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VIEW PAPER

Modern geochemical and molecular tools for monitoring in-situ biodegradation of MTBE and TBA

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Abstract Methyl *tert*-butyl ether (MTBE) is a major gasoline oxygenate worldwide and a widespread groundwater contaminant. Natural attenuation of MTBE is of practical interest as a cost effective and non-invasive approach to remediation of contaminated sites. The effectiveness of MTBE attenuation can be difficult to demonstrate without verification of the occurrence of in-situ biodegradation. The aim of this paper is to discuss the recent progress in assessing in-situ biodegradation. In particular, compound-specific isotope analysis (CSIA), molecular techniques based on nucleic acids analysis and in-situ application of stable isotope labels will be discussed. Additionally, attenuation of *tert*-butyl alcohol (TBA) is of particular interest, as this compound tends to occur alongside MTBE introduced from the gasoline or produced by (mainly anaerobic) biodegradation of MTBE.

Keywords Methyl *tert*-butyl ether (MTBE) · *Tert*-butyl alcohol (TBA) · Natural attenuation · Biodegradation · Groundwater · Stable isotope analysis · Stable isotope tracers · CSIA · Molecular tools · Bio-Sep

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1 Introduction

Methyl *tert*-butyl ether (MTBE) has been used in gasoline blends since mid-1970s, initially as an octane enhancer (Moyer 2004). In the early 1990s, reformulated gasoline containing as much as 15% of MTBE per volume was introduced in a number of metropolitan areas within the USA to comply with the Clean Air Act's requirements of gasoline oxygen content. With increased use, MTBE became one of the most commonly detected groundwater contaminants, typically associated with leaks at underground storage tanks (UST; Squillace et al. 1996). In the USA alone, there may have been as many as 250,000 individual releases of gasoline containing MTBE (Johnson et al. 2000). While there is a trend of substituting MTBE with alternative oxygenates (most notably in the USA), it remains a major component of gasolines throughout the world. In comparison with other major components of gasoline, MTBE stands out for its high solubility in water and hydrophilic behavior. These properties facilitate rapid transfer of MTBE into the aqueous phase, fast plume migration, and limit the significance of sorption or volatilization. Thus, a relatively small volume of spilled gasoline can lead to contamination of an extensive portion of an aquifer. Action levels for MTBE in groundwater are different in different states. California is among those with most demanding water quality regulations and in the case of MTBE no more than 13 μg/l is permitted in public water supplies.

Natural attenuation is defined as a sum of natural processes leading to contaminated site remediation through biodegradation or abiotic transformation to a less toxic form, or strong sorption onto the soil matrix to reduce contaminant mobility and bioavailability (EPA 1999). Natural attenuation of MTBE has been apparent at a number of sites where MTBE plumes were stabilized or receding (Reid et al. 1999; Kolhatkar et al. 2000; Wilson and Kolhatkar 2002). As discussed in the following section, work on the microbiology of MTBE degradation has been yielding an increasing amount of evidence that multiple aerobic and anaerobic microorganisms can efficiently biodegrade MTBE, providing a sound basis for in-situ bioattenuation solutions, natural or engineered (bioaugmentation or biostimulation). Apart from biodegradation, volatilization of MTBE from gasoline in the vadose zone may be locally significant, but volatilization from the dissolved MTBE plumes is not likely (Lahvis et al. 2004). Natural attenuation combined with a stringent site monitoring, i.e., monitored natural attenuation (MNA), may be appropriate where it can be demonstrated that the natural processes lead to site-specific remediation objectives (EPA 1999). MNA is a particularly attractive option for cost-effective treatment of low risk sites, or as a secondary element of remediation in combination with active techniques for high-risk sites (Cataldo 2004).

Several recent MTBE reviews are recommended. In particular, MTBE Remediation Handbook (edited by Moyer and Kostecki 2004) is a compendium of the science and technology of MTBE remediation. A technical report of Wilson et al. (2005b) is the most up-to date comprehensive source on attenuation of MTBE. Additionally, Schmidt et al. (2004a) provide an extensive review of microbial degradation of MTBE and TBA, Scow and Hicks (2005) discuss attenuation of various contaminants including MTBE, and Schmidt et al. (2004b) cover isotope techniques used for biodegradation studies. The present review will focus on MTBE biodegradation and the recent progress on the in-situ assessment of biodegradation. In addition, a separate section refers to the occurrence and fate of *tert*-butyl alcohol (TBA), a compound commonly detected at MTBE sites and genetically related to MTBE.

