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Carbon isotope fractionation during aerobic biodegradation of *n*-alkanes and aromatic compounds in unsaturated sand

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Abstract

Microcosm experiments were conducted to quantify carbon isotope fractionation during aerobic biodegradation of *n*-alkanes (from C_3 to C_{10}) and monoaromatic hydrocarbons in unsaturated alluvial sand. In single compound experiments with *n*-alkanes, the largest enrichment factor was obtained for propane (-10.8 ± 0.7‰). The magnitude of the enrichment factor decreased with increasing number of carbon atoms from propane to *n*-decane (-0.2 ± 0.1‰). This trend can partly be explained by the decreasing probability that a ^{13}C is located at the reacting site in the molecule with increasing chain length. After correcting for the presence of non-reacting positions, a chain length dependence of the calculated apparent isotope effect persisted. This observation suggests that transport and binding steps before the actual reaction step become increasingly rate limiting with increasing chain length. For aromatic compounds tested individually, the enrichment factor was the largest (-1.4 ± 0.1‰) for benzene (B), followed by toluene (T) (-0.8 ± 0.1‰) and *m*-xylene (X) (-0.6 ± 0.1‰). Enrichment factors for BTX were systematically smaller than for *n*-alkanes with equivalent number of carbons, which is likely related to different biodegradation mechanisms. The study demonstrates that significant carbon isotope fractionation occurs during aerobic biodegradation of *n*-alkanes and aromatic compounds under unsaturated conditions and that the magnitude of isotope enrichment is linked to molecule size and molecule structure.

1. Introduction

Strategies for remediation of petroleum hydrocarbon contaminated soil and groundwater increasingly rely on natural attenuation (Hinchee et al., 1995; NRC, 2000). Usually intrinsic biodegradation by indigenous microbial populations is the key process for contaminant elimination and methods are required to demonstrate its efficacy. In groundwater studies, compound-specific isotope analysis (CSIA) has been used increasingly to demonstrate *in situ* biodegradation of various types of organic contaminants (Kelley et al., 1997; Sturchio et al., 1998; Hunkeler et al., 1999; Sherwood Lollar et al., 2001; Kolhatkar et al., 2002; Mancini et al., 2002; Meckenstock et al., 2002; Song et al., 2002; Kirtland et al., 2003; Richnow et al., 2003a,b; Chu et al., 2004; Griebler et al., 2004; Steinbach et al., 2004; Hunkeler et al., 2005; Morrill et al., 2005). In contrast, only a few studies have investigated the use

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of CSIA in the unsaturated zone (Stehmeier et al., 1999; Kirtland et al., 2005).

The stable isotope method relies on a faster cleavage of chemical bonds between two light isotopes compared to bonds between a light and a heavy isotope. During biodegradation of hydrocarbon molecules, the slightly faster cleavage of ^{12}C–H bonds results in an accumulation of molecules with ^{13}C–H in the remaining substrate. The magnitude of ^{13}C accumulation is compound specific as the isotope fractionation depends on the type of bond that is broken, the reaction mechanism, and the extent of bond cleavage in the transition state (Gandour and Schowen, 1978; Huskey, 1991). It also depends on the molecule size. For larger molecules, the heavy isotope is located more frequently at a position other than the site where initial bond cleavage takes place, and hence no isotope effect occurs. Thus, the non-reactive positions "dilute" the isotope effect. Since no bond cleavage occurs during phase transfer processes, isotopic fractionation is expected to be absent or very small during sorption, dissolution, and volatilisation of compounds, as shown by several studies (Dempster et al., 1997; Harrington et al., 1999; Huang et al., 1999; Poulson and Drever, 1999; Slater et al., 1999; Hunkeler and Aravena, 2000). A small sorption isotope effect was recently reported for benzene and toluene in an organic matter rich matrix (Kopinke et al., 2005).

Numerous batch and column studies report isotope fractionation factors for benzene, toluene and xylene (BTX) under aerobic and anaerobic conditions (Meckenstock et al., 2004; Schmidt et al., 2004; Elsner et al., 2005). In contrast, fewer data are available on isotope fractionation during biodegradation of short chain alkanes (C_3 to C_9), which are common gasoline constituents (Riser-Roberts, 1998). A ^{13}C enrichment during biodegradation of short *n*-alkanes (C_1 and C_3) has been observed by Lebedew et al. (1969) and later by Stahl (1980) for chain lengths from C_3 to C_6 and by George et al. (2002) for chain lengths from C_5 to C_9. However, the reported data in the latter two studies do not allow calculation of enrichment factors for individual compounds. Studies that quantify isotope fractionation of individual compounds have focussed on methane, on *n*-alkanes with chain lengths $>C_{13}$ (Mansuy et al., 1997; Mazeas et al., 2002) and recently on *n*-alkanes from C_1 to C_4 (Kinnaman et al., 2007). The isotope enrichment factors reported during aerobic oxidation of methane were found to be dependent on temperature (Coleman et al., 1981; King et al., 1989), soil water content (Tyler et al., 1994), partial pressure of CH_4, cell numbers and type of methanotrophs (Templeton et al., 2006).

