

Hydrogen and Carbon Isotope Fractionation during Aerobic Biodegradation of Benzene

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The main aim of the study was to evaluate hydrogen and carbon isotope fractionation during biodegradation of benzene as a possible tool to trace the process in contaminated environments. Aerobic biodegradation of benzene by two bacterial isolates, *Acinetobacter* sp. and *Burkholderia* sp., was accompanied by significant hydrogen and carbon isotope fractionation with hydrogen isotope enrichment factors of $-12.8 \pm 0.7\%$ and $-11.2 \pm 1.8\%$, respectively, and average carbon isotope enrichment factors of $-1.46 \pm 0.06\%$ and $-3.53 \pm 0.26\%$, respectively. Inorganic carbon produced by *Acinetobacter* sp. was depleted in ^{13}C by 3.6–6.2‰ as compared to the initial $\delta^{13}\text{C}$ of benzene, while the produced biomass was enriched in ^{13}C by 3.8‰. The secondary aim was to determine isotope ratios of benzenes from different manufacturers with regard to the use of isotopes for source differentiation. While two of the four analyzed benzenes had similar $\delta^{13}\text{C}$ values, each of them had a distinct $\delta^2\text{H}$ – $\delta^{13}\text{C}$ pair and $\delta^2\text{H}$ values spread over a range of 66.5‰. Thus, combined analyses of hydrogen and carbon isotopes may be a more promising approach to trace sources and/or biodegradation of benzene than measuring carbon isotopes only.

Introduction

Contamination of soil and groundwater by petroleum hydrocarbons such as gasoline is a common environmental problem. For example, in the United States, release of gasoline has been documented for 380 000 underground fuel tanks (1). At gasoline spill sites, the presence of aromatic hydrocarbons such as benzene, ethylbenzene, toluene, and xylenes (BTEX) is usually of major concern since these compounds are toxic and have a relatively high mobility in the saturated and unsaturated zone due to their comparatively high solubility and vapor pressure (2). Furthermore, gasoline additives such as the highly soluble methyl *tert*-butyl ether (MTBE) can affect the quality of groundwater due to their strong taste and odor (1). In the past decade, it has increasingly been recognized that contaminant plumes at petroleum hydrocarbon spill sites are frequently short and stable (2). The limited spreading of petroleum hydrocarbon

compounds is usually attributed to the occurrence of biodegradation. Numerous laboratory studies have confirmed that BTEX compounds can be biodegraded under aerobic (3) and anaerobic conditions (4–6). However, it is often difficult to prove biodegradation at field sites since concentration changes can also be due to physical processes such as dispersion and sorption.

The use of compound-specific isotope analysis is a promising method to demonstrate biodegradation of organic contaminants at field sites (7, 8). The method relies on the frequent occurrence of a kinetic isotope effect during the initial transformation of organic compounds. Usually the reaction rate is slightly faster for molecules with light isotopes (^1H , ^{12}C) as compared to molecules with heavy isotopes (^2H , ^{13}C), and as a result, the degrading compound becomes increasingly enriched in the heavy isotopes as the reaction progresses. In contrast, physical processes such as dissolution, volatilization, or sorption are usually not accompanied by significant isotope fractionation (9–12). Large isotope effects with respect to carbon have been observed during reductive dechlorination (8, 13, 14) and oxidation (15, 16) of chlorinated solvents while much smaller isotope effects occurred during biodegradation of BTEX (13, 17, 18). As a consequence, carbon isotope ratios are not a very sensitive indicator for biodegradation of BTEX, and significant shifts of the carbon isotope ratio are only observed after a large fraction of the BTEX has been biodegraded. An alternative approach is to determine not only carbon isotope ratios but also hydrogen isotope ratios. From numerous studies in enzymology and organic chemistry, it is known that hydrogen isotope effects are much larger than carbon isotope effects due to the larger relative mass difference between the two isotopes in case of hydrogen. In particular, large isotope effects are expected when a bond between hydrogen and another element is broken or formed in the rate-limiting step (primary isotope effect). The different magnitudes of isotope effects for carbon and hydrogen isotopes can be illustrated by estimating the isotope effect for breaking a typical C–H bond (vibrational frequency $\nu = 2900 \text{ cm}^{-1}$). The magnitude of the isotope effect depends on the structure of the transition state (19). If a symmetrical transition state is assumed, the ratio of the reaction rate for the light isotope divided by the rate for the heavy isotope is 6.441 for hydrogen and only 1.021 for carbon (20). Even if no bond to a hydrogen atom is broken, a hydrogen isotope effect can occur. Isotope effects occurring although a bond to an atom of a specific element is neither broken nor formed are denoted as secondary isotope effects (21). Secondary isotope effects are caused by changes in the force field at the isotopic nucleus between reactant state and transition state.

