AN ABSTRACT OF THE THESIS OF

Dominik E. Reusser for the degree of Master of Science in Chemistry presented on August 30, 2001.

Title: In Situ Transformation of Toluene and Xylene to Benzylsuccinic Acid Analogs in Contaminated Groundwater.

Abstract approved: _Redacted for Privacy_

Jennifer A Field

The rate of removal of benzene, toluene, ethylbenzene and the xylene isomers (BTEX) from contaminated groundwater is needed to design remediation processes. Benzylsuccinic acid (BSA) and methyl-benzylsuccinic acid (methyl BSA) are unambiguous metabolites of anaerobic BTEX biodegradation. An analytical method for quantitative determination of BSA in groundwater samples was developed. Samples containing BSA and methyl BSA were extracted onto 0.5 g of styrene-divinylbenzene, eluted with ethyl acetate, and methylated with diazomethane. Gas chromatography coupled to mass spectrometry with electron impact ionization was used for separation and detection. The recovery from spiked 1 L groundwater samples was 88 to 100 %. The precision of the method, indicated by the relative standard error was $\pm 4\%$ with a method detection limit of 0.2 μg/L. The method was then used to analyze samples from single-well push-pull tests conducted by injecting deuterated toluene and xylene into BTEX-contaminated wells in order to demonstrate in-situ biodegradation. Unambiguous evidence for

deuterated toluene and xylene biodegradation was obtained with the observation of deuterated BSA and methyl BSA coupled with the utilization of nitrate presumably due to denitrification as terminal-electron-accepting process. Minimum first-order degradation rates for deuterated toluene estimated from formation of BSA were 0.0004 to 0.001 day^{-1}. Rates of methyl BSA formation were not calculated because methyl BSA, although detected, was not above the quantitation limit. Removal rates of deuterated toluene and o-xylene were not directly measurable because the rates were too low to measure significant changes in parent compound concentrations. Wells for which the formation of deuterated BSA and methyl BSA were observed had lower relative concentrations of toluene and xylenes relative to total BTEX than wells for which no deuterated BSA and methyl BSA were observed. Retardation factors for injected deuterated toluene and background toluene of 2 and 14, respectively, were obtained from push-pull tests conducted to determine toluene transport properties. Differences in retardation factors for injected and background toluene indicate differences between injected and background solute transport and is a topic that requires further study.

In Situ Transformation of Toluene and Xylene to Benzylsuccinic Acid Analogs in

Contaminated Groundwater

by

Dominik E. Reusser

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Major Professor, representing Chemistry

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Dominik E. Reusser, Author

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CONTRIBUTION OF AUTHORS

Dr. Jennifer Field was involved in the design, analysis, and writing of each manuscript. She also provided the laboratory. Dr. Jack Istok was involved in the design, analysis and interpretation of the push-pull tests. Dr. Harry Beller contributed to the design of the push-pull tests.

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This thesis is dedicated to Gertrud, Rudolf, Simon and Benjamin.

IN SITU TRANSFORMATION OF TOLUENE AND XYLENE TO BENZYLSUCCINIC ACID ANALOGS IN CONTAMINATED GROUNDWATER

1 GENERAL INTRODUCTION

1.1 HYDROCARBONS IN THE SUBSURFACE

Hydrocarbons from landfills and leaking underground storage tanks are common groundwater contaminants. Benzene, toluene, ethylbenzene and the xylene isomers, collectively known as BTEX, are the components of major concern (1). Their aqueous solubilities range from 152 to 1780 mg/L (2). Aromatic compounds form the major fraction (> 95%) of the water-soluble compounds in gasoline (3). While the carcinogenic potential of benzene is known (4), carcinogenicity of toluene and the xylene isomers is uncertain (5, 6). The EPA drinking water criteria are 0.005 mg/L for benzene, 1 mg/L for toluene, 0.7 mg/L for ethylbenzene, and 10 mg/L for xylene isomers (7, 8). The acute toxicity of aromatic hydrocarbons is due to interactions with cell membranes as shown for microorganisms (9, 10).

1.2 ANAEROBIC BIODEGRADATION OF AROMATIC HYDROCARBONS

Most field sites with gasoline spills are anaerobic because oxygen is rapidly utilized by microorganisms. For this reason, BTEX biodegradation in absence of oxygen is the process of major interest for BTEX bioremediation. The first

demonstrations of toluene degradation in anaerobic mixed cultures were performed by Grbić-Galić and Vogel and Kuhn et al. (11, 12). Pure and mixed cultures capable of BTEX degradation have been reported under varying conditions (**Table 1**). Enrichment cultures with the capability of degrading benzene, toluene, and p-xylene under methanogenic and manganese-reducing conditions have been reported (**Table 1**); however no pure cultures with this capability have been isolated (13).

The biochemical pathway for aromatic compound biodegradation under anaerobic conditions has been investigated over the last few years. I will focus on degradation of toluene, ethylbenzene and xylene to benzoyl-coenzyme A (benzoyl-CoA) because it is a major intermediate for most aromatic compounds (**Figure 1**). Mineralization of benzoyl-CoA was reviewed recently; for details see Heider et al and Harwood et al. (14, 15).

Many of the details of the degradation pathway were elucidated with toluene as substrate and pure cultures including *Azoarcus* strain T, *Thauera aromatica*, and *Thauera sp.* strain T1 (16-26) (**Table 1**). The key step is an unusual enzymatic reaction that results in the formation of a C-C bond with no net redox change. Fumarate is added to the methyl-C of toluene resulting in benzylsuccinic acid (BSA; **Figure 1**). The reaction starts inside the box with toluene {1}. BSA {2} was first reported from an enrichment culture, *T. aromatica* strain K172 and strain T1 (16, 27, 28). Beller and Spormann showed that formation of BSA is the initial step for toluene degradation (17).

Table 1   Pure and mixed microbial cultures reported to degrade BTEX compounds under varying conditions. Aromatic and aliphatic compounds other than BTEX are not included in this table.

Species and or strain	BTEX compound metabolized	Reference for isolation
Denitrifying bacteria		
Thauera aromatica K172	Toluene	(29, 30)
Thauera aromatica T1	Toluene	(31)
Azoarcus sp. strain T	Toluene, m-xylene	(32)
Azoarcus tolulyticus Tol4	Toluene	(33)
Azoarcus tolulyticus Td15	Toluene, m-xylene	(34)
Strain ToN1	Toluene	(35)
Strain EbN1	Ethylbenzene, toluene	(35)
Azoarcus sp. strain EB1	Ethylbenzene	(36)
Strain PbN1	Ethylbenzene	(35)
Strain mXyN1	Toluene, m-xylene	(35)
Strain T3	Toluene	(37)
Strain M3	Toluene, m-xylene	(37)
Strain pCyN1	Toluene	(38)
		(39)
Iron-reducing bacterium		
Geobacter metallireducens GS15	Toluene	(40)
Sulfate-reducing bacteria		
Desulfobacula toluolica Tol2	Toluene	(41)
Strain PRTOL1	Toluene	(42)
Desulfobacterium cetonicum	Toluene	(43)
Strain oXyS1	Toluene, o-xylene	(43)
Strain mXyS1	Toluene, m-xylene	(43)
Phototrophic Bacteria		
Blastochloris sulfoviridis ToP1	Toluene	(44)
Enrichment cultures		
Denitrifying bacteria	Benzene	(45)
Iron-reducing bacteria	Benzene	(46-48)
Manganese-reducing bacteria	Toluene	(49)
Sulfate reducing bacteria	Benzene	(47, 50-54)
	p-Xylene	(50, 55, 56)
Methanogenic bacteria	Benzene	(11, 47)
	o-Xylene	(57)
	Toluene	(11, 58)

The radical enzyme, which catalyzes the initial step of toluene degradation, was first isolated and characterized by Leuthner et al. (21). It is an $\alpha_2\beta_2\gamma_2$ heterohexamer (tertiary structure composed of six proteins; three times two equivalent proteins) with a flavin cofactor (electron acceptor/donor) and is represented as letter E (**Figure 1**). Work with the purified enzyme from *Azoarcus* strain T indicated that only the R-isomer of BSA was formed (23). The same stereospecificity was observed in *T. aromatica* (59). Investigations with deuterated toluene indicated that deuterium was retained in the BSA that formed (17, 18).
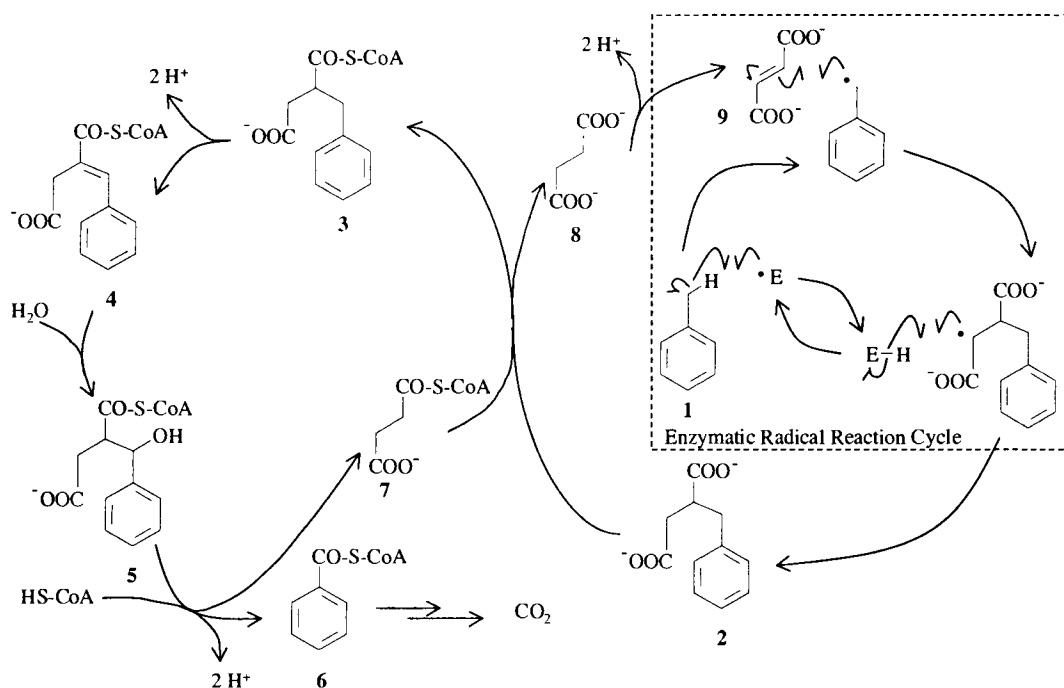
Figure 1 Degradation pathway of {1} toluene to {6} benzoyl-CoA. Additional observed intermediate products include {2} benzylsuccinic acid, {3} benzylsuccinyl-CoA, {4} E-phenylitaconyl-CoA, {5} 2-carboxymethyl-3-hydroxyphenylpropionyl-CoA, {7} succinyl-CoA, {8} succinate, and {9} fumarate.

Krieger et al. observed a kinetic isotope effect defined as the rate with ¹H-hydrogen divided by the rate with deuterium of k(H)/k(D) = 3 for deuterated toluene and m-xylene under denitrifying conditions (24), which indicates that cleavage of the C-H bond is the rate determining step. Isotopic fractionation was also observed under sulfate-reducing conditions for o- and m-xylene (60) and under methanogenic conditions for toluene (61). Studies of substrate specificity revealed that the substrate radical needs to be stabilized by delocalization and that replacement of fumarate by its cis-stereoisomer, maleate, also leads to R-BSA (23). Electron paramagnetic resonance spectroscopy measurements of the enzyme gave

signals characteristic of enzymes with a glycyl radical in the reactive center (62). Similarities of benzylsuccinate synthase with other enzymes containing a glycyl radical center were confirmed by gene sequencing (21, 22, 25).

The described initial activation of toluene apparently is widespread in the microbial environment. It was demonstrated in *Desulfobacula toluolica* strain PRTOL1, in the phototrophic *Blastochloris sulfoviridis*, and in a methanogenic enriched culture (44, 58, 63, 64). Similar activation reactions of m-xylene (24, Wilkes, 2000 #41, 42), p-cymene (38), m-cresol (65), p-cresol (66), m-toluic acid (67), n-hexane (68), and n-dodecane (69) were reported. Under sulfate-reducing conditions ethylbenzene (67) and 2-methylnaphtalene (70) also underwent addition to fumarate. Beller et al. proposed to use products of alkylbenzene addition to fumarate (e.g., BSA and methyl BSA) as unique indicators of anaerobic degradation processes (71). Despite their potential to indicate biodegradation, only a few reports have identified these metabolites in BTEX-contaminated aquifers (67, 71-73).

The degradation pathway of BSA follows enzymatic reactions similar to that in the β-oxidation of fatty acids and a hydrolytic cleavage to form benzoyl-CoA (59, 74, 75). Leutwein and Heider identified the enzyme producing BSA-CoA (3 in **Figure 1**) from *T. aromatica* and measured its activity (59, Leutwein, 2001 #582). Oxidation of BSA-CoA to E-phenylitaconyl-CoA (4 in **Figure 1**) was independent from ATP and acetyl-CoA (16, 17) and activity of BSA-CoA dehydrogenase was detected (74). To the best of my knowledge, enzymes have not been yet isolated

that catalyze the hydrolysis of E-phenylitaconate-CoA to 2-carboxymethyl-3-hydroxyphenylpropionyl-CoA (5 in **Figure 1**) and the subsequent cleavage of that molecule to benzoyl-CoA and succinyl-CoA (6 and 7, respectively, in **Figure 1**). The genes coding for all these enzymes were sequenced from *T. aromatica* strain T1 and K172 and the mechanism of gene expression was revealed recently. (20-22, 25, 74, 76).
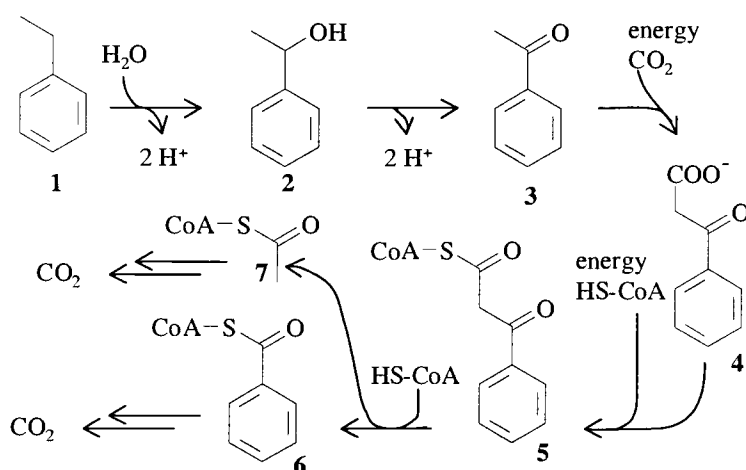
Figure 2   Degradation pathway of {1} ethylbenzene under denitrifying conditions to {6} benzoyl-CoA. Intermediate compounds are {2} 1-phenylethanol, {3} acetophenone, {4} benzoylacetate, {5} benzoylacetyl-CoA, {6} benzoyl-CoA, and {7} acetyl-CoA.

Under denitrifying conditions ethylbenzene seems not to be transformed by the same pathway (64, 77). Degradation of ethylbenzene to benzoyl-CoA is shown in **Figure 2**. The oxygen in 1-phenylethanol and acetophenone (2 and 3 in **Figure 2**) were derived from water as demonstrated with ^{18}O-labeled water (36). Johnson and Spormann measured the activity of the corresponding enzymes in cell-free extracts

(78). The electron acceptors were quinone and NAD^+ for the first and second step, respectively. The enzyme for the first reaction was purified and characterized independently by Johnson et al. (79) and Kniemeyer and Heider (80). It is a heterotrimer ($\alpha\beta\gamma$) containing molybdopterin (a cofactor consisting of a molybdenum bonded to a multi-ringed structure called pterin) and it occurs in the periplasm. Johnson et al also sequenced the corresponding genes (79). Carboxylation to benzoylacetate (4 in **Figure 2**) is assumed to be the next reaction since degradation of ethylbenzene and acetophenone was dependent on the presence of CO_2 (36, 77). The rest of the pathway to benzoyl-CoA is assumed to follow the pathway indicated in **Figure 2** (35, 36, 78). Propylbenzene was degraded by the same pathway as that identified for ethylbenzene (35, 78).

1.3 EVIDENCE FOR AROMATIC HYDROCARBON DEGRADATION IN THE SUBSURFACE

Clearly, laboratory experiments have elucidated the pathways and intermediates in the anaerobic biodegradation of toluene, ethylbenzene, and xylenes. Despite the availability of detailed information on the pathways and enzymes involved in the anaerobic degradation of these compounds, very little information is available on the in-situ biotransformation of these chemicals in contaminated groundwater. Current research is focused on exploiting our knowledge of the transformation pathway for the purpose of remediating BTEX-contaminated groundwater.

The limitations and costs of conventional ground water cleanup technologies and soil treatment methods have made monitored natural attenuation of BTEX an attractive alternative approach to site remediation (81). Monitored natural attenuation includes physical, chemical and biological processes that lead to reduction in contaminant concentrations. The National Research Council made recommendations how to apply and document monitored natural attenuation (81, 82).

According to the recommendations of the National Research Council, the assessment of monitored natural attenuation requires multiple, ideally converging approaches. Evidence of contaminant concentration reduction can be obtained by collecting and analyzing spatial and temporal field data on contaminant concentrations and biogeochemical indicators. Such field data can be collected as part of a synopsis or snapshot survey or as part of a long-term monitoring program (83). Spatial and temporal field data also are used to deduce the main physical, chemical and biological processes responsible for contaminant removal (84, 85). Unfortunately, heterogeneous distribution of contaminants in the subsurface can confound attempts to establish that in-situ degradation is occurring from spatial field data since heterogeneity introduces uncertainty into spatial measurements of contaminant concentrations. In addition, concentration changes due to non-biological processes including advection, dispersion, and sorption/desorption can obscure the effects of biological transformation processes (86). Normalizing contaminant concentrations to other internal chemical indicator species (markers)

within the system that have similar transport behavior but are not biodegraded is an approach used to determine if contaminant removal is occurring (87-90).

Reporting concentrations of indicators such as electron acceptors (e.g. O_2, NO_3^-, SO_4^{2-}) is a common approach to site characterization and assessment of biodegradation potential (91-94). However, this does not always allow for the determination of the predominant terminal electron accepting process (95). Chapelle et al. proposed to correlate hydrogen concentrations to terminal electron accepting processes (e.g. sulfate reduction corresponds to hydrogen concentrations of 1-4 nM) (96-100).

When specific biodegradation pathways and intermediates are known, the measurement of degradation products in field samples also provides strong evidence for biodegradation. (60, 71, 88, 89, 91, 99, 101, 102). In addition, evidence for biodegradation can be obtained from $\delta^{13}C$ measurements of contaminants and their degradation products. $\delta^{13}C$ measurements compare the isotopic enrichment in field samples with that of a standard (90, 99, 100, 103-107). Under sulfate-reducing and methanogenic conditions, bacteria preferentially use compounds with the low-mass isotopes, which is energetically favorable and known as the kinetic-isotope effect (61, 103, 108). Under anaerobic conditions, substrates become enriched in the heavier isotope ^{13}C whereas products become enriched in the lighter isotope ^{12}C. The disadvantage of $\delta^{13}C$ in BTEX biodegradation studies is that the magnitude of the fractionation effect is small, which limits is potential use in the field (61). Ward et al. found that the stable

isotopes of hydrogen may be a better indicator of hydrocarbon biodegradation (61). In addition to carbon and hydrogen, isotope ratios of oxygen (106) have also been used as evidence for diesel fuel biodegradation. Correlations between characteristic bacteria and contaminant distributions also have been used as evidence of contaminant biodegradation (83, 87, 91, 109-111).

As previously discussed, laboratory experiments with pure cultures were useful in elucidating transformation pathways. Laboratory studies with enrichment or mixed cultures were used to demonstrate the potential for contaminant biodegradation. Furthermore, laboratory microcosm studies containing sediment and groundwater provide estimates of transformation rates. Unfortunately, laboratory studies include only parts of the system and may not reproduce in-situ field conditions, such that the validity of extrapolating laboratory results to the field is questionable (100, 112). Moreover, microcosms utilize relatively small samples of groundwater or sediment that potentially are not representative of the field. The process of obtaining samples inherently disturbs sediments and possibly contaminates the samples with non-indigenous microorganisms. One field parameter that is not well understood and therefore difficult to reproduce is bioavailability. Availability of contaminants for microorganisms is determined by the interactions of contaminants with sediment surfaces, diffusion-limited transport to bacteria, and partitioning of compounds into soil organic matter (113).

