UNIVERSITAT DE BARCELONA

Universitat de Barcelona
Facultat de Química

Departament de Química Analítica

Institut d'Investigacions Químiques i Ambientals de Barcelona IIQAB-CSIC

Departament de Química Ambiental

Vigilància ambiental de metil *tert*-butil èter (MTBE), un additiu de la gasolina, en aigües i sòls

Mònica Rosell i Linares

Juliol 2006

4 ANÀLISI D'ISÒTOPS ESTABLES (CSIA) PER A L'AVALUACIÓ DE LA BIODEGRADACIÓ *IN SITU* DE MTBE EN AIGÜES SUBTERRÀNIES

4.1 INTRODUCCIÓ

4.1.1 Els processos d'atenuació natural (NA)

La intensa activitat industrial dels últims segles o dècades ha comportat l'aparició de nombrosos i extensos llocs contaminats arreu del món. En general, el compartiment ambiental més malmès han estat les aigües subterrànies. Degut a la seva importància com a reserva d'aigües pel consum humà, s'han fet nombrosos esforços per tal de pal·liar-ne la contaminació. No obstant, tot i les grans inversions monetàries i els avenços tecnològics en aquest camp, en la majoria dels casos és tècnica o econòmicament poc factible recuperar les aigües subterrànies contaminades a curt termini en condicions acceptables [157]. Així és com va sorgir la idea de l'atenuació natural (NA), sola, controlada o bé potenciada amb mesures de remeiació activa.

Bàsicament, els processos naturals que tenen lloc en el medi ambient (normalment, en el subsòl) com són la biodegradació microbiana, la dispersió i la difusió dels contaminants al llarg dels corrents d'aigua subterrània, poden, sense cap intervenció humana, disminuir la concentració, la massa, el volum, la toxicitat i/o la mobilitat del pol·luent a nivells que no siguin perjudicials per a la salut humana, pel medi ambient o que no excedeixen els límits acceptables establerts per les organitzacions reguladores.

En els últims anys, aquesta alternativa de remeiació, ha estat molt discutida. En primer lloc, perquè per a la seva aplicació real com opció racional de recuperació de llocs contaminats, calen mètodes fiables per a l'avaluació i valoració de l'eficiència dels processos naturals que hi tenen lloc. I en segon lloc, perquè cal un consens entre les organitzacions reguladores, les empreses i l'acceptació pública. L'aplicació de l'atenuació natural pot significar l'acord per totes les parts de que grans volums d'aigua subterrània estaran contaminades per llargs períodes de temps (possiblement dècades). Per això, un prerequisit per a la seva utilització, és la seguretat de que cap receptor o consumidor es veurà afectat [158]. Quan aquestes condicions es donen, la NA pot ser la

millor alternativa en termes de cost-benefici envers altres mesures tècniques molt més cares.

En els últims anys s'han identificat un significant nombre de llocs amb grans problemes de contaminació a l'antiga República Democràtica Alemanya (o Alemanya de l'est), fent necessari l'avaluació dels processos d'atenuació natural per tal de pal·liar les despeses econòmiques per a la seva remeiació [158]. Alguns d'aquests llocs, molts d'ells antigues refineries, pateixen grans contaminacions de les aigües subterrànies per fuites accidentals de gasolina, i on el MTBE és objecte d'estudi [159].

La clau per a la implementació de l'atenuació natural és trobar la relació causa-efecte que demostri que els processos desitjats estant tenint lloc o que probablement el tindran [160]. Els indicadors convencionals per a demostrar la biodegradació dels compostos al camp inclouen l'estudi de la distribució dels contaminants, la presència dels seus productes de degradació, les correlacions temporals i espaials amb els paràmetres geoquímics indicatius dels processos microbians com per exemple, la disminució dels acceptors d'electrons.

En el cas del MTBE, aquests indicadors tradicionals no són suficients per provar que s'estiguin produint els processos de biodegradació esperats. A continuació s'enumeren les raons.

- ☹ Altres processos naturals com poden ser la dilució, l'adsorció o la volatilització poden fer disminuir la concentració del MTBE a les aigües. La realització de balanços de massa fiables és impossible si no es té un coneixement profund dels règims i fluxos d'aigua subterrània, hi ha un nombre de pous limitat o el nombre de mesures al llarg del temps són insuficients.
- ☹ La mera presència dels seu principal compost de degradació, el TBA no és consistent (com es va comprovar a l'Art. 4 de la present tesi), doncs pot ser emprat com additiu oxigenat a la gasolina o aparèixer com a impuresa en la fabricació de MTBE [118,161]; pot provenir d'altres fonts de contaminació ja que és emprat com a dissolvent en nombrosos processos industrials [162] i com

a tal també està subjecte a processos de biodegradació que poden fer disminuir la seva concentració.

☹ La disminució d'oxigen o altres acceptors d'electrons a l'aqüífer, així com la producció de metà pot ser associat a altres components presents a la gasolina, en general, i no de manera específica amb el MTBE. A més, els additius aromàtics són més fàcilment biodegradables que no pas el MTBE [48].

Així doncs, tot i la quantitat d'estudis relacionats amb la presència, persistència o desaparició de MTBE en estudis de camp, el cert és que rarament donen idea de les condicions necessàries per a la degradació del compost o les vies metabòliques que hi tenen lloc.

4.1.2 Biodegradació de MTBE i ETBE

Tot i les dificultats inicials per aïllar microorganismes capaços de degradar el MTBE, i que van fer descriure el compost com a recalcitrant [33], el cert és que a l'última dècada experiments *ex situ* en condicions controlades de laboratori (microcosmos) han demostrat la biodegradació del compost tant en condicions aeròbiques com anaeròbies, com recullen alguns articles de revisió [44-47].

Tot i això els mecanismes metabòlics són encara incerts, fins i tot, en presència d'oxigen on s'han pogut aïllar i reproduir en cultius biològics alguns microorganismes capaços d'alimentar-se del compost, tant com a font de carboni com d'energia. És probable, que l'oxidació del MTBE produeixi un intermediari hemiacetal *tert*-butoxi metanol [45]. Aquest compost és molt inestable, fent impossible la seva detecció, i podria passar directament a TBA (desprenent un acetaldehid) o bé via la formació de TBF i àcid fòrmic (excloent la formació de formaldehid) [45,51,52], l'esquema d'aquestes reaccions es mostra a la *Figura 23*.

Els estudis consultats a la literatura mostren 3 tipus de biodegradació aeròbica del MTBE. La primera, és aquella en que els microorganismes utilitzen el compost com a única font de carboni i energia. S'han descrit alguns microorganismes Gram negatius

com (i) *Methylibium petroleiphilum* (anteriorment anomenat *Rubrivivax gelatinosus*) comunament conegut com PM1, que va ser aïllat d'un biofiltre de l'EDAR de la ciutat de Los Angeles (CA, EEUU) [163-166] (ii) la bactèria oxidadora d'hidrogen *Hydrogenophaga flava* (ENV735) d'una altra EDAR a New Jersey [167]. Ambdós organismes degradaven MTBE a TBA i posteriorment a HIBA. Aquestes reaccions involucraven almenys dos enzims diferents (monooxigenases induïdes), alliberant una modesta quantitat d'energia útil pel creixement cel·lular [45].

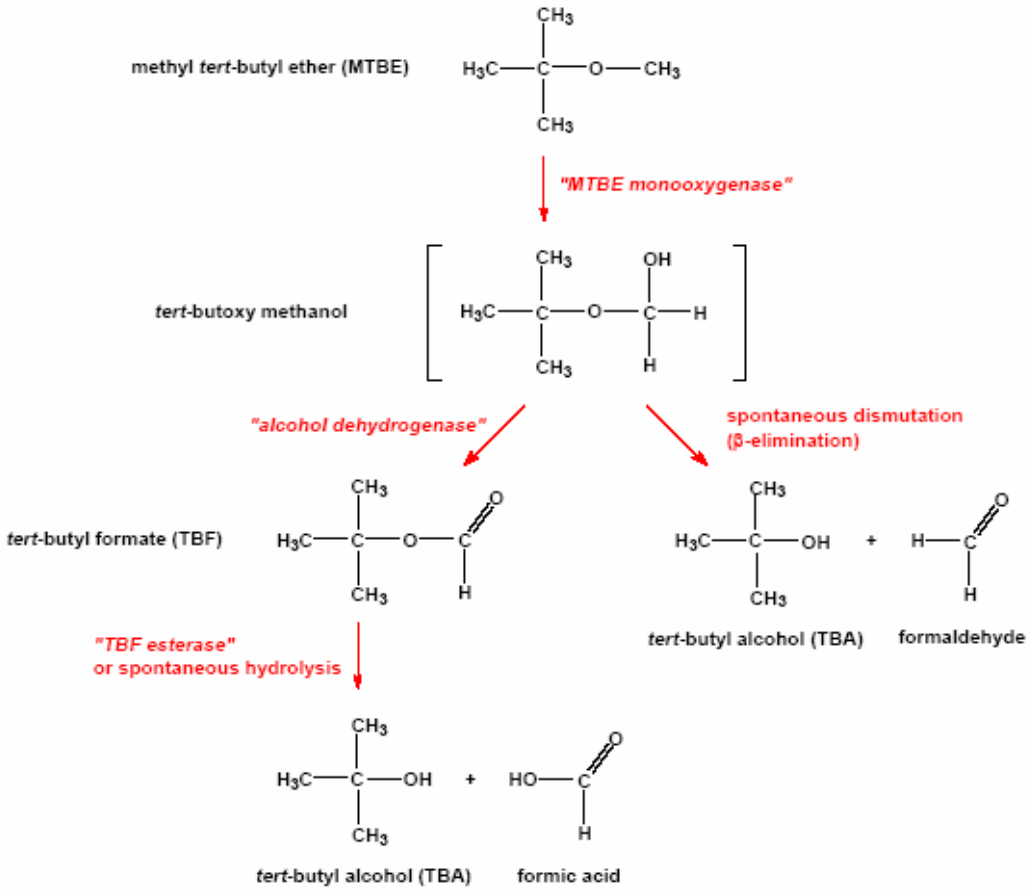

Figura 23.-Principals vies metabòliques proposades per a l'oxidació biològica del MTBE

Un segon mecanisme, era el catalitzat per organismes Gram positius com *Mycobacterium vaccae* (JOB5), que es va aïllar d'una zona contaminada per gasolina utilitzant isopentà [168,169] o metilotròfics com *Mycobacterium austroafricanum* (IFP 2012) procedent del fang de depuradora d'una EDAR urbana a prop de París i que es va

aïllar emprant TBA [170]. Ambdós organismes oxidaven MTBE a TBF, el qual s'hidrolitzava a TBA [171]. Però, en aquest cas, es va observar que la monooxigenasa involucrada podria oxidar tots dos compostos (MTBE i TBA), i el mecanisme metabòlic presentava una complicada regulació (a base d'inducció, inhibició, competició) entre el MTBE i els seus productes de degradació.

En tercer lloc, es trobaria la degradació co-metabòlica, en la qual s'han trobat nombrosos i variats microorganismes capaços d'oxidar propà [52] i n-alcans [172]. Bactèries com la *Pseudomonas putida* (GPo1) [173] o la *P. mendocina* (KR1) [168] han estat descrites a la literatura, però la degradació del compost es veu limitada per l'acumulació dels seus productes de degradació.

Pel que fa a altres additius oxigenats, existeixen alguns estudis [42,52], la majoria realitzats per grups d'investigacions francesos amb microorganismes de l'espècie *Rhodococcus ruber sp.* (IFP2001 and IFP2007) capaços de degradar ETBE [174-176].

En tots aquests estudis de laboratori, s'ha fet palesa la importància de les condicions ambientals específiques del lloc d'on es van extreure els microorganismes i també han posat de manifest que els estudis amb microcosmos són cars, llargs i molt sovint difícils d'aplicar a les condicions de camp. És per això, que es fan necessaris altres alternatives per a l'avaluació de la biodegradació *in situ* dels additius oxigenats en aigües subterrànies contaminades.

4.1.3 Anàlisi isotòpica dels elements estables (CSIA)

Des de que Thomson a l'any 1910-1912 identifiques els isòtops estables del Neó durant els seus experiments amb "raigs positius" i més tard (1927-1932), Aston descobrís pràcticament la resta d'isòtops estables, hi ha hagut molt avenços tecnològics que permeten mesurar les petites variacions d'abundància d'aquests a la natura o mesurar l'anomenat "fraccionament isotòpic" [93].

Els VOCs poden ser separats amb un GC connectat en línia amb una unitat de combustió on són completament convertits a CO_2 o H_2, per a l'anàlisi dels isòtops estables del carboni i l'hidrogen respectivament. Aquests gasos resultants passen a través d'una trampa que reté l'aigua i van a parar a un espectròmetre de masses per a la mesura de les relacions isotòpiques dels isòtops estables (IRMS). La determinació exacta d'aquestes relacions és de gran importància en nombroses àrees d'estudi com la geologia econòmica, la hidrologia, la meteorologia, l'agricultura, l'alimentació, la fisiologia vegetal, la medicina, la datació geològica, etc. [93].