2 Biodegradation of MTBE and TBA

The biodegradation potential of MTBE and TBA under aerobic conditions is well documented. The most extensively studied pure culture is a β-proteobacterium strain *Methylibium petroleiphilum* or PM1, growing on MTBE without transient accumulation of the putative intermediate, TBA (Hanson et al. 1999, Nakatsu et al. 2006). Molecular tools have been developed for identification of PM1-like gene sequences in environmental samples (Hristova et al. 2001). Results of a whole-genome analysis of PM1 have been presented by Kane et al. (2007). Multiple pure or mixed cultures are capable of growing on MTBE and/or TBA or can degrade MTBE co-metabolically while growing on short-chain alkanes (Schmidt et al. 2004a; Ferreira et al. 2006 and references therein). Unlike PM1, most of the studied organisms tend to produce a transient accumulation of TBA upon MTBE degradation. Enzymes involved in aerobic degradation of MTBE and TBA are discussed by Ferreira et al. (2006). PM1 and a number of other studied organisms employ monooxygenases for the initial degradation step (oxidation of the methyl group of MTBE molecule). Aerobic reaction pathways are discussed in more detail by Steffan et al. (1997) and Smith et al. (2003). While aerobes can efficiently mineralize MTBE, their attenuation potential is limited by oxygen availability. A comparative study of benzene and MTBE plumes in Florida suggested widespread MTBE attenuation (Reid et al. 1999). This high attenuation potential may be attributed to high oxygenation of groundwater in the study area (Stocking et al. 2001). Oxygen limitation has been demonstrated empirically—it has been observed that oxygen infusion activated indigenous PM1-like microbes to rapid in-situ MTBE biodegradation (Hristova et al. 2003; Smith et al. 2005).

Anaerobic degradation of MTBE and TBA has been studied exclusively in sediment microcosms and at present no degrading organisms have been identified. MTBE was found to degrade under a wide range of electron acceptor conditions (Schmidt et al. 2004a and references therein). Positive results were obtained with nitrate, sulfate, Fe^{3+}, Mn^{4+} and in methanogenic microcosms, and in the majority of cases MTBE was not completely mineralized. Mormile et al. (1994), Somsamak et al. (2001,

2005, 2006) and Wilson et al. (2005a) reported stoichiometric accumulation of TBA as MTBE was depleted (biotransformation to TBA occurred rather than complete mineralization). TBA biodegradation was apparent in a study by Yeh and Novak (1994), and in several microcosm studies with ^{14}C-MTBE or ^{14}C-TBA substrate (Bradley et al. 2002). Remarkably efficient mineralization of TBA under Fe^{3+} reducing conditions was observed by Finneran and Lovley (2001, 2004) and Bradley et al. (2002). The latter showed 75% mineralization of the initial 0.1 μmol ^{14}C-TBA. In another study of MTBE-degrading Fe^{3+} reducing cultures, Pruden et al. (2005) reported accumulation of TBA.

Current understanding of anaerobic degradation mechanisms of MTBE and TBA is limited. The data of Somsamak et al. (2006) indicate that conversion of MTBE to TBA under both methanogenic and sulfate reducing conditions is not directly coupled to the terminal electron acceptors. Stable isotope data from methanogenic (Kuder et al. 2005) and sulfidogenic (Somsamak et al. 2005; 2006) microcosms suggest cleavage of oxygen-methyl carbon bond (S_N2-type reaction, cf. Elsner et al. 2005), speculatively attributed to acetogenic bacteria (Somsamak et al. 2006).

3 Documenting in-situ attenuation

The major challenge in the application of MNA to MTBE is the difficulty in assessing the progress of attenuation. A standard approach (EPA 1999) calls for the primary line of evidence based on the reduction of contaminant concentrations over time. Secondary evidence of geochemical footprints (e.g., depletion of electron acceptors, change of alkalinity or accumulation of degradation products) confirms in-situ degradation (NRC 2000). If the above are not conclusive, the tertiary line evidence is verification of biodegradation potential in a microcosm study. While reduction of MTBE concentration is the expected outcome of successful attenuation, there are relatively few examples of a clear-cut relationship between contaminant concentration and the progress of biodegradation. The quality of hydrological data is of prime importance here. In a classic example, a network of several hundred wells at the Borden aquifer in Ontario, Canada, has been used to monitor the behavior of MTBE plume (Schirmer and Barker 1998). Reduction of MTBE mass after 6 years was 97% and aerobic biodegradation was proposed as the most plausible attenuation scenario. In a more recent example of high-density monitoring network, Mackay et al. (2007) demonstrate biotransformation of MTBE to TBA within a methanogenic footprint created by biodegradation of ethanol experimentally released at a sulfate reduction-dominated site. The main line of evidence was provided by high-resolution plume delineation and MTBE and TBA mass discharge trends. This level of sampling coverage is difficult to achieve at the majority of sites, potentially leading to incorrect conclusions on the progress of attenuation (Wilson 2004; Wilson et al. 2004). The secondary line of MNA evidence—the geochemical footprint approach—is applicable only if there are no co-occurring degradable compounds. Microcosm experiments remain the traditional final line of evidence, however they tend to be expensive, time consuming and often inconclusive (anaerobic microcosms in particular).