1.4 FIELD TRACER TESTS

Tracer tests conducted in the field provide information complementary to that obtained by field sampling and laboratory microcosm experiments. However, unlike long-term sampling and microcosm experiments, tracer tests provide direct measurements of transformation rates in relatively undisturbed groundwater environments (114). Careful design of the test solution and injection conditions allows specific questions on contaminant transport and transformation to be addressed. Tracer tests can be designed to interrogate contaminant biodegradation under natural or 'intrinsic' conditions. Alternately, test conditions can be selected to increase or enhance the rates of biodegradation (72, 115-117).

Despite their advantages, tracer tests that are conducted in a well-to-well format typically are more logistically complex and expensive to perform compared to synoptic field sampling or laboratory microcosm experiments. In particular, well-to-well tests are designed to interrogate processes occurring over large portions of aquifer. Such tests typically involve the injection of large volumes of test solution and extensive sampling from large arrays of downgradient monitoring wells (118-120). Intermediate-scale well-to-well tracer tests interrogate smaller portions of aquifers (121-123). Well-to-well tests, regardless of size, have the disadvantage that multiple wells are required, which are costly to install (114). In addition, mass balance is difficult to obtain with well-to-well tracer tests because the injected test solution is only partially captured by sampling the downgradient monitoring wells (114).

A-well "push-pull" tests to answer a wide range of questions (114). The test consists of an injection phase (**Figure 3a**), a reaction phase, and an extraction phase (**Figure 3b**). Test solutions containing conservative tracers and reactive compounds are injected into the saturated zone of aquifers through the screens of monitoring wells. During the injection, the test solution is radially distributed around the well (**Figure 3a**). Reactions continue during the reaction phase when the test solution drifts according to the regional groundwater flow field. During the extraction phase of the test, the test solution/groundwater mixture is recovered from the groundwater well (**Figure 3b**) and solute concentrations are measured in order to obtain breakthrough curves.

The advantages of push-pull tests are that they include the heterogeneity at site scale, they utilize existing wells, they do not require arrays of down-gradient monitoring wells and they are relatively fast to perform. When the extraction phase is conducted by continuous pumping, the injected solution is recovered and mass balance can be obtained, which makes it easier to gain regulatory approval for injecting contaminants and exotic materials.

a) injection phase   b) extraction phase
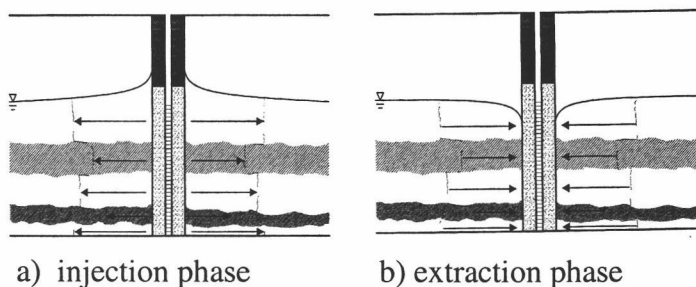
Figure 3   Injection and extraction phases of a single-well "push-pull" test.

Several characteristics of aquifers can be derived from breakthrough curves, depending on the specific push-pull test design. Push-pull tests were used to measure zero- (114) and first- (124) order reaction rates, enzyme kinetics (125), sorption of surfactants (126), retardation of solutes (127), and non-aqueous phase liquid saturation (128). Modified push-pull tests, in which the extraction phase is carried out over days to months, were used to investigate the in-situ transformation of TCE (86) and toluene and xylenes in BTEX-contaminated groundwater (72). In the test conducted by Reinhard, the background BTEX was first stripped from the groundwater and a buffer of groundwater was injected prior to the test (71, 72). These steps, which required time and more complex logistics, were taken to ensure that the injected solutes could be distinguished from native solutes, which was necessary to interpret the field data.

1.5 USE OF ISOTOPE-LABELED SURROGATES

Working at sites with background contaminant concentrations introduces problems because background contaminants potentially interfere with the interpretation of the experimental field data (129). To avoid the time and cost associated with removing background contaminants and the uncertainties of complete removal, alternative approaches are needed. Labeled surrogates allow for the distinction between introduced and background compounds. An ideal surrogate has the same physicochemical properties and the same chemical and biological reactivity as the original compound. Isotope enriched chemicals fulfill these requirements since they are still the same chemical while differing in mass.

Differences between contaminants and their isotopic analogs are in the rates of their chemical and biological reactions due to the kinetic-isotope effect as previously discussed. ^{14}C-labeled compounds have been widely used in laboratories (29, 32, 115) and in one controlled-ecosystem enclosure (130). However, approval for the release of radioactive compounds into the environment is difficult if not impossible to obtain. As reviewed earlier, δ^{13}C has been used as evidence of biodegradation, however ^{13}C-enriched substrates have not been used in tracer experiments. Both ^{13}C-enriched and ^{14}C-labeled chemicals are prohibitively expensive, which limits their use in field experiments.

Deuterated BTEX compounds were used extensively to determine the degradation pathways for toluene and xylenes in laboratory studies as discussed earlier (17, 18, 24). Advantages of deuterium-labeled compounds include relatively low cost, low background abundance of deuterium, no radioactivity, and that chemicals containing multiple deuterium atoms can be analytically separated and detected with commonly available gas chromatography/mass-spectrometry instrumentation. To the best of our knowledge, deuterated compounds have not been used as surrogates of hydrocarbons in field tracer tests even though this application was suggested in the National Research Council report (81). Because deuterated surrogates offer the mentioned advantages, the objective of this study was to combine the deuterated analogs of toluene and xylene with the push-pull test in order to determine the in-situ rates of toluene and xylene transformation in BTEX-contaminated groundwater.

2 QUANTITATIVE DETERMINATION OF BENZYLSUCCINIC ACID IN BTEX-CONTAMINATED GROUNDWATER BY SOLID PHASE EXTRACTION (SPE) COUPLED WITH GAS CHROMATOGRAPHY/MASS SPECTROMETRY

2.1 ABSTRACT

Methods are needed to determine the rate of benzene, toluene, ethylbenzene and the xylene isomers (BTEX) biodegradation in contaminated groundwater for the purpose of site characterization and bioremediation. Benzylsuccinic acid (BSA) and methyl-benzylsuccinic acid (methyl BSA) are unambiguous metabolites resulting from anaerobic toluene and xylene biodegradation. An analytical method for the quantitative BSA determination in groundwater samples was developed. Samples containing BSA and methyl BSA were extracted onto 0.5 g of styrenedivinylbenzene, eluted with ethyl acetate, and methylated with diazomethane. Gas chromatography coupled to mass spectrometry with electron impact ionization was used for separation and detection. The recovery from spiked 1 L groundwater samples was 88 to 100 %. The precision of the method, indicated by the relative standard error was ± 4%. The method detection limit was 0.2 µg/L. The concentration of BSA and methyl BSA in groundwater samples from anaerobic BTEX-contaminated sites ranged from below detection limit to 3 µg/L.

2.2 INTRODUCTION

Gasoline contamination of the subsurface, mainly from underground storage tanks, is a widespread problem. Benzene, toluene, ethylbenzene and the xylene isomers, collectively known as BTEX are the components of major concern due to their high solubility (2, 3) and their toxicity. (4-6). The EPA drinking water criteria are 0.005 mg/L for benzene, 1 mg/L for toluene, 0.7 mg/L for ethylbenzene, and 10 mg/L for xylene isomers (7, 8).

Limitations and costs of conventional ground water cleanup technologies and soil treatment methods has made monitored natural attenuation of BTEX an attractive approach for site remediation (81). Monitored natural attenuation includes physical, chemical and biological processes that lead to reduction in contaminant concentrations. In particular, biodegradation is important for transforming BTEX to less toxic products. Since most sites with gasoline spills are anaerobic, BTEX degradation in the absence of oxygen is the process of interest for bioremediation. BTEX-degrading cultures have been reported under denitrifying (29, 30 , 31-38, 45), sulfate-reducing (41-43, 47, 50-56), iron-reducing (39, 40, 46-48), manganese-reducing (49), phototrophic (44), and methanogenic (11, 47, 57, 58) conditions.

Documenting in-situ biodegradation ideally should include multiple lines of evidence (81, 82). Identification of degradation products if field samples is particularly compelling evidence, especially when the products detected are unambiguous indicators of a specific biodegradation pathway. The anaerobic degradation pathway for toluene has been investigated in detail during the last few

years (16-28, 59-62, 75). Benzylsuccinic acid (BSA) is the first intermediate during anaerobic microbial degradation of toluene. In a corresponding manner, methylbenzylsuccinic acid (methyl BSA) results from anaerobic xylene degradation. Beller et al. (71) proposed to use BSA and methyl BSA as unique indicators of the anaerobic degradation of toluene and the three xylene isomers. The relationship of BSA and methyl BSA to their respective parent compounds is well understood, they do not have commercial or industrial uses, and they occur at detectable levels (67). To the best of our knowledge, few reports document the occurrence of in-situ formation of these degradation products despite their potential for indicating in-situ biodegradation. (67, 71-73).

In previous studies, samples containing BSA and related compounds were extracted using liquid-liquid extraction, followed by methylation with diazomethane (71) or silanization with N,O-bis(trimethylsilyl)trifluoroacetamide (67). Unfortunately, liquid-liquid extraction is cumbersome and typically uses high volumes of solvents. Solid-phase extraction is an attractive alternative to liquid-liquid extraction due to decreased solvent usage. Currently many formats and sorbent materials are available for solid phase extraction. Classical solid-phase extraction utilizes packed columns of the sorbent of choice (typically 40 μm particles). Alternatively, the disk or membrane format has smaller particles (8 μm) embedded in a Teflon membrane, which allows for higher flow rates due to smaller particle size and higher cross-sectional area compared to packed columns (131).

However, capacity, which is dependent on the mass of sorbent, is limited due to smaller amount of sorbent embedded in membranes.

Solid-phase extraction can be carried out in normal phase, reversed phase, size exclusion or ion exchange mode. The classical approach for acid analytes is to acidify samples and extract the free acids onto reversed-phase sorbents. Alternatively, extraction onto a strong anion exchange (SAX) resin followed by in-vial derivatization and elution also has been applied to other carboxylic acids (132, Field, 1996 #553). The goal of this study was to develop and validate a solid-phase extraction method for analyses of BSA and methyl BSA compounds in BTEX-contaminated groundwater samples.

2.3 EXPERIMENTAL

2.3.1 Reagents and Standards

Benzylsuccinic acid (BSA; 99% purity) was purchased from Sigma Chemical (St. Louis, MO). 2-Chlorolepidine (2CL, 99% purity) and 4-(trifluoromethyl) hydrocinnamic acid (4TFM, 95% purity) were purchased from Aldrich Chemical (Milwaukee, WI). BSA, 2CL, and 4TFM were prepared in acetonitrile at 1 mg/mL. 4TFM was used as a surrogate standard and was spiked prior to the extraction while 2CL was used as internal standard and was spiked just prior to the derivatization reaction. Acetone, methanol, and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Fairlawn, NJ). Ethyl acetate (HPLC-grade) was obtained from Mallinckrodt (Paris, KY). Hydrochloric acid was obtained from J. T.

Baker (Phillipsburg, NJ). An diethyl ether solution of diazomethane was prepared from Diazald (Aldrich Chemical, Milwaukee, WI) according to standard procedures (Technical Bulletin AL113; Aldrich Chemical, Milwaukee, WI).

The solid-phase extraction sorbents in disk format evaluated for this study included the Empore styrenedivinylbenzene (SDB) (Varian, Harbor City, CA), Empore SDB-RPS (Fisher Scientific, Fairlawn, NJ), SDB-XC (Fisher Scientific, Fairlawn, NJ), and octadecyl-bonded silica or C18 (Varian, Harbor City, CA). The sorbents in cartridge format included SDB ENVI-Chrom P (Supelco, Bellefonte, PA) and Bond Elut C18 (Varian, Harbor City, CA).

2.3.2 Field Sites and Sampling

The first field site is a bulk-fuel terminal located in Willbridge near Portland, OR. The site hereafter will be referred to as the Northwest Terminal. The unconfined aquifer at this site consists of a layer of fill (medium dense to fine grained sand and silty sand) that rests on top of alluvium, which consists of clayey silt with sand interbedded with silty clays and clays. Total BTEX concentrations were between 2 and 30 mg/L (**Table 2**). A 71,900 L (19,000 gallons) release of ethanol occurred in 1999. The site had a pre-existing dissolved hydrocarbon plume. Groundwater was anaerobic due to the existing hydrocarbon contamination and nitrate and sulfate were depleted and methane was detected. The water table is approximately 2 to 3 m below land surface. Groundwater flows towards the east and the velocity is estimated to be about 100 m per year (133). A total of four wells were sampled at this site.

The second field site is a former petroleum refinery near Kansas City, KS and hereafter will be referred to as the Kansas City site. It was operated from 1930 until 1982. After installation of monitoring wells, presence of low-density non-aqueous phase liquid (LNAPL) was discovered over a 90-hectare area. Recovery operations initiated in 1984 recovered over 6 million L; however, recovery efficiency recently declined. Prior to this study, BTEX concentrations ranged from 0.065 to 5.28 mg/L (**Table 2**) (134). The unconfined aquifer consists of fine sand with clayey silt or silt (to ~3 m below land surface) covering sand (below ~3 m below land surface) (135). Although groundwater flows generally southeast towards the Missouri River, in the spring, high stage elevations of the Missouri River cause the hydraulic gradient to shift reverse inward toward the site. (136). At the time of the experiments, groundwater velocities were 0.05 m/day (137) and the water table was about 7 to 9 m below land surface, which were low water table conditions for this site. Tests were conducted in wells that had 3-m screened intervals with the top of the screen located at 4.6 to 7 m below land surface.

Samples were obtained from each site from 2-inch inner diameter PVC wells. Before taking the sample, three times the well volume was purged where possible. The low water level in well 207 at the Kansas City site made it impossible to purge three times the well volume; therefore, 3 L were purged prior to sampling this well. Samples were collected in glass bottles (250 mL to 1 L), preserved with 5 %(v/v) formalin, shipped on ice, and stored at 4 °C until analysis.

Table 2   Characterization of groundwater used for method development and demonstration. Data provided from the site responsible persons.

Parameter	Northwest Terminal		Kansas City site		
	Well CR12	Well CR15	Well 105 s	Well 106 s	Well 207
NAPL observed in sample	NO	YES	NO	NO	YES
Benzene mg/L	0.15	13	4.2	0.02	ND
Toluene mg/L	2.8	0.1	0.32	ND	ND
Ethylbenzene mg/L	0.6	0.5	0.38	0.032	1.9
Xylene mg/L	3.6	1.8	0.38	0.013	ND
Total BTEX mg/L	7.15	15.4	5.28	0.065	1.9
Ethanol mg/L	48	<2 [a]	N/A	N/A	N/A
DO mg/L	0.5	ND	0.65	1.81	ND
Nitrate	ND	ND	ND	ND	ND
Iron mg/L	N/A	N/A	39	45	N/A
Manganese mg/L	5	12.5	N/A	N/A	N/A
Sulfate mg/L	< 1 [a]	< 1 [a]	7.6	6.9	1.8
Methane mg/L	10	11	18	14.3	N/A

ND: not detected

N/A: not analyzed or not applicable

[a] detected below quantitation limit

2.3.3   Solid Phase Extraction

Prior to extraction, ground water samples were warmed to room temperature. The samples (0.5 - 1.0 L) were titrated to pH 2.0 with concentrated HCl. After acidification, the samples were spiked with 20 µL of 1 µg/µL of the 4TFM surrogate standard. The samples were then filtered under vacuum through 1 µm Whatman glass-fiber filters (Fisher Scientific, Fairlawn, NJ).

Glass columns, attached to a vacuum manifold, and fitted with Teflon frits were packed with 0.5 g SDB ENVI-Chrom P (Supelco, Inc., Bellefonte, PA). The columns were preconditioned in three steps. First, 2.5 mL acetone was applied and

the columns were allowed to dry. Second, 2.5 mL methanol was applied, afterwhich the columns were kept wet until the end of the extraction. Third, the columns were washed with 4 x 5 mL of 0.01 M HCl. Teflon transfer lines were attached to the top of each column and used to transfer samples from the filtration flasks to the columns. Extractions were performed using a 5 mL/min flow rate.

After extraction, the sorbent columns were dried overnight using vacuum to pull air through. To elute the columns, 2 x 2.5 mL of ethyl acetate was passed through each column, collected and concentrated to ~0.5 mL under a stream of dry nitrogen. The eluate was transferred to a 2 mL autosampler vial by rinsing with 2 x 0.2 mL ethyl acetate. The autosampler vial contents were concentrated to 0.5 mL under a stream of dry nitrogen and then spiked with 20 μL of 1 μg/μl of the 2CL internal standard. The vial was then capped after adding 1.0 mL of diazomethane. The reaction was allowed to proceed for 5 ± 1 min. The vial was then uncapped and the excess diazomethane was removed under a stream of dry nitrogen. The vial was recapped and placed on the autosampler for analysis.

2.3.4 Spike and Recovery

Three sets of spike and recovery experiments were performed. First, five replicate 1 L tap water samples were spiked to give a final concentration of 20 μg/L BSA. Tap water samples were the only samples not prefiltered before the extraction. To obtain quantitative recovery from tap water, glass columns for the extraction cartridges and beakers containing the acidified BSA-spiked tap water were silanized. Clean glassware was rinsed for about 15 s with DMDCS

(dimethyldichorosilane 5% in toluene, Supelco, Bellefonte, PA), then washed two times with toluene, three times with methanol, and then air dried. Note that this procedure was necessary only for tap water samples and not for ground water samples. Glassware for the elution and the derivatization reaction were not silanized.

The second set of spike and recovery experiments was performed with a groundwater composite obtained by combining aliquots of several samples from different wells from the Kansas City site. The composite sample first was determined to be blank. Seven 1 L aliquots of the composite sample were spiked with BSA to give a final concentration of 2 µg/L. Over the course of the study, a total of sixteen additional blank 1 L samples collected from a total of six wells from the Kansas City site and the Northwest Terminal were spiked to give a final concentration of 20 µg/L of BSA.

2.3.5   Gas Chromatography – Mass Spectrometry

All extracts were analyzed using a Hewlett Packard Model 5890 gas chromatograph (GC) equipped with a Model 5972 mass selective detector (MSD). The GC was equipped with a 30 m x 0.32 mm x 4 μm SPB-1 capillary column (Supelco Inc., Bellefonte, PA).   The injector was operated under splitless conditions at 250°C with a 1 µL injection volume and helium as carrier gas. The initial oven temperature of 130°C was increased at 2.5 °C/min to 225 °C and then raised at a rate of 10 °C/min up to 265 °C, and held for 3 min to give a total run

time of 45 min. The MSD was operated in electron impact mode with a source

temperature of 265 °C. Three ions were acquired in single ion monitoring mode

and used to identify and quantify each analyte in its methylated form (**Table 3**).

