AN ABSTRACT OF THE THESIS OF

Robert E. Alumbaugh for the degree of Master of Science in Chemistry presented on August 29, 2005.

Title: Measuring In Situ Biotransformation in BTEX-Contaminated Groundwater.

Abstract approved: Jennifer A. Field

Benzylsuccinate (BSA), methylbenzylsuccinate (methylBSA), and ethylbenzylsuccinate (ethylBSA) are unambiguous anaerobic biotransformation products from toluene, xylenes, and ethylbenzene decay, respectively, and may be used to indicate intrinsic bioremediation is occurring at hydrocarbon-contaminated sites. In order to improve upon current methods that detect and quantify anaerobic hydrocarbon metabolites in field samples, solid-phase-extraction (SPE) and direct sample injection methods coupled with liquid chromatography/tandem mass spectrometry (LC/MS/MS) were evaluated. In laboratory studies, recoveries of authentic standards of non-deuterated or deuterated benzylsuccinates and toluates ranged from 80 to 106% with relative standard errors ranging from 2 to 4%. The method detection limits for these analytes using SPE-LC/MS/MS ranged from 0.006 to 0.029 μg/L whereas those for direct injection-LC/MS/MS ranged from 0.61 to 1.5 μg/L. Given the increased sensitivity of using SPE coupled with LC/MS/MS, this technique was then used to analyze for the presence of putative anaerobic alkylbenzene metabolites in groundwater from a hydrocarbon-contaminated site where single-well push-pull tests were conducted using
deuterated aromatic hydrocarbons. Test solutions (250 L) containing the deuterium-labeled organic substrates toluene, ethylbenzene, and o-xylene, along with nitrate as an electron acceptor and bromide as a non-reactive tracer, were injected into each of four existing wells at an alkylbenzene-contaminated field site. Samples of the test solution/groundwater blend were then extracted over a 29-day period. Volatile alkylbenzenes were analyzed and quantified by gas chromatography/mass spectrometry (GC/MS). Metabolites were determined by solid-phase extraction (SPE) followed by high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis. Due to the rapid transport of the test solution through the aquifer, resulting in the loss of the tracer signal, no test solution was available for three wells. Of the wells tested, only the test conducted in well CR-20 gave samples with sufficient concentration of analytes to warrant full processing of the samples and data. Decreases in deuterated alkylbenzene substrates and the appearance of signature metabolites including deuterated ethylbenzylsuccinate (ethylBSA-d5) and o-methylbenzylsuccinate (o-methylBSA-d10), were observed while deuterated benzylsuccinate (BSA-d5) was not detected. Of the deuterated secondary metabolites monitored, m-toluate-d7 and benzoate-d5 also were determined in well CR-20. Although analytes were detected in well CR-20, erratic changes in analyte concentrations made it infeasible to determine rates.
Measuring In Situ Biotransformation in BTEX-Contaminated Groundwater

by
Robert E. Alumbaugh

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APPROVED:

Redacted for Privacy
Major Professor, representing Chemistry

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Chair of the Department of Chemistry

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CONTRIBUTION OF AUTHORS

Dr. Lisa Gieg provided analytical standards produced from microcosms and editorial assistance.
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Measuring *In Situ* Biotransformation in BTEX-Contaminated Groundwater
Chapter 1

Introduction

It is estimated that 2,560 billion liters of petroleum hydrocarbons are handled and consumed each year by industrialized nations [2]. Benzene, toluene, ethylbenzene, and xylenes (BTEX) are toxic components of petroleum hydrocarbons such as gasoline, aviation fuels, and solvents. These alkylbenzenes enter the hydrosphere through accidental spills, illegal dumping, and deteriorating infrastructure such as leaking underground storage tanks and transfer lines. Benzene is a known carcinogen while the U.S. EPA lists toluene, ethylbenzene and xylene as priority pollutants [3]. BTEX are also rated on CERCLA’s 2003 Priority List of Hazardous Substances, a list of contaminants

<table>
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a Percent by weight
b Values at 25°C from the Toxnet Hazardous Substance Database (HDSB)
c p-Xylene
d Total percent of branched pentanes
ranked by a combination of factors including: frequency found, toxicity and potential for human exposure [4]. Due to their relatively high water solubilities (Table 1.1) BTEX dissolve readily in water, compared to other fuel constituents. Although only 2-3% by weight of fuels are BTEX, they comprise a greater proportion of the dissolved fraction in fuel-contaminated water than do the other fuel components [2]. Because of this greater water solubility, alkylbenzenes are readily transported from the original source of contamination through advection and thus impact sources of domestic drinking water including groundwater aquifers and surface waters.

Remediating BTEX-contaminated groundwater often involves pump-and-treat strategies using ex-situ air sparging or activated carbon adsorption; however, these approaches are time consuming and costly, which makes them relatively inefficient. Clean-up times estimated up to and beyond 100 years render pump-and-treat systems more suitable as a containment strategy rather than a remediation strategy [5]. Engineered in-situ remediation technologies, such as soil vapor extraction and bioslurping were developed as an alternative to ex-situ treatment and have achieved varying levels of success [6]. Alternatively, bioremediation has emerged as an option of merit due to its simplicity, effectiveness, and low cost relative to other engineered systems and to the fact it can be employed in both aerobic and anaerobic environments [7].

Anaerobic microbial degradation of alkylbenzenes has been intensively studied for the past 20 years [2,8]. While microbial biodegradation occurs more quickly in aerobic systems than under anaerobic conditions, the introduction of petroleum
hydrocarbons quickly renders a BTEX-contaminated aquifer anaerobic upon the consumption of available oxygen [9]. Initially it was thought that simple aromatic hydrocarbons would be resistant to anaerobic microbial mineralization [6]. It was not until 1984 that indirect evidence of anaerobic microbial degradation was obtained [10]. In 1986, $^{14}C$-labeled toluene was found to mineralize to $^{14}CO_2$ [11] and it was shown that the oxygen inserted into simple mono-aromatic compounds under anaerobic conditions was obtained from water [12].

Figure 1.1 Suggested radical enzymatic pathway of fumarate addition to toluene, forming benzylsuccinate. The benzylsuccinate synthase radical ($\cdot E$) conserves the hydrogen through the radical addition.

Adapted from: Spormann and Widdel, 2000. [1]
Laboratory studies were used to elucidate the anaerobic pathway of alkylbenzene degradation in pure and mixed cultures [13-22]. During the first step of anaerobic mineralization of toluene, an enzymatic radical reaction using benzylsuccinate synthase catalyzes the formation of a toluene radical. The toluene radical causes the addition of fumarate and its subsequent reduction to form benzylsuccinate (BSA) [Fig. 1.1] [1,23,24]. A similar mechanism transforms xylene isomers and ethylbenzene to produce methylbenzylsuccinate (methylBSA) and ethylbenzylsuccinate (ethylBSA), respectively [Fig. 1.2] [22]. BSA, methylBSA, and ethylBSA are considered metabolites that unequivocally result from the anaerobic degradation of alkylbenzenes [22,25-28]. Because they are not 'dead-end' products of cometabolism, the detection of these 'signature' metabolites in BTEX-contaminated groundwater provides unequivocal evidence that anaerobic alkylbenzene degradation is occurring [22,29-34]. Secondary metabolites including toluates, phthalates, and benzoates also are produced. Unlike BSA and its derivatives, toluates, phthalates, and benzoates are not 'signature metabolites' because they are not unique to anaerobic alkylbenzene metabolism [21,35,36]. Of these secondary metabolites, m-toluate was found at higher concentrations in mixed culture microcosms than that of 3-methyl-BSA [22].

Microcosm experiments using contaminated sediment and/or groundwater provide information on the likelihood of contaminant transformation, the nature of products formed, and proof that appropriate microbial communities are present that can transform contaminants. However, due to the nature of the disturbed sediments, the small volumes of sediments used, and the temperature differences between field and lab, it is
not possible to directly relate rate information obtained from microcosm experiments to rates occurring \textit{in-situ}. Therefore, it is necessary to measure \textit{in-situ} biodegradation rates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Proposed pathways for the anaerobic degradation of toluene, xylene and ethylbenzene. Adapted from Elshahed, Gieg, McInerney, and Suflita, 2001. [22]}
\end{figure}
in order to provide quantitative information on bioremediation and to evaluate the potential of intrinsic bioremediation to meet restoration goals or to assess the success of engineered systems.

Although concentrations of both substrates and products (metabolites) in groundwater samples are reasonably easy to assess, in-situ transformation rates are more difficult to obtain [37]. The conventional approach for estimating contaminant transformation rates is to monitor changes through time in the concentration of contaminants and their transformation products along groundwater-flow paths through treatment zones [17,38]. However, this approach may give ambiguous or misleading information because, in addition to potential chemical or microbially-mediated reactions, observed concentration changes may be caused by a combination of confounding factors including: 1) spatially-variable initial concentrations of contaminants and their transformation products, 2) the release of contaminants from inadequately characterized nonaqueous phases (if present), 3) sorption, 4) volatilization, and 5) mass transport processes [39,40]. Mass transport processes include advection (bulk transport of a contaminant plume with groundwater flow) and dispersion (decreasing concentration as a contaminant plume spreads). Dispersion is caused by 1) differences in path lengths as groundwater flows around soil particles, 2) faster water velocities through larger subsurface pore spaces, and 3) groundwater flowing faster through the center of a pore than the edges [41].

Field studies are needed to provide rate information that can be used to evaluate the effectiveness of bioremediation [42-46]. Griebler et al. recently used carbon isotope
ratios ($\delta^{13}C$) to provide rate information on a BTEX contaminated aquifer [34]. Briefly, the $^{13}C/^{12}C$ ratio of substrates, relative to a standard, increases as biotransformation occurs while $^{13}C/^{12}C$ ratio of metabolites decreases because isotopically-distinct molecules react at different rates (kinetic-isotope effect), with biologically-activated reactions favoring transformation of the lighter isotopes [47]. A combination of a specialized high-resolution magnetic sector mass spectrometer and a catalytic combustion chamber are used to determine isotopic ratio and the isotopic ratios of the sample are then compared against a standard. The ratios are expressed as $\delta^{13}C$ values or $\%$ (parts per thousand) difference where $\delta^{13}C = ((^{13}C/^{12}C_{\text{sample}}) / (^{13}C/^{12}C_{\text{standard}}) - 1) \times 1000$ [48,49]. An increase in $^{13}C/^{12}C$ ratios of substrates ($\delta^{13}C > 0$) can be used as a line of evidence for in situ biotransformation of contaminants [47,48,50-52]. Lately attempts have been made to improve sensitivity and determine concentrations of BTEX metabolites through compound-specific isotope analysis and signature metabolites analysis [34]. A significant advantage of carbon isotope analysis is that abiotic processes such as sorption, dissolution, volatilization, advection, and dispersion do not affect the signature isotopic ratios [34,47].

The drawbacks to using carbon isotope ratio analysis are not insignificant. The small $\delta^{13}C$ values (described earlier) make the application challenging [49]. The high cost of the dedicated gas chromatography combustion isotope ratio mass spectrometry instrumentation and the expertise required to precisely handle sample preparation add to the downside of this type of environmental analysis. Because of the difficulties listed
above and the availability of analytical instrumentation, most labs will find the use of conventional tracer studies more suited to their needs.

Groundwater tracer studies involve the use of a non-reactive compound not found in the background, such as bromide, injected via a well into the groundwater along with a test solution. Upon extraction of the test solution, the non-reactive tracer is used to normalize the concentrations of reactants, metabolites, and any other amendments in the test solution by accounting for compound dilution caused by advection and dispersion. It is important to note that non-reactive tracers like bromide do not account for sorption of organic solutes to organic soil particles and, when used to normalize concentrations of sorbing organic compounds, will result in erroneous values [40]. Thus, it is not possible to accurately determine degradation rates from quantifying the loss of an organic substrate over time solely by normalizing reactants against a non-sorbing tracer. However, non-reactive tracers such as bromide are important as they allow for data processing the quantities of injected substrates such as ionic electron acceptors and other compounds that transport with little or no retardation. In addition, bromide can be used to determine the percentage of test solution present at the sampling well.

