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Evidence for *in situ* degradation of mono-and polyaromatic hydrocarbons in alluvial sediments based on microcosm experiments with ^{13}C-labeled contaminants

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A method based on ^{13}C-labeled substrates was developed to determine the intrinsic biodegradation potential of aromatic pollutants under oxic and under anoxic conditions.

Abstract

A microcosm study was conducted to investigate the degradation of mono- and polyaromatic hydrocarbons under in situ-like conditions using alluvial sediments from the site of a former cokery. Benzene, naphthalene, or acenaphthene were added to the sediments as ^{13}C-labeled substrates. Based on the evolution of ^{13}C-CO_2 determined by gas chromatography isotope-ratio mass spectrometry (GC-IRMS) it was possible to prove mineralization of the compound of interest in the presence of other unknown organic substances of the sediment material. This new approach was suitable to give evidence for the intrinsic biodegradation of benzene, naphthalene, and acenaphthene under oxic and also under anoxic conditions, due to the high sensitivity and reproducibility of ^{13}C/^{12}C stable isotope analysis. This semi-quantitative method can be used to screen for biodegradation of any slowly degrading, strongly sorbing compound in long-term experiments.

Keywords: Aromatic hydrocarbons; Bioremediation; Natural attenuation; Stable isotopes; Intrinsic biodegradation potential

1. Introduction

The assessment of biodegradation in contaminated aquifers has become an increasingly important issue. This is closely related to the acceptance of intrinsic bioremediation (or monitored natural attenuation) as a means to manage contaminated sites (Scow and Hicks, 2005; US-EPA, 1999). In this context, it is necessary to distinguish between mineralization leading to complete removal of pollutants, partial degradation to − possibly harmful − products, and physico-chemical processes such as sorption and dilution which locally reduce concentrations but do not remove pollutants (Norris, 1994; Wiedemeier et al., 1999). The differentiation becomes even more complicated, as in the majority of cases rather undefined mixtures of pollutants are present. Among the main contributors to environmental pollution are aromatic hydrocarbons. Although polyaromatic hydrocarbons (PAHs) are still continuously generated by combustion of organic matter and atmospherically deposited, immense contaminations of the environment typically originate from industrial sites, from coal gasification, landfill leachates, or accidental petroleum and fuel spills (US-EPA, 1999). The microbial degradation of aromatic hydrocarbons is hampered for three reasons: Firstly, because of the high activation energy needed to chemically attack aromatic rings (Boll et al., 2002), secondly by the substances' toxicities towards bacteria (Jonker et al., 2006; Loibner et al., 2004), and thirdly because aromatic hydrocarbons have a tendency to sorb on hydrophobic surfaces. This

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tendency is increasing with the number of their aromatic rings (Kleineidam et al., 2002; Kubicki, 2006). Sorption and poor solubility in water makes the major fraction of PAHs inaccessible for bacterial degradation (Bosma et al., 1997; Volkering et al., 1992). Although one controversial study reported on bacteria that directly accessed the sorbed substrate (Guerin and Boyd, 1997), presently it is believed that PAHs adsorbed on soil particles, solid PAH crystals, or hydrocarbons dissolved in non-aqueous phase liquids (NAPLs) remain unavailable to bacteria (Johnsen et al., 2005).

Until now, no standard procedure exists that has the potential to demonstrate biodegradation of organic environmental pollutants in contaminated environments. In practice, often two or more independent approaches are combined (Smets and Pritchard, 2003; Watanabe and Hamamuray, 2003), among these are evidence of concentration decrease of contaminants over time and distance (Wiedemeier et al., 1999), enrichment of heavy stable isotopes in the remaining fraction of organic contaminants (Hunkeler et al., 2002; Meckenstock et al., 2002; Sherwood Lollar et al., 1999), radiotracer studies (Bianchin et al., 2006; Conrad et al., 1997), succession of redox zones in the field (Kuhn and Suflita, 1989; Vroblesky and Chapelle, 1994); accumulation of signature metabolites (Beller, 2002; Elshahed et al., 2001), investigation of the intrinsic microbial biodegradation potential in microcosm studies − in parts with ^{14}C-labeled substrates − (Aelion and Bradley, 1991; Ambrosoli et al., 2005; Lovley, 2001); characterization of the bacterial community by molecular techniques (Amann et al., 1995; Bakermans et al., 2002), tracing ^{13}C in fatty acid profiles of bacteria (Geyer et al., 2005), and detection of bacterial enzymes (Hanson et al., 1999; Heinaru et al., 2005; Hendrickx et al., 2006; Löffler et al., 2000). Each of these methods has its advantages and limitations, and therefore it is advisable to base investigations on several approaches, even more when dealing with complex field sites. It must be noted that only isotope-based methods (^{13}C or ^{14}C) targeting CO_2 are suitable to prove complete mineralization of single pollutants. Minor amounts of substrate which are incompletely mineralized or used to synthesize cellular building blocks remain unconsidered. Since the use of radioactive markers such as ^{14}C is usually restricted, radio-labeled compounds were almost exclusively-applied in laboratory experiments (Chapelle et al., 1996; Conrad et al., 1997). Recovery of ^{14}C-CO_2 in the headspace of the respective test systems is a very sensitive tool for proving complete mineralization of the labeled substrate (Bradley et al., 2002). However, radioactivity causes difficulty in sample treatment, detection, and waste disposal, and as a consequence the interest in ^{13}C-based methods is increasing. Moreover, ^{13}C-labeled substances can be used in lab and field studies.

This study focuses on the evaluation of the intrinsic bacterial degradation potential of mono- and polyaromatic hydrocarbons at the site of a former cokery located in direct proximity to a river. In terms of risk assessment, it is imperative to investigate whether or not biodegradation is sufficiently rapid to prevent dissolved contaminants from reaching the river. Here, we present a new method based on the addition of ^{13}C-labeled contaminants and the subsequent analysis of ^{13}C-CO_2 generated during biodegradation. Hence this approach conveys compound-specific information on mineralization and ^{13}C was found to be an ideal marker to test the fate of one particular contaminant out of a mixture. Microcosm experiments with sediments from the former cokery site were performed under conditions resembling in situ conditions using benzene, naphthalene, and acenaphthene as substrates. All three substances were major contaminants at the field site and belong to the 16 priority PAHs designated by the United States Environmental Protection Agency (US-EPA; http://www.epa.gov/) and to the 33 priority substances recently defined in the EU Water Framework Directive (EC, 2006).