Since hydrogen isotope effects may frequently be larger than carbon isotope effects, measurement of compound-specific hydrogen isotope ratios may be a more sensitive tool to trace biodegradation of petroleum hydrocarbons than the use of carbon isotope ratios. Ward et al. (22) have recently shown that methanogenic toluene degradation is accompanied by a significant hydrogen isotope fractionation while carbon isotope fractionation is small. Furthermore, a combined use of carbon and hydrogen isotopes has been successfully applied to evaluate sources and processes affecting methane in aquatic systems (23, 24).

The main aim of this study was to compare hydrogen and carbon isotope fractionation during biodegradation of benzene in laboratory experiments. Benzene was chosen as a model compound since it is the most toxic of the BTEX compounds and is often the slowest to degrade at field sites

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(2, 25). A method to determine compound-specific hydrogen isotope ratios in BTEX was developed and tested using benzenes from different manufacturers. Carbon and hydrogen isotope fractionation was evaluated for aerobic biodegradation of benzene by pure cultures of two different bacteria.

Material and Methods

Microbial Cultures. Biodegradation experiments were performed using two pure cultures of bacteria that had been isolated from sediments from Hamilton Harbor, Hamilton, ON, Canada. One of the strains was identified as an *Acinetobacter* sp. (26). The second is tentatively a *Burkholderia* sp., identified as *Burkholderia* (formerly *Pseudomonas*) *cepacia* by biochemical test strip (Rapid NFT, API System, France). The cultures were grown in mineral medium containing 3.22 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.81 g/L KH_2PO_4 , 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.015 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The medium was supplemented with 1 mL/L trace element solution (27) and adjusted to pH 7.2 before sterilization by autoclaving. Afterward, 0.1 mL/L sterile vitamin solution (28) was added. The cultures were kept in 250-mL glass bottles, which contained 185 mL of medium and were closed with Mininert-Valves (Vici Precision Sampling, Baton Rouge, LA).

For the experiments, 15 mL of a previous subculture was added to bottles containing 170 mL of autoclaved medium. To remove dissolved inorganic carbon (DIC), the medium was purged for 2 h with helium using a sterile stainless steel needle. Afterward, 20 mL of pure O_2 and 11 μL of benzene in pure phase were added, and the bottles were placed on a shaker. Controls were obtained by adding HgCl_2 (25 ppm of Hg^{2+}). After 1 h of shaking, samples representing initial concentrations were taken. The cultures were incubated in duplicate (denoted as replicates A and B) at 23 °C and continuously shaken. At various times during the experiment, headspace samples (0.05 mL) were taken for concentration and $^{13}\text{C}/^{12}\text{C}$ ratio analyses of benzene. From one of the duplicate cultures, aqueous samples (5 mL) for $^2\text{H}/^1\text{H}$ isotope ratio analysis were taken (replicate A) and preserved using HgCl_2 (25 ppm of Hg^{2+}). In the experiment with *Acinetobacter* sp., additional aqueous samples (2.5 mL) were taken from replicate B at various times during the experiment for concentration and $^{13}\text{C}/^{12}\text{C}$ analysis of DIC. Furthermore, samples for biomass concentration and $^{13}\text{C}/^{12}\text{C}$ ratios were taken at the beginning and the end of the experiment. This made it possible to establish a complete carbon isotope balance for benzene degradation by *Acinetobacter* sp.

Determination of Carbon and Hydrogen Isotope Ratios of Pure Phase Benzene. Carbon and hydrogen isotope ratios of pure phase benzene were determined to evaluate the variability of hydrogen isotope ratios of benzene from different manufacturers and to establish standards for compound-specific isotope analysis. Isotope ratios of two different batches of benzene from BDH (97.7%, BDH Inc., Toronto, Canada), denoted as BDH I and BDH II; one batch from Aldrich (99.8%, Aldrich, Milwaukee, WI); and one batch from Baker (100%, J. T. Baker, Philipsburg, PA) were analyzed.