Many field tests to interrogate aquifers incorporate the use of multiple wells spaced along a groundwater flow path downgradient of the contaminant source. Although a multiple-well test, also known as a well-to-well test, can be a powerful tool it has disadvantages including: 1) the time and expense associated with drilling new wells [53], 2) large test solution volumes must be injected [54,55], 3) multi-well tests can take
excessive periods of time for test solutions to be transported to a sampling well [53], and 4) it is possible the injected test solution might bypass the sampling well entirely.

Alternatively, a type of single well test known as a "push-pull" test offers much in the way of interrogating an aquifer while eliminating many of the problems inherent in multi-well tests [53,56-59]. Push-pull tests are conducted by injecting (the "push") [Fig. 1.3a] an aqueous test solution containing a nonreactive tracer and one or more reactants into the saturated zone of an aquifer via an established monitoring well. Samples of the test solution/groundwater mixture are then extracted (the "pull") [Fig. 1.3b] from the same well over time and analyzed for tracer, reactant, and product concentrations. Test solutions typically contain a non-reactive, nonsorbing tracer and one or more reactive solutes (substrates) that vary with the type of assay being performed. Within the aquifer,
injected substrates are transformed to known products; the extent of transformation provides a quantitative measure of microbial activity. The in-situ transformation rate of an injected reactant is then determined by removing the effects of transport processes from measured reactant or transformation product concentrations of samples taken over time by using a data processing technique.

Beller, et al conducted a type of push-pull test to obtain information about the degradation of BTEX and corresponding appearance of metabolites (no rates were reported) that involved pumping a volume of groundwater (840L) from a contaminated site. The removed groundwater was then treated by deionization to remove non-volatile salts followed by sparging with helium to remove dissolved oxygen and volatile compounds [31]. The collected groundwater was then spiked with the aromatic compounds under study along with a conservatively-transported tracer before being reinjected back into the same well to create an aquifer test zone. In addition to the test solution, a ‘buffer’ solution was used, being injected prior to the test solution to separate the test solution from the native groundwater. Periodic samples were extracted for analysis to quantify the rate of aromatic mass removal and the appearance of signature metabolites. Although this carefully designed test provided valuable information, the groundwater pretreatment procedure was both costly and time consuming.

A variation of the push-pull test used by others [56,60] eliminated the need to remove and treat a volume of water from the aquifer by exploiting the use of deuterium or fluorine-labeled “surrogate” compounds. For these tests, a concentrated solution of the labeled surrogate(s) under study is injected into a contaminated aquifer. After a reaction
period, the test solution is periodically sampled and analyzed for concentrations of the
original deuterated or fluorinated surrogate(s), the deuterated or fluorinated metabolite(s),
electron acceptor(s) and the bromide conservative tracer. Advantages to this approach
include: 1) less complexity to prepare and conduct the test than multi-well tracer tests, 2)
the ability to distinguish the test solution components and their degradation products from
background contaminants without the time and cost expenditure of a pretreatment
routine, and 3) the completely unambiguous determination of ‘signature’ metabolites as
evidenced by their labeled form. Reusser, et al. successfully used this variation of the
push-pull test to determine in-situ rates of signature metabolite formation from a BTEX-
contaminated field site.

In order to quantify rates of in-situ anaerobic alkylbenzene biodegradation,
sensitive and selective analytical methods are needed to quantify the benzylsuccinate
metabolites of toluene, xylenes and ethylbenzene, in addition to toluates and benzoates.
Signature metabolites were first quantified by gas chromatography/mass spectrometry
(GC/MS); however, the non-volatile metabolites require derivatization prior to GC/MS
analysis, which is time consuming and involves the use of toxic reagents
[21,22,31,60,61]. Derivatization is achieved through methylation with diazomethane [31]
or through silanization with \(N,O\)-bis-(trimethylsilyl)-trifluoroacetamide [22]. A direct
injection method was developed by Beller [32] to quantify BSA and methylBSA using
high-pressure liquid chromatography coupled with tandem mass spectrometry
(HPLC/MS/MS). The use of HPLC/MS/MS for groundwater analysis greatly reduces the
steps required for sample preparation while maintaining sensitivity.
Because low concentrations of metabolites typically are observed [22,31,61], a concentration step prior to LC/MS/MS was deemed advantageous. Liquid-liquid extraction was first used to concentrate BSA and methyl-BSA [22,31]; however, it has the disadvantage of being unwieldy and of requiring large volumes of solvents. A solid-phase extraction (SPE) method devised by Reusser et al. [61] was an improvement over liquid-liquid extraction yet it still required the time-consuming step of packing SPE columns with ultra-clean sorbents. Moreover, the large sample volumes (1 L) extracted at low flow rates of 5 mL/min was both cumbersome and time consuming. Chapter 2 of this thesis describes an SPE method that was developed using commercially-available Empore® SPE discs. The ease of use coupled with the fast-flow rates possible with Empore® discs substantially decreased the time required for the concentration step while improving LC/MS/MS detection limits by a factor of 100.

Chapter 3 of this thesis describes a set of push-pull tests designed to investigate the in-situ transformation of deuterated ethylbenzene, toluene and xylenes. The formation of BSA, methylBSA, and ethylBSA (both deuterated and non-deuterated) along with selected additional secondary metabolites including toluates and benzoates, were determined in samples collected from the push-pull tests.

Literature Cited:


Chapter 2

Determination of Alkylbenzene Metabolites in Ground Water by Solid Phase Extraction and LC/MS/MS

Robert E. Alumbaugh, a Lisa M. Gieg, c and Jennifer A. Field b

aDepartment of Chemistry and bDepartment of Environmental and Molecular Toxicology
Oregon State University, 1007 ALS, Corvallis, OR 97331

Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019

Journal of Chromatogramph A
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1000 AR Amsterdam
The Netherlands
Abstract

Benzylsuccinate (BSA), methylbenzylsuccinate (methylBSA), and ethylbenzylsuccinate (ethylBSA) are unambiguous anaerobic biotransformation products from toluene, xylenes, and ethylbenzene decay, respectively, and may be used to indicate intrinsic bioremediation is occurring at hydrocarbon-contaminated sites. In order to improve upon current methods that detect and quantify anaerobic hydrocarbon metabolites in field samples, solid phase extraction (SPE) and direct sample injection methods coupled with liquid chromatography/tandem mass spectrometry (LC/MS/MS) were evaluated. In laboratory studies, recoveries of authentic standards of non-deuterated or deuterated benzylsuccinates and toluates ranged from 80 to 106% with relative standard errors ranging from 2 to 4%. The method detection limits for these analytes using SPE-LC/MS/MS ranged from 0.006 to 0.029 μg/L whereas those for direct injection-LC/MS/MS ranged from 0.61 to 1.5 μg/L. Given the increased sensitivity of using SPE coupled with LC/MS/MS, this technique was then used to analyze for the presence of putative anaerobic alkylbenzene metabolites in groundwater from a BTEX-contaminated site where single-well push-pull tests were conducted using deuterated aromatic hydrocarbons. Both deuterated and non-deuterated benzylsuccinates and toluates were successfully detected and quantified in field samples using this method.

Introduction

The U.S. dependence upon groundwater is underscored by the fact that 99% of rural drinking water and 46% of total drinking water is obtained from aquifers [1]. Unfortunately, contamination of groundwater is a persistent, ubiquitous problem throughout the U.S. [2]. Aromatic hydrocarbons including benzene, toluene,
ethylbenzene and the xylene isomers (BTEX) are introduced to the subsurface in the form of gasoline spills and leaks from underground storage facilities. The BTEX compounds are of particular interest due to their relative high water solubilities and toxicities [3,4] and because they are among the 33 synthetic organic contaminants most frequently found in drinking-water wells [5].

Limitations and costs associated with conventional remediation methods have generated interest in intrinsic bioremediation as an alternative to cleaning up contaminated groundwater [2,5,6]. Since available oxygen is quickly depleted in petroleum-impacted subsurface waters, anaerobic conditions often prevail [7], which limits intrinsic or enhanced bioremediation approaches to those relying on anaerobic metabolism.

The detection of metabolites that are unique to anaerobic alkylbenzene transformation are a definitive way to demonstrate that intrinsic or enhanced bioremediation is occurring at BTEX-contaminated sites [8,9]. Under anaerobic conditions, fumarate-addition reactions lead to the formation of unique metabolites including benzylsuccinate (BSA) from toluene, methylbenzylsuccinate (methylBSA) from xylenes, and ethylbenzylsuccinate (ethylBSA) from ethylbenzene, respectively [9-11]. While the transformation of these alkylbenzenes to their associated benzylsuccinate metabolites has been observed in numerous laboratory studies, as evidenced by numerous reviews [8,12-15], few reports describe the occurrence of BSA and methylBSA in groundwater at BTEX-contaminated sites [9,16-18]. To the best of our knowledge the detection of ethyl-BSA has not been reported for groundwater samples or groundwater
tracer tests. Fewer yet are the number of reports that document benzylsuccinate formation during groundwater tracer tests [19,20]. Beller et al. [20] conducted a groundwater slug test in which toluene and xylenes were injected and the subsequent formation of BSA and methylBSA metabolites was observed. Reusser et al. [19] injected deuterated toluene-d_{8} and o-xylene-d_{10} and monitored the formation of deuterated BSA-d_{8} and methylBSA-d_{10} and reported first-order rates of formation. Rates obtained from in situ tracer tests are more likely to be representative of actual aquifer conditions than those obtained from microcosm studies [21].

Laboratory studies indicate that BSA and methylBSA can be further biotransformed to toluate, phthalate, and benzoate [9,13]. While these metabolites are not unique to the anaerobic transformation of alkylbenzenes, the detection of their deuterated forms during groundwater tracer tests can be used to validate that alkylbenzenes are being metabolized in situ beyond the initial transformation to benzylsuccinates. To date, quantitative methods for the detection of the deuterated forms of toluate, phthalate, and benzoate have not been developed.

In support of previous laboratory and field studies, several analytical approaches were developed for the detection of BSA and methylBSA. Several studies have used liquid/liquid extraction coupled with gas chromatography/mass spectrometry (GC/MS) [20]; however, this method is time-consuming and uses relatively large volumes of solvent. Reusser and Field [19] coupled solid-phase extraction (SPE) with GC/MS analysis, which required extracting 1 L samples followed by derivatization with diazomethane, to achieve a method detection limit (MDL) of 0.2 μg/L. Subsequently,
Beller [16] achieved an MDL of 0.3 μg/L for the analysis of BSA and methylBSA by direct injection liquid chromatography/tandem mass spectrometry (LC/MS/MS). This latter technique is more promising for rapid analysis because no extraction or derivatization procedures are required. However, additional analytical methodology is needed because [1] the concentrations of in situ benzylsuccinates can be at or below the MDLs of the current GC- and LC-based methods (e.g. < 0.2-0.3 μg/L) and [2] methods are needed to detect metabolites resulting from the further decomposition of benzylsuccinates that may be produced during groundwater tracer tests.