2. Materials and methods

2.1. Field site

A microcosm study was conducted using alluvial sediments from the premises of a former cokery in the alluvial plain of the river Meuse. The area covering more than 7 ha of land is located in the region of Luik, Belgium, in direct proximity to the river (Fig. 1). The upper 2.5 m are partly made up of backfill material and the sediments below consist of silt, sand, clay, and small gravels. A gravel aquifer is below the depth of 5.5−7 m. The groundwater flow direction is South-East towards the Meuse (Fig. 1). Earlier site characterization campaigns performed by the Walloon Environmental Protection Agency (Sociéte Publique d'Aide à la Qualité de l'Environnement, SPAQuE) showed severe contamination with heavy metals, cyanides, mineral oils, aromatic compounds, and polyaromatic hydrocarbons (PAH) in groundwater and in sediments. A light NAPL (non-aqueous phase liquid) floating on the water table and a dense NAPL in a depth of about 12 to 13 m, directly above the bedrock, were localized. The dense NAPL consists, among else, of dibenzofurane and PAHs (with a large fraction of acenaphthene, methyl-, and dimethylnaphthalenes), monoaromatic hydrocarbons (BTEX), and cyanides. Maximum concentrations of benzene in groundwater of 210 mg/L were detected which was more than 1000 times above the defined limit at which according to Walloon legislation intervention is obligatory. PAHs were detected to be up to 70 times above their respective maximum acceptable levels (Décret Wallon du 1er Avril relatif à l'assainissement des sols pollués et aux sites d'activités économiques à réhabiliter, 2004).

A drilling campaign took place in March 2005 where 14 profound and 14 shallow piezometers were constructed. During the campaign, freshly drilled sediment material for microcosm studies was sampled, directly filled into brown-glass bottles of 250 mL with teflon-sealed caps (Infochroma AG, Zug, Switzerland), cooled immediately, and stored at 5 °C until usage.

2.2. Microcosm set up

Microcosms consisted of 25 g of sediment that was transferred into 50 mL-culture bottles (Ochs, Bovenden, Germany) working under an atmosphere of N_2 in a glove bag (Sigma-Aldrich, Buchs, Switzerland). Groundwater from the site was sterile filtrated using disposable syringe filters (0.22 μm; Schleicher & Schüll, Dassel, Germany) and diluted 5-fold with sterile nanopure water to result in concentrations of NO_3^- and SO_4^{2-} of 6 and 140 mg/L, respectively. Diluted groundwater was purged with N_2 for 0.5 h to remove dissolved O_2 and was filled into 250 mL-glass bottles (Infochroma AG). Benzene, naphthalene, or acenaphthene labeled with ^{13}C at 6 positions (99% purity; Cambridge Isotope Laboratories, Andover, MA) were added into these bottles. The same amounts of non-labeled benzene, naphthalene, and acenaphthene were equally dissolved in diluted groundwater and later used for parallel incubations with sediments sampled from location U13 (Fig. 1). Then N_2 was supplied as headspace and the bottles were closed with teflon-sealed caps (Sigma-Aldrich). To guarantee the dissolution of the aromatic hydrocarbons, the bottles with substrate-amended groundwater were shaken horizontally

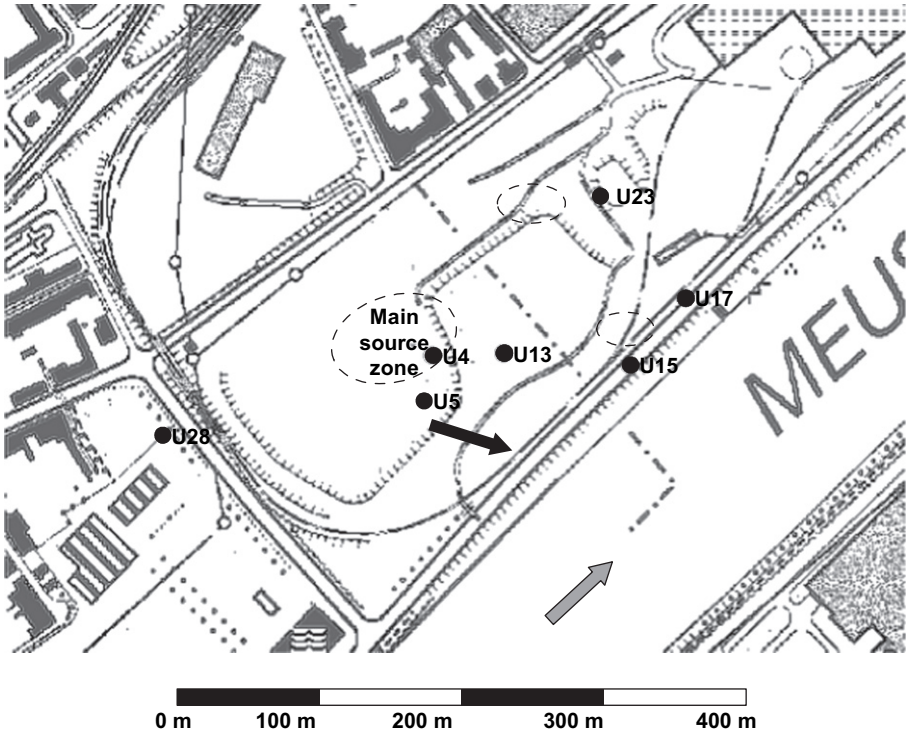

Fig. 1. Site map of the former cokery of Flémalle, Belgium, located in direct vicinity to the river bed of the Meuse. Locations of sediment sampling are labeled with circles. Putative source zones of aromatic hydrocarbons are surrounded by broken lines. The black and grey arrows depict the ground- and surface water flow directions, respectively.

2 h at 80 rotations per minute before being distributed to microcosms. Per bottle, 15 mL of anoxic substrate solution were added. Substrate concentrations were chosen as displayed in Table 1 and were high enough to ensure detection of mineralization products and on the other hand low enough to avoid inhibition and to guarantee complete dissolution. The microcosms containing sandy-silty material of 5−7 m depth were incubated in presence of air, according to the environmental conditions of the vadose zone, and alluvial gravel material from 7−11 m was incubated under an atmosphere of N_2. Bottles were closed with non-absorptive Viton stoppers (Maagtechnic, Duebendorf, Switzerland). The incubation temperature of 16 °C was close to natural conditions. Microcosms studies were carried out in duplicate. Controls were either without addition of substrate or were inhibited by addition of sodium azide to a final concentration of 0.1% (w/v).

2.3. Hydrocarbon analysis

Concentrations of benzene and PAHs were analyzed using a Varian 3800 gas chromatograph equipped with an autoinjector CP8410 for solid phase microextraction (SPME; Varian, Palo Alto, CA). Aromatic hydrocarbons were extracted from the headspace of 2 mL-vials filled with 0.5 mL of sample plus 0.1 g of NaCl with polydimethylsiloxane fibers (film thickness of 100 μm; Supelco, Bellefonte, PA). The extraction time was 18 min at room temperature. Fibers were desorbed at 250 °C for 8 min. The N_2 carrier gas flow was 1.2 mL/min. Separation was achieved using a DB-5 column of 15 m length. The temperature program was as follows: 50 °C isothermal for 2 min followed by a 20 °C/min increase to 200 °C held for 2 min followed by a second ramp of 20 °C/min to 220 °C.

