Article

In Situ Assessment of Biodegradation Potential Using Biotraps Amended with ^{13}C-Labeled Benzene or Toluene

R. Geyer, A. D. Peacock, A. Miltner, H.-H. Richnow, D. C. White, K. L. Sublette, and M. Kstner

Environ. Sci. Technol., **2005**, 39 (13), 4983-4989 • DOI: 10.1021/es048037x

Environ. Sci. Technol. **2005**, *39,* 4983−4989

In Situ Assessment of Biodegradation Potential Using Biotraps Amended with ^{13}C-Labeled Benzene or Toluene

R. GEYER,*,†,‡ A. D. PEACOCK,† A. MILTNER,§ H.-H. RICHNOW,§ D. C. WHITE,† K. L. SUBLETTE,∥ AND M. KÄSTNER§

Center for Biomarker Analysis, The University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, Tennessee 37932, Department of Environmental Microbiology, UFZ Centre for Environmental Research Leipzig-Halle, Permoserstrasse 15, D-04318 Leipzig, Germany, Department of Isotope Biogeochemistry, UFZ Centre for Environmental Research Leipzig-Halle, Permoserstrasse 15, D-04318 Leipzig, Germany, and Center for Applied Biogeosciences, The University of Tulsa, 600 South College Avenue, Tulsa, Oklahoma 74104

Stable isotope fractionation analysis of an aquifer heavily contaminated with benzene (up to 850 mg L^{-1}) and toluene (up to 50 mg L^{-1}) at a former hydrogenation plant in Zeitz (Saxonia, Germany) has suggested that significant biodegradation of toluene was occurring. However, clear evidence of benzene biodegradation has been lacking at this site. Determining the fate of benzene is often a determining factor in regulatory approval of a risk-based management strategy. The objective of the work described here was the demonstration of a new tool that can be used to provide proof of biodegradation of benzene or other organics by indigenous microorganisms under actual aquifer conditions. Unique in situ biotraps containing Bio-Sep beads, amended with ^{13}C-labeled or ^{12}C nonlabeled benzene and toluene, were deployed at the Zeitz site for 32 days in an existing groundwater monitoring well and used to collect and enrich microbial biofilms. Lipid biomarkers or remaining substrate was extracted from the beads and analyzed by mass spectrometry and molecular methods. Isotopic analysis of the remaining amounts of ^{13}C-labeled contaminants (about 15−18% of the initial loading) showed no alteration of the ^{12}C/^{13}C ratio during incubation. Therefore, no measurable exchange of labeled compounds in the beads by the nonlabeled compounds in the aquifer materials occurred. Isotopic ratio analysis of microbial lipid fatty acids (as methyl ester derivatives) from labeled benzene- and toluene-amended biotraps showed ^{13}C enrichment in several fatty acids of up to δ (^{13}C) 13400‰, clearly verifying benzene and toluene biodegradation and the transformation of the labeled carbon into biomass by indigenous organisms under aquifer conditions. Fatty acid profiles of total lipid fatty acids and the phospholipid fatty acid fraction and their isotopic composition showed significant differences between benzene- and toluene-amended biotraps, suggesting that different microbial communities were involved in the biodegradation of the two compounds.

Introduction

The most important mechanism of natural attenuation leading to destruction of the contaminants in groundwater is intrinsic bioremediation. It has now been clearly established that BTEX hydrocarbons are amenable to microbial degradation even in the absence of oxygen under suitable conditions with respect to geochemistry, environmental parameters, and microbial ecology (*1*−*6*). More recently, it has been shown that aliphatic hydrocarbons are also susceptible to anaerobic biodegradation (*7*, *8*). When clear proof of natural attenuation exists and environmental receptors will not be threatened during the life of a hydrocarbon plume, a risk-based management approach can be both cost-effective and protective of human health and ecological receptors. However, gathering the necessary evidence for natural attenuation can itself be a costly undertaking since operative mechanisms and rates of intrinsic bioremediation can be site specific. Conventional indicators of intrinsic bioremediation include the distribution of hydrocarbons and metabolites and the correlation of temporal trends with the concentrations and distributions of geochemical parameters (electron acceptors, products of reduction of electron acceptors, DO, redox potential, hydrogen, etc.). Another indicator of contaminant biodegradation may be the application of the stable isotope fractionation analysis along the groundwater flow path as shown for some aromatic compounds (*9*, *10*). All these data are typically collected over the entire plume and in suitable control areas over a period of time at significant cost. The actual extent of site characterization required to support a risk-based management strategy varies from site to site depending on the regulatory environment. However, the goals remain the same: to deduce the prevalent bioprocesses in the subsurface and to determine whether natural attenuation will prevent exposure of environmental receptors to the hydrocarbon plume. With respect to the prevalent bioprocesses, the key word here is "deduce"; that is, these data amount to circumstantial evidence of intrinsic bioremediation.

More direct evidence of biodegradation potential and the operative degradation pathways may be obtained from ex situ microcosms using groundwater or soils and sediments from the site as source material. In batch cultures (ex situ) stable isotope-labeled tracer compounds such as toluene (*1*, *11*) or phenanthrene (*12*) have been used to prove the degradation processes and to link microbial activity directly to organism groups or even specific organisms by detection of the label in biomolecules such as phospholipid fatty acid (PLFA)—or using ^{13}C-labeled phenol—even in RNA (*13*). More applications of stable isotope-labeled compounds and biomarkers in microbial ecology can be found elsewhere (*14*−*16*). However, ex situ microcosms require lengthy incubations and extensive analytical efforts and suffer from the criticism that the in situ environment may not necessarily be represented by the microcosms. Clear evidence of biodegradation of target compounds by indigenous microorganism under in situ conditions has the potential of significantly reducing the cost of site characterization.