La principal peculiaritat d'aquests equips de IRMS és que a diferència dels MS convencionals on es treballa fent escombrats sobre un interval més o menys ampli de masses, aquí es manté fix el camp magnètic i es col·loca un col·lector (normalment de tipus copa de Faraday o "channeltron") al final de la trajectòria de cada un dels isòtops d'interès [93]. Per exemple, es mesuren les *m/z* 44, 45 i 46 per l'anàlisi dels isòtops 12 i 13 del carboni convertit en CO_2 o les *m/z* 2 i 3 per l'hidrogen (H_2). A la *Figura 24* es pot veure un esquema global d'un equip de GC/C-IRMS habitualment utilitzat per a la realització d'aquest tipus de mesures.

Les mesures d'enriquiment isotòpic, també anomenades signatures o valors Delta (δ) s'obtenen de la comparació isotòpica de la mostra amb uns estàndards o valors de referència internacionals. Per això, els equips de IRMS disposen d'un dispositiu de doble entrada, que permet la introducció seqüencial de volums de mostra i de referència que produeixen nivells de senyals comparables.

En els últims anys, les tècniques per a la mesura dels isòtops estables específics dels compostos d'estudi (CSIA) s'han convertit en una de les eines més prometedores per a l'avaluació *in situ* de la biodegradació de compostos orgànics en general, com mostren els recents articles de revisió de Meckenstock et al. [177] i Schmidt et al. [178]; i pel MTBE en particular [179,180].

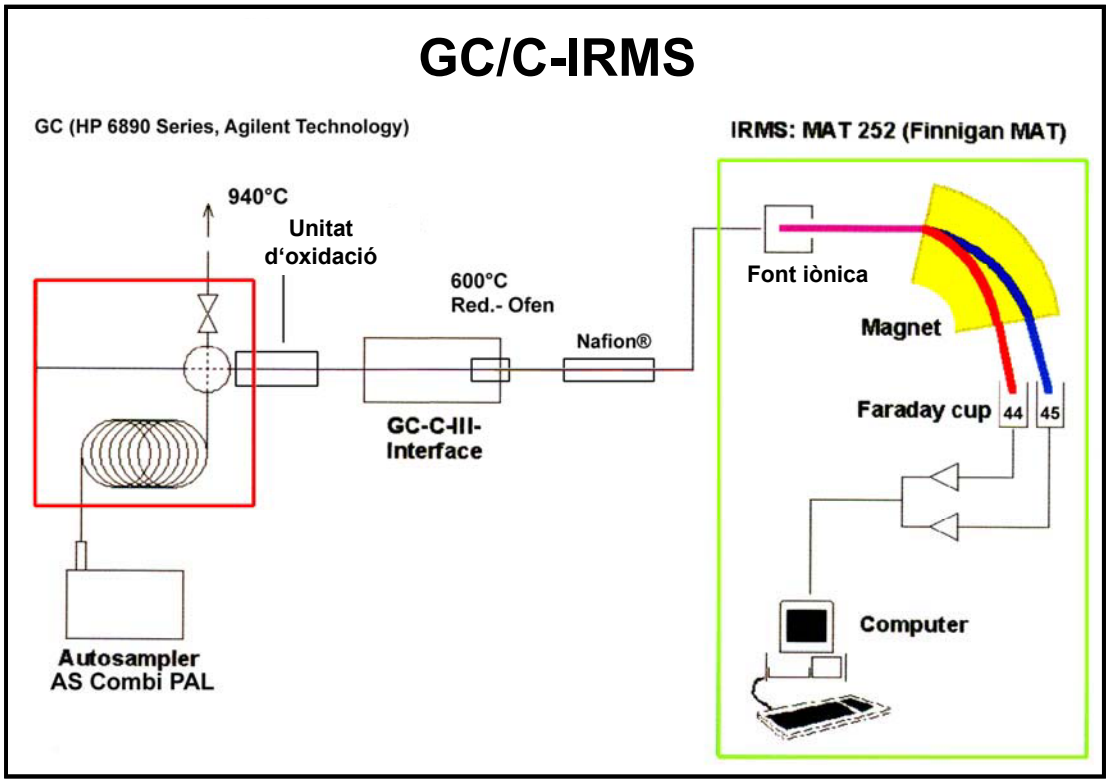

Figura 24.- Equip de GC/C-IRMS per a l'anàlisi dels isòtops estables del carboni

El principi bàsic per a la identificació dels processos de biodegradació, és el fet de que a aquests els hi acompanya un efecte isotòpic en la cinètica de la reacció entre isòtops pesats i lleugers d'un element. És a dir, els isòtops lleugers són consumits pels microorganismes més ràpidament que els pesats, donant lloc a un canvi en la relació entre ells. La fracció residual del compost d'estudi es va carregant de l'isòtop pesat, mentre el producte de degradació és fa més lleuger. Així doncs, en el cas del MTBE s'espera un enriquiment en isòtops pesats (^{13}C i ^{2}H o deuteri, D) conforme avança la biodegradació (mirar *Figura 25*).

En principi, les tècniques de preconcentració prèviament comentades per VOCs en els anteriors capítols, també serveixen per CSIA. Tot i això, algunes aplicacions ambientals que requereixen l'anàlisi a concentracions baixes (μg/L) necessiten de l'estudi de la influència que aquestes tècniques poden exercir sobre el fraccionament isotòpic dels compostos d'estudi. En un estudi recent, Zwank et al. [181] van observar els efectes de la SPME, P&T i algunes injeccions líquides sobre els resultats de fraccionament

isotòpic d'alguns VOCs (entre ells el MTBE). En general, els efectes observats per SPME i P&T van ser petits i força reproduïbles, fent possible la correcció dels resultats. Tot i això, aquests efectes es consideren gairebé menyspreables en comparació amb els valors de fraccionament observats pel MTBE en els estudis de biodegradació amb bactèries obtinguts al laboratori [178]. En el cas de les injeccions de líquids els límits de detecció van ser entre 4-6 ordres de magnitud superiors a les tècniques anteriors, fent-les no aptes per a l'anàlisi ambiental de traces [181].

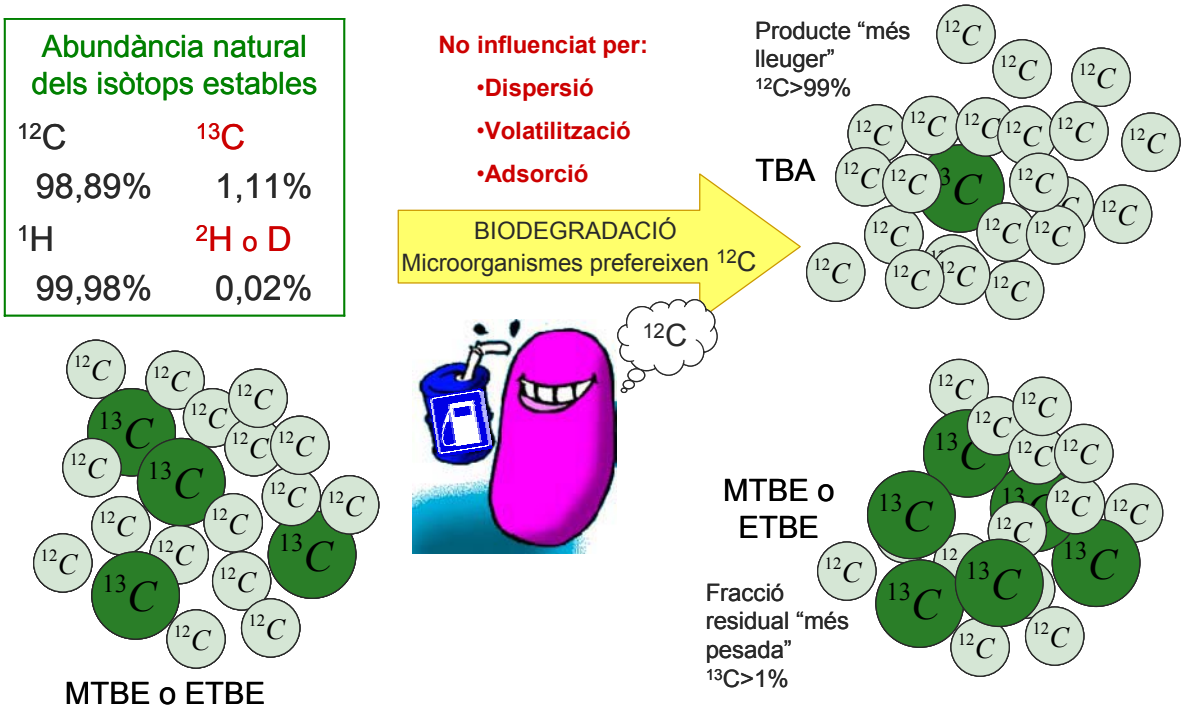

Figura 25.-Principis bàsics del fraccionament isotòpic del carboni durant la biodegradació de MTBE i ETBE.

Els estudis de degradació en el laboratori serveixen doncs, per a calcular els diferents factor de fraccionament isotòpic (α) a unes determinades condicions ambientals (amb diferents acceptors d'electrons). En general, els valors d'enriquiment isotòpic (ε) calculats a partir de l'equació $\varepsilon = (\alpha - 1) \times 1000$, resulten molt més intuitius i fàcilment comparables que no pas els de fraccionament (α). Expressats d'una manera o una altra, aquests factors no es veuen influenciats per altres processos ambientals com la

dispersió, l'adsorció o la volatilització dels compostos. I per això, un cop calculats poden servir per a la quantificació de l'estat de la biodegradació al camp en llocs contaminats de les mateixes característiques [178].

Fins el moment, pel MTBE s'han calculat aquests factors de fraccionament isotòpic pel carboni i l'hidrogen, tant en condicions aeròbiques [182,183] com anaeròbiques [179,180,184-187]. Tot i això, només un cultiu pur de la soca PM1 i un parell de cultius mixtes havien estat estudiats en condicions aeròbiques [182,183]. Els resultats havien mostrat una baixa variabilitat en la mesura del factor d'enriquiment isotòpic pel carboni (εC = -1,5‰ to -2,4‰), i es mostrava prou diferent del trobat habitualment en cultius anaeròbics mixtes (mitjana εC entre -8,63‰ [180] i -14,4‰ [187]), com per poder identificar i quantificar l'estat de la biodegradació en el camp.

4.2 ESTUDIS DE DEGRADACIÓ DE MTBE I ETBE AMB DIFERENTS CULTIUS BACTERIANS AEROBIS

4.2.1 Experiments amb bactèries (preparació de microcosmos)

L'aïllament i cultiu d'una nova bactèria (soca L108) procedent de les aigües contaminades d'una refineria a l'Alemanya de l'est (Leuna), capaç d'alimentar-se del MTBE com a font de carboni i energia en condicions aeròbiques, va ser el punt de partida de nombrosos experiments per a l'estudi de les vies metabòliques de degradació del compost [188], així com d'estudis filogenètics i del càlcul del fraccionament isotòpic respecte altres soques com la R8 (aïllada d'un cultiu mixte de procedència desconeguda del Dr. Arvin, Dinamarca) o la IFP 2001 (*Rhodococcus ruber* aïllat pel grup de la Dra. Fayolle, Institut Francais Du Petrole, França [174-176]), així com la comparació amb el seu substitut a la gasolina, el ETBE.

Aquests estudis de biodegradació es van portar a terme a una estada de 6 mesos (de maig a desembre de 2005) gràcies a la col·laboració entre els departaments de Microbiologia i de Biogeoquímica isotòpica del centre d'Investigació Ambiental UFZ-Leipzig-Halle, Alemanya, sota la supervisió dels Dr. Rohwerder i Dr. Richnow, respectivament.

Tota la preparació dels materials i les solucions minerals, així com la posterior manipulació i presa de mostres dels microcosmos, es va realitzar en condicions d'esterilitat. Els cultius es van realitzar en ampolles de 250 mL amb 50 mL de solució salina (amb els oligoelements, vitamines i cobalt necessaris pels microorganismes) que permetien un espai de cap amb suficient oxigen com per a no haver de ser obertes al llarg de l'estudi (minimitzar pèrdues volàtils del compost i contaminacions externes). Es van aplicar concentracions inicials elevades dels additius d'estudi (100-500 mg/L), tenint en compte, les condicions de contaminació trobades al camp (al Leuna fins a 40 mg/L) i la baixa sensibilitat dels instruments de mesura (LODs de 250, 10 and 20 µg/L pel TBA, MTBE i ETBE per a la determinació de la concentració per HS-GC-FID; i de 3 i 8 mg/L per la determinació isotòpica del C i H per MTBE i ETBE per HS-GC/C-IRMS).

