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New Evaluation Scheme for Two-Dimensional Isotope Analysis to Decipher Biodegradation Processes: Application to Groundwater Contamination by MTBE

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Compound-specific analysis of stable carbon and hydrogen isotopes was used to assess the fate of the gasoline additive methyl *tert*-butyl ether (MTBE) and its major degradation product *tert*-butyl alcohol (TBA) in a groundwater plume at an industrial disposal site. We present a novel approach to evaluate two-dimensional compound-specific isotope data with the potential to identify reaction mechanisms and to quantify the extent of biodegradation at complex field sites. Due to the widespread contaminant plume, multiple MTBE sources, the presence of numerous other organic pollutants, and the complex biogeochemical and hydrological regime at the site, a traditional mass balance approach was not applicable. The isotopic composition of MTBE steadily changed from the source regions along the major contaminant plume (−26.4‰ to +40.0‰ (carbon); −73.1‰ to +60.3‰ (hydrogen)) indicating substantial biodegradation. Constant carbon isotopic signatures of TBA suggest the absence of TBA degradation at the site. Published carbon and hydrogen isotope fractionation data for biodegradation of MTBE under oxic and anoxic conditions, respectively, were examined and used to determine both the nature and the extent of in-situ biodegradation along the plume(s). The coupled evaluation of two-dimensional compound-specific isotope data explained both carbon and hydrogen fractionation data in a consistent way and indicate anaerobic biodegradation of MTBE along the entire plume. A novel scheme to reevaluate empiric isotopic enrichment factors (ϵ) in terms of theoretically based intrinsic carbon ($^{12}k/^{13}k$) and hydrogen ($^{1}k/^{2}k$) kinetic isotope effects (KIE) is presented. Carbon and hydrogen KIE values, calculated for different potential reaction mechanisms, imply that anaerobic biodegradation of MTBE follows a S_N2-type reaction mechanism. Furthermore, our data suggest that additional removal process(es) such as evaporation contributed to the overall MTBE removal along the plume, a phenomenon that might be significant also for other field sites at tropic or subtropic climates with elevated groundwater temperatures (25°C).

Introduction

With an annual production of 21 million t in 1999 (*1*), MTBE is among the organic chemicals with the highest production volume worldwide. Despite its relatively recent introduction as gasoline additive (*2*), MTBE has become one of the most frequently detected groundwater contaminants (*3*). MTBE tends to form widespread contaminant plumes in groundwater due to its negligible sorption (*4*, *5*) and relatively slow biodegradation (*6*−*8*). As chemical transformation of MTBE is insignificant in soil and groundwater biodegradation commonly is thought to be the dominant removal mechanism in the subsurface.

Under oxic conditions, biodegradation of MTBE has been demonstrated in pure cultures (*9*−*13*). Although MTBE biodegradation may proceed co-metabolically in the presence of short-chain alkanes (*14*−*16*), the presence of other gasoline components such as BTEX may slow or even completely inhibit the degradation of MTBE (*17*). In the absence of oxygen, MTBE degradation was described for microcosms under denitrifying conditions (*18*, *19*), sulfate-reducing conditions (*18*, *20*), iron-reducing conditions (*18*, *21*, *22*) and methanogenic conditions (*19*, *23*, *24*). Degradation rates under methanogenic conditions are slow ($t_{1/2} = 2.2-5.0$ yr^{-1} in the field, 3.5 yr^{-1} in microcosms with added alkylbenzenes, and 3 yr^{-1} in unamended microcosms) (*25*). The reaction mechanism(s) for anaerobic biodegradation of MTBE are unknown, but enzyme-catalyzed hydrolysis has been suggested as a potential pathway (*26*).

tert-Butyl alcohol (TBA) is considered as the major product of MTBE biodegradation under both oxic and anoxic conditions. Compared to MTBE, little is known about the biodegradation of TBA (for an overview, see ref *27*). TBA often accumulates in the presence of MTBE and therefore has been used in field studies as an indicator of MTBE biodegradation. Biodegradation of MTBE without significant accumulation of TBA (*28*−*30*) or preferred biodegradation of TBA (*30*) has also been described. The potential lack of TBA accumulation and the fact that TBA may be present as a constituent of spilled gasoline (*21*, *31*) complicate the assessment of in-situ degradation of MTBE by a mass balance approach even under well-defined boundary conditions (*32*, *33*). At our and at many other contaminated field sites, a "traditional" mass balance approach to assess in-situ biodegradation is not applicable due to the widespread contamination plume, the existence of multiple contamination sources, and complex biogeochemical and hydrological conditions. Hence, alternative methods are necessary to assess quantitatively sources and the fate of MTBE and TBA in the field.

Here, we describe a two-dimensional compound-specific carbon and hydrogen stable isotope approach to evaluate biodegradation of MTBE and TBA in a contaminated groundwater plume at an industrial disposal site. Compound-specific isotope analysis (CSIA) has been applied to different organic groundwater contaminants including chlorinated solvents (*34*−*39*) or BTEX (*40*, *41*) and is recognized as a

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promising tool to elucidate transformation processes of such pollutants in the subsurface. Our two-dimensional stable isotope approach is based on a comparison of measured field data with carbon and hydrogen isotope fractionation data reported for biodegradation of MTBE under oxic and anoxic conditions (*42*, *43*). Specifically, a novel evaluation procedure allowed for the first time the interpretation of observed empirical fractionation of MTBE in terms of theoretically based intrinsic kinetic isotope effects (KIE). It was therefore not only possible to assess the nature and extent of in-situ biodegradation but also to decipher the pathway of anaerobic MTBE degradation.

Theoretical Background

As bond cleavage may be accompanied by a kinetic isotope effect, the isotopic signatures of the reactant and the product(s) may change during a given transformation reaction. The remaining fraction of the parent compound may thus become progressively enriched in heavier isotopes since bonds containing light isotopes are slightly less stable and thus more reactive than those with heavy isotopes. Isotopic enrichment factors (ϵ, in per mil) are commonly used to quantify the apparent isotopic shift during a reaction. ϵ values (in per mil) can be derived from a linearized form of the Rayleigh eq 1. A detailed derivation of eq 1 can be found in ref *44*:

$$\ln\left(\frac{\delta^{13}C_x + 1000}{\delta^{13}C_0 + 1000}\right) = \frac{\epsilon}{1000}\cdot\ln f \qquad (1)$$

Here, $\delta^{13}C_x$ and $\delta^{13}C_0$ refer to the carbon isotopic signatures of MTBE at well x and in the contamination source, respectively. The average carbon isotopic signature of MTBE within the source zone was used as $\delta^{13}C_0$. f is the remaining fraction of the reacting compound and is defined as the ratio of the MTBE concentration at well x as compared to a reference concentration of MTBE (e.g., at the source of contamination):

$$f = \frac{[\mathrm{MTBE}]_x}{[\mathrm{MTBE}]_0} \qquad (2)$$

Solving eq 1 for f yields

$$f = \left(\frac{\delta^{13}C_x + 1000}{\delta^{13}C_0 + 1000}\right)^{1000/\epsilon} \qquad (3)$$

which allows the calculation of the expected concentration ratio using the determined isotopic signatures and assuming that the difference between C_0 and C_x is only due to a transformation reaction with a given ϵ. On the basis of this ratio, the extent of (bio)degradation (B) can be estimated (*45*, *46*):

$$B = (1 - f) \times 100 \quad (\%) \qquad (4)$$

Assuming (i) similar transport processes for TBA and MTBE, (ii) MTBE degradation as the only source of TBA, and (iii) the absence of TBA (bio)transformation, the expected TBA concentration in well x may be estimated using B and the MTBE concentration found at this well using eq 5:

$$[\mathrm{TBA}]_x = \frac{B}{1 - B}[\mathrm{MTBE}]_x \quad (\mu\mathrm{M}) \qquad (5)$$

Determination of $^{12}k/^{13}k$ and $^{1}k/^{2}k$ Values from Enrichment Factors. Isotopic signatures determined in degradation studies reflect the average isotopic composition over the entire molecule and not only of the atom(s) involved in the reaction. It is, however, the isotopic fractionation of the atom(s) at the reactive position that has to be known in order to use this information for mechanistic interpretations. Such intrinsic fractionation is commonly expressed as kinetic isotope effect (KIE), which is the ratio between the reaction rate constant of the light isotope and the heavy isotope (e.g., $^{1}k/^{2}k$ and $^{12}k/^{13}k$ for hydrogen and carbon KIEs, respectively). Hence, if molecules contain more than one atom of the element for which isotope fractionation is studied, two different effects must be taken into account. One effect is the "dilution" of the measured isotopic shift due to the presence of atoms of the studied element at nonreacting positions in a molecule. In the case of MTBE oxidation for example, ^{13}C and ^{2}H isotope fractionation will occur solely at the methoxy group, whereas the isotopic composition of the *tert*-butyl group will remain unchanged. This invariant isotope ratio in the nonreacting group will, however, contribute to a great extent to the average isotopic signature of the whole molecule; hence, the observed effect will be much smaller than the intrinsic isotope effect occurring at the reactive bond.