Table 1
Initial substrate concentrations added to microcosms and the respective amounts of ^{13}C-labels added

Substrate	Concentration [mg/L]	^{13}C/microcosm [μmol]
$^{13}C_6$-Benzene	35	40
$^{13}C_6$-Naphthalene	10	7.0
$^{13}C_6$-Acenaphthene	4	2.3

2.4. Stable isotope analysis of CO_2

Two milliliters of headspace sample was taken through the Viton stoppers with a gas tight syringe (Hamilton, Bonaduz, Switzerland) and subsequently injected into a closed 22 mL-headspace vial (Infochroma AG) filled with 100% N_2. Measurements for concentration of CO_2 and the corresponding enrichments in ^{13}C-CO_2 were carried out by gas chromatography isotope-ratio mass spectrometry (GC-IRMS) using a gas chromatograph (Trace GC; Thermo Finnigan; Waltham, MA) connected via a combustion interface (Thermo Combustion III; Thermo Finnigan) to an isotope-ratio mass spectrometer (Delta plus XP; Thermo Finnigan). The system was connected to a headspace autosampler (Tekmar Dohrmann 7000; Teledyne, Los Angeles, CA) and 100 μL of sample were transferred for analysis. The helium carrier gas flow was 1.7 mL/min. CO_2 was separated from other gases using a PORA-PLOT-Q column of 30 m length, an inner diameter of 0.32 mm, and a film thickness of 10 μm (Varian). The temperature was held isothermally at 40 °C. CO_2 eluted at 230 s. Isotope measurements were performed in duplicate. The $\delta^{13}C$ [‰] values were calculated relative to external CO_2 reference gas (Eq. (1)):

$$\delta^{13}C[‰] = \left(\frac{{}^{13}C_{sample}/{}^{12}C_{sample}}{{}^{13}C_{standard}/{}^{12}C_{standard}} - 1\right) \times 1000. \quad (1)$$

Changes in the ^{13}C-CO_2 content of samples were calculated using Eq. (2):

$$\frac{R_t}{R_0} = \frac{\delta^{13}C_t + 1000}{\delta^{13}C_0 + 1000} \quad (2)$$

where R_t is the stable isotope ratio at point t and R_0 is the initial $^{13}C/^{12}C$ composition.

3. Results

3.1. Aerobic intrinsic degradation potential at the field site

The distribution of the intrinsic biodegradation potential was investigated in sediments sampled from the vadose and from the saturated zone of 7 different drilling locations (Fig. 1). Microcosms were set up with non-labeled benzene, naphthalene, or acenaphthene (U13) and with the respective ^{13}C-labeled compounds (all 7 locations). In parallel, controls of all 7 locations were investigated that were either without addition of substrate or that were inhibited by sodium azide but contained ^{13}C-labeled substrates. In controls the ^{13}C/^{12}C carbon isotope ratio in CO_2 remained stable within 135 days (Fig. 2). Maximum δ^{13}C-deviations were recorded to be $\Delta\delta^{13}C\ [‰] \leq 5$. Microbiologically active incubations without labeled substrates did not show a more pronounced enrichment or depletion in ^{13}C-CO_2 over time than growth-inhibited controls (Fig. 3). Equally, ^{13}C-CO_2 remained stable in those microcosms of location U13 amended with non-labeled benzene, naphthalene, or acenaphthene. Microcosms with non-labeled aromatic compounds or without substrate addition served to confirm that changes in ^{13}C-CO_2 in the microcosm headspace were attributed to the mineralization of ^{13}C-labeled contaminants and not to the ca. 1% of ^{13}C being naturally present in organic or inorganic material. In microcosms that degraded ^{13}C-benzene, -naphthalene, or -acenaphthene, enrichments in ^{13}C-CO_2 were significantly larger, so e.g. an increase of $\Delta\delta^{13}C = 902‰$ was documented during aerobic ^{13}C-naphthalene degradation by the intrinsic bacterial community in sediment sampled from location U17 which is located 240 m downgradient of the major source zone (Fig. 1). This corresponds to an increase in ^{13}C-CO_2 per total

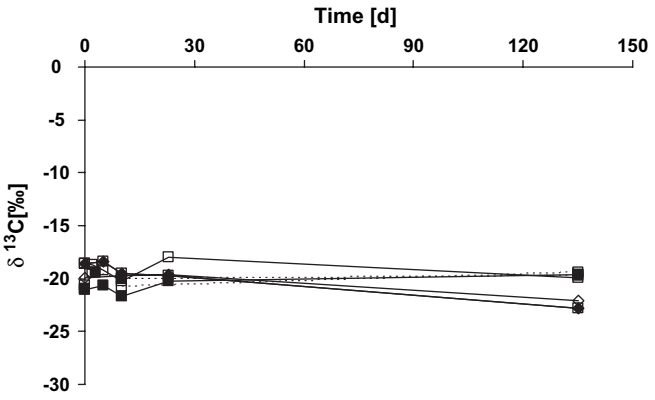

Fig. 2. Temporal variations of δ^{13}C [‰] in CO_2 in controls set up with sediments from locations U4 (squares) and U17 (diamonds). □ and ◇ depict microcosms with sediment from the non-saturated, ■ and ◆ microcosms with sediment from the saturated zone of U4 and U17, respectively, without addition of substrate. Curves with broken lines show killed controls that contained ^{13}C-benzene and were set up with sediments from location U4, both from the non-saturated and saturated zone. Temporal variations of δ^{13}C [‰] in CO_2 in aerobic and anaerobic microcosms of locations U4 and U17 are displayed in Table 2A,B.

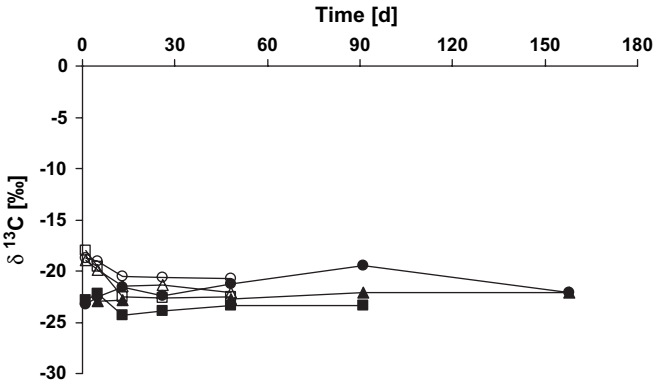

Fig. 3. Temporal evolution of δ^{13}C [‰] in CO_2 over time shown for aerobic and anaerobic microcosms containing oxic (open symbols) and anoxic sediments (filled symbols) from drilling location U13 with non-labeled benzene (□, ■), naphthalene (○, ●) and acenaphthene (△, ▲) as substrates. (For comparison, evolution of δ^{13}C [‰] in CO_2 in microcosms with ^{13}C-labeled benzene, naphthalene, and acenaphthene are shown in Table 2A,B.)

