Carbon and Hydrogen Isotopic Fractionation during Biodegradation of Methyl *tert*-Butyl Ether

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Carbon and hydrogen isotopic fractionation during aerobic biodegradation of MTBE by a bacterial pure culture (PM1) and a mixed consortia from Vandenberg Air Force Base (VAFB) were studied in order to assess the relative merits of stable carbon versus hydrogen isotopic analysis as an indicator of biodegradation. Carbon isotopic enrichment in residual MTBE of up to 8.1‰ was observed at 99.7% biodegradation. Carbon fractionation was reproducible in the PM1 and VAFB experiments, yielding similar enrichment factors (ϵ) of $-2.0‰ \pm 0.1‰$ to $-2.4‰ \pm 0.3‰$ for replicates in the PM1 experiment and $-1.5‰ \pm 0.1‰$ to $-1.8‰ \pm 0.1‰$ for replicates in the VAFB experiment. Hydrogen isotopic fractionation was highly reproducible for the PM1 pure cultures, with ϵ values of $-33‰ \pm 5‰$ to $-37‰ \pm 4‰$ for replicate samples. In the VAFB microcosms, there was considerably more variability in ϵ values, with values of $-29‰ \pm 4‰$ and $-66‰ \pm 3‰$ measured for duplicate sample bottles. Despite this variability, hydrogen isotopic fractionation always resulted in ^{2}H enrichment of the residual MTBE of >80‰ at 90% biodegradation. The reproducible carbon fractionation suggests that compound-specific carbon isotope analysis may be used to estimate the extent of biodegradation at contaminated sites. Conversely, the large hydrogen isotopic fractionation documented during biodegradation of MTBE suggests that compound-specific hydrogen isotope analysis offers the most conclusive means of identifying in-situ biodegradation at contaminated sites.

Introduction

Methyl *tert*-butyl ether (MTBE) is a fuel oxygenate whose addition to reformulated gasoline has resulted in the contamination of surface waters, groundwater, soil, and sediments. MTBE was originally produced as an octane enhancer to replace tetraethyl lead in premium grade gasoline. It is now the most commonly used fuel oxygenate (*1*) and is used in over 30% of gasoline in the United States (*2*). MTBE's properties have led to its widespread occurrence as a groundwater contaminant (*3*, *4*). As a result of MTBE's high solubility, infiltrating water can easily carry dissolved MTBE through the vadose zone and into the saturated zone, where it migrates at rates approximately equal to the groundwater velocity (*3*). Volatilization and sorption are not major attenuation processes for MTBE in groundwater (*3*, *5*). Although MTBE was originally thought to be resistant to biodegradation and highly persistent in the subsurface (*6*, *7*), it has recently been suggested that biodegradation may, in some cases, play a significant role in the natural attenuation of MTBE in groundwater (*8*).

Biodegradation of MTBE has been documented in laboratory microcosms conducted with materials collected from the field (*9*−*12*). Despite this documentation of biodegradation in the laboratory, there are few studies to date documenting in-situ biodegradation of MTBE in groundwater at field sites (*8*, *12*, *13*). Verification of intrinsic biodegradation at contaminated field sites involves documenting the loss of the contaminants on the field scale, the presence of biogeochemical indicators, and direct microbiological evidence (*14*). Although MTBE concentration distributions may suggest a loss of contaminant over space and time, conclusive proof of biodegradation via mass balances can be problematic (*15*). Because *tert*-butyl alcohol (*t*BA), a common metabolic intermediate (*16*), is also a fuel additive, in many cases, its presence cannot be conclusively used as an indicator of biodegradation (*10*). Finally, documentation of biodegradation in laboratory microcosms does not ensure that biodegradation at field sites would occur or would occur at rates sufficient for risk management, because the laboratory tends to offer optimum conditions of oxygen and nutrient availability.

Compound-specific isotopic analysis (CSIA) has the potential to overcome some of the challenges presented by the current methods used to identify biodegradation at contaminated field sites. The technique relies on the reaction of the lighter isotopes at a slightly faster rate during biodegradation, because of differences in the activation energies between ^{12}C- and ^{13}C-containing molecules (*17*). For the residual contaminant pool, the result can be an isotopic enrichment in the heavier isotope as biodegradation proceeds. CSIA allows for the rapid determination of carbon and hydrogen isotopic signatures of organic compounds over the course of biodegradation by measuring the two stable isotopes of carbon (^{13}C and ^{12}C) and of hydrogen (^{2}H and ^{1}H). These ratios are expressed relative to an international standard as δ^{13}C and δ^2H (in ‰) respectively (*17*),

$$\delta^{13}\mathrm{C} = \left[\frac{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{sample}} - (^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}}{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard}}}\right] \times 1000 \qquad (1)$$

$$\delta^{2}\mathrm{H} = \left[\frac{(^{2}\mathrm{H}/^{1}\mathrm{H})_{\mathrm{sample}} - (^{2}\mathrm{H}/^{1}\mathrm{H})_{\mathrm{standard}}}{(^{2}\mathrm{H}/^{1}\mathrm{H})_{\mathrm{standard}}}\right] \times 1000 \qquad (2)$$

In this study, δ^{13}C is reported relative to V-PDB and δ^2H values are reported relative to V-SMOW (*17*). Laboratory studies have shown that, under equilibrium conditions, stable carbon isotope signatures do not fractionate significantly (>0.5‰) during dissolution (*18*, *19*), adsorption (*20*, *21*), and volatilization (*19*, *20*, *22*) for petroleum hydrocarbons and chlorinated ethenes. As such, stable isotope analysis offers

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the potential to differentiate between biological and physical processes of contaminant attenuation, provided that the isotopic fractionation associated with biodegradation is large as compared to fractionation effects associated with dissolution, volatilization, and adsorption. Large carbon isotopic fractionation associated with biodegradation of chlorinated solvents has been documented in the laboratory (*23*−*26*). At a contaminated field site, stable carbon isotopic enrichment of chlorinated ethenes was found to correlate with the appearance of biological breakdown products (VC, ethene), and the extent of biodegradation was estimated on the basis of stable carbon isotope values measured for TCE (*27*). Conversely, during the anaerobic biodegradation of toluene, a total isotopic shift of just 2.0‰ was measured for $\delta^{13}C$ of residual toluene (*28*). The order of magnitude larger fractionation (enrichment >60‰) associated with the hydrogen isotopic fractionation of toluene by the same microbial consortium suggests that, for aromatic hydrocarbons such as toluene, measurement of $\delta^{2}H$ values may provide a more reliable means of validating biodegradation than $\delta^{13}C$ measurements (*29*).