We have demonstrated that biofilms characteristic of aquifer conditions can be rapidly and efficiently collected

* Corresponding author phone: +49 (341) 235 2347; fax: +49 (341) 235 2247; e-mail: roland.geyer@ufz.de.
† The University of Tennessee.
‡ Department of Environmental Microbiology, UFZ Centre for Environmental Research Leipzig-Halle.
§ Department of Isotope Biogeochemistry, UFZ Centre for Environmental Research Leipzig-Halle.
∥ The University of Tulsa.

10.1021/es048037x CCC: $30.25
Published on Web 06/04/2005

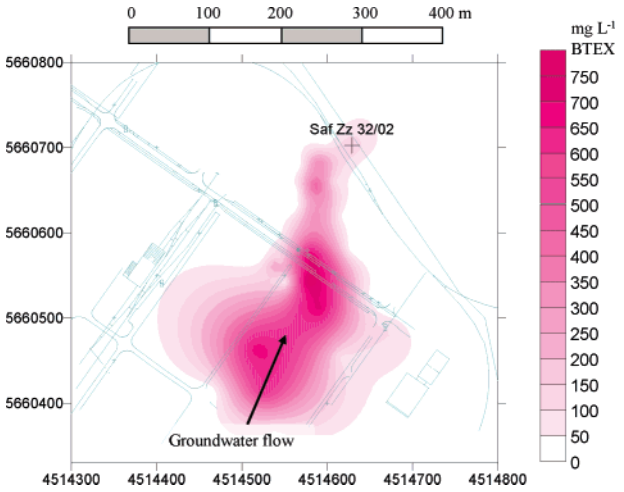

FIGURE 1. BTEX-contaminated plume in the Zeitz aquifer. The toluene concentration was below 0.05 mg L^{-1} at the monitoring well.

using a biofilm-sampling system or biotrap-based porous polymer beads impregnated with powdered activated carbon (PAC) (*17*, *18*). The feasibility of using Bio-Sep biotraps suspended down-well for microbial biofilm formation and collection under in situ conditions has been successfully tested at PCE-contaminated sites, at a site where uranium reduction and precipitation was affected by acetate injection (*19*), and in BTEX-contaminated aquifers where sulfate was being injected (K. Sublette, unpublished results) (*20*). Biotraps amended with ^{13}C-labeled contaminants and exposed under in situ conditions in a contaminated aquifer may provide more reliable data than ex situ microcosm studies and with less effort.

Here we describe the results of the incubation of biotraps based on Bio-Sep beads amended with ^{13}C-labeled benzene and toluene exposed in situ in a benzene- and toluene-contaminated anaerobic aquifer. An existing groundwater monitoring well at the test site was selected for the biotrap exposure on the basis of previous stable isotope fractionation surveys that indicated toluene degradation (*21*). After exposure, lipids extracted from biofilms which developed in the beads were enriched in ^{13}C, proving biodegradation under aquifer conditions.

Materials and Methods

Chemicals. All chemicals used were of the highest available purity. Stable isotope compounds [$^{13}C_6$]benzene (>98 atom %) and [^{13}C]-α-toluene (14 atom %), both with a chemical purity >99%, as well as nonlabeled benzene and toluene, were purchased from Sigma-Aldrich (St. Louis, MO).

Site Description. The site used in these experiments was a benzene- and toluene-contaminated plume in the Zeitz aquifer (Saxony, Germany) resulting from contamination from a former hydrogenation plant. The aquifer is virtually free of dissolved oxygen and contains benzene concentrations of up to 850 mg L^{-1} and toluene concentrations of up to 50 mg L^{-1}. Biodegradation of toluene has been demonstrated at the site by isotope fractionation analysis along the groundwater flow path (*21*, *22*). However, clear evidence of biodegradation of benzene has been lacking. Sulfate is considered to be the most likely terminal electron acceptor at the site due to its abundance (20−1930 mg L^{-1}) compared to nitrate (0−90 mg L^{-1}) and Fe^{3+} (0−1.3 mg L^{-1}) (*23*). The multilevel well Zz 32/02, located downstream of the contamination source but within the plume (Figure 1), was used to test the in situ biofilm sampling. The location at the edge of the plume was selected (i) to avoid a codegradation of toluene and benzene by accumulating both contaminants from the plume and (ii) to exclude alterations of the isotopic ratio of benzene and toluene in the plume at the test site. The groundwater in the well area contained approximately 44 mg L^{-1} benzene but less than 0.05 mg L^{-1} toluene at the time of incubation. The average concentrations of SO_4^{2-}, NO_3^-, and Fe^{3+} were 586 ± 235, 30 ± 14, and 0.25 ± 0.21 mg L^{-1}, respectively, measured at depths between 10 and 20 m. The groundwater table was 9.9 m below the surface, and the groundwater flow rate was around 2 m day^{-1} in this area.

In Situ Biotraps. Bio-Sep beads (University of Tulsa, Tulsa, OK) were employed as a substrate and amended with isotopically labeled contaminants. Bio-Sep consists of 3−4 mm diameter spherical beads engineered from a composite of 25% aramid polymer (Nomex) and 75% PAC with a porosity of 75%. The beads have an internal surface area greater than 600 m^2 g^{-1} and are surrounded by an ultrafiltration-like membrane with pores of 1−10 μm. The beads were heated to 300 °C for at least 4 h for sterilization and to render the beads free of fossil biomarkers. The efficiency of biofilm formation in the beads has been attributed to the high internal surface area, low-shear conditions within the bead, and the concentration of limiting nutrients by the PAC (*17*, *18*). Perforated Teflon tubes were filled with 50 beads each and plugged at both ends with glass wool. Assembled biotraps were autoclaved at 121 °C for sterilization and hydration of the beads and then air-dried for 24 h. The biotraps with beads were amended with benzene or toluene by vapor exposure under partial vacuum by placing prepared biotraps in small glass containers together with 200 μL of either labeled or nonlabeled benzene or toluene. The gas phase in the containers was removed by a vacuum pump until the organics started boiling at approximately 60 mbar, and the outlet valve was then closed. Microcosms were held under these conditions until deployed (at least for 48 h). Biotraps, containing beads amended with [^{13}C]benzene, [^{13}C]toluene, [^{12}C]benzene, or [^{12}C]toluene, and control biotraps containing nonamended beads were prepared in triplicate. Replicates were fastened to a tether with 20 cm of space between differently amended biotraps (total of 15) and suspended down well Zz 32/02 to between 14 and 15 m depth (4 m below the groundwater table) for a period of 32 days.