CO_2 of up to 1.01% in the microcosm headspace (Table 2A). Microcosms with sediments sampled downgradient of the source and also from U28 − located upgradient and therefore not affected by the groundwater contamination of the cokery − showed the highest enrichment in ^{13}C-CO_2 already after two days of incubation at 16 °C when ^{13}C-benzene or ^{13}C-naphthalene were supplied as substrates. Aerobic degradation of ^{13}C-acenaphthene was initiated between day 2 and 10. The increase in ^{13}C-CO_2 was slower and the maximum enrichment obtained was $\Delta\delta^{13}C\ [‰] = 281$ corresponding to 0.32% of ^{13}C-CO_2 per total CO_2 (Table 2A). Sediments from U4 which is in proximity to the source zone fed with ^{13}C-labeled substrates did not show any mineralization of these compounds within 135 days. Contaminant degradation in aerobic incubations with material from 5−7 m depth of U5 was found to be slower than in the other microcosms with sediments from drilling locations further downgradient from the main source (Fig. 4A). Duplicates showed good agreements (Fig. 4A, B).

3.2. Anaerobic intrinsic degradation potential at the field site

^{13}C/^{12}C ratios in CO_2 of living controls without added substrates and in CO_2 of those microcosms with sediments from location U13 amended with non-labeled benzene, naphthalene, or acenaphthene remained stable within $\Delta\delta^{13}C\ [‰] \leq 5$. The maximum δ^{13}C-CO_2 enrichment in anaerobic microcosms was obtained during degradation of ^{13}C-naphthalene with sediments from the saturated zone of drilling location U17 and was $\Delta\delta^{13}C\ [‰] = 970$. That corresponds to an increase in ^{13}C-CO_2 of 1.09% per total CO_2 in the headspace of the microcosm bottle (Table 2B). Compared to aerobic microcosms, ^{13}C-CO_2 increased more slowly and in most of the bottles gently began to rise after 3 days (Fig. 4B). The intrinsic community was able to utilize ^{13}C-benzene and ^{13}C-naphthalene in sediments of all 7 locations. Again, sediment from

Table 2
Absolute enrichment in ^{13}C in CO_2 of the headspace of aerobic microcosms (**A**) and anaerobic microcosms (**B**) over time

(**A**)	Time [d]	0		2		10		23		40		135	
		$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]
U4	^{13}C Benzene	0	0	0	0.00	−1	0.00	0	0.00	0	0.00	1	0.00
	^{13}C Naphthalene	0	0	0	0.00	0	0.00	−1	0.00	0	0.00	0	0.00
	^{13}C Acenaphthene	0	0	2	0.00	2	0.00	2	0.00	2	0.00	2	0.00
U5	^{13}C Benzene	0	0	30	0.03	**384**	**0.43**	237	0.27				
	^{13}C Naphthalene	0	0	20	0.02	**370**	**0.42**	285	0.32				
	^{13}C Acenaphthene	0	0	0	0.00	10	0.01	28	0.03	**57**	**0.06**	40	0.05
U13	^{13}C Benzene	0	0	**667**	**0.75**	373	0.42	256	0.29				
	^{13}C Naphthalene	0	0	**640**	**0.72**	448	0.50	421	0.47				
	^{13}C Acenaphthene	0	0	5	0.01	85	0.10	98	0.11	**106**	**0.12**	87	0.10
U15	^{13}C Benzene	0	0	**411**	**0.46**	362	0.41	270	0.30				
	^{13}C Naphthalene	0	0	390	0.44	**720**	**0.81**	688	0.77	675	0.76	465	0.52
	^{13}C Acenaphthene	0	0	−1	0.00	12	0.01	25	0.03	42	0.05	65	**0.07**
U17	^{13}C Benzene	0	0	773	0.87	369	0.41	234	0.26				
	^{13}C Naphthalene	0	0	**902**	**1.01**	548	0.61	543	0.61				
	^{13}C Acenaphthene	0	0	73	0.08	262	0.29	279	0.31	**281**	**0.32**	218	0.24
U28	^{13}C Benzene	0	0	**511**	**0.57**	361	0.41	269	0.30				
	^{13}C Naphthalene	0	0	**556**	**0.62**	405	0.46	360	0.40				
	^{13}C Acenaphthene	0	0	−1	0.00	45	0.05	59	0.07	78	0.09	**186**	**0.21**
(**B**)	Time [d]	0		3		9		23		41		136	
		$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]	$\Delta\delta^{13}C$ [‰]	$^{13}CO_2$ [%]
U4	^{13}C Benzene	0	0	1	0.00	14	0.02	22	0.03	43	0.05	**44**	**0.05**
	^{13}C Naphthalene	0	0	0	0.00	764	0.86	521	0.58	702	0.79	**852**	**0.96**
	^{13}C Acenaphthene	0	0	2	0.00	1	0.00			2	0.00	1	0.00
U5	^{13}C Benzene	0	0	15	0.02	**215**	**0.24**	166	0.19				
	^{13}C Naphthalene	0	0	4	0.00	315	0.35	435	0.49	655	0.74	**681**	**0.76**
	^{13}C Acenaphthene	0	0	2	0.00	0	0.00			2	0.00	4	0.00
U13	^{13}C Benzene	0	0	4	0.00	193	0.22	150	0.17				
	^{13}C Naphthalene	0	0	2	0.00	347	0.39	427	0.48	560	0.63	**785**	**0.88**
	^{13}C Acenaphthene	0	0	1	0.00	1	0.00			18	0.02	**71**	**0.08**
U15	^{13}C Benzene	0	0	−1	0.00	106	0.12	143	0.16	106	0.12	**289**	**0.32**
	^{13}C Naphthalene	0	0	0	0.00	19	0.02	192	0.22	400	0.45	**744**	**0.84**
	^{13}C Acenaphthene	0	0	3	0.00	4	0.00			7	0.01	16	0.02
U17	^{13}C Benzene	0	0	107	0.12	106	0.12	70	0.08				
	^{13}C Naphthalene	0	0	11	0.01	63	0.07	239	0.27	530	0.59	**970**	**1.09**
	^{13}C Acenaphthene	0	0	2	0.00	2	0.00			11	0.01	39	0.04
U23	^{13}C Benzene	0	0	6	0.01	205	0.23	214	0.24	258	0.29	**292**	**0.33**
	^{13}C Naphthalene	0	0	15	0.02	110	0.12	278	0.31	492	0.55	**682**	**0.77**
	^{13}C Acenaphthene	0	0	2	0.00	1	0.00			6	0.01	8	0.01
U28	^{13}C Benzene	0	0	155	0.17	**764**	**0.86**	320	0.36				
	^{13}C Naphthalene	0	0	27	0.03	73	0.08	**594**	**0.67**				
	^{13}C Acenaphthene	0	0	2	0.00	2	0.00			28	0.03	**147**	**0.17**

$\Delta\delta^{13}C$ [‰] values were converted to the percentage of ^{13}C-CO_2 in total CO_2 with $^{13}C\text{-}CO_2/CO_{2,tot}$ [%] = $(R_t\text{-}R_0) * 100$ and assuming $[CO_{2,tot}] \approx [^{12}C\text{-}CO_2]$. Highest enrichments obtained are shown in bold.

