 μM (Table 1) (*23*). Samples were not analyzed for BSA and methyl-BSA prior to this study. Tests were conducted in 10-cm-diameter PVC wells with 3-m screened intervals starting at 5−7 m below land surface.

Push−Pull Tests. Tests were performed in two wells at each site (Table 2). Test solutions consisted of tap water containing bromide (from KBr), nitrate (from $NaNO_3$), toluene-d_8 (injected at both field sites), and *o*-xylene-d_{10} (Northwest Terminal site only). For the concentrations of each analyte used in the individual tests, refer to Table 2. For these tests, *o*-xylene was selected for injection because previous reports indicated that *o*-methyl-BSA was the dominant xylene transformation product detected at other field sites (*5*). Sulfate was also present in the tap water used

TABLE 2. Test Solution Composition for Push−Pull Tests Conducted in Four BTEX-Contaminated Wells[a]

chemical	well CR15	well CR12	well 105s	well 106s
Br^- (mM)	1.5	1.3	1.4	1.3
toluene-d_8 (μM)	8.7	21.6	14.1	14.1
o-xylene-d_{10} (μM)	3.8	9.2	na	na
nitrate (mM)	3.7	3.6	4.6	4.6
sulfate (mM)	0.08	0.06	2.2	2.2

[a] n/a, not applicable.

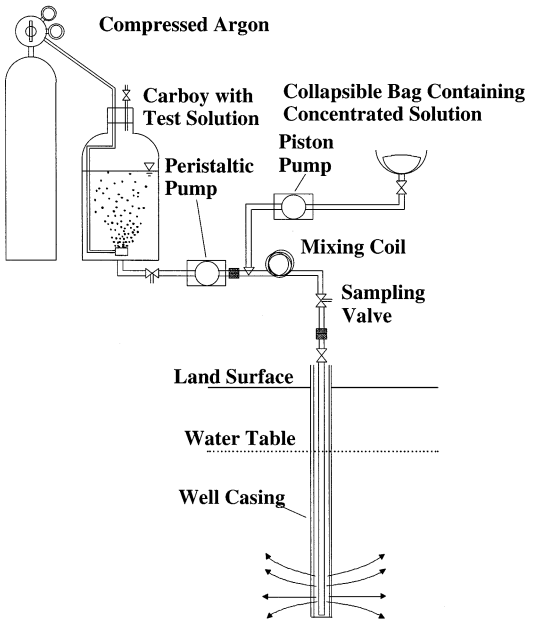

FIGURE 1. Schematic of equipment used in single-well push−pull field tests.

to prepare test solutions for the Kansas City site (2 mM) and the Northwest Terminal (0.1 mM). Test solutions were prepared and mixed in plastic tanks and then stripped of dissolved oxygen by vigorous bubbling with compressed Ar gas prior to injection. Toluene-d_8 and *o*-xylene-d_{10} were then introduced into the test solution during injection by a second pump that delivered a concentrated aqueous solution that had been prepared in a collapsible metallized bag (Figure 1). A 250-L aliquot of the test solution was injected at a rate of 0.5−2 L/min. Ten samples were collected during the injection phase and analyzed for bromide, nitrate, sulfate, toluene-d_8, and *o*-xylene-d_{10}.

During the extraction phase, samples were taken daily to biweekly for up to 30 days. With this approach, <10% of the injected test solution was recovered. Prior to sampling, wells were purged 1−2 well casing volumes using a peristaltic pump. Samples for volatiles were collected in 40-mL acid-preserved VOA vials without headspace. Samples for bromide analysis were collected in 40-mL VOA vials without acid preservation. Samples for BSA-d_8 and methyl-BSA-d_{10} were collected in 1-L baked glass bottles with Teflon-lined lids and preserved with HCl. All samples were shipped on ice and stored <1 month at 4 °C until analysis.