Els cultius, junt amb ampolles control (estèrils) s'incubaven a un agitador a 120 rpm i 30 ºC de temperatura. Periòdicament es prenien mostres en vials per HS encapsulats de 10 mL mitjançant xeringues de plàstic de 2,5 mL. Els vials contenien prèviament la mateixa quantitat (1 mL) de solució saturada de NaCl per tal de matar els microorganismes un cop presa la mostra i facilitar el pas dels VOCs a la fase gas per l'anàlisi per HS. Amb els 0,5 mL restants es mesuraven els canvis de pH.

Figura 26.- Material necessari per a la realització d'estudis de degradació aeròbica en microcosmos.

4.2.2 Anàlisi CSIA de $^{13}C/^{12}C$ i D/H

A continuació l'**article 6** "*Variations in $^{13}C/^{12}C$ and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation"* recull els estudis de degradació (microcosmos), anàlisi dels isòtops estables tant del carboni com l'hidrogen, i el càlcul dels respectius factors de fraccionament isotòpics per MTBE i ETBE amb la utilització de diferents soques bacterianes amb capacitat per biodegradar aquests additius de la gasolina. Els resultats obtinguts es comparen amb la resta de factors trobats a la literatura i es plantegen hipòtesis sobre els possibles mecanismes de degradació dels compostos oxigenats que poden tenir lloc a la natura i la seva aplicació com a indicadors dels processos de biodegradació *in situ*.

Article científic (Art. 6):

"Variations in $^{13}C/^{12}C$ and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation"

Mònica Rosell, Damià Barceló, Thore Rohwerder, Uta Breuer, Roland H. Müller, Matthias Gehre, Hans Hermann Richnow

Environmental Science and Technology (2006) tramès

Environ. Sci. Technol. ***2006****, xx, xx-xx*

Variations in $^{13}C/^{12}C$ and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation

MÒNICA ROSELL, DAMIÀ BARCELÓ
Department of Environmental Chemistry, IIQAB-CSIC. Jordi Girona 18-26, 08034 Barcelona, Catalonia, Spain

THORE ROHWERDER
Department of Environmental Microbiology, Aquatic Biotechnology, Biofilm Center, University Duisburg-Essen, Geibelstr. 41, 47057 Duisburg, Germany

UTA BREUER
Department of Environmental Microbiology

MATTHIAS GEHRE, HANS HERMANN RICHNOW*
Department of Isotope Biogeochemistry UFZ-Centre for Environmental Research Leipzig-Halle. Permoserstrasse 15, 04318 Leipzig,Germany

Reliable compound specific isotope fractionation factors are needed for a quantitative assessment of *in situ* biodegradation in contaminated groundwater. In order to obtain information on the variability on carbon and hydrogen fractionation factors (εH, εC) the isotope fractionation of methyl tertiary (tert-) butyl ether (MTBE) and ethyl tert-butyl ether (ETBE) upon aerobic degradation was studied with different bacterial isolates. Methylibium sp. R8 shows a carbon and hydrogen isotope fractionation upon MTBE degradation of -2.35 ±0.05‰ and -40 ±2‰, respectively, which is in the range of previous studies with pure cultures (Methylibium petroleiphilum PM1) as well as mixed consortia. In contrast, carbon isotope fractionation of the ß-proteobacterium L108 (-0.48 ±0.02‰) and Rhodococcus ruber IFP 2001 (-0.28 ±0.03‰) was much lower and hydrogen isotope fractionation was negligible (≤ -0.2‰). The varying isotope fractionation pattern indicates that MTBE is degraded by different mechanisms by the strains R8 and PM1 compared to L108 and IFP 2001. The carbon and hydrogen isotope fractionation of ETBE by L108 (-0.68 ±0.03‰ and -14±1‰) and IFP 2001 (-0.73 ±0.04‰ and -11±2‰) was very similar and significantly higher than the fractionation of MTBE upon degradation by the same strains. This may indicate that these strains use different mechanisms for degrading MTBE and ETBE although the compounds are structurally quite similar. The carbon and hydrogen fractionation factors may be applied for interpreting isotope pattern of fuel oxygenates in contamination plumes.

Keywords: MTBE; ETBE; fuel oxygenates; CSIA (compound-specific stable isotope analysis); aerobic biodegradation; pure cultures.

** Corresponding author. Tel.: +49-341-235-2810, Fax: +49-341-235-2552, e-mail: hans.richnow@ufz.de*

Introduction

Since the 1970s, fuel oxygenates (commonly ethers and alcohols) were used as octane enhancers. These compounds promote cleaner combustion and reduce vehicle air emissions. Methyl tertiary (tert-) butyl ether (MTBE) is by far the most commonly used oxygenate. Worldwide, about 20 Mt of MTBE are manufactured and used each year (60% in the USA and

15% in Europe), which puts it into the category of high production volume chemicals (1). As a result of this extensive use, and due to its high water solubility, considerable mobility and slow degradation rates, MTBE has become one of the most frequently detected volatile organic compounds in groundwater (2). Therefore, an increasing demand for the protection of drinking water resources can be assumed as odor and taste thresholds for MTBE are low and it is a possible carcinogen (3,4). Today, MTBE is substituted by ethyl tert-butyl ether (ETBE) in Europe due to tax incentives for the application of biomass-derived ethanol which is synthesized to produce the ethyl group of ETBE (5). Thus, the production and consumption of ETBE will increase and it is expected that ETBE will be one of the emerging fuel derived contaminants in Europe in the future.

Groundwater sites heavily contaminated with fuel oxygenates have been identified in many countries (5-8) and natural attenuation is currently discussed as a management option (9,10). In aquifers *in situ* biodegradation is the only sustainable sink of MTBE and ETBE. One strategy to elucidate *in situ* biodegradation may be the analysis of metabolites, however, the mere presence of the intermediate tert-butyl alcohol (TBA) is inappropriate for providing evidence for natural attenuation of fuel oxygenates in many cases because it is a component of the original gasoline (11) or can originate from other industrial processes (12).

During the last decade, laboratory degradation studies showed the potential of microbial communities to degrade fuel oxygenates under oxic and anoxic conditions (13). Aerobic bacterial strains capable of productive degradation of MTBE are e. g. *Methylibium petroleiphilum* PM1 (14) and the ß-proteobacterium strain L108 (15). In addition, growth on ETBE has been demonstrated with several aerobic strains such as strain L108 and *Rhodococcus ruber* IFP 2001 (16). The latter strain is not capable of growing on MTBE and shows only an incomplete degradation of ether oxygenates resulting in TBA accumulation. While the anaerobic pathway is practically unexplored there is general agreement that the initial step of the aerobic pathway is a hydroxylation reaction resulting in the cleavage of the ether bond. In case of R. ruber IFP 2001, the responsible ETBE monooxygenase has been identified (17,18). Further aerobic degradation may proceed via tert-butyl formate (TBF) and TBA (19). The analysis of metabolites can be useful to characterize degradation pathways, however, quantitative information on the *in situ* degradation is difficult to obtain.

Therefore, approaches are urgently needed to monitor the *in situ* biodegradation of fuel oxygenates in polluted groundwater tables. Recently, compound-specific stable isotope analysis (CSIA) has become a promising tool for evaluating *in situ* MTBE degradation (20,21). CSIA makes use of kinetic isotope fractionation processes upon biodegradation leading to an enrichment of heavy isotopes (^{13}C and ^{2}H) in the residual fraction. For quantitative assessment of *in situ* degradation the compound specific isotope fractionation factor (α) is needed (22,23) which is obtained in controlled

laboratory studies. Knowledge of the variability of α is required to evaluate the uncertainty of quantitative field work. Moreover, the biochemical reaction may be characterized by the isotope fractionation pattern (24) which may allow identification of the degradation pathway in the field (20,21,25). This characterization relies on variations of isotope fractionation pattern correlating to particular microbial degradation mechanisms.

MTBE carbon and hydrogen isotope fractionation has been found under oxic (26,27) and anoxic conditions (20,21,25,28,29). Carbon enrichment factors for aerobic MTBE degradation were determined only from one pure culture, strain PM1, and two enrichment cultures (26,27), and a limited number of experiments for stable hydrogen isotope fractionation is documented (27). Isotope fractionation studies of other ether oxygenates such as tert-amyl methyl ether (TAME) or ETBE are scarce (28). Therefore a more systematic study on hydrogen and carbon isotope fractionation under defined conditions is needed for evaluating the CSIA concept for characterizing and assessing *in situ* biodegradation of fuel oxygenates.

The objective of this study is to investigate the variability of isotope fractionation upon aerobic biodegradation of fuel oxygenates (MTBE and ETBE). We examined the carbon and hydrogen enrichment factors obtained by strains L108, R. ruber IFP 2001, and the newly isolated strain Methylibium sp. R8 under controlled laboratory conditions. The results were interpreted with respect to the variability of isotope fractionation to investigate degradation pathways and to quantify the *in situ* biodegradation.

Experimental Section

Chemicals. All chemicals were purchased from Sigma-Aldrich Chemie (Munich, Germany) at the highest purity available unless stated elsewhere. MTBE was obtained from Merck in p. a. quality and ETBE (purum, ≥97.0%, GC) purchased from Fluka (Buchs, Switzerland).

Bacterial strains and cultivation conditions. Strain L108 belongs to β-Proteobacteria and was isolated from MTBE-contaminated groundwater (Leuna, Germany) (15) and showed a 95.6 % identity of the 16S rRNA gene to M. petroleiphilum PM1 (supporting information). Methylibium sp. R8 was isolated from a MTBE-degrading enrichment culture provided by E. Arvin (DTU, Denmark). The strains L108 and R8 were aerobically cultured in a mineral salt medium on MTBE as sole source of carbon and energy (15). Strain R8 did not grow productively on ETBE.

Rhodococcus ruber IFP 2001 was provided by F. Fayolle (IFP, France) and was cultured in the above described mineral salt medium on ETBE. For isotope fractionation experiments the microorganisms were aerobically incubated with 50 mL mineral salt solution in 240-mL serum bottles. MTBE or ETBE was added to a final concentration of 100-500 mg/L. Two percent inoculum (v/v) was added and the cultures were incubated on a rotary shaker at 30 °C. A negative control without

inoculum was incubated under identical conditions in each series (see supporting information for further details).

Experiments with resting cells. Strains L108 and IFP 2001 were used for isotope fractionation with resting cells. Bacteria were grown on either MTBE (strain L108) or ETBE (both strains) and harvested by centrifugation. Cells were washed with mineral salt solution and suspended at a concentration of 1-2 g/L (biomass dry weight). 50 mL of this cell suspension was supplemented with MTBE or ETBE at 100-200 mg/L and incubated on a rotary shaker at 30 °C. Over an 8-h period up to 99.9% of the substrate was decomposed. In co-metabolic degradation experiments with MTBE and strain IFP 2001, glucose at a final concentration of 350 mg/L was added as a co-substrate (see also Supporting information).

Analytical Methods. Specific cultivation conditions are detailed in the Supporting Information. For sampling, aliquots (2.5 mL) of the medium were removed with a syringe. From this sample, 1 mL sub-sample each was transferred into 10-mL headspace vials (i) for headspace GC analysis of the ETBE, MTBE, and TBA concentration (15) and (ii) for the stable isotope analysis. In both cases, bacterial activity was stopped by mixing the sample with the same volume of aqueous saturated NaCl solution. Samples not immediately analyzed were stored at -20 °C until further processing. External five-point linear regression curves were employed for quantitative GC analysis. Reproducibility of concentration values was ±5% and detection limits were 250, 10 and 20 µg/L for TBA, MTBE and ETBE, respectively.

Stable isotope composition was determined using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry system (GC-C-IRM-MS). The system consisted of a GC (6890 series; Agilent Technology) coupled with a combustion or high temperature pyrolysis interface (GC-combustion III or GC/C-III/TC; ThermoFinnigan, Bremen, Germany) to a MAT 252 IRMS for the carbon and to a MAT 253 IRMS for hydrogen analysis (both from ThermoFinnigan, Bremen, Germany). Headspace samples were injected in split or splitless mode. The method had detection limits for MTBE and ETBE of approximately 3 mg/L for carbon and 8 mg/L for hydrogen. The error associated to the system (accuracy and reproducibility) was ±0.5‰ for carbon and ±4‰ for hydrogen (see Supporting Information for further details).

