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Monitoring Biodegradation of Methyl *tert*-Butyl Ether (MTBE) Using Compound-Specific Carbon Isotope Analysis

D. HUNKELER,*,† B. J. BUTLER,‡ R. ARAVENA,† AND J. F. BARKER†

Departments of Earth Sciences and Biology, University of Waterloo, Waterloo, Ontario, Canada

Methyl *tert*-butyl ether (MTBE), the most common gasoline oxygenate, is frequently detected in surface water and groundwater. The aim of this study was to evaluate the potential of compound-specific isotope analysis to assess in situ biodegradation of MTBE in groundwater. For that purpose, the effect of relevant physical and biological processes on carbon isotope ratios of MTBE was evaluated in laboratory studies. Carbon isotope fractionation during organic phase/gas-phase partitioning (0.50 ± 0.15‰), aqueous phase/gas-phase partitioning (0.17 ± 0.05‰), and organic phase/aqueous-phase partitioning (0.18 ± 0.24‰) was small in comparison to carbon isotope fractionation measured during biodegradation of MTBE in microcosms based on aquifer sediments of the Borden site. In experiments with MTBE as the only substrate and a cometabolic experiment with 3-methypentane as primary substrate, MTBE became enriched in ^{13}C by 5.1 to 6.9‰ after 95 to 97% degradation. For both experiments, similar isotopic enrichment factors were obtained (−1.52 ± 0.06 to −1.97 ± 0.05‰). Biodegradation of TBA, which accumulated transiently in the cometabolic microcosms, was also accompanied by carbon isotope fractionation, with an isotopic enrichment factor of −4.21 ± 0.07‰. This study suggests that carbon isotope analysis is a potential tool to trace in situ biodegradation of MTBE and TBA and thus to better understand the fate of these contaminants in the environment.

Introduction

In recent years, many industrialized nations have attempted to minimize air pollution from vehicular emissions through the use of reformulated gasoline. One approach has been the addition of methyl *tert*-butyl ether (MTBE) to gasoline, initially as an octane enhancer after the elimination of tetraalkyl lead. Since the mid-1990s MTBE has been widely used in the U.S.A. as a fuel oxygenate, to reduce CO emissions and lower ground ozone levels (*1*). While beneficial to the atmosphere, MTBE has become a frequently detected environmental contaminant. MTBE has been detected in urban air (*2*), surface water (*3*), and shallow groundwater (*4, 5*). It has been estimated that MTBE may have been released from up to 250,000 leaking underground storage tanks in the United States (*1*). MTBE is affecting the quality of drinking water due to its strong taste and odor and its possible carcinogenic effects. As a result, the U.S. EPA has issued a drinking water advisory of 20 to 40 μg/L for MTBE (*1*).

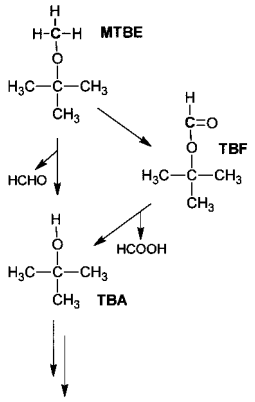

FIGURE 1. Proposed pathways for initial transformation of methyl *tert*-butyl ether (MTBE) to *tert*-butyl formate (TBF) and *tert*-butyl alcohol (TBA).

MTBE has a much greater solubility in water than other gasoline compounds of concern (e.g. BTEX). For example, the solubility of MTBE from reformulated gasoline (11.1% MTBE by volume) is 4700 mg/L at 20 °C, while the solubility of benzene from conventional gasoline is only 18 mg/L (*1*). Since it only sorbs weakly on solids (K_{oc} = 11 (*2*)) and appears difficult to biodegrade (*5*), it can travel nearly unretarded in groundwater and long plumes of dissolved MTBE are frequently observed (*6, 7*). In several laboratory studies, biodegradation of MTBE by aerobic bacteria has been observed (*8*−*11*), while in others, MTBE was reported to be recalcitrant, in particularly under anaerobic conditions (*12*−*15*). Biodegradation occurred in the presence of a cosubstrate such as linear and branched short-chain alkanes (*10, 16, 17*) or with MTBE as sole carbon and energy source (*8, 9, 11*). Cometabolic degradation of MTBE by a fungus has also been reported (*16*). Two routes for the initial transformation of MTBE have been proposed (Figure 1), direct transformation of MTBE to *tert*-butyl alcohol (TBA) (*10*) and transformation of MTBE to *tert*-butyl formate (TBF) (*16*), which subsequently undergoes abiotic or biotic hydrolysis to TBA. Abiotic hydrolysis of TBF is a fast process with a half-life of 5 days at neutral pH and a temperature of 22 °C (*18*). After the initial transformation, the carbon of the methoxy methyl group and the carbon of the *tert*-butyl group may be further oxidized to CO_2 (*10*). While biodegradation of MTBE has often been observed in laboratory experiments, there is less evidence for biodegradation of MTBE at field sites (*19*−*22*), possibly due to the lack of adequate tools to monitor biodegradation. Since MTBE frequently migrates over long distances and degradation rates may be low, it is difficult to assess biodegradation based on concentrations or using a mass balance approach. Furthermore, occurrence of TBA, the most stable intermediate product, may not be a reliable indicator for biodegradation of MTBE since biodegradation can occur without significant TBA accumulation (*23*) or TBA may already be present in the gasoline (*13*). A better means to obtain evidence of in situ MTBE degradation would be of great value for the investigation of the fate of MTBE in the environment and to evaluate the performance of biodegradation-based remediation technologies.