Table 3   Ions used to detect and quantify analytes of interest. The molecular
ion [M$^+$] is marked with a *

Analyte	Quantitation Ion (m/z)	Qualifier Ion(s) (m/z)
BSA	236*	176 / 91
Methyl BSA	250*	190 / 105
4TFM	172	232* / 159
2CL	177$^{*\ a)}$	179$^{*\ a)}$ / 142

a) chlorine isotopes

Calibration curves for BSA were constructed from standards prepared in 0.5

mL ethyl acetate that also ontained 0.2 to 25 µg of BSA, 20 µg of the surrogate

standard (4TFM), and 20 µg of the internal standard (2CL). Standards were

methylated in a procedure similar to that for samples; however, that only 0.5 mL

diazomethane was used. Surrogate-standard quantitation was used and gave linear

calibration curves, typically with r^2 values of 0.999. No internal standard was

available for methyl BSA. However, fragmentation patterns for BSA and methyl

BSA were similar except that ions from methyl SA were heavier by 14 mass units

which corresponded to the additional methyl group (**Figure 4**). Mass spectra were

similar to those reported by Beller et al. (24, 42, 71). Since no standard for methyl

BSA was available, the calibration curve of BSA (m/z 236) was used for methyl

BSA (m/z 250) quantification and a response factor of 1 was assumed. Multiple peaks with similar fragmentation patterns were observed, which were likely to correspond to the different isomers of methyl BSA. Since we were not able to distinguish between isomers, peak areas for methyl BSA were summed up.
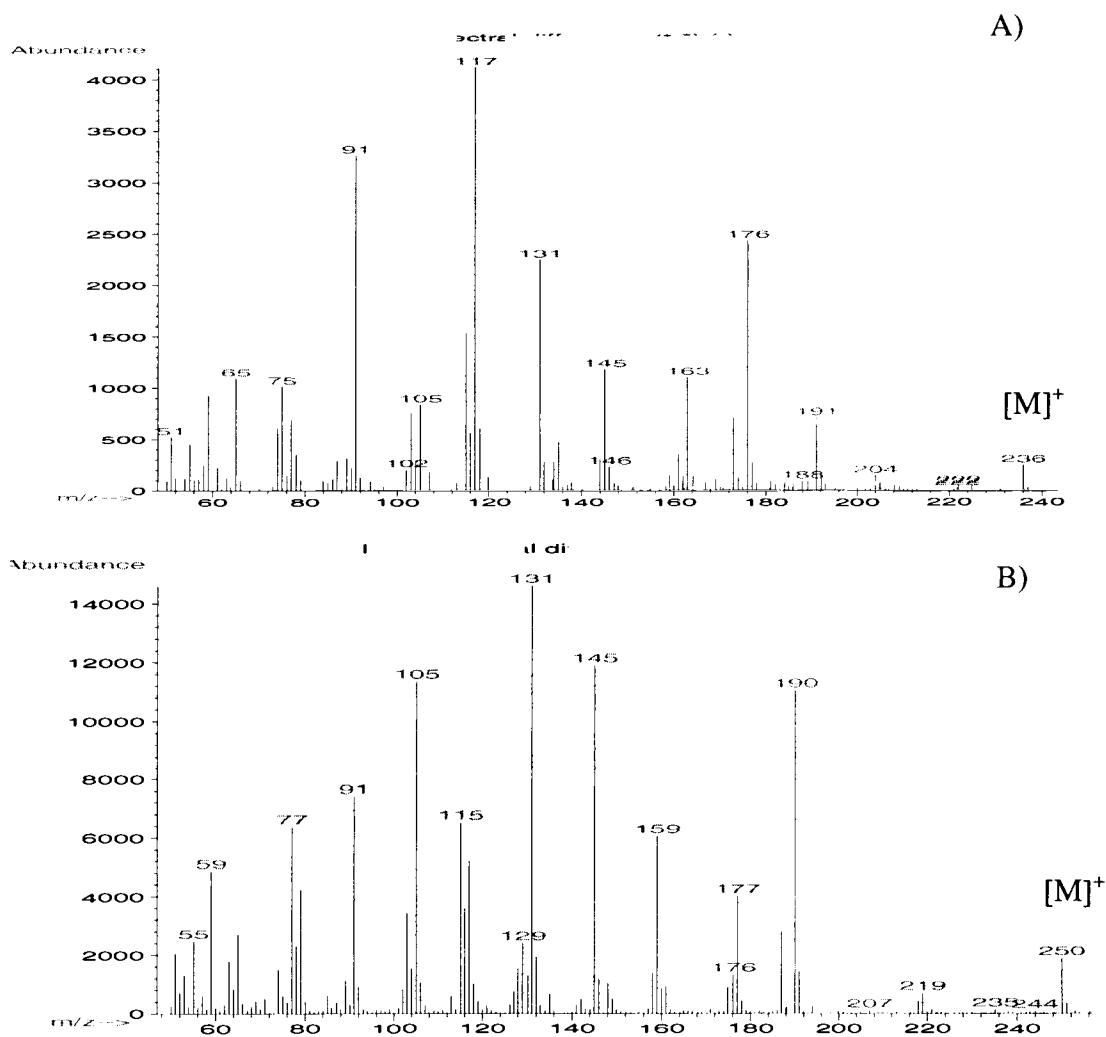
Figure 4 Mass spectra of A) BSA and B) methyl BSA from a groundwater sample from the Northwest Terminal well CR13

2.4 RESULTS AND DISCUSSION

2.4.1 Gas Chromatography – Mass Spectrometry

The temperature gradient of the gas chromatograph was optimized towards fast separation while minimizing the variability of the BSA/2CL ratio. Chromatograms obtained for the same sample using temperature gradients ranging from 1 to 10 °C/min indicated that decreasing the gradient from 10 °C/min to 2.5 °C/min significantly reduced the baseline signal (**Figure 5**). In addition the BSA/2CL atio became more reproducible and reached the value expected from standards. Decreasing the gradient further to 1 °C/min did not further improve the reproducibility. Therefore, a temperature gradient of 2.5 °C/min was used for all subsequent experiments.
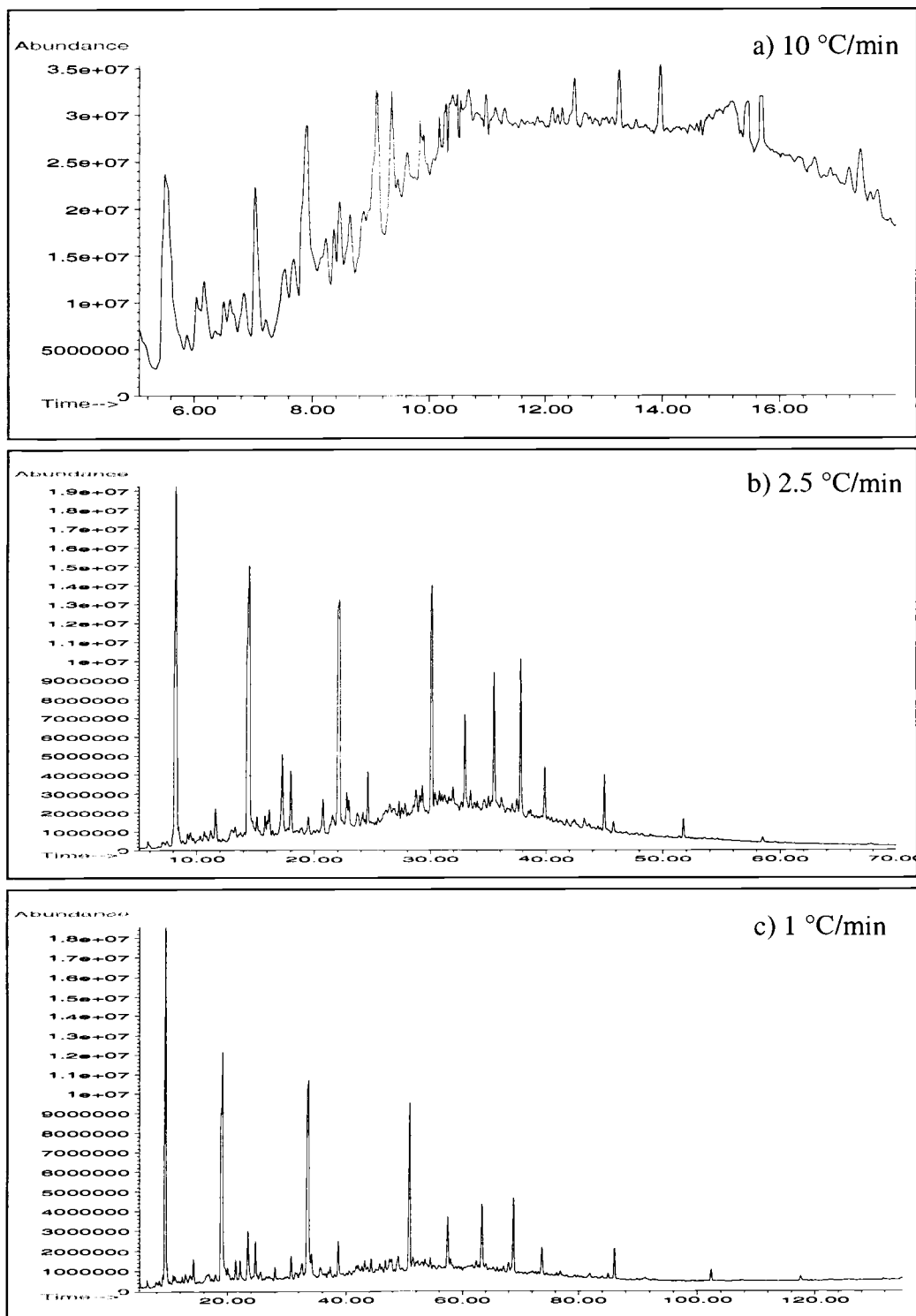
Figure 5   Total ion chromatograms for three different temperature gradients on the gas chromatograph for a single sample from the Kansas City site.

2.4.2 Methylation Reaction

To evaluate the influence of the reaction solvent, 20 μg each of BSA and 2CL were spiked into separate vials containing 0.5 mL of the following solvents: acetonitrile, methanol, diethyl ether, and ethyl acetate. The solution was allowed to react for 30 min with 0.5 mL diazomethane. After removing the diazomethane under a flow of dry nitrogen, the samples were analyzed by GC/MS. Methyl ester yields, determined as peak area of BSA relative to the area of the internal standard, were greatest for reactions conducted in ethyl acetate and methanol and lower for reactions conducted in diethyl ether and acetonitrile (**Table 4**). Except for reactions conducted in acetonitrile and diethyl ether, standard deviations were not significantly different (F-test, 95%). Since ethyl acetate is less polar and has a smaller expansion factor (138), it is the better injection solvent than methanol. Therefore, ethyl acetate was used as reaction solvent for all subsequent reactions and experiments.

Table 4   Influence of the reaction solvent on methyl-ester formation. The average and standard deviation from triplicate samples were computed as the area of BSA relative to the area of internal standard. Areas from single ions were used for quantification: BSA m/z 236, 2CL (internal standard) m/z 177.

Solvent	Relative peak area BSA
Acetonitrile	0.0399 ± 0.0028 (7.1%)
Ethyl acetate	0.0880 ± 0.0014 (1.6%)
Diethyl ether[a]	0.0684 ± 0.0001 (0.1%)
Methanol	0.0856 ± 0.0009 (1.0%)

a) Ethyl acetate was used as injection solvent since diethyl ether is not a good injection solvent.

Once the reaction solvent was selected, the reaction time was optimized using BSA and a range of chemicals selected for evaluation as potential surrogate standards, including 4-fluorobenzoic acid, which was used by Beller et al. (71) and Elshahed et al. (67) (**Figure 6**). Reaction time was investigated by spiking a mixture of 20 μg of each BSA, 4TFM, 2CL, 4-fluorobenzoic acid (Sigma Chemical), 3-chlorocinnamic acid (Sigma Chemical, St. Louis MO), and 4-fluorocinnamic acid (Sigma Chemical) into 0.5 mL ethyl acetate and adding 0.5 mL diazomethane. Areas for the internal standard 2CL were stable over time as were the areas for the methyl esters of BSA, 4TFM and 4-fluorobenzoic acid as indicated by stable ion ratios (**Figure 7**). However, the methyl esters of 4-fluorocinnamic acid and 3-chlorocinnamic acid, which have a double bond, disappeared with first order degradation rates of 0.018 and 0.05 min^{-1}, respectively. Reaction of the double bond with diazomethane may be the cause for the decreasing concentrations of the methyl esters with time. Although not evaluated in this study, phenylitaconic acid, the second intermediate of toluene degradation (**Figure 1**) has a double bond in its structure. If a degradation products with a double bond are of interest, a reaction time of 5 ± 1 min should be used. 5 min was used for all subsequent experiments in this study. Careful control of reaction time is required to retain all analytes of interest during the reaction step. Due to their reactivity, 3-chlorocinnamic acid and 4-fluorocinnamic acid were excluded as potential surrogate standards, which left 4TFM and 4-fluorobenzoic acid for further evaluation during the development of the solid phase extraction approach.

1   2   3   4   5
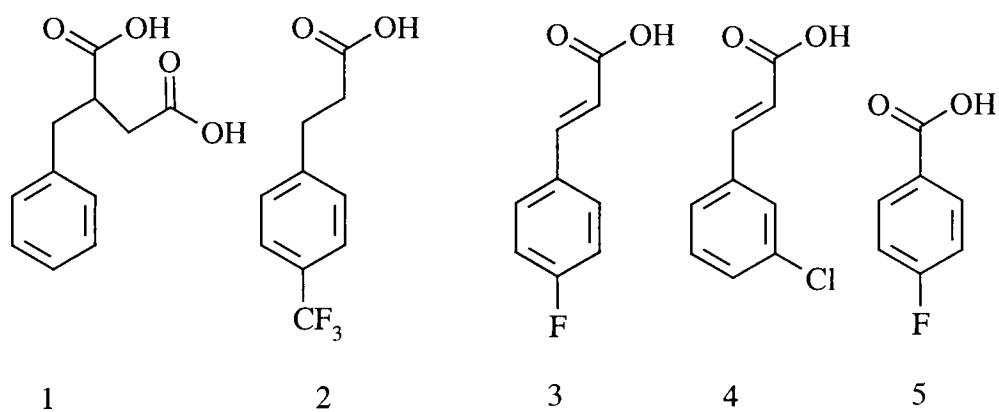
Figure 6   Structures of possible surrogate standards compared to {1} benzylsuccinic acid (BSA). {2} 4-(trifluoromethyl)hydrocinnamic acid (4TFM), {3} 4-fluorocinnamic acid, {4} 3-chlorocinnamic acid, and {5} 4-fluorobenzoic acid.
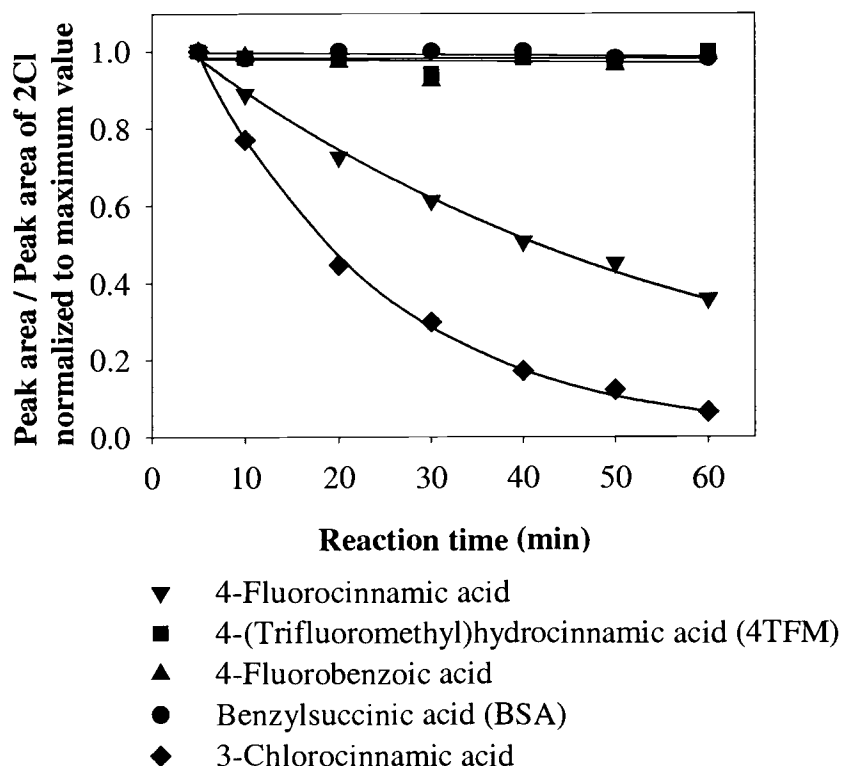
Figure 7   Time dependence of the derivatization reaction for possible surrogate standards and benzylsuccinic acid (BSA). Results are shown relative to the internal standard (2-Chlorolepidine, 2CL, m/z 177) and were normalized to maximum value observed for each individual analyte.

2.4.3   Solid Phase Extraction

Initial experiments focused on evaluation of strong anion exchange (SAX) disks for BSA isolation using procedures similar to those developed for other carboxylic acids (132, 139). With this procedure, 4-fluorobenzoic acid was not retained on 25-mm disks when 0.5-L groundwater samples were extracted, which indicated problems with breakthrough. To investigate the influence of the ionic composition of the groundwater, spike and recovery experiments were performed

with an artificial sample composed of deionized water enriched with the same anion concentrations as that of the groundwater from the Kansas City site (100 mg/L Br^-, 20 mg/L Cl^-, 270 mg/L NO_3^-, 200 mg/L SO_4^{2-}; added as sodium salts). Experiments were performed on a smaller scale with 50 mL samples extracted onto 13-mm disks instead of a 650-mL sample onto a 25-mm disk. Sixty-five percent of the 2 mg/L BSA was not retained. Thurman and Mills suggest to estimate the volume for extraction based on the charge density (meq/mL) in the sample and the exchange capacity of the disk (140). Calculations with the field sample anion concentrations and an exchange capacity of the 13-mm disk of 0.015 meq (0.2 meq for 47-mm disk (141)) indicate that the disk's exchange capacity is exceeded after about 2 mL. Ion affinities should be included into the calculations, but the necessary data were not available. As expected, the problem was not observed when BSA was extracted from 50 mL deionized water. Dilution of the sample as suggested by Ledin et al (142) and precipitation of the sulfate with barium hydroxide did not solve the problem; therefore, SAX was abandoned.

Breakthrough capacities for reversed-phase sorbents were determined by measuring breakthrough curves. To develop breakthrough curves, outflow concentrations relative to the applied concentrations were plotted versus the cumulative volume extracted. Groundwater samples from the Kansas City site, previously determined to be blank, were acidified with concentrated HCl to pH 2. The groundwater samples were then spiked to give a final concentration of 10 mg/L of BSA; high concentrations were used to achieve good detection. The spiked

groundwater samples were then continuously applied to separate columns or disks. Samples for measurement of the outflow concentration were taken every 50 to 100 mL during the extraction and were analyzed by either high performance liquid chromatography with diode-array detection (HPLC/DAD) or GC/MS. For some experiments, HPLC/DAD was used to simplify sample analysis because no derivatization step was required and the separation method was faster. Samples were analyzed on a Waters 2690 instrument with a Phenomenex Luna C_{18} column (150 mm ^{x} 4.6 mm ^{x} 5 µm). The mobile phase was a methanol/potassium phospate (25 mM) buffer at pH 2.5. A solvent gradient was used changing from 30 % to 60 % methanol in 5 min. A Waters 996 diode array detector was used to monitor at 203 nm. For 4TFM and other selected experiments, GC/MS was used for analyses of 1-mL. For GC the aqueous 1-mL sample was dried under a stream of dry nitrogen, redissolved in 0.5 mL ethyl acetate.

The 0.5-g SDB cartridge exhibited the highest breakthrough capacity (**Figure** 8) with no evidence of breakthrough up to 1.1 L and only 10% breakthrough at 1.4 L. The 0.5-g C_{18} cartridge showed breakthrough of 0.5 at about 500 mL. The higher capacity of the 0.5-g SDB cartridge was expected since SDB is a 100 % polymer sorbent compared to the 0.5 g octadecyl, which is about 20 % polymer and 80 % silica by weight. Different sorbents in the 47-mm disk format gave 0 to 60% breakthrough at ~200 mL (**Figure** 8), which was insufficient for the expected sample volume required to detect environmental concentrations (67, 71, 73). The C18 in the disk format had a lower capacity than the C18 in the cartridge format

even though both formats had about the same actual sorbent loading of ~0.1 g. The

breakthrough capacity of SDB in disk format was also lower than that expected

based on comparison of the mass of sorbent loading in cartridge (~0.5 g) and disk

(~0.3 g) format. Subsequent breakthrough experiments with 4TFM and 4-

fluorobenzoic acid indicated that 4-fluorobenzoic acid broke through more quickly

than BSA. On the other hand, 4TFM was retained more strongly than BSA. Based

on these experiments, sample volumes up to 1 L, 0.5 g SDB cartridges, and the

surrogate standard of 4TFM were used in all subsequent experiments.
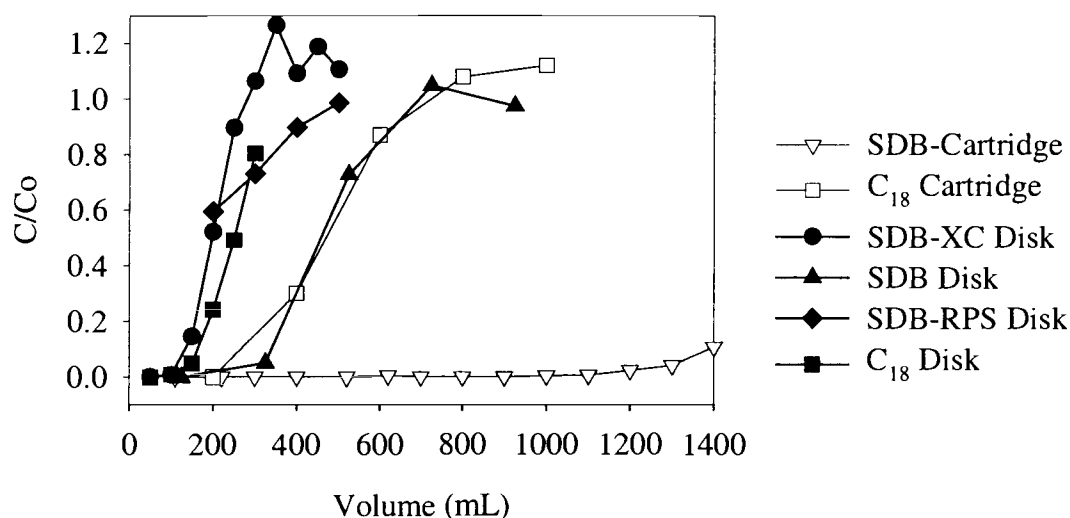
Figure 8   Breakthrough curve for benzylsuccinic acid (BSA) extracted from spiked groundwater by different types of sorbent in cartridge (0.5 g) and disk format (47 mm).