Thus, the objective of this study was to develop and evaluate a SPE method coupled with LC/MS/MS for the determination of BSA, methylBSA, ethylBSA and their deuterated forms, and for toluates, deuterated toluates and deuterated benzoate. As part of this study, the SPE method was examined alongside a direct injection method similar to that outlined by Beller [8] in order to compare MDLs and to expand the methodology to include a greater suite of putative metabolites. To limit the scope of this project, the m-methylBSA isomer was selected; however, o- and p-methylBSA also could be determined by the methods described herein. Furthermore, because phthalates formed only at low concentrations in microcosm experiments [9], phthalates and their deuterated forms were not evaluated for this project. The developed and validated SPE method for the selected metabolites and their deuterated forms was then applied to the analysis of aromatic hydrocarbon-impacted groundwater samples.
Experimental Methods

Reagents and Standards. Standards of DL–benzylsuccinic acid (BSA, 99% purity) and benzoic acid (99.5%) were obtained from Alfa Aesar (Ward Hill, MA). Standards of m-Toluic acid (99%), o-toluic acid (99%), 2,4-difluorobenzoic acid (2,4-DFBA, 98%), and 2,4-dichlorobenzoic acid (2,4-DCBA, 98%) were obtained from Aldrich Chemical (Milwaukee, WI). The surrogate standard, p-fluorobenzoic acid (4-FBA, 98%), was purchased from Sigma Chemical (St. Louis, MO). The commercially-available deuterated standards, o-toluic acid-d7 (99.3%) and benzoic acid-d5 (99.2%) were purchased from CDN Isotopes (Quebec, Canada). Toluene-d5, m-xylene-d10, and ethylbenzene-d5 for push-pull tests were also acquired from CDN isotopes. Acetone (HPLC grade) and methanol (Optima grade) were obtained from Fisher Scientific (Fairlawn, NJ). Hydrochloric acid was obtained from J.T. Baker (Phillipsburg, NJ). Ethylbenzylsuccinic acid (ethylBSA) and m-methylbenzylsuccinic acid (m-methylBSA) were synthesized and purified according to published methods [22,23].

Benzylsuccinate-d5 (d5-BSA) and m-methylbenzylsuccinate-d10 (m-methylBSA-d10) were produced by enrichment cultures capable of biodegrading toluene or m-xylene under sulfate-reducing conditions [9]. Approximately 40 µmol (5 µL) of either d5-toluene or m-xylene-d10 were amended to the enrichment cultures which were incubated until approximately half of the given alkylbenzene had been consumed. At this time, half of the culture supernatant was acidified, extracted with ethyl acetate, concentrated, silylated, and analyzed by GC-MS according to published methods [9] in order to confirm the presence of BSA-d5 or m-methylBSA-d10. Mass spectral profiles indicated that these deuterated metabolites were indeed formed (data not shown). The remaining
culture supernatants were then subjected to SPE to concentrate metabolites. These concentrated metabolites were used as biologically-generated reference materials to confirm the presence of BSA-d$_5$ and \( m \)-methylBSA-d$_{10}$ in groundwater samples analyzed by the LC/MS/MS method.

**Groundwater Samples.** Groundwater samples were obtained from a BTEX-contaminated aquifer beneath a bulk fuel terminal near Portland, OR. Previous investigations at this site had shown evidence for the *in situ* degradation of alkylbenzenes under anaerobic conditions [16,19]. Indeed, site groundwater was confirmed to be anoxic with measured dissolved oxygen levels \( \leq 0.1 \) mg/L. In addition to background groundwater samples, groundwater samples containing nondeuterated and deuterated hydrocarbons and their putative anaerobic metabolites were obtained during a single well push-pull experiments conducted at this site in a manner similar to those described by Reusser et al. [18]. Briefly, 250 L of a test solution containing toluene-d$_5$, \( m \)-xylene-d$_{10}$ and ethylbenzene-d$_5$ at concentrations ranging from 1210 – 1921 \( \mu \)g/L, along with bromide as a conservative tracer (100 mg/L), were injected into four hydrocarbon-contaminated wells, designated CR-12, CR-13, CR-14, and CR-20 [16,24]. Groundwater samples, collected from the wells over a 25-day period, were obtained using a Masterflex peristaltic pump (Barnant Co., Barrington, IL) that was coupled to 0.6 mm nylon-braided PVC tubing (Kuriyama, Santa Fe Springs, CA) after first purging 3 well-casing volumes. Samples were collected in 1 L glass bottles preserved with 1.5% (vol/vol) 6M HCl to achieve a pH of 2, and stored at 4°C until analysis. An uncontaminated background well, CR-4, was also similarly sampled and used in method development experiments.
**Solid Phase Extraction (SPE).** Groundwater samples were brought to room temperature and vacuum filtered through a Whatman GDF 150, 1μm glass microfibre filter (Whatman Int. Ltd., Maidstone, U.K.). The filtered samples were spiked with 0.05 μg of the 4-FBA surrogate standard. An Empore SDB-XC 47 mm solid-phase extraction disk (3M, Inc., Minneapolis, MN) was placed in an MFS PP47, 47 mm polypropylene filter holder (Advantec/MFS, Inc., Dublin, CA) that was fitted to a Supelco vacuum manifold (Sigma-Aldrich Inc., Milwaukee, WI). The disk was preconditioned by first wetting with 10 mL of acetone and allowing the disk to dry. The disk was further conditioned by passing 10 mL of isopropanol, 10 mL methanol and 5 mL of pH 2 reagent water sequentially without allowing the disk to go dry. A sample volume of 100 mL was then applied and the disk was allowed to dry for a minimum of 2 h under vacuum.

Compounds adhering to the SDB-XC disks were eluted by passing three 3 mL aliquots of methanol through the disk and collected in 15 mL vials (Supelco, Bellefonte, PA). A 1 mL aliquot of reagent water was added to each vial along with 1 μg of 2,4-DCBA as an internal standard prior to reducing the volume to ~1 mL with heat (85 °C) under a steam of dry nitrogen using an N-Evap analytical evaporator (Organomation Associates, Berlin, MA). During the concentration process, care was taken to ensure samples were not allowed to go dry, otherwise significant analyte losses were observed (data not shown). The sample vials were capped, cooled to room temperature, then the contents were transferred to a 2 mL autosampler vial.

**SPE Spike and Recovery.** Spike and recovery experiments were performed to determine the accuracy and precision of the SPE method. For these experiments,
groundwater obtained from well CR-4 was used as the sample matrix since it was devoid of the analytes of interest. Five replicate 100 mL CR-4 groundwater samples were spiked to give a concentration of 1 µg/L of the following six analytes: [1] benzoate-d5, [2] m-toluate, [3] o-toluate-d7, [4] BSA, [5] ethylBSA and [6] m-methylBSA and 0.05 µg of the 4-FBA surrogate standard. All SPE extracts were spiked with 1 µg of the 2,4-DCBA internal standard prior to LC/MS/MS analysis. The concentrations of the analytes spiked into the CR-4 groundwater and processed through SPE and LC/MS/MS analysis were determined from calibration curves constructed from standards prepared in CR-4 groundwater.

**SPE Detection and Quantitation Limits.** To determine the MDL for the SPE method, 8 replicate 100 mL CR-4 groundwater samples were spiked to give 0.05 µg/L of each analyte and 0.5 µg/L of 4-FBA surrogate standard. An analyte concentration of 0.05 µg/L was selected because that concentration was estimated to be less than 5 times the predicted MDL. The MDLs for the SPE method were calculated as described by Glaser et al. [25]. Quantitation limits were defined as the concentrations that gave signal-to-noise values ≥ 10.

**Direct Injection.** A set of spike and recover experiments was performed to determine the accuracy and precision of the direct injection LC/MS/MS method. To this end, a set of 7 replicate, 1 mL samples of CR-4 groundwater were spiked to contain 100 µg/L of each analyte and 1 µg of the 2,4-DCBA internal standard. Concentrations were quantified from calibration curves constructed from standards prepared in CR-4 groundwater. For purposes of comparison, the accuracy and precision of direct injection
(25 μL injection volume) was determined. The MDL for direct injection was determined by spiking a set of 8 replicate 1 mL samples of blank CR-4 groundwater to contain 5 μg/L of each analyte and 1 μg of 2,4-DCBA internal standard and analyzing the samples by direct injection.

**Liquid Chromatography/Tandem Mass Spectrometry.** All compounds were separated by a Waters 2690 Separations Module (Waters Inc., Milford, MA) liquid chromatograph (LC) fitted with on a 150 x 2 mm Betasil C18 column (Thermo Electron Corp., Bellefonte, PA). A mobile phase consisting of 43:57 (vol/vol) mixture of methanol and 1 mM ammonium acetate buffer (pH 4) prepared in reagent water was used in isocratic mode with a 0.2 mL per min flow rate. A sample injection volume of 25 μL was used for all samples. The LC was interfaced to a Quattro Micro triple quadrupole mass spectrometer (Micromass Inc., Manchester, U.K.). The mass spectrometer was operated in the negative electrospray ionization (ESI) mode with a desolvation temperature of 300 °C and a source block temperature of 80 °C. The desolvation and cone gas flows were set at 575 L/hr and 92 L/hr, respectively. For all compounds, the capillary voltage was set to 2.75 kV, the cone voltage was set to 24 V, and the multiplier voltage was set to 650 V. Argon (purity 99.998%) was used as the collision gas and the collision energy was set at a value between 12 eV and 14 eV, with the values optimized for each analyte. Collision cell pressure was set to be between 2.1 e⁻³ mBar and 2.2 e⁻³ mBar. A maximum of 6 transitions were monitored to achieve maximum sensitivity. For this reason, separate LC/MS/MS analyses were conducted to determine the deuterated and non-deuterated analyte concentrations.
Benzylsuccinic acid was spiked into ammonium salt buffers ranging from pH 3 to pH 7 and directly infused to compare signal strength for determination of the optimal buffer pH to be used in the mobile phase. A buffer pH of 4 gave the largest signal and was thus selected for all subsequent infusion experiments. To optimize tune settings and determine the appropriate transitions for quantitation, each analyte was prepared at a concentration ranging between 20 and 50 μg/L in a 50:50 solution of methanol and 10 mM ammonium acetate buffer in reagent water (pH 4). The standard solutions were directly infused into the mass spectrometer at a rate of 10 μL/min via the syringe pump. Precursor ions of [M-H]+ corresponded to the molecular ion minus an ionizable hydrogen atom. The product ions [M-H-CO2]+, corresponded to the loss of the CO2 carboxyl moiety (m/z 44) and were used for quantitation [16].

**Quantitation.** Quantitation was performed by conventional internal standard calibration using calibration standards prepared both in reagent water and, where noted, in CR-4 groundwater. Calibration standards ranged from 5 μg/L to 500 μg/L for each analyte and contained 0.05 μg of the 4-FBA surrogate standard and 1 μg of the 2,4-DCBA internal standard. Absolute recoveries of analytes following SPE were determined from calibration curves constructed from analytes and the 2,4-DCBA internal standard while relative recoveries were determined from curves developed from analytes and the 4-FBA surrogate standard. Weighted linear regression (1/x) was used to generate calibration curves [26,27] from 5 calibration standards. Calibration curves were not forced through zero and R² values typically were 0.999.
Results And Discussion

**Chromatography/Mass Spectrometry.** Initial experiments were aimed at establishing the criteria for the detection and quantitation of deuterated analytes when authentic standards of deuterated analytes were not available. During the initial infusion experiments, precursor ions of [M-H]⁻ with transition to product ions [M-H-COO]⁻ were obtained for all the carboxylated analytes of interest and used for quantitation (Table 2.1). The transitions obtained for BSA and m-methylBSA were consistent with those reported by Beller [16]. Transitions of precursor ions to product ions were obtained for the nondeuterated analytes and their deuterated counterparts including benzoate/benzoate-d5, o-toluate/o-toluate-d7, and for m-methylBSA/m-methylBSA-d10. The consistent (e.g., predictable) difference in masses between the deuterated and nondeuterated analytes indicates that transitions can be reliably predicted for deuterated analytes from their nondeuterated counterparts when authentic standards are not available.