Analysis. Benzene and toluene were extracted from biotrap beads in triplicate with dichloromethane (5 mL per trap) and were quantified by headspace GC−MS. The isotopic composition of the ^{13}C-enriched organics was determined by comparing the abundance of the respective molecular ions of the labeled and the nonlabeled compound. Gas chromatography coupled to isotopic ratio monitoring mass spectrometry (GC−IRM-MS) was not applicable for a direct [^{13}C]benzene and [^{13}C]toluene analysis given the high ^{13}C enrichment of >98 and 14 atom %, respectively, but was used to measure ^{13}C incorporation into total lipid fatty acid (TLFA) as described below.

Beads from amended biotraps and nonamended controls were lyophilized and then lipids extracted with a single-phase chloroform−methanol−buffer system (*24*), as modified by White et al. (*25*). The chloroform phases (after phase separation by addition of water and chloroform) were dried under a gentle stream of nitrogen and subjected to derivatization with trimethylchlorosilane (TMCS) in methanol, yielding fatty acid methyl esters (FAMEs) (*26*).

For the fatty acids derived from the total microbial lipid extracts, isotope compositions were determined using a gas chromatography−combustion isotope ratio monitoring mass spectrometry (GC−C-IRM−MS) system. The system consisted of a gas chromatograph (6890 series, Agilent Technology, Palo Alto, CA) coupled via a Conflo III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan). The performance and experimental details of this system have been described

TABLE 1. Concentrations and ^{13}C Abundance of Contaminants in Amended in Situ Biotraps before and after Exposure in Well Zz 32/02

	before in situ deployment		after 32 days in situ		
	^{13}C abundance (atom %)	load (mg biotrap^{-1})	^{13}C abundance (atom %)	load (mg biotrap^{-1})	loss (%)
benzene	0.1	44 ± 0.5	0.1	7.9 ± 0.1	82
toluene	0.1	56 ± 0.5	0.1	8.4 ± 0.7	85
[$^{13}C_6$]benzene	98.0	44 ± 0.1	98.0	7.9 ± 0.1	82
[^{13}C]-α-toluene	14.0	56 ± 0.6	14.0	8.9 ± 0.7	84
control	N/A[b]	0.0	0.1	0.23 (benzene)[a]	N/A

[a] Accumulation from the aquifer contaminant plume, represents 0.5% of the maximum achieved with amendment. [b] N/A = not applicable.

elsewhere, showing that the systematic error due to nonlinearity of the instrument was ≤0.1 δ unit (*9*). Aliquots of 1−4 μL of *n*-hexane containing the FAMEs were injected into a split/splitless injector held at 280 °C. On the basis of the expected concentrations, the injection mode was set to splitless or the split ratio was adjusted up to 1:5. A BPX-5 column (50 m length × 0.32 mm i.d., 0.5 μm film thickness; SGE, Darmstadt, Germany) was used for chromatographic separation with helium as carrier gas at a flow rate of 1.5 mL min^{-1}. The temperature program was 2 min at 60 °C isothermally, then heat at a rate of 20 °C min^{-1} to 120 °C, and then heat at a rate of 2 °C min^{-1} to 300 °C with a terminal 20 min isothermal hold (*27*).

The carbon isotope ratio of fatty acids is reported in the δ notation (per mil) relative to Vienna Pee Dee Belemnite standard (PDB) according to eq 1. The natural abundance

$$\delta_t(^{13}C)\ (\%) = \left(\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} \times 1\right) \times 1000 \qquad (1)$$

of ^{13}C is 1.11% (*28*) and $(^{13}C/^{12}C)_{standard}$ = 0.0112372 ± 0.0000090. The $\delta(^{13}C)$ values of the fatty acids reported here are the average of at least three individual measurements and are corrected for the methyl group introduced during derivatization although it was negligible compared to the high enrichment of ^{13}C in the substrate and the FAMEs.

Absolute concentrations of TLFAs were determined by parallel injections on a Hewlett-Packard 6890 gas chromatograph, coupled to a Hewlett-Packard 5970 mass spectrometer containing a BPX-5 column (30 m × 0.32 mm × 0.25 μm, splitless injection at 280 °C). The temperature program was 4 min at 120 °C, heat at 4 °C min^{-1} to 250 °C, then heat at 20 °C min^{-1} to 300 °C, hold for 10 min. FAME 21:0 (54.9 pmol (μL of hexane)$^{-1}$) was added as internal standard to the samples prior to measurement.

The fatty acid nomenclature used here is of the form "*A*:*B*ω*C*" where *A* designates the total number of carbon atoms, *B* the number of double bonds, and *C* the distance of the closest double bond (unsaturation) from the aliphatic end (ω-nomenclature) of the molecule. The suffixes "c" for cis and "t" for trans refer to double bond isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching, respectively, whereas "br" assigns an undetermined position of the methyl branching; "cy" assigns a cyclopropyl FA (*29*).