The hydrogen isotope ratio of pure phase benzene was determined by using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS). The EA-IRMS system consisted of an Euro Vector elemental analyzer (EuroVector, Milan, Italy) coupled to a Micromass Isoprime isotope ratio mass spectrometer (Micromass, Manchester, U.K.). The pyrolysis column of the elemental analyzer was packed with glassy carbon and nickelized carbon and operated at 1290 °C. Between 0.15 and 0.45 μL of each benzene was injected 7–9 times into a syringe injection port attached to the elemental analyzer, and mass-3 to mass-2 ratios were recorded. In addition, 0.25–0.75 mL of methane with a known $^2\text{H}/^1\text{H}$ isotope ratio was injected. For hydrogen isotope

analyses, the mass-3 ion abundance must be corrected for the presence of H_3^+ ions, which are formed by reaction of H_2 with H_2^+ (29–32). The formation of H_3^+ is the main factor that contributes to a peak size dependence of the mass-3 to mass-2 ratio. The abundance of H_3^+ is proportional to the square of the H_2 pressure in the source and can account for as much as 5–30% of the recorded mass-3 signal (31). To correct for the H_3^+ contribution to mass-3, the mass-3 to mass-2 ratios were plotted versus the peak heights. A linear relation between mass-3 to mass-2 ratio and peak height was obtained for all benzenes and for methane. The benzene hydrogen isotope ratios were linearity corrected and normalized relative to methane and are reported in delta notation ($\delta^2\text{H}$). The $\delta^2\text{H}$ value is defined as $\delta^2\text{H} = (R_s/R_r - 1) \times 1000$, where R_s and R_r are the $^2\text{H}/^1\text{H}$ ratios of the sample and the international standard VSMOW (Vienna Standard Mean Ocean Water), respectively.

The carbon isotope ratios of benzene were determined as described by Hunkeler and Aravena (11) using a gas chromatograph coupled to an isotope ratio mass spectrometer via a combustion interface (GC-C-IRMS). Each benzene was injected 7–10 times into the GC-C-IRMS system. All $^{13}\text{C}/^{12}\text{C}$ ratios are reported in delta notation ($\delta^{13}\text{C}$). The $\delta^{13}\text{C}$ value is defined as $\delta^{13}\text{C} = (R_s/R_r - 1) \times 1000$, where R_s and R_r are the $^{13}\text{C}/^{12}\text{C}$ ratios of the sample and the international standard VPDB (Vienna Pee Dee Belemnite), respectively. All $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values are reported with the corresponding standard uncertainty. The standard uncertainty corresponds to the standard deviation of the mean (33).

Analysis of Samples from Pure Culture Studies. Compound-specific hydrogen isotope ratios were determined using a gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IRMS) system and solid-phase microextraction (SPME). The GC-P-IRMS system consisted of a Hewlett-Packard GC 6890 (Hewlett-Packard, Palo Alto, CA) coupled to a PDZ Europa Geo 20-20 isotope ratio mass spectrometer via a ceramic pyrolysis tube and an Orchid interface module. The oven was operated at 1200 °C when samples of the biodegradation experiment were analyzed and at 1300 °C when the pure phase benzenes were analyzed. Benzene was extracted by immersing a 100- μm poly(dimethylsiloxane) (PDMS) fiber into the aqueous samples for 15 min, which had been supplemented with 25 wt % NaCl. The split/splitless injector was equipped with a SPME inlet liner (Supelco, Bellefonte, PA) and held at 270 °C. The injection was performed in splitless mode, and the inlet was purged for 1 min after starting the run. The GC oven was kept at a constant temperature of 70 °C. The SPME method was tested by dissolving the four reference benzenes in distilled water at concentrations between 15 and 60 ppm and extracting the solutions as described above. The relationship between peak height and mass-3 to mass-2 ratio was nonlinear and was approximated with a second-order polynomial, similar to a previous study (34). The measured isotope ratios were corrected based on the fitted polynomial function and normalized to the $\delta^2\text{H}$ of Aldrich benzene. When analyzing samples, standards containing between 7.5 and 60 ppm of Aldrich benzene were analyzed in parallel with the samples. The average standard uncertainty of the $\delta^2\text{H}$ measurement was $\pm 1.1\%$.