As derived in the Supporting Information, this dilution of the observed isotopic shift $\Delta\delta^{13}C = \delta^{13}C_x - \delta^{13}C_0$ (and $\Delta\delta^{2}H = \delta^{2}H_x - \delta^{2}H_0$) can be corrected by an appropriate factor n/x, where n is the total number of atoms of one element present in the molecule and x is the number of atoms of this element that are located at reactive positions. In the case of MTBE oxidation mentioned above, the correction factor would then equal 5/1 and 12/3 for ^{13}C and ^{2}H isotope fractionation, respectively (see Table 3 for correction factors n/x for all three possible reaction mechanisms). It should be mentioned that the underlying assumption for the application of this correction is a statistical distribution of the heavy isotopes in the parent compound, here MTBE. The validity of this assumption has been discussed to some detail in ref *44*. The term n/x may be replaced by the experimentally determined isotopic distribution if such data are available. For example, as discussed below, an analysis of commercially available MTBE in this study showed that the *tert*-butyl group was enriched in $\delta^{13}C$ by about 2.6‰ and the methoxy group was depleted by about 8‰ as compared to the average $\delta^{13}C$ of MTBE (−28.13‰). Consequently, because the relative abundance of ^{13}C was 0.8% lower in the methoxy group, a correction factor of $n/x = 5/(1-0.008) = 5.04$ would be appropriate instead of $(n/x = 5/1) = 5.00$ if a sample of this particular production batch reacted by microbial oxidation. The fact that the deviation between the two values is very low illustrates that with carbon isotopes the effect of a nonstatistical isotope distribution is generally negligible; hence, the factor n/x is a good approximation.

If eq 1 is rewritten as:

$$\ln\left(\frac{1000 + \delta^{13}C_0 + \Delta\delta^{13}C}{1000 + \delta^{13}C_0}\right) = \frac{\epsilon}{1000}\ln f \qquad (6)$$

Applying the correction factor n/x leads to:

$$\ln\left(\frac{1000 + \delta^{13}C_0 + \frac{n}{x}\Delta\delta^{13}C}{1000 + \delta^{13}C_0}\right) = \frac{\epsilon_{\text{reactive position}}}{1000}\ln f \qquad (7)$$

The whole correction procedure will be discussed in greater detail in a critical review of Elsner et al. (currently in preparation), and a mathematical derivation of eq 7 is included as Supporting Information.

Note that for reevaluation, original data of $\delta^{13}C_0$ and $\Delta\delta^{13}C$ (or $\delta^{2}H_0$ and $\Delta\delta^{2}H$) rather than published enrichment factors ϵ must be used. Whereas this is a straightforward process if

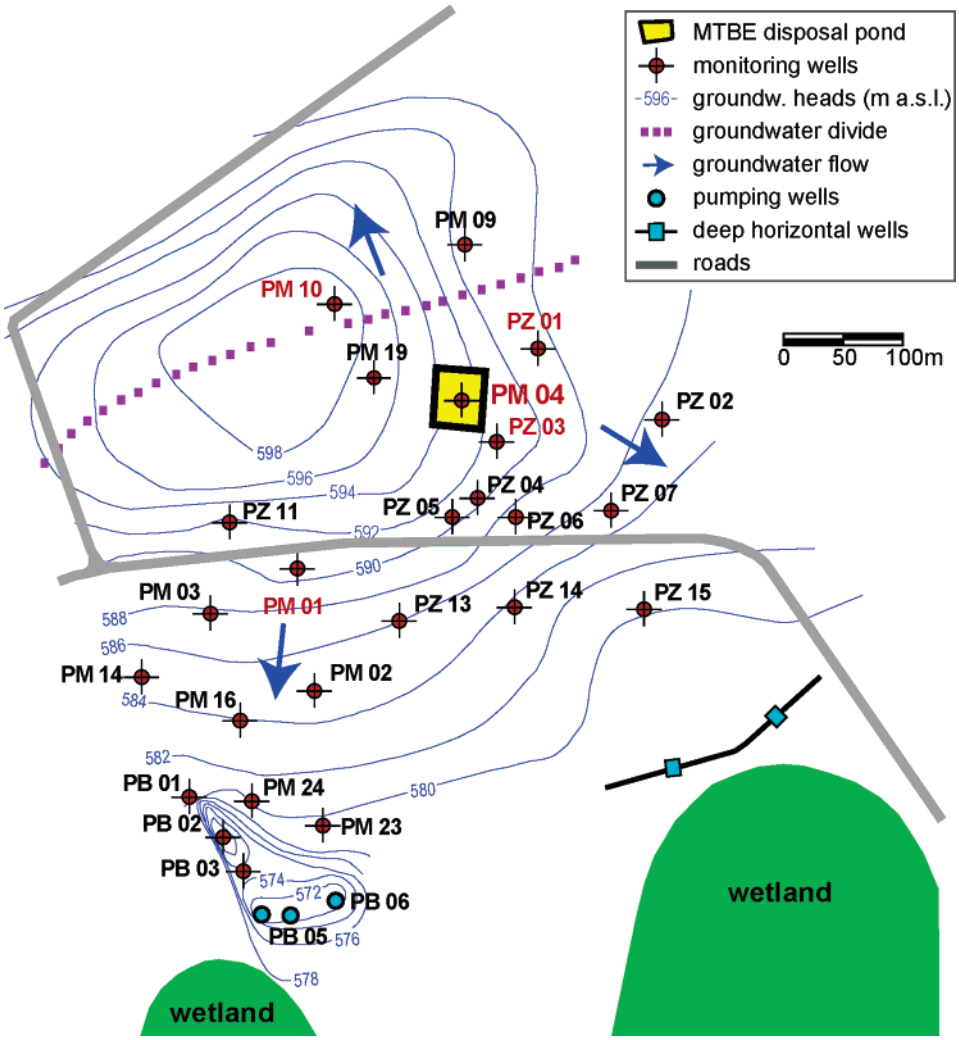


FIGURE 1. Map of the investigated field site depicting the major MTBE disposal pond, monitoring wells, and groundwater heads. The main MTBE source is located at well PM 04. In addition to the major disposal pond PM04, there are other infiltration ponds of minor significance for MTBE input where phenol and other organic compounds were disposed.

newly acquired experimental data are available, in past studies these values are generally not reported. In such cases it is convenient to use an approximate equation:

$$\epsilon_{\text{reactive position}} \approx n/x \times \epsilon \tag{8}$$

where $\epsilon_{\text{reactive position}}$ is an approximate estimate of the position-specific enrichment factor, and ϵ is the "traditional" value reported in most literature studies. Equation 8 gives good approximations as long as changes in isotopic signatures $\Delta\delta^2$H or $\Delta\delta^{13}$C do not become too large (see Supporting Information).

The second effect besides dilution that has to be taken into account is intramolecular competition. Depending on the symmetry of the molecule, it is possible that the investigated element is present at z chemically equivalent, reactive positions in the molecule. In the case of the oxidation of MTBE, this is the case for the three hydrogen atoms of the methoxy group. At low natural isotopic abundance only one of the three positions at most is occupied by deuterium. Hence, if a bond to this heavy isotope is broken, the reaction is in competition with breakage of bonds to the two light hydrogen isotopes in equivalent positions. To correct for this effect, the $\epsilon_{\text{reactive position}}$ value resulting from eq 7 has to be multiplied by z (see Supporting Information and review by Elsner et al. (in preparation)).