Stable isotope calculations and definitions. The carbon and hydrogen isotopic compositions (R) are reported as delta notation ($\delta^{13}C$ and $\delta^{2}H$) in parts per thousand (‰) relative to an international standard; Vienna Pee Dee Belemnite standard (V-PDB) and Vienna Standard Mean Ocean Water (V-SMOW), respectively (30). δ values were calculated as follows:

$$\delta[‰] = \left(\frac{R_{sample}}{R_{reference}} - 1 \right) \times 1000 \quad (1)$$

where R_{sample} and $R_{reference}$ are the ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$ or D/H) in the sample and an international standard, respectively. Calculation of the isotopic fractionation factor (α) was based on the Rayleigh equation (31) simplified for a closed system (32):

$$\ln\left(\frac{R_t}{R_0}\right) = \left(\frac{1}{\alpha} - 1\right) \times \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

where R is the isotope ratio, C is the concentration, and the index (0 and t) describes the incubation time at the beginning (0) and during the reaction time of experiment (t). When $\ln(R_t/R_0)$ versus $\ln(C_t/C_0)$ was plotted the isotopic enrichment factor (ε) could be determined from the slope of the curve (b), with $b = (1/\alpha) - 1$ and $\varepsilon = 1000 \times b$. The experiments were repeated for the various strains until representative biodegradation points were reached. Linear regression was used to estimate the slope of each data set.

Results and Discussion

Concentration Profiles. The time course of MTBE and ETBE degradation of the strain L108 under oxic conditions was studied (see Figure S1 in the Supporting Information). With an initial MTBE concentrations between 200 to 500 mg/L, up to 15 days were necessary for a degradation of 99.9%. 70 to 250 mg/L ETBE was degraded within the same time period to a similar extent. ETBE was not degraded by strain R8 and for similar initial MTBE concentrations as in experiments with L108, approximately twice of the time was required. In 26 days this strain degraded about 500 mg/L to 99.9% (data not shown). Conversely, strain IFP 2001 (that only grew on ETBE) was found to be the most effective. In less than 5 days 250 mg/L of ETBE were consumed. As indicated in previous studies a concomitant accumulation of TBA was detected (16). During each experiment, MTBE and ETBE concentrations remained constant in negative controls. In addition, TBA was not detected under sterile conditions.

Carbon Isotopic Profiles. The initial $\delta^{13}C$ for MTBE and ETBE (-29.35 ±0.03‰ and -24.3 ±0.1‰, respectively) were calculated as the average of three different samples at time zero and these values remained constant in the negative controls during the whole period of incubation. In live cultures, $\delta^{13}C$ of MTBE and ETBE increased as biodegradation proceeded (as shown in Figure S1, Supporting information) for three batch experiments with strain L108), indicating the enrichment of ^{13}C in the residual compound fraction. The investigation of ETBE fractionation by strain R8 was not possible, since the strain was not able to degraded ETBE.

The carbon enrichment factors (εC) were calculated with the Rayleigh equation (Figure 1, A and B). Results for all strains and substrates are summarized in Table 1. The relatively good correlation between concentration and isotope composition with correlation factors (R^2) between 0.81 and 0.98 suggested that carbon isotope fractionation during aerobic MTBE and

ETBE degradation can be modeled by the Rayleigh equation. The degradation experiments with L108 and IFP 2001 gave a similar εC between -0.48 ±0.02‰ to -0.28 ±0.03‰, respectively. In contrast, strain R8 showed one order of magnitude higher εC of -2.35 ±0.05‰ which are very similar to values obtained with PM1 ranging between -2.0 to -2.4‰ (εC) reported by Gray et al. (27). Although mixed cultures showed a slightly lower carbon isotope fractionation between -1.5 to -1.97‰ (26,27), the factors obtained in experiments with strains L108 and IFP 2001 showed the lowest isotope fractionation observed for MTBE thus far. The εC obtained in ETBE degradation experiments with strains L108 and IFP 2001 were nearly identical (-0.68 ±0.03‰ and -0.73 ±0.04‰, respectively) and quite similar to those obtained for MTBE. Moreover, ETBE degradation assay with L108 resting cells (experimental details are given in the Supporting Information) did not show apparent differences in εC value (-0.76 ±0.04‰) in comparison to the fractionation in growing cultures. However, to our knowledge, no other values are available to date in the literature for comparison.

Hydrogen Isotopic Profiles. The initial hydrogen isotope composition of MTBE and ETBE was -177 ±2‰ and -223 ±3‰, respectively, and remained stable in the negative control over 14 days of incubation. The hydrogen isotope fractionation of strain R8 was much stronger (-40‰) than the carbon isotope fractionation (-2.35‰) upon biodegradation as expected. Surprisingly, the two strains L108 and IFP 2001 did not exhibit any detectable hydrogen isotope fractionation upon MTBE degradation (Table 1). For example, in the experiment with strain L108 the average δ^2H value after 82% of MTBE degradation was -180 ±2‰ and similar to the negative control (Figure S1 A, Supporting information). However, slight enrichment in δ^2H as observed for carbon isotopes may not be detectable for hydrogen as a higher error is associated with the determination of hydrogen isotopes. The hydrogen isotope fractionation of R8 upon MTBE degradation (-40 ±2‰) was in the same range as reported for PM1 ranging from -33 to -37‰ (εH) but even lower than reported for a mixed consortium (up to -66‰) (27) (Table 1). In contrast to MTBE, in all live cultures an enrichment in δ^2H upon ETBE degradation could be detected (Figure 1 C and D).

The hydrogen enrichment factor (εH) of strain L108 and IFP 2001 was between -11 and -14‰. The isotope fractionation was slightly lower in the experiment with resting cells of strain L108 (-11‰) compared to growing cells, however, in the same order of magnitude. The hydrogen and carbon ETBE fractionation by growing cells of the strains L108 and IFP 2001 were positively correlated similar to the experiment with resting cells of strain IFP 2001. Nearly identical hydrogen as well as carbon isotope fractionation factors were obtained, indicating that no significant difference in isotope fractionation were detected with growing or resting cells, respectively. The times of substrate consumption changed from 2 weeks in growth experiments to some hours in

resting cell experiments and indicate that the overall kinetics of degradation as well as the amount of biomass did not effect the isotope fractionation in this case. Therefore, degradation assays with resting cells may be a good alternative in place of long incubation experiments which are time-consuming and often do not allow to monitor the progress in biodegradation steadily.

Kinetic Isotope Effects (KIE). Recently kinetic carbon and hydrogen isotope fractionation patterns were used to gain information on the biochemical mechanism governing *in situ* biodegradation. The direct comparison of isotopic enrichment factors (εbulk) obtained for different compounds requires normalization to the reactive position since only the atom where the reaction takes place is involved in the isotope fractionation process and other parts of the molecule remains unaltered (24). Starting from basic rate laws, a quite general derivation of the Rayleigh equation was obtained, taking into account the effects of (i) non-reacting positions and (ii) intra-molecular competition and leading to position-specific apparent kinetic isotope effects (KIE) values rather than bulk enrichment factors (εbulk).

The factor describing the fractionation at the reactive position (εreactive position) and the apparent KIE of the reaction (AKIE) were calculated according to Elsner et al. (24). Since intramolecular competition was considered negligible during oxidation process ($z = x$), the approximated equations 3 and 4 were applied to the oxidation of methoxy and ethoxy group of MTBE and ETBE, respectively, as follows:

$$\varepsilon_{reactive\ position} \approx \frac{n}{x} \times \varepsilon_{bulk} \quad (3)$$

$$\mathrm{AKIE_E} = \frac{1}{1 + \left(\frac{z}{x} \cdot \frac{n}{x} \varepsilon_{bulk} / 1000\right)} \approx \frac{1}{1 + \left(n \cdot \varepsilon_{bulk} / 1000\right)} \quad (4)$$

where n is the number of the atoms of the element considered of which x is the atom located at the reactive site and z the number of atoms which are involved in the intramolecular isotopic competition. Hence, for carbon fractionation, 1 of 5 atoms of MTBE is located at the reacting position, whereas 1 of 6 atoms of ETBE. For correcting the hydrogen fractionation of MTBE, we assume that 3 out of 12 atoms are bound to the methoxy group and are involved in intramolecular isotopic competition (z), whereas for ETBE, 2 out of 14 atoms are bound to the ethoxy group in the reactive position (z).

Table 1 shows the corrected values in comparison with values available in the literature. εCreactive position and εHreactive position for strains PM1 and R8 are similar suggesting a similar biochemical mechanism in the degradation of MTBE. PM1 and R8 are close relatives and therefore their phylogenetic relationship is reflected by the fractionation pattern (see Supporting Information). In contrast, strains L108 and IFP 2001 show a significantly lower carbon fractionation (nearly one order of magnitude) and almost no hydrogen fractionation indicating a

different biochemical degradation mechanism. In the latter case, carbon and hydrogen isotope composition is not correlated indicating that, in contrast to carbon, a cleavage of a hydrogen bond is not a limiting step in the overall reaction kinetics. In previous studies, the uptake of substrate has been shown to affect the isotope composition (33-35), however, in this case the transport limitation or diffusion through the microbial cell membrane should affect both elements in a similar way. This is not the case because a slight carbon but no hydrogen isotope fractionation was observed. In addition, the gram-negative strains R8, PM1, and L108 have different cell wall structures than the gram-positive IFP 2001, which could affect the isotope fractionation. However, possible differences in uptake of substrates cannot be employed here to explain the isotope fractionation pattern because it is unlikely that kinetic limitations due to uptake can selectively affect carbon or hydrogen isotope fractionation. Thus, it is more likely that a different biochemical mechanism is responsible for the observed difference in the isotope fractionation.

εCreactive position of MTBE by L108 and IFP 2001 was more than 40% lower than for ETBE indicating different pathways may be at work as well. This assumption is supported by the fact that for ETBE hydrogen bond cleavage is involved in a rate limiting step of the reaction but not for MTBE. The biodegradation mechanisms can significantly influence the isotope fractionation pattern (35-37). In summary, the fractionation pattern gives first indications on the biochemical mechanism which takes place in the initial steps of the transformation reactions. Because a slight carbon but no significant hydrogen isotope fractionation, we propose that a different biochemical mechanism is responsible for the observed difference in the isotope fractionation.

A further method for interpreting isotope fractionation is to calculate the AKIE (eq 4). The AKIE values for breaking hydrogen, carbon or oxygen bonds can be compared to the possible maximal theoretical fractionation called Streitwieser Semiclassical Limits (38). The cleavage of the ether bond leading to TBA as a product may occur via different mechanisms for example initiated by (I) the cleavage of the C-H bond by oxidation, (II) an acidic hydrolysis (S_N1) or (III) by a nucleophilic reaction (S_N2) which may be distinct by their fractionation effects on hydrogen and carbon (24). Experimental KIE values for chemical reaction are often 50% lower as the predicted theoretical values which has to be considered for interpretation (24).

In the case of MTBE degradation by strains L108 and IFP 2001, the hydrogen isotope fractionation was very low (< -0.2) and due to the uncertainty a meaningful $AKIE_H$ values could not be calculated. The found $AKIE_C$ was relatively low. Therefore, a cleavage of the C-H bond as the initial rate limiting biochemical reaction is unlikely, as this reaction would result in a significant primary hydrogen (expected $KIE_H = 2 – 50$) and carbon isotope effect (expected $KIE_C = 1.01 – 1.03$) (24). A hydrolysis reaction cleaving the C-O bond of the ether linkage in the kinetic reaction step did not lead to a primary hydrogen isotope effect and would

result in a low hydrogen isotope fractionation. Thus, a hydrolysis of an ether bond may explain the isotopic fractionation pattern. In this case, higher KIE_C (1.03 to 1.09) compared to KIE_H (0.95 to 1.05) should be expected (24). In contrast, the positive correlation between carbon and hydrogen isotope fractionation upon MTBE degradation by PM1 and R8 and the order of $AKIE_H$ (1.66 to 1.92) as well as the $AKIE_C$ (1.010 to 1.012) may indicate a monooxygenase reaction cleaving a C-H bond of the methyl group. Although AKIE values are not very high, they still fall in the order of the Streitwieser Semiclassical Limits of a monoxygenase like reaction. The rather small KIE for hydrogen (1.5 to 4.8) calculated from MTBE aerobic degradation studies by Gray et al. (27) were previously explained as an indication for a slow non-fractionating step preceding the isotopically sensitive bond cleavage (24). Our results indicated that the strains L108 and IFP 2001 employ surprisingly a different mechanism to attack MTBE as the strains PM1 and R8. Moreover, in the ETBE degradation studies with strains L108 and IFP 2001, the obtained $AKIE_C$ (1.004-1.005) is still lower than the theoretical KIE value (1.021) for the cleavage of C-H (38). However, the $AKIE_C$ may be quite low and the $AKIE_H$ too high to assume again a hydrolysis reaction. Therefore, it seems to be more likely that an oxidation of the ethyl group of ETBE may explain the carbon and hydrogen isotope fractionation observed in strains L108 and IFP 2001 although their extent is low. A different degradation mechanism for MTBE and ETBE by the same strain may be surprising but the increase in hydrogen and carbon isotope fractionation provides evidence for this assumption. Because isotope fractionation studies provide only indications for a mechanism further investigation to decipher the reaction mechanisms are needed. In addition, this example may show the complications and uncertainty when interpreting reaction mechanism using isotope fractionation data.