The $\delta^{13}\text{C}$ values of benzene in headspace samples were determined using GC-C-IRMS. The degree of carbon isotope fractionation during aqueous-phase/gas-phase partitioning was evaluated by adding benzene to a medium-containing bottle and, after 6 h of shaking, by analyzing the carbon isotope ratio of benzene in the headspace. The difference in $\delta^{13}\text{C}$ between the added benzene and benzene in the headspace was $-0.02 \pm 0.06\%$, indicating that no significant carbon isotope fractionation occurs during aqueous-phase/gas-phase partitioning. Concentrations of benzene were

determined based on the peak areas of the mass-44 ion trace of samples and external standards. The relative standard uncertainty of the concentration measurement was $\pm 8\%$ for benzene ($n = 3$).

Carbon isotope ratios and concentrations of DIC and biomass were determined as described previously (15). The $\delta^{13}\text{C}$ of the total inorganic carbon (DIC plus CO_2 in the headspace) was calculated based on concentrations and $\delta^{13}\text{C}$ values of DIC in the aqueous phase and the calculated concentration and $\delta^{13}\text{C}$ of CO_2 in the headspace. The $\delta^{13}\text{C}$ of the produced biomass was calculated based on amount, carbon content, and the $\delta^{13}\text{C}$ of biomass at the beginning and end of the experiment. The standard uncertainty of the $\delta^{13}\text{C}$ measurement was $\pm 0.15\%$ for DIC ($n = 6$) and $\pm 0.2\%$ for biomass ($n = 4$). The relative standard uncertainty of the concentration measurement was $\pm 4\%$ for DIC ($n = 6$); the relative standard uncertainty of the carbon content was $\pm 5\%$ ($n = 4$). The ^{13}C mass balance was calculated by multiplying concentrations of benzene, inorganic carbon, and biomass with their $\delta^{13}\text{C}$; adding the contributions; and dividing the sum by the total concentration of these components. Bacterial culture turbidity was monitored as optical densities determined at 600 nm using an Amersham Pharmacia Ultraspec Plus UV/visible spectrophotometer (Amersham Pharmacia Biotech, USA).

Quantification of Isotope Fractionation. Isotope fractionation during biodegradation was quantified using a Rayleigh-type evolution model (35–37). According to this model, the isotopic composition of the substrate is given by

$$R_S = R_{S_0} f^{(\alpha-1)} \quad (1)$$

where f is the fraction of substrate remaining, R_S is the isotope ratio of substrate at a remaining fraction f , R_{S_0} is the initial isotope ratio of the substrate, and α is the fractionation factor. The fractionation factor is defined by (36)

$$\alpha = \frac{d^{13}\text{P}/d^{12}\text{P}}{^{13}\text{S}/^{12}\text{S}} \quad (2)$$

where $d^{13}\text{P}$ and $d^{12}\text{P}$ are increments of product containing ^{13}C and ^{12}C , respectively, which appear in an infinitely short time (instantaneous product), and ^{13}S and ^{12}S are the concentration of substrate with ^{13}C and ^{12}C , respectively.

By using the $\delta^{13}\text{C}$ notation for carbon isotope ratios, eq 1 transforms to

$$\ln \frac{\delta^{13}\text{C}_S + 1000}{\delta^{13}\text{C}_{S_0} + 1000} = (\alpha - 1) \ln f \quad (3)$$

where $\delta^{13}\text{C}_S$ is the carbon isotope ratio of the substrate at a remaining fraction f , and $\delta^{13}\text{C}_{S_0}$ is the initial carbon isotope ratio of the substrate. The fractionation factor α was quantified using linear regression based on eq 3 and is given as enrichment factor (ϵ) that is related to the fractionation factor α by

$$\epsilon = (\alpha - 1) \times 1000 \quad (4)$$

Results

The isotope data for benzene from different manufacturers show a range between $-24.93 \pm 0.03\%$ and $-27.92 \pm 0.02\%$ for carbon isotopes and between $-27.9 \pm 1.5\%$ and $-94.4 \pm 1.7\%$ for hydrogen isotopes (Table 1). While two of the benzenes have similar $\delta^{13}\text{C}$ values, each of them has a distinct pair of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values. The $\delta^{13}\text{C}$ values are within a similar range as observed in a previous study, which reported $\delta^{13}\text{C}$ for benzenes between -23.87 and -29.40% (38). The $\delta^2\text{H}$ values of dissolved benzenes from different manufac-

TABLE 1. Carbon and Hydrogen Isotope Ratio of Benzene from Different Manufacturers