Hunkeler et al. (*30*) demonstrated a reproducible carbon enrichment of 5.1−6.9‰ in residual MTBE after 95−97% aerobic biodegradation using laboratory microcosms from an unconfined aquifer at C.F.B. Borden, Ontario, suggesting that carbon isotope analysis offers the potential to assess in-situ biodegradation of MTBE. Biodegradation effects were large as compared to the isotopic fractionation occurring during dissolution of MTBE into water from the organic phase and volatilization from the aqueous phase, which involved only a minimal carbon isotope fractionation effect (*30*). To date, carbon isotopic studies have not been carried out in microcosm studies for other contaminated sites, and it remains to be determined if microbial communities indigenous to different contaminated sites will produce similar degrees of fractionation. Most importantly, the potential for hydrogen isotopes as an indicator of MTBE biodegradation has not been evaluated.

The first objective of this study was to determine the relative merits of stable carbon versus hydrogen isotope analysis of MTBE to identify effects of biodegradation and their potential application at contaminated field sites. The second objective was to compare isotopic fractionation of MTBE during biodegradation, by a pure culture, to isotopic fractionation due to biodegradation by microcosms containing a mixed microbial community derived from a contaminated field site. To meet these objectives, experiments were conducted using the pure bacterial strain PM1 and using the native microbial consortia from sediment and groundwater within an MTBE plume at Vandenberg Air Force Base (VAFB), CA. Strain PM1 is capable of utilizing MTBE as its sole carbon and energy source (*31*). PM1 was isolated from a mixed microbial consortium in a compost biofilter capable of degrading MTBE (*31*). When inoculated into a contaminated sediment core that had previously showed no evidence of MTBE biodegradation, PM1 successfully degraded 20 μg of MTBE/mL (*31*). The MTBE-contaminated site at VAFB is the subject of studies to assess in-situ biodegradation by native microorganisms supported by diffusive oxygen release (*32*, *33*). Microcosm studies carried out with samples from this site demonstrated that native aerobic MTBE-degrading microbes are capable of degrading MTBE concentrations of up to 16 mg/L solely by the addition of oxygen (*32*).

Experimental Section

Experiment One: Biodegradation of MTBE by Bacterial Pure Culture (PM1). PM1 bacteria and 50 mL of media were sealed with Teflon-lined Mininert valves in 250 mL experimental bottles and amended with MTBE. Killed controls were created for each experiment by preparing bottles in the same way as the degrading samples and then adding 4 mL of a 40% sodium azide stock solution. The experiments assessing carbon and hydrogen isotopic fractionation used PM1 in Wagner media (Tris-HCl, 0.5 g/L; $NaHCO_3$, 0.42 g/L; NH_4Cl, 0.36 g/L; $MgSO_4 \cdot 7H_2O$, 0.15 g/L; K_2HPO_4, 0.11 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; KH_2PO_4, 0.08 g/L; $FeCl_3 \cdot 6H_2O$, 0.003 g/L; EDTA, 0.003 g/L). Samples and killed controls were analyzed for both MTBE concentrations and isotopic composition as biodegradation proceeded. All samples were stored in the dark at room temperature.

Experiment Two: Biodegradation of MTBE by VAFB Consortia. The microcosms used in this study were constructed using sediment and groundwater obtained from an MTBE-contaminated aquifer located at VAFB Site 60, CA. The unconfined aquifer is shallow, with the water table located 1.8−2.4 m below ground surface. The source of the MTBE plume is an underground storage tank that spilled approximately 2000 L of gasoline in 1985. Concentrations vary from 0 to 80 mg/L in the source area, and the plume currently spans an area approximately 520 m long × 75−90 m wide. The aquifer is weakly anaerobic with dissolved oxygen levels of <0.5 mg/L (*32*).

To construct the microcosms, sediment samples were collected approximately 200 ft downgradient of the source area. MTBE concentrations at the sampling site range from 0.1 to 5 mg/L, with no detectable *t*BA. Sediment was obtained from a depth of 2.4−2.7 m using a sterile hollow core 3.8 cm in diameter. This core was emptied into a sterile mason jar, topped with groundwater, sealed, and shipped to the laboratory on ice. Sediment and groundwater were later transferred into 250 mL bottles and sealed with Teflon-lined Mininert valves to construct the experimental microcosms. Three samples were set up containing 46.5, 45.5, and 21.5 g of sediment, respectively. The amount of liquid in each microcosm was determined by the moisture content measurements of the sediment and the amount of liquid added during MTBE amendments, which yielded final liquid volumes of 13, 13, and 10 mL for the three microcosms. To ensure sufficient oxygen during the course of the biodegradation experiments, a large headspace volume was provided (220−240 mL). The microcosms were initially amended with MTBE to a concentration of 10 mg/L, and the concentrations were increased slowly on subsequent biodegradation experiments up to the starting concentrations used in the carbon and hydrogen isotope biodegradation experiments. A killed control containing 21.5 g of sediment and 10 mL of groundwater was created by autoclaving for 1 h at 120 °C on 2 consecutive days. Sterile water and sufficient sodium azide to create 4% sodium azide by mass of sediment was added to compensate for water losses upon autoclaving. Microcosms were stored in the dark at room temperature on an orbital shaker set to 100 rpm. The microcosms were amended with MTBE and monitored over the course of biodegradation. All three microcosms were monitored during the carbon isotope experiment. During the hydrogen experiment, only two microcosms were still available. Samples and killed controls were analyzed for both MTBE concentrations and isotopic composition as biodegradation proceeded.

Analytical Methods. *Chemical Analyses.* All MTBE concentrations were determined by removing 600 μL of headspace from each bottle using a 1000 μL Pressure-Lok gastight syringe (Vici Precision Sampling Inc.). Samples were injected into a Varian 3300 gas chromatograph (GC) equipped with a 0.53 mm × 30 m DB 624 column (J&W Scientific) and a flame ionization detector. The GC column temperature was first held at 40 °C for 1 min, increased to 120 °C at a rate of 10 °C/min, and then held for 2 min. MTBE concentrations of injected samples were determined using a three-point calibration. Reproducibility on concentration analyses is ±5%.