After applying both corrections, the intrinsic kinetic isotope effect (KIE) is then obtained by:

$$\frac{1}{\text{KIE}} = \frac{^{13}k}{^{12}k} = z\frac{\epsilon_{\text{reactive position}}}{1000} + 1 \tag{9}$$

For the reevaluation of literature data, the corresponding data sets were obtained from graphs found in the cited publications using an Excel-based digitizing software (GrabIt! XP, Datatrend Software, Raleigh, NC).

Experimental Section

Site Description. The investigated field site (Figure 1) is a former industrial landfill in South America where phenol and MTBE have been disposed in open ponds. Phenol had been disposed for over 20 yr until 1989. Over a span of several years, MTBE was used as solvent in chemical synthesis and was also disposed. The ponds are located on a shallow hill, leading to a radial groundwater flow pattern (Figure 1).

The water table varies between 10 and 20 m below the topsoil; the aquifer is shallow (10−15 m) and has a low hydraulic conductivity (10^{-5} cm/s). It is confined by an underlying zone of basaltic rock from volcanic origin. This impermeable zone is fractured, and preferential flow along these fissures is likely to occur. The average annual ground-

water temperature is between 25 and 30 °C. The assessment of the biodegradation of MTBE is difficult at this particular site, because 13 other disposal ponds (not shown in Figure 1) are located in the area between wells PM 10 and PM 04. Production wastes containing organic contaminants such as BTEX, phenol, and isopropylbenzene have been disposed in these additional ponds. MTBE was also disposed near well PM01 although apparently of minor significance to the overall MTBE contamination resulting primarily from disposal in the pond near PM04. Two wetlands are situated 400−600 m downstream of the disposal site. To protect these wetlands from contamination, three pumping wells and two deep horizontal pumping wells have been installed as hydraulic barriers. The installation of these wells has changed the hydrological regime at the site, leading to potentially higher transport velocities of the contaminants. Forty-seven permanent monitoring wells have been installed at the site and are sampled semiannually. The monitoring wells do not all extend to the bottom of the aquifer, hence varying dilution effects during sampling cannot be excluded.

Sample Collection and Storage. The samples used for this study were taken during two sampling campaigns. Before sampling, the different wells had been pre-pumped for several hours. Water temperature and pH data were determined on site. Measurements with an oxygen-sensitive electrode on selected samples indicated an oxygen depletion (<1 mg/L) of the groundwater. The samples for the determination of inorganic parameters were collected in polyethylene flasks, acidified, and kept at 4 °C until analysis. Samples for the determination of concentrations and isotopic signatures of MTBE and TBA were filled without headspace into 40 mL glass vials. The vials were closed with PTFE-sealed screw caps and stored at 4 °C until analysis.

Analytical Methods. Concentrations of MTBE and of its major degradation products TBA as well as BTEX were determined using direct aqueous injection gas chromatography/mass spectrometry (DAI-GC/MS) using the procedure described by Zwank et al. (*31*), on a gas chromatograph (GC 8000, Fisons, Manchester, U.K.) coupled to a mass spectrometric detector (single quadrupole MD 800, Fisons, Manchester, U.K.).

To achieve a sufficient sensitivity for carbon and hydrogen isotopic analysis, the samples were extracted using a solid-phase microextraction (SPME) procedure (*47*). The analytes were extracted for 30 min from the samples (to which 4 M NaCl was previously added) by directly immersing a Carboxen-PDMS fiber (75 μm, Supelco, Bellefonte, PA) into the sample. The fractionation of the carbon isotopic signature caused by SPME extraction was very small and highly reproducible (*47*). After SPME extraction, the analytes were thermally desorbed for 1 min in a split−splitless injector (270°C) equipped with a deactivated SPME liner. To reduce the peak tailing, a splitless time of only 0.75 min was used. The chromatographic separation of the analytes for IRMS was achieved on a gas chromatograph (Trace GC, Thermo Finnigan) equipped with a 60 m × 0.32 mm Stabilwax fused silica column (1 μm film cross-bonded Carbowax poly(ethylene glycol)) purchased from Restek (Bellefonte, PA) with the following temperature program: 2 min at 45°C, then to 90°C at 7.5 °C/min, 2 min at 90 °C, then to 200 °C at 30 °C/min, and 6 min at 200 °C. For carbon isotopic measurements the analytes were combusted after separation in a combustion interface (GC Combustion III, Thermo Finnigan MAT, Bremen, Germany) maintained at 940 °C, and the resulting CO_2 was analyzed in an isotope ratio mass spectrometer (DeltaPLUSXL, Thermo Finnigan MAT, Bremen, Germany). The catalyst in the combustion interface was oxidized regularly after ∼40 samples. For hydrogen isotopic analysis, the analytes were pyrolyzed at 1400 °C, and the resulting H_2 was analyzed in the isotope ratio mass spectrometer. H_3^+ is formed in the ion source of the mass spectrometer, and hence the precise detection at $m/z = 3$ of HD^+ is hampered. The production of H_3^+ is correlated to the amount of H_2 entering the source and can be corrected with the H_3^+ factor. To allow a good DH/H_2 determination, a low and stable H_3^+ factor is a prerequisite. This factor was determined daily by measuring a set of nine reference gas peaks of increasing amplitudes. This factor was constant (3.21 ± 0.14 ppm/nA). To verify the reproducibility of the isotopic measurements during one sequence of samples as well as throughout the measurement campaign, external standards of known carbon isotopic signature were measured after 3−5 samples. If not stated otherwise, the isotopic signatures correspond to the average of at least three replicate measurements.

Complete MTBE Hydrolysis under Acidic Conditions. MTBE of known isotopic composition was hydrolyzed to TBA under acidic conditions. The hydrolysis reaction was carried out in 20-mL headspace vials sealed with PTFE-lined crimp caps. The vials were filled with 17 mL of deionized water, and subsequently 1.7 mL of concentrated HCl was added. The vials were placed in the agitator of a CombiPAL Autosampler (CTC, Zwingen, Switzerland) heated at 60 °C. After an equilibration time of 1 h, 0.5 μL of a pure MTBE standard was spiked into the reaction vials. To determine the reaction kinetics of this reaction, 75 μL of sample was taken at appropriate time intervals from the reaction vials and neutralized with NaOH. A control reaction at neutral pH was sampled in the same way. Instead of adding NaOH, the control samples were diluted accordingly with distilled water. The concentrations of MTBE and TBA were measured using DAI-GC/MS. The mass spectrometer was acquiring in the scan mode (m/z 40−250) in order to verify that no additional products formed during hydrolysis. The high methanol concentrations produced during the reaction led to an overestimation of the TBA concentrations, due to peak broadening, but since no additional products were formed, complete MTBE hydrolysis can be expected. For the isotopic determination of the MTBE *tert*-butyl group, one vial was spiked with 1 mL of pure MTBE of known isotopic signature, and one vial was spiked with 200 mL of a methanolic solution (1:10 v/v) of a field sample from the MTBE source that contained no TBA. To ensure complete hydrolysis, the vials were kept at 60 °C for 5 h. After hydrolysis the samples were neutralized using NaOH (1 M) and kept at 4°C until analysis. Carbon isotopic compositions were determined using purge-and-trap extraction coupled on-line to a GC−IRMS system following a method described elsewhere (*47*).

Results and Discussion

Position-Specific Isotope Signatures in MTBE. To evaluate whether the isotopic signature of the *tert*-butyl group of MTBE is significantly different from the isotopic composition of the methoxy group, MTBE of a known isotopic composition was hydrolyzed to TBA under acidic conditions. As MTBE was completely transformed to TBA, which was stable under the given conditions, the carbon isotopic signature of TBA corresponds to the one of the *tert*-butyl group of MTBE. MTBE was hydrolyzed with an observed pseudo-first-order constant of $-0.0233\ min^{-1}$ ($R^2 = 0.98$) corresponding to a half-life of 30 min (at pH 0 and 60 °C). The reaction kinetics were in rather good agreement with rates and activation energies determined by O'Reilly et al. (*26*) ($t_{1/2} = 41$ min).