In the past decade, new site assessment techniques have become available. The novel geochemical techniques are identification of biodegradation effects by determination of natural abundance stable isotope ratios of MTBE by compound-specific isotope analysis or CSIA and in-situ application of isotopically labeled surrogates (e.g., ^{13}C-MTBE). Developments in molecular microbiology (DNA fingerprinting) opened new possibilities in characterization of microbes in the environment. In contrast to the traditional lines of evidence, CSIA provides a footprint of biodegradation that is MTBE-specific. On the other hand, CSIA does not determine whether the biodegradation process is active at the time of sampling, i.e., it is not a direct equivalent of a microcosm experiment. The isotope-labeled substrate studies are a functional equivalent of microcosm studies ("in-situ microcosms"), providing the real-time snapshot of degradation. Molecular tools can offer specific information on the presence and/or activity of specific degrading organisms. Benefits and limitations of these techniques in assessing biodegradation are discussed in the following sections.

3.1 CSIA applications—using changes in the stable isotope ratios of MTBE to demonstrate in-situ biodegradation

Compound-specific isotope ratio analysis (CSIA) indicates biodegradation (or chemical degradation) if stable isotope ratios measured for a given contaminant is enriched in the heavy isotope species relative to the initial values (cf. *Isotope Geochemistry Primer*). possible to determine the pre-degradation isotope ratios by analysis of contaminant samples from the plume source zone (e.g., a study of monoaromatic hydrocarbons by Richnow et al. 2003). If representative material is not available, either due to uncertain site history (e.g., multiple spills may have had different initial isotope ratios over time) or due to the potential of degradation of the contaminant in the source zone, the range of feasible pre-degradation

Isotope geochemistry primer

In environmental sciences, isotope ratios are reported in δ units, representing deviation of the measured isotope ratio vs. interntnl. standard, where, for C isotopes, R is $^{13}C/^{12}C$, and the standard is VPDB, or Vienna Pee Dee Belemnite)

$\delta^{13}C = (R_{sample}/R_{standard} - 1) * 1000$ (note that a unit is ‰ or permil)

Changes of isotope ratios due to chemical or physical processes are known as isotope fractionation or isotope effects. A "heavy" isotope (e.g., ^{13}C) present in the cleaved bond increases the bond activation energy and, in effect, reduces the rate of degradation. In a closed system, the residual substrate becomes enriched in ^{13}C with degradation progress ($^{13}C/^{12}C$ increases). Isotope effects in unidirectional reactions follow the Rayleigh fractionation model. E.g., where ^{13}k is the rate of degradation of ^{13}C-substituted bond, and ^{12}k is the rate of degradation of ^{12}C-substituted bond, the ratio of $^{12}k/^{13}k$ is a fractionation factor (α). The same can be said if instantaneous ratios $R = ^{13}C/^{12}C$ are substituted for the reaction rates:

$\alpha = {}^{12}k/{}^{13}k = R_{product}/R_{substrate}$ Equation 1

Substitution of the instantaneous R by a readily measurable R and concentr. of the substrate yields Eq. 2. R_t is $^{13}C/^{12}C$ at time t;. $R_{t=0}$ is the initial $^{13}C/^{12}C$; f is the ratio of substrate remaining at time = t ($concentr._t/concentr._{t=0}$); ε is the isotopic enrichment factor.

$1000 * \ln(R_t/R_{t=0}) = \varepsilon * \ln f$ Equation 2

A transposition of Eq. 2 (δ notation used) yields Eq. 3, where $\delta^{13}C_t$ is $\delta^{13}C$ at time t; $\delta^{13}C_{t=0}$ is the initial $\delta^{13}C$.

$f = \exp[1000 * \ln\{(10^{-3}\,\delta^{13}C_t + 1)/(10^{-3}\,\delta^{13}C_{t=0} + 1)\}/\varepsilon]$ Equation 3

Non-degradative processes, in particular volatilization from organic or aqueous phase and vapor migration can result with measurable changes of isotope ratios, however based on published experimental data (Wang and Huang 2003, Kopinke et al. 2005b and references therein) the magnitudes of such changes of carbon isotope ratios is small and will not interfere with the signatures of biodegradation. More significant fractionation of hydrogen isotopes is feasible (Wang and Huang 2003). Hydrogen isotope CSIA should be always considered a secondary line of evidence and evaluated in the context of carbon isotope data and of the position of the specific sample within the contaminant plume. In few case studies, it has been isotope ratios can be obtained by surveying the compound of interests in various commercial products. Smallwood et al. (2001) and O'Sullivan et al. (2004) measured $\delta^{13}C$ of commercial MTBE from the USA (10 samples) and worldwide (25 samples from Europe, Asia, South America and Africa), respectively. Carbon isotope ratios of MTBE in the gasolines were falling between –31.7 and –28.3 and –32 to –27.4, respectively. No systematic differences have been observed between samples from different geographic areas. For any MTBE spill with an unknown original $\delta^{13}C$, values more positive than –27 (± instrumental precision) would suggest in-situ degradation (cf. Kuder et al. 2005).