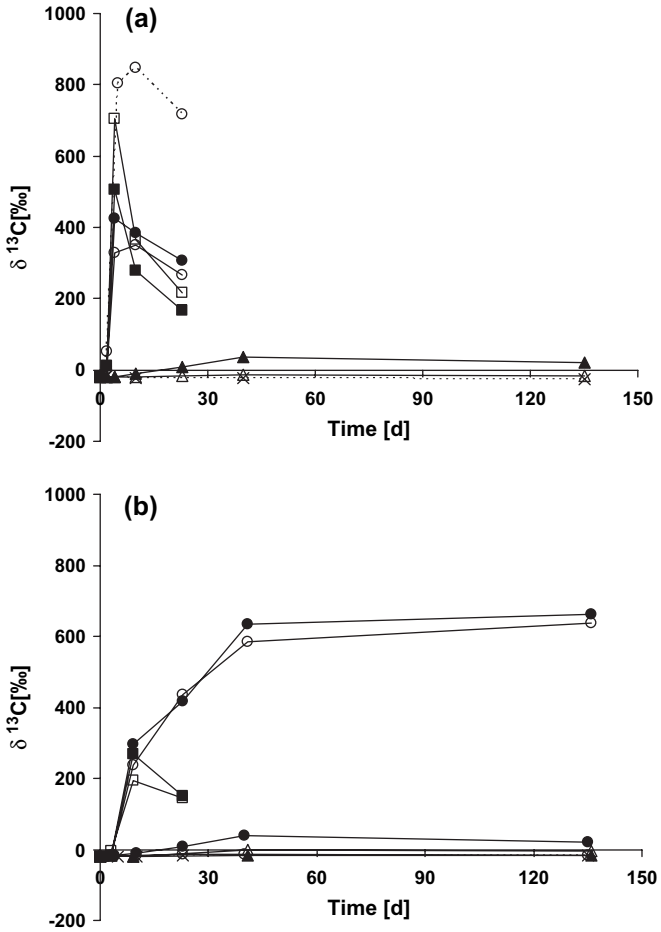

Fig. 4. Temporal variation of $\delta^{13}C$ [‰] in CO_2 shown for aerobic microcosms containing sediments from the unsaturated zone (A) and for anaerobic microcosms containing sediments from the saturated zones of drilling location U5 (B). Duplicates of microcosms with ^{13}C-benzene (□, ■), ^{13}C-naphthalene (○, ●), and ^{13}C-acenaphthene (△, ▲) as substrates are displayed. In parallel, anoxic sediment from the saturated zone was incubated with air as headspace and ^{13}C-naphthalene as substrate (- - ○ - -) (A). Curves with broken lines (- - × - -) show killed controls (A, B).

upgradient from the contaminated site (U28) contained bacteria that were capable of degradation of ^{13}C-benzene and ^{13}C-naphthalene without considerable lag phases. Anaerobic microcosms with sediments from U13 and U28 with ^{13}C-acenaphthene as labeled carbon source showed a significant increase in ^{13}C in the headspace of $\Delta\delta^{13}C$ [‰] = 71 and $\Delta\delta^{13}C$ [‰] = 147, respectively. The intrinsic biodegradation potential for acenaphthene was less pronounced but present in sediments of U15, U17, and probably also in U23 (Table 2B).

To investigate the effect of fluctuating groundwater tables or changing redox conditions on contaminant degradation, saturated sediments (9 m depth) from location U5 were in parallel incubated under a headspace of air. These microcosms showed that in formerly anoxic sediments the presence of O_2 accelerated substrate mineralization, here defined as increase in $\delta^{13}C$-CO_2. Degradation was comparably fast as in aerobic microcosms with non-reduced sediments from 5–7 m depth (Fig. 4A).

4. Discussion

4.1. Isotope shifts in CO_2 in experiments with non-labeled substrates

One condition to be met in order to apply the ^{13}C-based screening method, was to verify whether the ^{13}C-CO_2 in the headspace was a degradation product of the individual labeled compounds or of any organic or inorganic substance containing about 1% of naturally abundant ^{13}C. Parallel incubations of sediments from drilling location U13 with ^{13}C-labeled and non-labeled substrates (Fig. 3) were compared to each other and served to quantify the increase that came from labeled compounds. In former studies, biochemical degradation of aromatic hydrocarbons has been shown to cause stable isotope fractionation, meaning an unequal distribution of ^{13}C in substrates and products of a reaction (Ahad et al., 2000; Meckenstock et al., 1999, 2004). As a consequence, not only the ^{13}C-content in the remaining substrate fraction but also the $^{13}C/^{12}C$ ratios in CO_2 as the final oxidation product of hydrocarbon mineralization might change over time. More recently, a field test on aerobic biodegradation with mixtures of monoaromatic hydrocarbons and *n*-decane was conducted (Bugna et al., 2004). The authors concluded that the carbon stable isotope signatures in respired CO_2 reflect the isotope ratios in the parent compounds. However it has to be mentioned that the investigated reactions are known to be often accompanied by only small isotope effects (Meckenstock et al., 2004; Morasch et al., 2002). Another group investigated changes in $^{13}C/^{12}C$ in CO_2 during degradation of crude oil that was very depleted in ^{13}C and had an initial overall signature of $\delta^{13}C$ [‰] = − 30.6 (Medina-Bellver et al., 2005). Here, results pointed into the same direction. The signature of the substrate determined the signature in the daughter products. For example, relative to the initially more positive signature of $\delta^{13}C_0$[‰] = − 2 in CO_2 the degradation of ^{13}C-depleted crude oil compounds led to a decrease $\Delta\delta^{13}C$ [‰] = 13 in ^{13}C-CO_2 over time (Medina-Bellver et al., 2005) The authors assumed a signature of $\delta^{13}C$ [‰] = 0 in marine DIC and could therefore exclude inorganic carbon pools as origin of the negative $\delta^{13}C$-CO_2. In this study, for those of our microcosms that were set up with non-labeled benzene, naphthalene, or acenaphthene, GC-IRMS analyses yielded stable isotope ratios in CO_2 over time (Fig. 3). Probably this was not entirely due to the relatively negative initial $^{13}C/^{12}C$ signatures in CO_2 in the headspace of our microcosms which were close to typical isotope signatures for aromatic contaminants of around $\delta^{13}C$ [‰] = − 25. Also, these contaminated sediments partly contained several other hydrocarbons that − apart from the single compound added − might have been degraded by the intrinsic bacterial community. However, major impacts on stable carbon isotope signatures in CO_2 in bottles without labeled substrate can be expected from the DIC pool with initial carbon isotope signatures of approximately −15‰ (Clark and Fritz, 1997) when the microcosms have both a high proportion of sediment material and groundwater rich in HCO_3^-. Stable ^{13}C-CO_2 signatures over time demonstrated that microcosms

conducted with ^{12}C-substrates and controls without substrate addition were appropriate to rule out significant isotope shifts in CO_2 caused by the mineralization of non-^{13}C-labeled compounds and carbon exchange with DIC.