Under the chromatographic conditions used for this study, the deuterated analogs gave shorter (0.2 to 0.8 min) retention times than their non-deuterated counterparts (for example, compare m-toluate (Figs. 2.1a & b) and d7-m-toluate (Fig. 2.1c). Earlier elution of deuterated analytes is well documented and is due to the lower vibrational energy of the C-D bond compared to the C-H bond. The shorter, stronger C-D bonds are slightly less polarizable than C-H bonds and exhibit less affinity for the non-polar stationary phase [28-31].
Response factors and/or retention times for equimolar concentrations of selected deuterated analytes were determined relative to their non-deuterated analytes. The

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Molecular Weight</th>
<th>Precursor Ion [M-H]^- (m/z)</th>
<th>Product Ion [M-H-COO]^- (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>122</td>
<td>121</td>
<td>77</td>
</tr>
<tr>
<td>d5-Benzoate</td>
<td>127</td>
<td>126</td>
<td>82</td>
</tr>
<tr>
<td>o-, m-, p-Toluate</td>
<td>136</td>
<td>135</td>
<td>91</td>
</tr>
<tr>
<td>o-Toluate-d7</td>
<td>143</td>
<td>142</td>
<td>98</td>
</tr>
<tr>
<td>Benzylsuccinate</td>
<td>208</td>
<td>207</td>
<td>163</td>
</tr>
<tr>
<td>Benzylsuccinate-d5*</td>
<td>213</td>
<td>212</td>
<td>168</td>
</tr>
<tr>
<td>Ethylbenzylsuccinate</td>
<td>222</td>
<td>221</td>
<td>177</td>
</tr>
<tr>
<td>m-Methylbenzylsuccinate</td>
<td>222</td>
<td>221</td>
<td>177</td>
</tr>
<tr>
<td>m-methylbenzylsuccinate-d10*</td>
<td>232</td>
<td>231</td>
<td>187</td>
</tr>
<tr>
<td>2,4-Dichlorobenzoate (DCBA)</td>
<td>191</td>
<td>189</td>
<td>145</td>
</tr>
<tr>
<td>(Internal Std)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Fluorobenzoate (4-FBA)</td>
<td>140</td>
<td>139</td>
<td>95</td>
</tr>
<tr>
<td>(Surrogate Std)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* biologically-generated (see Experimental Section)
Fig. 2.1 Chromatograms for (a) a calibration standard with analytes (5 μg/L) spiked into CR-4 groundwater, (b) non-deuterated metabolites in CR-20 groundwater processed by solid phase extraction, and (c) deuterated metabolites in CR-20 groundwater processed by solid phase extraction.
deuterated/non-deuterated analyte pairs evaluated included benzoic/ benzoic-d₅ and o-toluic/d₇-o-toluic acid. The BSA/ BSA-d₅ and m-methylBSA/ m-methylBSA-d₁₀ pairs were not used to determine response factors because the biologically-generated d₅-BSA and m-methylBSA-d₁₀ concentrations in the microcosm were not known precisely. Response factors for deuterated analytes were within 10% of those obtained for non-deuterated analytes. In addition, the linear regressions of response factor versus analyte concentration for the deuterated analytes were ± 10% of those for the nondeuterated analytes (data not shown). For these reasons, deuterated analytes for which authentic standards were not available (e.g., m-toluate-d₇) were quantified from calibration curves constructed from the corresponding authentic standards of non-deuterated analytes (e.g. m-toluate).

Attention was paid to the determination of benzoate-d₅ because it is a metabolite in common to ring-deuterated toluene and ethylbenzene. Although d₄-benzoate would be expected from the transformation of ring-deuterated xylenes, sensitivity was maintained by keeping the number of multiple reaction monitored (MRM) ions to a minimum. For this reason, we chose to eliminate d₄-benzoate from our list of analytes. In addition, (nondeuterated) benzoate also was eliminated from further consideration because we were unable to obtain water samples that were blank with respect to benzoate for method development purposes.

**Ion Suppression.** Signal suppression due to matrix effects have been reported for ESI mass spectrometry [32-35]. Signal suppression can occur because of competition between analyte and matrix-component ions for access to the droplet surface in the spray
Fig. 2.2 Infusion chromatogram (20 min infusion of a mixture that was 100 μg/L each of analyte) of a) a 25 μL injection of reagent water and b) a 25 μL injection of blank CR-4 groundwater. Loss of signal between 2 and 8 min indicates ion suppression due to the groundwater matrix.

for emission as the gas-phase ion [33]. Since the goal of this research was to develop methods to detect and quantitate metabolites in groundwater samples, it was necessary to confirm that quantifiable analyses could be conducted with such matrices. Initially, 2,4-DFBA was selected for use as an internal standard for quantitation purposes for its structural similarity to the analytes under investigation and the low probability it would
occur in groundwater from aromatic hydrocarbon-contaminated sites. However, further investigations revealed a significant loss of 2,4-DFBA signal when this standard was added to CR-4 groundwater. To test for ion suppression, the effluent line from the HPLC column was fit with a PEEK t-fitting and connected to a syringe pump that was used to continuously infuse (post-column) a 100 µg/L standard mixture of analytes into the mass spectrometer over a 20 min period in an approach described by Bonfliglio et al. [32]. Blank reagent water (Fig. 2.2a) and groundwater from well CR-4 (Fig. 2.2b) were then injected (25 µL each) separately into the HPLC and the signal recorded over 20 min. Signal suppression, detected as a drop in the baseline beginning around 2 min, was greatest for the groundwater sample (Fig. 2.2a). It is likely that polar matrix components are responsible for the observed ion suppression because neither filtration nor solid phase extraction lessened the level of ion suppression for 2,4-DFBA, which eluted at ~4.2 min. Alternatively, 2,4-DCBA, which eluted at ~6.5 min, was selected as the internal standard because its retention time was beyond the period of greatest ion suppression. Therefore, unless otherwise noted, 2,4-DCBA was used as the internal standard for all subsequent analyses.

**Solid Phase Extraction Method.** Empore extraction disks were selected for the solid-phase extraction procedure because of their ease of use and fast flow rates. The 47 mm SDB-XC disks were selected for this study because strong anion-exchange disks and C18 disks gave poor performance in earlier studies [18]. Initial breakthrough experiments indicated that up to 150 mL of sample could be passed through the 47 mL SDB-XC disk without analyte breakthrough including benzoate-d5, o-toluate-d7,
Table 2.2 Precision and accuracy of the solid phase extraction and direct injection methods for analytes spiked into blank CR-4 groundwater.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Solid Phase Extraction</th>
<th>Direct Injection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute (%)</td>
<td>Relative (%)</td>
<td>Absolute (%)</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>RSE</td>
<td>Recovery</td>
</tr>
<tr>
<td>Benzoate-(d_5)</td>
<td>80 ± 7</td>
<td>3</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>(m)-Toluate</td>
<td>96 ± 5</td>
<td>2</td>
<td>116 ± 6</td>
</tr>
<tr>
<td>(\alpha)-Toluate-(d_7)</td>
<td>84 ± 7</td>
<td>3</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>Benzylsuccinate</td>
<td>91 ± 9</td>
<td>4</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>EthylBSA</td>
<td>106 ± 6</td>
<td>2</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>(m)-MethylBSA</td>
<td>101 ± 7</td>
<td>2</td>
<td>117 ± 4</td>
</tr>
<tr>
<td>4-FBA (Surrogate Std)</td>
<td>86 ± 5</td>
<td>2</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* 5 replicate samples of blank CR-4 groundwater spiked to contain 1.0 μg/L of each analyte; injection volume 25 μL

*b* 7 replicate samples of blank CR-4 groundwater were spiked to contain 100 μg/L of each analyte; (direct) injection volume 25 μL

*c* Absolute recovery relative to the internal standard; reported at the 95% confidence interval

*d* RSE = relative standard error = \((\text{StDev}/\sqrt{n})/\text{Average Recovery}\) x 100

*e* Recovery relative to the surrogate standard; reported at the 95% confidence interval

benzylsuccinate, ethylbenzylsuccinate, and m-methylbenzylsuccinate (data not shown). A conservative volume of 100 mL was selected for subsequent use in method development.
The accuracy and precision of SPE was determined from spike and recovery experiments performed with analytes at high (1 μg/L) and low (0.05 μg/L) concentration in CR-4 groundwater. The absolute recoveries of analytes, determined relative to the internal standard, at a concentration of 1 μg/L ranged from 80 to 106% (Table 2.2). The precision of the method, as indicated by the relative standard error (RSE), ranged from 2 to 4% for the 5 replicate samples analyzed. The relative recovery of analytes, determined relative to the 4-FBA surrogate standard, ranged from 93 to 124% with RSE ranging from 1 to 4% (Table 2.2). From these data, 4-FBA was determined to be a suitable surrogate standard for these analytes in the SPE method. The SPE method detection limit (MDL) was determined for blank CR-4 groundwater that was spiked with each analyte to a final concentration of 0.05 μg/L. The calculated MDL determined from these samples ranged from 0.007 to 0.022 μg/L (Table 2.3) and are a factor of 10 lower than that reported by others [16,18]. At a concentration of 0.05 μg/L, the chromatographic peaks for the analytes gave signal-to-noise ratios (S/N) that ranged from 3 to 4 for BSA, ethylBSA, and methylBSA and from 13 to 19 for benzoate-d₅, m-toluate, and o-toluate-d₇ (data not shown). The quantitation limits, defined as the concentration needed to produce a S/N ≥ 10, was defined as 0.05 μg/L for benzoate-d₅, m-toluate, and o-toluate-d₇ (Table 2.3).

The estimated quantitation limit, determined as 3 times the 0.05 μg/L concentration that gave the observed S/N of ≥ 3, was 0.15 μg/L for BSA, ethylBSA, and m-methylBSA (Table 2.3).
Table 2.3 Method detection limit (MDL) and quantitation limit (QL) for analytes by solid phase extraction and direct injection into blank CR-4 groundwater.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Solid Phase Extraction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Direct Injection&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDL (µg/L)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Quantitation Limit&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Benzoate-d&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.007</td>
<td>0.05</td>
</tr>
<tr>
<td>m-Toluate</td>
<td>0.020</td>
<td>0.05</td>
</tr>
<tr>
<td>o-Toluate-d&lt;sub&gt;7&lt;/sub&gt;</td>
<td>0.016</td>
<td>0.05</td>
</tr>
<tr>
<td>Benzy1succinate</td>
<td>0.022</td>
<td>0.15</td>
</tr>
<tr>
<td>EthylBSA</td>
<td>0.020</td>
<td>0.15</td>
</tr>
<tr>
<td>m-MethylBSA</td>
<td>0.019</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> 8 replicate samples of blank CR-4 groundwater were spiked to contain 0.05 µg/L of each analyte. Concentrations were determined from calibration curves constructed from standards prepared in blank CR-4 groundwater.

<sup>b</sup> 8 replicate samples of blank CR-4 groundwater were spiked to contain 5 µg/L of each analyte. Concentrations were determined from calibration curves constructed from standards prepared in blank CR-4 groundwater.

<sup>c</sup> MDL = t<sub>n-1, 1-α=0.99</sub> * StDev [25]

<sup>d</sup> Quantitation limit = concentration required to produce S/N ≥ 10.

**Direct Injection Method.** The recovery of analytes, when spiked into blank CR-4 groundwater at 100 µg/L, ranged from 99 to 104% with relative standard errors of 1 to 4% (Table 2.2). When analytes were spiked into blank CR-4 groundwater at a concentration of 5 µg/L, recovery ranged from 100 to 116% with relative standard errors of 1 to 4% (data not shown). The MDL for direct injection, determined from the replicate samples containing 5 µg/L of each analyte in blank CR-4 groundwater, ranged from 0.73
to 1.5 µg/L (Table 2.3). These MDLs are 2 to 5 times higher than those obtained in a previous direct injection study (0.3 µg/L) [16]. Thus, given that the SPE method described was found to be more sensitive than the direct injection method in our studies, we selected SPE coupled with LC/MS/MS to detect and quantify metabolites in hydrocarbon-contaminated groundwater.