To determine the position-specific carbon isotopic composition of MTBE, the isotopic composition of the MTBE standard used in the hydrolysis experiment was measured ($\delta^{13}C = -28.13 \pm 0.15‰$). After complete hydrolysis of MTBE, the $\delta^{13}C$ of the produced TBA was $-25.49 \pm 0.10‰$, reflecting the isotopic composition of the *tert*-butyl group of the parent MTBE. With the help of an "isotopic mass balance" the $\delta^{13}C$

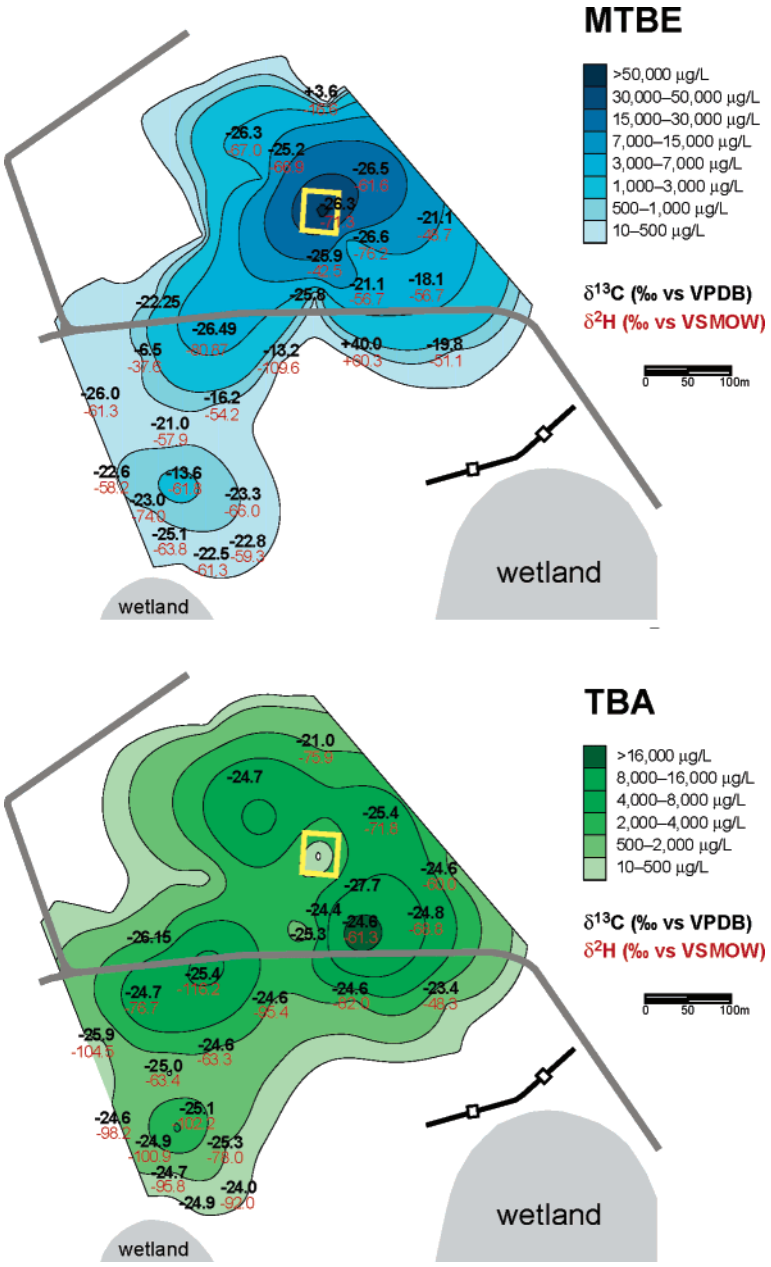


FIGURE 2. Extension of the MTBE (upper part) and TBA (lower part) plume as well as carbon and hydrogen isotopic signatures of these two compounds. The upper isotopic value corresponds to δ^{13}C vs VPDB and the lower value to δ^{2}H vs VSMOW.

of the methoxy group was calculated to be $\delta^{13}C = -36.35 \pm 1.11$‰. Thus, the isotopic signature of the methoxy group and the *tert*-butyl group of MTBE differed by more than 10 ‰. To our knowledge, this is the first indication of position-specific isotope signatures in MTBE.

Qualitative Assessment of in-situ Biodegradation of MTBE. As can be seen from Figure 2, the MTBE plume at the contaminated site was very widespread (over a length of >500 m) with a maximum concentration of 1.7 g/L (aqueous solubility of MTBE = 48 g/L) (*5*) at the major source zone (PM 04). At this well the TBA concentration was below the detection limit (e.g., 2 μg/L) but increased with distance from the MTBE source, suggesting that TBA was not present as co-contaminant but rather was formed by biodegradation of MTBE. Highest TBA concentrations (>22.6 mg/L) were measured downstream of the highest MTBE concentrations. The MTBE concentration contours depicted in Figure 2 show a major hotspot (PM 04) from where the plume stretches out radially. The fact that several wells near the suspected major MTBE source near PM 04 (PM 10, PM01, PZ01, and PZ03) show almost identical carbon (−26.44 ± 0.15‰) and similar hydrogen signatures (−71.4. ± 7.6‰) indicates that these wells share MTBE of the same isotopic composition and that biodegradation is not significant next to the source zone.

Aerobic degradation of MTBE is known to be accompanied by a slight and robust carbon isotopic enrichment ($-1.5 \pm 0.1‰ < \epsilon_c < -2.0 \pm 0.1‰$) (*28*) regardless of whether MTBE was the sole carbon source or degraded co-metabolically with 3-methylpentane as substrate. Similar carbon enrichment factors were obtained in microcosms enriched from contaminated sites ($-1.5 \pm 0.1‰ < \epsilon_c < -1.8 \pm 0.1‰$) and in pure cultures (strain PM1; $-2.0 \pm 0.1‰ < \epsilon_c < -2.4 \pm 0.3‰$) (*48*). Although these ϵ_c values are relatively small, they differ significantly from those caused by physical processes such as organic phase/gas-phase partitioning ($\epsilon_c = 0.50 \pm 0.15‰$), aqueous phase/gas-phase partitioning ($\epsilon_c = 0.17 \pm 0.05‰$), and organic phase/aqueous phase partitioning ($\epsilon_c = 0.18 \pm 0.24‰$) (*28*). Hydrogen enrichment factors (ϵ_H) for aerobic MTBE biodegradation ranged from $-29 \pm 4‰$ to $-66 \pm 3‰$ in microcosm experiments and from $-33 \pm 5‰$

TABLE 1. Concentrations and Isotopic Signatures ($\delta^{13}C$ and $\delta^{2}H$) of MTBE and TBA