	$\delta^{13}\text{C}$ GC ^a (‰ VPDB)	$\delta^2\text{H}$ EA ^b (‰ VSMOW)	$\Delta\delta^2\text{H}$ GC ^c (‰ VSMOW)
Aldrich	-27.92 ± 0.02	-27.9 ± 1.5	-27.9 ± 1.1
Baker	-26.10 ± 0.06	-69.2 ± 0.9	-68.8 ± 3.3
BDH I	-24.93 ± 0.03	-94.4 ± 1.7	-96.8 ± 3.1
BDH II	-27.90 ± 0.04	-74.6 ± 1.4	-70.5 ± 2.4

^a Determined using a gas chromatograph coupled to an isotope ratio mass spectrometer via a combustion interface. ^b Determined using an elemental analyzer coupled to an isotope ratio mass spectrometer. ^c Determined using SPME and a gas chromatograph coupled to an isotope ratio mass spectrometer via a pyrolysis interface.

turers that were determined by EA-IRMS or by GC-P-IRMS with SPME are in close agreement (Table 1).

In the pure culture studies with benzene, the added benzene was mineralized within 8 h by *Acinetobacter* sp. and within 30 h by *Burkholderia* sp. (Figure 1A). In both cases, a similar increase in the optical density was observed (Figure 1B). In the experiment with *Acinetobacter* sp., the $\delta^{13}\text{C}$ increased from $-27.50 \pm 0.14\%$ to $-24.84 \pm 0.16\%$ (replicate A) and from $-27.48 \pm 0.17\%$ to $-24.05 \pm 0.23\%$ (replicate B), respectively after $>80\%$ of benzene degradation (Figure 1C). The $\delta^2\text{H}$ increased from $-27.9 \pm 0.4\%$ to $-7.3 \pm 1.6\%$ (replicate A, Figure 1D). The $\delta^{13}\text{C}$ of inorganic carbon was $-33.64 \pm 0.17\%$ in the first sample and increased to $-31.04 \pm 0.15\%$ over the course of the experiment (Figure 1C). The biomass formed during the experiment was enriched in ^{13}C as compared to the substrate with a $\delta^{13}\text{C}$ of $-23.7 \pm 0.2\%$. The average $\delta^{13}\text{C}$ of the produced biomass and inorganic carbon at the end of the experiment ($-27.3 \pm 0.4\%$) corresponded well to the $\delta^{13}\text{C}$ of benzene at the beginning of the experiment ($-27.5 \pm 0.3\%$). The carbon recovery in form of inorganic carbon and biomass was $86 \pm 13\%$ of the carbon initially added as benzene. In the experiment with *Burkholderia* sp., the $\delta^{13}\text{C}$ increased from -27.86 ± 0.15 to $-23.84 \pm 0.43\%$ (replicate A) and from $-27.92 \pm 0.15\%$ to $-22.43 \pm 0.23\%$ (replicate B), respectively, after $>70\%$ of benzene degradation (Figure 1C). The $\delta^2\text{H}$ increased from $-27.9 \pm 0.4\%$ to $-14.8 \pm 1.1\%$ (Figure 1D).

An approximately linear relationship between the shift in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ can be observed (Figure 2). The $\Delta\delta^2\text{H}/\Delta\delta^{13}\text{C}$ ratio is larger for *Acinetobacter* sp. (8.7) than for *Burkholderia* sp. (3.3). Enrichment factors were determined for carbon and hydrogen isotopes using eqs 3 and 4. A good correlation between $\ln[(\delta^{13}\text{C}_S + 1000)/(\delta^{13}\text{C}_{S_0} + 1000)]$ and $\ln f$ was observed ($R^2 > 0.9051$) indicating that the enrichment factors remained constant during the experiment (Table 2). The carbon isotope enrichment factors for duplicate experiments agreed within the range of uncertainty, and average enrichment factors of $-1.46 \pm 0.07\%$ for *Acinetobacter* sp. and $-3.53 \pm 0.26\%$ for *Burkholderia* sp. were obtained. Similar enrichment factors were obtained with respect to hydrogen for *Acinetobacter* sp. ($-12.8 \pm 0.7\%$) and *Burkholderia* sp. ($-11.2 \pm 1.8\%$).