Because sorbents from breakthrough experiments were not eluted and sorbed

masses not determined, it was important to test the compatibility of eluates from

SDB cartridges with diazomethane derivatization and GC/MS analyses. Plastic cartridges containing 0.5 g SDB used to extract acidified water led to formation of a precipitate during the methylation reaction step with diazomethane. Formation of the precipitate caused generally low and variable recoveries of BSA in the range from 2 to 140 % with an average of 62% (n=29). As an alternative, the sorbent was packed into a glass tube fitted with PTFE frits. Four spiked 500-mL samples from the Kansas City site were extracted and the sorbent was eluted with 2x2 mL ethyl acetate. To demonstrate completeness of elution, an additional third eluate (0.5 mL) was collected. In all four cases, the third fraction contained < 1 % 4TFM and BSA of the amount collected in the first two fractions. Furthermore, no precipitation was observed during the methylation reaction step. Average recoveries from the Kansas City site groundwater were higher (86%) and less variable (standard error 2%) than those obtained during experiments with plastic columns. Therefore, glass tubes with PTFE frits filled with 0.5 g SDB were eluted with 2 x 2.5 mL ethyl acetate for all subsequent experiments.

Ground water samples contained varying amounts of brown-colored precipitate (from nearly absent to very turbid, brown-orange colored sample). The precipitate, which was probably formed from iron, was not present at sampling time and did not form in acid-preserved samples. In order to check if BSA co-precipitated, non-acidified samples were spiked with BSA in the field and stored for three days during which varying amounts of precipitate formed. Filtered samples where then analyzed for BSA by HPLC (faster analyses) as described before in this Chapter.

Average recovery for four replicates from each of four different wells were 100 ± 5 % (1 % standard error; n = 16), which indicated that BSA is not sorbed to the precipitate and therefore, that BSA concentrations are not affected by iron precipitation. However samples were filtered in all subsequent experiments to achieve faster flow rates during the extraction process.

2.4.4   Accuracy and Precision

Quantitative recovery from tap water was only obtained if glass columns for the extraction cartridge and beakers containing the acidified BSA-spiked tap water were silanized. We suspect that interactions of the BSA with the glass surface caused losses from acidic tap water whereas the organic acids and phenols present in the groundwater samples may compete with BSA for sorption sites on the glass. The surrogate standard 4TFM was measured relative to the internal standard 2CL. The average recovery for 4TFM from tap water was $84 \pm 1\%$ (average recovery $\pm$ standard error, n = 5, **Table 5**). The precision, indicated by the relative standard error (RSE), was $\pm$ 1%. The recovery of BSA was measured relative to that of the surrogate standard 4TFM. Recovery of BSA from tap water was $100 \pm 1\%$ (1% RSE).

Spike and recovery experiments also were performed using a groundwater composite because no single sample of sufficient volume was available to perform the necessary seven replicate analyses (143). The recoveries for 4TFM and BSA were 98 ± 1 % (1% RSE) and 88 ± 1 % (1% RSE), respectively. The recoveries for 4TFM and BSA were 86 ± 1 % (1 % RSE) and $100 \pm 1\%$ (1 % RSE), respectively,

from 16 blank groundwater samples collected over the course of the study. Note that the samples contained various amounts of iron precipitate. The equally good precision for these samples and the groundwater composite further supports the conclusion that the iron precipitate did not affect method performance.

Table 5   Accuracy and precision of the surrogate 4TFM (relative to internal standard 2CL) and BSA (relative to 4TFM) at two concentrations in 1 L tap water and groundwater composite samples. Results are given as average ± standard error (relative standard error).

Sample	n	BSA µg/L	Recovery Surrogate %	Recovery BSA %
Tap water	5	20	84 ± 1 (1 %)	100 ± 1 (1 %)
Groundwater composite	7	2	98 ± 1 (1 %)	88 ± 1 (1 %)

Knowledge of the limit of detection is critical for trace analyses. The detection limit is determined from the noise at or near zero concentration. The conventional method obtains detection limits from signal to noise values estimated from baseline variation and peak height (143). Due to the complex sample matrix, no steady baseline was available to estimate signal to noise ratios. Alternatively, the detection limit can be calculated as the standard deviation of repeated measurements of peak area (144). Therefore, we used the seven replicate analyses of the groundwater composite spiked with 2 µg/L; the signal at this concentration was low but above detection. The standard deviation of the seven replicates was multiplied by a factor of 3.14 (the Student's t value for a one-tailed test at the 99% confidence interval with 6 degrees of freedom) to obtain the method detection limit

of 0.2 µg/L (143). The upper and lower confidence levels of 0.5 and 0.1 µg/L respectively were obtained by multiplying the method detection limit by 2.2 and 0.64 respectively (percentiles of the chi square distribution at the 95% confidence interval with 6 degrees of freedom) (143). The method quantitation limit of 0.7 was calculated by multiplying the method detection limit by three (144). (Confidence interval of 0.4 to 1.4 µg/L, calculated in the same way as confidence interval for the method detection limit). To determine the source of the method variability, repeated injection (n=7) of two of the samples with 2 µg/L were performed. The standard deviation for seven injections of the same sample was not significantly lower (F-Test on a 99% confidence level) than the standard deviation for the seven extractions. Therefor, variations in the method are likely due to variations caused by the instrument, which includes injection variability.

2.4.5   Application to Groundwater Samples

Samples from a total of six different wells from the Northwest Terminal and the Kansas City site were analyzed for BSA and methyl BSA. Methy BSA, the degradation product of the three xylene isomers, was included in the demonstration phase of the methods development. Due to the limited amount of sample, replicate extraction analyses were not performed. A typical chromatogram (Northwest Terminal, CR13) is shown in **Figure 9**. Benzylsuccinic acid was eluted towards the end of the chromatographic run with a retention time window from 31.4 to 31.7 min. The methyl BSA isomers eluted later with peaks from 34.9 to 35.2 min and

35.3 to 35.6 min. Concentrations for BSA were below detection limit (0.2 µg/L) in

wells CR 15, 105s, 106s and 207 and below quantitation limit (<0.7 µg/L) in well

CR 12 and 3.2 µg/L in well CR 13 (**Table 6**). These concentrations were equivalent

to less than 0.01 mol % of background toluene concentrations (**Table 2**).

Concentrations were in general higher for methyl BSA. No methyl BSA was

detected in well 207 and methyl BSA concentrations were below quantitation limit

(<0.7 µg/L) in well 107s. Concentrations were 5.7, 6.6, 10.4, and 155 µg/L for

wells CR 12, 105s, CR 15 and CR 13 respectively (**Table 6**). These concentrations

of methyl BSA corresponded to 0.1 to 0.8 mol % of the background xylene

concentrations in each well. The mol % are nearly in the same range as reported by

the group of Suflita who found degradation product concentrations that were 3 to 4

orders of magnitude lower than their parent BTEX concentrations (67, 73). As

expected, degradation products were not detected in well 207, which did not

contain background toluene and xylene (**Table 2**).
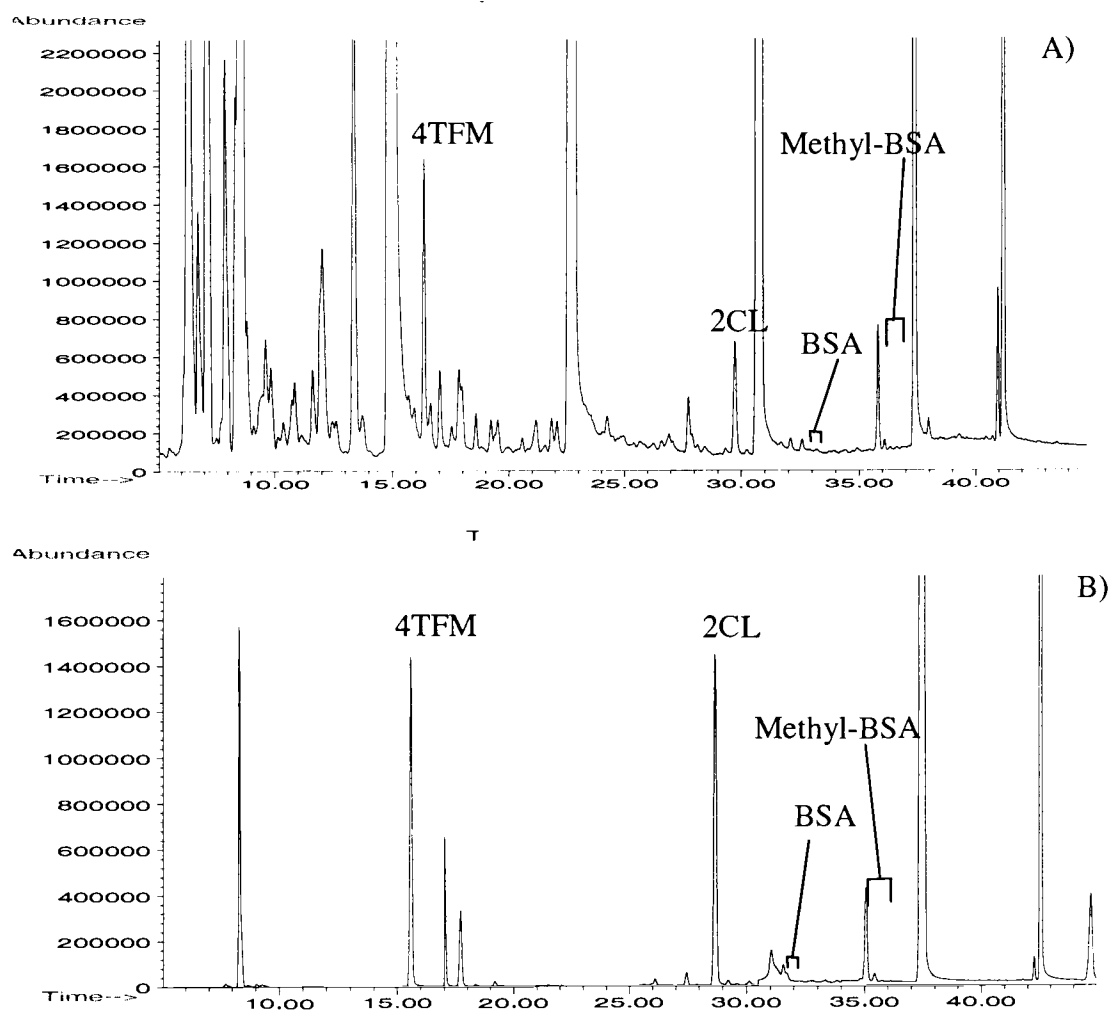
Figure 9 A) Total ion chromatogram obtained under A) scan mode and B) in single ion monitoring mode for a groundwater sample from the Northwest Terminal well CR13. Unlabeled peaks have mass spectra corresponding to isomers of hydrocarbons, phenolic compounds and carboxylic acids.

Table 6   Concentrations of BSA in single 0.25 to 1 L groundwater samples.

Site	Well	BSA µg/L	Methyl BSA µg/L
Northwest Terminal	CR12	<0.7[a]	5.7
	CR13	3.2	155
	CR15	ND	10.4
Kansas City Site	105s	ND	6.6
	106s	ND	<0.7
	207	ND	ND

ND: compound was not detected in this sample (detection limit = 0.2 µg/L)

[a] concentration was below quantitation limit (0.7 µg/L) but above detection limit of 0.2 µg/L.

2.5   CONCLUSIONS

A solid phase extraction method was developed for the isolation of BSA from BTEX-contaminated groundwater. A method detection limit of 0.2 µg/L was obtained using a total of 10 mL solvent per sample. Method conditions were optimized including the chromatographic conditions, the derivatization reaction, and the solid phase extraction process. For GC temperature gradients higher than 2.5 °C/min, increased variability in peak area ratios. The methylation reaction with diazomethane was complete after 5 min; however, compounds with double bonds were not stable during the reaction. A styrenedivinylbenzene sorbent in cartridge format was selected; however, commercially-available plastic columns interfered with the derivatization reaction. Therefore, plastic cartridges were replaced by glass tubes fitted with PTFE frits. Although quantitative recovery of BSA was obtained from BTEX-contaminated groundwater, for clean systems like tap water,

glassware had to be silanized in order to prevent sorption of BSA to glassware from acidified solution. It was demonstrated that iron precipitation did not affect concentrations of BSA.

To reduce the number of handling steps and time required for sample analysis samples should be acidified in the field. With this change in protocol, adjustment of the pH in the laboratory would not be necessary and the use of formalin as preservative, which is carcinogenic, could be avoided. Moreover, prefiltration of the samples would not be necessary since the red-brownish iron precipitates wold not form in acidified samples. Diazomethane could be eliminated by using N,O-bis(trimethylsilyl)trifluoroacetamide as demonstrated by Elshahed et al. (67). It is possible that this reagent would not interfere with the eluate from commercially available plastic columns so that packing of glass columns could be eliminated. Finally, derivatization and spiking of the internal standard could be performed in the elution vial and an aliquot could be transferred to the autosampler vial, thus avoiding the rinsing steps and recapping of autosampler vials.

2.6   ACKNOWLEDGEMENTS

3 DETERMINATION OF TRANSPORT BEHAVIOR AND BIODEGRADATION POTENTIAL OF TOLUENE AND XYLENE IN AQUIFERS WITH SINGLE-WELL TESTS AND DEUTERATED SURROGATES

3.1 ABSTRACT

Measuring fate and transport of benzene, toluene, ethylbenzene and xylene (BTEX) in the subsurface is a difficult task. Benzylsuccinic acid (BSA) and its methylated analog (methyl BSA) were suggested as unambiguous evidence of toluene and xylene biodegradation. Single-well push-pull tests with deuterated BTEX surrogates were used to assess transport and biodegradation of toluene and xylene in BTEX-contaminated wells. Retardation factors for injected deuterated toluene and background toluene were 2 and 14, respectively. This difference in retardation factors indicated that sorption is important for BTEX transport in these aquifers but that those processes affecting injected solutes and background contaminants are still not well understood. Even though we were not able to observe direct removal of injected toluene-d_8 and o-xylene-d_{10}, unambiguous evidence for biodegradation was obtained from (I) formation of unambiguous degradation products coupled with (II) utilization of nitrate. Zero-order degradation rates for deuterated toluene estimated from formation of BSA were 0.0004 to 0.001 day^{-1}. Wells where formation of deuterated BSA and methyl BSA were observed had lower concentrations of toluene and xylene relative to total BTEX than did

wells in which no deuterated BSA and methyl BSA were observed. This study is the first to report the use of deuterated BTEX surrogates in field tracer experiments.

3.2 INTRODUCTION

Gasoline contamination of the subsurface, mainly from underground storage tanks, is a widespread problem. Benzene, toluene, ethylbenzene and the xylene isomers, collectively known as BTEX are the components of major concern due to their high solubility (2, 3) and their toxicity. (4-6). The EPA drinking water criteria are 0.005 mg/L for benzene, 1 mg/L for toluene, 0.7 mg/L for ethylbenzene, and 10 mg/L for xylene isomers (7, 8).

Limitations and costs of conventional ground water cleanup technologies and soil treatment methods has made monitored natural attenuation of BTEX an attractive alternative approach to site remediation (81). Monitored natural attenuation includes physical, chemical and biological processes that lead to reduction in contaminant concentrations. Biological processes or "biodegradation" are important for decreasing concentrations of BTEX compounds. Since most sites with gasoline spills are anaerobic, BTEX degradation in absence of oxygen is the process of interest for bioremediation. BTEX-degrading cultures have been reported under denitrifying (29, 30 , 31-38, 45), sulfate-reducing (41-43, 47, 50-56), iron-reducing (39, 40, 46-48), manganese-reducing (49), phototrophic (44), and methanogenic (11, 47, 57, 58) conditions.

The National Research Council made recommendations on how to apply and document monitored natural attenuation (81, 82). Ideally multiple lines of evidence

are used to establish evidence for biodegradation occurring in the field. For example, identification of degradation products in-situ is one possible line of evidence. Another desirable line of evidence for in-situ biodegradation includes determination of in-situ contaminant transformation rates. Unfortunately rates are difficult to measure because of the limited accessibility of the subsurface. The anaerobic degradation pathway for toluene has been investigated in detail during the last few years (16-28, 59-62, 75). Benzylsuccinic acid (BSA) is the first intermediate during anaerobic microbial degradation of toluene. Beller et al. (71) proposed to use BSA and methyl BSA as unique indicators of the anaerobic degradation of toluene and the three xylene isomers. The relationship of BSA and methyl BSA to their respective parent compounds is well understood, they do not have commercial or industrial uses, and they have been detected outside of microorganisms. Beller et al. identify these characteristics as ideal indicators of anaerobic toluene and xylene degradation (71). To the best of our knowledge, few reports document occurrence of formation in BTEX-contaminated aquifers of these degradation products despite their potential for indicating in-situ biodegradation. (67, 71-73).

The typical approach is microcosm test studies (67), while fewer field tracer tests have been performed (71, 72). Istok et al. suggested to use single-well "push-pull" tests (114) to interrogate chemical and biological aquifer characteristics. Physical, chemical and biological aquifer characteristics have been measured this way (72, 86, 124-127). Push-pull tests can be conducted in existing monitoring

wells with relatively simple and inexpensive, and commercially-available materials. A push-pull test consists of an injection (push) of a test solution containing conservative tracers and selected reactive compounds (**Figure 10a**). This solution expands radially from the well, penetrating a volume of subsurface material that is determined by the volume of injected test solution and aquifer porosity. After a test period where the test solution drifts according to the regional groundwater flow field, the solution is extracted (pulled) and concentrations of solutes are measured in order to obtain breakthrough curves (**Figure 10b**).

a) injection phase   b) extraction phase
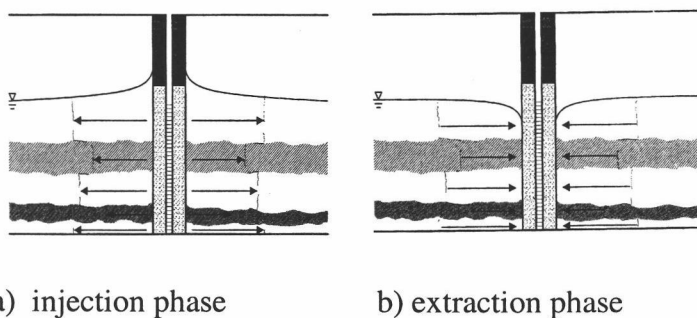
Figure 10   Injection and extraction phases of a single-well "push-pull" test.

Working at sites with background contaminant concentrations introduces problems because background contaminants potentially interfere with the interpretation of the experimental field data (129). Beller et al. used push-pull tests but took extra measures to remove background concentrations and injected a 'buffer'-solution to separate the test solution from the background (71). An

alternative to removing background concentrations is to use labeled surrogates to measure degradation rates. Good surrogates ideally have the same physicochemical, chemical and biological properties as the contaminant of interest. Isotope-labeled chemicals fulfill this requirement, however differences in rates of chemical and biological reaction can occur, due to the kinetic isotope effect. Tests with isotope-labeled surrogates allow for the quantitative evaluation of contaminant transformation during in-situ tests despite background contaminant concentrations. Beller et al. used deuterated BTEX compounds in laboratory studies to elucidate degradation pathways (17, 18, 24). The goal of the present study was to use deuterated surrogates in push-pull tests to measure transport behavior and in-situ degradation rates of toluene and o-xylene to benzylsuccinic acid analogues. To the best of our knowledge, deuterated compounds have not been used in-situ as surrogates of hydrocarbons even though the possibility is mentioned in the report of the National Research Council (81).