One potential tool for monitoring in situ biodegradation of organic contaminants is the determination of compound-specific isotope ratios of contaminants. This method has

* Corresponding author phone: (519)885-1211; fax: (519)746-7484; e-mail: dhunkele@sciborg.uwaterloo.ca.
† Department of Earth Sciences.
‡ Department of Biology.

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shown promise for gaining insight into the origin and fate of chlorinated solvents in aquifers (*24*, *25*). The method relies on the occurrence of a kinetic isotope effect during biodegradation of inorganic or organic compounds, whereby molecules with light isotopes react faster than molecules of the same compound with heavy isotopes. As a result, a characteristic difference in the isotope ratio between precursor and product occurs (isotope fractionation), and the precursor becomes increasingly enriched in the heavy isotopes as degradation proceeds. Large shifts in the carbon isotope composition of remaining contaminant have been observed during reductive dechlorination of chlorinated ethenes (*24*, *26*, *27*), aerobic oxidation of dichloromethane (*28*), and aerobic oxidation of 1,2-dichloroethane (*29*). In contrast, smaller shifts occurred during biodegradation of aromatic hydrocarbons (*30*). One of the reasons for this difference is the fact that the kinetic isotope effect mainly occurs with respect to the atoms of the bond that is formed or broken in the initial transformation step, while the measurement yields the average isotope ratio of all atoms of an element in the compound. Therefore, an enrichment of heavy isotopes is more likely to be detectable for small molecules. Given that MTBE and TBA only contain 5 and 4 carbon atoms, respectively, it is reasonable to expect detectable carbon isotope fractionation during their degradation.

Whether stable isotope analysis is diagnostic for biodegradation not only depends on the occurrence of a kinetic isotope effect during biodegradation but also on the magnitude of potential isotope fractionation associated with physical processes that affect organic contaminant concentrations in groundwater. Previous studies on isotope fractionation during volatilization and sorption of chlorinated solvents and aromatic hydrocarbons have suggested that only a small carbon isotope fractionation occurs during these processes (*31*−*34*).

In this study, a method for carbon isotope analysis of MTBE and TBA was developed, and the occurrence and magnitude of carbon isotope fractionation was evaluated for biodegradation of MTBE and partitioning of MTBE between organic phase, aqueous phase and gas phase. The effect of sorption on isotope ratios was not investigated since MTBE sorbs only weakly on aquifer solids. The biodegradation studies included microcosms amended with MTBE and microcosms with MTBE and 3-methylpentane as cosubstrate. In the cometabolic microcosm, the isotope ratio of accumulating TBA was also determined to evaluate if TBA degradation is accompanied by isotope fractionation as well.

Material and Methods

Partitioning Experiments. For the partitioning experiments, a toluene/MTBE mixture with 12 vol % MTBE was used since aromatic compounds such as toluene are important gasoline constituents and since MTBE is typically added to gasoline at 10−15 vol % (*1*). All experiments were performed at 23 ± 1 °C. For the organic phase/aqueous phase partitioning experiment, 4 mL of organic phase was dispensed into a 22 mL vial containing organic-free water and a magnetic stirrer, displacing an equivalent amount of water. During the entire experiment, including the addition of organic phase, the vial was kept upside down to prevent contact of the organic phase with the Teflon-lined septum and with the needle during sampling. After 1, 2, and 4 h of stirring, 100 μL of aqueous phase was removed, and the $^{13}C/^{12}C$ ratio of dissolved MTBE was determined using the method described below. For the organic phase/gas-phase partitioning experiment, 2 mL of organic phase mixture was dispensed into a 22 mL vial, which had been filled with helium and closed with an open screw cap containing a Teflon-lined septum. The vial was kept under static conditions. After 1, 2, and 4 h, 425 μL of gas phase was removed, and the $^{13}C/^{12}C$ ratio of MTBE was determined using a method described by Hunkeler and Aravena (*32*). For aqueous phase/gas-phase partitioning, MTBE was added to 125 mL bottles (150 mg/L) which had been completed filled with organic-free water and closed with open screw caps containing Teflon-lined septa. After 10 h of stirring, a 20 mL headspace was introduced by replacing aqueous solution by helium. The bottles were placed upside down in a rotary shaker at 150 rpm, and after 1, 2, and 4 h headspace samples were analyzed for $^{13}C/^{12}C$ ratio in MTBE using a method described by Hunkeler and Aravena (*32*).