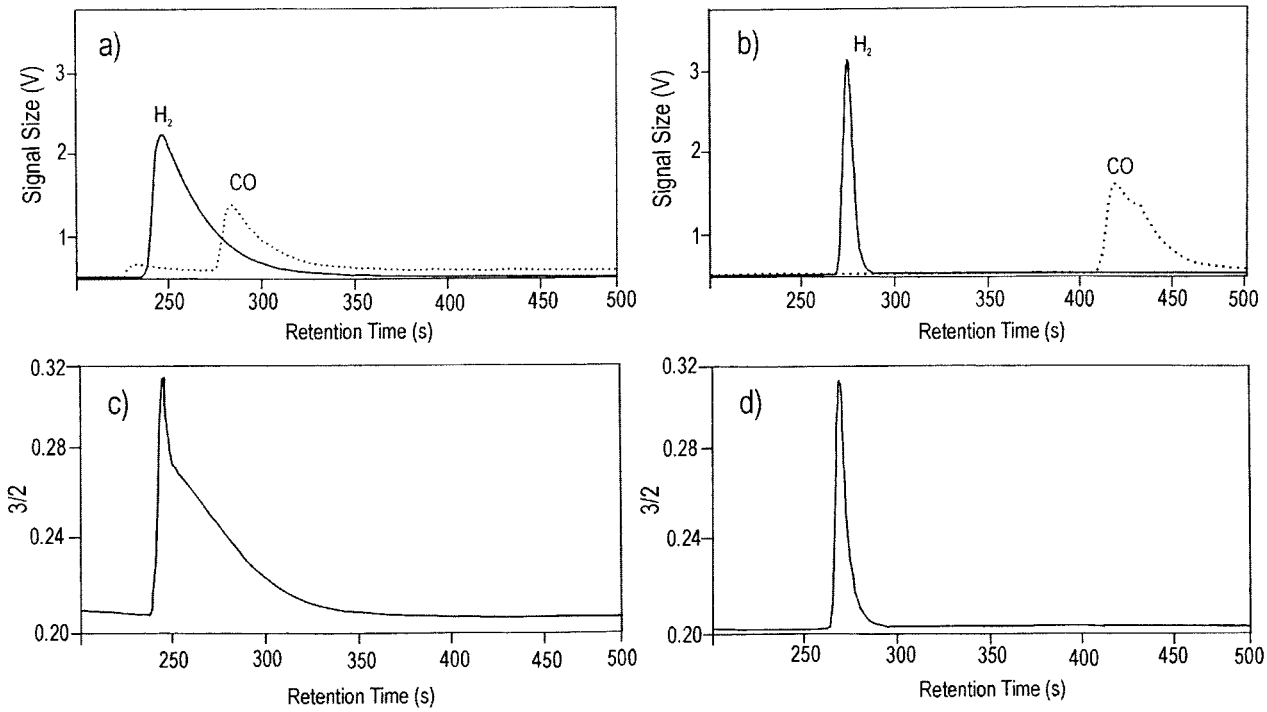

FIGURE 1. (a) Superposition of GC traces for mass spectrometer peaks of H_2 (solid line) and CO gas (dashed line), which coelute at retention times of 250 and 275 s, respectively. (b) Schematic of the mass 3 to mass 2 trace affected by coelution of H_2 and CO. (c) Superposition of GC traces for mass spectrometer peaks of H_2 (solid line) and CO gas (dashed line). Addition of a molecular sieve column between the pyrolysis oven and mass spectrometer effectively separates the peaks of H_2 and CO at retention times of 275 and 425s, respectively. (d) Schematic of the mass 3 to mass 2 trace no longer affected by coelution of H_2 and CO.

Carbon Isotope Analyses. All MTBE carbon isotope ratios were determined using a gas chromatography/combustion/isotope ratio mass spectrometer (GC/C/IRMS). The GC/C/IRMS system consists of a Varian 3400 GC equipped with 30 m × 0.25 mm i.d. DB 624 capillary column (J&W Scientific) coupled with a Finnigan MAT 252 gas source isotope ratio mass spectrometer. The GC column temperature was first held at 40 °C for 6 min, increased to 120 °C at a rate of 10 °C/min, and then held for 2 min. Split size varied from 6:1 to splitless mode during the latter stages of biodegradation, to optimize signal size in the mass spectrometer. Headspace injection volumes ranged from 0.5 to 2 mL. MTBE aqueous standards run by direct headspace injection over the course of the experiments had a mean of −28.7‰ ± 0.2‰ (n = 52). The direct headspace method has a detection limit of approximately 5 mg/L. To improve detection in the late stages of biodegradation during the VAFB microcosm experiment, samples with concentrations <5 mg/L were analyzed using headspace solid-phase microextraction (hSPME) (*30*), which is described later. Total error (accuracy and reproducibility) on all samples run by GC/C/IRMS is ±0.5‰ (*18*, *19*).

Hydrogen Isotope Analyses. MTBE hydrogen isotope ratios were determined using an HP 6890 GC equipped with a DB 624 capillary column (30 m × 0.25 mm i.d.; J&W Scientific) interfaced with a micropyrolysis furnace (1440 °C), in line with a Finnigan MAT Delta$^+$ XL gas source isotope ratio mass spectrometer (IRMS). The DB 624 column temperature was first held at 30 °C for 3 min, increased to 120 °C at a rate of 20 °C/min, and then held for 1 min.

During pyrolysis, nonoxygenated hydrocarbons are converted to carbon (graphite) and H_2 gas (*34*). The H_2 gas then passes through a capillary into the mass spectrometer. For oxygenated compounds such as MTBE, CO is produced as an additional product of pyrolysis (MTBE → C + CO + H_2). This CO peak coelutes with the H_2 peak on the mass spectrometer (Figure 1a) causing artifacts in the mass 3 to mass 2 signal (Figure 1b) and, hence, the measured δ^2H value of the sample. To eliminate this coelution, a second GC column (4 m × 0.32 μm i.d.; molecular sieve, 5 Å) was added to the system between the micropyrolysis furnace and the mass spectrometer. The addition of this second GC retards the elution of the CO peak relative to the H_2 pyrolysis product (Figure 1c), effectively separating the H_2 peak and CO peak and producing an optimized mass 3 to mass 2 signal (Figure 1d).