3.3   EXPERIMENTAL SECTION

3.3.1   Reagents and standard

Toluene-d_8 (99.95 atom % D) and o-xylene-d_{10} (>99 atom % D) were used for transformation tests and were purchased from Aldrich Chemical (Milwaukee, WI) Toluene-d_5 (99.5 atom % D) was used for the transport test and was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). 2,4-Dichlorobenzoic acid from Aldrich Chemical (Milwaukee, WI) and potassium bromide (Fisher, Fair Lawn, NJ)

were used as conservative tracers (145). Sodium nitrate (Mallinckrodt, Paris, KY) was used as electron acceptor. HCl 36.5-38 % (J. T. Baker, Phillipsburg, NJ) and formaldehyde solution 37 % (formalin, Fisher, Fair Lawn, NJ) were used to preserve samples. 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 (BSA; 99% purity) was purchased from Sigma Chemical (St. Louis, MO). Deuterated benzylsuccinic acid (BSA-d_8) and deuterated 2-methyl benzylsuccinic acid (o-methyl BSA-d_{10}) were obtained from Harry Beller (Lawrence Livermore Laboratory, CA). 2-Chlorolepidine (2CL, 2-chloro-4-methyl quinoline; 99% purity) and 4-(trifluoromethyl)hydrocinnamic acid (4TFM, 95% purity) were purchased from Aldrich Chemical (Milwaukee, WI). BSA, 2CL, and 4TFM were prepared in acetonitrile at 1 mg/mL. 2CL and 4TFM were used as the internal and surrogate standards, respectively. Acetone, methanol, and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Fairlawn, NJ). Ethyl acetate (HPLC-grade) was obtained from Mallinckrodt (Paris, KY).

3.3.2 Site Description

Experiments were performed at two field sites. The first site is a bulk fuel terminal located in Willbridge near Portland, OR (**Figure 11**). It will hereafter be referred to as the Northwest Terminal. The unconfined aquifer at this site consists of a layer of fill (medium dense to fine grained sand and silty sand) that rests on top

of alluvium consisting of clayey silt with sand interbedded with silty clays and clays. Total BTEX concentrations in the wells at the site range between 2 and 30 mg/L. A 71,900 L (19,000 gallons) release of ethanol occurred in 1999 from tank 58 (133). The site had a pre-existing dissolved hydrocarbon plume (133). Strongly-reducing conditions exist at this site and soluble nitrate and sulfate are below detection (**Table 7**). The water table was at about 2 to 3 m below land surface and it did not change more than 0.1 m in each single-well during the time of this study. Groundwater flow was towards east and was estimated to be about 100 m per year (133). Tests were conducted in 5 cm inner diameter wells with 3-m screened intervals starting at 1.6 m below land surface. Aerobic respiration and denitrification rates (**Table 7**) at the Northwest Terminal were measured in August 2000 by Istok with push-pull tests as described by Istok et al. (114). Harry Beller (Lawrence Livermore Laboratory, CA) determined that BSA was < 4µg/L (detection limit) while methyl BSA was 10 –230 µg/L in May 2000 (**Table 7**, unpublished data).
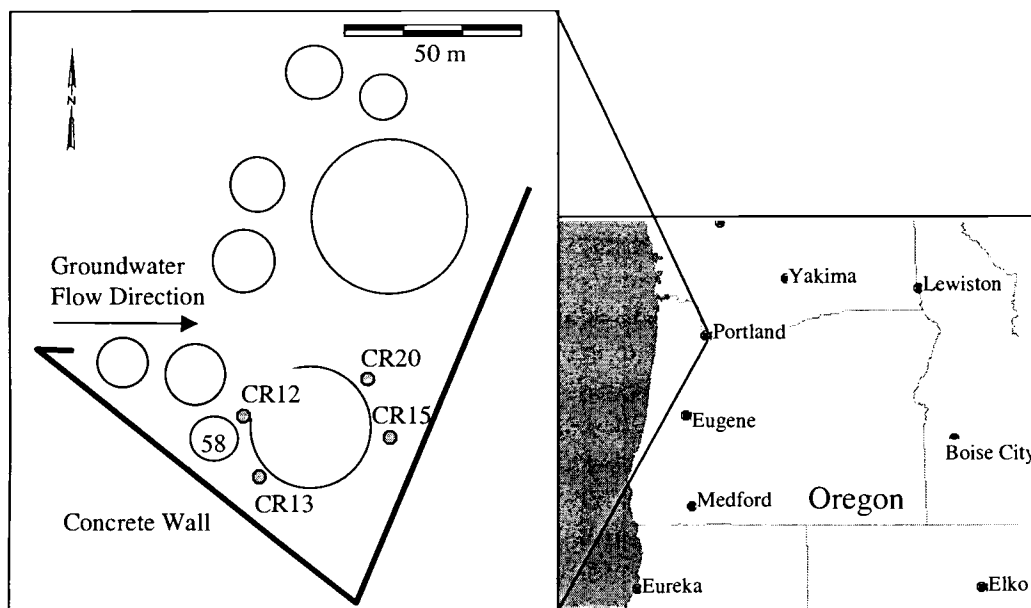
Figure 11   Map of the Northwest Terminal including the locations of the wells. The circles represent storage tanks. Ethanol was released from tank 58 in 1999.

The second field site is a former petroleum refinery near Kansas City, KS and hereafter will be referred to as the Kansas City site. It was operated from 1930 until 1982. After installation of monitoring wells, the presence of low-density non-aqueous phase liquid (LNAPL) was discovered over a 90 hectare area. Recovery operations initiated in 1984 recovered over 6 million L; however, recovery efficiency recently declined. Prior to this study, BTEX concentrations ranged from 0.065 to 5.28 mg/L (**Table 7**) (134). The unconfined aquifer consists of fine sand with clayey silt or silt (to ~3 m below land surface) covering sand (below ~3 m below land surface) (135). Although groundwater flows generally southeast towards the Missouri River, in the spring, high stage elevations of the Missouri River cause the hydraulic gradient to shift reverse inward toward the site. (136). At the time of the experiments, groundwater velocities were 0.15 ft/day (137) and the

water table was about 7 to 9 m below land surface, which were low water table conditions for this site. Tests were conducted in wells that had 3-m screened intervals with the top of the screen located at 4.6 to 7 m below land surface.
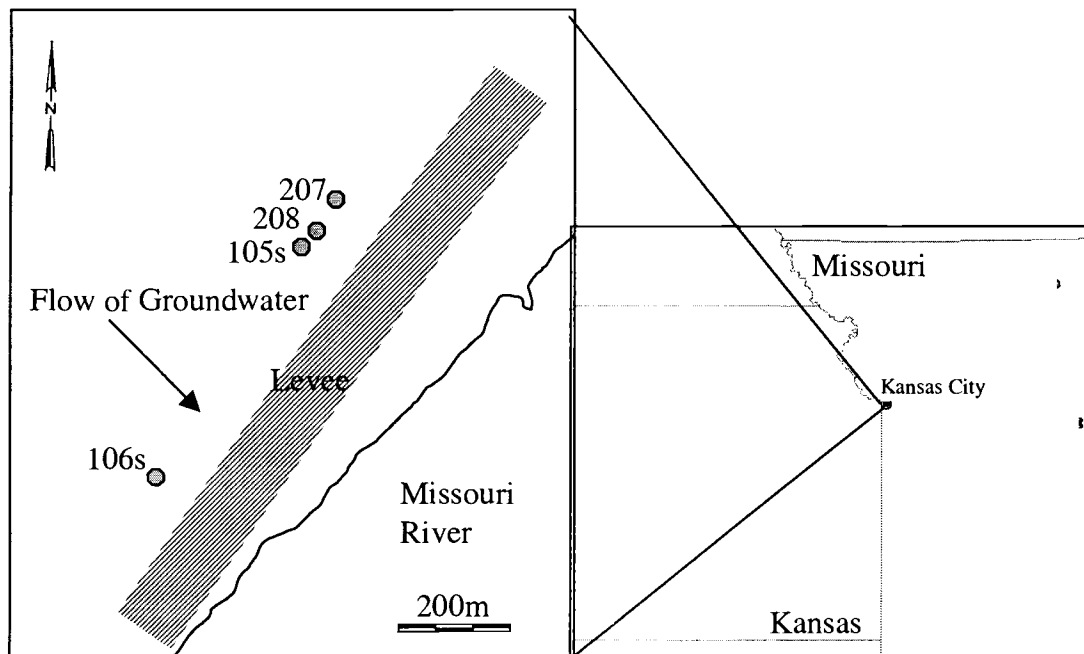
Figure 12   Map of the Kansas City site including locations of the wells and general groundwater flow direction.

3.3.3   Push-Pull Tests

Push-pull tests were conducted to obtain information on the transport and transformation of deuterated toluene and xylene..

Table 7   Characterization of groundwater in wells used for transport and transformations tests.

Location	Northwest Terminal		Kansas City site		
	Well CR12	Well CR15	Well 105 s	Well 106 s	Well 207
NAPL observed in sample [b]	NO	YES	NO	NO	YES
Benzene mg/L [b]	0.15	13	4.2	0.02	ND
Toluene mg/L [b]	2.8	0.1	0.32	ND	ND
Ethylbenzene mg/L [b]	0.6	0.5	0.38	0.032	1.9
Xylene mg/L [b]	3.6	1.8	0.38	0.013	ND
Total BTEX mg/L [b]	7.15	15.4	5.28	0.065	1.9
Toluene relative to background BTEX %	39	1	6	0	0
Xylene relative to background BTEX %	50	12	7	20	0
Ethanol mg/L [b]	48	<2 [a]	N/A	N/A	N/A
DO mg/L [b]	0.5	ND	0.65	1.81	ND
Nitrate [b]	ND	ND	ND	ND	ND
Iron mg/L [b]	N/A	N/A	39	45	N/A
Manganese mg/L [b]	5	12.5	N/A	N/A	N/A
Sulfate mg/L [b]	< 1 [a]	< 1 [a]	7.6	6.9	1.8
Methane mg/L [b]	10	11	18	14.3	N/A
Respiration µM/h [c]	84	43	N/A	N/A	N/A
Denitrification µM/h [c]	4	16	N/A	N/A	N/A
BSA µg/L [d]	ND	ND	N/A	N/A	N/A
Methyl BSA µg/L [d]	230	10	N/A	N/A	N/A

ND: not detected

N/A: not analyzed

[a] Detected below quantitation limit

[b] From site responsible person

[c] Measured by J. Istok in August 2000

[d] Measured by H. Beller in May 2000

3.3.3.1 Transport Test

A single transport test was conducted in Well CR-12 at the Northwest Terminal. Toluene-d_5 was selected for the transport test since it was distinguishable

from background toluene and toluene-d_8, which would be used in subsequent transformation push-pull tests. In order to prevent volatilization of toluene-d_5, a concentrated aqueous solution (containing all the solutes) was prepared and then pumped into a collapsible metalized-film gas sampling bag (Chromatography Research Supplies, Addison, IL), which hereafter will be further referred to as metalized bag.

In order to get stable injection concentrations, the test solution was transferred into the metalized bag at least 12 hours before a test began and allowed to equilibrate. Once the metalized bag was connected to the injection lines the lines were purged to allow equilibration with the test solution for at least 10 minutes prior to injection. The two equilibration times were determined in preliminary laboratory experiments. The contents of the metalized bag were metered and diluted (1:50) into the main flow (**Figure 13**) with a piston pump (Fluid Metering Inc., Oyster Bay, NY). The tap water in the carboy was not purged of oxygen for this test since the residence time of the solution was less than 4.5 hours and no biological transformation was expected for this time frame. A total of 50 L test solution consisting of 2 mg/L 2,4-dichlorobenzoic acid as a conservative tracer (145), 0.8 mg/L toluene-d_5, and 2.5 mg/L BSA in tap water was injected into the well through 6-mm nylon-braided tubing (Kuryama Co., Santa Fe Springs, CA) with a Masterflex peristaltic pump (Barnant Co., Barrington, IL) at a flow rate of 0.5 L/min. Over the course of the injection, five samples were taken from the sampling valve and analyzed for 2,4-dichlorobenzoic acid, BSA, and toluene-d_5 to

determine the concentrations in the test solution (C_0). The elevation of the water table was measured over the course of the injection and did not increase more than 0.2 m.

Immediately after the injection ended, the test solution/groundwater mixture was extracted at a flow rate of 0.5 L/min. Samples were collected every 2 to 4 L and analyzed for BSA, 2,4-dichlorobenzoic acid, toluene-d_5, and background BTEX. A total of 100 L of groundwater were extracted. Samples for determination of volatile organic compounds were collected in 40 mL volatile organic analysis vials without headspace, preserved with 0.75% (v/v) concentrated HCl, shipped on ice, and stored at 4 °C until analysis. Kovacs and Kampbell showed that this sample treatment is acceptable for BTEX but losses might occur for more hydrophobic hydrocarbons (146). Samples for BSA and 2,4-dichlorobenzoic acid were collected in glass bottles (250 mL), preserved with 5%(v/v) formalin, shipped on ice, and stored at 4 °C until analysis.
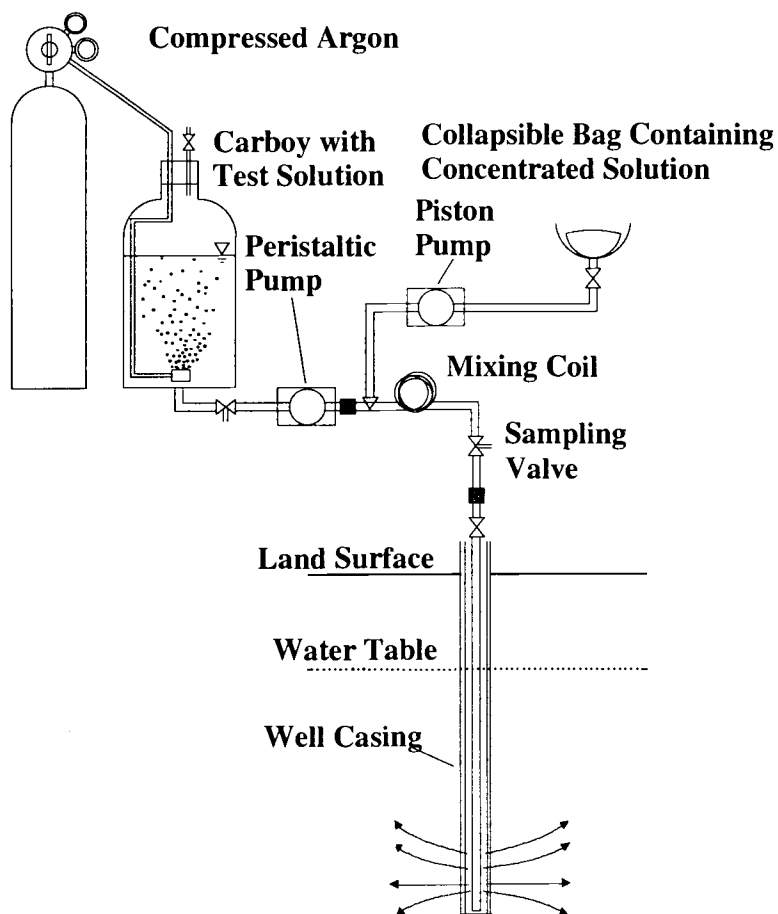
Figure 13   Experimental setup for injection of test solution containing volatile compounds (not drawn to scale)

3.3.3.2 Transformation Tests

A series of transformation tests was performed to assess the potential for in-situ biodegradation of toluene-d_8 and o-xylene-d_{10} in five wells at two different sites (listed in **Table 7**). In order to prevent volatilization of toluene-d_8 and o-xylene-d_{10}, a concentrated solution of toluene/bromide or toluene/xylene/bromide in water was pumped from a metalized bag and combined in-line with nitrate-

amended tap water that had been sparged overnight with argon to remove dissolved oxygen (DO) (**Figure 13**).

A total of two hundred and fifty liters of the test solution was injected at a rate of 0.5 to 2 L/min. The final test solution consisted of 100 mg/L bromide, 250 mg/L nitrate, 0.4 to 2.5 mg/L toluene-d_8, and 0.4 to 1.0 mg/L o-xylene-d_{10}. o-Xylene-d_{10} was only injected during tests conducted at the Northwest Terminal. Sulfate was present in the tap water used for the tests at the Kansas City site (200 mg/L) and the Northwest Terminal (10 mg/L). Over the course of the injection ten samples were taken from the sampling valve (**Figure 13**) and analyzed for bromide, nitrate, sulfate, DO, toluene-d_8, and o-xylene-d_{10}. During the injection phase, the water level in each well increased by less than 0.3 m.

During the extraction phase, samples were taken daily to biweekly for up to 30 days. Before the sample was taken, three times the well casing volume was purged if possible and DO was measured (CHEMetrics, Calverton, VA). Samples for volatile organic analytes and BSA and methyl BSA were treated the same way as described for the transport test. Samples for anion analysis were collected in a 40-mL volatile organic analysis (VOA) vials without headspace, shipped on ice, and stored at 4 °C until analysis.

3.3.4   Analytical Methods

3.3.4.1   Quantitative Determination of Volatile Organic Analytes (VOA)

Two analytical methods were used to determine the concentrations of toluene-d_8 and o-xylene-d_{10} in groundwater samples. For higher concentrations in the injected test solutions, an 'in-vial' method was used. Samples containing lower concentrations (e.g., extraction-phase samples) were analyzed by purge and trap with gas chromatography/mass selective detection (GC/MSD).

For the 'in-vial' method, 0.5 mL methylene chloride was placed in a 2 mL autosampler vial with 5 μL of the 4-BFB (2 ng/μL) internal standard. To this mixture, 1.0 mL of a groundwater sample was added. The vials were capped and mixed on a Vortex mixer for 15 s. This mixing time was determined in preliminary experiments to allow equilibration of solute concentrations between the two phases. The phases separated in 1 min and the sample vial was placed on the autosampler tray for analysis by GC/MSD. Calibration curves were constructed for BTEX, toluene-d_8 and o-xylene-d_{10} with concentrations ranging from 0.02-50 mg/L.

For purge and trap analyses, samples in 40 mL VOA vials (no headspace) were placed on a Tekmar-Dohrmann 3100 Sample Concentrator (Cincinnati, OH) equipped with an AQUATek 70 autosampler and a Tenax/Silica Gel/Charcoal trap (Tekmar-Dohrmann). Prior to analysis, 2 μL of 4-BFB internal standard (100 ng/μL) was added to a sample volume of 25 mL. The following conditions were used for 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. Calibration curves

were constructed from BTEX, toluene-d_8, and o-xylene-d_{10} standards ranging in concentration from 0.4 μg/L to 600 μg/L.

Separation and detection were performed on a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5972 mass selective detector (MSD). The GC was equipped with a 30 m x 0.32 mm x 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. Helium was the carrier gas. 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 electron impact mode with a source temperature of 265 °C. For quantification in single ion monitoring mode, one ion was used for identification and quantification and a second ion was used for confirmation (**Table 8**). Retention times were determined from injection of authentic standards.

Table 8   Ions used to detect and quantify volatile analytes of interest.

Analyte	Quantitation Ion	Qualifier Ion(s)
Benzene	78	51
Toluene	91	65, 51
Ethylbenzene	106	91
Xylene isomers	106	91
Toluene-d_8	100	98
O-xylene-d_{10}	116	98
4-BFB	174	95

3.3.4.2 Quantitative Determination of BSA and methyl BSA

Samples (≤ 1 L) were acidified to pH 2, spiked with 20 µg 4TFM and prefiltered through a 1 µm Whatman glass-fiber filters. Samples were then extracted onto a preconditioned styrenedivinylbenzene column (0.5 g). Degradation products were eluted with 2x2.5 mL ethyl acetate, concentrated under a stream of nitrogen, spiked with 20 µg of the internal standard (2CL), and methylated with diazomethane.

Samples were analyzed on a Hewlett Packard Model 6890 gas chromatograph equipped with a Model 5972 mass selective detector (MSD) and a 30 m x 0.32 mm x 4 µm SPB-1 capillary column (Supelco Inc., Bellefonte, PA). The injector was operated at 250°C under splitless conditions with a 1 µL injection volume. An initial oven temperature of 130°C was increased at 2.5 °C/min to 217 °C and then ramped at 10 °C/min up to 265 °C, which gave a total run time of 45 min.