Aerobic MTBE-Degrading Enrichments. The aerobic microcosm enrichments investigated in this study were based on three original MTBE-degrading aquifer microcosms. The original microcosms had been prepared using aquifer sediments and groundwater from the Borden aquifer, a shallow, sandy unconfined aquifer located near Alliston, ON, to assess the potential for MTBE biodegradation at the site (*35*, *36*). In these studies, MTBE degradation in the absence of a cosubstrate occurred in only a few of the microcosms and after a lag period of >200 days, suggesting that MTBE-metabolizing microorganisms are relatively rare at the site. In other microcosms and sterile controls no significant changes in the MTBE concentration occurred. Two of the active microcosms used in this study (microcosms 8b, 10a; 150 g sand/200 mL groundwater) had originally been prepared in 1997 using material from a zone through which a slug of MTBE-contaminated groundwater had passed in 1995/1996 (*19*) and the other in 1999 using material from an uncontaminated zone of the aquifer (microcosm 18; 25 g sand/70 mL groundwater). The sand slurry of each parent microcosm has since been divided, replenished with site groundwater, and amended with MTBE and mineral medium as follows. The parent microcosms 8b and 10a were split into two second generation microcosms (75 g sand/60 mL groundwater) in 1999 and twice amended with MTBE (6 and 5 mg/L) and mineral medium (2 and 1.5 mL). One of each second generation microcosm pair was split again into third generation microcosms (37 g sand/30 mL groundwater) and used for this study (8b-2i and ii, 10a-2i and ii). The parent microcosm 18 was split into three second generation microcosms (8 g sand/22 mL groundwater) and amended three times with MTBE (5, 3, and 11 mg/L) and once with mineral medium (2 mL). For this study, two of the second generation microcosms (18-1 and 18−3) were replenished with groundwater (total volume 30 mL) and mineral medium (2 mL). All second and third generation microcosms were contained in 110-mL screw-cap bottles sealed with screw-cap Mininert valves (Vici Precision Sampling, Baton Rouge, LA, U.S.A.) and were incubated in the dark at room temperature (22−25 °C). The mineral medium used in this study contained per mL 1 mg of K_2HPO_4, 1 mg of KH_2PO_4, 1 mg of NH_4NO_3, 0.2 mg of $MgSO_4 \cdot 7H_2O$, 0.02 mg of $CaCl_2 \cdot 2H_2O$, and 0.005 of mg $FeCl_3$. The repeated addition of MTBE has undoubtedly increased the population of MTBE degraders relative to the native population and may have led to other modifications to the indigenous conditions as well. To recognize this, we refer to the six sand and groundwater-containing microcosms used in this study as "microcosm enrichments". At the outset of the experiments reported here, the microcosm enrichments were amended with a filter-sterilized MTBE stock solution to a concentration of 10−13 mg/L. After MTBE addition, all the MTBE-amended bottles were initially shaken for 1.5 h at 100 rpm and then allowed to settle for 1.5 h, before the initial sample was taken. Thereafter, the microcosm enrichments were incubated without shaking, in the dark at room temperature (22−25 °C). Samples of microcosm water were obtained periodically using a glass syringe. The samples were preserved with mercuric chloride (25 mg/L Hg) and analyzed immediately

for MTBE concentration or stored at 4 °C for later TBA concentration analysis and carbon isotope analysis.

Aerobic MTBE Cometabolism Experiment. In contrast to MTBE-metabolizing microcosms, alkane-degrading systems that evidently cometabolize MTBE are readily initiated with Borden aquifer sediments (*35*). For this study, a series of aerobic microcosms was prepared with Borden aquifer materials never exposed to MTBE or 3-methylpentane, the alkane chosen for use as the primary substrate. Each microcosm condition (sterile control, MTBE, MTBE plus 3-methylpentane) was prepared in duplicate. The microcosms consisted of 110-mL screw-cap bottles equipped with Mininert valve closures. They contained 25 g of aquifer sand, 70 mL of groundwater, 1 mL of mineral medium, and 0.5 mL of a filter-sterilized MTBE stock solution, to provide an initial MTBE concentration of about 10 mg/L. The 3-methylpentane-containing microcosms were also injected with 3 μL of neat 3-methylpentane, initially and three additional times (immediately following analyses on days 8, 14, 24) during the experiment, using a 10-μL gastight glass syringe. An additional 1 mL of mineral medium was added to all (i.e. including sterile control) microcosms on day 21. Sterile control microcosms contained aquifer sand autoclaved for 1 h on three successive days, groundwater poisoned with 0.1% (w/v) Na-azide, and were amended with the MTBE stock solution, mineral medium, and 3 μL of 3-methylpentane. The microcosms were incubated and sampled as described in the previous section.