**SPE Method Demonstration on Groundwater Samples.** Selected samples taken from single-well push-pull tests conducted at the Northwest Terminal site in which toluene-d₅, ethylbenzene-d₅, and m-xylene-d₁₀ were injected into 4 wells. Samples from wells CR-12, CR-13, and CR-14 did not contain any of the target analytes above method detection limits (data not shown). This was surprising, since methyl-BSA was detected in these wells in previous examinations [16,18]. However, both non-deuterated (Fig. 2.1b) and deuterated (Fig. 2.1c) analytes were detected in samples collected from well CR-20 at various time points after injection. Of the non-deuterated benzylsuccinates, m-methylBSA was detected at the highest levels (0.20 to 1.14 µg/L) followed by ethylBSA (0.16 to 0.67 µg/L), while BSA was below the detection limit of 0.02 µg/L (Table 2.4; Fig. 2.1b). In well CR-20, m-toluate was detected (0.14 – 0.61 µg/L) as were o-toluate and p-toluate, although the latter were not quantified. The most abundant deuterated metabolite
Table 2.4 Concentrations (µg/L) of non-deuterated and deuterated metabolites of toluene, ethylbenzene, and xylenes and their deuterated analogs in groundwater obtained from well CR-20.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CR-20 Sample 6</th>
<th>CR-20 Sample 16</th>
<th>CR-20 Sample 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate-d$_5$</td>
<td>0.64</td>
<td>&lt; QL</td>
<td>&lt;QL</td>
</tr>
<tr>
<td>m-Toluene</td>
<td>0.61</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>m-Toluene-d$_7$</td>
<td>1.2</td>
<td>0.82</td>
<td>0.64</td>
</tr>
<tr>
<td>Benzylsuccinate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Benzylsuccinate-d$_5$</td>
<td>&lt; QL</td>
<td>0.18</td>
<td>0.21</td>
</tr>
<tr>
<td>Ethylbenzylsuccinate</td>
<td>0.16</td>
<td>0.53</td>
<td>0.67</td>
</tr>
<tr>
<td>Ethylbenzylsuccinate-d$_5$</td>
<td>&lt; QL</td>
<td>0.28</td>
<td>0.29</td>
</tr>
<tr>
<td>m-Methylbenzylsuccinate</td>
<td>0.20</td>
<td>1.02</td>
<td>1.14</td>
</tr>
<tr>
<td>m-Methylbenzylsuccinate-d$_{10}$</td>
<td>&lt; QL</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

< QL =Detected but at less than quantitation limit given in Table 3.
ND =Not detected above MDL of BSA (0.022 µg/L).

detected was m-toluate-d$_7$ (0.64 to 1.2 µg/L), followed by m-methylBSA-d$_{10}$ (< QL- 0.40 µg/L), ethylBSA-d$_5$ (< QL to 0.29 µg/L), and BSA-d$_5$ (< QL - 0.21 µg/L) (Table 2.4).

The identification of deuterated metabolites in CR-20 groundwater samples was verified by spiking the samples with deuterated standards. Authentic standards of benzoate-d$_5$ (Fig. 2.3a) and m-toluate (Fig. 2.3b) added to a CR-20 extract increased the peak area relative to that of the pre-spiked sample. The apparent recovery of benzoate-d$_5$ and m-toluate, spiked into extracts, ranged from 81 to 90% and from 90 to 113%, respectively (data not shown). The additions of BSA-d$_5$ (Fig. 2.3c) and m-methylBSA-
Fig. 2.3 Typical chromatograms for a single sample of CR-20 before and after spiking with deuterated and non-deuterated analytes including (a) benzoate-d_5, (b) m-toluate, (c) BSA-d_5, and (d) m-methylBSA-d_{10}.

d_{10} (Fig. 2.3d) increased the corresponding peak areas of these compounds in the CR-20 groundwater extracts relative to the pre-spiked sample, with recoveries ranging from 83 to 92% and from 110 to 156%, respectively (data not shown).

While Reusser and co-workers [18,19] were able to detect and quantify BSA in groundwater samples from the Northwest Terminal site, we were not able to detect its presence using SPE coupled with LC/MS/MS in our push-pull tests. Such a result may not be too uncommon, since other studies have mentioned that putative hydrocarbon
metabolites are not always detected at every sampling event, even in the same wells [9,36]. However, we did detect and quantify \textit{m}-methylBSA at concentrations lower than those possible using different methods followed in earlier studies [16,18].

In addition to our detection of benzylsuccinates as unique indicators of anaerobic alkylbenzene metabolism, we also detected toluates and benzoates. Although not considered unique to the anaerobic degradation of xylenes [9,13] since they may also be produced aerobically [20], these putative hydrocarbon metabolites were detected at higher concentrations than the signature benzylsuccinates at anoxic field sites [9]. The fact that we detected deuterated benzoate and \textit{m}-toluate in addition to deuterated BSA and \textit{m}-methylBSA in a push-pull test at an anoxic site does suggest that they were produced anaerobically and indicates further transformation of the benzylsuccinates thereby suggesting BSA and \textit{m}-methylBSA are not dead-end products in this system. Detection of the unambiguous deuterium-labeled succinate metabolites coupled with the detection of similarly labeled biodegradation products of those succinate metabolites should be useful for obtaining unequivocal evidence for \textit{in situ} alkylbenzene mineralization.

\textbf{Acknowledgments}

We thank Tim Buscheck of CRTC and Gerry Koshal of Redhill Environmental and PNG Environmental for field support. Thank you to Kim Hageman, Melissa Schultz and Ralph Reed from Oregon State University. Funding for this research was provided by National Science Foundation Environmental Engineering Directorate Grant 2004-06.
Literature Cited:

Chapter 3

Detection and Quantification of Signature Metabolites of Anaerobic Alkylbenzene Biotransformation During Single-Well Push-Pull Tests

Abstract

Field tests to determine in-situ biodegradation rates of alkylbenzenes including, toluene, ethylbenzene and xylene, are needed to evaluate the effectiveness of bioremediation in contaminated aquifers. Single-well, push-pull tracer tests were performed to determine the rate of signature metabolite appearance as well as the loss of selected deuterated alkylbenzene substrates. Test solutions (250 L) containing the deuterium-labeled organic substrates toluene, ethylbenzene, and xylene, along with nitrate as an electron acceptor and bromide as a non-reactive tracer, were injected into each of four existing wells at an alkylbenzene-contaminated field site. Samples of the test solution/groundwater blend were then extracted over a 29-day period. Volatile alkylbenzenes were analyzed and quantified by gas chromatography/mass spectrometry (GC/MS). Metabolites were determined by solid-phase extraction (SPE) followed by high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis. Due to the rapid transport of the test solution through the aquifer, resulting in the loss of the tracer signal, no test solution was available for three wells. Of the wells tested, only the test conducted in well CR-20 gave samples with sufficient concentration of analytes to warrant full processing of the samples and data. Decreases in deuterated alkylbenzene substrates and the appearance of signature metabolites including deuterated ethylbenzylsuccinate (ethylBSA-d5) and o-methylbenzylsuccinate (o-methylBSA-d10),
were observed while deuterated benzylsuccinate (BSA-d5) was not detected. Of the deuterated secondary metabolites monitored, m-toluate-d7 and benzoate-d5 also were determined in well CR-20. Although analytes were detected in well CR-20, erratic changes in analyte concentrations made it infeasible to determine rates.

Introduction

Large volumes of petroleum hydrocarbons are handled and consumed each year by industrialized nations [1]. Recent news articles report the introduction of petroleum hydrocarbons into the aqueous environment through accidental spills, through deteriorating infrastructure such as leaking underground storage tanks and transfer lines, and through illegal dumping [2-4]. Benzene, toluene, ethylbenzene and the xylene isomers (BTEX) are among the alkylbenzene components in many of the petroleum products used as solvents, lubricants and to power internal combustion engines. The toxicity of benzene, a known carcinogen, as well as toluene, ethylbenzene, and xylene are listed by the U.S. EPA as priority pollutants [5] and rated on CERCLA’s 2003 Priority List of Hazardous Substances [6]. Because of the relative high water solubility of BTEX [Table 3.1.] they are found in higher concentrations than other petroleum constituents and are readily transported by groundwater advection. As a result, BTEX reaches and contaminates water sources used for domestic and agricultural use, as well as reaching aquatic habitats.

Although many different engineered approaches to ex-situ groundwater cleanup strategies have been proposed over the years they have largely been found to be ineffective at restoring contaminated groundwater sites; estimated remediation times up
### Table 3.1 Water Solubilities of Selected Automotive Gasoline Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
<th>Water Solubility&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>(mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>3.2</td>
<td>1800</td>
<td></td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>1.4</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>4.8</td>
<td>526</td>
<td></td>
</tr>
<tr>
<td>Xylenes</td>
<td>6.6</td>
<td>162&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>n-Hexane</td>
<td>11.6</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Isopentane</td>
<td>15.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>3.0</td>
<td>insoluble</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent by weight  
<sup>b</sup> Values at 25°C from the Toxnet Hazardous Substance Database (HDSB) [7]  
<sup>c</sup> p-Xylene  
<sup>d</sup> Total percent of branched pentanes

Due to the ineffectiveness of ex-situ remediation of groundwater, bioremediation has emerged as an option of interest because of its simplicity, effectiveness, and low cost (relative to other engineered systems) and because it can be employed under both aerobic and anaerobic conditions [9].

Anaerobic degradation of alkylbenzenes is well documented in reports that describe microbial microcosms studies [10-19]. Benzylsuccinate, methylbenzylsuccinate
and ethylbenzylsuccinate are considered to be unequivocal ‘signature’ metabolites of BTEX mineralization [19-23]. Of further significance, these signature metabolites have been found in groundwater [19,24-27].

Although not considered unequivocal evidence of anaerobic BTEX degradation, secondary metabolites are formed. Toluates and phthalates form from the degradation of methylbenzylsuccinate, while benzoate is a degradation product common to benzylsuccinate, methylbenzylsuccinate and ethylbenzylsuccinate [18,28,29]. With the exception of toluates, secondary metabolites typically are found at concentrations lower than those of the primary benzylsuccinate metabolites in microcosms [19].

Differences in rates for processes in laboratory and field studies underscore the need for field studies to provide realistic rate information for evaluating in-situ biotransformation [30-34]. While a number of field studies providing qualitative assessments of in-situ biodegradation of alkylbenzenes (e.g. detection of unambiguous metabolites) are reported [19,25,26,35,36], few studies report rates of biotransformation [24,37,38]. This is most likely due to the difficulty in determining in-situ transformation rates.

To evaluate if remediation goals are being met, it is necessary to determine degradation rates yet, because of groundwater processes such as advection and diffusion that simply transport rather than eliminate contaminant mass, it is difficult to determine in-situ degradation rates. Rates for in-situ bioremediation of contaminated groundwater can be investigated using multi-well tracer tests where changes in reactant, product, and tracer concentrations are measured, temporally and spatially, at a series of wells arranged
as transects. Alternatively, the use of single-well tests can be used, where reactant, product, and tracer concentrations are measured at a single well over time. The use of the single-well push-pull test has many advantages over the multi-well test including: (1) push-pull tests can be done at an established well, eliminating the expense and time to drill multiple wells, (2) quantifiable recoveries of the test slug can be achieved in order to address regulatory concerns and, (3) the time required to run a test can be tailored to the experimental design rather than relying on groundwater flow rates to transport a test solution to a downgradient well [39-43]. Due to these advantages we used push-pull tests to evaluate bioremediation in four wells at a bulk fuel transfer site in Portland Oregon.