Membrane-derived PLFAs were obtained from total lipid extracts by fractionation into neutral lipids, glycolipids, and polar (mainly phosphoglycero) lipids by silicic acid column chromatography with chloroform, acetone, and methanol (*25*). The PLFAs were transesterified and acyl chains methylated to the respective FAMEs with TMCS in methanol (1:9, v/v) at 60 °C for 1 h. The solvent was evaporated with a gentle stream of nitrogen, and residues were dissolved in hexane containing 2.5 pmol μL^{-1} 21:0 FAME as an internal standard. FAMEs were analyzed by GC−MS (Agilent 6890 gas chromatograph, coupled to an Agilent 5973 mass spectrometer) with a 60 m nonpolar column (DB-1, Agilent, 0.25 mm i.d., 0.25 μm film thickness), with a temperature program of 100 °C initial temperature for 1 min, heat at 10 °C min^{-1} to 150 °C, hold for 1 min, heat at 3 °C min^{-1} to 282 °C, and hold for 5 min, an injector temperature of 270 °C, and a detector temperature of 230 °C. This column and temperature program resolved FAMEs according to their carbon chain length and, within a group containing the same number of carbons, in the following order: terminally branched, polyunsaturated, monounsaturated (i.e., retention time 18:1ω12 < ω9 < ω7 < ω5 fatty acids, and cis < trans fatty acids), normal saturated, midchain methyl-branched fatty acids. FAMEs eluted in an order comparable to that of the BPX-5 column used for GC−MS analysis of total lipids. Absolute and relative amounts of fatty acids in the samples were determined according to the concentration of the added internal FAME standard. GC−MS of bacterial PLFA has a limit of quantification (LOQ) of approximately 25 pmol or 2 × 10^5 cells, whereas 10^7 to 10^9 cells are necessary to obtain results that include some rare PLFAs with sufficient signal-to-noise ratios and standard deviations of less than 5% (*30*−*32*).

Results and Discussion

Reliable assessment of microbial in situ activity requires that the transformation of a target compound occurs under aquifer conditions by indigenous organisms. The decrease in concentration of the target compound can be linked to an increase in microbial biomass if the carbon was used as a substrate for growth. Stable carbon isotope-labeled compounds (i.e., toluene) have been used as substrates with biodegradation demonstrated by measuring the resulting isotopic abundance in microbial fatty acids (*1*, *11*, *12*). The application of this approach under in situ aquifer conditions requires the deployment of labeled compounds in a manner in which the substrates are available to microorganisms, not exchanged with aquifer material, and microbial biomass is retained for analysis of isotopic abundance in microbial biomarkers. The beads used here will be shown to be an ideal material from which to fabricate an in situ biotrap with these characteristics.

Exchange of Benzene and Toluene with the Aquifer. Vapor-phase adsorption of benzene and toluene by the beads resulted in loadings of 44 mg of benzene or 56 mg of toluene per biotrap (50 beads) with less than 5% standard deviation. Following 32 days of incubation in the Zeitz aquifer, 7.90 (±0.03) mg of benzene and 8.45 (±0.69) mg of toluene remained in the biotraps (Table 1). This represents 18% and 15% of the initial loading, respectively. The nonamended control biotrap contained a total of 0.226 mg of benzene, but no measurable toluene. Benzene accumulation due to the PAC in the control microcosm was influenced by equilibrium sorption in the water phase and may have affected the degradation processes within the developing microbial biofilm in the nonamended beads. As shown in Table 1 the

TABLE 2. Relative Abundance and ^{13}C Incorporation of Total Lipid Fatty Acids Extracted from [^{13}C]Benzene- or [^{13}C]Toluene-Amended Biotraps after in Situ Exposure for 32 Days[a]

	[^{13}C]benzene biotrap		[^{13}C]toluene biotrap	
fatty acid[b]	relative abundance (%)	isotopic ratio $\delta(^{13}C)$[c] (‰)	relative abundance (%)	isotopic ratio $\delta(^{13}C)$[c] (‰)
12:0	21.55	−24.6	0.18	ND[d]
14:0	13.56	46.0	1.23	805.0
i15:0	0.04	ND	0.87	60.3
a15:0	0.59	ND	0.72	15.5
15:0	1.13	ND	0.55	7946
br16:1	0.31	ND	ND	ND
i16:0	0.44	ND	0.67	−24.1
16:1ω7c	3.22	13410	13.44	13360
16:1ω7t	0.58	ND	0.44	ND
16:0	26.49	998.0	11.99	6283
10me16:0	ND	ND	0.48	ND
18:2ω6	ND	ND	0.32	ND
18:1ω9c	7.24	191.0	2.27	16.0
18:1ω7c	3.03	ND	2.99	9684
18:0	13.38	264.0	5.31	92.9
10me18:0	0.93	ND	0.27	ND

[a] The $\delta(^{13}C)$ values for the isotopic composition of the respective methyl esters represent the isotopic shift compared to the PDB standard. [b] The total amounts were 10510 and 26240 pmol in the benzene and toluene microcosms, respectively; for the fatty acid nomenclature, see the Material and Methods. [c] The isotope ratio is given as $\delta(^{13}C)$ on the basis of the PDB standard; the 21:0 fatty acid methyl ester used as internal standard (IS) for quantification of abundances showed $\delta(^{13}C)$ -29.2 ± 0.4‰. [d] ND means the ratio could not be determined because the concentration was too low to provide adequate material to analyze the stable carbon isotope ratio (limit of detection approximately 150−350 pmol for an individual FA).

^{13}C isotopic composition (atom %) of residual benzene and toluene remained unchanged, indicating that there was neither a measurable exchange with the aquifer nor cross-talk between biotraps.