The aim of this study was to determine the carbon isotope enrichment factors during aerobic degradation of several aliphatic hydrocarbons (from C_3 to C_{10}) in soil microcosms with an indigenous micro-organism population under unsaturated experimental conditions and to evaluate how isotope enrichment factors vary as a function of chain length. Enrichment factors for BTX compounds were determined to compare the variations in carbon isotope fractionation between saturated and aromatic hydrocarbons having the same number of carbons. Unsaturated conditions were chosen because compound concentrations in the gas phase are greater than in dissolved media for many of the selected compounds facilitating isotope ratio measurement and in view of using isotope analysis to evaluate biodegradation in the unsaturated zone of contaminated sites.

2. Materials and methods

2.1. Microcosm experiments

Biodegradation of organic contaminants was carried out in serum bottles of 63 ml volume. Alluvial sand from Lake Geneva near the Rhone River delta, Switzerland, was used, which had been shown to degrade diverse petroleum hydrocarbons (Höhener et al., 2003). The microbial and physical properties of this sand were described by Pasteris et al. (2002). Prior to the experiments, the sand was amended with NH_4NO_3 and K_2HPO_4 (Fluka, Buchs, Switzerland) based on C:N and C:P ratios of 25:1 and 250:1, respectively (Zhou and Crawford, 1995) to make sure that the nutrients were not rate limiting. The soil water content measured prior the experiment was below 10% w/w. The sand was compacted as much as possible in the bottles, which were then closed with Mininert® valves (VICI, Schenkon, Switzerland), avoiding any change in water saturation. Experiments were carried out at 23 °C. The first gas sample was taken after waiting for at least 15 min once the compounds had been added. Preliminary experiments with sterile sand demonstrated that this period is sufficient to reach stable concentrations. Furthermore, the calculated characteristic diffusion length $x = \sqrt{D \cdot t}$, where D is the diffusion coefficient and t the time, amounts

to 7 cm after 15 min for the most slowly diffusing compound, which corresponds to the height of the bottles.

2.2. Compounds

The gaseous compounds propane (C_3) and *n*-butane (C_4) were obtained from Messer (Lenzburg, Switzerland). All other liquid compounds were obtained from Fluka (Sigma–Aldrich, Switzerland) in their purest form. Biodegradation of *n*-alkanes (C_3, C_4, C_5, C_6, C_7, C_8 and C_{10}) and BTX was first tested individually in bottles containing the compound of interest and a tracer in order to check for gas leaks. For C_3 and C_4 *n*-alkanes, 0.5 mg of compounds was injected simultaneously with methane as tracer, which was previously shown not to degrade within the time frame of the experiments. For longer chain *n*-alkanes (C_5 to C_8) and for BTX, 0.6 mg of gaseous compound was injected simultaneously with isooctane (2,2,4,-trimethylpentane) as a tracer. For C_{10}, only 0.1 mg was added due to its low vapour pressure. In every microcosm, the quantity of O_2 was high enough to sustain biodegradation under oxic conditions throughout the experiments. Injections and sampling were performed using gas tight syringes (VICI, Schenkon, Switzerland). Each compound was tested in duplicate and repeated twice.

Biodegradation experiments with two different hydrocarbon mixtures were also carried out. Mixture 1 (called the 5-compound mixture) consisted of five typical constituents of gasoline (*n*-hexane, *n*-octane, benzene, toluene, and *m*-xylene). Mixture 2 (called the *n*-alkane mixture) consisted of four *n*-alkane compounds (*n*-pentane, *n*-hexane, *n*-octane, and *n*-decane). Both liquid mixtures were prepared in flasks with a large headspace. The proportion of compounds in the liquid phase was chosen such that equal partial pressures of each compound in the gas phase were obtained. Ten ml of vapour of each mixture was taken from the headspace and injected in the microcosm along with a tracer. Approximately 0.2 mg of each compound was added in the microcosm with the 5-compound mixture and, due to the low vapour pressure of *n*-decane, only 0.1 mg of each compound was added with the *n*-alkane mixture. Experiments were conducted in duplicate and repeated twice for each mixture. For control microcosms, the same procedure as described above was followed with sand autoclaved three times at 120 °C for 20 min within a three day period, and with an additional 2 ml of NaN_3 (10% solution) to ensure total inhibition of microbial activity. Two control microcosms were prepared for each type of incubation to determine whether processes other then biodegradation can change the isotope ratio.