	MTBE			TBA		
sampling well	concn (μg/L)	$\delta^{13}C$ (‰ vs VPDB)	$\delta^{2}H$ (‰ vs VSMOW)	concn (μg/L)	$\delta^{13}C$ (‰ vs VPDB)	$\delta^{2}H$ (‰ vs VSMOW)
PM 04[a]	1 700 000	−26.35 ± 0.73	−71.3 ± 1.9	<2[b]		
PZ 01	23 700	−26.47 ± 0.25	−61.6 ± 3.3	6 610	−25.37 ± 0.63	nd[c]
PZ 04	10 800	−25.92 ± 0.19	−42.5 ± 5.4	8 790	−24.42 ± 0.34	nd[c]
PM 01	6 060	−26.49 ± 0.02	−80.9 ± 1.4	10 200	−25.44 ± 0.13	−116.2 ± 11
PZ 06	5 740	−21.11 ± 0.06	−56.5 ± 1.2	22 600	−24.61 ± 0.20	−61.3 ± 7.0
PZ 03	4 690	−26.64 ± 0.17	−76.2 ± 3.8	5 190	−27.63[d]	nd[c]
PZ 07	4 590	−18.12 ± 0.02	−56.7[d]	8 740	−24.76 ± 0.02	−68.8 ± 0.6
PM 10	3 900	−26.26 ± 0.44	−67.0 ± 3.1	6 140	−24.75 ± 1.00	nd[c]
PZ 02	3 870	−21.07 ± 0.07	−48.6 ± 1.6	2 120	−24.57 ± 0.16	−60.0 ± 18
PM 19	2 690	−25.18 ± 0.54	−66.8 ± 9.5	10 300	−25.26[d]	nd[c]
PM 24	1 420	−13.63 ± 0.07	−61.8 ± 2.8	4 440	−25.10 ± 0.08	−102.2 ± 3.8
PM 23	726	−23.35 ± 0.08	−66.0 ± 3.0	459	−25.29 ± 0.11	−78.0 ± 1.1
PB 01	626	−22.57 ± 0.38	−58.2 ± 4.3	1 270	−24.64 ± 0.17	−98.2 ± 4.3
PZ 11	575	−22.25 ± 0.17	nd[c]	2 030	−26.15[d]	nd[c]
PM 03	506	−6.54 ± 0.07	−37.6 ± 10	7 130	−24.69 ± 0.08	−76.7 ± 10
PB 02	493	−23.03 ± 0.33	−74.0 ± 6.3	1 730	−24.91 ± 0.13	−100.9 ± 6.3
PM 14	401	−25.98 ± 0.44	−61.3 ± 2.2	309	−25.90 ± 0.34	−104.5 ± 14
PB 03	348	−25.12 ± 0.09	−63.8 ± 1.2	296	−24.69 ± 0.12	−95.8 ± 7.6
PZ 15	318	−19.81 ± 0.07	−51.1 ± 1.8	746	−23.37 ± 0.13	−48.3 ± 4.4
PZ 14	278	+40.04 ± 1.15	+60.3 ± 11	2 270	−24.62 ± 0.07	−82.0 ± 3.1
PM 02	270	−16.21 ± 0.15	−54.2 ± 1.9	1 540	−24.61 ± 0.11	−63.3 ± 3.6
PZ 05	265	−25.83 ± 0.06	nd[c]	1 780	−25.31 ± 1.33	nd[c]
PM 16	204	−20.97 ± 0.18	−57.9 ± 4.5	363	−24.99 ± 0.52	−63.4 ± 21
PB 06	198	−22.77 ± 0.05	−59.3 ± 2.5	183	−24.95 ± 0.13	−92.0 ± 11
PB 05	88	−22.53 ± 0.06	−61.3 ± 10	43	−24.92 ± 1.39	nd[c]
PM 09	58	+3.52 ± 0.05	−16.6 ± 1.3	1 820	−21.98 ± 0.09	−75.5 ± 9.7
PZ 13	46	−13.20 ± 0.83	−109.6 ± 11	1 520	−24.56 ± 0.17	−95.4 ± 13

[a] Source well. [b] Below method detection limit. [c] Not determined. [d] $n = 1$.

to −37 ± 4‰ for the pure culture (strain PM1) (*48*). It was proposed that the larger but less reproducible hydrogen isotopic fractionation was a sensitive qualitative indicator of in-situ degradation, whereas the more reproducible carbon isotopic fractionation was best suited for quantification of biodegradation in the field. Aerobic co-metabolic degradation of TBA in the laboratory resulted in a carbon isotopic enrichment (ϵ_c) of −4.21 ± 0.07‰ (*28*). In a "mainly" anoxic plume, TBA degradation was accompanied by a change of $\delta^{13}C$ values from −28.6‰ in the source zone to −22‰ with decreasing TBA concentrations (*49*).

Anaerobic biodegradation of MTBE appears to also cause a consistent but substantially higher carbon isotopic enrichment ($\epsilon_c = -8.1 \pm 0.85$‰ in the field and $\epsilon_c = -9.2 \pm 5.0$‰ in microcosms) (*50*) as compared to aerobic conditions. Provided that the few studies available in the peer-reviewed literature are representative, this difference of carbon isotope fractionation may indicate that different reaction mechanisms are involved in aerobic and anaerobic biodegradation of MTBE and thus may be used to characterize the nature of biodegradation in the field. Furthermore, the hydrogen isotopic fractionation under anaerobic field conditions appears to be less pronounced than under aerobic conditions ($\epsilon_H = -11.4$‰) (*51*).

In both studies $\delta^{13}C$ values of TBA did not show a pronounced fractionation. Under oxic conditions, the initial step of MTBE degradation takes place at the methoxy group which is eliminated during the course of the reaction. Thus, a substantial carbon isotopic change of the product TBA is not expected.

Table 1 shows measured concentrations of MTBE and TBA together with their carbon and hydrogen isotopic signatures. A significant isotopic enrichment of MTBE with increasing distance from the source is evident with $\delta^{13}C$ values of up to +40.04‰ versus VPDB and $\delta^{2}H$ of up to +60.3‰ versus VSMOW. MTBE in well PM24 was present at high concentrations but at the same time showed a significant

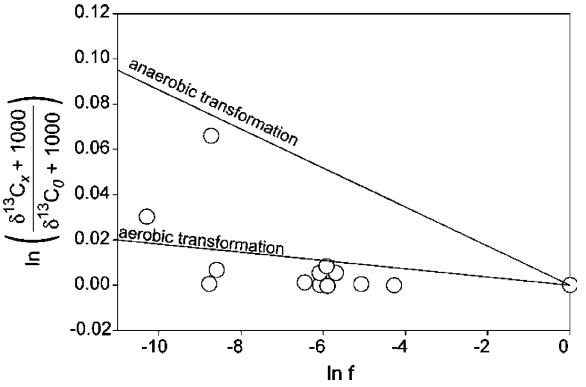


FIGURE 3. Rayleigh plot of measured $\delta^{13}C$-field data for MTBE. Open circles represent data from the major plume near well PM 04. The concentration decline ($f = C/C_0$) in the field does not match with carbon isotopic shifts expected for anaerobic ($\epsilon = -8.62$‰) or aerobic ($\epsilon = -1.82$‰) biodegradation.

enrichment in ^{13}C as compared to the wells downstream that had lower concentrations, suggesting substantial biodegradation despite high concentrations. The apparent mismatch of MTBE concentration and $\delta^{13}C$ value in this particular well might be due to changes in flow patterns and mixing imposed by the intense pumping in wells PB 04, PB 05, and PB 06 (see Figure 1). With the exception of well PM 09 (−20.98 ± 0.09‰), the carbon isotopic signature of TBA was more or less invariable within the plume (−25.02 ± 0.75‰) but slightly enriched in ^{13}C as compared to MTBE at the source. This effect can be explained by a position-specific difference in the carbon isotopic signature of the methoxy and *tert*-butyl group in MTBE as found for a MTBE standard (see above). The hydrogen isotopic signature of TBA did not show a consistent trend possibly due to a variable hydrogen isotopic signature of the *tert*-butyl group of MTBE

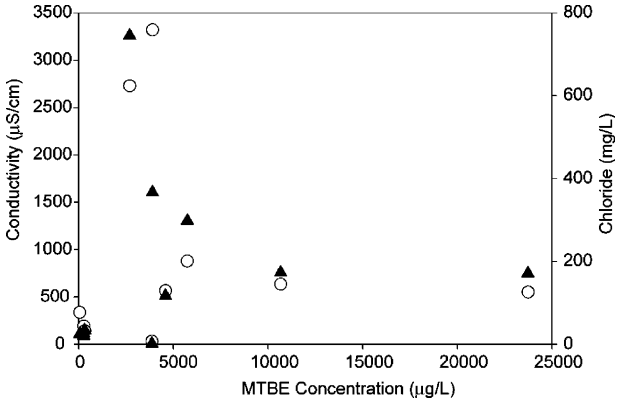


FIGURE 4. Plot of MTBE concentration versus chloride concentration (▲) and conductivity (○).