Owing to strong enrichment of ^{13}C and deuterium (D) observed in anaerobic microcosms, CSIA is a particularly attractive option to monitor anaerobic biodegradation of MTBE. While isotope enrichments due to aerobic biodegradation have been observed in microcosms (Hunkeler et al. 2001, Gray et al. 2002), to date, no case studies documenting aerobic biodegradation in the field have been published (Lesser et al. 2005, shows results that are essentially inconclusive).

Isotope effects measured in aerobic microcosms (Table 1 and references therein) yield a consistent range of carbon ε (enrichment factors; cf. *Isotope Geochemistry Primer*) with values clustering around –2. In one study, a very low isotope effect has been observed for aerobic biodegradation of MTBE by two aerobic strains (Rosell et al. 2007), indicating that CSIA may be not sensitive enough for certain types of aerobic bacteria. Hydrogen ε presented by Gray et al. (2002) were –29 to –66 (PM1 and sediment microcosms). The values shown by Rosell et al. were lower, proportional to the reported low carbon enrichment factors. For anaerobic MTBE degradation, carbon isotope effects were significantly higher, ranging from –8.2 ± 3.1 (±95% confidence level) to –15.6 ± 4.1 in several published studies on methanogenic and sulfate reducing sediment microcosms (Table 1 and references therein). Hydrogen ε (–16 ± 5) was determined on one methanogenic sample set (Kuder et al. 2005). Accurate determination of ε in a sediment microcosm can only be achieved when there is no restriction of MTBE bioavailability. Net isotope effect measured under reduced MTBE bioavailability will be proportionally lower than that expected under ideal conditions (for a discussion of a related artifact see Kopinke et al. 2005a). In static sediment microcosms, the rate of diffusive or advective redistribution of MTBE may not assure uniform distribution of the substrate, thus reducing its bioavailability and resulting with underestimation of ε. Two methanogenic and three sulfate reducing microcosm sets constructed from soil collected at different gasoline spill sites were incubated under mechanical agitation to assure uniform distribution of microcosm content (Kuder et al. unpublished). The obtained carbon ε values ranged from –16.5 ± 0.6 to –19.3 ± 0.6 (average ε was –18). The average value of hydrogen ε was –25. These results imply even

Table 1 Isotope enrichment factors in MTBE and TBA biodegradation

References	Substrate	Culture & redox	Enrichment factor
Kolhatkar et al. (2002)	MTBE	Methanogenic	ε_C –8.2 ± 3.1
Kuder et al. (2005)	MTBE	Methanogenic	ε_C –13.0 ± 1.1 ε_H –16 ± 5
Somsamak et al. (2005)	MTBE	Methanogenic	ε_C –15.6 ± 4.1
		Methanogenic with BES inhibitor	ε_C –14.6 ± 5.2
Somsamak et al. (2006)	MTBE	Methanogenic CC sediment	ε_C –14.4 ± 1.5 and –14.0 ± 1.5
		Sulfate reducing CC sediment	ε_C –13.7 ± 1.5 and –14.4 ± 3.6
		Sulfate reducing AK sediment	ε_C –13.9 ± 5.6 and –14.5 ± 2.5
Kuder et al. (unpublished)	MTBE	Methanogenic, methanogenic with BES inhibitor, sulfate reducing	ε_C –16.5 ± 0.6 to –19.3 ± 0.6 $\varepsilon_C/\varepsilon_H$ 1.4 (average ε_H –25)
Hunkeler et al. (2001)	MTBE	Aerobic; soil microcosms	ε_C –1.6 ± 0.1 to –2.0 ± 0.1
		Aerobic; cometabolic soil microcosms	ε_C –1.5 ± 0.1
Hunkeler et al. (2001)	TBA	Aerobic; cometabolic soil microcosms	ε_C –4.2 ± 0.1
Gray et al. (2002)	MTBE	Aerobic; PM1	ε_C –2.0 ± 0.1 to –2.4 ± 0.3 ε_H –33 ± 5 to –36 ± 6
		Aerobic; soil microcosm	ε_C –1.4 ± 0.1 to –1.8 ± 0.1 ε_H –29 ± 4 to –66 ± 3
Lesser et al. (2005)	MTBE	Aerobic; soil microcosms	ε_C –1.4 (average from multiple sets)
Rosell et al. (2007)	MTBE	Aerobic; *Methylibium* sp. R8	ε_C –2.1 ± 0.1 ε_H –42 ± 4
		Aerobic; β-proteobacterium L108	ε_C –0.5 ± 0.1 ε_H –0.2 ± 8
		Aerobic; *Rhodococcus ruber* IFP 2001	ε_C –0.3 ± 0.1 ε_H +5 ± 17

stronger isotope effect in anaerobic MTBE degradation than that apparent from the previous studies and higher degree of similarity of isotope effects produced by different consortia under sulfate reducing and methanogenic conditions.