Push-pull tracer tests are accomplished by injecting a test solution (the “push”) followed by a reaction period that is dependent on the test goals and finally culminating in the extraction phase (the “pull”), the length of which is also determined by the test goals. The test solution usually contains substrates of interest, a non-reactive tracer used to normalize concentration changes of non-sorbing compounds to account for dilution, and any chemical amendments such as electron acceptors or donors. Ideally, injected substrates and any expected products need to be differentiated from any background contaminants. Beller et al. [24] injected a blank buffer, or isolation slug, followed by a test solution spiked with toluene, o-xylene and m-xylene in order to assess in-situ biodegradation. A somewhat simpler solution takes advantage of labeled compounds that can be differentiated from the contaminants found on site. Hageman et al. successfully used trichlorofluoroethene (TCFE), a fluorine-labeled compound, to study in-situ reductive dechlorination of trichloroethene [40]. Reusser et al. used an analogous
approach to obtain alkylbenzene biodegradation rates by injecting toluene-$d_8$ and o-xylene-$d_{10}$ and observing the loss of the deuterated substrates and corresponding appearance of the deuterated metabolites [37]. To the best of my knowledge, in-situ biodegradation of ethylbenzene has not been studied.

To expand on the work done by Reusser et al [36,37], a push-pull test was designed to determine the in-situ degradation rates of ethylbenzene-$d_5$, toluene-$d_5$, and xylene-$d_{10}$. In addition to the signature metabolites BSA-$d_5$ methylBSA-$d_{10}$, and ethylBSA-$d_5$, selected secondary metabolites including toluate-$d_7$, and benzoate-$d_5$ were quantified in extraction-phase samples for additional evidence to confirm in-situ biodegradation of the deuterated alkylbenzenes.

**Experimental Methods**

**Reagents and Standards.** The test solution included ring-deuterated toluene-$d_5$, ring deuterated ethylbenzene-$d_5$ and perdeuterated m-xylene-$d_{10}$ purchased from CDN Isotopes (Quebec, Canada). Internal standards used for GC analysis included 4-bromofluorobenze purchased from Chem Service (West Chester, PA) and 1-chlorobutane from Mallinckrodt, Inc. (St. Louis, MO). A BTEX standard (2000 $\mu$g/mL in methanol) also was purchased from Chem Service. Toluene (ACS reagent grade, 99.5%), ethylbenzene (99%), and o-xylene (97%) were purchased from Aldrich Chemical (Milwaukee, WI). The reagent grade potassium nitrate used as an electron acceptor in the test solution, was obtained from JT Baker (Phillipsburg, NJ) and the conservative tracer potassium bromide, certified grade, was purchased from Fisher Scientific (Fair Lawn, NJ).
Field Site and Background Sampling. A group of push-pull experiments were conducted at “Northwest Terminal”, a bulk fuel terminal in Portland Oregon [Fig. 3.1]. The unconfined aquifer at this site consists of a layer of fill (medium dense to fine-grained sand and silty sand) overlying alluvium consisting of clayey silt with sand and organics. Groundwater flow in the fill material is approximately 0.25 to 0.34 meters per day, east toward the river [44]. Flow rates and water table heights vary with precipitation.

Fig. 3.1 Schematic of Northwest Bulk Fuel Terminal. Circles represent fuel storage tanks.
Groundwater was anaerobic with dissolved oxygen levels of 0.1 mg L\(^{-1}\) or less. Wells ranged from 4.6 – 4.9 m in depth below land surface and were constructed from 2-inch schedule-40 PVC screened over the last 3.05 m.

Background samples were collected from the four contaminated wells selected for the push-pull experiments (CR-12, CR-14, CR-15 and CR-20) immediately prior to the injection phase of push-pull tests. All background samples were obtained after first purging 2 to 3 well-casing volumes (5-10 L) using a Masterflex peristaltic pump (Barnant Co., Barrington, IL) coupled to ¼-inch, nylon-braided PVC tubing (Kuriyama, Santa Fe Springs, CA,). Metabolite samples were collected in 1 L glass bottles and preserved with 1.5% (vol/vol) 6 M HCl (15 mL) while volatile samples were collected with zero headspace in 40 mL VOA (volatile organic analysis) vials (VWR, So. Plainfield, NJ) preserved with 1.5% (vol/vol) 6 M HCl (0.6 mL). Samples for anion analysis, including bromide and nitrate were collected in 15 mL vials without preservation. All samples were stored on ice until transfer to cold storage at 4 °C where they were kept until analysis.
**Push-Pull Transformation Tests.** The concentrated test solutions were made by spiking 531 mg of each of the deuterated compounds into 4.25 L of reagent water containing 39.6 g of potassium bromide in cleaned and baked 4-L solvent bottles with minimal headspace. Each bottle of solution was mixed using a Teflon stir bar with a magnetic stir plate and allowed to continuously mix for four days before being transferred by siphon to Teflon bags. Four, custom-made, collapsible 5-L Teflon bags (Alltech Associates, Deerfield, IL) fitted with a push-pull type valve (2-N-1) and a septum port were filled with the concentrated test solutions. The last 50 mL of mixed solution was left in the bottle to reduce the likelihood of transferring any non-aqueous phase liquid. After the test solutions in their Teflon bags were allowed to equilibrate overnight, concentrations of the deuterated volatiles [Table 3.2.](#) were determined by purge and trap, gas chromatography/mass spectrometry analysis of samples taken through the septum ports.

At the selected test site a 285-L carboy was filled with 250 L of tap water into

<table>
<thead>
<tr>
<th>Well</th>
<th>d$_5$Ethylbenzene</th>
<th>d$_{10}$m-Xylene</th>
<th>d$_5$Toluene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR-12</td>
<td>73.4</td>
<td>77.5</td>
<td>100.8</td>
</tr>
<tr>
<td>CR-13</td>
<td>75.7</td>
<td>78.8</td>
<td>100.6</td>
</tr>
<tr>
<td>CR-14</td>
<td>75.2</td>
<td>79.4</td>
<td>100.7</td>
</tr>
<tr>
<td>CR-20</td>
<td>74.8</td>
<td>78.7</td>
<td>100.4</td>
</tr>
</tbody>
</table>
which 40.8 g of potassium nitrate (KNO₃) was dissolved. The filled carboy was continuously sparged with argon for a minimum of 12 hours to remove dissolved oxygen and to thoroughly mix the KNO₃. Argon sparging continued throughout the injection phase. The injection apparatus [Fig. 3.2] consisted of a Masterflex peristaltic pump connected to the 250-L carboy by Tygon tubing, and a piston pump connected to the Teflon collapsible bag by Tygon tubing; the two Tygon lines exiting the pumps were joined with a ¼-in. nylon tee-fitting that lead to a 25-m Teflon mixing-coil that was connected to a section of braided nylon tubing long enough to reach the bottom of the injection well. The targeted injection rate was 1 L min⁻¹. The group of four wells (CR-12, CR-13, CR-14 and CR-20) each received 250 L of the test solution [Table 3.3.] containing 1.0-1.9 mg L⁻¹ toluene-d₅, o-xylene-d₁₀, ethylbenzene-d₅ as well as 1.2 mM bromide (as a conservative tracer) and 1.5 mM nitrate (as the electron acceptor). A small valve fitted immediately after the mixing coil allowed for sampling the test solution as it was being injected. Injection-phase samples for volatile, metabolite,
and bromide analyses were taken at the beginning of the injection and every 50 L thereafter. Dissolved oxygen concentrations in the test solution were determined every sample session by colorimetric determination using a Chemets kit (Chemetrics, Calverton, VA).

Following the injection phase, extraction-phase sampling occurred over a 29-day period. Samples were collected twice per day during the first week after injection followed by a two-week period of collecting groundwater samples once every other day (Monday-Wednesday-Friday) with no samples collected during the weekends. After first purging 2 to 3 well-casing volumes (5-10 L) extraction-phase samples were obtained using a Masterflex peristaltic pump (Barnant Co., Barrington, IL) that was coupled to an extraction line made of Masterflex silicone tubing joined with 3/8-inch, low-density polyethylene tubing (VWR Scientific Products, So. Plainfield, NJ). Extraction-phase samples for metabolite analysis were collected in 1-L glass bottles and preserved with

Table 3.3 Average Concentrations of Analytes Injected in Selected Wells at Northwest Terminal.

<table>
<thead>
<tr>
<th>Well</th>
<th>Ethylbenzene-d5</th>
<th>m-Xylene-d10</th>
<th>Toluene-d5</th>
<th>Bromide</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR-12</td>
<td>1.3</td>
<td>1.3</td>
<td>1.9</td>
<td>110</td>
<td>88</td>
</tr>
<tr>
<td>CR-13</td>
<td>1.2</td>
<td>1.3</td>
<td>1.8</td>
<td>112</td>
<td>81</td>
</tr>
<tr>
<td>CR-14</td>
<td>1.0</td>
<td>1.1</td>
<td>1.5</td>
<td>84</td>
<td>78</td>
</tr>
<tr>
<td>CR-20</td>
<td>1.3</td>
<td>1.4</td>
<td>1.9</td>
<td>120</td>
<td>90</td>
</tr>
</tbody>
</table>
1.5% (vol/vol), 6 M HCl (15 mL) while samples for volatile analysis were collected with zero headspace in 40-mL VOA vials and preserved with 1.5% (vol/vol), 6 M HCl (0.6 mL). Extraction-phase samples for anion analysis, including bromide and nitrate, were collected in 15-mL vials without preservation. Dissolved oxygen determinations made by colorimetric analysis (Chem-Ets) were made to confirm anaerobic conditions. Extraction-phase samples collected for analysis of volatiles, metabolites, and bromide were stored on ice and transferred to cold storage at 4 °C where they remained until analysis.

**Test Solution Collapsible Bags.** To contain the concentrated volatile, deuterated alkylbenzenes test solution, collapsible bags were used for the push-pull apparatus to avoid mass loss due to volatilization Collapsible, metallized-film gas-sampling bags (Chromatography Research Supplies, Addison, IL) were used in previous studies [37,40], although mass loss from metallized bags due to outgassing and/or sorption is known to occur. A Teflon collapsible bag was found through Alltech Chromatography (Deerfield, IL) and a study was undertaken to determine the best type of collapsible bag to use to minimize the mass loss of the volatile compounds. Mass loss was traced by injecting a known aqueous concentration of ethylbenzene, toluene and o-xylene (data not shown) into a collapsible bag and quantifying the test solution over time. From work done by D. Reusser [45] it was determined the metallized bag lost 66-80% of the initial mass within 5 hours [Fig. 3.3.a]. From my work it was determined the Teflon bag lost 9-16% of the initial mass over 10 hours [Fig. 3.3.b]. I speculate the initial mass loss is due to sorption on the inner surface of the bags. The Teflon bag continued to loose mass over an
Fig. 3.3 Comparison of Teflon bags to metallized bags. Concentrations of alkylbenzenes normalized to initial concentrations over time.

extended period, possibly through outgassing, eventually losing 20-33% over 5 days,
still better than the metallized bag performance. The mass loss of toluene, xylene and ethylbenzene in aqueous solution from metallized bags compared to solutions in Teflon bags resulted in the decision to use the Teflon bags during the push-pull tests. It is recommended that transfer of mixed volatile solutions from a mixing bottle to the collapsible bag be as close to injection time as possible.

**Volatile Organic Compound Analysis.** Volatile concentrations in groundwater samples were determined using purge-and-trap/GC-MS analysis. A Tekmar-Dohrmann (Cincinnati, OH) 3100 purge-and-trap sample concentrator fitted with a Tenax/Silica Gel/Charcoal trap was used. For ease and efficiency of sample handling an AQUATek 70 auto sampler (Tekmar-Dohrmann) was employed. Aqueous samples were purged with grade 4.7 helium for 11 min. to strip volatiles from the groundwater which are then captured on the trap. A desorption temperature of 245 °C for 1 min. was used to desorb the target analytes off the trap for transfer the GC. Line and valve temperatures were set at 150 °C.