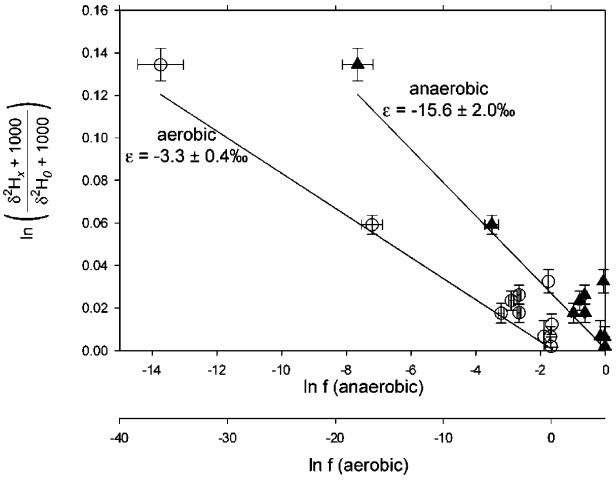


FIGURE 5. Hydrogen isotopic enrichment vs relative concentrations of MTBE calculated from carbon isotopic enrichment factors for anoxic conditions (▲) and oxic conditions (○). *y*-axis: measured hydrogen enrichment; *x*-axis: fraction of remaining MTBE calculated from measured carbon isotopic fractionation according to eq 3. The lines correspond to a linear regression model; their slopes represent the corresponding hydrogen enrichment factors. Note the different scales of the *x*-axis for aerobic and anaerobic biodegradation.

disposed over the years, and/or hydrogen exchange at the hydroxyl group of TBA.

Quantification of Biodegradation. In the evaluation of field isotope data usually "field enrichment factors" are calculated by constructing a Rayleigh plot from measured concentration and isotope ratio data according to eq 1. The ϵ values thus determined are subsequently compared to those obtained from laboratory experiments conducted under controlled conditions. Clearly, because along a contaminant plume in the field concentrations do not only decrease due to transformation processes but also owing to other processes such as dilution and dispersion, the true isotope fractionation might be underestimated if concentration data were not corrected appropriately.

In a tentative approach to determine "true" field enrichment factors, we tried to minimize effects that can be caused by the above-mentioned variable hydrological conditions as well as pumping and focused on the wells that are located around the major contamination source (PM 04). As can be seen from Figure 3, there is no apparent simple correlation between the MTBE concentration and the carbon isotopic enrichment patterns ($R^2 = 0.25$). The field data did not match with the range expected for carbon isotopic fractionation of MTBE under aerobic or anaerobic conditions. In most cases, the observed relative concentrations were too low to explain the observed isotopic values. Hence other processes such as dilution and elimination processes (e.g., volatilization) that do not cause significant isotopic fractionations also have to be taken into account.

To correct for dilution processes, MTBE concentrations can be normalized to a suitable conservative tracer. At this site, however, due to the multiple contamination sources and the long contamination history of the disposal site, no suitable conservative tracer could be identified. As an example, concentrations of MTBE and chloride together with conductivity data are shown in Figure 4. Hence, the traditional Rayleigh-type approach was not appropriate to delineate biodegradation of MTBE at this complex field site.

An alternative approach to determine the extent of (bio)-degradation has been suggested by refs *40*, *41*, *52*, and *53*. Assuming a constant ^{13}C signature of the source, the fraction of MTBE is calculated that results from the enrichment in ^{13}C of MTBE downgradient of the source using eqs 3 and 4. This calculated f represents the concentration decrease that would be expected along a streamline assuming plug flow (no mixing) and one degradation process with a constant enrichment factor. However, because the nature of the degradation process (i.e., aerobic or anaerobic biodegradation) cannot be determined in our case, and because both processes are associated with significantly different enrichment factors (ϵ), an unambiguous determination of B by eq 4 was not possible. For example, assuming aerobic biodegradation associated with an average isotopic enrichment factor of $\epsilon = -1.82‰$ (*28*, *48*), the extent of expected biodegradation in wells PZ 06 and PM 23 is 95% and 82%, respectively. However, if the average isotopic enrichment factor for anaerobic biodegradation is used ($\epsilon = -8.63‰$) (*51*), biodegradation only accounted for 46% and 30% in the same wells. Thus, an approach has to be developed that allows differentiating between anaerobic and aerobic biodegradation of MTBE at hydrogeologically and biogeochemically complex field sites.

A first useful approach is to subject the values of f that may be calculated according to eq 3 to a plausibility test. For example, in well PZ 14 a highly enriched carbon isotope ratio of $\delta^{13}C = +40‰$ was measured (see Table 1). If one assumes that this value has been reached by fractionation during anerobic degradation of MTBE, a calculation according to eq 3 with ($\epsilon_{C(anaerobic)} = -8.63‰$) would give a value of $\ln f \approx -7$ (see value of x-axis belonging to the black triangle farthest to the left in Figure 5). Conversely, under the assumption that fractionation leading to $\delta^{13}C = +40‰$ had taken place during aerobic fractionation, the calculation according to eq 3 with ($\epsilon_{C(aerobic)} = -1.82‰$) would give a value of $\ln f \approx -37$ (see value of x-axis belonging to the open circle farthest to the left in Figure 5). The latter concentration would be clearly too small to be analyzed by CSIA so that for this particular point the aerobic pathway can be excluded. An analysis, however, is less conclusive for wells where more moderate enrichment has taken place so both aerobic and anaerobic transformation are possible scenarios. (Calculated $\ln f$ then corresponds to the x-axis values associated with triangles and circles farther to the right in Figure 5.) Clearly, in such cases an alternative "plausibility test" is needed. Fortunately, the microbial transformation of MTBE under oxic and anoxic conditions is not only associated with different carbon isotopic enrichments but also simultaneously with significantly different hydrogen isotopic enrichments. Our key to the solution, therefore, was to correlate on one hand the $\ln f$ values that were calculated from measured carbon isotope ratios according to eq 3 (x-axis of Figure 5) and on the other the hydrogen isotope ratios that were measured in the same samples (y-axis of Figure 5). (Note that Figure 5 has two scales for the x-axis, one for anaerobic degradation (associated with ▲) and one for aerobic degradation (associated with ○).)

TABLE 2. Calculated Extent of Biodegradation and Measured vs Predicted MTBE and TBA Concentrations

sampling well	*f* (MTBE) measd	*f* (MTBE)[a] calcd	extent of biodeg (%)	measd TBA concn (μM)	pred TBA concn (μM)	$[TBA]_{meas}/[TBA]_{pred}$
PM 04[b]	1.00E+00	0.99E+00	0		38	
PZ 03[c]	2.75E−03	1.03E+00	−3	70	−2	−35
PZ 01[c]	1.39E−02	1.01E+00	−1	89	−3	−29
PM 01[c]	3.56E−03	1.01E+00	0	140	−1	−140
PM 10[c]	2.29E−03	9.87E−01	1	83	1	83
PM 14	2.36E−04	9.55E−01	4	4	0	
PZ 04	6.26E−03	9.48E−01	5	120	7	17
PZ 05	1.55E−04	9.38E−01	6	24	0	
PM 19	1.58E−03	8.68E−01	13	140	5	28
PB 03	2.05E−04	8.62E−01	14	4	1	4
PM 23	4.25E−04	6.99E−01	30	6	4	1.5
PB 02	2.90E−04	6.73E−01	33	23	3	7.6
PB 06	1.16E−04	6.52E−01	35	2	1	2
PB 01	3.68E−04	6.37E−01	36	17	4	4.2
PB 05	5.18E−05	6.34E−01	37	1	1	1
PZ 11	3.38E−04	6.13E−01	39	27	4	6.7
PZ 06	3.37E−03	5.36E−01	46	300	56	5.4
PZ 02	2.27E−03	5.33E−01	47	29	38	0.8
PM 16	1.20E−04	5.27E−01	47	5	2	2.5
PZ 15	1.87E−04	4.59E−01	54	10	4	2.5
PZ 07	2.69E−03	3.76E−01	62	120	86	1.4
PM 02	1.59E−04	3.00E−01	70	21	7	3
PM 24	8.35E−04	2.22E−01	78	60	56	1.1
PZ 13	2.71E−05	2.11E−01	79	21	2	10
PM 03	2.98E−04	9.67E−02	90	96	54	1.8
PM 09	3.40E−05	3.01E−02	97	25	21	1.2
PZ 14	1.63E−04	4.78E−04	100	31	6600	0

[a] Calculated fraction of remaining MTBE under the assumption that biodegradation is the only major elimination process. [b] Concentration set as initial value (*f* (MTBE) = 1) for the downgradient wells. [c] Well within the estimated source zone next to the major MTBE disposal pond at PM04.