Combined carbon and hydrogen CSIA (2-D CSIA; Kuder et al. 2005; Zwank et al. 2005) can readily discriminate between the aerobic and anaerobic processes (note that the information provided by an XY plot of $\delta^{13}C$ and δD such as the one in Fig. 1 is essentially identical to that of the $\varepsilon_H/\varepsilon_C$ ratio). In summary, aerobic biodegradation of MTBE results with low carbon isotope fractionation and proportionally higher hydrogen isotope fractionation, as expected for oxidation of the MTBE methyl group. Anaerobic biodegradation results in strong carbon isotope fractionation and proportionally lower hydrogen isotope fractionation indicative of demethylation of the methoxy group of MTBE. The different patterns of isotope fractionation correspond to the different biochemical reactions (cf. Elsner et al. 2005).

Within the framework of the Rayleigh fractionation model (Eq. 3, *Isotope Geochemistry Primer*), it is possible to estimate the mass of biodegraded contaminant (applications to various VOC contaminants have been shown by Sherwood Lollar et al. 2001; Richnow et al. 2003; Kuder et al. 2005) and field attenuation (biodegradation) rates (Wilson et al. 2005b). Stringent application of the Rayleigh model is practical if ε is accurately determined and the site

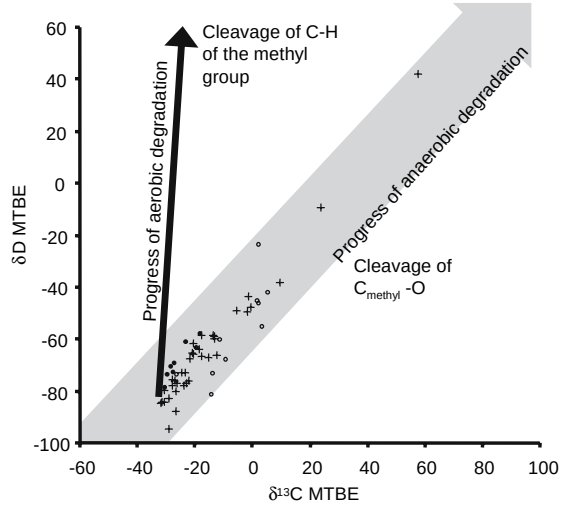

Fig. 1 Bimodal distribution of 2-D CSIA data for aerobic and anaerobic degradation of MTBE

hydrology and history are well known. Richnow et al. (2003) have shown that the concentration profile of toluene along the plume flow line was closely matched by the attenuation predicted from $\delta^{13}C$ of toluene. Application of the Rayleigh model to evaluation of field CSIA data has been formally discussed by Abe and Hunkeler (2006) and Van Breukelen (2007a), with the conclusion that underestimation of the calculated degradation rate and of the extent of biodegradation is to be expected due to uncertainties of the model parameters and the influence of advection/dilution. Van Breukelen (2007b) proposed a scheme for application of the Rayleigh model to a situation where competing degradation mechanisms contribute to the net isotope fractionation. For most contaminated sites the monitoring network is not adequate to meet these criteria, necessitating a conservative approach for CSIA data evaluation (Kuder et al. 2005). To avoid overestimating the extent of biodegradation, two parameters of Eq. 3 have to be carefully selected: (1) ε—the maximum effect representative of anaerobic MTBE biodegradation; and, (2) the pre-degradation $\delta^{13}C$—most positive feasible value of $\delta^{13}C$. Considering the results from the microcosm studies, –20 appears to be a reasonable projection of ε value for anaerobic biodegradation (extreme range of the regression at 95% confidence). The suggested upper range of commercial MTBE's $\delta^{13}C$ is –26.5 (accounting for CSIA precision of ±0.5 unit of $\delta^{13}C$). Solution of Eq. 3 (*Isotope Geochemistry Primer*) for these parameters yields an estimate of the minimum fraction of degraded MTBE.

Wilson et al. (2005b) used carbon CSIA data from an anaerobic plume to determine in-situ MTBE biodegradation rate. The attenuation rate, expressed as first order rate of attenuation with distance or with time, is derived from the extent of biodegradation (calculated from isotope data as discussed in the previous paragraph) and the distance from the contaminant source along the plume flow path or seepage velocity of ground water.

Applications of CSIA to the assessment of MTBE biodegradation in-situ have been published by Kolhatkar et al. (2002), Kuder et al. (2005) and Zwank et al. (2005). Kolhatkar et al. (2002) demonstrated enrichment of ^{13}C in a methanogenic MTBE plume at a gasoline station in New Jersey, USA. Kuder et al. (2005) showed $\delta^{13}C$ and δD data for nine MTBE sites

(methanogenic and sulfate reducing). The values of $\delta^{13}C$ from all of the sites were indicative of biodegradation, and the 2-D CSIA data confirmed an anaerobic biodegradation pathway for all of the studied sites. Rayleigh model-based assessment of the progress of in-situ biodegradation confirmed significant removal of MTBE, exceeding 90% of the original mass for some of the samples from the abovementioned sites. The same data augmented by additional sites have been discussed by Wilson et al. (2005c), in the context of MTBE-TBA transformation (cf. section 4). Zwank et al. (2005) presented $\delta^{13}C$ and δD values from an industrial site in Brasilia, with similar conclusions regarding anaerobic degradation of MTBE.