Analytical Methods. A Tekmar-Dohrmann 3100 sample concentrator (Cincinnati, OH) equipped with an AQUATek 70 autosampler and a Tenax/Silica Gel/Charcoal trap (Tekmar-Dohrmann) was used to analyze volatiles. Prior to analysis, 2 μL of 4-BFB internal standard (500 μM) was added to 25-mL samples. The following conditions were used for purge-and-trap analysis: a line and valve temperature of 150 °C, a purge time of 11 min, a 245 °C desorption temperature, and a 1-min desorption time. Separation and detection were performed on a Hewlett-Packard model 6890 gas chromatograph equipped with a model 5972 mass-selective detector (MSD). The GC was equipped with a 30 m × 0.32 mm × 4 μm SPB-1 capillary column (Supelco Inc., Bellefonte, PA). The injector temperature was 250 °C and operated under splitless conditions with a 1-μL injection volume. The initial oven temperature of 50 °C was held for 2 min and then increased at 15 °C/min to 180 °C. The MSD was operated in single ion monitoring, electron impact mode with a source temperature of 265 °C. Calibration curves were constructed from BTEX, toluene-d_8, and *o*-xylene-d_{10} standards ranging in concentration from 0.002 to 2.8 μM.

TABLE 3. Ions Used To Detect and Quantify the Deuterated and Nondeuterated Dimethyl Esters of Benzylsuccinic Acid and Methylbenzylsuccinic Acid

analyte	quantitation ion (*m/z*)	qualifier ion(s) (*m/z*)
BSA	176	236/91
BSA-d_8	183	244/98
methyl-BSA	190	250/105
o-methyl-BSA-d_{10}	199	260/114

For determination of BSA, BSA-d_8, and *o*-methyl-BSA-d_{10}, samples were analyzed by solid-phase extraction followed by derivatization to their methyl esters (*24*). Briefly, acidified samples were filtered and extracted onto a 0.5-g column of styrenedivinylbenzene; the acid analytes were eluted and methylated with diazomethane. Samples were analyzed by GC/MS operated in single ion monitoring mode (Table 3). Due to the number of ions acquired, sample extracts were each analyzed twice to obtain maximum instrumental sensitivity. Even though the molecular ions for BSA and methyl-BSA were only 20% of the base peak intensity, the molecular ions were selected for purposes of maximum selectivity. Due to the lack of sufficient quantities of authentic standards, concentrations of BSA-d_8, *o*-methyl-BSA-d_{10}, and *o*-methyl-BSA were estimated from BSA calibration curves by assuming a response factor of 1.

Samples containing BSA-d_8 had m/z 244/183 ion ratios ranging from 0.07 to 0.13, which was similar to the value of 0.12 obtained for the authentic BSA-d_8 standard. In addition, the ion ratio m/z 183/98 obtained for BSA-d_8 in groundwater samples ranged from 0.55 to 0.94, whereas the authentic BSA-d_8 standard gave a value of 1.06. For *o*-methyl-BSA-d_{10} detected in groundwater samples, the ion ratio m/z 260/199 ranged from 0.19 to 0.26 while that of the deuterated *o*-methyl-BSA-d_{10} standard was 0.26. For the ion ratio m/z 199/114, *o*-methyl-BSA-d_{10} detected in groundwater samples had values ranging from 0.20 to 0.23 while the deuterated *o*-methyl-BS-d_{10} standard gave a value of 0.30.

The method detection limit determined for BSA was 0.0009 μM. This detection limit also was assumed for BSA-d_8 and for *o*-methyl-BSA-d_{10} because insufficient quantities were available for spike and recovery studies. Precision of the analytical method for BSA was determined by spiking seven replicate samples of a groundwater composite sample to give a final concentration of 0.092 μM 4TFM (surrogate standard) and 0.01 μM BSA (*24*). The absolute recoveries of 4TFM and BSA from the composite sample were 84 ± 2% with a 2% relative standard deviation (RSD) and 86 ± 2 (2% RSD), respectively (*24*). Over the course of the study, the absolute recoveries of 4TFM and BSA, both spiked to give a final concentration of 0.09 μM, from 16 blank, individual groundwater samples were 86 ± 3 (4% RSD) and 88 ± 4% (5% RSD), respectively. The average recovery of BSA relative to 4TFM was 100 ± 4% (4% RSD) (*24*).