4.2. Intrinsic biodegradation potential under oxic and anoxic conditions

Benzene, naphthalene, and acenaphthene as model compounds for mono- and polyaromatic hydrocarbons have been shown to be degradable under oxic and anoxic conditions by the intrinsic bacteria of (contaminated) alluvial sediments. Generally, aerobic biodegradation of benzene or naphthalene was initiated within two days and occasionally started immediately after microcosm set up. This emphasized that bacteria capable of mineralization of aromatic compounds in presence of O_2 were widespread and active in contaminated sediments but were also present upgradient from the source and in an area unaffected by the pollution of the former cokery. As the site is located in a highly-industrialized region, it cannot be ruled out that sediment at the reference point was affected by contaminants from other sources in the past. Equally, ^{13}C-acenaphthene was well-degradable by the aerobic intrinsic bacterial community, however the turnover was significantly slower as measured by the increase in $\delta^{13}C$ in CO_2 over time. Biodegradation was found to be either completely inhibited in sediments sampled within the contaminant source zone or slow in sediments sampled close to the source. This led to the conclusion that bacteria that have the enzymatic repertoire for oxygenase reactions targeting mono- and polyaromatic rings are widespread in sediment material and the initiation times for this kind of reactions are short. Aromatic hydrocarbons in higher concentrations were toxic towards bacteria and no mineralization of ^{13}C-labeled substrates was observed. Bacteria capable of aerobic degradation of aromatic hydrocarbons were also found to be active in anoxic sediments sampled from the saturated zone. Presence of O_2 accelerated the degradation significantly compared to parallel anaerobic microcosms with the same sediment material. The high recovery of ^{13}C-CO_2 in the headspace of all aerobic microcosms with ^{13}C-labeled substrates also suggested that the substrates were either completely or in high percentage mineralized. Remarkably, in absence of O_2 ^{13}C-benzene and ^{13}C-naphthalene were also effectively degraded and recovery of ^{13}C-CO_2 gave clear evidence that ^{13}C-acenaphthene was mineralized by the intrinsic anaerobic bacterial communities. Particularly, these findings on anaerobic acenaphthene degradation exemplified the applicability of the ^{13}C-based method in cases with relatively small amounts of substrate and a high percentage of molecules probably sorbed to sediment particles. The not ubiquitous and less efficient degradation of ^{13}C-acenaphthene under anoxic conditions is in agreement with findings in preceding field studies (Steinbach et al., 2004; Zamfirescu and Grathwohl, 2001). These papers reported on long contaminant plumes with high contents of acenaphthene particularly under reduced conditions. Consequently, persistence to in situ biodegradation in absence of O_2 was proposed. In contrast this conclusion based on field experiments but in agreement with the clear increase in ^{13}C-CO_2 in our microcosm experiments, enrichment cultures served to demonstrate biodegradation of small concentrations of acenaphthene under denitrifying, sulfate-reducing, and methanogenic conditions, after incubation times longer than 80 days, (Chang et al., 2002; Mihelcic and Luthy, 1988).

4.3. Sensitivity of the ^{13}C-method

In control microcosms which were either inhibited or did not contain labeled substrate, a maximum variability of $\Delta\delta^{13}C\ [‰] = 5$ was determined (Figs. 2 and 3). An unambiguous evidence for biodegradation of one ^{13}C-labeled substrate of interest needs therefore an enrichment of $\delta^{13}C > 5‰$. This corresponds to an increase in ^{13}C-CO_2 per $CO_{2,tot}$ of 0.006% being equivalent to 2.5 μmol/L. Taking the dimensionless Henry's constant $K_{H'} = [CO_2]_g/[CO_2]_{aq} = 0.92$ at 16 °C (Yaws, 1999) it needs to be considered that about half of ^{13}C-CO_2 produced might be non-accessible to headspace analysis performed by GC-IRMS because it is present as $[CO_2]_{aq}$. When assuming a C:H ratio of 1:1 for aromatic hydrocarbons, the amount of 2.5 μmol/L corresponds to the degradation of 33 μg/L substrate. In our microcosm where ^{13}C-labeled substrates were supplied dissolved in 15 mL of groundwater this was fulfilled when 1 μg of substrate were degraded. This clearly illustrates that the method is very sensitive and appropriate to detect small amounts of environmental pollutants being degraded in slowly-growing incubations. Taking as an example $^{13}C_6$-benzene and $^{13}C_6$-acenaphthene, which had initial concentrations of 35 mg/L and 4 mg/L in the microcosms, respectively, mineralization of 0.2% of the added ^{13}C-benzene can produce a measurable signal as well as the mineralization of 1.4% of the added ^{13}C-acenaphthene (Table 1). Due to the fact that the isotope exchange between HCO_3^-, $[CO_2]_{aq}$ and $[CO_2]_g$ is fast, also carbon exchange reactions with the HCO_3^- pool should be taken into account (Clark and Fritz, 1997). Since the pK for HCO_3^-/CO_2 is 6.3, in our microcosms with neutral to slightly alkaline pH a considerable amount of 90% of the formed ^{13}C-CO_2 might have been converted into HCO_3^- and therefore not recorded by headspace analysis. Apart from reflections of pH effects on the definition of the limits of detection, additionally processing of headspace samples prior to their analysis and with that a dilution factor of − in our experiments 1:40 − must be taken into account.

4.4. Influencing factors

Three factors influencing the recovery of ^{13}C-CO_2 will be briefly discussed: The incomplete recovery of ^{13}C from labeled substrates in CO_2, a dilution effect of ^{13}C-CO_2 caused by geochemical processes biodegradation of background compounds that are present in sediment materials (see above), and finally the equilibrium isotope effects between CO_2 in the gas phase and HCO_3^- in the water phase. Degradation of organic substrates proceeds not necessarily to CO_2. Organic hydrocarbons are frequently transformed into metabolites which may be either stocked in bacterial cells or may be excreted. However, taking degradation of aromatic hydrocarbons, complete

mineralization can be expected to be dominating. One study based on experiments with pure cultures determined mineralization yields between 62 and 77% for naphthalene degradation under oxic conditions (Bouchez et al., 1996). The same paper reported on various three-ring aromatic hydrocarbons having mineralization yields of around 60%. ^{14}C-labeled toluene was used in experiments with sulfate-reducing pure cultures from which, 60% of the substrate's carbon was recovered in CO_2 (Pelz et al., 2001). Transferring those findings to our microcosm experiment, we can assume that 50 to 75% of ^{13}C-labeled carbon that was utilized by the bacteria was finally oxidized to CO_2.

Dilution of ^{13}C-CO_2 from mineralization of ^{13}C-labeled substrates by CO_2 of geochemical origin is a second factor potentially complicating the assessment. In this context, the most abundant geochemical process in sediments is the dissolution of $CaCO_3$ leading to formation of DIC (HCO_3^- and $[CO_2]_{aq}$) and with that also to a release of CO_2 into the gas phase. A former study aimed to characterize the origin of DIC using stable isotope analysis (Bolliger et al., 1999). The authors concluded that in groundwater of an anoxic aquifer 88% of the DIC produced actually originated from the biodegradation of petroleum hydrocarbons. These findings demonstrate that geochemical processes significantly contribute to an increase in DIC. Although, when denitrification or sulfate-reduction are the prevalent electron accepting processes dissolution of $CaCO_3$ is less important. Generally, the major process leading to formation of CO_2 can be expected to be the biodegradation of environmental contaminants. Difficulties may although arise when the mass of the contaminant of interest is small compared to other organic carbon degraded or $CaCO_3$ dissolved.