Analytical Methods. *Chemical Analysis.* For analysis of MTBE concentrations, 0.75 mL of aqueous sample was dispensed into a 2-mL screw-cap vial with a Teflon-lined septum. After equilibrating at 36 °C, 400 μL of headspace gas was injected into a Shimadzu GC-9A (Shimadzu Corp., Kyoto, Japan) gas chromatograph (GC) equipped with a 60 m Supelcowax 10 capillary column (Sigma-Aldrich, Oakville, ON, Canada) and flame ionization detection. Concentrations of TBA were determined in one microcosm of each pair by injecting 2 μL aliquots of aqueous solution into a HP 5840A gas chromatograph (Agilent, Palo Alto, CA). The GC was equipped with a flame ionization detector and a 10 ft × 0.125 in. i.d. column, packed with 3% SP−1500 on Carbopack B (80/100 mesh, Sigma-Aldrich, Oakville, ON, Canada). The detection limit was 0.1 mg/L for MTBE and 0.2 mg/L for TBA; the relative standard deviation was 2.5% for MTBE and 1.8% for TBA.

Isotope Analysis. Stable isotope analyses were performed in the Environmental Isotope Laboratory (EIL) of the University of Waterloo using a gas chromatography−combustion−isotope ratio mass spectrometry system (GC−C−IRMS). The GC−C−IRMS system consisted of an Agilent 6890 GC (Agilent, Palo Alto, U.S.A.) with a split/splitless injector, a Micromass combustion interface operated at 850 °C, and a Micromass Isochrom isotope-ratio mass spectrometer (Micromass, Manchester, U.K.). The $^{13}C/^{12}C$-ratio of reference MTBE and TBA was determined as previously described (*24*).

Dissolved MTBE and TBA were extracted by solid-phase microextraction using 75 μm poly(dimethylsiloxane)/carboxen fibers, which have been used in a previous study to quantify dissolved MTBE at low concentrations (*37*). Vials with 2 mL volume were chosen to minimize the required sample size, and since it has been shown that, when extracting MTBE by immersing the fiber into the aqueous phase (direct SPME), the extraction efficiency is higher with smaller vials (*37*). Before extraction, 0.3 mL of solution was removed from the completely filled vial (2 mL) to avoid contact of the sample with the fiber holder. Extractions were performed at 23 °C in continuously stirred samples. For practical reasons, the extraction time was limited to 20 min even though higher extraction efficiencies could be reached using longer extraction times (*37*). Since splitless injection, which is commonly used in SPME methods, resulted in tailing, split injection was used. Small split ratios of 2:1 and 5:1 were used to minimize loss of sensitivity. In regard to analysis of future field samples, additional tests were performed with the aim to further decrease the minimal required concentration. For that purpose, the effect of adding NaCl, a common method to improve SPME extraction efficiencies, and the use of headspace SPME was investigated. For volatile and semivolatile compounds, a higher amount of compound is extracted by headspace SPME than by direct SPME for a given extraction time since mass transfer of compounds to the fiber is faster in the gas phase than in the aqueous phase (*38*). In contrast, for less volatile compounds, mass transfer to the headspace becomes limiting, and headspace SPME provides no advantage compared to direct SPME. For headspace SPME, 40 mL vials were used to increase the amount of compound available for extraction. Prior to extraction, 4 mL of standard solution was removed, and the sample was stirred for 20 min at 1200 rpm on a magnetic stirrer to promote partitioning of the compounds into the headspace. Afterward the fiber was exposed to the headspace of the sample for 20 min while being stirred at 1200 rpm. For all tests, the degree of carbon isotope fractionation between the aqueous phase and the SPME fiber was evaluated as described by Hunkeler and Aravena (*32*). The detection limit was determined by calculating the required concentration to obtain a peak height of 0.75 V (low end of linear range). The $^{13}C/^{12}C$-ratios are reported in the usual delta notation ($\delta^{13}C$). The $\delta^{13}C$ value is defined as $\delta^{13}C = (R_s/R_r - 1) \times 1000$, where R_s and R_r are the $^{13}C/^{12}C$ ratios of the sample and the international standard, VPDB (Vienna Peedee Belemnite), respectively. Each sample was analyzed twice, the obtained values were corrected for isotope fractionation during extraction as described in ref *32*, and the average is reported. The analytical system was verified daily using reference MTBE and TBA dissolved in organic-free water. The uncertainty of the measurement was ±0.3‰ ($n = 2$).