Two-Dimensional Isotope Analysis. The combined use of hydrogen and carbon isotope analysis has been recently proposed as a tool to characterize the pathway of biodegradation in the field (21). Plots of δ^2H versus $\delta^{13}C$ signatures avoid masking effects which should affect both elements in the same way and provide valuable information on degradation state and whether aerobic or anaerobic pathways are employed (24). Our results were plotted in a δ^2H versus $\delta^{13}C$ diagram (Figure 2) and the slope of the linear regression of each data set were calculated and correspond approximately to the curve calculated by the $\varepsilon H/\varepsilon C$ ratio. The experiment with strain R8 showed a good correlation (R^2 value of 0.87) and the slope (14.8) was similar to results from PM1 ($\varepsilon H/\varepsilon C = -34.75/-2.20 = 15.8$) (27). MTBE degradation by strains IFP 2001 and L108 gives a low carbon fractionation and almost no hydrogen fractionation indicating that carbon vs. hydrogen plot cannot always clearly distinct between aerobic and anaerobic degradation pathways.

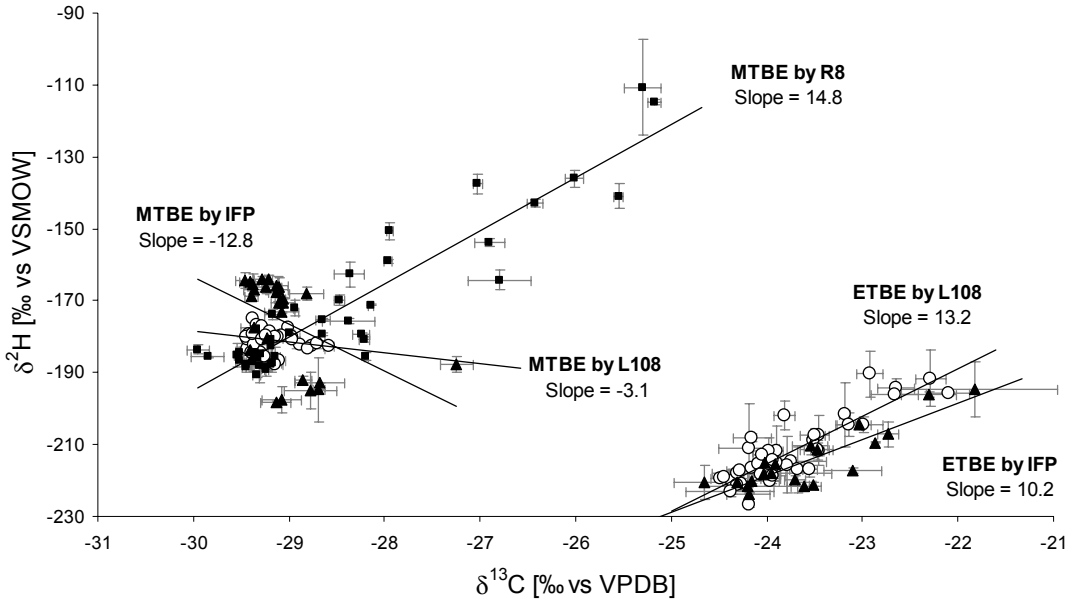

FIGURE 2. Plot of hydrogen versus carbon isotopic shifts for aerobic biodegradation of MTBE and ETBE by different pure cultures: (open circles) strain L108; (solid squares) strain R8 and (solid triangles) strain IFP 2001. The lines correspond to a linear regression model.

A very low fractionation is difficult to assess because field data typically show some variability in the isotope pattern due to source variations in addition to the analytical uncertainty. Our results illustrates the complications when using the two dimensional isotope analysis as suggested by Zwank et al. (21) to distinct anaerobic and aerobic MTBE degradation. The available data for carbon and hydrogen isotope fractionation for aerobic as well as for the only anaerobic degradation from laboratory enrichment cultures ($\varepsilon H/\varepsilon C = -16/-13 = 1.2$) were plotted (Figure 3). The anaerobic isotope fractionation pattern is similar to the pattern observed for aerobic degradation by L108 and IFP 2001 and therefore caution is needed for the interpretation of $\Delta\delta^2H/\Delta\delta^{13}C$ diagrams. The carbon and hydrogen fractionation upon ETBE degradation by strains L108 and IFP 2001 was positively correlation with regression curves between 10.2 and 13.2, respectively. In this case the hydrogen and carbon isotope fractionation pattern may be used to characterize the aerobic degradation in the field, if the correlation is significantly different from an anaerobic pathway which is still unknown.

Environmental implication. The present study highlights the isotope fractionation of strains that aerobically degrade MTBE. This work demonstrated that even if similar biochemical reaction may be involved leading to an ether cleavage of MTBE or ETBE to form the intermediate TBA, highly variable isotope fractionation factors are found. Carbon fractionation values (εC) of mixed cultures were lower than of pure

cultures, which on one hand show the variability. On the other hand, if organisms are present in a mixed culture that fractionate the substrate to a different extent as shown in our experiments with the strains L108 and R8 the average isotope fractionation factor might be lower. This makes the selection of appropriate isotope fractionation factors for quantitative work difficult and can lead to strong variable fractionation in mixed cultures depending on the dominance of organism actually growing best. Therefore, isotope fractionation factors from uncharacterized microcosm studies should always be used with caution and may not be representative for isotope fractionation in the field. For the field application of stable isotope fractionation, caution is also needed as fractionation may vary by more than an order of magnitude depending on the bacteria responsible for degradation (see Figure 3). To understand the diversity in isotope fractionation and the responsible biochemical mechanisms with respect to individual microbial strains, more systematic work with pure cultures is needed. At a field scale, the extent of biodegradation is also influenced by the relative contribution of aerobic and anaerobic conditions along the flow path. Under such circumstances, uncertainty is associated in applying isotope fractionation factors for quantification of natural degradation at contaminated sites. The correlation of carbon and hydrogen isotopes may contribute to characterize the degradation conditions to some extent and help to select a fractionation factor for a quantitative assessment of biodegradation. A selection of a larger fractionation factor (as found before in the literature) will give a conservative estimate and do not result in an overestimation of *in situ* biodegradation (35). However, investigation of the indigenous microbial consortium in the aquifer and the identification of the organisms by molecular biological methods in the environment might help to improve the selection of appropriate isotope fractionation factors suitable for the assessment of *in situ* degradation at MTBE and ETBE contaminated sites.

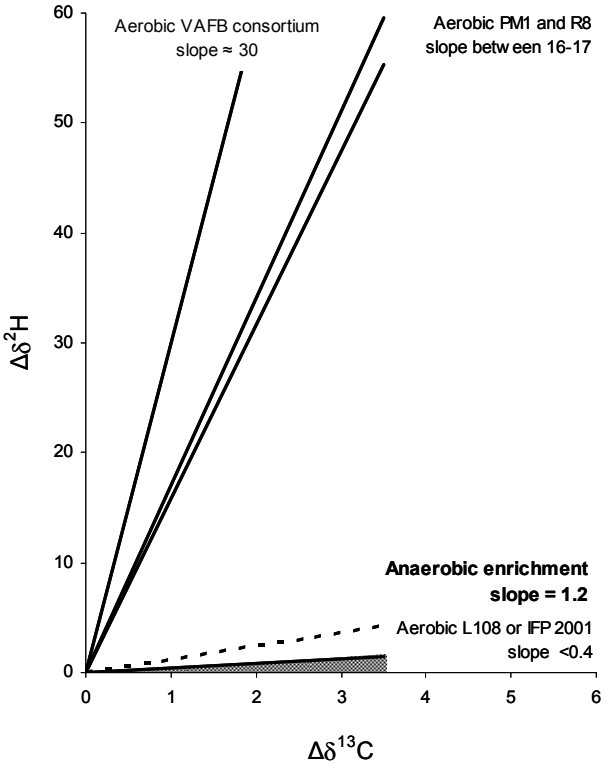

FIGURE 3. Plot of H versus C isotopic shifts for both (aerobic and anaerobic) biodegradation of MTBE which shows the total variability for field application. The slopes were calculated by the $\varepsilon H/\varepsilon C$ *ratio and the axis represent the difference between the isotope signature at time t and time zero* $\Delta\delta^{13}C = \delta^{13}C_t - \delta^{13}C_0$ *and* $\Delta\delta^{2}H = \delta^{2}H_t - \delta^{2}H_0$.

Acknowledgments

We thank Ursula Günther, Monika Neytschev and Cornelia Schumann for technical support at the UFZ. We are also grateful to Erik Arvin (Technical University of Denmark, Lyngby, Denmark) and Françoise Fayolle and Frédéric Monot (Institute Français du Petrole, Cedex, France) for providing an enrichment culture and strain IFP 2001, respectively.

Part of the work was financed by the German Federal Ministry of Education and Research (02 WN 0348) within the METLEN project and by the UFZ (SAFIRA). The fellowship of Mònica Rosell was supported by AXIOM-Marie Curie Host Fellowships For Early Stage Training (EST) (contract MEST-CT-2004-8332) and Catalonian fellowship for research in an outsider country (2005 BE 00008) in the framework of AXIOM project - "Assessment of *in situ* Transformation of Xenobiotic Organic Material". She also received a regular PhD grant from Department of Universities, Research and Information Society of La Generalitat de Catalunya (expedient 2005FIR 00348).

Supporting Information Available

Characterization of bacterial strains, cultivation conditions, short-term experiments with resting cells, and monitoring of concentration and isotope composition upon MTBE and ETBE biodegradation by strain L108 (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Ahlberg, R.; Gennart, J. P.; Mitchell, R. E.; Schulte-Koerne, E.; Thomas, M. E.; Vahervuori, H.; Vrijhof, H.; Watts, C. D. An environmental risk assessment of MTBE use in Europe; EFOA/ECETOC Task Force on Environmental Risk Assessment of MTBE; Brussels, Belgium, 2001.

(2) Squillace, P. J.; Pankow, J. F.; Korte, N. E.; Zogorski, J. S. Review of the environmental behavior and fate of methyl tert-butyl ether. Environ. Toxicol. Chem. 1997, 16, 1836-1844.

(3) USEPA Oxygenates in Water: Critical Information and Research Needs; U. S. Environmental Protection Agency, Office of Research and Development; EPA/600/R-98/048; Washington, DC, USA, 1998.

(4) Finnish Environment Institute In EUR 20417 EN - European Union Risk Assessment Report tert-butyl methyl ether; Hansen, B. G., Munn, S. J., Pakalin, S., Musset, C., Luotamo, M., de Bruijn, J., Berthault, F., Vegro, S., Pellegrini, G., Allanou, R., Scheer, S., Eds.; Office for Official Publications of the European Communities: Luxembourg, 2002; Vol. 19.

(5) Schmidt, T. C.; Morgenroth, E.; Schirmer, M.; Effenberger, M.; Haderlein, S. B. In Oxygenates in Gasoline: Environmental Aspects; Diaz, A. F., Drogos, D. L., Eds.: Washington, DC, 2002; pp 58-79.

(6) Rosell, M.; Lacorte, S.; Ginebreda, A.; Barcelo, D. Simultaneous determination of methyl tert.-butyl

ether and its degradation products, other gasoline oxygenates and benzene, toluene, ethylbenzene and xylenes in Catalonian groundwater by purge-and-trap-gas chromatography-mass spectrometry. J. Chromatogr. A 2003, 995, 171-184.

(7) Shih, T.; Rong, Y.; Harmon, T.; Suffet, M. Evaluation of the impact of fuel hydrocarbons and oxygenates on groundwater resources. Environ. Sci. Technol. 2004, 38, 42-48.

(8) Fischer, A.; Oehm, C.; Selle, M.; Werner, P. Biotic and abiotic transformations of methyl tertiary butyl ether (MTBE). Environ. Sci. Pollut. 2005, 12, 381-386.

(9) Schirmer, M.; Butler, B. J.; Barker, J. F.; Church, C. D.; Schirmer, K. Evaluation of biodegradation and dispersion as natural attenuation processes of MTBE and benzene at the Borden field site. Physics and Chemistry of the Earth, Part B: Hydrology, Oceans and Atmosphere 1999, 24, 557-560.

(10) Wilson, J. T.; Kaiser, P. M.; Adair, C. Monitored natural attenuation of MTBE as a risk management option at leaking underground storage tanks sites; U. S. Environmental Protection Agency; EPA/600/R-04/179; Ada, OK, 2005.

(11) Kramer, W. H.; Douthit, T. L. Water Soluble Phase Oxygenates in Gasoline From Five New Jersey Service Stations; Proc. of NGWA Conference, Petroleum Hydrocarbons and Organic Chemicals in Groundwater: Prevention, Detection and Restoration. Anaheim, CA, November 15-17, 2000.

(12) Clark, J. J. J. In Oxygenates in Gasoline: Environmental Aspects; Diaz, A. F., Drogos, D. L., Eds.; American Chemical Society: Washington, DC, 2002; pp 92-106.