2.3. Concentration and isotope analysis

Gas samples were periodically taken from the microcosms and the $\delta\ ^{13}C$ values of the compounds were determined using a TRACE™ gas chromatograph (GC) coupled to an isotope ratio mass spectrometer (IRMS) via a ThermoFinnigan™ GC combustion III interface. Injections were performed using a loop injector and varied from 10 to 250 µl to ensure a minimum delivery of 1 nmol of carbon to the column. The carrier gas was helium with a flow rate of 1.7 ml min^{-1} and the oxidation and reduction reactors were set to 940 °C and 650 °C, respectively. Isotopic ratios are reported in the δ notation relative to VPDB standard (Clark and Fritz, 1997):

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{reference}} - 1\right)1000 \quad (1)$$

where R_{sample} and $R_{reference}$ is the $^{13}C/^{12}C$ ratio of the measured sample and the reference material, respectively. Concentrations of the biodegradable substrate and the tracer were determined based on the combined peak area of all three CO_2 mass ions (44, 45 and 46) for every sample. Concentrations of biodegradable substrate were then normalised with respect to the concentration of the tracer. The reproducibility of the concentration measurements was ±5% and the average standard deviation of the mean for carbon isotopic ratio was ±0.2‰ (n = 12). Measurements were performed until less than 10% of the compound was remaining in the microcosm for pure compound experiments (<30% for propane) and less than 20% for experiments with mixtures. Isotope enrichment factors were quantified using the Rayleigh type evolution model for closed system according to (Clark and Fritz, 1997):

$$R_{sample} = R_0(C_{sample}/C_0)^{\alpha-1} \quad (2)$$

Rearrangement of Eq. (2) yields:

$$\ln(R_{sample}/R_0) = (\alpha - 1)\ln(C_{sample}/C_0 \quad 3)$$

where R_0 is the initial $^{13}C/^{12}C$ ratio, α is the fractionation factor, C_0 and C_{sample} is the initial concentration and the remaining concentration at time t, respectively. The fractionation factor was obtained

by plotting $\ln(R_{sample}/R_0)$ versus $\ln(C_{sample}/C_0)$, which yields $\alpha - 1$ as the slope of the linear regression. The fractionation factor α is more conveniently expressed as enrichment factor (ε) according to:

$$\varepsilon = (\alpha - 1) * 1000 \quad (4)$$

The kinetic isotope effect (KIE) refers to the variation in reaction rate between molecules with ^{12}C and ^{13}C at the reactive position, respectively. Using k to represent the degradation rate coefficient (first order), KIE is defined as:

$$\mathrm{KIE} = {}^{l}k/{}^{h}k \quad (5)$$

where "l" and "h" stand for light and heavy isotope at the reactive position, respectively. In enzymatic processes, the actually observed isotope effect (denoted as apparent kinetic isotope effect, AKIE) is frequently lower than the KIE because binding steps that precede bond cleavage can be rate limiting. In addition, the presence of atoms at non-reactive positions reduces the magnitude of the observed isotope effect as discussed above. Elsner et al. (2005) proposed an equation to correct for the effect of non-reactive positions and to calculate AKIE:

$$\mathrm{AKIE} \approx \frac{1}{1 + n\varepsilon/1000} \quad (6)$$

where n is the number of atoms of interest composing the molecule. This equation allows comparing the KIE between any molecules, independently of the size. However, the equation is only valid if the initial transformation step only involves a single atom of the element of interest.

3. Results

3.1. Biodegradation of compounds

The concentration decreases during aerobic biodegradation of *n*-alkanes (C_3, C_4, C_5, C_6, C_7, C_8 and C_{10}) and aromatic compounds (BTX) in single compound microcosms are presented in Fig. 1 for one representative repetition. The concentration decrease was approximately linear for most of the compounds, suggesting zero order kinetics.