Quantification of Isotope Fractionation. In all partitioning experiments, two phases are present. Isotope fractionation between the two phases can be quantified using the following equation *(32)*

$$\epsilon_{yx} \approx \delta^{13}C_y - \delta^{13}C_x = \frac{\delta^{13}C_y - \delta^{13}C_0}{r_x} \qquad (1)$$

where ϵ_{yx} is the isotopic enrichment factor, $\delta^{13}C_y$ and $\delta^{13}C_x$ are the carbon isotope ratios of MTBE in phase y and x, respectively, $\delta^{13}C_0$ is the initial carbon isotope ratio of the MTBE, and r_x is the fraction of MTBE in phase x. In the SPME tests, three phases are present (aqueous phase, SPME fiber coating, and gas phase). However, the amount of MTBE and TBA in the gas phase is negligible for both direct and headspace SPME, and therefore isotope fractionation between the aqueous phase and SPME fiber coating was also quantified using eq 1.

For the microcosm experiments, a Rayleigh type approach was used to evaluate if isotope fractionation remained constant throughout the experiments and to quantify isotope fractionation. The simplified Rayleigh equation was used which applies for enrichment factors $|\epsilon| < 20$‰ (*39*)

$$\delta^{13}C_S = \delta^{13}C_{S,0} + \epsilon \cdot \ln f \qquad (2)$$

where $\delta^{13}C_{S,0}$ is the initial isotope ratio of the substrate, $\delta^{13}C_S$ is the isotope ratio of a remaining fraction f of substrate, and ϵ is the isotopic enrichment factor.

Results

Isotope Analysis Using SPME. The extracted MTBE and TBA are depleted in ^{13}C compared to dissolved MTBE and TBA

TABLE 1. Isotopic Enrichment Factors with 95% Confidence Interval for Extraction of MTBE and TBA by Solid Phase Microextraction and Minimal Required Concentration (C_{Min}) for Carbon Isotope Analysis

	MTBE		TBA	
conditions[a]	ϵ_{fw} (‰)	C_{Min} (pbb)	ϵ_{fw} (‰)	C_{Min} (pbb)
direct/2 mL/0%/5:1	-0.47 ± 0.27	360	-1.26 ± 0.30	10200
direct/2 mL/0%/2:1	-0.45 ± 0.14	120	-1.30 ± 0.15	3400
direct/2 mL/25%/2:1	-0.49 ± 0.11	90	-1.18 ± 0.12	370
headspace/ 42 mL/25%/2:1	-0.67 ± 0.21	11	-1.49 ± 0.10	860

[a] Extraction mode/vial volume/NaCl concentration/split ratio of injector.

TABLE 2. Isotopic Enrichment Factors for Organic Phase/Gas Phase (ϵ_{go}), Aqueous Phase/Gas Phase (ϵ_{gw}), and Organic Phase/Aqueous Phase (ϵ_{wo}) Partitioning of MTBE with 95% Confidence Interval ($n = 4$)

hours	ϵ_{go} (‰)	ϵ_{gw} (‰)	ϵ_{wo} (‰)	$\epsilon_{gw} + \epsilon_{wo}$ (‰)
1	0.56 ± 0.23	0.14 ± 0.11	0.21 ± 0.21	0.35 ± 0.24
2	0.44 ± 0.10	0.24 ± 0.06	0.18 ± 0.28	0.42 ± 0.29
4	0.50 ± 0.12	0.14 ± 0.06	0.16 ± 0.23	0.30 ± 0.24

(Table 1). The magnitude of isotope fractionation is similar for all conditions. In previous studies, a depletion in ^{13}C of up to 1.5‰ was observed during extraction of organic acids using polar fibers (*40*), while no significant carbon isotope fractionation occurred during extraction of chlorinated solvents using poly(dimethylsiloxane) fibers (*32*). The minimal required concentration for carbon isotope analysis is lower for MTBE than TBA. Addition of NaCl strongly decreases the detection limit for TBA, while it has less effect on MTBE. Headspace SPME leads to a lower detection limit for MTBE, while the detection limit for TBA is higher than under comparable conditions for direct SPME.