Apart from limitations of poor detection limits (while purge and trap-GC-IRMS permits analysis of $\delta^{13}C$ in MTBE at low concentrations of a few µg/l, some of the published studies relied on headspace sampling or liquid-liquid extraction, Table 2) or chromatographic separation, CSIA may yield inconclusive results through inadequate sampling missing the active parts of a plume or "dilution" of biodegradation signal in a heterogeneous plume. The problem is illustrated by the results from an isotope study of Port Hueneme site (Lesser et al. 2005). While MTBE biodegradation at an aerobic biobarrier was evident in high-resolution concentration data, the increase of $\delta^{13}C$ was either not observed or lower than the anticipated value. It was proposed that spatial variations in MTBE biodegradation activity combined with preferential groundwater flow pathways permitted a small fraction of MTBE to break through the barrier escaping degradation. Spatial heterogeneity of subsurface microbiology at Port Hueneme, expressed in variable efficiency of MTBE degradation in microcosms constructed from sediment collected at different points of the aquifer or in fluctuations of cell density of PM-1-like bacteria, was shown at the same site by Lesser and Johnson (2005) and Smith et al. (2005), respectively. Consequently, a sample collected from the hydraulic radius of a monitoring well could be dominated by undegraded MTBE (cf. Kopinke et al. 2005a). Fresh MTBE partitioning from NAPL at a source zone may have a similar effect, overwhelming the signal from degraded MTBE present in adjacent groundwater (monitoring wells with gasoline sheen are not likely to yield degradation evidence). While positive evidence of biodegradation (isotope ratio enrichment) provided by CSIA is robust, the limitations discussed above imply that negative results (no isotope ratio enrichment) of CSIA should be considered as inconclusive and that the calculated amount of biodegraded MTBE will be often underestimated. The differences in isotope fractionation patters between aerobic and anaerobic organisms suggest that different approach is needed for sites where the expected attenuation mechanism is aerobic biodegradation. While carbon CSIA should be sufficient to detect strong isotope ratio enrichments at anaerobic MTBE sites, combined 2-D CSIA is recommended for aerobic sites. Moreover, quantitation of aerobic MTBE biodegradation based on aerobic enrichment factors is tricky as dramatic overestimation of the extent of biodegradation is possible if MTBE has been previously partially degraded in an anaerobic process. Conservative approach, i.e., using the largest reported isotope effect (the most negative enrichment factor for the anaerobic process ε_C –20, as opposed to the aerobic ε_C –2.4) at an aerobic site would underestimate the real extent of biodegradation by one order of magnitude. To validate the use of aerobic ε_C for biodegradation calculation, the MTBE plume hydrogeology would have to be studied well enough to permit delineation of the study area so that the baseline (pre-degradation) and degraded samples are clearly past the influence of an anaerobic processes. Assessment of aerobic biodegradation based on ε_H rather than on ε_C may be possible in the future when more cultures are studied for their hydrogen isotope effects.

3.2 Molecular tools—detection of nucleic acid signatures specific of MTBE-degrading organisms

The potential of molecular tools for microbial population characterization or identification of genes specific for certain organism can be hardly overestimated. Water (planktonic cells suspended in water), natural biofilms (soil samples) or in-situ microcosms can be analyzed. The field techniques described in section Sect. 3.3 can be combined with various variants of molecular analysis. A significant body of work has been focused on aerobic MTBE degradation. In particular, development of a 16S ribosomal RNA quantitative PCR for PM1 bacteria permitted

Table 2 Analytical quantitation limits of various CSIA techniques used for aqueous MTBE and TBA samples

References	Sample introduction technique	CSIA quantitation limits (QL) (μg/l)
Zwank et al. (2003)	Purge and trap[a]	MTBE δ^{13}C 0.6
		MTBE δD 6–12[f]
Kuder et al. (2005)	Purge and trap[b]	MTBE δ^{13}C 2.5
		TBA δ^{13}C 25
		MTBE δD 20
Hunkeler et al., (2001)[c]	SPME (carboxen/PDMS)[c, d]	MTBE δ^{13}C 11
		TBA δ^{13}C 370
Gray et al. (2002)	SPME (carboxen/PDMS)[c]	MTBE δ^{13}C 350
		MTBE δD 1000
Zwank et al. (2003)[g]	SPME (carboxen/PDMS)[c]	MTBE δ^{13}C 16
		MTBE δD 160–320[f]
Gray et al. (2002)	Direct headspace	MTBE δ^{13}C 5000
		MTBE δD 20000
Rosell et al. (2007)	Direct headspace[e]	MTBE δ^{13}C 3000
		MTBE δD 8000

[a] GC program optimized for quantitation limits—QL applicable for samples with low coelution potential

[b] GC program optimized for MTBE separation—applicable for samples with high coelution potential

[c] Carboxen/PDMS SPME is sensitive to matrix effects resulting with a reduction of QL, in particular at high concentrations of gasoline-range aromatic hydrocarbons (Black and Fine, 2001)

[d] Similar method used by Lesser et al. (2005a)