^{13}C Incorporation into Total Lipid Fatty Acids. In the [^{12}C]benzene-amended biotraps the isotopic ratios of carbon in extracted fatty acids were between $\delta(^{13}C)$ −40‰ and −10‰, which is comparable with $\delta(^{13}C)$ -31.7 ± 2.8‰ for fatty acid 21:0 added as an internal standard (*25*). Similar natural $\delta(^{13}C)$ values have been reported for nonlabeled microbial fatty acids in aquifer microcosms (-28.9 ± 2.8‰) (*11*), for fatty acids produced during growth on a range of different nonlabeled carbon sources (*33*), and for growth on benzene (with a natural $^{12}C/^{13}C$ ratio of -26.6 ± 0.2‰) (*34*). Positive $\delta(^{13}C)$ values in microbial fatty acids were detectable only if assimilation of labeled carbon occurred from a source artificially enriched in ^{13}C. In another study the in situ deployment of bacteria enriched in ^{13}C resulted in increased $\delta(^{13}C)$ values of fatty acids in the samples, allowing specific and sensitive detection and enumeration of the bacteria of interest (*35*).

Our [^{13}C]benzene-amended biotraps showed significant incorporation of the ^{13}C isotope into several microbial lipid fatty acids. This undoubtedly verifies the in situ biodegradation of the ^{13}C-labeled compound (Table 2). The highest ^{13}C enrichments were found in the 16:0 and 16:1ω7c fatty acids with $\delta(^{13}C)$ of 998‰ and 13410‰ vs PDB, respectively, in the IRMS trace. The relative mass abundances within all detected fatty acids were 26.5% and 3.2%, respectively. Fatty acids 14:0, 18:1ω9c, and 18:0 were characterized by high mass abundance but lower ^{13}C incorporation (Table 2). Fatty acid 12:0 had no ^{13}C incorporation despite a high abundance of 21.5%. Both 12:0 and 14:0 are typical fatty acids in lipid A of Gram-negative bacteria (*29*). The 18-carbon fatty acids, typical of membrane-derived polar lipids, showed a greater abundance for the ω9c than for the ω7c form in the benzene biotrap. Concentrations of the longer fatty acids and terminally branched fatty acids were insufficient for obtaining isotopic ratio values.

In general, total lipid extracts from beads amended with [^{13}C]toluene showed incorporation of ^{13}C into roughly the same fatty acids as found in the [^{13}C]benzene-amended biotraps. Differences included higher abundance of the 16:1ω7c fatty acid and much greater isotopic shift for the 18:1ω7c than for the 18:1ω9c fatty acid in the toluene-amended biotrap, compared to the benzene-amended biotrap. The 16:0 and 16:1ω7c fatty acids, with isotopic ratios of $\delta(^{13}C)$ 6283‰ and 13360‰, respectively, both had a relative abundance greater than 10% (Table 2), indicating clear differences compared to the biotraps amended with [^{13}C]-benzene (26% and 3%). The similar $\delta(^{13}C)$ of around 13400‰ in the 16:1ω7c fatty acid indicates that ^{13}C from [^{13}C]-α-toluene (14 atom %) was incorporated into the microbial biomass to a greater extent than ^{13}C from [$^{13}C_6$]benzene (98 atom %). We calculated for the fatty acid 16:1ω7c ($\delta(^{13}C)$ 13410‰) that approximately 17% of the carbon was derived from the labeled benzene, and the balance of the carbon was derived from other sources such as nonlabeled benzene, other organic substrates from the aquifer, or CO_2. However, a quantitative estimation of benzene and toluene degradation or a mass balance of the label incorporation into the microbial biomass was not a focus of the experiment. Last, in the [^{13}C]-toluene-amended biotraps, the terminally branched 16:0 fatty acid showed no measurable shift in the ^{13}C isotope ratio ($\delta(^{13}C)$ −24.1‰ for i16:0) and several other terminally branched fatty acids (i15:0, a15:0, i17:0) showed only a low abundance and a small isotopic shift. This indicates that some cell carbon was derived from carbon sources other than the labeled toluene.

Further studies are needed to obtain more microbial biomass to investigate the labeling pattern of fatty acids in more detail and to improve the interpretation of isotope and fatty acid data. However, our results have clearly demonstrated that microorganisms indigenous to the aquifer were able to biodegrade benzene and toluene and use metabolites as carbon sources under in situ aquifer conditions. Comparison of the fatty acid profiles of the benzene- and the toluene-amended biotraps indicated differences in the microbial communities grown with benzene or toluene.

PLFA as an Indicator for Viable Cells. Using total lipid extracts for isotope ratio analysis accesses all possible fatty acid pools such as free fatty acids and fatty acids from diglycerides of nonviable microbial biomass (*36*), from glycolipids of Gram-negative bacteria, and from microbial phosphoglycerolipids (*37*). Therefore, total lipid extraction yields the highest overall fatty acid concentrations for detection. However, phthalates, hydrocarbons, and other organic compounds were also concentrated during the extraction process and compromised fatty acid profile comparisons. Therefore, PLFA profiles were analyzed for comparison of the viable microorganisms (microbial communities) in biotraps amended with benzene or toluene. PLFAs are biomarkers for microbial membrane phosphoglycerolipid content, which is an established indicator for viable cells (*38*). Total amounts of PLFA were highest in the [^{13}C]toluene-amended biotraps (8870 pmol per biotrap) and [^{12}C]toluene-amended biotraps (8480 pmol per biotrap). The biotraps amended with [^{13}C]- or [^{12}C]benzene and the control contained 5860, 2410, and 860 pmol of PLFA per biotrap, respectively. Utilizing the conversion factor of $(1.4-4.0) \times 10^4$ cells (pmol of PLFA)$^{-1}$ (the factor used was 2.5×10^4 cells pmol^{-1}), these values correspond to 2.3×10^7 to 2.4×10^8 bacteria cells the size of *Escherichia coli* (*30*, *32*, *36*), indicating a significant microbial colonization in the bead material of the biotraps.