Isotope Fractionation during Partitioning Processes. For all partitioning processes evaluated in this study, the magnitude of the enrichment factor did not depend on the equilibration time (Table 2), which demonstrates that isotope equilibrium is reached rapidly. MTBE in the gas phase was enriched in ^{13}C compared to MTBE in the organic phase. Similarly, MTBE in the gas phase is slightly enriched in ^{13}C compared to MTBE dissolved in water, although the effect is minimal. No significant isotope effect occurred during partitioning of MTBE between the organic and the aqueous phase.

Isotope Fractionation during Aerobic Biodegradation. In the microcosm enrichments with MTBE as the only substrate, 10 to 13 mg/L MTBE were degraded within 8 to 12 days (Figure 2). Similar concentration profiles were observed in duplicate microcosms. TBA was only detected in one of the microcosm pairs at low concentrations. In microcosm enrichments 18-1 and 18-3, degradation of MTBE began after a lag period of about 2 days, while in the 8b-2 and 10a-2 microcosm enrichments, degradation started immediately after adding MTBE. Since similar MTBE concentrations were measured in duplicate microcosm enrichments, isotope ratios were only determined for one microcosm of each pair, except pair 18-1 and 18-3. For this pair, isotope ratios were determined in both microcosms, and similar results were obtained for both microcosms. In all microcosm enrichments, the $\delta^{13}C$ of MTBE steadily increased, and a total shift in $\delta^{13}C$ of 5.1 to 6.9‰ after 95 to 97% of degradation was observed. In a sterile control, no significant changes of concentration and $\delta^{13}C$ of MTBE were observed.

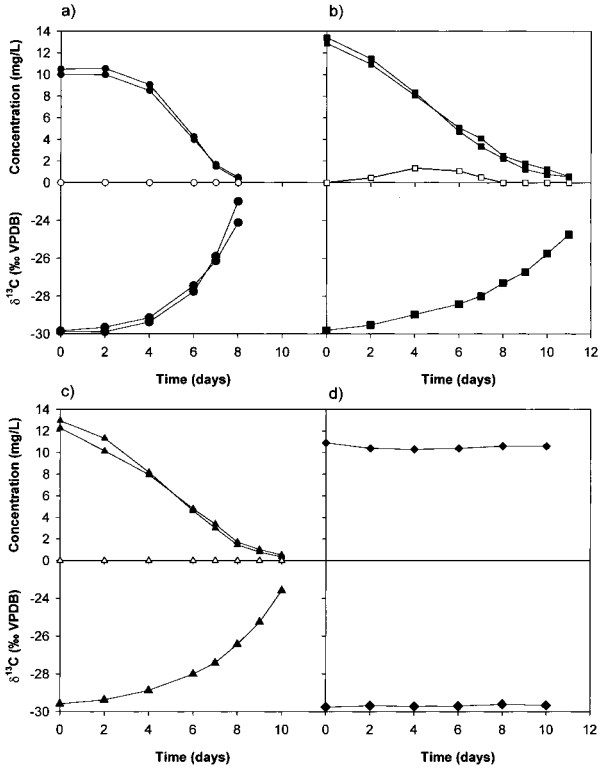

FIGURE 2. Concentration of MTBE, concentration of TBA (open markers) and $\delta^{13}C$ of MTBE in microcosm enrichments with MTBE as sole carbon and energy source: (a) 18−1 and 18−3, (b) 8b-2i and 8b-2ii, (c) 10a-2i and 10a-2ii, and (d) sterile control. The uncertainty of the $\delta^{13}C$ measurement corresponds approximately to the size of the marker.

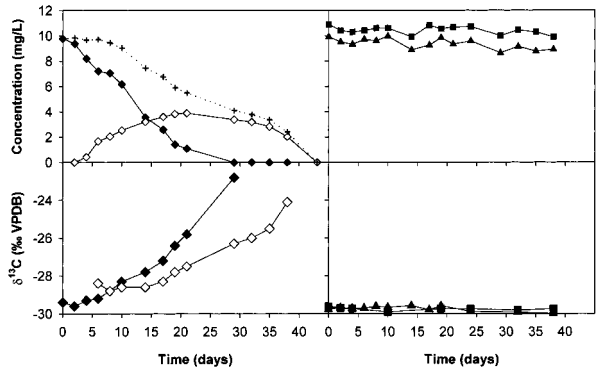

FIGURE 3. Left: Concentration and $\delta^{13}C$ of MTBE (filled diamond) and TBA (open diamond) in microcosm with 3-methylpentane as primary substrate and sum of MTBE and TBA (cross-hair) in mg/L MTBE equivalent. Right: Concentration and $\delta^{13}C$ of MTBE in sterile control (square) and microcosm with no cosubstrate (filled triangle). The uncertainty of the $\delta^{13}C$ measurement corresponds approximately to the size of the marker.