[e] Similar method used by Somsamak et al. (2005, 2006)

[f] Hydrogen CSIA QL is a theoretical extrapolation of carbon CSIA QL

[g] Similar method used by Zwank et al. (2005). Zwank et al. also report TBA data but no details on quantitation limits are given

detection of PM1-like genes at field sites (Hristova et al. 2003; Smith et al. 2005). Kane et al. (2001) identified PM1-like bacteria in several MTBE-degrading microcosms. Biggerstaff et al. (2007) demonstrated an application of fluorescence in situ hybridization (FISH) probes for quantitative detection of bacteria (eubacteria and δ-proteobacteria) from MTBE and benzene-contaminated and apparently anaerobic sites (no specific identification of degrading organisms has been proposed). More molecular applications for identification anaerobic MTBE-degrading microorganisms would be particularly welcome at this point.

Molecular techniques are invaluable as a tool to detect known degrading organisms. On the other hand, the molecular approach may be inconclusive for monitoring in-situ attenuation, as the presence of degradation potential is not enough to prove that the attenuation is occurring. Combination of molecular techniques with field application of isotope-labeled degradation substrates (see the following section) can be particularly powerful for characterization of in-situ biodegradation, e.g., as shown in a study linking respiration of ^{13}C-labeled substrates (glucose, phenol, caffeine and naphthalene) with incorporation of ^{13}C into biomass of several classes of bacteria identified by 16s rRNA sequencing (Padmanabhan et al. 2003). While incorporation of isotope label into biomass is a direct evidence of actively occurring biodegradation, the species intercepting the label via degradation products are not necessarily the same as the degraders. Unless the probe targets a confirmed degrading organism, specific attribution of degradation based on the distribution of isotope label throughout the microbial population may be uncertain.

3.3 Detection of biodegradation by in-situ applications of isotope tracers

Injection of stable isotope-labeled contaminant (e.g., ^{13}C-MTBE) and identification of isotope-labeled

metabolites or incorporation of the isotope label into the microbial biomass is a direct and real-time indication of active degradation. Alternatively, stable isotope-labeled tracers can be used to distinguish the tracer plume from the existing plume of the same chemical species in mass balance studies (cf. Amerson and Johnson 2003). In such studies, it is assumed that the properties of labeled and unlabeled chemicals are identical. It is worth to stress the difference between stable isotope-labeled and radiolabeled tracers. While the latter are commonly used in laboratory setting, field applications are few (e.g., Bianchin et al. 2006), primarily due to strict regulations controlling environmental release of radioactive materials. Stable isotope-labeled chemicals can be applied in field experiments with the same restrictions as those for unlabeled chemicals.

Labeled compounds can be applied as aqueous tracers, with monitoring for degradation products along the flow line of the contaminant plume (Fischer et al. 2006, a study of BTEX biodegradation) or in the push-pull technique, where the injection is followed by recovering the remaining substrate and the degradation products from the hydraulic radius of the sampling well (Kleikemper et al. 2005 and references therein). Tracer techniques are feasible when biodegradation occurs without a lag phase over the duration of the experiment, so that the degradation effect are present and can be measured. Deuterated MTBE flow line tracer was used in an MTBE plume mass-balance study of Amerson and Johnson (2003), however, in this case only advective contaminant transport and no biodegradation were observed. There is a significant body of work on in-situ application of isotope tracers to study biodegradation, but no examples of MTBE or TBA applications. A recent review by Kreuzer-Martin (2007) gives an up to date list of references on the progress in the field.

A novel approach is the use of solid matrix (e.g., Bio-Sep beads) "baited" with the labeled compound and/or with appropriate electron acceptors or nutrients, to collect biofilm sample for molecular or geochemical characterization (Geyer et al. 2005; Stelzer et al. 2006; Sublette et al. 2006; Biggerstaff et al. 2007). The idea is to stimulate colonization of the matrix with bacteria degrading the target contaminant. While the structure of Bio-Sep bead biofilm is probably not identical to that in the ambient soil, sampling of microbial biomass through Bio-Sep has the advantage of higher cell density as compared with recovery of bacteria from groundwater and is more representative of sediment surface-attached community. Incorporation of the isotope label into the microbial biomass can be measured on the extractable fatty acid or nucleic acid fractions (Chang et al. 2005) and the active members of the microbial population may be thus identified. An application of Bio-Sep beads to collect biofilm samples for detection of aerobic PM1-type MTBE degrader has been shown by Tornatore et al. (2004). Busch-Harris et al. (2006) showed results from Bio-Sep beads "baited" with ^{13}C-labeled MTBE and ^{13}C-labeled TBA, respectively, from several locations. Beads incubated at contaminated sites at various redox conditions yielded microbial fatty acids profiles enriched in ^{13}C, indicative of incorporation of the labeled substrate into microbial biomass. The ^{13}C-enrichment suggests that the local bacteria are capable of biodegradation of MTBE and TBA under local redox conditions.