TABLE 3. PLFAs Obtained from the Control Biotrap and Biotraps Amended with Benzene ($n = 2$) or Toluene ($n = 2$) Deployed down Well Zz 32/02 in the Zeitz Aquifer for 32 Days

	relative abundance (%)		
PLFA[b]	control[a]	benzene	toluene
12:0	ND[c]	0.3	ND
14:0	ND	1.7	ND
16:1ω7c	38.6	44.9	66.7
16:1ω7t	5.5	3.0	1.8
16:1ω5c	ND	ND	1.0
16:0	11.3	16.3	24.1
i17:0	ND	0.1	ND
a17:0	ND	0.2	ND
cy17:0	ND	0.2	ND
17:0	ND	0.1	ND
18:2ω6	ND	0.4	ND
18:1ω9c	31.1	23.2	1.0
18:1ω7c	13.5	7.1	5.2
18:0	ND	1.2	ND

[a] Note that the control microcosm accumulated benzene (0.23 mg microcosm^{-1}) derived from the aquifer contamination (approximately 44 mg L^{-1}), whereas toluene was detectable neither in the aquifer at well Zz 32/00 nor in the control microcosm. [b] The amounts of PLFA in the control, benzene, and toluene microcosms were 933, 4130, and 8770 pmol, respectively, corresponding to 2.3×10^7 to 2.2×10^8 cells (2.5×10^4 cells (pmol of PLFA)$^{-1}$) (*30*, *32*). [c] ND = not detectable.

Despite different total PLFA concentrations, differences in the relative abundances of fatty acids were very similar in the benzene-amended biotraps and the control biotraps, whereas both were different from those in the toluene-amended biotraps. With benzene (benzene-amended biotraps and control biotraps which accumulated low amounts of benzene from the aquifer) the fatty acid 18:1ω9c had a higher abundance than 18:1ω7c, and the abundances of both were in the range of the abundances of 16:1ω7c and 16:0 (Table 3). Fatty acids 14:0 and 12:0 were detectable in the benzene-amended microcosm, whereas in the control the expected concentrations were below the detection limit of the analysis. We propose that in both the benzene-amended and the nonamended biotraps (controls) a comparable community developed. With toluene the unsaturated fatty acid 18:1ω9c was of lower abundance than 18:1ω7c, and relative abundances of both fatty acids were 10 times lower than those of fatty acids 16:1ω9c and 16:0 (Table 3). These significant variations in the PLFA composition indicate growth of different microbial communities in the benzene- and the toluene-amended biotraps (examples of PLFA profiles are in Supporting Information Figure 1).

Several sediment cores from the Zeitz aquifer, analyzed earlier (9B/00, 12A/00, 14/00), typically contained bacterial fatty acid concentrations of less than 1000 pmol (g of sediment)$^{-1}$ in the core layers below 14 m. The sediments from the more contaminated areas showed higher abundances of 18:1ω9c than 18:1ω7c and low amounts of 16:1 (R. Geyer, unpublished results) similar to what was found in the current study in the benzene-amended biotrap. Principal component analysis of sediment data revealed that horizontally and vertically the bacterial community composition was determined mainly by the concentration of pollutants in the aquifer rather than the sediment characteristics. In addition, some sediment samples had large amounts of long-chain normal-saturated fatty acids (n-sats) due to a lignite layer and polyunsaturated fatty acids characteristic of eukaryotes (18:2ω6c, 18:3ω6c). Neither of these fatty acid groups was detectable in our biotrap experiments. The small pore size in the membrane surrounding the beads generally omits eukaryotes, and the internal bead structure produces resistance to diffusion that will limit accumulation of free fatty acids on the activated carbon embedded in the beads. Therefore, we propose that the in situ biotraps facilitated the enrichment of bacteria indigenous in the aquifer sediments although the enrichment of an artificial community usually developing in the well water itself (*39*, *40*) cannot be completely excluded. Nevertheless, the biotraps were inoculated by indigenous well or aquifer bacteria definitely able to degrade the labeled toluene and benzene. This confirms results obtained in the isotopic fractionation survey with respect to toluene (*21*). In another study nonamended biotraps were used to monitor the microbial ecology of a BTEX-contaminated aquifer receiving injections of sulfate as a remediation amendment (K. Sublette, unpublished results). Over a six-month period biotrap biofilms were compared to well water microbial communities using PLFA analysis. Throughout this period biotrap community structures were consistent with changes in aquifer geochemistry while the well water community structure remained unchanged.

Last, this method has now been used at a second site, a BTEX-contaminated aquifer near Ft. Lupton, CO. Biotraps amended with [$^{13}C_6$]benzene and [^{12}C]benzene in combination (9 atom % ^{13}C in the mixture) were deployed 15−20 cm below the water table for 30 and 80 days. Even at this lower specific activity of ^{13}C in the benzene amendment, fatty acids were readily detected which were enriched in ^{13}C ($\delta(^{13}C)$ from +36.4‰ to +153.1‰). The enriched fatty acids were 16:1ω7c, 16:1ω7t, 16:0, and 18:1ω7c.

In summary, the in situ biotrap approach described in this paper can be used to verify the biodegradability of a variety of ^{13}C-labeled organic compounds under aquifer conditions by indigenous microorganisms and to evaluate the natural attenuation potential. The only requirement is sufficient affinity of the compound for PAC to allow the compound to be retained in the beads during incubation. However, many other adsorbents can be incorporated into the beads during fabrication, and other chemicals such as nutrients, electron acceptors, or electron donors can also be incorporated. Such growth-supporting substances produce slow-release repositories within the biotraps (K. Sublette, unpublished results). Biofilms will be formed which are reflective of the available carbon sources as well as prevailing environmental conditions (e.g., temperature, pH, redox potential) in the aquifer (*17*, *20*) and may thus be suitable to delineate operative mechanisms of natural attenuation and evaluate remediation amendments. Therefore, in situ biotraps based on these porous, adsorptive beads can potentially provide much the same type of data as ex situ microcosms but in less time, with less expense, and without artificial incubation conditions, providing a more reliable picture of subsurface processes. The stable isotope approach may also help to shed light on the complex pathway network of carbon sharing (*41*). Analysis of nonlabeled or labeled nucleic acid biomarkers (RNA, DNA) (*13*, *16*, *42*), which may also be obtained from biotrap biofilms (*17*), may ultimately reveal the identity of the actual organisms involved in the biodegradation of benzene, toluene, and other organics.