To assess the accuracy and reproducibility of δ^2H analysis of MTBE by CSIA, a series of standard experiments were conducted over a range of signal sizes, concentrations, and split settings after Ward et al. (*29*). A series of aqueous MTBE standards were made over a concentration range of 50−200 mg/L. Each standard was made by adding a known amount of neat MTBE to 50 mL of distilled water in 250 mL bottles sealed with screw-cap Mininert valves. The first experiment assessed the effect of signal size on the δ^2H values of MTBE with a constant split of 1:1 (Figure 2a). Concentrations (100−200 mg/L MTBE) and injection volumes (0.2−1 mL) were varied to produce signal sizes in the range of 0.5−2.5 V. Samples were run over several weeks, thereby incorporating any random effects associated with hydrogen CSIA. As shown in Figure 2a, there is no significant linearity effect as a result of signal size. Values of δ^2H averaged −100‰ ± 4‰ (1σ, n = 42), over the entire range of the dataset. The second experiment addressed the effect of varied concentration. A series of standards from 50 to 200 mg/L were run at a split of 1:1 by injecting 0.5−1.0 mL of each sample (Figure 2b). Once again, no systematic effect on δ^2H was found. Values of δ^2H averaged −100‰ ± 4‰ (1σ, n = 48). The final experiment addressed the effects of different split settings by injecting 0.2−1 mL of headspace from 200 mg/L MTBE standards over a range of splits from 2:1 to 0.1:1. A slight trend toward more depleted values does occur as split increases, but the overall average δ^2H remains −100‰ ± 4‰ (1σ, n = 29), and the variability for any given split setting is almost as large as the overall variability for the data.

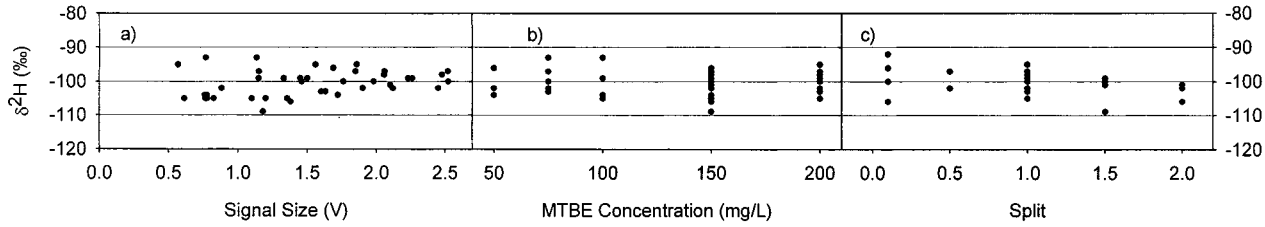

FIGURE 2. Results for δ^2H analysis of aqueous MTBE standards as a function of (a) signal size, (b) concentration, and (c) split setting. Mean values for the MTBE standard for these three experiments were −100‰ ± 4‰ (n = 42), −100‰ ± 4‰ (n = 48), and −100‰ ± 4‰ (n = 29), respectively.

Figure 2 (parts a−c) indicates that the error is largely a function of random effects associated with compound-specific isotope analysis rather than because of any systematic variable or set of variables. The same degree of variability (±4‰) was obtained when tests were repeated for MTBE standards run by hSPME (data not shown). On the basis of all datapoints, the δ^2H value of the laboratory MTBE standard is −100‰ ± 4‰ (1σ, n = 71). For samples, reproducibility based on replicate injections is typically between 1‰ and 4‰, but is generally too small to be an appropriate representation of total error (accuracy and reproducibility) with respect to standards run under a wider range of normal operating conditions. The standard deviation of the standard runs (Figure 2, parts a−c) run under a range of typical daily operating conditions is, therefore, the minimum estimate of total error (accuracy and reproducibility) that should be applied to any given δ^2H MTBE analysis. For all samples then, error bars representing total error are set at ±4‰. This magnitude of error is consistent with Sessions et al. (*35*), who reported errors of ±2.1‰ to 5.2 ‰ for C17−C30 *n*-alkanes and Li et al. (*36*), who reported ±3‰ for most *n*-alkanes. The finding is also consistent with Ward et al. (*29*), who found an accuracy and reproducibility of ±5‰ for toluene over a range of injection volumes, concentrations, split settings, and syringes.

For the VAFB and PM1 experiments, split was decreased over the course of biodegradation from 2:1 to 0.2:1 to ensure sufficient signal size in the mass spectrometer. Injection volumes ranged from 0.5 to 1 mL. The headspace method has a detection limit of approximately 20 mg/L for hydrogen isotope analysis. As with carbon, all VAFB samples with concentrations less than the headspace detection limit were run by hSPME.

hSPME Analyses. MTBE in headspace was extracted using a 75 μm PDMS-carboxen fiber (*37*). The fiber was exposed in the headspace for 20 min, removed and inserted into the injection port, and thermally desorbed for 2 min (*38*). The detection limit for hSPME was 350 μg/L for δ^{13}C and 1 mg/L for δ^2H. Small offsets have been noted for SPME analysis of semivolatile organics compounds (*39*), gasoline hydrocarbons in oils (*40*), and for a range of chlorinated methanes, ethanes, and ethenes (*41*). Hunkeler et al. (*30*) reported that δ^{13}C values for MTBE sampled via direct SPME inserted into the dissolved phase were depleted in ^{13}C as compared to dissolved MTBE and, like Dias and Freeman (*39*), suggest this is due to preferential partitioning of ^{12}C onto the polar SPME fiber. Table 1 compares δ^{13}C and δ^2H values for MTBE via hSPME (SPME inserted into the headspace over an aqueous sample) and, similarly, finds a depletion in δ^{13}C and δ^2H values as compared to isotopic analysis by direct headspace injection. In the two experiments where hSPME was used, all samples analyzed by this technique are corrected to account for this depletion with respect to direct headspace. As discussed previously, for samples run by hSPME, total error is ±0.5‰ and ±4‰ for carbon and hydrogen isotope analysis, respectively.

TABLE 1. δ^{13}C (‰) and δ^2H (‰) Values for MTBE Aqueous Standards Analyzed Using HSPME and Direct Headspace Injection[a]

carbon	δ^{13}C (‰)	SD (‰)	n	hydrogen	δ^2H (‰)	SD (‰)	n
headspace	−28.7	0.2	42	headspace	−100	4	71
hSPME	−29.6	0.3	52	hSPME	−117	4	20
Δ	0.9			Δ	17		

[a] Δ represents the isotopic depletion in ^{13}C (and ^{2}H) observed for hSPME analysis as compared to direct headspace (see text); n = number of analyses. SD = standard deviation.