The MSD was operated in electron impact mode with a source temperature of 265 °C. For quantification in single ion monitoring mode, two ions were used to identify and quantify each analyte in their methylated forms (**Table 9**). Because only very small masses (~2 µg) of authentic standards of BSA-d_8 and o-methyl BSA-d_{10} were available, they were only used to obtain mass spectra and retention times. Since mass spectra of BSA and BSA-d_8 indicated similar fragmentation patterns (data not shown), concentrations of BSA-d_8 were determined from calibration curves for BSA assuming a response factor of 1. Furthermore, concentrations of o-methyl BSA-d_{10} and background methyl BSA also were

determined from BSA calibration curves assuming a response factor of 1. The calibration curve for BSA was linear from 0.05 µg/L to 50 µg/L. The method detection limit for BSA was 0.2 µg/L as described in Chapter 2.

Some samples from the transport test were analyzed for metabolites using an extraction method based on strong anion exchange SAX similar to the method described by Field and Monohan (147). In short, surrogate-spiked (4-fluorobenzoic acid, Sigma Chemical, St. Louis, MO) samples were extracted onto a preconditioned 13-mm SAX disk. For in-vial elution and derivatization, the disk was placed in a 2-mL autosampler vial together with 20 µg internal standard (2CL), 1 mL acetonitrile, and 200 µL methyl iodide (Aldrich Chemical, Milwaukee, WI). The vial was then capped and heated to 80 °C for 1 h. Note that excess methyl iodide was not removed as described by Field and Monohan but samples were analyzed without further manipulation after reaction. Samples were then analyzed by the same GC/MS method as previously described.

Table 9   Ions used to detect and quantify benzylsuccinic acid (BSA) and related compounds.

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
4TFM	172	232 / 159
2CL	177 / 179	142

3.3.4.3 Quantitative Determination of Anions

Bromide, nitrate, and sulfate concentrations were determined by ion chromatography on a Dionex DX-120 (Sunnyvale, CA) with electrical conductivity detector and a Dionex AS14 column. External calibration was used for standards containing between 5 and 100 mg/L bromide and sulfate and 20 and 450 mg/L nitrate. The quantitation limit was at about 1 mg/L.

3.4 RESULTS AND DISCUSSION

3.4.1 Transport test

A single transport test was performed in well CR12 at the Northwest Terminal (**Figure 11**) in order to get information on the transport of toluene-d_5 and BSA. Breakthrough curves for the extraction phase show the relative concentration (C/C_0) for each solute, where C is the measured concentration and C_0 is the injected concentration, vs. the (cumulative) extracted volume divided by the total injected volume of test solution (**Figure 14**). Fitting parameters of the advection-dispersion equation to obtain a good agreement with the data for the conservative tracer 2,4-dichlorobenzoic acid (145) using the method described by Schroth et al. resulted in an estimated dispersivity of 0.15 cm (127). The breakthrough curve for toluene-d_5 indicated deviations relative to the conservative tracer. Deviations were negative at the beginning and positive at the end of the test, because retardation occurred during the test. A retardation factor of 2 was calculated using the method described by Schroth et al. (127). Retardation reduced the recovery of toluene-d_5

(97.5 % of mass injected) relative to the recovery of the conservative tracer (99.6%), which indicates that essentially no biodegradation of toluene-d_5 occurred during the short time frame of the experiment (4.5 hours). However, background toluene indicated a different sorption behavior (**Figure 14**), which resulted in a retardation factor of 14 (127). Different rates for sorption and desorption as observed here have been reported in laboratory and field (113, 148, 149) and this phenomena has been reviewed elsewhere (150).

O   2,4-Dichlorobenzoic acid (conservative tracer)
——— Analytical solution for a conservative tracer
■   Toluene-d_5
—— Analytical solution for a sorbed compound
      with retardation factor 2
◇   Benzylsuccinic acid
▲   Background Toluene
—— - Analytical solution for a background
      compound with retardation factor 14
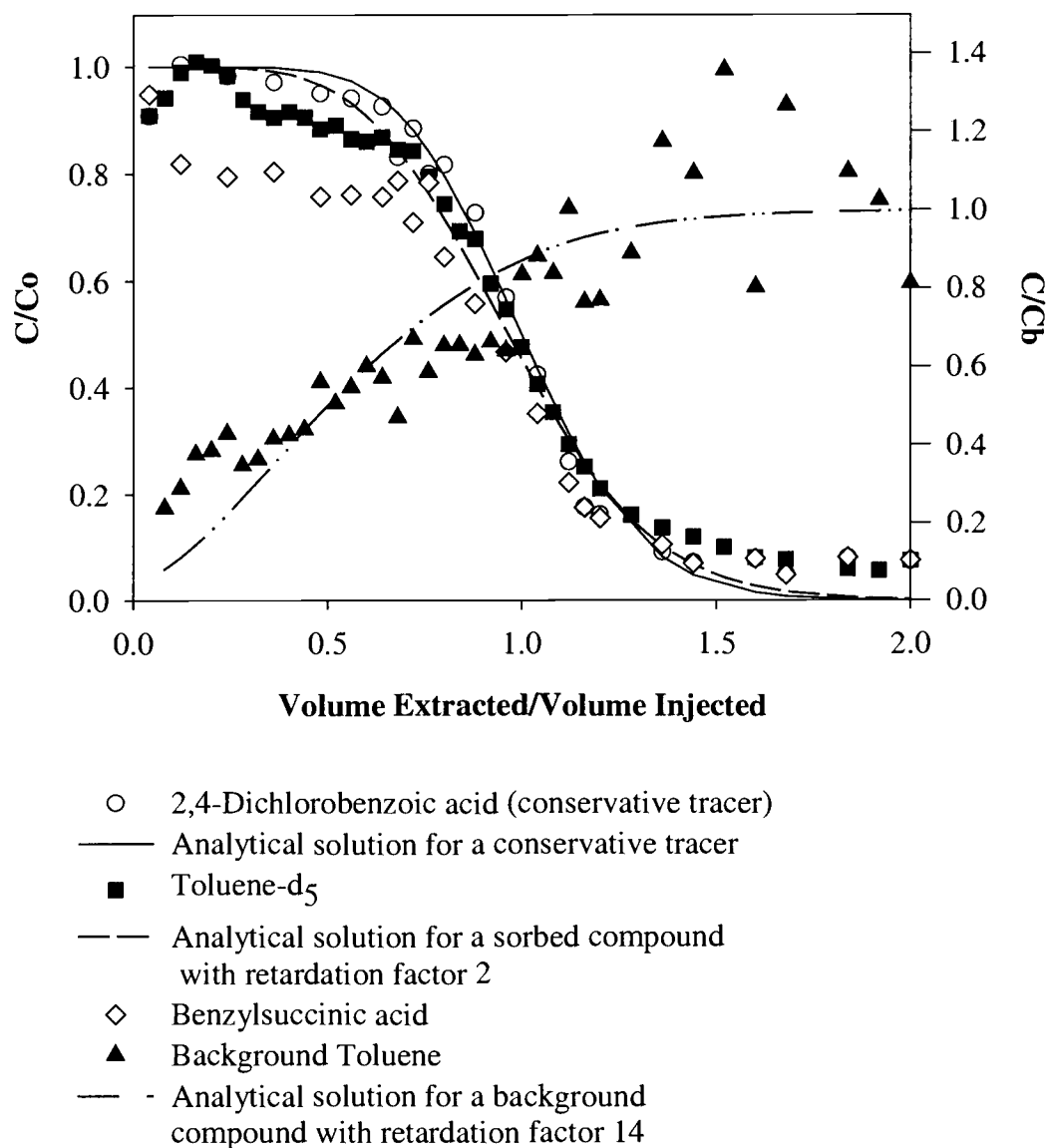
Figure 14   Transport test breakthrough curves for 2,4-dichlorobenzoic acid (conservative tracer), BSA, toluene-d_5, and background toluene in well CR12 at the Northwest Terminal. Lines correspond to fitted analytical solutions.

Concentrations of BSA in the second part of the test followed the conservative tracer closely, indicating conservative transport. However, a maximum relative concentration of only 0.8 was observed for BSA during the first part of the test and

relative concentrations of BSA were less than those of 2,4-dichlorobenzoic acid up to (Volume extracted/Volume injected) ~ 0.7. Accordingly, recovery of injected BSA mass was smaller (84.5%) than that of 2,4-dichlorobenzoic acid. No explanation was found for the loss of compound. Loss by chemical or biological reactions does not explain the observed curve because lower concentrations for BSA due to biodegradation would be expected especially during the last part of the experiment and not only during the first part. Overall the shape of the breakthrough curve and physicochemical properties of BSA indicated no retardation for BSA. Conservative BSA transport has previously been observed for push-pull tests conducted in a BTEX-contaminated well near OSU (unpublished data).

3.4.2 Transformation test

The purpose of the transformation tests was to evaluate the potential of deuterated surrogates to detect and quantify in-situ biodegradation of toluene and xylene in BTEX-contaminated aquifers. Transformation tests were performed in wells CR-12 and CR-15 at the Northwest Terminal and in wells 105s, 106s and 207 at the Kansas City site. All wells had a history of BTEX contamination and were under oxygen depleted conditions (**Table 7**). BTEX concentrations were greater at the Northwest Terminal and concentrations for xylene isomers were higher than concentrations for toluene (**Table 7**).

3.4.2.1 Well CR 12 Northwest Terminal

The transformation test performed in well CR 12 at the Northwest Terminal hereafter will be referred to as CR12 - transformation test. A decrease in relative bromide concentrations from 1 to 0 within 8 days indicated transport and dilution of the test solution (**Figure 15**). Relative concentrations of toluene-d_8 were similar to those of bromide for sampling times < 3 days and greater at times $\geq$ 3 days, which indicated retardation of the toluene-d_8.

Optimal correction factors for dilution are the relative concentrations (C/C_0) of a non-reactive tracer that has similar transport behavior (124). Since no such tracer was available for toluene-d_8 and o-xylene-d_{10}, their relative concentrations were corrected for dilution by dividing by relative bromide concentrations (**Figure 16A**). Dilution-adjusted values equivalent to 1 indicate that a solute behaved in a manner similar to that of bromide. Values less than 1 indicate removal relative to bromide while values greater than 1 indicate that the samples are enriched in the solute relative to bromide. Increasing dilution-adjusted concentrations for toluene-d_8 reflect the higher relative concentrations at sampling times $\geq$ 3days and indicated retardation (**Figure 16A**). Since the transport of toluene-d_8 was not similar to that of bromide, the dilution-adjusted toluene-d_8 data could not be used to determine if biodegradation had occurred.
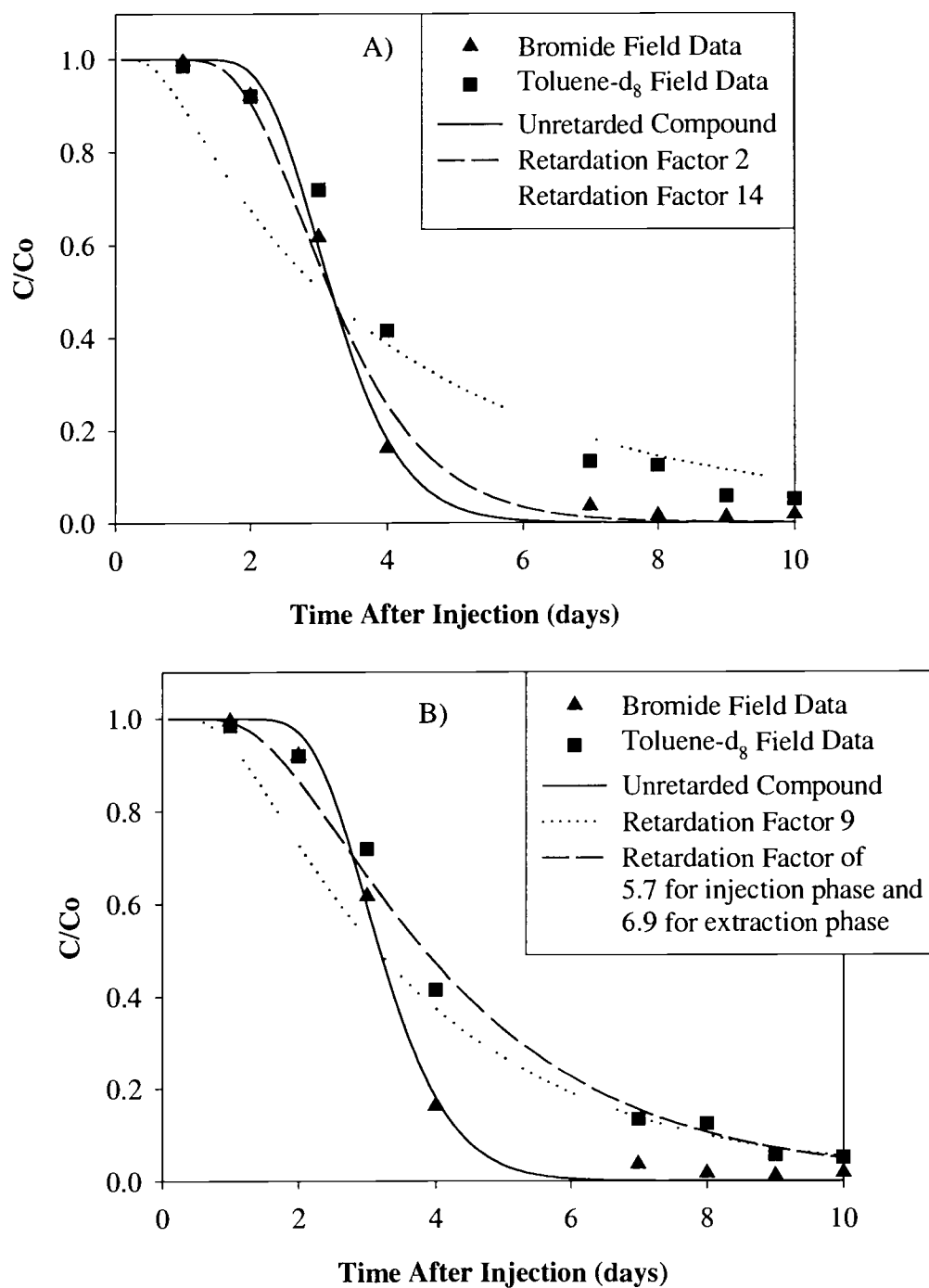
Figure 15 Breakthrough curves for bromide and toluene-d$_8$ from the transformation test in well CR 12 at the Northwest Terminal. The lines show analytical solutions for the one dimensional advection-dispersion equation with a pulse as initial condition.
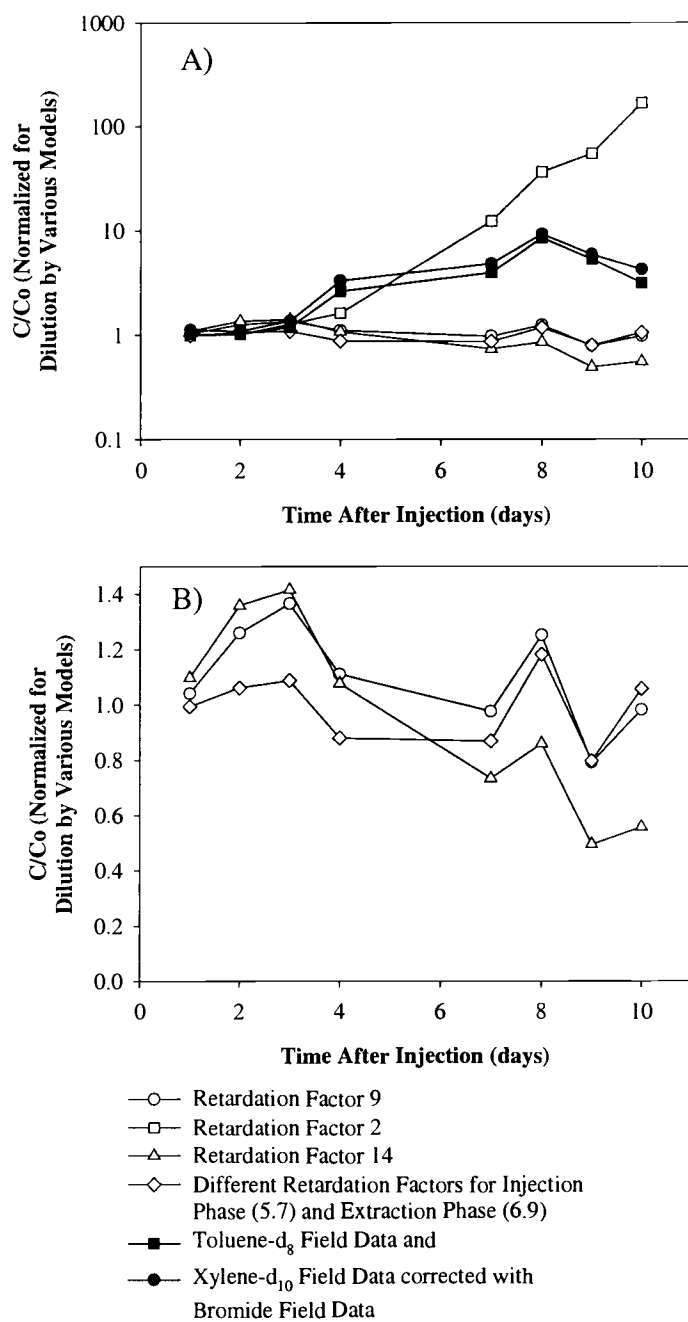
Figure 16 Dilution-adjusted breakthrough curves for toluene-d$_8$ and xylene-d$_{10}$ from the transformation test in well CR 12. Relative concentrations (C/Co) from different analytical solutions for the one-dimensional advection-dispersion equation with a pulse as initial condition (outlined symbols, toluene-d$_8$ data only) and actual bromide field data (filled symbols, toluene-d$_8$ and xylene-d$_{10}$ data) were used as correction factors. Panel A shows the relative concentrations on a logarithmic scale whereas panel B shows a detail section of panel A on a linear scale.

Even though the breakthrough curves of toluene-d_8 and xylene-d_{10} could not be used as one line of evidence for in-situ biodegradation, further analyses were performed on the breakthrough curves to understand transport better. An approximate analytical solution for the linear, one-dimensional advection-dispersion equation (ADE) with a pulse as initial condition (151) was used to model field data. The approximation was valid at all times.

The length of the pulse was the only parameter that was calculated from test conditions. The pulse length was assumed to be equal to twice the radius of the injected solution which in turn was calculated from the injected volume, the screened interval, and the porosity (assumed to be 0.3). The parameters (dispersivity and groundwater velocity) of the ADE were fitted (least squares fit) to get good agreement between the analytical solution and the data for the breakthrough curve for bromide (**Figure 15A**). The fit resulted in a dispersivity of 0.9 cm. A higher dispersivity than that observed during the transport test (0.15 cm) was expected based on the longer test duration and the differing flow fields. The flow field during the short-term transport test was radial, dominated by the injection and extraction of the test solution, whereas the flow field was mainly influenced by the natural gradient during the long-term transformation test. The estimated groundwater flow of 10 m/year estimated from the transformation test was one order of magnitude lower than that estimated by Buscheck and O'Reilly (133).

Dispersivity, groundwater velocity and the radius of the injectate were divided by the retardation factor in order to get ADE solutions for toluene-d_8 and xylene-d_{10}. The retardation factors obtained from the transport test (2 and 14 for injected and background toluene, respectively) gave solutions that circumscribed the actual data (**Figure 15A**). The two curves were used to correct toluene-d_8 values for dilution and resulted in increasing dilution-adjusted concentrations for the retardation factor of 2 (**Figure 16A**) and decreasing concentrations for the retardation factor of 14 (**Figure 16B**). In an alternative approach, biodegradation was assumed to be zero and a least square fit revealed that a retardation factor of 9 was necessary to reach dilution-adjusted toluene-d_8 concentrations of one (**Figure 16B**). Note that these dilution-adjusted concentrations increased during the first three days. The same difference between analytical solution and actual data is also indicated by higher relative concentrations at times < 4 days (**Figure 15B**). At times > 4 days relative toluene-d_8 concentrations were well matched by the analytical solution. No better match was obtained because the model requires the relative concentration of 0.5 for retarded and unretarded compounds to be at the same time. However, data from the push-pull test did not meet that requirement (**Figure 15**).