As is evident from Figure 5, the calculation of *f* based on carbon isotopic fractionation resulted in a strong correlation ($R^2 = 0.94$) suggesting that this approach allows to distinguish MTBE concentration decrease associated with in-situ biodegradation from dilution or other nonfractionating processes. From the slope of the correlations shown in Figure 5, hydrogen isotopic enrichments factors (ϵ_H) could be determined for both aerobic (-3.3 ± 0.4‰) and anaerobic (-15.6 ± 2.0‰) conditions. The obtained ϵ_H (−3.3‰) for aerobic biodegradation, however, is much lower than those previously reported (−29‰ to −66‰) (*48*). Also, the extremely low ln *f* values calculated for aerobic biodegradation ($\ln f \ll -10$) are not realistic because, as discussed above, the resulting concentrations are far below those that can be utilized or detected by both microorganisms and available analytical instrumentation.

Assuming anaerobic biodegradation (filled symbols in Figure 5), however, the obtained hydrogen enrichment factor ($\epsilon_H = -15.6 \pm 2.0$‰) corresponds fairly well with the enrichment factors published by Kuder et al. (*51*) for anoxic conditions (−11.5‰), and the calculated *f* values appear more appropriate.

Hence, the analysis of Figure 5 did not only exclude aerobic biotransformation of MTBE as major degradation pathway, it also allowed us to calculate the extent of biodegradation using eqs 3 and eq 4 assuming an isotopic enrichment factor (ϵ_C) of −8.63‰ that is proposed for anaerobic biodegradation of MTBE (*51*). The results of this calculation are shown in Table 2.

Importance of a Two-Element CSIA Approach for the Evaluation Scheme. This example illustrates the potential of using a multi element approach in CSIA especially in the case of field studies. If the data of only one element (e.g., carbon isotopic signatures) were present, a quantification of in-situ biodegradation would have been impossible. Due to the fact that we applied the isotopic data of one element (carbon in this case) for the correction of the concentration, we were able to use isotopic information of the second element to get hints on the process responsible for the observed isotopic fractionation. This new approach relies on the combined evaluation of carbon and hydrogen isotopic data collected at the field site and allows to identify (i) whether the observed isotopic enrichment of carbon and hydrogen isotopes at the site is associated with one single process as well as (ii) the nature of this process.

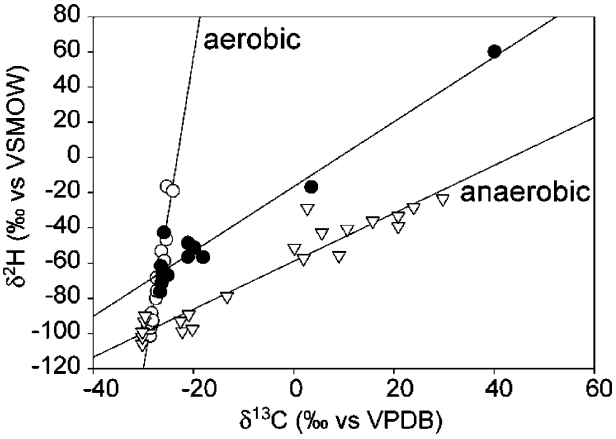
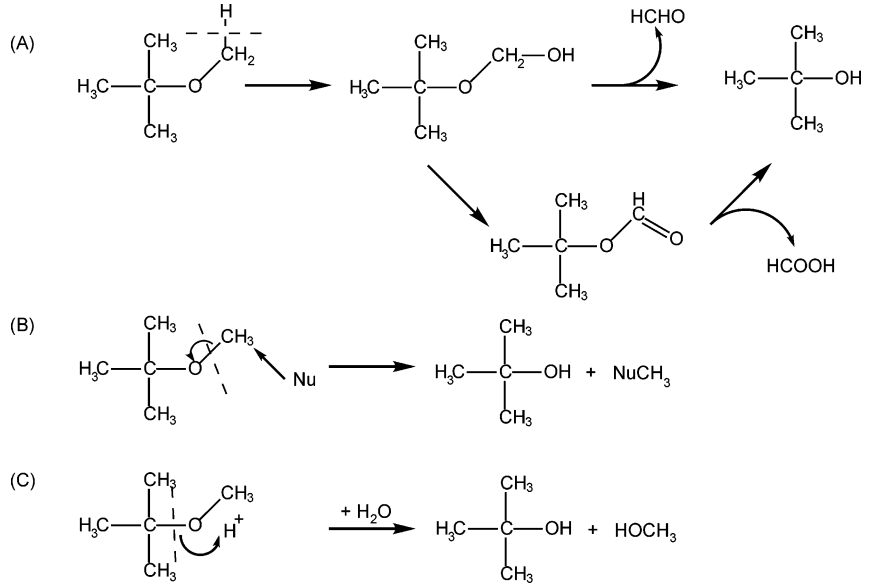


FIGURE 6. Plot of hydrogen versus carbon isotopic shifts for MTBE. (○) aerobic biodegradation in a batch experiment (*48*); (△) anaerobic biodegradation at different field sites (*51*); (●) field data from the major MTBE plume of our study.

Hence, based on a plausible hypothesis for the type of process causing the monitored fractionation, it was possible to calculate the extent of biodegradation using eq 4. Furthermore, expected TBA concentrations were calculated for each sampling well, using eq 5. The results of this calculation are also shown in Table 2 and are based on the following assumptions: (i) no differences in the transport behavior of MTBE and TBA, (ii) MTBE biodegradation is the only source of TBA formation, (iii) constant isotopic signatures of TBA, and (iv) the absence of TBA biodegradation.

The TBA concentrations were generally underestimated with this approach. Only in well PZ 14, where the highest TBA concentration would be expected based on the large isotopic enrichment of MTBE, much less TBA was found than would be expected. However, the general underestimation can be explained by the fact that the measured MTBE concentrations used for this calculation ([MTBE]x) are not only lowered by biodegradation (quantified using isotopic fractionation) or dilution (which should be roughly the same for TBA) but by an additional process that does not yield TBA and does not change the isotopic composition of the parent compound. Owing to the relatively high temperature of the groundwater (~25 °C) and the higher water−air partition constant of MTBE as compared to TBA (*5*), volatilization might be significant for the attenuation of the MTBE concentrations at this particular site. Volatilization is known to be associated with a small inverse isotopic effect, which means that molecules with the heavy isotope preferentially partition into the gas phase (*54*, *55*). For MTBE, this effect is, however, small ($+0.17 \pm 0.05$‰) (*28*) as compared to the overall fractionation observed at the field site. The calculated TBA concentration in the source wells is far off the measured value since even small deviations in isotopic signatures from the average carbon isotopic signature of the source wells lead to high deviations in the calculated TBA concentrations considering the high initial MTBE concentrations. Generally, however, the differences between measured and calculated TBA concentrations corroborate the hypothesis that alterna-

SCHEME 1. Reaction Mechanisms for Enzymatic Degradation of MTBE[a]



[a] Dashed line indicates the chemical bond at which isotopic fractionation will occur during the given reaction.

TABLE 3. Correction Factors Applied and Expected KIEs for Different Reaction Mechanisms for the Transformation of MTBE

	oxidation	S_N2	S_N1
	Carbon		
no. of carbon atoms (*n*)	5	5	5
no. of carbon atoms in reactive positions (*x*)	1	1	1[a]
correction for nonreacting sites[b]	$n/x = 5$	$n/x = 5$	$n/x = 5$
correction for intramolecular competition (*z*)[c]	none ($z = 1$)	none ($z = 1$)	none ($z = 1$)
expected KIE $^{12}k/^{13}k$	1.01−1.02	1.03−1.08	1.00−1.02
	Hydrogen		
no. of hydrogen atoms (n)	12	12	12
no. of hydrogen atoms in reactive positions (*x*)	3 (primary effect)	3 (secondary effects in α-position)	9 (secondary effects in β-position)
correction for nonreacting sites[b]	$n/x = 4$	$n/x = 4$	$n/x = 4/3$
correction for intramolecular competition (*z*)[c]	$z = 3$, three equal positions of which only one reacts	simultaneous secondary effects, no competition ($z = 1$)	simultaneous secondary effects, no competition ($z = 1$)
expected KIE $^1k/^2k$	3−8	0.95−1.05	1.1−1.2

[a] The three methyl carbon atoms that could show a secondary isotope effect are neglected. [b] n/x value used in eqs 7 and 8. [c] Correction factor *z* is used in eq 9.

tive MTBE sinks (such as evaporation) exist, consistent with too low MTBE concentrations in the "traditional" Rayleigh analysis (see Figure 3).