The evolution of concentrations in the microcosm receiving either the 5-compound mixture or the *n*-alkane mixture is presented in Fig. 2. The biodegradation of each compound in both mixtures followed approximately a zero order rate law and a sequential degradation was observed over the course of both experiments. After an acclimation period during the first series of tests (lag phase before occurrence of biodegradation), the 5-compound mixture was degraded with preferential utilisation in the following order: *n*-octane > *n*-hexane > aromatic compounds. The increase of the relative concentration of benzene to a value of more than 1 was likely due to a non-ideal partitioning of the compound between air and water phases. In the *n*-alkane mixture, sequential degradation was observed as well. The concentrations of *n*-decane and *n*-octane decreased to zero within the first 400 min, during which the concentrations of the other compounds varied little. After 400 min, *n*-hexane started to decrease quickly, followed by *n*-pentane.

3.2. Isotope fractionation

For *n*-alkanes, the maximal shift in $\delta\ ^{13}C$ decreased with increasing chain length (Fig. 1). The carbon isotope enrichment factors were calculated using Eqs. (3) and (4) (Table 1). For *n*-alkanes tested individually, the largest enrichment factor was obtained for the shortest *n*-alkane chain (propane, −10.8‰). With increasing chain length, the magnitude of the enrichment factors decreased. The smallest isotope enrichment factor (−0.2‰) was obtained for *n*-decane. It is still significantly different from a slope of zero (Student *t*-test at 99% confidence level). The carbon isotope enrichment factors obtained for *n*-alkanes were found to follow an empirical $\ln(\varepsilon)$ versus carbon number trend (Fig. 3). For individually tested compounds, the value for *n*-pentane (−2.4‰) is slightly smaller than the linear trend (Fig. 3a), while it follows the trend in the microcosm with *n*-alkane mixture (−3.4‰, Fig. 3b). For the aromatic compounds tested individually, benzene showed the largest enrichment factor (−1.4‰) and *m*-xylene the smallest (−0.6‰) (Table 1). In sterile microcosms, no significant changes in isotope ratios were observed for the 5-compound mixture (Fig. 4).

4. Discussion

4.1. Degradation rates and kinetics

The preferential utilisation of *n*-alkanes over aromatic compounds observed in this study is in agreement with previous studies (Bailey et al., 1973; Milner et al., 1977; Stahl, 1980) as well as for the