(13) Schmidt, T. C.; Schirmer, M.; Weiss, H.; Haderlein, S. B. Microbial degradation of methyl tert-butyl ether and tert-butyl alcohol in the subsurface. J. Contam. Hydrol. 2004, 70, 173-203.

(14) Nakatsu, C. H.; Hristova, K.; Hanada, S.; Meng, X.-Y.; Hanson, J.; Scow, K. M.; Kamagata, Y. Methylibium petrophilum PM1T gen. nov., sp. nov., a new methyl tert-butyl ether (MTBE) degrading methylotroph of the beta-Proteobacteria. Int. J. Syst. Evol. Microbiol. 2006, 56, 983-989.

(15) Rohwerder, T.; Breuer, U.; Benndorf, D.; Lechner, U.; Müller, R. H. The alkyl tertiary butyl ether intermediate 2-hydroxyisobutyrate is degraded via a novel cobalamin-dependent mutase pathway. Appl. Environ. Microbiol. 2006, 72, 4128-4135.

(16) Hernandez-Perez, G.; Fayolle, F.; Vandecasteele, J. P. Biodegradation of ethyl t-butyl ether (ETBE), methyl t-butyl ether (MTBE) and t-amyl methyl ether (TAME) by Gordonia terrae. Appl. Microbiol. Biotechnol. 2001, 55, 117-121.

(17) Chauvaux, S.; Chevalier, F.; Le Dantec, C.; Fayolle, F.; Miras, I.; Kunst, F.; Beguin, P. Cloning of a genetically unstable cytochrome P-

450 gene cluster involved in degradation of the pollutant ethyl tert-butyl ether by Rhodococcus ruber. J. Bacteriol. 2001, 183, 6551-6557.

(18) Beguin, P.; Chauvaux, S.; Miras, I.; Francois, A.; Fayolle, F.; Monot, F. Genes involved in the degradation of ether fuels by bacteria of the Mycobacterium/Rhodococcus group. Oil & Gas Science and Technology-Revue De L Institut Francais Du Petrole 2003, 58, 489-495.

(19) Fayolle, F.; Vandecasteele, J. P.; Monot, F. Microbial degradation and fate in the environment of methyl tert-butyl ether and related fuel oxygenates. Appl. Microbiol. Biotechnol. 2001, 56, 339-349.

(20) Kolhatkar, R.; Kuder, T.; Philp, P.; Allen, J.; Wilson, J. T. Use of compound-specific stable carbon isotope analyses to demonstrate anaerobic biodegradation of MTBE in groundwater at a gasoline release site. Environ. Sci. Technol. 2002, 36, 5139-5146.

(21) Zwank, L.; Berg, M.; Elsner, M.; Schmidt, T. C.; Schwarzenbach, R. P.; Haderlein, S. B. New evaluation scheme for two-dimensional isotope analysis to decipher biodegradation processes: Application to groundwater contamination by MTBE. Environ. Sci. Technol. 2005, 39, 1018-1029.

(22) Schmidt, T. C.; Zwank, L.; Elsner, M.; Berg, M.; Meckenstock, R. U.; Haderlein, S. B. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. Anal. Bioanal. Chem. 2004, 378, 283-300.

(23) Meckenstock, R. U.; Morasch, B.; Griebler, C.; Richnow, H. H. Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. J. Contam. Hydrol. 2004, 75, 215-255.

(24) Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P. A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. Environ. Sci. Technol. 2005, 39, 6896-6916.

(25) Kuder, T.; Wilson, J. T.; Kaiser, P.; Kolhatkar, R.; Philp, P.; Allen, J. Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: Microcosm and field evidence. Environ. Sci. Technol. 2005, 39, 213-220.

(26) Hunkeler, D.; Butler, B. J.; Aravena, R.; Barker, J. F. Monitoring biodegradation of methyl tert-butyl ether (MTBE) using compound-specific carbon isotope analysis. Environ. Sci. Technol. 2001, 35, 676-681.

(27) Gray, J. R.; Lacrampe-Couloume, G.; Gandhi, D.; Scow, K. M.; Wilson, R. D.; Mackay, D. M.; Lollar, B. S. Carbon and hydrogen isotopic fractionation during biodegradation of methyl tert-butyl ether. Environ. Sci. Technol. 2002, 36, 1931-1938.

(28) Somsamak, P.; Richnow, H. H.; Haggblom, M. M. Carbon isotopic fractionation during anaerobic biotransformation of methyl tert-butyl ether and tert-amyl methyl ether. Environ. Sci. Technol. 2005, 39, 103-109.

(29) Somsamak, P.; Richnow, H. H.; Haggblom, M. M. Carbon isotope fractionation during anaerobic degradation of methyl tert-butyl ether under sulfate-reducing and methanogenic conditions. Appl. Environ. Microbiol. 2006, 72, 1157–1163.

(30) Galimov, E. M. In The biological fractionation of isotopes; Academic Press: Orlando, FL, USA, 1985.

(31) Rayleigh, J. W. S. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 1896, 42, 493-498.

(32) Mariotti, A.; Germon, J. C.; Hubert, P.; Kaiser, P.; Letolle, R.; Tardieux, A.; Tardieux, P. Experimental-Determination of Nitrogen Kinetic Isotope Fractionation - Some Principles - Illustration for the Denitrification and Nitrification Processes. Plant and Soil 1981, 62, 413-430.

(33) O'Leary, M. H. Carbon isotope fractionation in plants. Phytochemistry 1981, 20, 553-567.

(34) Morasch, B.; Richnow, H. H.; Schink, B.; Meckenstock, R. U. Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspects. Appl. Environ. Microbiol. 2001, 67, 4842-4849.

(35) Nijenhuis, I.; Andert, J.; Beck, K.; Kastner, M.; Diekert, G.; Richnow, H. H. Stable isotope fractionation of tetrachloroethene during reductive dechlorination by Sulfurospirillum multivorans and Desulfitobacterium sp strain PCE-S and abiotic reactions with cyanocobalamin. Appl. Environ. Microbiol. 2005, 71, 3413-3419.

(36) Morasch, B.; Richnow, H. H.; Schink, B.; Vieth, A.; Meckenstock, R. U. Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons. Appl. Environ. Microbiol. 2002, 68, 5191-5194.

(37) Mazeas, L.; Budzinski, H.; Raymond, N. Absence of stable carbon isotope fractionation of saturated and polycyclic aromatic hydrocarbons during aerobic bacterial biodegradation. Org. Geochem. 2002, 33, 1259-1272.

(38) Huskey, W. P. In Enzyme mechanism from isotope effects; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, USA, 1991; pp 37-72.

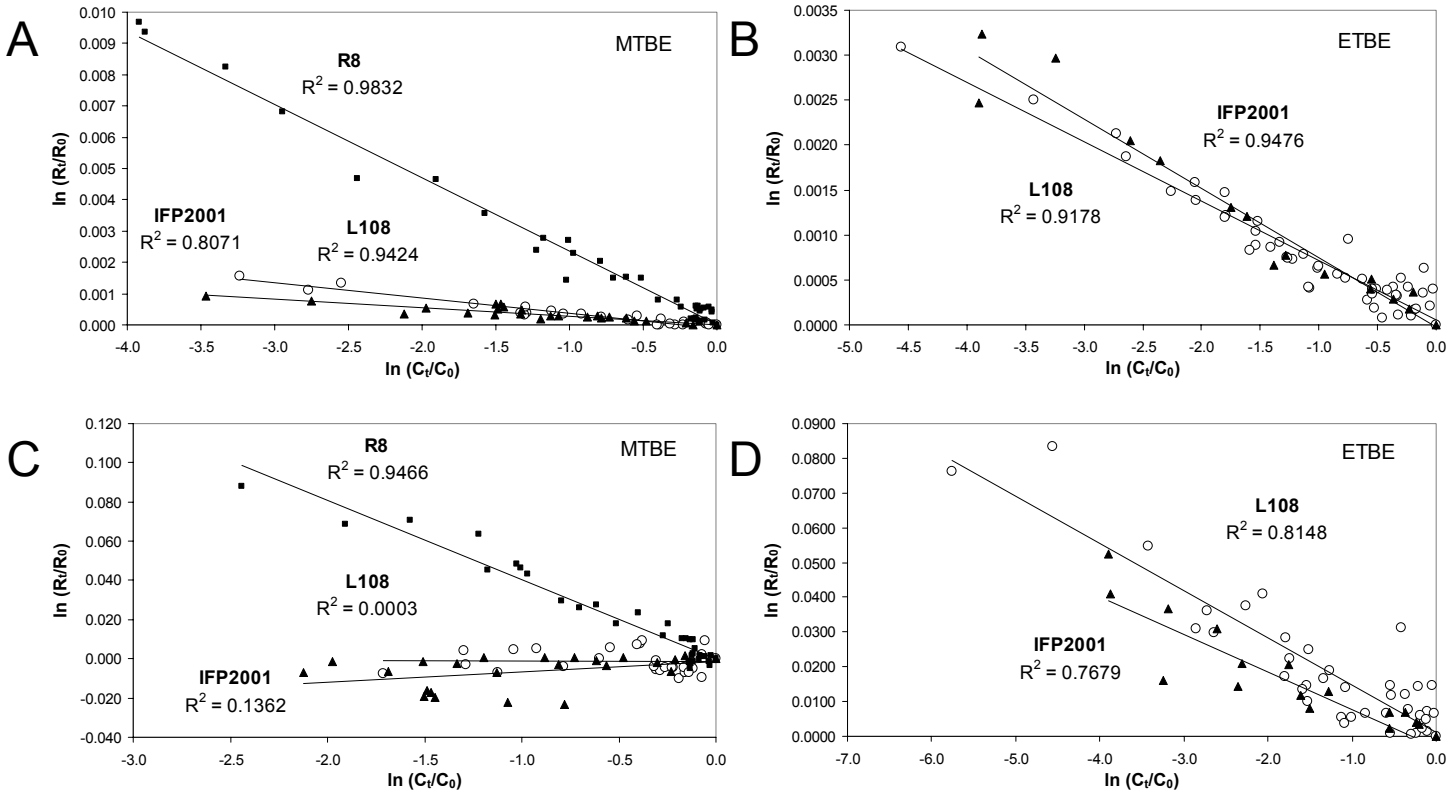

FIGURE 1. Double logarithmic plot according to the Rayleigh equation of the isotopic composition versus the residual concentration of substances (A) Carbon isotope fractionation of MTBE (B) Carbon isotope fractionation of ETBE (C) Hydrogen isotope fractionation of MTBE and (D) Hydrogen isotope fractionation of ETBE; by strain L108 (open circles); strain R8 (solid squares) and strain IFP 2001 (solid triangles). The lines correspond to a linear regression model.

TABLE 1. Comparison of isotopic enrichment factors (ε) and apparent kinetic isotope effects (AKIE) for aerobic biodegradation of MTBE and ETBE. The ε reactive position values were obtained by the approximated equation $\varepsilon_{reactive\ position} \approx n/x \times \varepsilon_{bulk}$ *where n: number of atoms of the element considered that are present in the molecule and x: of them are located at the reactive site. The standard deviation (SD) is given with a confidence interval of ±95% and (N) is the number of samples.*

MTBE

Culture	εC_{bulk} [‰]	SD [‰]	$\varepsilon C_{reactive\ position}$ (n/x = 5/1)	$AKIE_C$	N	εH_{bulk} [‰]	SD [‰]	$\varepsilon H_{reactive\ position}$ (n/x = 12/3)	$AKIE_H$	N	Reference
Enrichment culture (Borden aquifer)	-1.52 to -1.97	0.06	-7.6 to -9.85	1.008 to 1.010	38	na					Hunkeler et al. 2001
VAFB mixed consortium	-1.5 to -1.8	0.1	-7.5 to -9	1.008 to 1.009	55	-29 to -66	3 to 4	-116 to -264	1.53 to 4.81	24	Gray et al. 2002
strain PM1	-2.0 to -2.4	0.1 to 0.3	-10 to -12	1.010 to 1.012	39	-33 to -37	4 to 5	-132 to -148	1.66 to 1.80	26	Gray et al. 2002
strain L108	-0.48	0.02	-2.4	1.002	34	No enrichment (-0.2)	3			29	This work
strain IFP2001 (resting cells)	-0.28	0.03	-1.4	1.001	27	No enrichment (+5)	3			24	This work
strain R8	-2.35	0.05	-11.75	1.012	40	-40	2	-160	1.92	36	This work

ETBE

Culture	εC_{bulk} [‰]	SD [‰]	$\varepsilon C_{reactive\ position}$ (n/x = 6/1)	$AKIE_C$	N	εH_{bulk} [‰]	SD [‰]	$\varepsilon H_{reactive\ position}$ (n/x = 14/2)	$AKIE_H$	N	
strain L108	-0.68	0.03	-4.06	1.004	51	-14	1	-98	1.24	41	This work
strain L108 (resting cells)	-0.76	0.04	-4.56	1.005	10	-11	1	-77	1.18	13	This work
strain IFP2001	-0.73	0.04	-4.38	1.004	16	-11	2	-77	1.18	19	This work
strain R8	not applicable					not applicable					This work

Supporting Information

Variations in $^{13}C/^{12}C$ and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation

Mònica Rosell[1], Damià Barceló[1], Thore Rohwerder[2], Uta Breuer[3], Roland H. Müller[3], Matthias Gehre[4], Hans Hermann Richnow[4]*

[1] Department of Environmental Chemistry, IIQAB-CSIC. Jordi Girona 18-26, 08034 Barcelona, Catalonia, Spain

[2] Aquatic Biotechnology, Biofilm Center, University Duisburg-Essen, Geibelstr. 41, 47057 Duisburg, Germany

[3] Department of Environmental Microbiology and [4] Department of Isotope Biogeochemistry
UFZ-Centre for Environmental Research Leipzig-Halle. Permoserstrasse 15, 04318 Leipzig,Germany

* Corresponding author: phone: +49-341-235-2810, Fax: +49-341-235-2552, e-mail: hans.richnow@ufz.de

1. Bacterial strains and cultivation conditions

Strain L108 is a bacterial isolate from MTBE-contaminated groundwater (Leuna, Germany) (1); it showed 95.6 % identity to the 16S rRNA gene of *Methylibium petroleiphilum* PM1 as the closest MTBE-degrading species. Both strains phylogenetically belong to the β-Proteobacteria (1). Strain R8 was isolated newly in this study from an MTBE-degrading enrichment culture obtained from E. Arvin (DTU, Denmark). The strains L108 and R8 were aerobically cultured in a mineral salt medium on MTBE as sole source of carbon and energy as previously described (1). Contrary to strain L108 (1), productive growth on ETBE was not observed with strain R8.