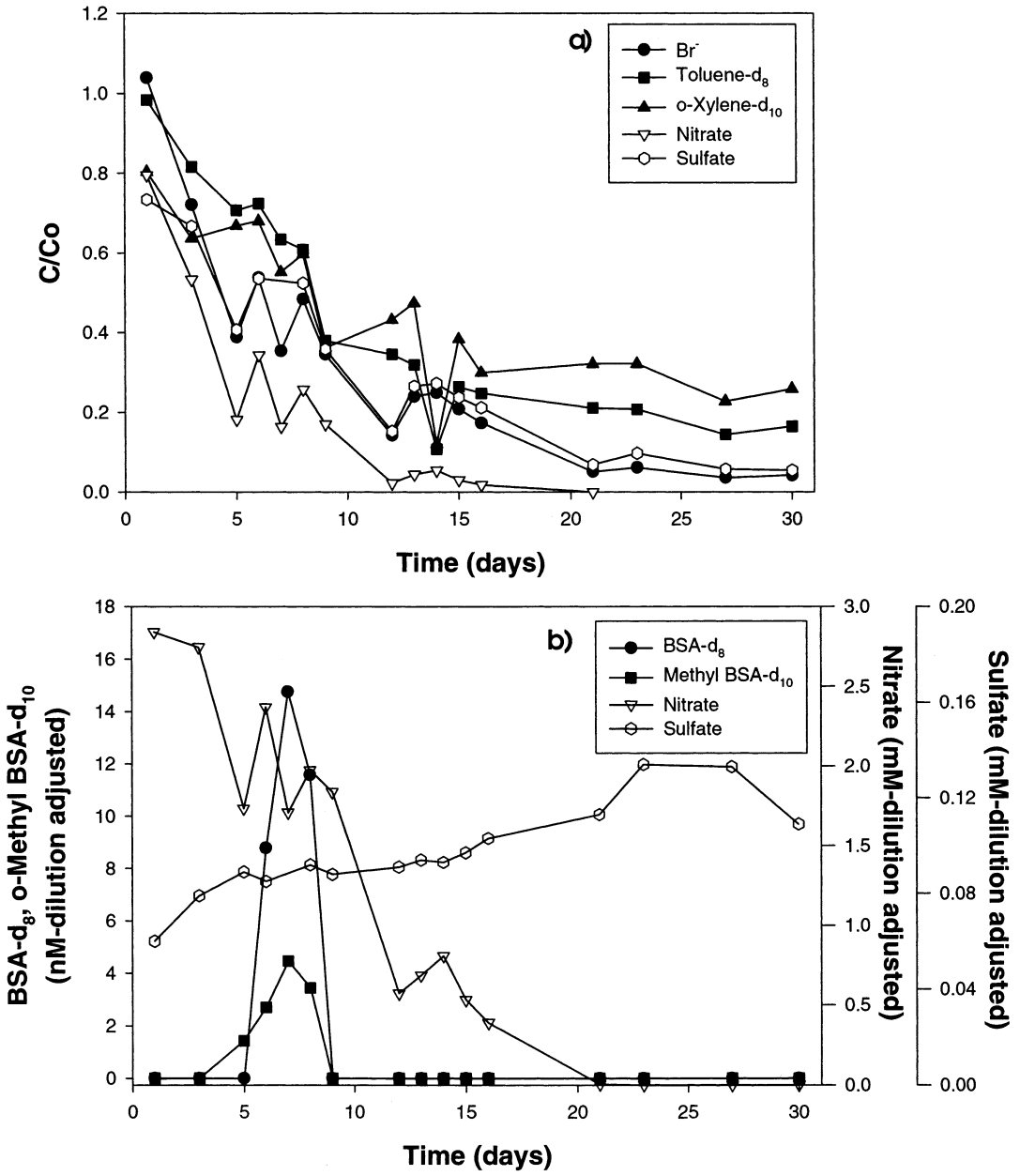

FIGURE 2. Extraction-phase breakthrough curve (a) for test in well CR15 at the Northwest Terminal site indicating dilution of injected toluene-d_8 and *o*-xylene-d_{10} and (b) utilization of injected nitrate and concurrent formation of BSA-d_8 and *o*-methyl-BSA-d_{10}.

Bromide, nitrate, and sulfate concentrations were determined by ion chromatography using a Dionex DX-120 (Sunnyvale, CA) with an electrical conductivity detector and a Dionex AS14 column. External standard calibration was used and the quantitation limit was ~10 μM.