The model was expanded based on the observation that retardation factors during the transport test were different for injected compounds and background compounds (**Figure 14**). The expanded model was fitted to obtain dilution-adjusted concentrations close to 1 (**Figure 16B**) by allowing different retardation factors for

injection phase and extraction phase. Retardation factors were 5.7 for the injection phase and 6.9 for the extraction phase and gave a better fit of the data at times < 4 days and resulted in an equally good match at times > 4 days (**Figure 15B**). Dilution-adjusted values obtained using two retardation factors did not show the increase during the first three days as observed for dilution-adjusted concentrations obtained from the solutions using a single retardation factor (**Figure 16B**). Results for the model indicated that transformation test results can be described by the linear, one-dimensional ADE with equilibrium sorption. The model also indicated that knowledge of the appropriate retardation factors is necessary to avoid increasing dilution-adjusted toluene-d_8 (and o-xylene-d_{10}) concentrations, which have no physical interpretation.

The relative concentrations for the degradation products (carboxylic acids), sulfate, and nitrate were corrected with the relative concentration of bromide. No BSA-d_8 or o-methyl BSA-d_{10} was observed during the transformation test, even though decreasing dilution-adjusted nitrate concentrations indicated denitrification (**Figure 17**). Nitrate was utilized within 8 days. A linear zero-order degradation rate of 22 μM/hr (**Table 10**) was calculated by fitting a line to the dilution-adjusted concentrations. The denitrification rate from the transformation test was within the range reported by Istok et al. for another BTEX-contaminated site (1-80 μM/hr) (114) but was higher than that measured with a one day test conducted in August 2000 in the same well (4 μM/hr **Table 7**). Increasing dilution-adjusted sulfate concentrations probably occurred because of sulfate in the background and

indicated absence of sulfate reduction. As expected, sulfate reduction did not occur when nitrate was present.

Background BTEX concentrations observed towards the end of the push-pull test (results not shown) agreed well with concentrations provided by the site owner (**Table 7**). Concentrations of non-deuterated BSA and methyl BSA in samples obtained at the end of the test were detected below the quantitation limit and 5 µg/L, respectively. The background toluene and xylene concentrations were 2.8 mg/L and 3.6 mg/L respectively (**Table 7**), which resulted in a molar ratio of c(BSA)/c(toluene) of < 0.01mol% and c(methyl BSA)/c(xylene) of 0.07 mol% respectively (**Table 12**).
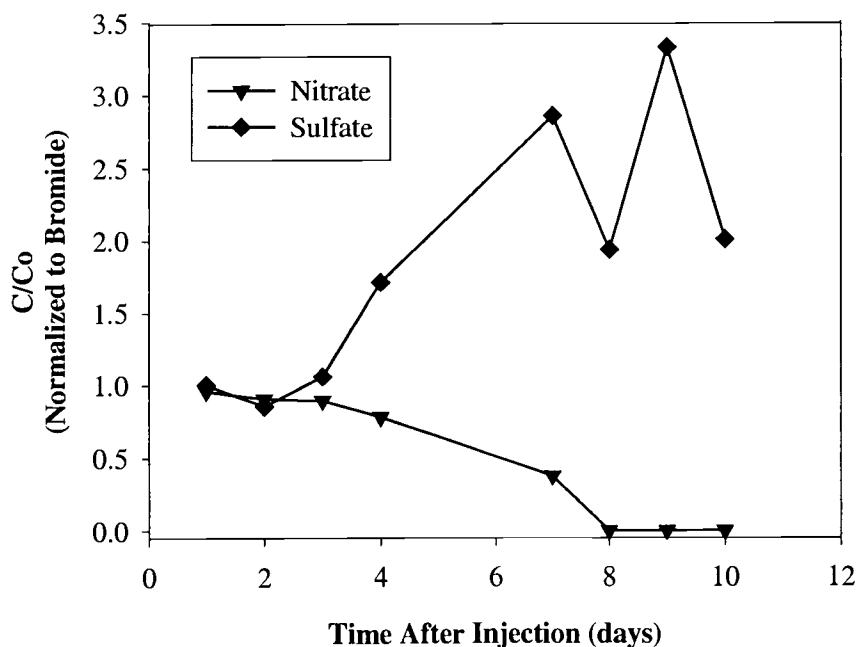

Figure 17   Dilution-adjusted breakthrough curves for nitrate and sulfate in well CR 12 at the Northwest Terminal.

Table 10   Transformation rates for nitrate removal and BSA-d$_8$ formation.

		CR12	CR15	105 s	106 s	207
Zero-order denitrification rate	μM/hr	22	9	18	24	4
Zero-order BSA-d$_8$ formation rate	μg/Lday	ND	1.5	ND	0.7	ND

Table 11   Maximum concentrations of deuterated metabolites, absolute (μg/L) and relative to parent compound (concentration in mol% on day of maximum metabolite concentration).

		CR12	CR15	105 s	106 s	207
BSA-d$_8$	μg/L	ND	1.2	ND	1.1	ND
BSA-d$_8$	mol%	N/A	0.1	N/A	0.1	N/A
o-Methyl BSA-d$_{10}$	μg/L	ND	<0.7	N/A	N/A	ND
o-Methyl BSA-d$_{10}$	mol%	N/A	<0.2	N/A	N/A	N/A

Table 12   Concentrations of background metabolites measured at the end of the test, absolute (μg/L) and relative to parent compound (mol%).

		CR12	CR15	105 s	106 s	207
BSA	μg/L	<0.7	ND	ND	ND	ND
BSA	mol%	<0.01	N/A	N/A	N/A	N/A
Methyl BSA	μg/L	5	10	5	<0.7	ND
Methyl BSA	mol%	0.07	0.3	0.6	<2	N/A

3.4.2.2   Well CR 15 Northwest Terminal

Dilution-adjusted concentrations for toluene-d$_8$ and o-xylene-d$_{10}$ increased in a similar way (**Figure 18**) as described for the CR12 - transformation test, so that that

data could not be used to determine if biodegradation occured. However, strong evidence was provided by appearance of BSA-d_8 and o-methyl BSA-d_{10} (**Figure 18**). The formation of these distinctive degradation products is direct evidence for the presence of indigenous microorganisms with the capability to degrade toluene and xylene at these sites. BSA-d_8 and o-methyl BSA-d_{10} concentrations increased from day 5 through day 7 and then decreased to zero on day 9. Molar ratios at the maximum BSA-d_8 and o-methyl BSA-d_{10} concentrations were calculated as c(BSA-d_8)/c(toluene-d_8) of 0.1 mol% and c(o-methyl BSA-d_{10})/c(o-xylene-d_{10}) of <0.2 mol% (**Table 11**) indicate that only a small parts of toluene-d_8 and o-xylene-d_{10} were transformed to BSA-d_8 and o-methyl BSA-d_{10} during the test. The small parts of toluene-d_8 and o-xylene-d_{10} transformed support the assumption of no significant transformation that was made for the analytical solution fitting well CR12 data. An initial zero-order formation rate for BSA-d_8 of 1.5 µg/Lday (**Table 10**) was calculated from the dilution-adjusted values during the time when concentrations increased and corresponded to a minimum toluene-d_8 degradation rate. Since o-methyl BSA-d_{10} was detected below the quantitation limit, no rate was determined. Dilution was not the reason for decreasing concentrations, since values were adjusted to bromide. Therefore, further degradation is the suggested reason for loss of deuterated degradation products.
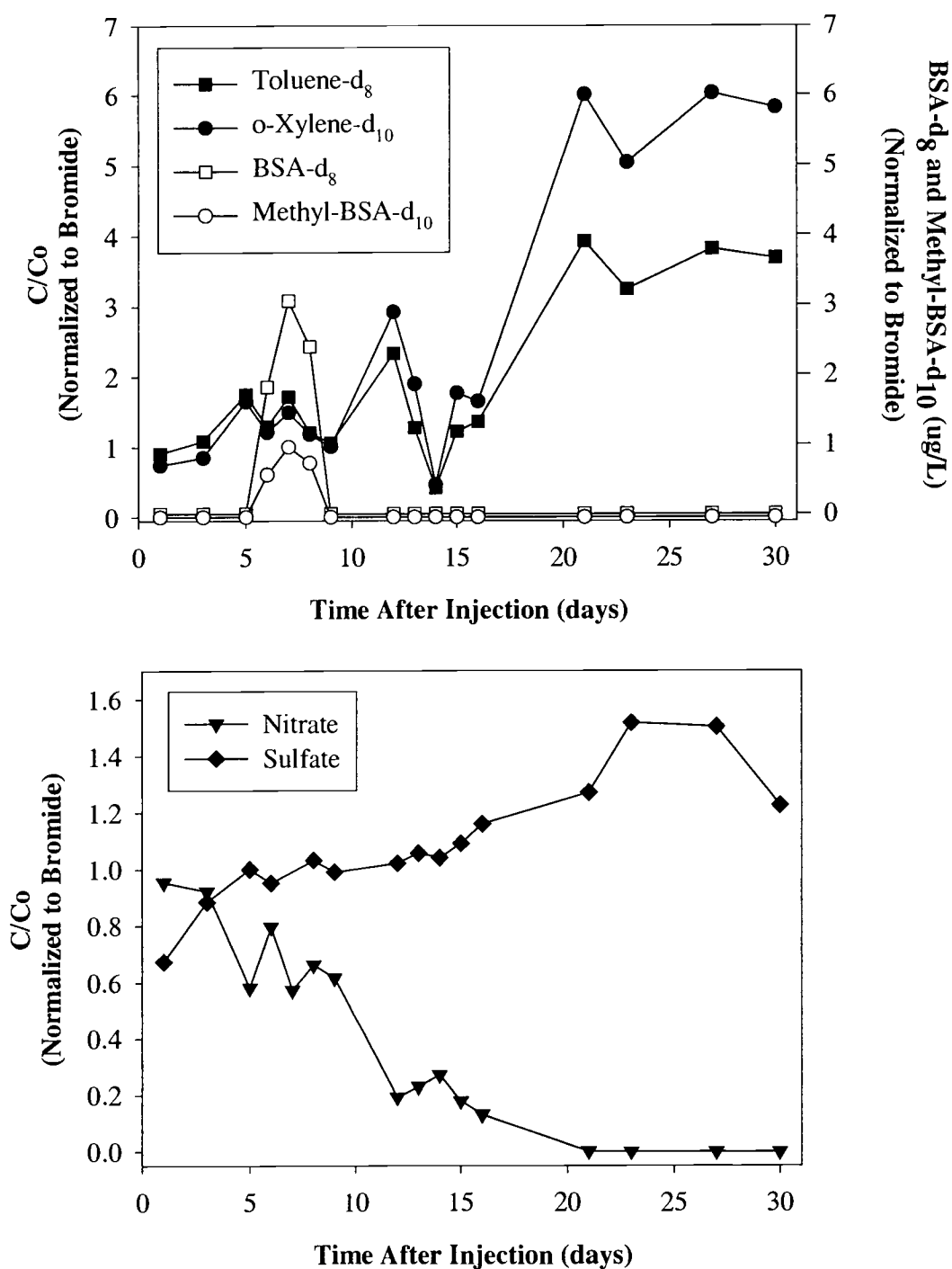
Figure 18 Formation of BSA-d$_8$ and o-methyl BSA-d$_{10}$ from injected deuterated toluene and o-xylene under denitrifying conditions in well CR 15 at the Northwest Terminal.

The actual in-situ rate of non-deuterated toluene transformation is likely faster than that of toluene-d_8 because of the kinetic-isotope effect. For example Krieger et al. (24) observed 3 times faster transformation of non-deuterated m-xylene compared to the deuterated m-xylene analog. BSA-d_8 and o-methyl BSA-d_{10} were detected on three days only.

The appearance of BSA-d_8 and o-methyl BSA-d_{10} coincided with nitrate removal, presumably due to denitrification (**Figure 18**). A rate of 9 μM/hr was calculated by linear regression of data thorough day 21 (**Table 10**). The denitrification rate from the transformation test was within the range reported by Istok et al. for another BTEX-contaminated site (1-80 μM/hr) (114) and was lower than that measured (16 μM/hr) with a one day test conducted in well CR 15 in August 2000 (**Table 7**). The observed BSA-d_8 and o-methyl BSA-d_{10} formation and the corresponding toluene-d_8 and o-xylene-d_{10} degradation did not represent a major fraction of the nitrate used, indicating that other compounds contributed to the removal of nitrate. As expected, sulfate reduction did not occur when nitrate was present. This is indicated by dilution-adjusted sulfate concentration of ~1 (**Figure 18**).

Background BTEX concentrations observed towards the end of the push-pull test (results not shown) agreed well with concentrations provided by the site owner (**Table 7**). Methy BSA was detected at 10 μg/L in samples from the end of the test, which corresponded to a molar ratio of 0.3 mol% (**Table 12**). BSA was not detected.

Compared to well CR 12, absolute concentrations of toluene and xylene were lower in well CR 15 groundwater whereas total BTEX was higher (**Table 7**). This is also apparent from relative concentrations of toluene and xylene to BTEX which are lower in well CR 15. Since well CR 12 falls close to the center of the ethanol spill in 1999 (133), ethanol concentrations were higher in well CR 12 (**Table 7**). The presence of ethanol could be a reason for the absence of toluene and xylene degradation, since ethanol is more easily degraded than BTEX and would represent an important competitor for electron acceptors. This is in agreement with the faster denitrification rate observed in well CR 12 compared to well CR 15 (**Table 10**).

3.4.2.3   Well 105s Kansas City Site

The dilution-adjusted concentrations for toluene-d_8 increased to values > 1 in a similar way (**Figure 19**) as those described for the CR12 - transformation test. Note that o-xylene-d_{10} was used in tests at the Northwest Terminal only. No BSA-d_8 was observed during the transformation test even though decreasing dilution-adjusted nitrate concentrations indicated denitrification (**Figure 19**) occurred at a rate of 18 μM/hr (**Table 10**). As expected, sulfate reduction did not occur when nitrate was present, which is indicated by dilution-adjusted sulfate concentration of 1 (**Figure 19**). Background BTEX concentrations observed towards the end of the push-pull test (results not shown) agreed well with concentrations provided by the site owner (**Table 7**). Non-deuterated methyl BSA was detected at 5 μg/L, in samples from the end of the tests, which corresponded to a molar ratio of 0.6 mol% (**Table 12**).

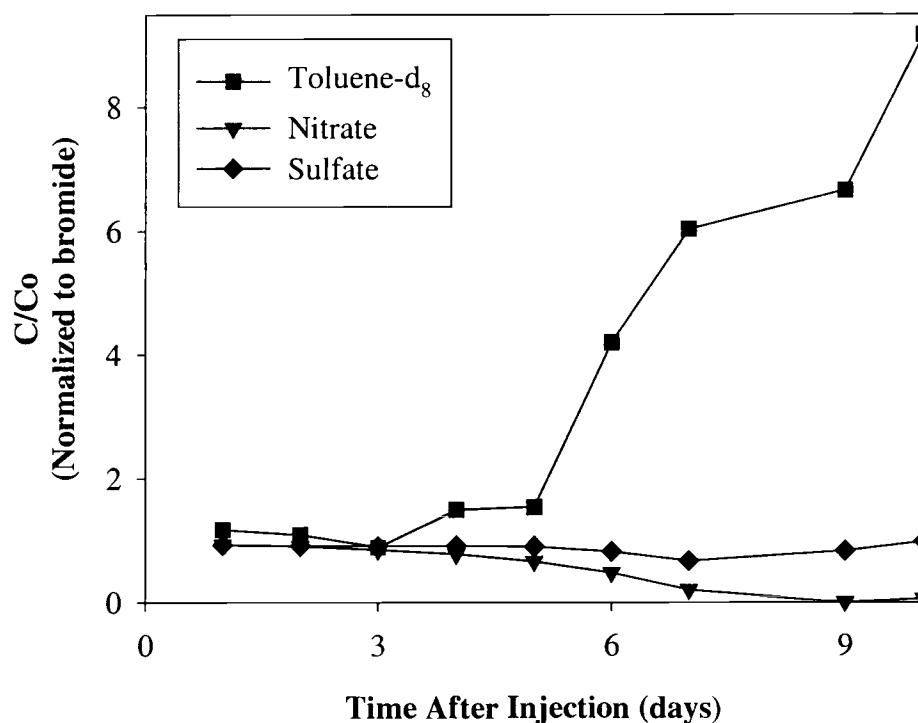**Figure 19** Dilution-adjusted breakthrough curves of toluene-d_8, nitrate and sulfate for a push-pull test conducted in well 105s at the Kansas City site.

3.4.2.4 Well 106s Kansas City Site

The dilution-adjusted concentration for toluene-d_8 in the test performed in well 106s at the Kansas City also increased to values >1 (**Figure 20**). However, strong evidence for biodegradation was provided by BSA-d_8 concentrations above the detection limit on days 1-3 (**Figure 20**) A zero-order formation rate calculated from these three concentrations was 0.7 μg/L/day (**Table 10**). The maximum concentration of 1.1 μg/L corresponded to 0.1 mol% of the toluene-d_8 at day 3 (**Table 11**). As in the CR 15-test, only a small fraction of the injected toluene-d_8 was transformed to BSA-d_8 during the time of the test.
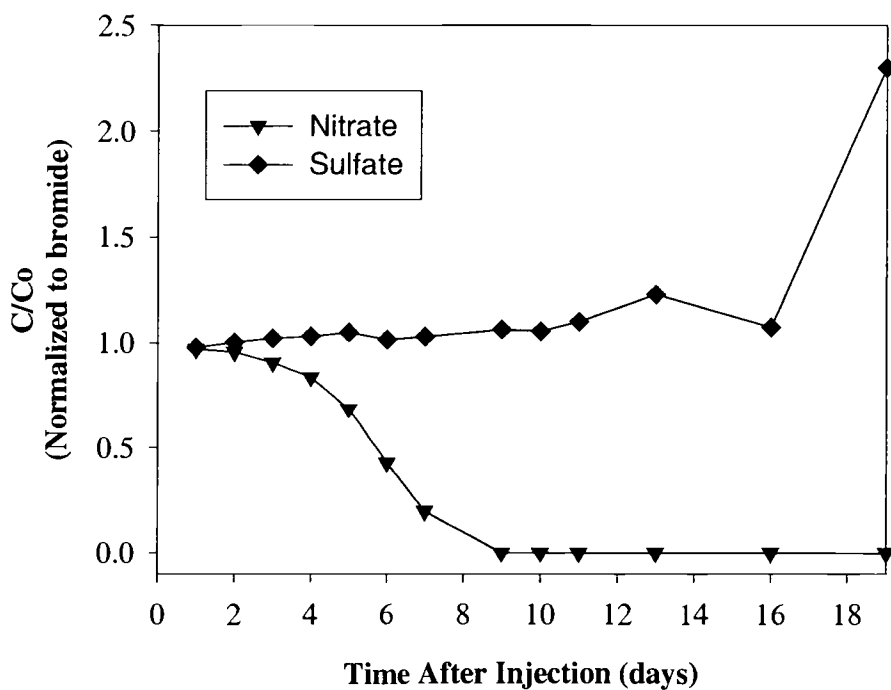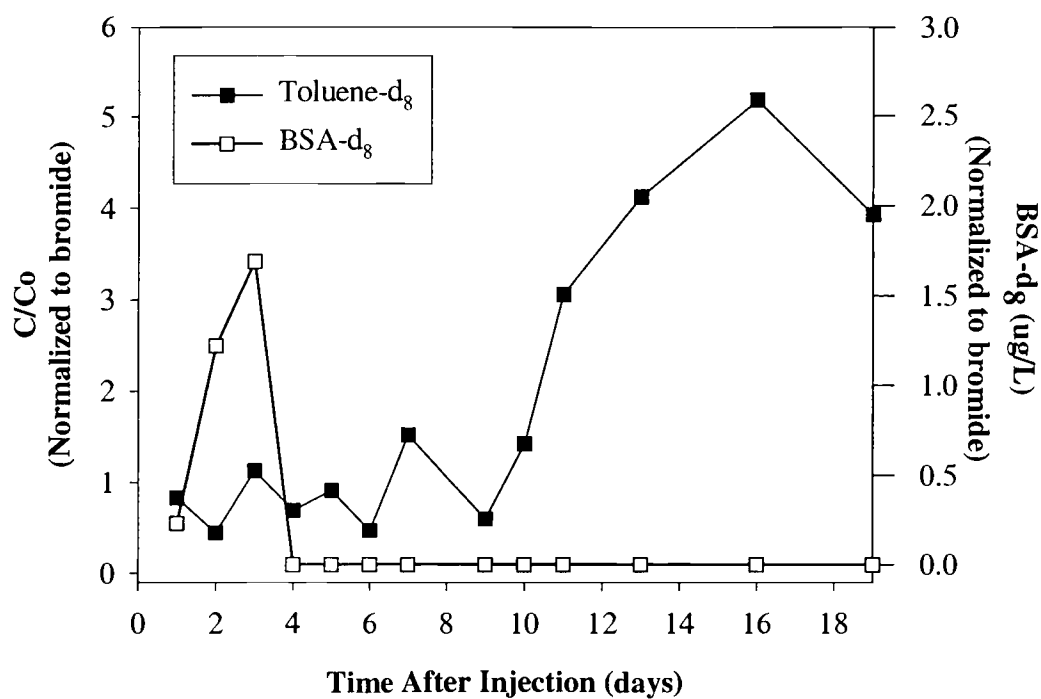
Figure 20 Dilution-adjusted breakthrough curves of toluene-d_8, BSA-d_8, nitrate and sulfate for a push-pull test conducted in well 106s at the Kansas City site.