Discussion

The good agreement between GC-C-IRMS and EA-IRMS $\delta^2\text{H}$ values for different benzenes demonstrates that the SPME method can be used to accurately quantify shifts in $\delta^2\text{H}$ during biodegradation of benzene. The average standard uncertainty of the $\delta^2\text{H}$ values was 2.5% when analyzing the benzenes of the different manufacturers and 1.1% when analyzing the samples from the biodegradation experiment. The difference in the average standard uncertainty may have been due to slight changes in the peak shape after replacement of the pyrolysis oven between measurement of the pure phase

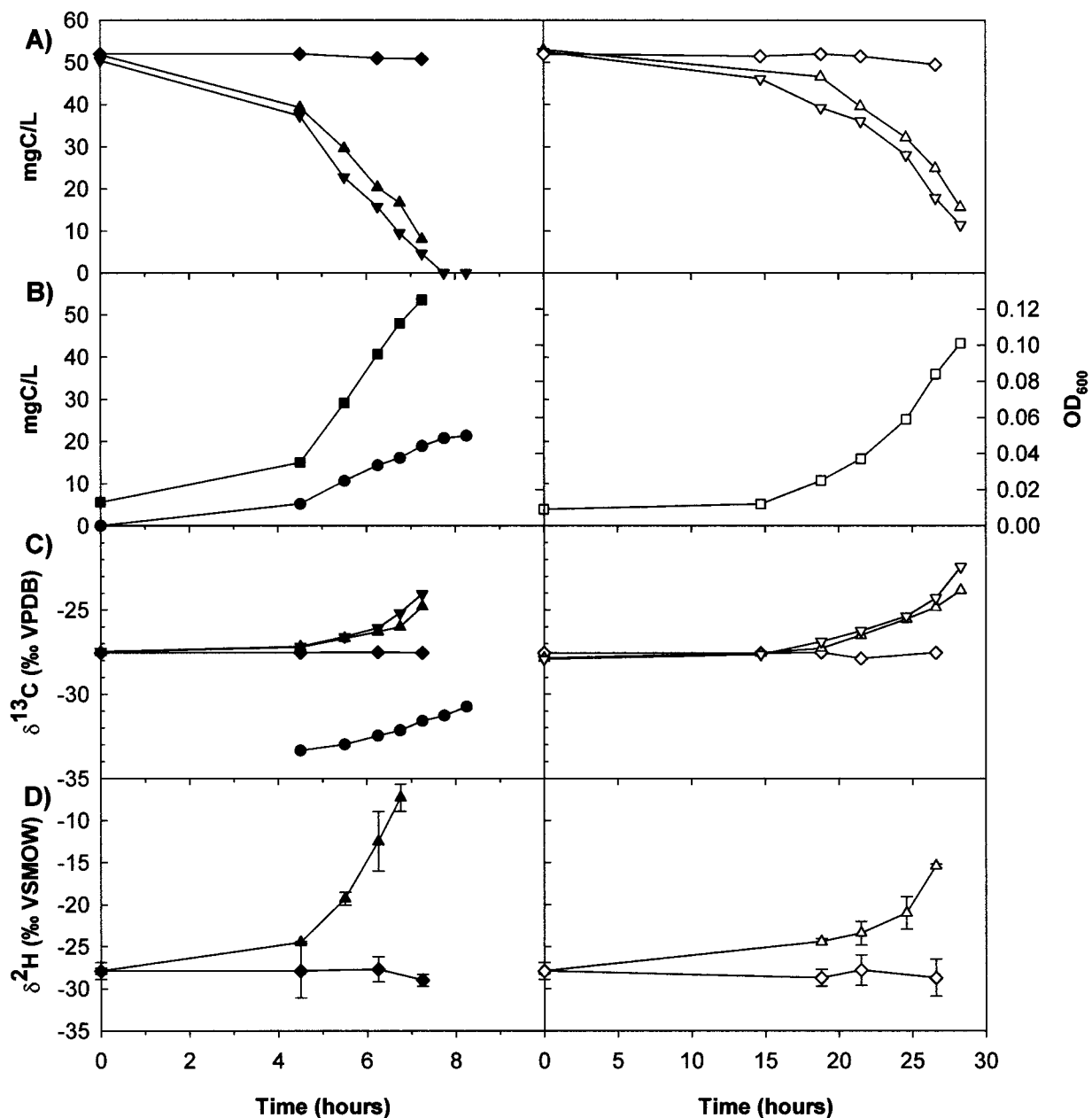


FIGURE 1. Aerobic biodegradation of benzene by *Acinetobacter* sp. (solid symbols) and *Burkholderia* sp. (open symbols). (A) Concentration of benzene in active cultures (triangle up, replicate A; triangle down, replicate B) and control (diamond). (B) Concentration of inorganic carbon in active culture (circle) and optical density of culture (square). (C) $\delta^{13}\text{C}$ of inorganic carbon in active culture (circle). The standard uncertainty of the $\delta^{13}\text{C}$ measurement corresponds approximately to the size of the symbols. (D) $\delta^2\text{H}$ of benzene in active culture (triangle) and control (diamond). Concentration and $\delta^{13}\text{C}$ of inorganic carbon and $\delta^2\text{H}$ of benzene were only measured in one of the two replicates.

benzenes and samples of the biodegradation experiment. The precision of the measurement is in the same range as the precision reported for $\delta^2\text{H}$ analysis of organic compounds in previous studies (31, 39).

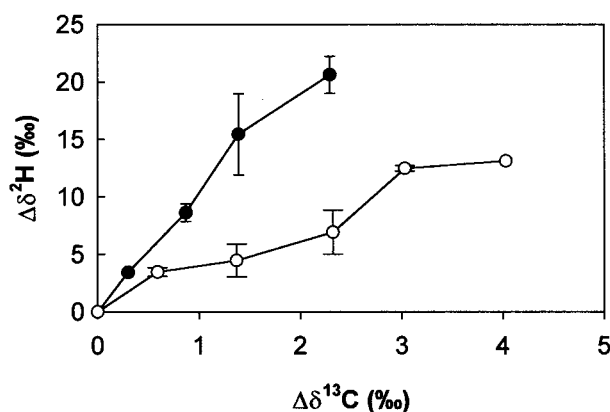
The isotopic enrichment factors for carbon during aerobic biodegradation of benzene are similar to those previously observed in other BTEX studies with individual bacterial strains. For example, Meckenstock et al. found an enrichment factor of $-2.6 \pm 0.17\text{‰}$ for aerobic degradation of toluene by *Pseudomonas putida* (17), which is in good agreement with our finding from aerobic biodegradation of benzene by *Burkholderia* sp. ($-3.53 \pm 0.26\text{‰}$). Our result for *Acinetobacter* sp. ($-1.46 \pm 0.07\text{‰}$) is similar to isotopic enrichment factors for carbon (-1.7 to -1.8‰) observed during biodegradation of toluene by three anaerobic species (17).