Results and Discussion

Concentration Profiles. Figure 3 (parts a and b) shows the concentration of MTBE through time for the PM1 experiments. Biodegradation proceeded over 11−30 days at significantly different rates in samples both within and between experiments. Initial concentrations (C_0) were measured for all of the bottles and averaged 249 mg/L ± 3% for the carbon experiment and 251 mg/L ± 10% for the hydrogen experiment. Initial concentrations for sample bottles are always slightly lower than concentrations of killed controls because of the high sodium azide content of the killed controls, which increases MTBE in the headspace. Mean values for the killed controls were 261 mg/L ± 6% (n = 7) and 287 mg/L ± 7% (n = 5), respectively, reflecting a slight loss over the course of the experiments. Aqueous standards showed no loss throughout the experiments. Replicates remained within ±4% for the carbon experiment (n = 43) and ±4% for hydrogen experiment (n = 13).

Figure 3 (parts c and d) shows the concentration of MTBE through time for the VAFB microcosms. Initial concentrations were 93, 101, and 115 mg/L MTBE for the carbon experiment (Figure 3c) and 93 and 125 mg/L for the hydrogen experiment (Figure 3d). Mean values for the killed controls were 160 mg/L ± 9% (n = 7) for the carbon experiment (reflecting a slight loss over the course of the experiment) and 131 mg/L ± 1% (n = 6) for the hydrogen experiment. Aqueous standards showed no loss over the experiments. Replicates remained within ±4% for the carbon experiment (n = 43) and ±4% for hydrogen experiment (n = 42). The killed controls and samples were spiked at different concentrations (~160 mg/L vs ~100 mg/L) by mistake, but this small difference in concentration does not affect the isotope experiment results.

Carbon Isotopic Profiles. For the PM1 experiment, the mean $\delta^{13}C_0$ of the three sample bottles was −28.9‰ ± 0.3‰, within error of the MTBE standard (−28.7‰ ± 0.2‰). Significant δ^{13}C enrichment occurred over the course of biodegradation in the PM1 experiments. Figure 4a shows the δ^{13}C values of the residual MTBE plotted against the fraction of MTBE remaining. δ^{13}C values became progressively more enriched up to a value of −23.4‰ at 93% biodegradation by PM1, corresponding to a total shift of 5.2‰. For the VAFB experiment, initial $\delta^{13}C_0$ of the three sample bottles was −28.3‰ ± 0.3‰, also within error of the

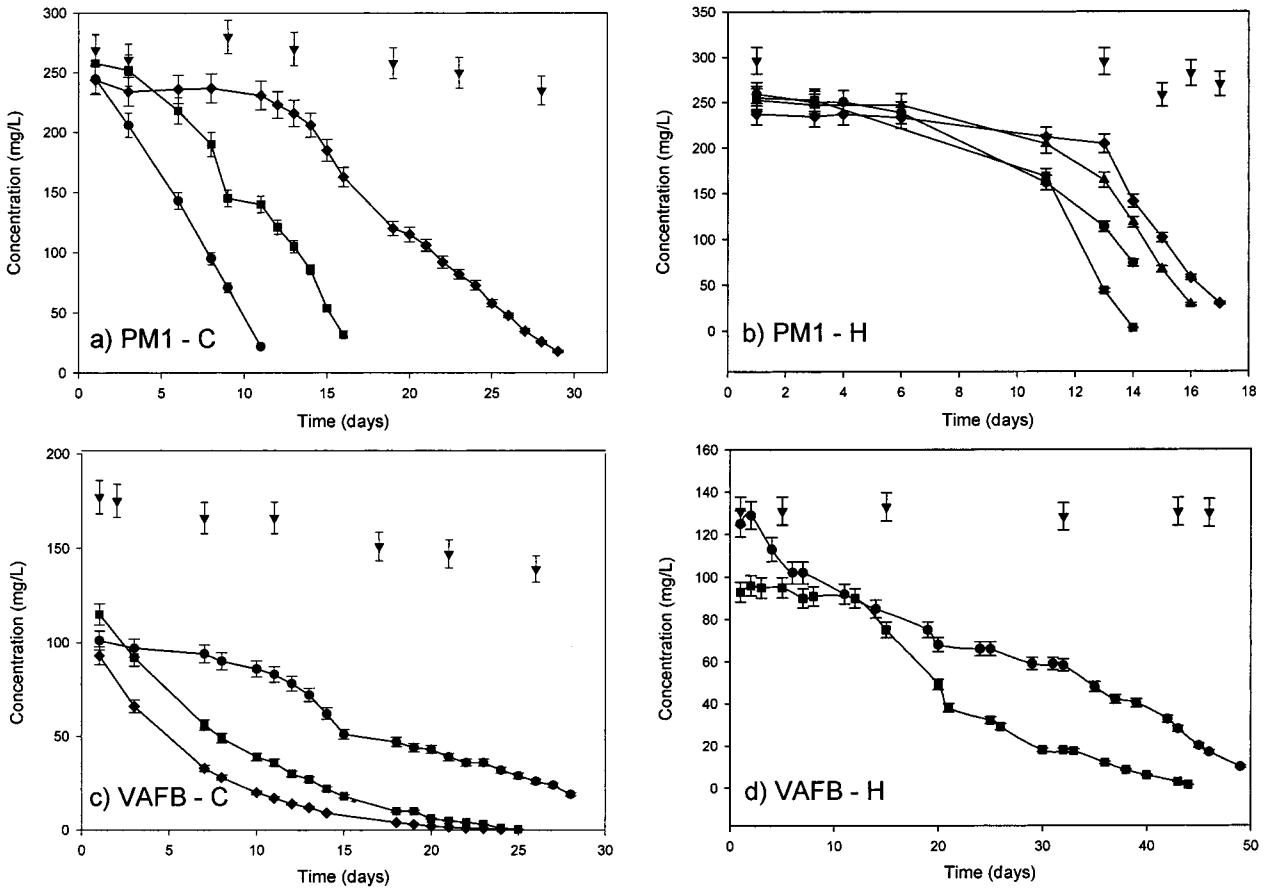

FIGURE 3. MTBE concentrations (mg/L) through time for the biodegradation experiments: (a) PM1 carbon isotope experiment, (b) PM1 hydrogen isotope experiment, (c) VAFB carbon isotope experiment, (d) VAFB hydrogen experiment; (▼) represents killed controls. Degrading samples are represented by different symbols. Error bars represent ±5% on concentrations.