In the cometabolic microcosms, complete degradation of MTBE took longer, and TBA accumulated transiently (Figure 3). The sum of the MTBE and TBA concentration started to decrease after day 8 and the TBA concentration after day 20, indicating that TBA was consumed, too. In the cosubstrate-free microcosm, the MTBE concentration remained constant throughout the experiment confirming that MTBE degradation only took place in the presence of a cosubstrate. The MTBE became enriched in ^{13}C by 6.6‰, similarly as in the microcosm enrichments, while no significant change in the $\delta^{13}C$ of MTBE was observed in the cosubstrate-free and the sterile microcosms. The $\delta^{13}C$ of initial TBA was larger than the $\delta^{13}C$ of MTBE (Figure 3). After day

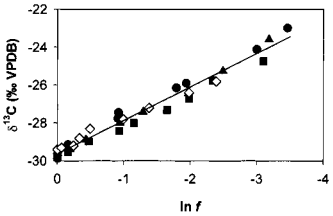

FIGURE 4. Rayleigh type plot for MTBE for microcosm enrichments 18−1 and 18−3 (filled circle), 8b-2i (filled square), 10a-2i (filled triangle), and cometabolic microcosm (open diamond). Line: Calculated isotope ratio using eq 2 and the average enrichment factor of all experiments. The uncertainty of the $\delta^{13}C$ measurement corresponds approximately to the size of the marker.

15, the $\delta^{13}C$ of TBA steadily increased indicating that TBA was degraded.

Quantification of Enrichment Factors. To evaluate if the enrichment factors for MTBE degradation remained constant throughout the experiments and to compare enrichment factors from different experiments, the $\delta^{13}C$ values of MTBE were plotted against ln *f* (Figure 4), and the enrichment factors were quantified based on eq 2 using linear regression. Similar enrichment factors were observed for the cometabolic microcosm and the microcosm enrichments with MTBE as the only substrate (Table 3). The enrichment factors were between -1.52 ± 0.06 and -1.97 ± 0.05‰, and the R^2 values were between 0.9880 and 0.9942.

The enrichment factor for TBA degradation was estimated based on $\delta^{13}C$ values of TBA between day 29 and 38, after complete degradation of MTBE. In this period, the $\delta^{13}C$ of TBA was probably controlled by TBA degradation only, while between day 6 and 29 it was affected by both TBA production and degradation. An enrichment factor of -4.21 ± 0.07‰ was obtained for TBA degradation.

Discussion

The analytical tests demonstrate that SPME coupled to GC−C−IRMS is a very sensitive method to analyze carbon isotope ratios of MTBE with a detection limit below the EPA advisory limit. The method is less sensitive for TBA, and a further reduction of the detection limit may be required for field measurement since TBA concentrations are typically lower than MTBE concentrations. For MTBE, the detection limit is lower for headspace SPME than direct SPME, while for TBA, direct SPME is more sensitive. The different behavior of the two compounds can be explained by the lower volatility of dissolved TBA compared to dissolved MTBE. Due to the low volatility of TBA, slow mass transfer of TBA from the aqueous phase to the headspace probably limits the extraction efficiency during headspace SPME.

The partitioning experiments demonstrate that carbon isotope fractionation during partitioning of MTBE between the organic, aqueous, and gas phase is very small. The largest effect occurred for organic phase/gas-phase partitioning, whereby molecules with ^{13}C were slightly more volatile than molecules with ^{12}C. Such an inverse isotope effect with respect to carbon has previously been reported for evaporation of chlorinated solvents (*31, 33*) and aromatic hydrocarbons from pure nonaqueous phase (*34*), and its origin has been explained using principles of statistical thermodynamics (*41*). Furthermore, a small inverse isotope effect with respect to carbon has also been observed during aqueous phase/gas-phase partitioning of chlorinated solvents (*32*). The three enrichment factors for the partitioning processes can be related using the following equation:

$$\epsilon_{go} = \epsilon_{gw} + \epsilon_{wo}$$

The calculated $\epsilon_{gw} + \epsilon_{wo}$ agrees with the measured ϵ_{go} within the range of uncertainty (Table 2), which demonstrates that the measured enrichment factors are consistent among each other.