Results and Discussion

For all tests, breakthrough curves were constructed as relative concentrations (C/C_o) versus time, where C is the measured concentration in a sample and C_o is the concentration of the same solute in the injected test solution. In all tests, the C/C_o for bromide, toluene-d_8 (both sites), and *o*-xylene-d_{10} (Northwest Terminal only) decreased during the extraction phase as the injected test solution was transported away from the vicinity of the well by regional groundwater flow (Figures 2a–5a). For example, for the test in well CR15 at the Northwest Terminal, relative concentrations for bromide, toluene-d_8, and *o*-xylene-d_{10} decreased from 1 to 0.02 during the 30 days following injection (Figure 2a).

To account for the effect of dilution on toluene-d_8 and *o*-xylene-d_{10} concentrations due to advection and dispersion, the C/C_o values for toluene-d_8 and *o*-xylene-d_{10} were divided by the corresponding Br^- C/C_o values to give dilution-adjusted C/C_o values. For example, on day 30 for the test in CR15 at the Northwest Terminal (Figure 2a), the C/C_o for Br^- was 0.042, which represents 95.8% dilution of the test solution, and the C/C_o for toluene-d_8 was 0.164. Dividing the C/C_o of toluene-d_8 by that of Br^- gives a dilution-adjusted C/C_o value of 3.9 for toluene-d_8. Retardation of both toluene-d_8 and *o*-xylene-d_{10} relative to bromide are indicated by dilution-adjusted C/C_o values greater than 1. Dilution-adjusted values of C/C_o for toluene-d_8 or *o*-xylene-d_{10} greater than 1 for all wells precluded quantifying anaerobic transformation rates from dilution-adjusted toluene-d_8 or *o*-xylene-d_{10} concentration data.

Sorption to aquifer sediments is likely responsible for the observed retardation of toluene-d_8 and *o*-xylene-d_{10} because factors such as volatilization were considered negligible and