MTBE standard (−28.7‰ ± 0.2‰). The VAFB microcosms also showed significant fractionation over the course of the experiment, with values as enriched as −20.0‰ at 99.7% biodegradation, corresponding to a total isotopic enrichment of 8.1‰ (Figure 4b). Killed controls in the PM1 and VAFB experiments had mean values of −28.9‰ ± 0.4‰ ($n = 6$) and −28.6‰ ± 0.2‰ ($n = 7$), respectively. Hence, isotopic shifts in the sample bottles can be attributed to biodegradation because $\delta^{13}C$ values on the killed controls always remained within analytical error of the aqueous MTBE standard (−28.7‰ ± 0.2‰). Hunkeler et al. (*30*) found that the isotopic fractionation occurring during dissolution of MTBE into water from the organic phase and volatilization from the aqueous phase involved only a minimal carbon isotope fractionation effect (*30*), similar to what has been found for other organic compounds (*19*, *20*, *22*). Similarly, adsorption under equilibrium conditions has not been shown to cause significant carbon isotope fractionation for aromatic and chlorinated hydrocarbons (*20*, *21*). While the results on adsorption for other compounds cannot necessarily be extrapolated to MTBE without further testing, the bulk of the literature identifies no significant fractionation effects associated with nondegradative processes at equilibrium. Given that sorption and volatilization in general play only a small role in the attenuation of MTBE (*12*), carbon isotope enrichment of MTBE in these experiments is attributed to biodegradation.

To compare results between the various experiments, enrichment factors were calculated according to the Rayleigh model. The model assumes a constant isotopic preference during biodegradation and has been shown to be applicable to carbon isotope fractionation during biodegradation of petroleum hydrocarbons (*28*, *42*), chlorinated solvents (*23*, *25*, *26*), and MTBE (*30*). Enrichment factors (ϵ) were calculated after Mariotti et al. (*43*) by plotting $\ln(f)$ versus $\ln[(\delta^{13}C_f/1000 + 1)/(\delta^{13}C_0/1000 + 1)]$, where f is the fraction of MTBE remaining. The linear regression of this plot yields a slope m, which is related to the fractionation factor (α) such that $m = \alpha - 1$ and $\epsilon = 1000(\alpha - 1)$. If a straight-line relationship results from such a plot, the isotopic fractionation associated with biodegradation is controlled by a first-order one-step reaction (*40*). Enrichment factors and 95% confidence intervals (CI) were calculated for all experiments using the measured initial value of C_0 and $\delta^{13}C_0$ for each individual sample bottle and are reported in Table 2. The ϵ values for the PM1 experiment ranged from −2.0‰ to −2.4‰ and were all within 95% confidence intervals of each other. The ϵ values for the VAFB microcosm experiments ranged from −1.4‰ to −1.8‰. The r^2 values for all calculated enrichment factors (ϵ) were between 0.88 and 0.99, indicating that carbon isotope fractionation during biodegradation can indeed be modeled as a Rayleigh process. Carbon fractionation during biodegradation is similar between experiments and is consistent with results reported by Hunkeler et al. (*30*), who reported ϵ values between −1.5‰ to −2.0‰ with r^2 values of 0.98 and 0.99.

Hydrogen Isotopic Profiles. For the PM1 experiment, the mean δ^2H_0 of the four sample bottles was −98‰ ± 2‰, within error of the MTBE standard (−100‰ ± 4‰). Figure 5a shows the progressive isotopic enrichment in δ^2H in the residual MTBE over the course of biodegradation by PM1. Substantial shifts in δ^2H values were observed with δ^2H values as enriched as −16‰ at 87% degradation, corresponding with a total isotopic shift of 80‰. For the VAFB experiment,

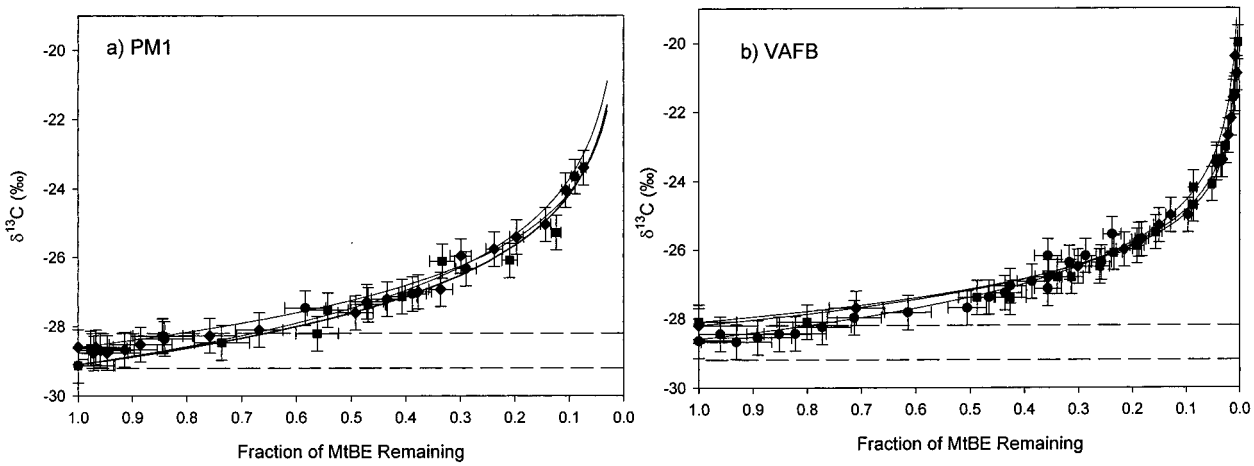

FIGURE 4. $\delta^{13}C$ values of residual MTBE versus the fraction of undegraded MTBE in the carbon isotope experiments: (a) PM1 experiment, and (b) VAFB experiment. The area between the dashed lines depicts the total error associated with the $\delta^{13}C$ value of the MTBE standard ($-28.7‰ \pm 0.5‰$). Samples (triplicate bottles) are represented by different symbols. Solid lines represent Rayleigh fractionation curves based on ϵ values and r^2 calculated for each sample bottle based on initial C_0 and $\delta^{13}C_0$ (see text). Vertical error bars represent total error (accuracy and reproducibility) of $\pm 0.5‰$ on the $\delta^{13}C$ values. Horizontal error bars represent $\pm 7\%$ on the fraction of MTBE remaining.