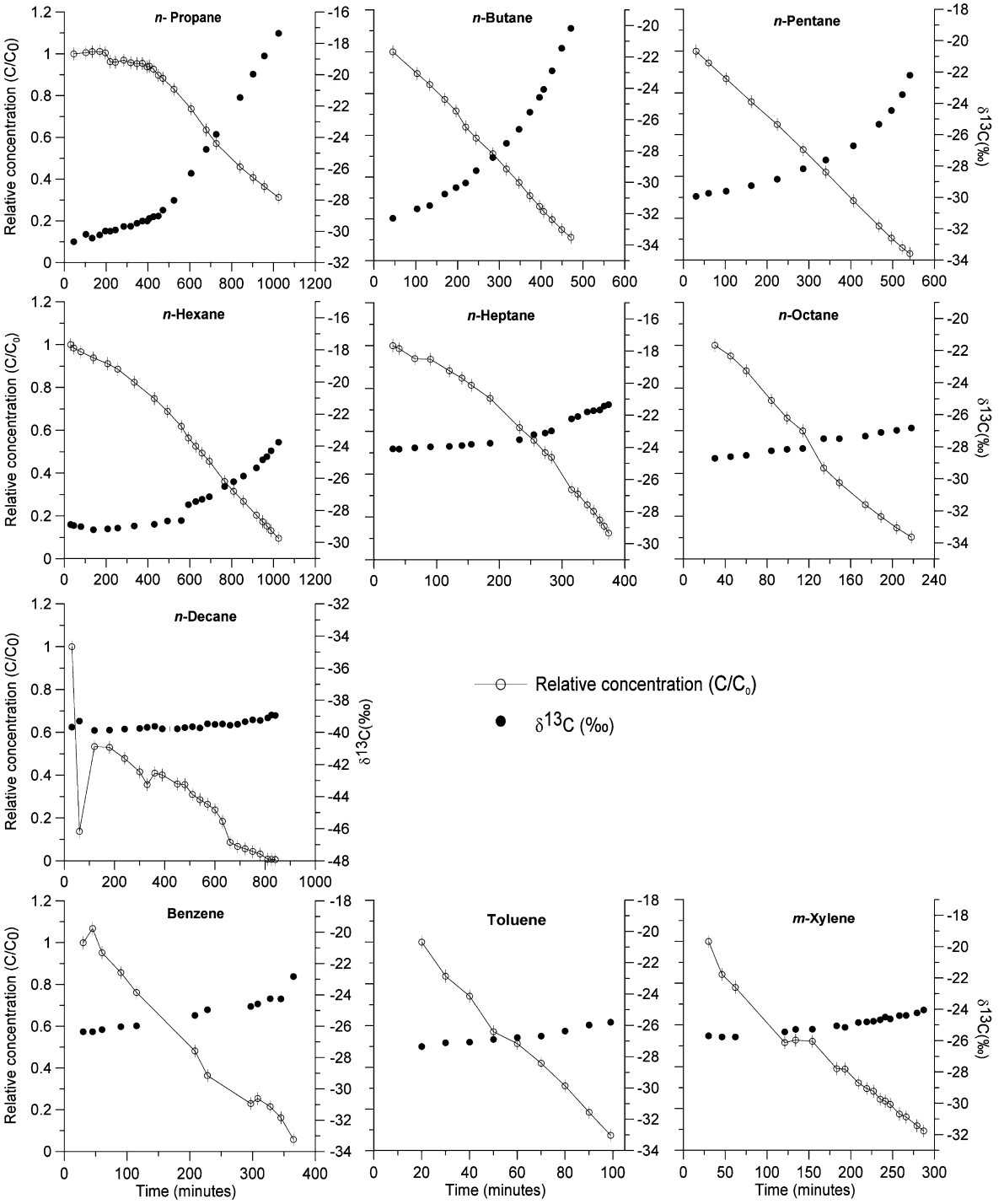

Fig. 1. Relative concentration and evolution of the δ ^{13}C values of the remaining substrate with time during aerobic biodegradation of different *n*-alkanes and monoaromatic hydrocarbons in microcosms.

preferential removal of long *n*-alkane chains over short chains (Stahl, 1980). An increase in biodegradation rates with increasing number of carbon atoms for gaseous *n*-pentane to *n*-decane was observed in previous laboratory (Ostendorf et al., 2000; Höhener et al., 2003) and field studies (Pasteris et al., 2002; Höhener et al., 2006), where first order kinetics were observed when compounds were

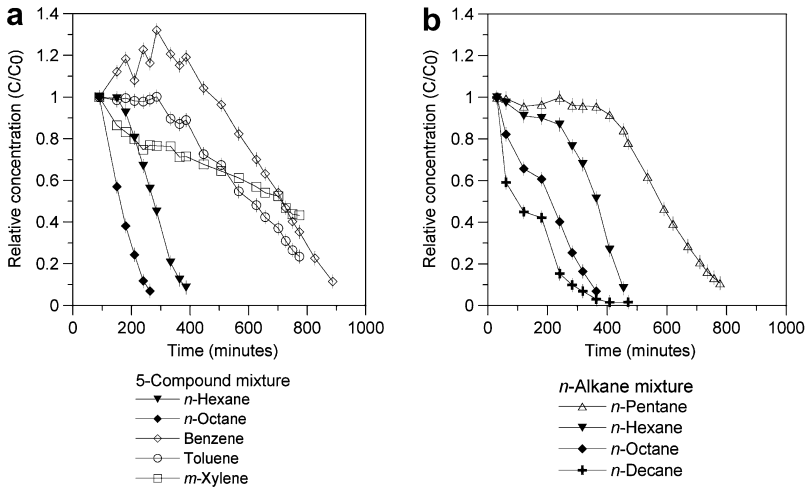

Fig. 2. Concentration evolution of hydrocarbon compounds during aerobic biodegradation of the 5-compound mixture (a) and the *n*-alkanes mixture (b). Compound concentration data are corrected relative to the tracer.

Table 1
Carbon isotope enrichment factors obtained during the biodegradation of saturated and aromatic hydrocarbons under oxic conditions in microcosms

Compound	Carbon enrichment factors (‰)		
	5-compound mixture[a]	*n*-Alkane mixture[a]	Pure compound[a]
Propane	nd	nd	-10.8 ± 0.7
n-Butane	nd	nd	-5.6 ± 0.1
n-Pentane	nd	-3.8 ± 1.3	-2.4 ± 0.2
n-Hexane	-2.2 ± 0.4	-2.4 ± 0.3	-2.3 ± 0.6
n-Heptane	nd	nd	-1.4 ± 0.1
n-Octane	-0.7 ± 0.2	-1.1 ± 0.1	-0.9 ± 0.1
n-Decane	nd	-0.3 ± 0.1	-0.2 ± 0.1
Benzene	-2.1 ± 0.1	nd	-1.4 ± 0.1
Toluene	-0.7 ± 0.1	nd	-0.8 ± 0.1
m-Xylene	-0.8 ± 0.1	nd	-0.6 ± 0.1