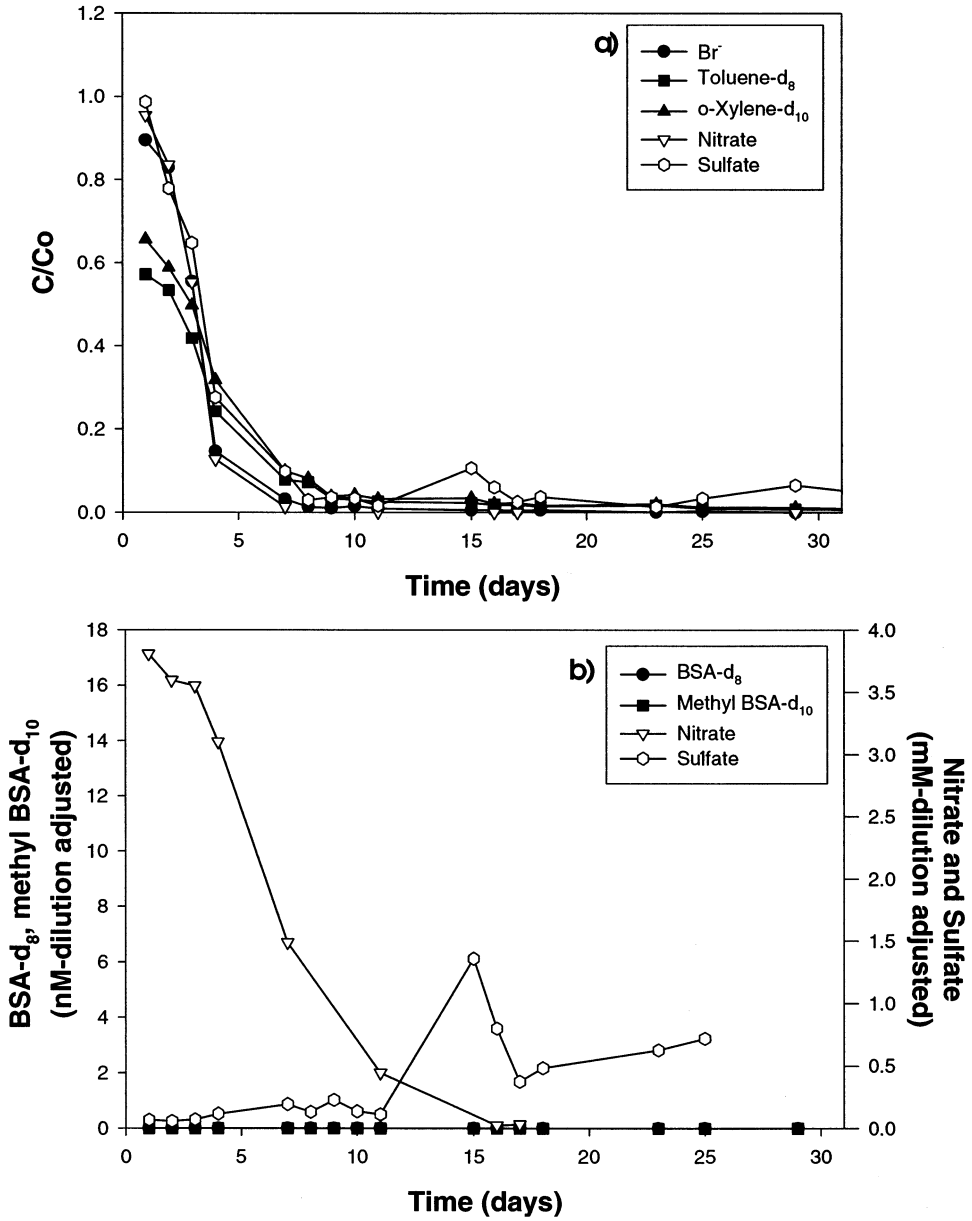

FIGURE 3. Extraction-phase breakthrough curve (a) for test in well CR12 at the Northwest Terminal site indicating dilution of injected toluene-d_8 and *o*-xylene-d_{10} and (b) utilization of injected nitrate. Neither BSA-d_8 nor *o*-methyl-BSA-d_{10} was detected.

dilution due to advection and dispersion was accounted for by normalization to bromide. An independent, short-duration (1 day) transport test was conducted in CR15 at the Northwest Terminal site, and estimated retardation factors for toluene-d_8 and *o*-xylene-d_{10} ranged from 6 to 14 (*25*). No short-duration transport tests were conducted at the Kansas City site; however, dilution-adjusted C/C_o values for toluene-d_8 of >1 for tests conducted in wells 106s and 105 (data not shown) also indicated the retardation of toluene-d_8 relative to bromide.

Unambiguous evidence for in situ anaerobic transformation of injected toluene-d_8 and *o*-xylene-d_{10} was obtained from the observed production of their deuterated transformation products, BSA-d_8 and *o*-methyl-BSA-d_{10}, respectively, in well CR15 (Figure 2b). On the other hand, neither BSA-d_8 nor *o*-methyl-BSA-d_{10} was observed during the test conducted in CR12 (Figure 3b). For the test conducted in well CR15 at the Northwest Terminal, BSA-d_8 and *o*-methyl-BSA-d_{10} were detected beginning on day 5 with measured concentrations of 4.7 and 1.5 nM, respectively. The concentrations increased until day 8 with measured BSA-d_8 and *o*-methyl-BSA-d_{10} concentrations of 5.6 and 1.7 nM, respectively, which then decreased to below detection on day 9. Dilution-adjusted concentrations of BSA-d_8 and *o*-methyl-BSA-d_{10} were calculated by dividing their measured concentrations by the corresponding C/C_o for Br^- (Figure 2b). For example, on day 7 in the test conducted in CR 15 (Figure 2b), the measured BSA-d_8 concentration of 5.2 nM was divided by the C/C_o for Br of 0.354 (which indicates 64.6% dilution of the test solution) to obtain a dilution-adjusted BSA-d_8 concentration of 14.8 nM. Initial zero-order formation rates (Table 4) for BSA-d_8 (7.4 nM/day) and *o*-methyl-BSA-d_{10} (1.0 nM/day) were estimated by linear regression of the increasing dilution-adjusted concentrations determined on days 5−7. The dilution-adjusted BSA-d_8 concentrations are lower than BSA concentrations reported by others for groundwater samples that range from 30 to 3000 nM (*5*, *8*, *11*). Molar ratios at the maximum BSA-d_8 and *o*-methyl-BSA-d_{10} concentrations of 14.8 and 4.5 nM, respectively, were calculated as 0.7 (BSA-d_8/toluene-d_8) and 0.2 mol % (*o*-methyl-BSA-d_{10}/*o*-xylene-