4 Sources and fate of *tert*-butyl alcohol

Tert-butyl alcohol is completely soluble in water, strongly partitions into water both from residual gasoline and from vapor and it is weakly retarded by sorption (Schmidt et al. 2004a). TBA has been sporadically used as a gasoline oxygenate and is found as impurity in commercial MTBE (at concentration of 2% or higher, Wilson et al. 2005c), therefore it can be expected to be present alongside MTBE in contaminated water (Wilson 2004). TBA is also an intermediate of aerobic and anaerobic MTBE biodegradation, with a potential environmental yield far exceeding the original gasoline content. TBA occurs at contaminated sites at similar frequency and concentration as MTBE does (Kolhatkar et al. 2000; Shih et al. 2004; Wilson et al. 2005c). Widespread occurrence and unclear human health hazard status (McGregor 2006) make TBA a potential emergent contaminant.

As discussed in the context of MTBE biodegradation, TBA is often inert under anaerobic conditions, so that biodegradation of MTBE leads to accumulation of TBA, significantly exceeding the amounts originally present in gasoline. A study by Wilson et al. (2005c) illustrated this scenario. Twelve

gasoline retail sites were selected for their high TBA concentrations in groundwater exceeding the values predicted by gasoline-water partitioning. Anaerobic biodegradation of MTBE was confirmed by CSIA at all locations. The samples with the strongest apparent excess of TBA were also those for which MTBE biodegradation was indicated by CSIA, while those with relatively low TBA concentrations tended to yield little evidence of biodegradation. The actual concentrations of TBA were higher than those reconstructed from CSIA results. Zwank et al. (2005) observed similar excess of TBA in their study. As discussed in the Sect. 3.1, CSIA tends to underestimate the extent of biodegradation. Another speculative possibility is preferential volatilization of MTBE leading to the apparent excess of TBA (Kuder et al. 2005; Zwank et al. 2005).

TBA appears to be readily degradable in the presence of oxygen and almost all studied aerobic MTBE degraders can mineralize TBA. On the other hand, the prevalence of in-situ biodegradation of TBA in anaerobic plumes remains uncertain. Some of the microcosm studies discussed in Sect. 2 confirmed anaerobic TBA biodegradation except under methanogenic conditions (e.g., Bradley et al. 2002; Finneran and Lovley 2001, 2004). There is relatively little evidence of natural attenuation of TBA. Three out of 74 gasoline plumes studied by Kolhatkar et al. (2000) permitted extraction of TBA attenuation rate constants. Day and Gulliver (2004) measured $\delta^{13}C$ of TBA from a plume at a chemical plant site in Texas. The trend of concentration reduction was correlated with a trend of $\delta^{13}C$ enrichment as expected for in-situ biodegradation. The plume was generally anaerobic, but microaerophilic status was not excluded, (within the plume, dissolved oxygen was 0.8 mg/l versus background value of 2 mg/l). At this point, isotope fractionation of TBA has been studied in one aerobic microcosm yielding ε of –4.2 (Hunkeler et al. 2001). The published CSIA data for TBA at several anaerobic gas station sites show very little variability in $\delta^{13}C$ values (Kolhatkar et al. 2002; Kuder et al. 2005, Zwank et al. 2005), indicating either negligible extent of in-situ TBA degradation or a degradation process resulting with negligible isotope effect. CSIA will not yield conclusive data on TBA attenuation unless TBA-degrading anaerobic cultures are available for laboratory study. Recently, Busch-Harris et al. (2006) reported strong ^{13}C enrichment of microbial fatty acids recovered from Bio-Sep probes loaded with ^{13}C-TBA, incubated at gasoline-contaminated sites, indicating potential for anaerobic biodegradation of TBA.

5 Conclusions

The potential for biodegradation of MTBE in aerobic or anaerobic conditions has been amply demonstrated in laboratory studies. Aerobic bacteria, in particular, are capable of complete mineralization of the contaminant. Natural attenuation by aerobic bacteria has been observed, but limited to the locations where oxygen supply is sufficient. Aerobic biodegradation is most significant for engineered remediation solutions, as limited oxygen supply in untreated plumes typically prevents effective attenuation of the high-concentration cores of MTBE plumes. Anaerobic MTBE biodegradation has been demonstrated for a number of contaminated sites, however, most laboratory studies point to significant or even stoichiometric accumulation of recalcitrant TBA intermediate, leading to net accumulations exceeding the original contributions from gasoline by one-two orders of magnitude. TBA biodegradation has been reported from microcosms under anaerobic conditions. On the other hand, field evidence for TBA attenuation is limited and equivocal. TBA remains a weakly regulated contaminant, however, considering its behavior, it may soon become a considerable environmental concern.

Novel monitoring tools, such as CSIA and Bio-Sep combined with molecular and/or stable isotope label techniques permit to identify biodegradation of MTBE at a contaminated site, providing robust support for MNA. Development of molecular tools for anaerobic MTBE and TBA degraders will be particularly welcome. The single most apparent challenge for future research is to understand the fate of TBA in anaerobic environments.

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