[a] Mean of four repetitions ± standard deviation.

at low concentrations. In order to have enough substrate for isotope analysis, experiments in this study were performed with high initial vapour concentrations. Thus, zero order rate kinetics were expected since maximum specific substrate utilisation rates are reached when the vapour concentration C_a is much larger than HK_s (Höhener et al., 2003), where H is the Henry's coefficient, and K_s is the half-saturation constant in the aqueous phase (also denoted as Monod constant). *n*-Alkanes have very low K_s (Höhener et al., 2003), and although they have high H's, the product HK_s is small.

4.2. n-Alkane isotope analysis

Enrichment factors obtained for *n*-alkanes tested either as a single compound or in a mixture pro-

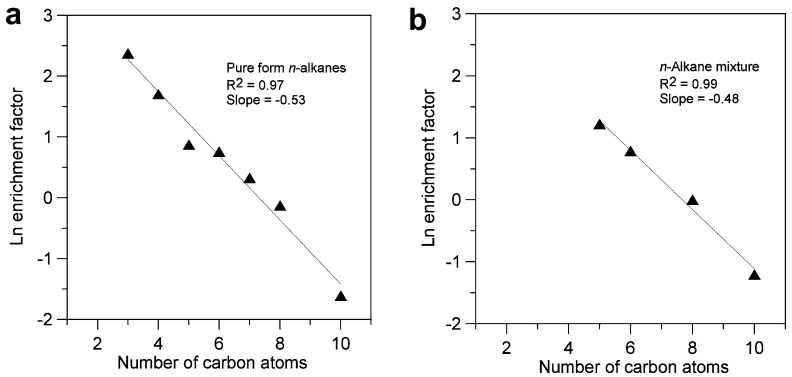

Fig. 3. Relationships between the natural logarithm of the enrichment factors and the number of carbon atoms included in the *n*-alkane chain for experiment with individual compounds (a) and for the *n*-alkane mixture (b).

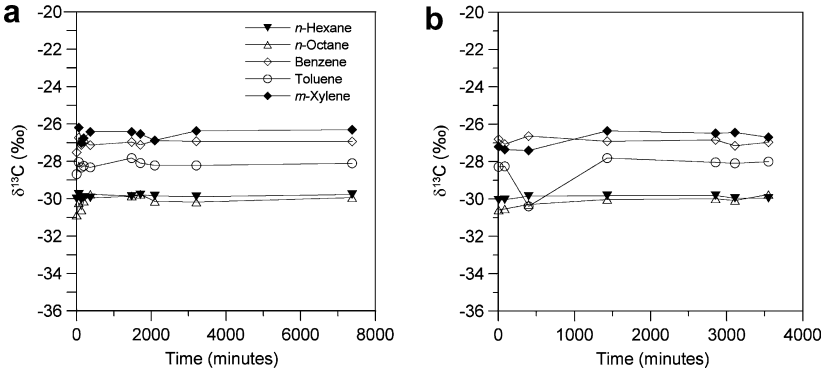

Fig. 4. Evolution of the δ ^{13}C for the 5-compound mixture with sterilized sand in function of time for two replicates.

vided similar values suggesting a similarity between the dominating biodegradation pathways expressed by the active microbial populations of both microcosms. Works of Kinnaman et al. (2007) also showed a similarity between enrichment factors measured during individual and gas mixture incubations for C_3 and C_4. However, enrichment factors for C_3 and C_4 measured in this current study with unsaturated alluvial sand are significantly larger than those measured with marine sediments in Kinnaman et al. (2007), probably due to a different microbial population adapted to saline water in their study. The magnitude of isotope fractionation decreased with increasing number of carbon atoms in the molecule (Fig. 4) can partially be explained by the "dilution" effect. With increasing number of carbon atoms, a ^{13}C is less likely to be involved in the initial enzymatic bond cleavage, which is required to cause an isotope effect. The dilution effect was suggested in George et al. (2002) and now experimentally demonstrated. When correcting for the number of carbon atoms, a relatively large range of AKIE values reaching from 1.002 to 1.033 is obtained (Table 2). This indicates that factors other than dilution of the isotope effect by non-reacting positions are responsible for the observed trend to smaller isotope enrichment factors for larger molecules. For small molecules, the AKIE corresponds well to the expected KIE, which is in the range of 1.021 for C–H bond cleavage according to the Streitwieser approximation (Elsner et al., 2005). Microbial oxidation reactions are catalysed either by P450 monooxygenase or by multiprotein monooxygenase systems (Berthe-Corti and Fetzner, 2002) and a direct H abstraction mechanism is expected for saturated hydrocarbons (Yoshizawa, 2002). However, the AKIE values for larger molecules are significantly lower.