TABLE 4. Estimated Zero-Order Rates for Nitrate Removal and BSA-d_8 and *o*-Methyl-BSA-d_{10} Production

	Northwest Terminal		Kansas City site	
	CR15	CR12	105s	106s
BSA-d_8 production (nM/day)	7.4	nd	nd	3.4
o-methyl-BSA-d_{10} production (nM/day)	1.0	nd	na	na
nitrate removal (μM/h)	13	39	25	25
nitrate removal without added toluene or *o*-xylene (μM/h; data not shown)	16	4	na	na

[a] nd, not detected; na , not applicable.

d_{10}). In a previous study conducted at the Northwest Terminal site, Beller found a background methyl-BSA/xylene ratio of 0.3 mol % (21). Beller et al. (*8*) reported less than 5 mol % conversions of injected toluene and xylenes to benzylsuccinic or (*E*)-phenylitaconic acid transformation products.

Formation of BSA-d_8 was also observed in the test conducted in well 106s at the Kansas City site (Figure 4b). Note that *o*-xylene-d_{10} was not injected in tests conducted at this site. As in the test in well CR15 at the Northwest Terminal, only a small fraction of the injected toluene-d_8 was detected as BSA-d_8 during the time of the test. The maximum dilution-adjusted BSA-d_8 concentration observed on day 3 (7.8 nM) corresponded to 0.01 mol % of the injected toluene-d_8 concentration. The initial zero-order rate of BSA-d_8 formation (3.4 nM/day; Table 4) was estimated from dilution-adjusted BSA-d_8 concentrations determined for days 1−3 (Figure 4b).

First-order rates for BSA-d_8 and *o*-methyl-BSA-d_{10} production were computed by dividing the zero-order rates reported in Table 4 by the average toluene-d_8 or *o*-toluene-d_{10} concentration that was observed during the time of BSA-d_8 or *o*-methyl-BSA-d_{10} formation, respectively. The computed first-order rates of 0.003- and 0.0001/day for BSA-d_8 production in the tests conducted in wells CR15 and 106s, respectively, were smaller than toluene degradation rates reported in laboratory studies conducted by others, which ranged from 0.003- to 0.04/day (*26*−*29*). The first-order rate for *o*-methyl-BSA-d_{10} formation in well CR15 was 0.004/day.

The BSA-d_8 and *o*-methyl-BSA-d_{10} observed during this study occurred within 5 days after injection in tests conducted in wells CR15 and 106s. In contrast, Beller et al. (*8*) found detectable concentrations of BSA after ~10 days in the study conducted at a BTEX-contaminated site in Seal Beach, CA, where sulfate was added as the electron acceptor. The more rapid formation of BSA-d_8 in the CR15 and well 106s tests in this study may have resulted from the utilization of nitrate as the electron acceptor. Reinhard et al. (*9*) found more rapid in situ transformation of toluene, ethylbenzene, and xylenes under nitrate-reducing conditions compared to sulfate-reducing conditions at the Seal Beach site (*8*). The dilution-adjusted concentrations of the deuterated transformation products decreased over time, which indicates that they were depleted during the tests. For this reason, it is likely that the rates of BSA-d_8 and *o*-methyl-BSA-d_{10} production reported in Table 4 underestimate actual in situ production rates. Furthermore, because extracellular BSA yields typically represent <3 mol % of the toluene consumed (*5*), the in situ toluene degradation rates could be 30 times greater than the measured BSA production rates. Finally, it is also important to recognize that deuterated substrates may be more slowly utilized than nondeuterated substrates due to the kinetic isotope effect. For example, Krieger et al. (*7*) observed 3-fold slower metabolism of deuterated *m*-xylene-d_{10} relative to nondeuterated *m*-xylene.

The formation of BSA-d_8 and *o*-methyl-BSA-d_{10} in the test conducted in well CR15 at the Northwest Terminal and the formation of BSA-d_8 in the test conducted in well 106s at the Kansas City site occurred concomitantly with nitrate removal (Figures 2b and 4b). Nitrate removal is attributed to microbial nitrate reduction with injected hydrocarbons and background organic compounds serving as electron donors. It should also be noted that nitrate removal observed during these tests does not necessarily indicate that nitrate reduction was the dominant terminal electron-accepting process at these sites, especially since background (pretest) geochemistry is consistent with more reducing (methanogenic) conditions (Table 1). Regardless, it was possible to obtain rates of denitrification from the data obtained from these tests.