The identification of strain R8 was based on 16S ribosomal DNA nucleotide sequence. A partial 16S rRNA gene sequence of that strain was determined as previously described (2). The BLASTN program (http://www.ncbi.nlm.nih.gov/blast) (3) was used to search for similar sequences in the nucleotide sequence databases GenBank, EMBL, and DDBJ respectively, and the Sequence Match tool was used to search for similar sequences compiled by the Ribosomal Database Project II Release 9 (http://rdp.cme.msu.edu) (4). The sequence was submitted to GenBank (DQ786030). Strain R8 shows very similar 16S rRNA pattern to PM1 (99 % identity) and we suggest it to belong to the same genus (*Methylibium sp.* R8).

Rhodococcus ruber IFP 2001 was provided by F. Fayolle (IFP, France) and was cultured in the above-mentioned mineral salt medium on ETBE. For isotope fractionation experiments with

fuel oxygenate-growing cells, bacteria were aerobically incubated with 50 mL mineral salt solution in 240-mL serum bottles sealed with butyl rubber stoppers. MTBE and ETBE were added from aqueous stock solutions at final concentrations of 100-500 mg/L. Generally, a 2 % (v/v) inoculum was applied and cultures were incubated on a rotary shaker at 30 °C. One bottle without inoculum was used as negative control in each series.

2. Stable Isotope analysis

Stable isotope composition was determined using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry system (GC-C-IRM-MS). The system consisted of a GC (6890 series; Agilent Technology) coupled with a combustion or high temperature pyrolysis interface (GC-combustion III or GC/C-III/TC; ThermoFinnigan, Bremen, Germany) to a MAT 252 IRMS for the carbon and to a MAT 253 IRMS for hydrogen analysis (both from ThermoFinnigan, Bremen, Germany). A GC-column ID-BPX5 (50 m × 0.32 mm ID, 0.5 µm film thickness; SGE, Darmstadt, Germany) was used for separation. Helium at a flow rate of 1.5 mL/min was used as carrier gas. The GC temperature program was held at 70°C isothermally for 60 min for injecting manually every 4 min; afterwards the temperature increased to 220°C at a rate of 20°C/min, and held for 2 min isothermally for cleaning purposes. Samples were injected by µL-Lock valve-gastight syringe (SGE, Darmstadt, Germany) into a split/splitless injector held at 250°C. Headspace injection volume and split mode were set based on the concentration of MTBE or ETBE determined previously according to sensitivity of carbon or hydrogen isotope analysis. For the carbon measurements, headspace volumes ranging from 30 to 100 µL were injected with split ratios set at 1:1–1:3; whereas for the analysis of hydrogen, headspace volumes ranging from 100 to 1000 µL and were injected in splitless mode. All samples were measured in at least three replicates for carbon and hydrogen isotope signatures. This headspace method had detection limits for MTBE and ETBE of approximately 3 mg/L for carbon and 8 mg/L for hydrogen. The error associated to the system (accuracy and reproducibility) was ±0.5‰ for carbon and ±4‰ for hydrogen.

3. Short-term experiments with resting cells

In case of strains L108 and IFP 2001, isotope fractionation was also determined with resting cells. For this purpose, bacteria were grown on either MTBE (strain L108) or ETBE (both strains) and harvested by centrifugation (6000 x g, 4 °C, 10-15 min). Cells were washed twice with mineral salt solution and finally suspended at 1-2 g biomass (dry weight) per liter. For each experiment, 50 mL cell suspension supplemented with MTBE or ETBE at 100-200 mg/L was incubated in 240-mL serum bottle on a rotary shaker at 30 °C. The degradation was monitored over an 8-h period in which up to 99.9% of the substrate was decomposed. In experiments with strain IFP 2001 and MTBE, glucose was added as supplementary substrate at a final concentration of 350 mg/L enabling co-metabolic degradation of the fuel oxygenate.

4. Results

Concentration Profiles. The time course of MTBE and ETBE degradation of the strain L108 under aerobic conditions was studied (Figure S1). With initial MTBE concentrations from 200 to 500 mg/L, up to 15 days were necessary for 99.9% of degradation. ETBE was degraded within the same time period, although it was applied at lower concentrations between 70-250 mg/L. ETBE was not degraded by strain R8 and for similar initial MTBE concentrations as in experiments with L108, approximately twice of the time was required. In 26 days this strain degraded about 500 mg/L to 99.9% (data not shown). Conversely, strain IFP 2001 (that only grows on ETBE) was found to be the most effective. In less than 5 days 250 mg/L of ETBE were consumed. As indicated in previous studies a concomitant accumulation of TBA was detected (5). During each experiment, MTBE and ETBE concentrations remained constant in negative controls. In addition, TBA was not detected under sterile conditions.

Carbon Isotopic Profiles. The initial $\delta^{13}C$ for MTBE and ETBE (-29.4 ±0.03‰ and -24.3 ±0.1‰, respectively) were calculated as the average of three different samples at time zero and these values remained constant in the negative controls during the whole period of incubation. In live cultures, $\delta^{13}C$ of MTBE and ETBE increased as biodegradation proceeded (as shown in Figure 1 for three batch experiments with strain L108), indicating the enrichment of ^{13}C in the residual compound fraction. The investigation of ETBE fractionation during degradation by strain R8 was not possible, since the strain was not able to grow on ETBE.

References of the Supporting Information

(1) Rohwerder, T.; Breuer, U.; Benndorf, D.; Lechner, U.; Müller, R. H. The alkyl tertiary butyl ether intermediate 2-hydroxyisobutyrate is degraded via a novel cobalamin-dependent mutase pathway. Appl. Environ. Microbiol. 2006, 72, 4128-4135.

(2) Kleinsteuber, S.; Riis, V.; Fetzer, I.; Harms, H.; Muller, S. Population Dynamics within a Microbial Consortium during Growth on Diesel Fuel in Saline Environments. Appl. Environ. Microbiol. 2006, 72, 3531-3542.

(3) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 1997, 25, 3389-3402.

(4) Cole, J. R.; Chai, B.; Marsh, T. L.; Farris, R. J.; Wang, Q.; Kulam, S. A.; Chandra, S.; McGarrell, D. M.; Schmidt, T. M.; Garrity, G. M.; Tiedje, J. M. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 2003, 31, 442-443.

(5) Hernandez-Perez, G.; Fayolle, F.; Vandecasteele, J. P. Biodegradation of ethyl t-butyl ether (ETBE), methyl t-butyl ether (MTBE) and t-amyl methyl ether (TAME) by Gordonia terrae. Appl. Microbiol. Biotechnol. 2001, 55, 117-121.

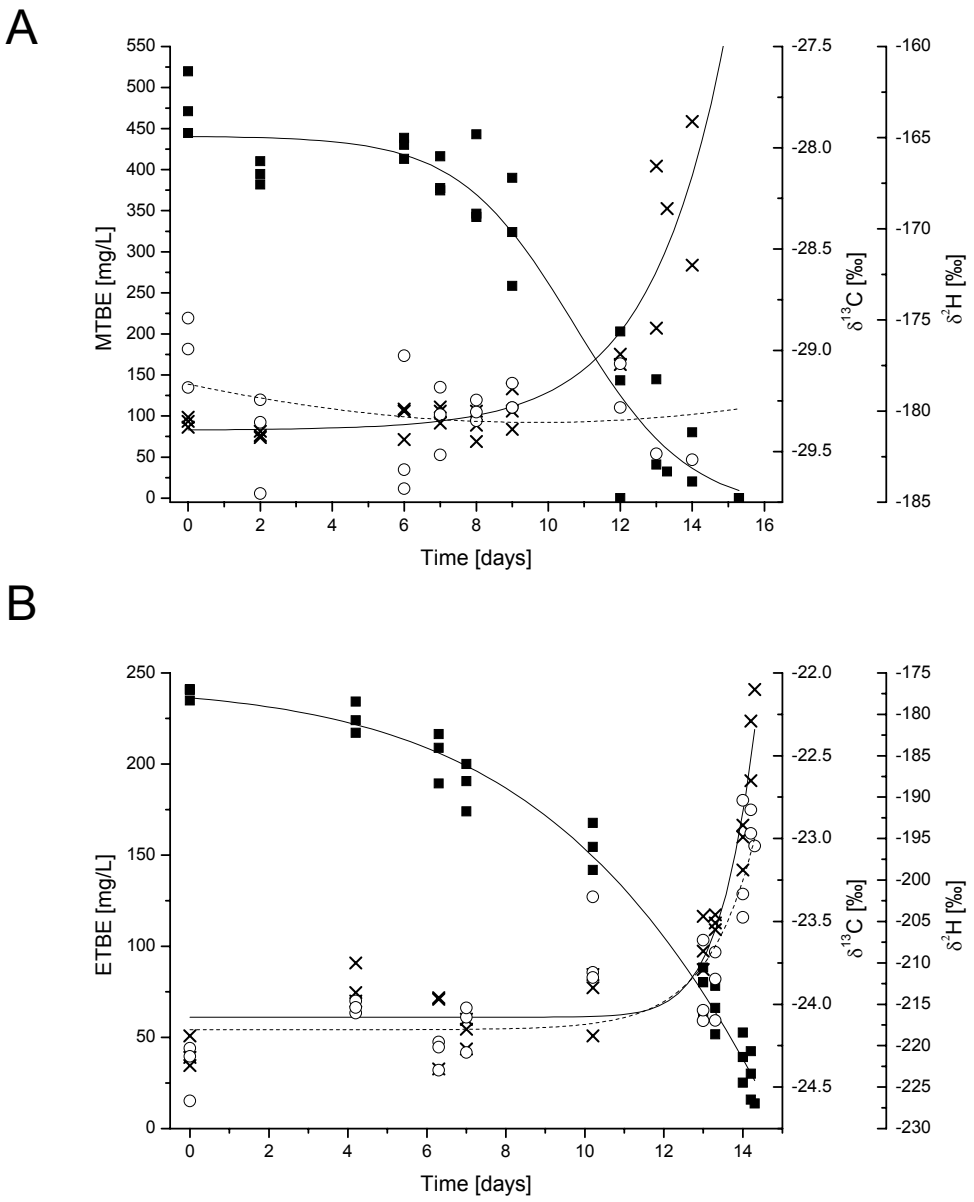

FIGURE S1. Monitoring of ether oxygenates biodegradation, (A) MTBE and (B) ETBE, by strain L108 in three batch experiments following the concentrations (solid squares) and the isotope signatures, $\delta^{13}C$ (crosses) and $\delta^{2}H$ (circles).

4.3 TREBALL EXPERIMENTAL ADDICIONAL: AVALUACIÓ DE LA BIODEGRADACIÓ DE MTBE *IN SITU* A UNA REFINERIA DE L'ALEMANYA DE L'EST

Es van agafar 21 mostres d'aigua subterrània (16 procedents de pous convencionals i 5 a través d'un pou multinivell) per a l'avaluació de l'estat de la biodegradació *in situ* del MTBE als terrenys d'una refineria en funcionament a l'Alemanya de l'est.