Kinetic and equilibrium carbon isotope effects during gas-water exchange and dissolution of CO_2 have been thoroughly studied before (Zhang et al., 1994). For dissolution, CO_2 in the headspace was found to be slightly depleted ($-2.26‰$) in ^{13}C compared to hydratized CO_2. The equilibrium fractionation effect between HCO_3^- and CO_2 gas at a temperature of 25 °C was 7.9‰. A more recent study based on numerical models investigated isotope effects between δ^{13}C-CO_2 and $\delta^{13}C_{(\text{total dissolved carbon})}$ and results were consistent with the preceding study of Zhang (Lesniak and Zawidzki, 2006). The isotope fractionation effects of CO_2 phase transfers and carbon equilibrium are relatively small compared to the enrichments we report on here. Although the isotope signatures in the distinct carbon species are substantially different, they behave generally in a conservative way and isotope effects are negligible. Only when the pH changes significantly their proportions towards each other shift and influences the ^{13}C-CO_2/^{12}C-CO_2 composition. Equally, for the interpretation of ^{13}C-based microcosm experiments, putative fractionation effects might be an issue when the ^{13}C-CO_2 enrichments are rather small.

5. Conclusions

A semi-quantitative screening method based on ^{13}C-CO_2 analysis by GC-IRMS has been developed and evaluated. This new approach permitted to investigate the intrinsic biodegradation potential under in situ-like conditions, e.g. low temperature, no light and no shaking, using a high sediment to groundwater ratio and groundwater from the site. In situ biodegradation of benzene, naphthalene, and acenaphthene in alluvial sediments from the site of a former cokery was evident in aerobic and anaerobic microcosms amended with ^{13}C-labeled substrates. The fate of one particular compound out of a complex mixture could be followed even in long-term incubations. Substrate-specificity was guaranteed, when the distinct ^{13}C label was recovered in the CO_2 moiety. A particular strength of this approach is its applicability with substances that tend to sorb. Contrastingly, classical microcosm experiments rely on the analysis of contaminant concentrations over time which is unviable for PAHs in presence of sediment. Often, the initial concentrations are unknown and PAHs are strongly sorbed. Based on ^{13}C-labeled substrates, determination of absolute contaminant concentrations could be circumvented. Successful application of this technique was underlined by the high ^{13}C recovery in CO_2 and the high sensitivity reached by GC-IRMS analysis. The technique is restricted to compounds artificially labeled with a high proportion of ^{13}C. During in the last years however more and more ^{13}C-labeled compounds became commercially available.

This newly-developed screening approach has the potential to be used for the evaluation of intrinsic biodegradation of further contaminants even when they are slowly-degraded or partly sorbed on surfaces. Results on in situ degradation of contaminants by the intrinsic bacterial community and under in situ conditions can be elegantly used to round up field studies, e.g. in terms of the evaluation of the natural attenuation potential. To systematically investigate parameters that may limit biodegradation in situ, incubation conditions can be varied easily in microcosm experiments.

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References

Aelion, C.M., Bradley, P.M., 1991. Aerobic biodegradation potential of subsurface microorganisms from a jet fuel-contaminated aquifer. Applied and Environmental Microbiology 57, 57−63.

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: implications for intrinsic bioremediation. Environmental Science and Technology 34, 892−896.

Amann, R., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. FEMS Microbiology Reviews 59, 143−169.

Ambrosoli, R., Petruzzelli, L., Minati, J.L., Marsan, F.A., 2005. Anaerobic PAH degradation in soil by a mixed bacterial consortium under denitrifying conditions. Chemosphere 60, 1231−1236.

Bakermans, C., Hohnstock-Ashe, A.M., Padmanabhan, S., Padmanabhan, P., Madsen, E.L., 2002. Geochemical and physiological evidence for mixed aerobic and anaerobic field biodegradation of coal tar waste by subsurface microbial communities. Microbial Ecology 44, 107−117.

Beller, H.R., 2002. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring in situ BTEX biodegradation. Environmental Science and Technology 36, 2724−2728.

Bianchin, M., Smith, L., Barker, J.F., Beckie, R., 2006. Anaerobic degradation of naphthalene in a fluvial aquifer: a radiotracer study. Journal of Contaminant Hydrology 84, 178−196.

Boll, M., Fuchs, G., Heider, J., 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Current Opinion in Chemical Biology 6, 604−611.

Bolliger, C., Höhener, P., Hunkeler, D., Häberli, K., Zeyer, J., 1999. Intrinsic bioremediation of a petroleum hydrocarbon-contaminated aquifer and assessment of mineralization based on stable carbon isotopes. Biodegradation 10, 201−217.

Bosma, T.N.P., Middeldorp, P.J.M., Schraa, G., Zehnder, A.J., 1997. Mass transfer limitation of biotransformation: quantifying bioavailability. Environmental Science and Technology 31, 248−252.

Bouchez, M., Blanchet, D., Vandecasteele, J.-P., 1996. The microbial fate of polycyclic aromatic hydrocarbons: carbon and oxygen balances for bacterial degradation of model compounds. Applied Microbiology and Biotechnology 45, 556−561.

Bradley, P.M., Landmeyer, J.E., Chapelle, F.H., 2002. TBA biodegradation in surface-water sediments under aerobic and anaerobic conditions. Environmental Science and Technology 36, 4087−4090.

Bugna, G.C., Chanton, J.P., Kelley, C.A., Stauffer, T.B., MacIntyre, W.G., Libelo, E.L., 2004. A field test of $\delta^{13}C$ as a tracer of aerobic hydrocarbon degradation. Organic Geochemistry 35, 123−135.

Chang, B.V., Shiung, L.C., Yuan, S.Y., 2002. Anaerobic biodegradation of polycyclic aromatic hydrocarbon in soil. Chemosphere 48, 717−724.

Chapelle, F.H., Bradley, P.M., Lovley, D.R., Vroblesky, D.A., 1996. Measuring rates of biodegradation in a contaminated aquifer using field and laboratory methods. Ground Water 34, 691−698.

Clark, I., Fritz, P., 1997. Environmental Isotopes in Hydrogeology. Lewis Publishers, Boca Raton, FL.

Conrad, M.E., Daley, P.F., Fischer, M.L., Bucahnan, B.B., Leighton, T., Kashgarian, M., 1997. Combined ^{14}C and $\delta^{13}C$ monitoring of *in situ* biodegradation of petroleum hydrocarbons. Environmental Science and Technology 31, 1463−1469.