For separation of the volatile compounds, a Hewlett-Packard model 6890 GC was used. A 30-m X 0.32-mm X 4-μm SPB-1 Sulfur capillary column (Supelco Inc., Bellefonte, PA) was used for all separations under splitless-injection conditions with an injector temperature of 175 °C. A gradient temperature program was run with an initial oven temperature of 50 °C held for 2 min. before ramping up at 15 °C min\(^{-1}\) to a final temperature of 180 °C. The total run time was 17 min. A Hewlett-Packard model 5972 mass selective detector (MSD) was used for detection and operated in electron-impact (70 eV), scan mode over the range 30 – 300 m/z with the source temperature set at 265
Table 3.4 GC/MS Quantifying and Qualifying Ions for Volatile Analytes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R.T. (min)</th>
<th>Quantifying Ion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Qualifying Ion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Qualifying Ion&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Bromofluorobenzene&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.13</td>
<td>173.95</td>
<td>95.00</td>
<td>175.90</td>
</tr>
<tr>
<td>1-Chlorobutane&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.94</td>
<td>56.00</td>
<td>41.0</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>5.39</td>
<td>78.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>6.90</td>
<td>91.05</td>
<td>92.00</td>
<td></td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>8.33</td>
<td>91.05</td>
<td>106.05</td>
<td></td>
</tr>
<tr>
<td>m&amp;p-Xylene</td>
<td>8.44</td>
<td>106.05</td>
<td>91.05</td>
<td></td>
</tr>
<tr>
<td>o-Xylene</td>
<td>8.79</td>
<td>91.05</td>
<td>106.05</td>
<td></td>
</tr>
<tr>
<td>Benzotrifluoride</td>
<td>6.10</td>
<td>146.00</td>
<td>96.00</td>
<td>127.00</td>
</tr>
<tr>
<td>Toluene-d&lt;sub&gt;5&lt;/sub&gt;</td>
<td>6.87</td>
<td>95.15</td>
<td>97.1</td>
<td></td>
</tr>
<tr>
<td>Ethylbenzene-d&lt;sub&gt;5&lt;/sub&gt;</td>
<td>8.30</td>
<td>96.0</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>m-Xylene-d&lt;sub&gt;10&lt;/sub&gt;</td>
<td>8.35</td>
<td>98.15</td>
<td>116.15</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Quantifying Ion.

<sup>b</sup>Primary qualifying ion used for confirmation of analyte peak.

<sup>c</sup>Secondary qualifying ion used for confirmation of analyte peak.

<sup>d</sup>Internal standard.

Calibration standards were made by spiking 40-mL VOA vials filled with reagent water (zero-headspace) from a concentrated stock solution containing ethylbenzene-d<sub>5</sub>, toluene-d<sub>5</sub>, and m-xylene-d<sub>10</sub> standards to give final aqueous concentrations ranging from 10-300 μg L<sup>-1</sup>. Linear regression of the calibration data entered into MS Excel gave
typical $r^2$ values ranging between 0.993 and 0.996. An internal standard of 8 µg (2 µL of 4 mg mL$^{-1}$ in methanol) of 1-chlorobutane was added by the AQUATek 70 auto sampler. A 20-µg spike of 4-bromofluorobenzene (20 µL of a 1 mg mL$^{-1}$ in methanol stock solution) was added as a second internal standard to each VOA vial prior to analysis to be used in the event of an auto sampler failure. Volatile compounds were quantified by conventional internal standard calibration using integration of selected target ion peak areas [Table 3.4]. Qualifier ion ratios were used to confirm identification of analytes.

**Anion Analysis.** Bromide, nitrate and sulfate concentrations were determined by ion chromatography with a Dionex DX-120 (Sunnyvale, CA) ion chromatograph using a Dionex AS14 column and an electrical conductivity detector. External calibration was used for standards containing 5-100 mg L$^{-1}$ in bromide and sulfate and 20-450 mg L$^{-1}$ in nitrate. Linear regression plots typically gave $r^2$ values of 0.999. Anion quantitation limit for this method is ~1 mg/L.

**Metabolite Analysis.** When improved detection limits were necessary because metabolite concentrations fell below a direct-injection method detection limit of 0.73-1.5 µg L$^{-1}$, depending on the analyte, a solid-phase-extraction (100:1) procedure (Chapt.2) utilizing a 47mm Empore SDB-XC (3M Corp., St. Paul, MN) disk was used. Briefly, pH 2 groundwater samples were filtered through a Whatman GDF 150, 1µm glass-microfibre filter (Whatman Int. Ltd., Maidstone, U.K.) after which a 100-mL aliquot of the filtered sample is spiked with 0.05 µg of 4-fluorobenzoic acid (4-FBA) as a surrogate standard. An Empore SDB-XC 47 mm SPE disk (3M, Inc., Minneapolis, MN) was preconditioned,
the 100-mL sample volume was passed through the disk using vacuum suction and allowed to dry for a minimum of 2 h under vacuum.

Analytes adsorbing to the Empore disks were eluted by passing three 3 mL aliquots of methanol through the disk and collecting the eluent. A 1 mL aliquot of reagent water was added to each collection vial along with 1 μg of 2,4-dichlorobenzoic acid (2,4-DCBA) as an internal standard prior to reducing the volume to ~1 mL with heat (85 °C) under a stream of dry nitrogen. The sample vials were capped, cooled to room temperature and the contents transferred to a 2 mL high performance liquid chromatography (HPLC) autosampler vial.

Metabolites were separated using a Waters Alliance 2690 (Waters, Milford, MA) HPLC with autosampler that was interfaced with atmospheric-pressure electrospray ionization (AP-ESI) to a Quattro Micro triple-quadrupole mass spectrometer (MS/MS) (Micromass Inc, Manchester, U.K.). Separations were performed on a Betasil C18 column (Thermo Electron Corp., Bellefonte, PA). A mobile phase of 43:57 (vol/vol) of Optima methanol (Fisher Scientific, Fairlawn, NJ) and 1 mM ammonium acetate buffer (pH 4) prepared in reagent water was used in isocratic mode with a 0.2 mL min.⁻¹ flow rate. A sample injection volume of 25 μL was used for all samples. The mass spectrometer was operated in electrospray, negative-ionization mode with the desolvation temperature set at 300 °C and a source temperature of 80° C. The desolvation and cone gas flows were set at 575 L hr⁻¹ and 92 L hr⁻¹ respectively. Capillary voltage was set to 2.75 kV, cone voltage was set at 24 V, and the multiplier voltage was set to 650 V. Collision cell pressure was set to be between 2.1 e⁻³ mBar and 2.2 e⁻³ mBar.
Multiple reaction monitoring (MRM) mode was used. Product ions were the deprotonated molecular ion minus a carboxylate \([\text{M-H-COO}^-]\) for a mass loss of 44 amu from the precursor ion \([\text{46}]\). Confirmation of analyte peak identification and quantification was achieved by comparing retention times of selected product ion peaks to those of authentic standards. Linear regression lines typically achieved \(r^2\) values of 0.999. Metabolite quantitation limits for this method ranged between 0.007-0.02 \(\mu\text{g L}^{-1}\).

**Results and Discussion.**

Background concentrations of bromide, nitrate, or sulfate were not detected in any well that was tested [Table 3.5.]. BTEX background concentrations, determined prior to the test, ranged from below the method detection limit (0.5 \(\mu\text{g L}^{-1}\)) to 5700 \(\mu\text{g L}^{-1}\) [Table 3.6.]. Well CR-13 had the highest concentration of total BTEX, suggesting either an upgradient source of BTEX contamination or poor conditions for bioremediation.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Chloride (mg/L)</th>
<th>Bromide (mg/L)</th>
<th>Nitrate (mg/L)</th>
<th>Sulfate (mg/L)</th>
<th>Nitrite (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR12</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CR13</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CR14</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CR20</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND – Not Detected
No deuterated metabolites were found in the background samples of any of the four wells tested [Table 3.7], although BSA, m-methylBSA and m-toluate were found in some of the wells at low concentrations [Table 3.8]. The detection of these non-deuterated metabolites indicates that anaerobic microbial degradation occurs at this site, but is likely limited by the lack of suitable electron acceptors.

Production of detectable concentrations of the primary metabolites may experience a lag time of 5 days or longer [37]. Since this time period is critical to analyte detection it is important to determine if test solution remains at the well for a
Table 3.7 Background Concentrations of Deuterated Metabolites in Test Wells at Northwest Terminal.

<table>
<thead>
<tr>
<th>Well</th>
<th>Location</th>
<th>BSA-d$_5$ ($\mu$g/L)</th>
<th>EtBSA-d$_5$ ($\mu$g/L)</th>
<th>m-MeBSA-d$_{10}$ ($\mu$g/L)</th>
<th>m-TA-d$_7$ ($\mu$g/L)</th>
<th>BA-d$_5$ ($\mu$g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR12</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CR13</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CR14</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CR20</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*nd = Undetected above laboratory reporting limit.

period longer than 5 days. To determine the percentage of test solution remaining at the well, breakthrough curves of the relative concentrations (C/Co) of bromide versus time were constructed, where C is the extraction phase sample concentration and Co is the

Table 3.8 Background Concentrations of non-Deuterated Metabolites in Test Wells at Northwest Terminal.

<table>
<thead>
<tr>
<th>Well Location</th>
<th>BSA ($\mu$g/L)</th>
<th>EtBSA ($\mu$g/L)</th>
<th>m-MeBSA ($\mu$g/L)</th>
<th>m-TA ($\mu$g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR12</td>
<td>nd $^a$</td>
<td>nd</td>
<td>0.08</td>
<td>0.32</td>
</tr>
<tr>
<td>CR13</td>
<td>Detect $^b$</td>
<td>nd</td>
<td>0.15</td>
<td>8.90</td>
</tr>
<tr>
<td>CR14</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Detect $^a$</td>
</tr>
<tr>
<td>CR20</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.23</td>
</tr>
</tbody>
</table>

$^a$ nd = Undetected above laboratory reporting limit.

$^b$ Detected below quantification limit of 0.15 $\mu$g/L.
average injected concentration. Extraction-phase breakthrough curves indicate the degree of test solution has been diluted with background groundwater due to advection and dispersion. Less than 1\% of the injected test solution remained in Wells CR-12, CR-13 and CR-14 by day 5 due to advection and dispersion, which is likely caused by high porosity of the fill and an elevated hydraulic gradient that contributes to rapid groundwater flow rates; because of this no further analysis of wells CR-12, CR-13 and CR-14 was performed. Well CR-20 had up to 3\% of the test solution present up to day 17 [Fig. 3.4], and was chosen for further determination of deuterated BTEX and
metabolite concentrations in extraction-phase samples. Although it is unknown why advection and dispersion are less in well CR-20 than the other three wells tested it is likely a heterogeneity exists in the fill material giving rise to a zone bounded by less permeable materials.

The breakthrough curve for well CR-20 indicated that the relative concentrations \( \frac{C}{C_0} \) of the injected deuterated BTEX compounds decreased over time at a rate greater than the relative concentrations of bromide [Fig. 3.5]. Since the reduction in mass of the conservative tracer, bromide, tracks concentration changes solely due to advection and dispersion, any loss of mass at rates greater than that of the conservative tracer are due to
processes such as sorption or transformation [37,41]. As time after injection increases, the bromide peak (not sorbed) travels a greater distance from the well than the alkylbenzene compounds that experience retardation, resulting in a higher percentage of the injected, sorbing compounds remaining at or near the well than that of the non-sorbing tracer. Attempts to mathematically correct concentrations for the sorbing compounds through dividing by the \( \frac{C}{C_0} \) value of bromide concentrations will result in artificially high concentrations of the sorbing compounds [47]. For this reason, relative concentrations of the labeled injectates were not dilution-corrected by normalization.