Mechanisms of MTBE Biodegradation. A meaningful application of the two-element isotopic approach presented above requires that correlations between carbon and hydrogen isotopic fractionations are not coincidental but reflect the underlying reaction mechanism(s).

In other words, the approach—using carbon isotope data to calculate the extent of biodegradation $(1 - f)$ combined with the use of calculated f to determine hydrogen isotopic enrichments—should only work if both isotope signatures are influenced by the same reaction. In Figure 6, carbon and hydrogen isotopic signatures reported during aerobic and anaerobic biodegradation (*51*) are plotted together with the data collected from the investigated field site.

A good correlation of the hydrogen and carbon isotopic shifts was obtained for all three data sets. Aerobic biodegradation causes a small carbon isotopic fractionation but a very strong shift in hydrogen isotopic signatures. Conversely, anaerobic biodegradation results in strong isotopic enrichment for both carbon and hydrogen. The slopes of the regression lines of our field data and the data published for anaerobic biodegradation are similar, corroborating our hypothesis of anaerobic in-situ biodegradation. Note that an alternative explanation for a similar slope due to commitment to catalysis could be ruled out (see Supporting Information).

Conversely, the significant difference between the fractionation patterns under oxic versus anoxic conditions is a strong indicator for the existence of different reaction mechanisms. In the presence of oxygen, the most likely degradation pathway from MTBE to TBA starts with an oxidation of the methoxy group by mono-oxygenases (Scheme 1) (*12*, *14*, *56*). The so formed *tert*-butoxy methanol either reacts directly to TBA and formaldehyde or is further oxidized to *tert*-butyl formate, which then hydrolyses to form TBA. The reaction mechanism for the formation of TBA from MTBE under anoxic conditions has not been elucidated so far. One possibility is a nucleophilic substitution reaction as depicted in Scheme 1 either via an S_N2 mechanism or via an S_N1 mechanism. These three different cases are also associated

TABLE 4. Reevaluation[a] of Published Carbon and Hydrogen Isotope Effects by Conversion of Published Enrichment Factors ϵ to KIE Values for the Biodegradation of MTBE

original data from	experimental conditions	reported carbon ϵ (‰)	calcd $^{12}k/^{13}k$ after correction of reported ϵ[b]	reported hydrogen ϵ (‰)	calcd $^{1}k/^{2}k$ after correction of reported ϵ[c]
Gray et al. (*48*)	aerobic, pure strain PM1	−2.0 to −2.4	1.010 to 1.012[e]	−33 to −37	oxidation: 1.70[f,g] S_N2: 1.16[f,g] S_N1: 1.05[f,g]
Gray et al. (*48*)	aerobic, mixed microcosm	−1.5 to −1.8	1.008 to 1.009[e]	−29 to −66	oxidation: 1.69/2.54[f] S_N2: 1.16/1.25[f] S_N1: 1.05/1.09[f]
Hunkeler et al. (*28*)	aerobic, direct or cometabolic degradation	−1.52 to −1.97	1.008 to 1.009[e]	nd[d]	
Kolhatkar et al. (*50*)	anaerobic, field and microcosm data	−8.1 to −9.2	1.042 to 1.048[e]	nd[d]	
Kuder et al. (*51*)	anaerobic	−8.9 to −10.2	1.047 to 1.054[e]	−11.5	oxidation: 1.16[e] S_N2: 1.05[e] S_N1: 1.02[e]
this work	anaerobic			−15.6	

[a] The parameters *n*, *x*, and *z* used for the reevaluation are listed in Table 3. [b] For $^{12}k/^{13}k$, identical *n*, *x*, and *z* values are obtained for all three reaction mechanisms. [c] For $^{1}k/^{2}k$, different *n*, *x*, and *z* values must be applied leading to three different calculated $^{1}k/^{2}k$. [d] Not determined. [e] Determined according to the approximate eq 8. [f] Determined based on the reevaluation of original isotope data provided by the authors according to eq 7. [g] Reevaluation was done with data of both duplicate experiments;

with different kinetic isotope effects (KIE, i.e.,$^{12}k/^{13}k$ or $^{1}k/^{2}k$). To compare the isotopic fractionation observed during the biodegradation of MTBE with KIEs for the different reaction mechanisms, the published data must first be reevaluated using the corrections (eqs 7−9). Table 3 shows the correction factors applied for the different reaction mechanisms.

In case of an oxidation at the methoxy group (i.e., the formation of *tert*-butoxy methanol), a carbon−hydrogen bond is broken in the first step of the reaction; hence, one would expect a very high primary hydrogen isotope effect combined with a primary carbon isotope effect. The Streitwieser Semiclassical Limits for the breaking of a C−H-bond are 6.4 for $^{1}k/^{2}k$ and 1.02 for $^{12}k/^{13}k$ (*57*). In the case of an S_N2 reaction mechanism, the expected carbon isotope effects are relatively large (*58*). The secondary hydrogen isotope effects, in contrast, are very small and may even be inverse due to a more constrained bending motion of the hydrogen in the transition state (*58*, *59*). The results for S_N1 reaction mechanisms show the opposite trend (i.e., relatively small carbon isotopic effects and quite large secondary hydrogen isotope effects). The extent of the hydrogen fractionation depends on the nature of the leaving group (*58*).

The reevalution was applied to isotopic enrichment factors reported in the literature and results are shown in Table 4. The carbon kinetic isotope effects calculated for the aerobic oxidation of MTBE are comparable to the expected $^{12}k/^{13}k$ values shown in Table 3. The suggested reaction mechanism is also supported by the hydrogen data. Even though the $^{1}k/^{2}k$ values based on our reevaluation of experimental literature data are significantly smaller than the predicted values in Table 3, they still show a primary isotope effect in case of a methyl group oxidation. In contrast, the hydrogen KIEs from aerobic experiments in Table 4 calculated for a S_N2 reaction are too large to be in agreement with an S_N2 reaction mechanism. They do however agree with results expected for a S_N1 mechanism. While, hence, a S_N1 mechanism cannot be ruled out based on kinetic isotope effects, an oxidation mechanism seems to be most plausible in the presence of molecular oxygen. A possible explanation for the reduced hydrogen KIE observed might be an enzymatic effect known as commitment to catalysis (see Supporting Information).

The carbon isotope effects calculated from reported fractionation for the anoxic reaction (KIE = 1.04−1.05) are very similar to those expected for a S_N2 reaction mechanism (expected KIE = 1.03−1.08) but mismatch with KIE values expected for oxidation (1.01−1.02) or for S_N1 (1.00−1.02) reactions. This first indication of an S_N2 reaction based on carbon isotope effects is corroborated by our reevaluation of hydrogen isotope fractionation data. A comparison of measured and predicted hydrogen KIEs for an oxidation (1.16 vs expected 3−8) and for a S_N1 reaction (1.02 vs expected 1.1−1.2) ruled out a primary isotope effect as well as a S_N1 reaction. The hydrogen isotope effect obtained for a S_N2 mechanism (KIE = 1.05), however, matches very well with the theoretical KIE range (0.95−1.05) for this type of reaction. This reevaluation of isotopic fractionation data illustrates that the differing isotopic enrichments found for aerobic and anaerobic biodegradation indeed reflect the underlying reaction mechanisms and therefore can be used as a powerful tool for the quantification of in-situ biodegradation of MTBE. A major advantage of this approach is its applicability at biogeochemically and hydrologically complex field sites as it is independent of mass balances, as is illustrated by this case study.

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Note Added After Print Publication

Due to a production error, some units of measure were incorrect in the version published on the web December 22, 2004 (ASAP) and in the February 15, 2005 issue (Vol. 39, No. 4, pp 1018−1029); the correct version of the paper was published on August 12, 2005 and an Addition and Correction appears in the September 15, 2005 issue (Vol. 39, No. 18).

Supporting Information Available

Detailed mathematical derivation of eq 7 that is essential for the described data reevaluation and a discussion of commitment to catalysis as a rationale for similar slopes of regression lines shown in Figure 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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