Table 2
Apparent kinetic isotope effect calculated for biodegradation of n-alkanes for this study as well as from Kinnaman et al. (2007), and for chemical oxidation from Anderson et al. (2004)

Compound	AKIE		
	Biodegradation this study	Biodegradation Kinnaman et al. (2007)	Chemical oxidation Anderson et al. (2004)
Methane	nd	1.027	nd
Ethane	nd	1.021	1.017
Propane	1.033	1.013	1.014
n-Butane	1.022	1.010	1.013
n-Pentane	1.019[a]	nd	1.014
n-Hexane	1.013	nd	1.021
n-Heptane	1.010	nd	1.017
n-Octane	1.007	nd	1.017
n-Decane	1.002	nd	nd

[a] Using value obtained with *n*-alkane mixture.

The trend to smaller AKIE for larger molecules could be due to rate limiting steps before the actual transformation step other than phase transfer processes such as sorption, dissolution, and volatilisation of compounds for which no or very small isotopic fractionation was shown (Dempster et al., 1997; Harrington et al., 1999; Huang et al., 1999; Poulson and Drever, 1999; Slater et al., 1999; Hunkeler and Aravena, 2000). Biodegradation of compounds is a multi-step procedure (O'Leary and Yapp, 1978) that includes transport of the compound into the cell (step 1), binding of the substrate to the enzyme (step 2), transformation at the reaction centre (step 3) and finally, dissociation of the product with the enzyme (step 4). Although transport and binding steps are generally assumed not to be associated with significant carbon isotope fractionation, they influence the AKIE value indirectly.

If the actual bond cleavage step is fast compared to the preceding transport steps, nearly each molecule that reaches the enzyme is transformed irrespective of its isotopic composition and no isotope fractionation is apparent. In contrast, if reversible transport and binding is fast compared to bond cleavage, the isotope discrimination associated with bond cleavage becomes fully apparent outside of the cell. Hence, the different AKIE values for different molecules could reflect a varying influence of transport and binding steps. The smaller AKIE for larger molecules could be due to an increasing influence of slower transport and binding on the reaction rate due to their large size. An approximately linear relationship between AKIE-1 and the square root of the molecule mass is observed (data not shown) suggesting that rate limiting transport by diffusion could explain the observed AKIE trend.

Unlike enzymatic transformation, homogeneous chemical processes have no potentially slow step preceding the initial bond cleaving step (Paneth, 1994) and, hence, should be less affected by molecule size effects other than dilution. To evaluate this hypothesis, the enrichment factors of this study were compared with those measured during gas phase reactions with OH radicals. The magnitude of enrichment factors obtained by chemical reactions (reported in ‰ according to $\varepsilon = (k_{13}/k_{12} - 1) * 1000$) were decreasing from C_2 (−8.57‰) to C_8 (−2.13‰) (Anderson et al., 2004). Once transformed to AKIEs, the obtained values become independent of the chain length unlike the AKIE values determined in this study and calculated based on the study by Kinnaman et al. (2007), which retain a chain length dependence (Table 2). This comparison emphasizes the indirect effect of transport and binding steps in biological processes on the observed isotope fractionation. Studies with whole cells and cell extracts for toluene (Morasch et al., 2001) and tetrachloroethene (Nijenhuis et al., 2005) degradation demonstrated that isotope fractionation tends to be somewhat larger when cell walls no longer limit the uptake of substrates. Kinnaman et al. (2007) also suggest a transport-limitation effect to explain their results for biodegradation of *n*-alkanes (C_1 to C_4).

The relationship between carbon isotope enrichment factors and carbon number observed in this study (Figs. 3a and b) is consistent with previous observations for methane and longer chain alkanes. Extrapolating our data from C_3–C_{10} *n*-alkanes to methane, values of −27.9‰ and −24.3‰, respectively, are obtained, similar to those observed in previous studies (Table 3). The extrapolation toward longer chains (Fig. 3) provides enrichment factors close to zero for chain C_{11}, in agreement with previous studies that reported absence of measurable isotope fractionation during biodegradation of *n*-C_{13} (Mansuy et al., 1997) and *n*-C_{14} (Mazeas et al., 2002).