EC, 2006. Directive of the European Parliament and of the Council on Environmental Quality Standards in the Field of Water Policy and Amending Directive 2000/60EC. COM (2006) 398final, vol. 2006/0129 (COD). Brussels, pp. 1−25.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., Suflita, J.M., 2001. Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. Environmental Science and Technology 35, 682−689.

Geyer, R., Peacock, A.D., Miltner, A., Richnow, H.-H., White, D.C., Sublette, K.L., Kästner, M., 2005. In situ assessment of biodegradation potential using biotraps amended with ^{13}C-labeled benzene or toluene. Environmental Science and Technology 39, 4983−4989.

Guerin, W.F., Boyd, S.A., 1997. Bioavailability of naphthalene associated with natural and synthetic sorbents. Water Research 31, 1504−1512.

Hanson, J.R., Macalady, J.L., Harris, D., Scow, K.M., 1999. Linking toluene degradation with specific microbial populations in soil. Applied and Environmental Microbiology 65, 5403−5408.

Heinaru, E., Merimaa, M., Viggor, S., Lehiste, M., Leito, I., Truu, J., Heinaru, A., 2005. Biodegradation efficiency of functionally important populations selected for bioaugmentation in phenol- and oil-polluted area. FEMS Microbiology Ecology 51, 363−373.

Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Rüegg, I., Bucheli-Witschel, M., Faber, F., Egli, T., Mau, M., Schlömann, M., Brennerova, M., Brenner, V., Pieper, D.H., Top, E.M., Dejonghe, W., Bastiaens, L., Springael, D., 2006. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. Journal of Microbiological Methods 64, 250−265.

Hunkeler, D., Höhener, P., Zeyer, J., 2002. Engineered and subsequent intrinsic in situ bioremediation of a diesel fuel contaminated aquifer. Journal of Contaminant Hydrology 59, 231−245.

Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution 133, 71−84.

Jonker, M.T.O., Brils, J.M., Sinke, A.J.C., Murk, A.J., Koelmans, A.A., 2006. Weathering and toxicity of marine sediments contaminated with oils and polycyclic aromatic hydrocarbons. Environmental Toxicology and Chemistry 25, 1345−1353.

Kleineidam, S., Schüth, C., Grathwohl, P., 2002. Solubility-normalized combined adsorption-partitioning sorption isotherms for organic pollutants. Environmental Science and Technology 36, 4689−4697.

Kubicki, J.D., 2006. Molecular simulations of benzene and PAH interactions with soot. Environmental Science and Technology 40, 2298−2303.

Kuhn, E.P., Suflita, J.M., 1989. Microbial degradation of nitrogen, oxygen and sulfur heterocyclic compounds under anaerobic conditions: studies with aquifer samples. Environmental Toxicology and Chemistry 8, 1149−1158.

Lesniak, P.M., Zawidzki, P., 2006. Determination of carbon fractionation factor between aqueous carbonate and CO2(g) in two-direction isotope equilibration. Chemical Geology 231, 203−213.

Löffler, F.E., Sun, Q., Li, J., Tiedje, J.M., 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Applied and Environmental Microbiology 66, 1369−1374.

Loibner, A.P., Szolar, O.H.J., Braun, R., Hirmann, D., 2004. Toxicity testing of 16 priority polycyclic aromatic hydrocarbons using Lumistox. Environmental Toxicology and Chemistry 23, 557−564.

Lovley, D.R., 2001. Anaerobes to the rescue. Science 293, 1444−1446.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., Richnow, H.H., 1999. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environmental Microbiology 1, 409−414.

Meckenstock, R.U., Morasch, B., Kästner, M., Vieth, A., Richnow, H.H., 2002. Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air and Soil Pollution: Focus 2, 141−152.

Meckenstock, R.U., Morasch, B., Griebler, C., Richnow, H.H., 2004. Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. Journal of Contaminant Hydrology 75, 215−255.

Medina-Bellver, J.I., Marin, P., Delgado, A., Rodriguez-Sanches, A., Reyes, E., Ramos, J.L., Marques, S., 2005. Evidence for *in situ* crude oil biodegradation after the *Prestige* oil spill. Environmental Microbiology 7, 773−779.

Mihelcic, J.R., Luthy, R.G., 1988. Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Applied and Environmental Microbiology 54, 1188−1198.

Morasch, B., Richnow, H.H., Schink, B., Vieth, A., Meckenstock, R.U., 2002. Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons. Applied and Environmental Microbiology 68, 5191−5194.

Norris, R.D., 1994. In-situ bioremediation of soils and groundwater contaminated with petroleum hydrocarbons. In: Norris, R.D. (Ed.), Handbook of Bioremediation. Lewis Publishers, Boca Raton, FL, pp. 17−37.

Pelz, O., Chatzinotas, A., Zarda-Hess, A., Abraham, W.-R., Zeyer, J., 2001. Tracing toluene-assimilating sulfate-reducing bacteria using ^{13}C-incorporation in fatty acids and whole-cell hybridisation. FEMS Microbiology Ecology 38, 123−131.

Scow, K.M., Hicks, K.A., 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Current Opinion in Biotechnology 16, 246−253.

Sherwood Lollar, B., Slater, G.F., Ahad, J., Sleep, B., Spivack, J., Brennan, M., MacKenzie, P., 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. Organic Geochemistry 30, 813−820.

Smets, B.F., Pritchard, P., 2003. Elucidating the microbial component of natural attenuation. Current Opinion in Biotechnology 14, 283−288.

Steinbach, A., Seifert, R., Annweiler, E., Michaelis, W., 2004. Hydrogen and carbon isotope fractionation during anaerobic biodegradation of aromatic hydrocarbons, a field study. Environmental Science and Technology 38, 609−616.

US-EPA, 1999. Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. OSWER directive 9200, 4−17.

Volkering, F., Breure, A.M., Sterkenburg, A., van Andel, J.G., 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Applied Microbiology and Biotechnology 36, 548−552.

Vroblesky, D.A., Chapelle, F.H., 1994. Temporal and spatial changes of terminal electro-accepting processes in a petroleum hydrocarbon-contaminated aquifer and the significance for contaminant biodegradation. Water Resources Research 30, 1561−1570.

Watanabe, K., Hamamuray, N., 2003. Molecular and physiological approaches to understanding the ecology of pollutant degradation. Current Opinion in Biotechnology 14, 289−294.

Wiedemeier, T.H., Rifai, H.S., Newell, C.J., Wilson, J.T., 1999. Natural Attenuation of Fuels and Chlorinated Solvents in the Subsurface. John Wiley & Sons, Inc, New York.

Yaws, C.L., 1999. Chemical Properties Handbook. McGraw-Hill, New York.

Zamfirescu, D., Grathwohl, P., 2001. Occurrence and attenuation of specific organic compounds in the groundwater plume at a former gasworks site. Journal of Contaminant Hydrology 53, 407−427.

Zhang, J., Quay, P.D., Wilbur, D.O., 1994. Carbon isotope fractionation during gas-water exchange and dissolution of CO_2. Geochimica et Cosmochimica Acta 59, 107−114.