Formation of degradation products coincided with nitrate removal, presumably due to denitrification (**Figure 20**) which was estimated at a rate of 24 µM/hr (**Table 10**). The observed BSA-d_8 formation and the corresponding toluene-d_8 degradation did not represent a major fraction of the nitrate used, indicating that other background compounds utilized the available nitrate. As expected, sulfate reduction did not occur when nitrate was present as indicated by slightly increasing dilution-adjusted sulfate concentrations > 1 (**Figure 20**) Values > 1 were due to sulfate present in the background groundwater (**Table 7**).

Background BTEX concentrations observed towards the end of the push-pull test (results not shown) were in agreement with historical concentrations provided by the site owner (**Table 7**). Methy-BSA was detected below the quantitation limit, in samples from the end of the tests, which corresponded to a molar ratio of <2 mol% (**Table 12**). BSA was not detected.

3.4.2.5   Well 207 Kansas City Site

Free NAPL was observed in well 207 at the Kansas City site during the time of the test. Since the well was dry after day 8 no samples could be obtained thereafter. Unlike for all other tests, the relative concentrations of toluene-d_8 were not higher than these of bromide (**Figure 21**). Decreasing relative concentrations of toluene-d_8 could be due to sorption into the NAPL. An analytical solution for the ADE was fit to the data and indicated a retardation factor of 64 for toluene-d_8 (**Figure 21**). Since transport behavior of toluene-d_8 in this well was not determined prior to the test, decreasing concentrations of toluene-d_8 could provide evidence for biodegradation.

No BSA-d_8 was observed during the transformation test in well 207, indicating absence of anaerobic degradation of toluene-d_8. However, decreasing dilution-adjusted nitrate concentrations indicated denitrification (**Figure 21**) at a rate of 4 μM/hr (**Table 10**). As expected, sulfate reduction did not occur when nitrate was present as indicated by slightly higher relative concentrations of sulfate than bromide (**Figure 21**). Background toluene and xylene were absent (**Table 7**) and as expected, BSA and methyl BSA were also not detected.
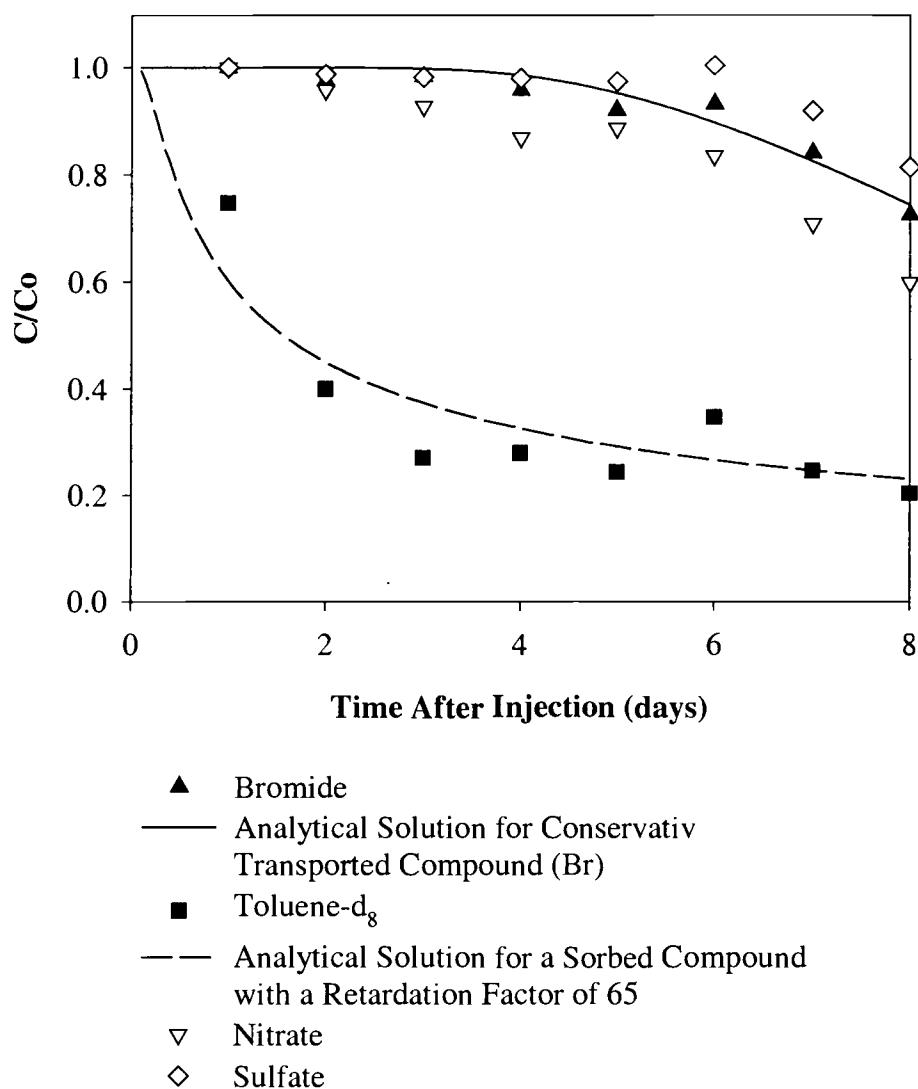
Figure 21 Breakthrough curves (not corrected for dilution) for bromide, toluene-d_8, nitrate, and sulfate from the transformation test in well 207 at the Kansas City site. The lines show analytical solutions for the one-dimensional advection-dispersion equation with a pulse as initial condition.

3.4.2.6 Comparison of All Tests

In all the tests, decreasing relative bromide concentrations indicated transport

and dilution of the test solution. Therefore, concentrations were adjusted for

dilution using relative bromide concentrations as correction factors. However, this caused dilution-adjusted toluene-d_8 and o-xylene-d_{10} concentrations to increase in most tests which suggested toluene-d_8 and o-xylene-d_{10} retardation. Toluene-d_8 showed decreasing dilution-adjusted concentrations only in the well 207 test, where free NAPL was present. However, for all tests the relative toluene-d_8 and o-xylene-d_{10} concentrations could be explained by retardation. An analytical solution for the ADE equation fit to the data from well CR 12 and well 207 gave varying retardation factors. Therefore, breakthrough curves for toluene-d_8 and o-xylene-d_{10} could not be used as evidence for biodegradation in any of the tests.

BSA-d_8 and o-methyl BSA-d_{10} formed from toluene-d_8 and o-xylene-d_{10} were detected within 6 days after injection in wells CR 15 and 106s. In a field study at a BTEX-contaminated site in California, Beller et al. (71) found detectable concentrations of BSA after approximately 10 days. The more rapid formation of BSA-d_8 in the CR15 and well 106s tests may be due to the fact that nitrate was utilized as the electron acceptor whereas sulfate was the electron acceptor at the site studied by Beller et al.

Initial zero order formation rates of BSA-d_8 (**Table 10**) represent minimum toluene-d_8 degradation rates. In order to compare to literature values, which are reported as first order rates, zero order formation rates were divided by the average toluene-d_8 concentration that was observed during the time of BSA-d_8 formation.

The estimated minimum first order rates (0.0004 and 0.001 day^{-1} for the well 106 test and the CR15 test, respectively) were lower than first order rates reported elsewhere from field and laboratory studies (0.003-0.04 day^{-1} (84, 100, 106, 129)).

Denitrification was observed in all tests. The rates (**Table 10**) were lowest (below 10 μM/hr) in wells CR15 and 207 where free NAPL was observed. Rates of about 20 μM/hr were observed during all the other tests. Observed rates were similar to values reported by Istok et al. for another site (1-80 μM/hr, (114)) even though they used a different test design with a duration of 1 day only. As expected, sulfate reduction did not occur when nitrate was present.

Background BSA was detected only in well CR-12 (**Table 11**) where the concentration of toluene was the highest (2.8 mg/L) for all tests (**Table 7**). Methylbenzylsuccinic acid ranged from <0.7 to 10 μg/L (**Table 11**) in the wells where xylene was present. BSA and methyl BSA were present in the molar ratio range of <0.01 to 0.8%, which is in the same order of magnitude as molar ratios observed for BSA-d_8 and o-methyl BSA-d_{10}. No correlation between the formation of deuterated BSA and methyl BSA and the presence of background BSA and methyl BSA existed. This lack of correlation could be due to several reasons. First, background metabolites observed in wells without biodegradation during the test could have been produced up-gradient from the well. Second, deuterated degradation products might not have been released from the microorganisms above the detection limit. Third, pretest redox conditions may have been sulfate-reducing or methanogenic (**Table 7**), whereas nitrate reducing conditions were promoted

during the test. As suggested by Chapelle et al. (100) mismatched terminal-electron-accepting processes could result in no observed biodegradation. Available field data did not provide conclusive evidence to select among these alternatives.

A connection between background BTEX concentration and biological activity seemed not to exist because biodegradation of toluene-d_8 and o-xylene-d_{10} was observed in wells with high (well CR15) and low (well 106) BTEX concentrations (**Table 7**). However toluene concentrations relative to total BTEX were low (~1%) in wells where biodegradation was observed (**Table 7**). This suggests that relative toluene concentration could be an indicator for biodegradation. Madsen et al have observed a similar inverse correlation between microbial activity of polyaromatic hydrocarbon degrading bacteria and polyaromatic hydrocarbon concentrations (83).

3.5 ACKNOWLEDGEMENTS

4 SUMMARY AND CONCLUSIONS

A solid phase extraction method for benzylsuccinic acid (BSA) and methyl BSA was developed. The method detection limit was 0.2 μg/L and only 10 mL of solvent per sample was used. The conditions for solid phase extraction, derivatization reaction and chromatography were optimized. A styrene-divinylbenzene sorbent in cartridge format was selected for the extraction of sample volumes up to one liter. Because commercially available plastic columns interfered with the derivatization reaction, the plastic cartridges were replaced by glass tubes with PTFE frits. For clean systems like tap water, glassware had to be silanized in order to prevent sorption of BSA from acidified solution to the glassware. It was demonstrated that iron precipitation did not affect aqueous concentrations of BSA. The methylation reaction with diazomethane was complete after 5 min; however, compounds with non-aromatic double bonds were unstable during the reaction.

The method was then applied to field samples obtained during push-pull tests designed to determine if in-situ transformation of toluene and xylene is occurring. Deuterated surrogates for toluene and o-xylene were used in order to distinguish from existing background toluene and xylene. Transport behavior of toluene in the subsurface was determined in a contaminated well and analysis of breakthrough curves indicated different processes for sorption and desorption. Retardation factors of 2 and 14 were determined for injected deuterated toluene and background toluene, respectively. Sorption should be explicitly considered in future push-pull

tests with the selection of suitable non-reactive transport surrogates. Because of the uncertainty in the mass balance of deuterated toluene and xylene due to non-conservative transport, we were not able to observe direct removal of deuterated toluene and o-xylene. Unambiguous evidence for biodegradation was obtained in two tests from formation of deuterated BSA and o-methyl BSA that coincided with nitrate removal, presumably due to denitrification.

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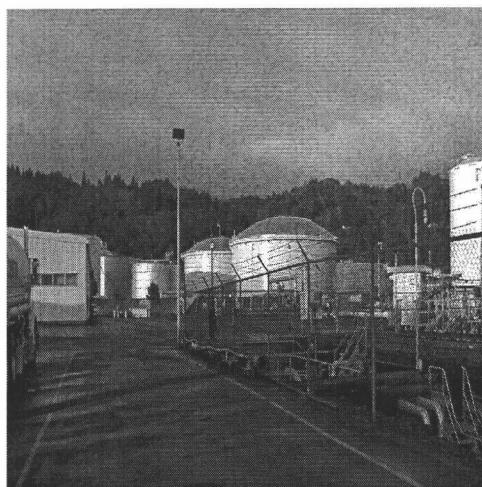(150) Brusseau, M. L.; Rao, P. S. C. *Crit. Rev. Environ. Control* **1989,** *19,* 33-99.

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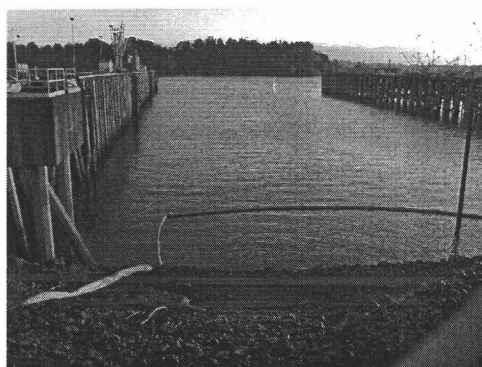
APPENDIX

APPENDIX: PHOTOGRAPHS

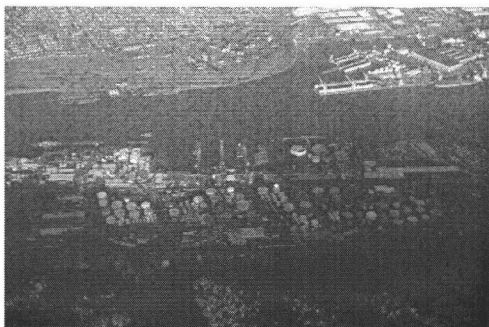**Photograph 1 Tank Farm at the Kansas City Site, Kansas City, KS.**

Photograph 2 Monitoring Well Next to Missouri River.

Photograph 3 Aerial View of the Northwest Terminal, Portland, OR.

Photograph 4 Tank Farm at Northwest Terminal, Portland, OR.

Photograph 5 Oil Barrier in Columbia River, Portland, OR.

Photograph 6 Calibrating Tube Before Injection.
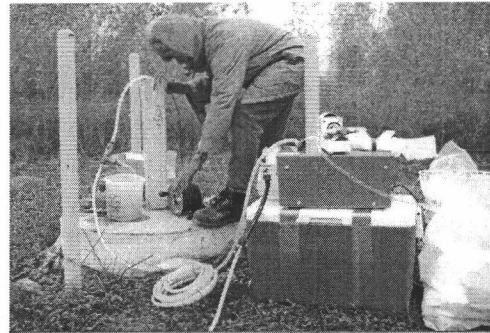
Photograph 9 Measuring Water Level During Injection Phase.
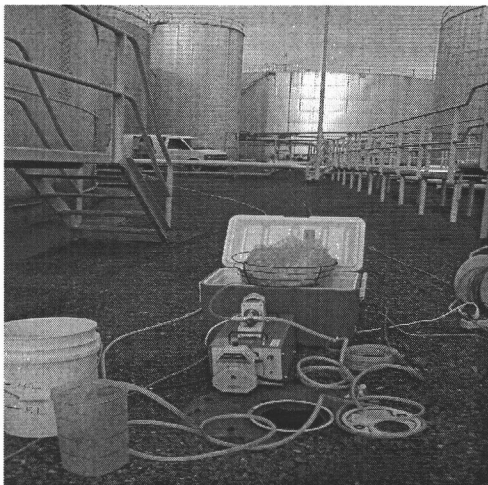
Photograph 7 Injection Setup.
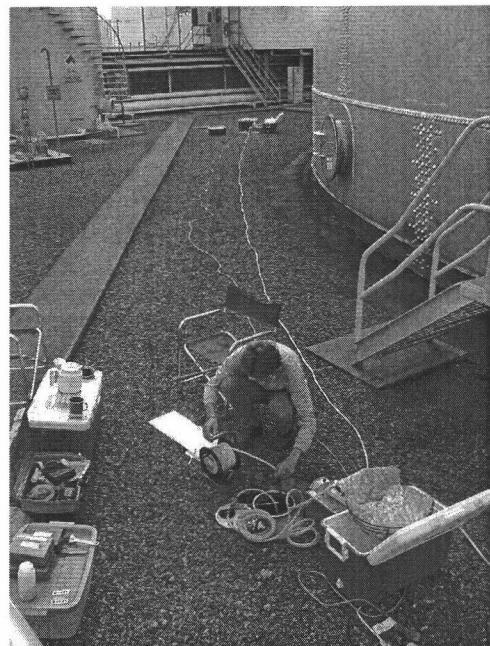
Photograph 10 Measuring Water Level During Injection Phase. Second Injection in the Background.

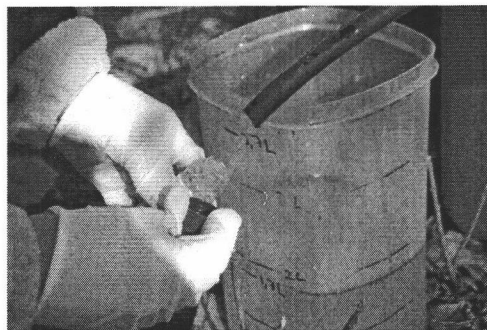Photograph 8 Running Tubing from Truck to Well.
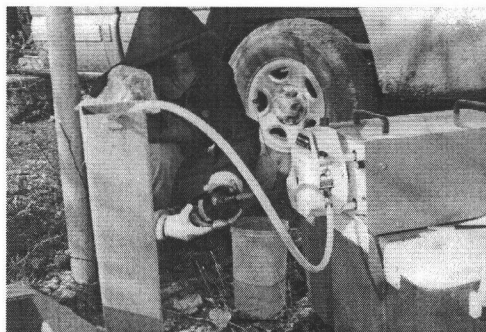
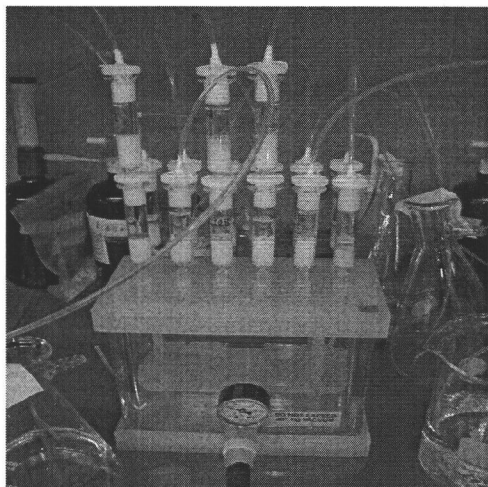**Photograph 11   Taking   a Metabolite   Sample   During Extraction Phase.**

Photograph 13   Taking a VOA Sample.

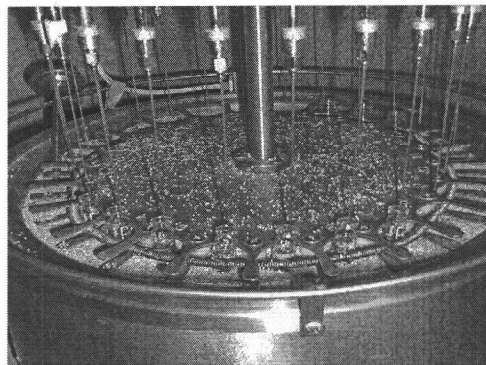**Photograph 12   Taking   a Metabolite   Sample   During Injection Phase.**
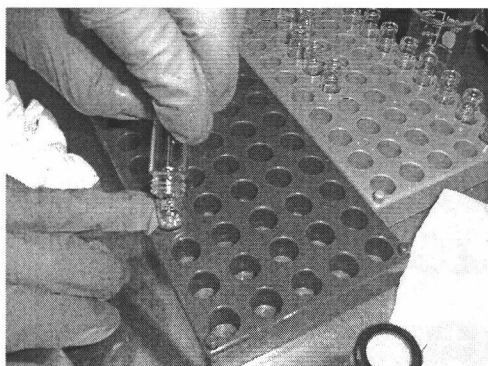

Photograph 14   Bad   Example for   Taking   a   VOA   Sample. Analytes Might Volatilize.

Photograph 15 Solid Phase Extraction Setup With Glass Columns and Transfer Lines.

Photograph 17 Concentrating Sample Before Derivatization.

Photograph 16 Transferring Sample to Autosampler Vial.