To account for the effect of dilution on nitrate concentrations, dilution-adjusted nitrate concentrations were computed by dividing the observed nitrate concentration by the corresponding C/C_o value for Br^- (Figures 2−4b). For example, a measured nitrate concentration on day 3 in the test conducted in well CR15 of 0.67 mM was divided by the C/C_o for Br^- of 0.388, which corresponds to 61.2% dilution of the test solution, to obtain a dilution-adjusted nitrate concentration of 1.7 mM (Figure 2b). Computed zero-order rates of nitrate removal were calculated from the linear relations between dilution-adjusted nitrate concentrations and time; correlation coefficient values ranged from 0.8988 to 0.9808, which were statistically significant at an α level of 0.01. Rates of nitrate utilization ranged from 13 to 39 μM/h for the four tests conducted in this study (Table 4).

The quantity of nitrate that would be required for mineralization of the toluene-d_8 and *o*-xylene-d_{10} metabolized (based on BSA-d_8 and *o*-methyl-BSA-d_{10} formation) is <1% of the injected nitrate that was reduced. Although injected nitrate was rapidly utilized in all tests at both sites (Figures 2b−4b), BSA-d_8 and *o*-methyl-BSA-d_{10} were not detected in the tests conducted in well CR12 at the Northwest Terminal (Figure 3b) or in well 105s at the Kansas City site (data not shown). Independent tests to determine the rates of denitrification in the absence of exogenous toluene or xylene were conducted in wells CR12 and CR15 2 months prior to the experiments described in this article. The rates of denitrification in the absence of added toluene or xylene were 4 and 16 μM/h for wells CR12 and CR15, respectively (Tables 1 and 4). In the case of well CR12, the rate of denitrification in the absence of exogenous toluene or xylene was lower than that obtained for the experiment described in this study with toluene-d_8 and *o*-xylene-d_{10}, while in the case of well CR15, the rate was similar (Table 4). The observed rates of denitrification for all the field tests were comparable to rates obtained for a different BTEX-contaminated site (*12*).

Although sulfate was present in the test solutions for all the tests, dilution-adjusted sulfate concentrations actually increased during the tests conducted in wells CR15 (Figure 2b), CR12 (Figure 3b), and 106s (Figure 4b), which indicates that the sulfate concentrations in the background groundwater were greater than that in the test solution. Although no evidence for sulfate reduction was obtained for these wells, this finding does not preclude the possibility that sulfate reduction occurred during the tests. In well 105s, the dilution-adjusted sulfate concentrations decreased during the test; however, during this test no BSA-d_8 was formed.

Although tap water was used to formulate the test solutions for this study, background (nondeuterated) BTEX compounds were detected in all extraction-phase samples. Note that dilution of the test solution ranged from 0 to 100% during the tests as evidenced by the decrease in Br^- C/C_o values from 1 to 0. As a consequence, the organic contaminants in the background groundwater mixed with the test solution during its residence time in the aquifer. Therefore, contaminants in the background groundwater in the vicinity of