However, for mixed microbial consortia, none or only a small carbon isotope fractionation ($|\epsilon| < 0.8\text{‰}$) was observed during aerobic (13) and anaerobic (40) biodegradation of toluene and aerobic biodegradation of benzene (18).

A significant hydrogen isotope fractionation has recently been observed during anaerobic degradation of toluene by a mixed methanogenic microbial culture with a shift of the $\delta^2\text{H}$ by $>60\text{‰}$ for $>95\%$ degradation (22). Furthermore, aerobic and anaerobic oxidation of methane is known to be accompanied by a large hydrogen isotope fractionation with isotopic enrichment factors between -95 and -285‰ (41, 42). The hydrogen isotope fractionation during benzene oxidation is smaller than that during anaerobic toluene degradation and methane oxidation. Furthermore, the $\Delta\delta^2\text{H}/\Delta\delta^{13}\text{C}$ ratio is smaller for aerobic benzene degradation (8.7

TABLE 2. Isotopic Enrichment Factors for Carbon (ϵ_C) and Hydrogen (ϵ_H) during Aerobic Benzene Degradation

	replicate	ϵ_C (‰)	R^2	ϵ_H (‰)	R^2
<i>Acinetobacter</i> sp.	A	-1.47 ± 0.07	0.9917	-12.8 ± 0.7	0.9913
	B	-1.44 ± 0.06	0.9944		
<i>Burkholderia</i> sp.	A	-3.41 ± 0.32	0.9656	-11.2 ± 1.8	0.9051
	B	-3.65 ± 0.19	0.9872		

FIGURE 2. Shift of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ during aerobic biodegradation of benzene by *Acinetobacter* sp. (solid circle) and *Burkholderia* sp. (open circle).

for *Acinetobacter* sp.; 3.3 for *Burkholderia* sp.) than has been reported for anaerobic toluene degradation (30; 22) or aerobic methane oxidation (8–14; 42) despite the fact that benzene has a smaller H/C ratio than toluene and methane. The differences in $\Delta\delta^2\text{H}/\Delta\delta^{13}\text{C}$ ratios may be a result of differences in the enzymatic reaction mechanism.