Table 3
Observed enrichment factors during aerobic oxidation of methane with soil indigenous microbial population

Enrichment factor (‰)	Soil	Reference
−22.0 to −28.0	Forest soil	Tyler et al. (1994)
−21.5 to −24.4	Forest soil	Reeburgh et al. (1997)
−24.4	Laboratory culture	Coleman et al. (1981)
−16.0 to −27.0	Tundra soil	King et al. (1989)
−24.9 to −36.5	Marine sediments	Kinnaman et al. (2007)
−27.9	Alluvial sand	Extrapolation from current study (Fig. 3a)
−24.3	Alluvial sand	Extrapolation from current study (Fig. 3b)

4.3. BTX Isotopes analysis

The values obtained for benzene in this study are in between the values reported by Hunkeler et al. (2001) for two pure cultures (−1.4 and −3.5‰). For toluene, a relatively small isotope enrichment factor was observed (Table 1). Under oxic conditions, the biodegradation of toluene can be initiated by a variety of oxygenase reactions and a previous study suggested that the enrichment factor is related to the enzyme mechanism (Morasch et al., 2002). An isotope enrichment factor of −3.3‰ was observed for a pure culture using a methyl monooxygenase reaction and enrichment factors of −1.1‰ and −0.4‰ for a ring monooxygenase and a ring dioxygenase reaction, respectively. The values of this study (−0.7 and −0.8‰) suggest a domination of the ring transformation mechanism in the active soil population. This is in agreement with data reported by Keener et al. (2001), where the majority of isolates from a contaminated aquifer degraded toluene via the toluene 3-monooxygenase pathway. For *m*-xylene, the enrichment factor observed in this study is much smaller than the value observed (−1.7‰) for a pure strain culture with initial bond cleavage on the methyl group (Morasch et al., 2002). Hence, data obtained in this study for *m*-xylene also suggest a ring oxygenation.

Isotope enrichments factors for BTX were systematically smaller than for *n*-alkanes with equiva-

lent number of carbons (C_6, C_7 and C_8, respectively) (Table 1). The smaller isotope enrichment factors are likely related to the difference in reaction mechanism. In contrast to degradation of *n*-alkanes that involves a C–H bond cleavage in the initial step, aromatic rings are transformed via formation of an arene oxide without breaking the C–H bonds (Tomaszewski et al., 1975; Wilkins et al., 1994). The mechanism without H abstraction alters the bonds strength to carbon atoms less during formation of the transition state and hence the isotope effect is smaller than for *n*-alkanes.

No differences in the enrichment factors were measured for toluene and *m*-xylene when individually tested or mixed with other compounds. However, larger enrichment factors were obtained for benzene when included in a mixture (Table 1). Several studies reported enhanced biodegradation of benzene with the presence of toluene, suggesting that toluene acts as an enzyme inducer (Arvin et al., 1989; Chang et al., 1993; Alvarez and Vogel, 1995; Gülensoy and Alvarez, 1999). As a result, the presence of toluene in the 5-compound mixture could induce the activity of some enzymes that were not active during benzene degradation in single compound experiments.

5. Conclusions

Isotope enrichment factors during biodegradation of petroleum hydrocarbons were determined in batch experiments with alluvial sand under unsaturated conditions. For *n*-alkanes, the magnitude of enrichment factors decreased from propane (C_3) to *n*-decane (C_{10}), indicating that the magnitude of the enrichment factor is inversely proportional to the number of carbon atoms composing the saturated hydrocarbons. No measurable enrichment is expected for *n*-alkanes with chain lengths longer than C_{10}. The magnitude of decrease in the isotope enrichment factor is larger than expected based on the "dilution" effect suggesting that other factors such as rate limiting transport and enzyme binding step play a role as well. Isotope enrichment factors for BTX were also measured and were systematically smaller than the *n*-alkane with equal number of carbons. The difference is related to different enzymatic mechanisms, as the direct H abstraction during oxidation of saturated hydrocarbons leads to a larger carbon isotope effect than transformation of aromatic compounds.

The substantial carbon isotope fractionation during degradation of small *n*-alkanes suggests that carbon isotope ratios can be used to assess biodegradation of these compounds similarly as for BTX. In contrast to some BTX, which can be degraded by different pathways under aerobic conditions leading to different isotope enrichment factors, isotope enrichment factors for *n*-alkanes are expected to be more robust. Hence, it should be possible to evaluate field isotope data of *n*-alkanes quantitatively. Thus combining concentration data with isotope data for short chain *n*-alkanes could provide more detailed insights into their fate in the environment.

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