Es van trobar elevades concentracions del compost al llarg de la ploma de contaminació. A partir d'uns 100 m de la font de contaminació, es van quantificar de 0,9 fins a 7.200 mg/L de MTBE, i fins i tot podia observar-se (i olorar-se) MTBE en fase lliure per sobre de la capa d'aigua freàtica.

Els paràmetres mesurats al camp, mostraven unes marcades condicions d'anaerobiosi a les aigües subterrànies amb presència de nitrats i sulfats, com a possibles acceptors d'electrons.

Les mostres es van agafar amb ampolles de 250 mL de vidre amb NaCl per a la completa saturació de la mostra, taps de goma negra i coberta d'alumini per a ser encapsulades (com es mostra a les fotografies de la *Figura 27*).

Figura 27.-Material per la presa de mostres per a l'anàlisi de MTBE per CSIA.

Depenent de la concentració de MTBE a les mostres es van injectar diferents volums de l'espai de cap de les ampolles (escalfades a 60 ºC) als respectius equips de IRMS per a l'anàlisi del isòtops de carboni i hidrogen.

Taula 14.-Resultats de les concentracions de MTBE i els respectius valors de Delta (C i H) mesurats per CSIA (n=3) a les mostres ambientals

Pou	**MTBE conc. [mg L^{-1}]**	**$\delta^{13}C$**	**± STDV**	**$\delta^{2}H$**	**± STDV**
A1	510	-32.785	0.02	-239.492	3.14
A2	550	-32.727	0.02	-238.676	0.46
A3	290	-32.602	0.08	-239.136	1.02
B1	530	-32.742	0.08	-238.312	0.55
B2	750	-32.773	0.04	-234.121	4.38
B3	140	-32.255	0.22	-239.908	1.45
C1	490	-32.826	0.07	-236.443	2.42
C2	600	-32.499	0.21	-233.753	1.14
C3	280	-32.663	0.08	-241.379	0.97
P9	360	-32.757	0.19	-243.704	1.17
P20	7200	-32.370	0.06	-240.267	0.56
P21	33	-32.929	0.18	-251.468	6.29
P25	0.29	-30.983	0.60	nd	-
P28	310	-32.656	0.07	-242.369	2.78
P29	6000	-32.188	0.05	-232.797	2.02
P30	770	-32.565	0.03	-236.808	0.95

Els resultats de les Deltes es donen a la *Taula 14* i mostren valors molt constants a prop de la font de contaminació (mitjanes: $\delta^{13}C$ = -32.7 ± 0.2‰ i $\delta^{2}H$ = -238.5 ± 3.1‰). Només es va observar un lleuger fraccionament isotòpic al pou P25 pel carboni ($\delta^{13}C$ = -30.98 ± 0.60‰), però que no es va poder comprovar amb l'hidrogen que no es podia mesurar per sobre dels límits de detecció del instrument; però sí al pou multinivell com es mostra a la *Figura 28*.

Tot i això, els resultats d'aquest estudi no van ser prou concloents com per a afirmar que una biodegradació del MTBE estava tenint lloc en aquests terrenys. En aquest sentit, serien necessàries més campanyes de presa de mostres per tal d'observar l'evolució dels valors de concentració respecte els valors Delta (δ).

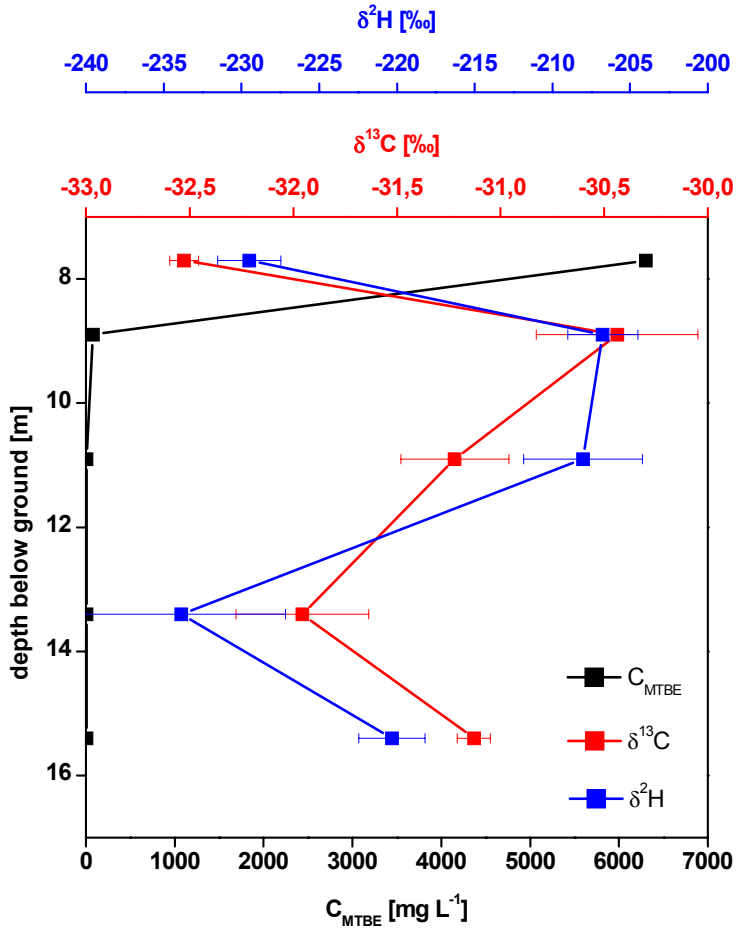

Figura 28.-Fraccionament isotòpic pel C i H observat a diferents profunditats que alhora mostren concentracions de MTBE molt diverses.

4.4 DISCUSSIÓ DELS RESULTATS

Els estudis de degradació del MTBE i el ETBE han demostrat que la biodegradació d'aquests additius oxigenats a partir de cultius purs és possible en períodes de temps factibles (de 5 a 20 dies) en les condicions d'incubació estudiades (presència d'oxigen, minerals, vitamines, 30 ºC, etc.). No només això, el cultiu de L108 en grans bioreactors (400 L) sota condicions òptimes controlades (temperatura, oxigen, pH, etc.), ja s'estudia com a alternativa per a la seva aplicació al camp [189].

No obstant, quan es van provar cultius mixtes extrets del Leuna (resultats no mostrats en l'Art. 6), els períodes d'activació dels microorganismes augmentaven considerablement (algunes ampolles van estar més de 300 dies a la incubadora). Tot i això, quan la degradació es donava, els factors de fraccionament resultants eren força semblants als obtinguts pels cultius purs pel carboni (εC =-0,71 ±0,6‰), però lleugerament superiors per l'hidrogen (εH =-11 ±2‰).

Aquestes discrepàncies en els factors de fraccionament comporten finalment dificultats pràctiques per a la seva aplicació al camp quan es vol quantificar la biodegradació *in situ* de MTBE a partir dels valors de concentració i $\delta^{13}C$ obtinguts en mostres reals. Per exemple, utilitzant els resultats observats al pou multinivell P26 del lloc contaminat estudiat.

El percentatge de biodegradació (B) es pot calcular com:

$$B[\%] = \left(1 - \left(\frac{R_t}{R_0}\right)^{\left[\frac{1}{\left(\frac{1}{\alpha}-1\right)}\right]}\right) \times 100$$

On α és el factor de fraccionament isotòpic i el quocient de les raons isotòpiques (R) en el temps (t) respecte el temps zero (0) es calcula com:

$$\frac{R_t}{R_0} = \frac{(\delta_t + 1000)}{(\delta_0 + 1000)}$$

Es va considerar com a signatura isotòpica inicial o Delta a temps zero (δ_0) el valor mitjà trobat a prop de la font de contaminació (-32,7‰).

Tot i que, en principi les condicions trobades a l'aqüífer farien més probable una degradació en condicions anaeròbiques (s'ha pres el valor mitjà trobat per Somsamak et al. [187]), a la *Taula 15* també es mostren els càlculs de la biodegradació amb el factor de fraccionament aeròbic mitjà proposat per Zwank et al. [180] i basat en els trobats anterioritat per Hunkeler et al. [182] i Gray et al. [183] en condicions aeròbiques i el que s'ha trobat en la present tesi amb els experiments amb L108 (**Art. 6**). Aquesta comparació serveix per fer evident les grans diferències entre la quantificació entre uns i altres factors. El que en condicions anaeròbiques només significaria una biodegradació menor del 15%, considerant condicions aeròbiques arribaria al 72% i en el cas del L108 fins un 99%.

Taula 15.-Càlcul de la biodegradació in situ de MTBE a partir de diferents factors de fraccionament

Fondària pou multinivell P26 (m)	Conc. MTBE (mg/L)	$\delta^{13}C$ (‰)	**Biodegradació calculada (B, %)**		
			Anaeròbica	***Aeròbica***	
			α= 1,0144 [187]	α = 1,00182 [180]	α = 1,0005 (soca L108)
7.7	6300	-32,53	1	9	30
8.9	80	-30,44	15	72	99
10.9	3,50	-31,22	10	57	95
13.4	8,00	-31,96	5	35	79
15.4	3,90	-31,13	11	59	96

De fet, si apliquem la teoria de l'anàlisi de les dues dimensions isotòpiques (la signatura del carboni respecte la de l'hidrogen) desenvolupada per Zwank et al. [180] per tal de diferenciar la via de degradació principal amb tots els punts estudiats al camp, ens trobem una bona correlació (R^2 = 0,8), com es mostra a la *Figura 29*. El pendent aproximadament d'un valor de 15, s'aproxima més als valors trobats pels estudis amb cultius aeròbics (PM1=15,8 i R8=14,8) que no pas a l'únic estudi que presenta ambdues mesures (C, H) en anaerobiosi amb un pendent de 1,2 [185].

Es demostra així que mesurar l'estat de la biodegradació del MTBE al camp en aquestes circumstàncies es fa molt difícil i pot comportar un gran marge d'error, més encara quan es poden trobar casos en els quals les condicions d'oxigen i altres acceptors d'electrons variïn al llarg de la ploma de contaminació o en el temps.

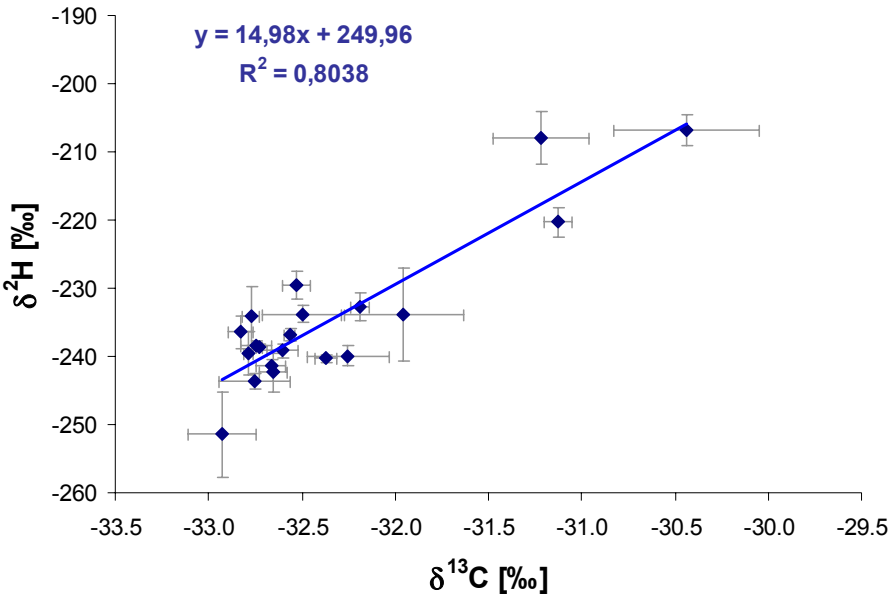

Figura 29.- Correlació de les signatures isotòpiques (δ) del carboni respecte l'hidrogen trobades al camp

Les línies d'investigació actual s'han adreçar, en primer lloc a un coneixement més exhaustiu dels mecanismes metabòlics que fan servir els microorganismes en cultius purs i en comparació amb cultius mixtes en condicions controlades de laboratori i d'altra banda, en el desenvolupament de noves tècniques que serveixin per esbrinar què està passant al camp (*in situ*) i quins són els microorganismes que estan prenent part en la degradació en cas de donar-se.

Actualment han aparegut metodologies avançades per a la identificació dels processos de degradació i assimilació de substrats o compostos d'estudi enriquits isotòpicament amb ^{13}C com les anomenades tècniques SIP (*Stable Isotope Probing*). Aquestes tècniques es basen en el fet que la transformació del compost amb ^{13}C derivarà amb

productes de degradació més pesats que acabaran incorporats a la biomassa o bioindicadors cel·lulars específics com són els àcids grassos o els àcids nucleics (ADN, ARN) i que es poden fer servir per a la identificació dels microorganismes responsables de la biodegradació al camp.