Studies on benzene oxidation by mammalian monooxygenases have shown that only a small or no hydrogen isotope effect occurs during benzene transformation, which was explained by the absence of C–H bond breaking in the initial step, the formation of an arene oxide (43–45). In contrast, during transformation of methane by methane monooxygenases, a large kinetic isotope effect with respect to hydrogen has been observed indicating cleavage of a C–H bond in the initial oxygenation step (46). Although the exact enzymatic mechanism utilized by the two strains investigated in this study is not known and may involve dioxygenases (3) or monooxygenases (47), the absence of C–H bond breaking in the initial transformation step may have contributed to the observed relatively small hydrogen isotope effect. For other strains, the magnitude of isotope fractionation may be different due to different enzymatic mechanisms.

In the experiment with *Acinetobacter* sp., the carbon isotope ratios for inorganic carbon and biomass were different, which indicates that carbon isotope fractionation not only occurs during initial substrate transformation but also at branch points of the carbon flow in the cell. Both effects contribute to a depletion of ^{13}C in the inorganic carbon as compared to the substrate. The difference in $\delta^{13}\text{C}$ between substrate and inorganic carbon has to be taken into account if carbon isotope ratios of DIC are used to trace mineralization of BTEX. A depletion of ^{13}C in inorganic carbon of similar magnitude has previously been observed during biodegradation of benzoate and phenol (48). A much larger depletion occurred during mineralization of 1,2-DCA (15). The good agreement between the $\delta^{13}\text{C}$ of the sum of inorganic carbon and biomass at the end of the experiment and the $\delta^{13}\text{C}$ of added benzene confirms that benzene was completely transformed to biomass and inorganic carbon in our experiments.

Implications for Application of Isotopes to Trace Biodegradation. A primary goal of this study was to evaluate

whether hydrogen isotopes might be a more sensitive tool to trace biodegradation of benzene than carbon isotopes. The greater the sensitivity of a given isotope method to trace biodegradation, the less substrate has to be transformed before a significant change in isotope ratio occurs. A high sensitivity is achieved with a combination of a large enrichment factor and a high precision of the analytical method. The isotopic enrichment factors for hydrogen are 8.8 (*Acinetobacter* sp.) and 3.2 (*Burkholderia* sp.) times larger than those for carbon. However, the uncertainty associated with the analytical method is at least 4 times larger for hydrogen than for carbon. Thus, the benefit of higher isotopic enrichment factors for hydrogen is offset by the higher uncertainty associated with the analytical method. In field situations, effects of physical processes on isotope ratios and differences in detection limits for the two isotopes could influence the ability of the isotope method to trace biodegradation. Previous studies have shown that physical processes such as volatilization, air/water partitioning, and adsorption are not accompanied by significant carbon isotope fractionation under equilibrium conditions ($<0.5\%$; 9, 38). However, larger effects may occur under kinetic conditions.

Even though this study suggests that the sensitivity of carbon and hydrogen isotopes to trace aerobic biodegradation of benzene is possibly in the same range, it may be useful to analyze isotope ratios of both elements. Observing shifts in the isotope ratios of both elements would provide stronger evidence for biodegradation. Furthermore, for other enzymatic mechanisms, larger hydrogen isotope effects may occur. For example, from studies with methane monooxygenases and mammalian enzymes it is known that, in contrast to oxidation of the aromatic ring, oxidation of alkanes or alkyl groups by monooxygenases is usually accompanied by a substantial primary hydrogen isotope effect due to cleavage of C–H bonds during oxygenation (43, 45).

In addition to tracing biodegradation, isotopes may also be used to distinguish between different sources of contamination in aquifers. Given that each commercial benzene analyzed in this study had a distinct pair of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values, analyzing isotope ratios of both elements is a more promising approach for distinguishing different sources of BTEX in aquifers than measuring carbon isotope ratios only. However, possible changes of the isotope ratio due to biodegradation have to be taken into account. In conclusion, this study suggests that a combination of hydrogen and carbon isotope analyses may help to trace biodegradation of benzene and/or to distinguish different sources of benzene in aquifers. Further studies are required to evaluate how carbon and hydrogen isotope fractionation of BTEX depends on redox conditions and degradation pathways.

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