TABLE 2. Carbon Enrichment Factors (ϵ) and 95% Confidence Intervals for Biodegradation of MTBE in PM1 and VAFB Microcosm Experiments[a]

experiment	ϵ (‰)	95% CI	r^2	n
PM1-1	−2.0	0.1	0.98	22
PM1-2	−2.2	0.3	0.88	11
PM1-3	−2.4	0.3	0.98	6
VAFB-1	−1.5	0.1	0.99	16
VAFB-2	−1.4	0.1	0.99	18
VAFB-3	−1.8	0.1	0.95	21

[a] Values of r^2 indicate how well the fractionation fits the Rayleigh model (see text); n = number of analyses.

the mean δ^2H_0 of the duplicate bottles was $-99‰ \pm 4‰$, also within error of the MTBE standard ($-100‰ \pm 4‰$). Hydrogen isotope results for the VAFB samples are shown in Figure 5b. Over the course of biodegradation, all VAFB samples showed a substantial enrichment in ^2H. However, the extent of hydrogen fractionation varied significantly between sample bottles. At ~90% biodegradation, isotopic shifts in the microcosms were 80‰ and 156‰ for the duplicate sample bottles.

Measurements of aqueous MTBE standards over the course of the PM1 experiment had a mean δ^2H value of $-101‰ \pm 2‰$ ($n = 13$). The mean of the PM1 killed control was $-99‰ \pm 2‰$ ($n = 5$). During the VAFB experiment, measured aqueous MTBE standards had a mean δ^2H value of $-100‰ \pm 3‰$ ($n = 14$). The mean of the killed control was $-97‰ \pm 4‰$ ($n = 5$). For the PM1 experiment and the VAFB experiment, the aqueous standards and killed controls showed no isotopic shifts with respect to the MTBE standard value of $-100‰ \pm 4‰$ (Figure 2). Hence, the isotopic shifts in the samples are attributable to the effects of biodegradation and are not simply due to other physical processes such as sorption or volatilization within the sample bottles or H exchange between MTBE and water. To confirm that hydrogen exchange does not occur between MTBE and water, multiple MTBE aqueous standards were prepared by dissolving MTBE standard into two isotopically distinct waters: one more enriched water (δ^2H, $-53‰$) and one more depleted water (δ^2H, $-206‰$). The δ^2H value of MTBE in the enriched water was $-99‰ \pm 3‰$ ($n = 6$), identical to the δ^2H value of MTBE in the more depleted water $-99‰ \pm 4‰$ ($n = 8$). These values did not change during the period of 3 months over which they were tested. This is consistent with literature on the δ^2H values of petroleum hydrocarbons which retain isotopic values indicative of their depositional environment and source rocks and are not significantly altered by hydrogen isotope exchange with the formation water of reservoir rocks, even over geologically long time periods (*36*).

Similar results were noted by Ward et al. (*29*), who documented hydrogen isotopic fractionation an order of magnitude larger than carbon isotopic fractionation during anaerobic biodegradation of toluene. In the anaerobic biodegradation of toluene, the first step is believed to be the addition of fumarate to the toluene methyl group and the breakage of a C−H bond (*44*), resulting in hydrogen isotopic fractionation of the residual toluene. In the bacterial biodegradation of MTBE, MTBE is first transformed to *t*BA (*16*). The resulting hydrogen fractionation associated with this transformation is consistent with studies that have documented secondary kinetic hydrogen isotope effects, even in pathways that do not involve C−H bond breakage or involve hydroxylation of the methoxy carbon (*45*, *46*).

Hydrogen enrichment factors (ϵ) for the experiments were calculated using the measured C_0 and δ^2H values for each individual sample bottle and are reported in Table 3. The extent of hydrogen fractionation in the PM1 experiments is quite reproducible, with ϵ ranging from $-33‰$ to $-37‰$ (with a mean ϵ value of $-35‰$). Values of r^2 were between 0.91 and 0.99. Variability within the VAFB microcosm experiments was much larger, and ϵ values of $-29‰$ and -66 ‰ were calculated for the individual sample bottles. These values fall outside of the 95% confidence interval of each other. For both VAFB and PM1 bottles, values of r^2 range from 0.90 to 0.99, indicating that, like carbon isotopic fractionation, hydrogen isotopic fractionation during biodegradation of MTBE can be described by a Rayleigh model.

The variability within the VAFB groundwater microcosms may reflect the higher level of complexity in these samples as compared to the pure cultures (PM1). The heterogeneity of the sediment may result in microenvironments that differ with respect to microbial community composition, which may contribute to the isotopic variability observed in the microcosms. Degradation rate may also affect the extent of isotopic fractionation. Goldhaber and Kaplan (*47*) found an inverse correlation between the rate of sulfate reduction and the magnitude of the sulfur isotope effect, suggesting that fractionation factors decreased with increasing degradation rate. No such relationship is observed in these experiments. At this time, no specific parameter can be identified to