Acknowledgments

The work was partially supported by the Centre of Environmental Research UFZ and by the German Research Fund (DFG). We thank involved colleagues at the UFZ, in particular M. Gehre for analytical assistance and O. Böhme (GFE-Consult) for providing the chemical data about the aquifer and the monitoring well. R.G. and A.D.P. were partially supported by Grant DE-FC02-96ER62278 from the Office of Biological and Environmental Research (OBER) of the Office of Science (SC), U.S. Department of Energy (DOE), Natural and Accelerated Bioremediation Research (NABIR) Program (Assessment Element).

Supporting Information Available

A sample calculation of ^{13}C carbon concentration in fatty acids from the $^{13}C/^{12}C$ ratio and Figure 1 giving examples for GC−MS profiles of fatty acids in total lipid extracts obtained from (A) nonamended control biotraps, (B) biotraps amended with natural benzene, and (C) biotraps amended with natural toluene deployed down-well in the aquifer. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Hanson, J. R.; Macalady, J. L.; Harris, D.; Scow, K. M. Linking toluene degradation with specific microbial populations in soil. *Appl. Environ. Microbiol.* **1999**, *65*, 5403−5408.

(2) Coates, J. D.; Chakraborty, R.; Lack, J. G.; O'Connor, S. M.; Cole, K. A.; Bender, K. S.; Achenbach, L. A. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas. *Nature* **2001**, *411*, 1039−1043.

(3) Coates, J. D.; Chakraborty, R.; McInerney, M. J. Anaerobic benzene biodegradation—a new era. *Res. Microbiol.* **2002**, *153*, 621−628.

(4) Lovley, D. R.; Coates, J. D.; Woodward, J. C.; Phillips, E. J. P. Benzene Oxidation Coupled to Sulfate Reduction. *Appl. Environ. Microbiol.* **1995**, *61*, 953−958.

(5) Anderson, R. T.; Lovley, D. R. Anaerobic Bioremediation of Benzene under Sulfate-Reducing Conditions in a Petroleum-Contaminated Aquifer. *Environ. Sci. Technol.* **2000**, *34*, 2261−2266.

(6) Bolliger, C.; Schroth, M. H.; Bernasconi, S. M.; Kleikemper, J.; Zeyer, J. Sulfur isotope fractionation during microbial sulfate reduction by toluene-degrading bacteria. *Geochim. Cosmochim. Acta* **2001**, *65*, 3289−3298.

(7) Elshahed, M. S.; Gieg, L. M.; McInerney, M. J.; Suflita, J. M. Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. *Environ. Sci. Technol.* **2001**, *35*, 682−689.

(8) Zengler, K.; Richnow, H. H.; Rossello-Mora, R.; Michaelis, W.; Widdel, F. Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **1999**, *401*, 266−269.

(9) Richnow, H. H.; Annweiler, E.; Michaelis, W.; Meckenstock, R. U. Microbial in situ degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation. *J. Contam. Hydrol.* **2003**, *65*, 101−120.

(10) Griebler, C.; Safinowski, M.; Vieth, A.; Richnow, H. H.; Meckenstock, R. U. Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. *Environ. Sci. Technol.* **2004**, *38*, 617−631.

(11) Pelz, O.; Chatzinotas, A.; Zarda-Hess, A.; Abraham, W. R.; Zeyer, J. Tracing toluene-assimilating sulfate-reducing bacteria using C-13-incorporation in fatty acids and whole-cell hybridization. *FEMS Microbiol. Ecol.* **2001**, *38*, 123−131.

(12) Johnsen, A. R.; Winding, A.; Karlson, U.; Roslev, P. Linking of microorganisms to phenanthrene metabolism in soil by analysis of C-13-labeled cell lipids. *Appl. Environ. Microbiol.* **2002**, *68*, 6106−6113.

(13) Manefield, M.; Whiteley, A. S.; Griffiths, R. I.; Bailey, M. J. RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. *Appl. Environ. Microbiol.* **2002**, *68*, 5367−5373.

(14) Boschker, H. T. S.; Middelburg, J. J. Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiol. Ecol.* **2002**, *40*, 85−95.

(15) Zhang, C. L. L. Stable carbon isotopes of lipid biomarkers: analysis of metabolites and metabolic fates of environmental microorganisms. *Curr. Opin. Biotechnol.* **2002**, *13*, 25−30.

(16) Radajewski, S.; McDonald, I. R.; Murrell, J. C. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr. Opin. Biotechnol.* **2003**, *14*, 296−302.

(17) Peacock, A. D.; Chang, Y.-J.; Istok, J. D.; Krumholz, L.; Geyer, R.; Kinsall, B.; Watson, D.; Sublette, K. L.; White, D. C. Utilization of Microbial Biofilms as Monitors of Bioremediation. *J. Microbiol. Ecol.* **2004**, *47*, 284−292.

(18) White, D. C.; Gouffon, J. S.; Peacock, A. D.; Geyer, R.; Biernacki, A.; Davis, G. A.; Pryor, M.; Tabacco, M. B.; Sublette, K. L. Forensic analysis by comprehensive rapid detection of pathogens and contamination concentrated in biofilms in drinking water systems for water resource protection and management. *Environ. Forensics* **2003**, *4*, 63−74.

(19) Istok, J. D.; Senko, J. M.; Krumholz, L. R.; Watson, D.; Bogle, M. A.; Peacock, A.; Chang, Y. J.; White, D. C. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. *Environ. Sci. Technol.* **2004**, *38*, 468−475.