While a decrease in concentration of the labeled compounds cannot be construed as evidence of biotransformation, the appearance of labeled metabolites provide unambiguous proof of biotransformation. The primary metabolites ethylBSA-d\(_5\) and \( m \)-methylBSA-d\(_{10}\) were detected in extraction-phase samples from well CR-20 [Fig. 3.6a] as were the secondary metabolites \( m \)-toluate-d\(_7\), and benzoate-d\(_5\) [Fig. 3.6b]. As no analyte signals for the MS/MS transitions corresponding to the deuterated metabolites were detected in the well CR-20 background sample it provides evidence these signals are metabolites produced from the anaerobic decomposition of the deuterated alkylbenzenes in the injected test solution. Reusser found a maximum dilution-adjusted concentration of \( o \)-methylBSA-d\(_{10}\) in well CR-15 at the Northwest terminal of 0.9 µg/L [37] whereas the maximum dilution-adjusted concentration of \( m \)-methylBSA-d\(_{10}\) in well CR-20 found during this test was 78 µg/L. Additional evidence confirming the identification of the metabolites BSA-d\(_5\), \( m \)-methylBSA-d\(_{10}\), benzoic acid-d\(_5\) and \( m \)-toluic acid was obtained through spike-addition using biologically-generated reference standards provided by Dr.
Figure 3.6 Concentrations normalized to bromide of a) EthylBSA-d₅ and \textit{m}-MethylBSA-d₁₀, and b) Benzoate-d₅ and \textit{m}-Toluate-d₇ formed over time in well CR-20 after injection of the deuterated test solution.

Lisa Gieg (University of Oklahoma) and commercially-obtained authentic standards.

The spike-additions were done using an extraction-phase sample obtained 13.8 days
after injection. Peak areas at the corresponding retention times for the analytes being confirmed increased relative to the amount spiked.

Except for BSA, the non-deuterated analogs were also found to have formed in well CR-20 [Fig. 3.6], although at lower concentrations. As noted earlier, \textit{m}-toluic acid was found in the background sample of well CR-20 at 0.23 \textmu g L\textsuperscript{-1}. Although it is likely that some of this background signal contributes at an increasing degree in the later stages of the experiment as the test solution is displaced by the native groundwater, the amount of background signal would be minimal compared with the total signal.

It is interesting to note that benzylsuccinates, both deuterated and non-deuterated did not occur until late in the test when both bromide and alkylbenzene concentrations are very low (< 3\% of injectate). Sorbed alkylbenzenes with a longer residence time could be the source of the late appearing benzylsuccinates. Secondary metabolites are formed early, between days 1-8 and this time overlaps with the test period when there are deuterated alkylbenzenes from the test solution still in the well. However, the difference in the time of the appearance of the secondary metabolites (\textit{m}-TA and BA) relative to the primary is not likely due to differences in transport through the aquifer. At normal groundwater pH of 6.5 - 8.5, both compound classes (benzoates and benzylsuccinates) will be in their highly water-soluble ionic forms. The appearance of secondary metabolites before the benzylsuccinates, and at higher concentrations, was observed in a microcosm study by Elshahed, et al [19]. Beller reported evidence that benzylsuccinate does not move readily into the microbial cell [17]. It is possible that the secondary metabolites are transported out of the microbial cell and into solution more effectively
than the benzylsuccinates thereby appearing in groundwater earlier and at higher concentrations. Due to the difficulty of BSA uptake, and by analogy MethylBSA, it is unlikely the early appearance of toluate is due to metabolic decomposition of MethylBSA.

Early in the extraction period a sinusoidal pattern of concentration variation for the benzoate and toluate is observed between days 1-8 [Figs. 3.6b and 3.7]. Although the range of concentrations for each species is quite different, the sinusoidal pattern remains
the same. Differences in sampling technique, temperature variation, or systematic aquifer variations were ruled out, as this sinusoidal pattern is not observed in concentration changes of the bromides or the volatiles over time. Source of analyte signals determined as m-toluate-d$_7$ and benzoate-d$_5$ are unlikely coming from the background because m-toluate-d$_7$ and benzoate-d$_5$ concentrations in the background were below detection limits of 0.016 µg L$^{-1}$ and 0.007 µg L$^{-1}$, respectively. In addition, they appear early when background water is 35-80% of samples rather than later when background water contributes an even higher proportion. Although it is apparent the signal for the secondary metabolites is not coming from the background the possibility exists that the compounds responsible for the signal are coming from the injected solution. Although injectate samples were not analyzed using SPE method, the concentration behavior pattern of deuterated benzoate and toluate does not follow that of the consistent (flat) bromide and nitrate injection, suggesting the toluate-d$_7$ and benzoate-d$_5$ signals are not coming from the injectate.

One possible explanation for this sinusoidal pattern of extraction concentrations could lie with the experimental design. In this experiment, both the injection phase and subsequent extraction (sampling) phase was performed over the entire 3 m. screened interval of the well. Because of this large interval there exists the possibility different zones along this 3 m screened interval are more heavily sampled during the extraction phase, based upon the location of the end of the extraction line in relation to its position along the screened interval. Zones with differing levels of microbial activity could
influence the concentration of the metabolites in the extraction sample, giving rise to non-linear changes in concentration.

The spike addition experiments performed on samples from days 6, 16 and 17 increased confidence in analyte identification. However, the high degree of complexity of the gasoline-contaminated groundwater gives rise to the possibility that there are additional chemicals in the samples that responded to the transition used to acquire data for the metabolites. For example, the transitions for the succinates and benzoates correspond to the loss of m/z 44, likely to be CO₂ - a carboxylic acid group, which could apply to a number of carboxylates. However, a search of the National Library of Medicine (NLM) ChemID Plus Advanced database [48], which allows for searches by molecular weight including substructure searching, gives a return of 292 compounds that fall within the mass range of 121.6-122.6 amu; the mass of benzoic acid within the resolution of the mass spectrometer. Adding a substructure search to include a carboxylate group drops this number to 242 found compounds. Of these, many of the carboxylate functionalities are not terminal and would not cleave to give a [M-H-COO]⁻ product ion. Compounds that are ionizable by AP-ESI in negative ionization mode further reduces the possible number of compounds that have a mass selected for as a precursor ion detected by the mass spectrometer as that of the precursor molecule. A similar search by the molecular mass of BSA (207.7 – 208.7 amu) returns a list of 662 compounds. This list is reduced to 6 that have a succinic acid substructure and the molecular mass of BSA.
For an additional increase in confidence of these metabolite identifications, a second MS/MS transition is desirable. However, at these low molecular weights, there are fewer unique ions to select than compounds with higher molecular weights. Additionally, the few available secondary product ions have very low abundances, making them difficult to detect.

Another method to increase confidence in identification is to derivatize samples by silanization and then analyze by electron-impact GC/MS to confirm characteristic fragmentation and ion ratios as done by Elshahed [18]. Alternatively, the use of high mass accuracy mass spectrometry may provide additional evidence for confirmation of compound identification.

Additional evidence of biotransformation is provided by an analysis of the rate of nitrate removal. Nitrate, injected to provide an electron acceptor, is removed at a rate greater than that of the bromide tracer suggesting processes other than advection and dispersion are taking place. The dilution-corrected rate of nitrate removal from well CR-20 is 8.0 μM h⁻¹, with a linear regression of the data returning an $r^2$ value of 0.78 which, when using a two-tailed significance level of correlation test proves to be significant at the 99% confidence level.

The detection of deuterated metabolites demonstrates the viability of using deuterated substrates as surrogates for non-labeled contaminants to provide one important line of evidence for ongoing bioremediation. Using push-pull tests at groundwater systems with lower conductivities will likely allow for determination of rates of
biotransformation leading to more accurate estimates of alkylbenzene-contaminated site remediation.

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Literature Cited


Chapter 4

Conclusion

Worldwide contamination of aquifers by toxic alkylbenzene components of fuel hydrocarbons is a ubiquitous, persistent problem. Components of particular concern are the BTEX compounds, an acronym for benzene, toluene, ethylbenzene and xylene, respectively. Because of their relatively high water solubility (162–1800 mg L⁻¹) the BTEX compounds pose greater risks to water supplies than other fuel hydrocarbon components by impacting large portions of aquifers, often far from the point source of contamination. Since BTEX contamination can readily spread through aquifers it becomes imperative to limit transport by remediating the aquifer after the source of contamination has been eliminated. Due to the rapid depletion of oxygen by microbial activity, aquifers develop anoxic environments and anaerobic respiration becomes the dominant microbial process. Research has shown that aqueous ethylbenzene, toluene and the xylenes can be anaerobic degraded through the enzymatic addition of fumarate to form benzylsuccinate derivatives.

The benzylsuccinate products from the metabolism of ethylbenzene, toluene and xylene are unique to anaerobic respiration and provide evidence for the anaerobic remediation of these compounds in contaminated aquifers. It is possible to investigate anaerobic alkylbenzene metabolism in contaminated aquifers by adding deuterated analogs of the contaminants in-situ, and following the formation of the deuterated metabolites. Previous efforts to determine these benzylsuccinates involved a complicated
routine involving solid phase extraction (SPE), derivatization of the non-volatile metabolites to a volatile form before being analyzed by GC/MS. A faster, more efficient method with improved sensitivity was required. As a result, the use of liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was selected for the detection and quantification of deuterated benzylsuccinates and secondary metabolites including toluate and benzoate. An improved SPE method using Empore discs was developed to improve detection and quantification limits for these signature metabolites. The use of SPE followed by LC/MS/MS resulted in recoveries of authentic standards of non-deuterated or deuterated benzylsuccinates and toluates ranging from 80-106%. The method detection limits for these analytes ranged from 0.006-0.029 µg L⁻¹, a ten-fold improvement over previously published methods.

The newly developed SPE and LC/MS/MS method was applied in field applications of the single-well, push-pull test in which deuterium-labeled alkylbenzenes were injected. Test solution volumes (250 L) containing of the labeled alkylbenzene compounds (1–2 mg L⁻¹), nitrate (100 mg L⁻¹) as a terminal electron acceptor and bromide (106 mg L⁻¹) as a conservative tracer and were injected into four wells at a BTEX-contaminated site. Of the four wells, only one (CR-20) was selected for a complete analysis of metabolite formation. Of the primary metabolites produced from the test solution, up to 2.6 µg L⁻¹ of o-methylbenzylsuccinate-d₁₀ (o-methylBSA) and 0.56 µg L⁻¹ of ethylbenzylsuccinate-d₅ (ethylBSA) were detected in well CR-20, while benzylsuccinate-d₅ (BSA) was absent throughout the test. Non-deuterated forms of the primary metabolites were also found and quantified with the exception of BSA. The
secondary labeled metabolites toluate-d$_7$ and benzoate-d$_5$ were found at concentrations of up to 13.9 $\mu$g L$^{-1}$ and 3.0 $\mu$g L$^{-1}$ respectively. The non-deuterated forms of the same secondary metabolites were also found. The appearance of these deuterated metabolites provides unambiguous evidence that anaerobic biotransformation of alkylbenzene compounds is occurring at this site. Continued anaerobic microbial metabolism of the alkylbenzenes has potential to completely remove these contaminants from the site.

The use of HPLC with tandem mass spectrometry provides a powerful tool for analyzing and quantifying metabolites of alkylbenzene biotransformation. By utilizing deuterium-labeled compounds, inexpensive and relatively easy field tests are available for the investigation of biotransformation in contaminated aquifers that can provide valuable information on remediation.
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