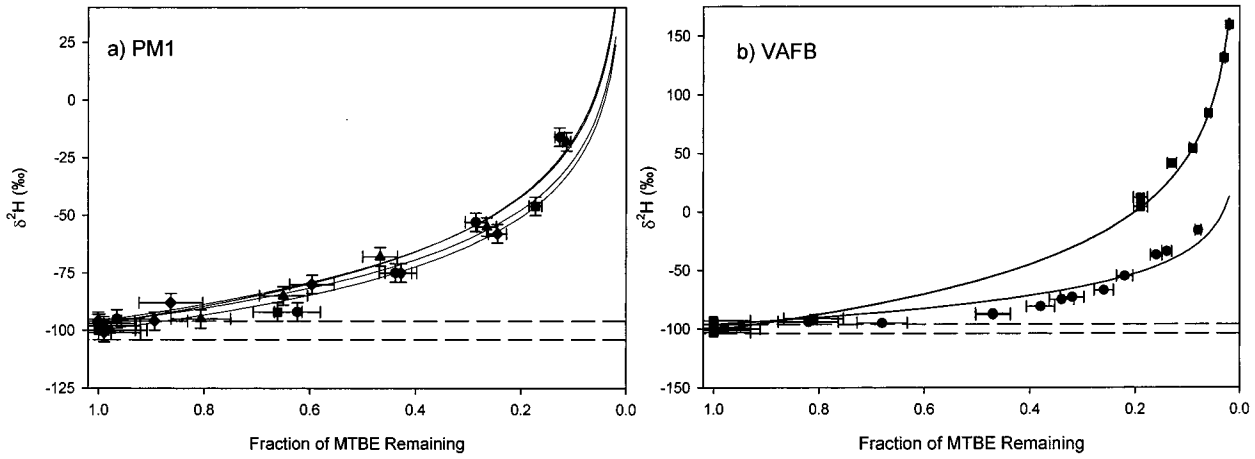

FIGURE 5. δ^2H values of residual MTBE versus the fraction of undegraded MTBE in the hydrogen isotope experiments: (a) PM1 experiment, and (b) VAFB experiment. (Note difference in scale for the *y* axis). The area between the dashed lines depicts the total error associated with the δ^2H value of the MTBE standard (−100‰ ± 4‰). Samples are represented by different symbols. Solid lines represent Rayleigh fractionation curves based on ϵ values and r^2 calculated for each sample bottle based on initial C_0 and δ^2H_0 (see text). Vertical error bars represent total error (accuracy and reproducibility) of ±4‰ on the δ^2H values. Horizontal error bars represent ±7% on the fraction of MTBE remaining.

TABLE 3. Hydrogen Enrichment Factors (ϵ) and 95% Confidence Intervals for Biodegradation of MTBE in PM1 and VAFB Microcosm Experiments[a]

experiment	ϵ (‰)	95% CI	r^2	*n*
PM1-1	−36	6	0.95	8
PM1-2	−33	5	0.99	4
PM1-3	−33	11	0.91	5
PM1-4	−37	4	0.98	7
VAFB-2	−66	3	0.99	12
VAFB-3	−29	4	0.90	14

[a] Values of r^2 indicate how well the fractionation fits the Rayleigh model (see text); *n* = number of analyses.

account for the differences in observed extent of fractionation between the two VAFB samples. The results underscore the difficulties in extrapolating from what may be highly reproducible laboratory derived values of ϵ to more complex field situations. Similarly, Slater et al. (*26*) identified variability in carbon isotopic fractionation during reductive dechlorination of chlorinated ethenes by different microbial consortia. The fact that the measured enrichment factors for the chlorinated ethenes nonetheless fell into distinct ranges indicated that stable carbon isotopic fractionation could still be used at a field site to estimate the relative extent of degradation from one portion of a contaminated plume to another with confidence (*27*). Similarly, despite ranges in enrichment factors of 10‰ to >20‰ for carbon isotopic fractionation and 95‰ to 285‰ for H isotopic fractionation, a pronounced isotopic enrichment in residual CH_4 has traditionally been used as a definitive indicator of the effects of microbial oxidation (*48*). In the present study, despite variability in hydrogen enrichment factors during biodegradation of MTBE, in all cases, the extent of fractionation is very large (>80‰ at 90% biodegradation). This large hydrogen isotopic fractionation provides an important new parameter to identify the occurrence of in-situ biodegradation of MTBE at contaminated sites.

Potential for Application of Carbon and Hydrogen Isotopic Fractionation. Carbon enrichment factors (ϵ) of −2.0‰ to −2.4‰ for PM1 and −1.5‰ to −1.8‰ for VAFB microcosms are similar to those reported by Hunkeler et al. (*30*) of −1.5‰ to −2.0‰ for the Borden Aquifer. Thus, to date, a total range of −1.5‰ to −2.4‰ has been reported for carbon enrichment factors during biodegradation of MTBE, corresponding to a total range of less than 1‰. This small variability in ϵ suggests that carbon isotope analysis has the potential to provide quantitative assessment of the extent of both natural biodegradation and bioremediation efforts at contaminated sites. Biodegradation can be quantified using the relationship between the concentration of the residual contaminant (or the fraction remaining *f*), the isotopic composition of the residual MTBE ($\delta^{13}C_{MTBE}$), and the Rayleigh equation expressed in δ ‰ notation after Mariotti et al. (*43*)

$$\ln(f) = \frac{\ln[(\delta^{13}C_{MTBE}/1000 + 1)/(\delta^{13}C_0/1000 + 1)]}{(\epsilon/1000)} \quad (3)$$

where ϵ is the enrichment factor, $\delta^{13}C_0$ is the isotopic composition of the contaminant source, and $\delta^{13}C_{MTBE}$ is the isotopic composition of the contaminant in the downgradient well. The fractionation factors (ϵ) for MTBE biodegradation of −1.5‰ to −2.4‰ can be used with eq 3 to determine the relative extent of biodegradation between the MTBE source ($\delta^{13}C_0$) and a downgradient well ($\delta^{13}C_{MTBE}$). This approach assumes that the MTBE in the downgradient well is derived from the source area and that the isotopic difference occurring between the two wells is solely a result of biodegradation and not mixing with a different MTBE source (*27*).

Although very large and reproducible hydrogen isotope ϵ values between −33‰ and −37‰ were found during biodegradation of MTBE by PM1, a wide range of ϵ was found for the VAFB microcosms (−29‰ to −66‰). The variability observed in the VAFB microcosms suggests that quantitative application of hydrogen enrichment factors to field sites to determine the extent of biodegradation should be done with caution. However, despite the observed variability in ϵ, in all cases, hydrogen fractionation was >80‰ at 90% biodegradation, an order of magnitude larger than that of carbon. This suggests that compound-specific hydrogen isotope analysis offers a sensitive indicator of in-situ biodegradation at contaminated sites. Even if applied only qualitatively, stable hydrogen isotope analysis has the potential to confirm biodegradation when geochemical and microbiological evidence are inconclusive.

MTBE concentrations in municipal groundwater monitoring wells range from 10 to 50 mg/L on the high end (*49*) to less than the USEPA drinking water advisory of 20−40

μg/L (*2*). Because MTBE has a solubility of 50 000 mg/L, concentrations in close proximity to source areas containing gasoline NAPL with 10% MTBE could be as high as 5000 mg/L (*3*). Current hSPME isotopic detection limits of 350 μg/L for $\delta^{13}C$ and 1 mg/L for $\delta^{2}H$ are not adequate for sites with very low MTBE concentrations. However, at any site where concentrations range from tens of mg/L to >350 μg/L downgradient, the extent to which decreased concentrations are a function of biodegradation and not simply contaminant dispersion and dilution can be reliably assessed by CSIA. Given the difficulties in assessing the occurrence of MTBE biodegradation at field sites and evaluating its extent, carbon and hydrogen isotopic fractionation offers significant potential as a new approach for the assessment of in-situ biodegradation at contaminated sites.

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