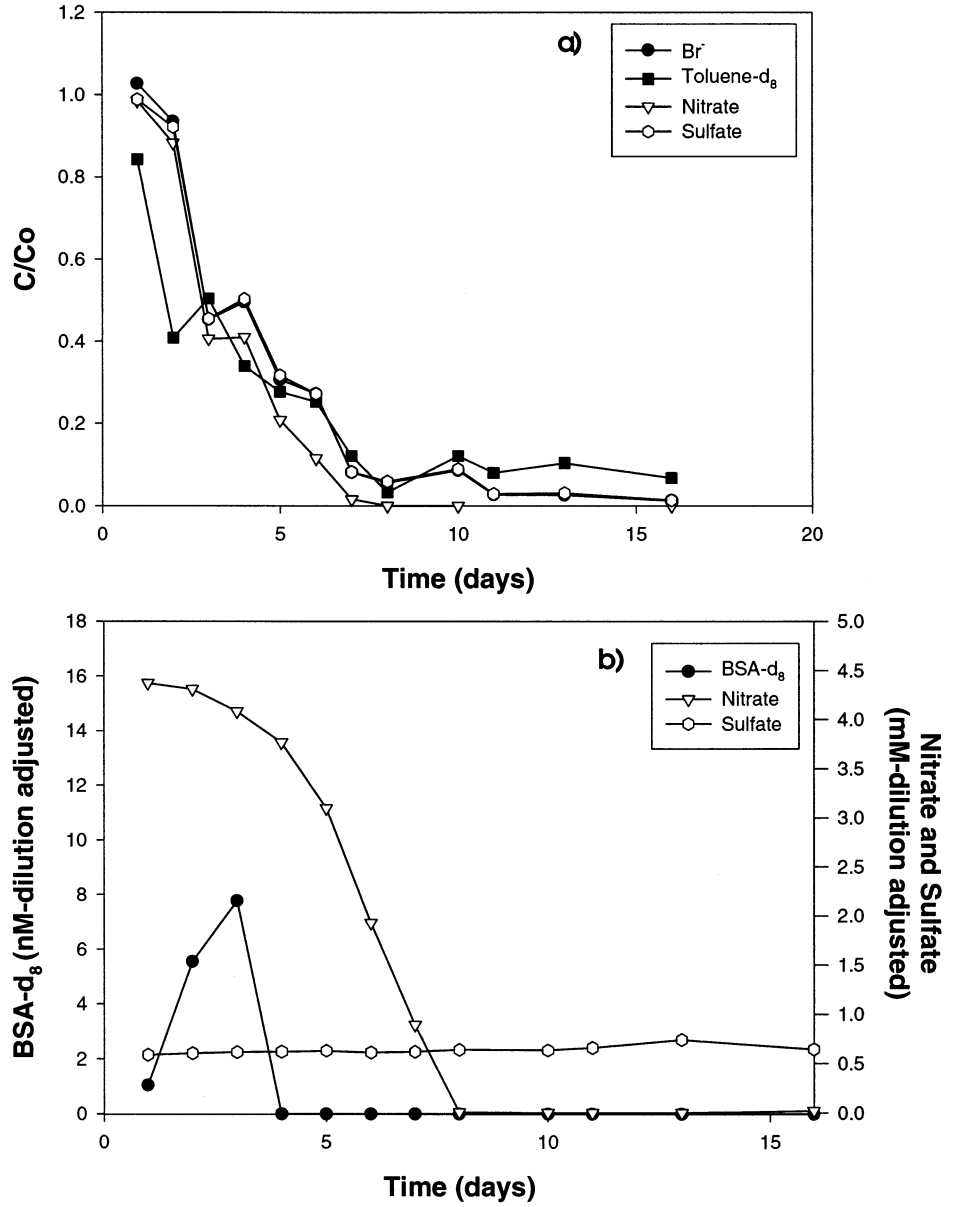

FIGURE 4. Extraction-phase breakthrough curve (a) for test in well 106s at the Kansas City site indicating dilution of injected toluene-d_8 and (b) utilization of injected nitrate and concurrent formation of BSA-d_8. No *o*-methyl-BSA was expected because *o*-xylene-d_{10} was not injected into this well.

these wells may have contributed to the observed variability in the occurrence and rate of BSA-d_8 and *o*-methyl-BSA-d_{10} formation and rates of nitrate removal.

The field tests conducted in this study represent the first reported use of deuterated toluene and xylene surrogates to detect and quantify anaerobic BTEX transformation product formation in BTEX-contaminated groundwater. Formation of deuterated transformation products including BSA-d_8 and *o*-methyl-BSA-d_{10} provided unequivocal evidence for anaerobic BTEX metabolism. Moreover, it was possible to measure a conservative rate of deuterated transformation product formation in the presence of background BTEX contamination. Using this approach, it is possible to design tests to detect anaerobic BTEX transformations in situ for a variety of defined conditions (e.g., with or without exogenous electron acceptors), which should prove useful for site characterization and remedial design. Future work will focus on expanding the number of injected deuterated surrogates and quantifying a wider range of transformation products including deuterated toluic, phthalic, and benzoic acids.

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