TABLE 3. Enrichment Factors for Biodegradation of MTBE in Microcosm Enrichments (ME) and MTBE and TBA in Cometabolic Microcosm (CM)

culture	compound	ϵ (‰)	stdev (‰)	R^2	*n*
ME 18-1	MTBE	−1.97	0.05	0.9877	6
ME 18-3	MTBE	−1.87	0.10	0.9890	6
ME 8b-2	MTBE	−1.64	0.05	0.9942	9
ME 10a-2	MTBE	−1.84	0.06	0.9927	8
CM	MTBE	−1.52	0.06	0.9880	9
CM	TBA	−4.21	0.07	0.9995	4

During biodegradation, the $\delta^{13}C$ of MTBE and TBA significantly increased in all experiments, which demonstrates that reaction rates are slightly faster for molecules with ^{12}C than for molecules with ^{13}C. The good correlation between $\delta^{13}C$ and ln *f* during biodegradation of MTBE and TBA (Figure 4, Table 3) indicates that the enrichment factors remained constant throughout the experiments. A similar observation has previously been made for isotope fractionation associated with reductive dechlorination of chlorinated ethenes (*24, 26*) and oxidation of dichloromethane (*28*), toluene (*30*), and 1,2-dichloroethane (*29*). The enrichment factors for MTBE degradation were similar for microcosm enrichments with MTBE as the only substrate and the cometabolic microcosm (Table 3), which may be due to a similarity in the enzymatic mechanism used by the bacteria for initial transformation of MTBE. They were in the same range as enrichment factors obtained for aerobic and anaerobic oxidation of toluene (*30*) and smaller than the enrichment factors observed for biodegradation of chlorinated solvents (*24, 26−28*).

Since reaction rates are slightly faster for bonds with ^{12}C than molecules with ^{13}C, the product is expected to be depleted in ^{13}C relative to the precursor, as has previously been observed for biodegradation of chlorinated solvents (*24, 26*). However, in this study, an opposite relationship is observed between initial TBA and MTBE, whereby TBA is enriched in ^{13}C compared to MTBE. This apparent contradiction arises because kinetic isotope effects occur mainly with respect to the bond that is formed or broken in the initial transformation step, while isotope analysis yields the average of all carbon atoms in the molecule. In the case of MTBE, the initial transformation step involves only the carbon of the methoxy methyl group, independent of which reaction mechanisms are used (Figure 1). Since this group is removed as MTBE is transformed to TBA, the $\delta^{13}C$ of TBA is not expected to reflect the isotope effect associated with MTBE transformation. Rather it should mainly reflect the $\delta^{13}C$ of the *tert*-butyl group in MTBE and isotope fractionation during biodegradation of TBA. As the sum of the MTBE and TBA concentration decreased, the $\delta^{13}C$ of TBA became more positive with time, confirming that TBA was degraded and degradation was accompanied by carbon isotope fractionation. In conclusion, the biodegradation experiments indicate that transformation of MTBE and transformation of TBA, which are probably the rate-limiting steps in MTBE mineralization, are accompanied by significant carbon isotope fractionation.

Implications for Field Application. Compound-specific isotope analysis can potentially be used to trace biodegradation of organic contaminants at field sites, if isotope fractionation associated with biodegradation is much larger than isotope fractionation associated with physical processes. Furthermore, shifts of isotope ratios due to biodegradation should also be larger than variations in the isotopic com-

position of spilled compounds, which might occur if gasoline enters the subsurface from leaking storage tanks over a prolonged period of time. Important physical processes that affect concentrations of MTBE in groundwater are dissolution of MTBE from gasoline and advective and dispersive transport. Sorption of MTBE to aquifer solids and volatilization from the aqueous phase are of minor importance due to the low organic carbon/water partitioning coefficient and low Henry's coefficient of MTBE (*2*). For TBA originating from MTBE degradation, advective and dispersive transport are probably the most important physical processes affecting its concentration. Advection and dispersion are not expected to significantly affect the isotope ratios of dissolved compounds. The data presented in this study demonstrate that dissolution of MTBE from an organic phase is not accompanied by significant carbon isotope fractionation, at least not under equilibrium conditions. Thus, physical processes are not expected to lead to significant changes of the $\delta^{13}C$ of dissolved MTBE. In contrast, biodegradation of MTBE and TBA is accompanied by a systematic shift of the $\delta^{13}C$ values. For MTBE a significant shift (two times uncertainty of measurement) occurs after 33% degradation, for TBA after 13% degradation based on the enrichment factors determined in this study. Since the kinetic isotope effect for MTBE probably mainly occurs with respect to the carbon in methoxy methyl group, an even smaller fraction of biodegradation could be detected by position-specific isotope analysis. However, for that purpose a method to remove and recover the methoxy methyl group from MTBE without isotope fractionation would have to be developed. The analytical method developed in this study makes it possible to determine compound-specific $\delta^{13}C$ values of MTBE to concentrations below the EPA advisory level and therefore is well suited for practical applications. In conclusion, this study shows that compound-specific isotope analysis of MTBE and TBA is a potential tool to trace in situ biodegradation of these compounds and thus could contribute to a better understanding of the fate of MTBE and TBA in the environment.

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