(20) Sublette, K. L.; Peacock, A. D.; Davis, G. A.; Harrison, M. C.; Geyer, R.; White, D. C. *In situ monitoring of the remediation of chlorinated hydrocarbons using "Bug Traps"*, Vol. X; Alleman, B. C., Leeson, A., Eds.; Battelle Press: Columbus, OH, 2003.

(21) Vieth, A.; Kästner, M.; Schirmer, M.; Weiss, H.; Gödeke, S.; Meckenstock, R. U.; Richnow, H. H. Monitoring in-situ biodegradation of benzene and toluene by stable carbon isotope fractionation. *Environ. Toxicol. Chem.* **2004**, *24*, 153−162.

(22) Fischer, A.; Vieth, A.; Knöller, K.; Wachter, T.; Dahmke, A.; Richnow, H. H. Charakterisierung des mikrobiellen Schadstoffabbaus mit Hilfe von isotopenchemischen Methoden. *Grundwasser* **2004**, *9*, 159−172.

(23) Wachter, T.; Dethlefsen, F.; Gödeke, S.; Dahmke, A. Räumlich-statistische Charakterisierung der Hydrogeochemie einer BTEX-Grundwasserkontamination am Standort "RETZINA"/Zeitz. *Grundwasser* **2004**, *1*, 21−32.

(24) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1954**, *37*, 911−917.

(25) White, D. C.; Ringelberg, D. B. Signature Lipid Biomarker Analysis. In *Techniques in Microbial Ecology*; Burlage, R. S., Atlas, R., Stahl, D., Geesey, G., Sayler, G., Eds.; Oxford University Press: New York, 1998; pp 255−272.

(26) Thiel, V.; Peckmann, J.; Richnow, H. H.; Luth, U.; Reitner, J.; Michaelis, W. Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and a microbial mat. *Mar. Chem.* **2001**, *73*, 97−112.

(27) Miltner, A.; Richnow, H. H.; Kopinke, F. D.; Kästner, M. Assimilation of CO2 by soil microorganisms and transformation into soil organic matter. *Org. Geochem.* **2004**, *35*, 1015−1024.

(28) Hoefs, J. *Stable isotope geochemistry*, 2nd ed.; Springer-Verlag: Berlin, Germany, 1980.

(29) Radledge, C.; Wilkinson, S. G. An overview of microbial lipids. In *In Microbial Lipids Volume*; Radledge, C., Wilkinson, S. G., Eds.; Academic Press: London, 1988; Vol. 1, pp 3−21.

(30) Pinkart, H. C.; Ringelberg, D. B.; Piceno, Y. M.; Macnaughton, S. J.; White, D. C. Biochemical approaches to biomass measurements and community structure analysis. In *Manual of Environmental Microbiology*, 2nd ed.; Stahl, D. E., Hurst, C. H., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D., Walter, M. V., Eds.; American Society for Microbiology Press: Washington, DC, 2000; pp 91−101.

(31) White, D. C.; Ringelberg, D. B.; Macnaughton, S. J.; Alugupalli, S.; Schrum, D. P. Signature lipid biomarker analysis for quantitative analysis in situ of environmental microbial ecology. In *Application of Molecular Markers in Environmental Geochemistry*; Eagenhouse, R., Ed.; ACS Symposium 671; American Chemical Society: Washington, DC, 1996; pp 22−34.

(32) Balkwill, D. L.; Leach, F. R.; Wilson, J. T.; McNabb, J. F.; White, D. C. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. *Microb. Ecol.* **1988**, *16*, 73−84.

(33) Abraham, W. R.; Hesse, C.; Pelz, O. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. *Appl. Environ. Microbiol.* **1998**, *64*, 4202−4209.

(34) Mancini, S. A.; Ulrich, A. C.; Lacrampe-Couloume, G.; Sleep, B.; Edwards, E. A.; Lollar, B. S. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. *Appl. Environ. Microbiol.* **2003**, *69*, 191−198.

(35) Holben, W. E.; Ostrom, P. H. Monitoring bacterial transport by stable isotope enrichment of cells. *Appl. Environ. Microbiol.* **2000**, *66*, 4935−4939.

(36) White, D. C.; Stair, J. O.; Ringelberg, D. B. Quantitative comparisons of in situ microbial biodiversity by signature biomarker analysis. *J. Ind. Microbiol.* **1996**, *17*, 185−196.

(37) Radledge, C.; Wilkinson, S. G. Fatty acids, related and derived lipids. In *In Microbial Lipids Volume*; Radledge, C., Wilkinson, S. G., Eds.; Academic Press: London, 1988; Vol. 1, pp 23−54.

(38) Guckert, J. B.; Antworth, C. P.; Nichols, P. D.; White, D. C. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol. Ecol.* **1985**, *31*, 147−158.

(39) Alfreider, A.; Krossbacher, M.; Psenner, R. Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Res.* **1997**, *31*, 832−840.

(40) Griebler, C.; Mindl, B.; Slezak, D.; Geiger-Kaiser, M. Distribution patterns of attached and suspended bacteria in pristine and

contaminated shallow aquifers studied with an in situ sediment exposure microcosm. *Aquat. Microb. Ecol.* **2002**, *28*, 117−129.
(41) Pelz, O.; Tesar, M.; Wittich, R. M.; Moore, E. R. B.; Timmis, K. N.; Abraham, W. R. Towards elucidation of microbial community metabolic pathways: unravelling the network of carbon sharing in a pollutant-degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. *Environ. Microbiol.* **1999**, *1*, 167−174.
(42) Manefield, M.; Whiteley, A. S.; Ostle, N.; Ineson, P.; Bailey, M. J. Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2179−2183.

Received for review December 12, 2004. Accepted March 14, 2005.

ES048037X