Published in Water Research 45, 4459-4469, 2011
which should be used for any reference to this work

Intrinsic biodegradation potential of aromatic hydrocarbons in an alluvial aquifer – Potentials and limits of signature metabolite analysis and two stable isotope-based techniques

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Keywords:
Groundwater contamination
Natural attenuation
(Poly-) aromatic hydrocarbons
Signature metabolites
Stable isotopes
Biodegradation rates

A B S T R A C T

Three independent techniques were used to assess the biodegradation of monoaromatic hydrocarbons and low-molecular weight polyaromatic hydrocarbons in the alluvial aquifer at the site of a former cokery (Flémalle, Belgium).

Firstly, a stable carbon isotope-based field method allowed quantifying biodegradation of monoaromatic compounds *in situ* and confirmed the degradation of naphthalene. No evidence could be deduced from stable isotope shifts for the intrinsic biodegradation of larger molecules such as methylnaphthalenes or acenaphthene. Secondly, using signature metabolite analysis, various intermediates of the anaerobic degradation of (poly-) aromatic and heterocyclic compounds were identified. The discovery of a novel metabolite of acenaphthene in groundwater samples permitted deeper insights into the anaerobic biodegradation of almost persistent environmental contaminants. A third method, microcosm incubations with ^{13}C-labeled compounds under *in situ*-like conditions, complemented techniques one and two by providing quantitative information on contaminant biodegradation independent of molecule size and sorption properties. Thanks to stable isotope labels, the sensitivity of this method was much higher compared to classical microcosm studies. The ^{13}C-microcosm approach allowed the determination of first-order rate constants for ^{13}C-labeled benzene, naphthalene, or acenaphthene even in cases when degradation activities were only small. The plausibility of the third method was checked by comparing ^{13}C-microcosm-derived rates to field-derived rates of the first approach. Further advantage of the use of ^{13}C-labels in microcosms is that novel metabolites can be linked more easily to specific mother compounds even in complex systems. This was achieved using alluvial sediments where ^{13}C-acenaphthyl methylsuccinate was identified as transformation product of the anaerobic degradation of acenaphthene.

1. Introduction

Biodegradation is claimed to be the key process leading to decontamination of many abandoned industrial sites impaired with coal- and tar oil-derived compounds. In practice, it is often difficult to judge whether degradation is taking place or not. Under environmental conditions, particularly in the absence of oxygen, mono- and polyaromatic contaminants are

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biodegraded at low rates or are supposedly persisting (Zamfirescu and Grathwohl, 2001; Foght, 2008). Consequently, solid information is needed on the *in situ* biodegradation of coal- and tar oil-derived pollutants. Due to low solubility in water and tendencies to sorb, turnover of (poly-) aromatic compounds (PAHs) may often be limited by mass transfer and not by microbial activity (Bosma et al., 1997). It is controversial whether biodegradation of aromatic hydrocarbons can be distinguished at all from partitioning when only a small decrease in concentration is measured (Foght, 2008). Even though it is considered as an issue of increasing importance, no universal technique exists to measure biodegradation at contaminated sites. In this study, three independent approaches were used in combination to assess the intrinsic biodegradation of aromatic and heterocyclic environmental contaminants. Particular attention was paid to useful approaches for the quantification of *in situ* biodegradation of compounds that show small degradation activities or have the tendency to sorb. The potentials and limits of the three techniques are compared and discussed.

1.1. *Compound-specific stable isotope analysis of individual pollutants in groundwater samples of contaminated sites*

This technique has proven appropriate for the direct assessment of biodegradation at those field sites where a contaminant plume has established. Along the centerline of a contaminant plume, *in situ* biodegradation is resolved over distance in the stable isotope shifts of individual groundwater pollutants. Stable isotope fractionation during degradation of monoaromatic hydrocarbons, naphthalenes, alkanes, chlorinated solvents, and gasoline additives was studied under various redox conditions (Hunkeler and Morasch, 2010). Larger molecules of high environmental relevance (e.g. three and more ring PAHs) have been investigated much less extensively and no enrichment of heavier isotopes above the analytical error was reported (Mazeas et al., 2002). Furthermore, compound-specific stable isotope analysis (CSIA) was used in field studies to calculate the percentage of *in situ* biodegradation and first-order rate constants (Richnow et al., 2003b; Batlle-Aguilar et al., 2009).

1.2. *Screening for signature metabolites indicating contaminant biodegradation in groundwater samples*

Signature metabolites are highly specific reaction intermediates produced only during biodegradation of target contaminants. They need to be excluded as contaminants themselves in order to be indicative of on-site remediation (Phelps et al., 2002). In previous studies, various signature metabolites were either identified in batch culture experiments or were extracted from groundwater samples directly. Recognized molecules include intermediates of the anaerobic degradation of alkylated aromatic compounds, e.g. benzylsuccinates that are formed in addition reactions with fumarate (Elshahed et al., 2001; Beller, 2002). Apart from methylated benzenes and naphthalenes, methylated heterocyclic compounds are anaerobically degraded also via initial fumarate addition (Annweiler et al., 2000; Safinofski et al., 2006).

1.3. *Incubations of field material in the lab under* in situ*-like conditions adding isotopically labeled contaminants*

The intrinsic biodegradation potential of individual compounds can be assessed by incubating field material in the laboratory under *in situ*-like conditions. Even in complex systems, biodegradation can be specifically tracked when isotopically labeled compounds are supplied as markers. Isotope labels circumvent difficulties related to classical microcosm experiments with field material that potentially contains organic background contaminants. Briefly, the method is based on the recovery of isotope labels in CO_2 produced from mineralization of isotope-labeled substrates. Complete oxidation of naphthalene under anoxic conditions e.g. was shown by adding ^{14}C-labeled naphthalene to aquifer material and to marine harbor sediments (Chapelle et al., 1996; Coates et al., 1996; Langenhoff et al., 1996). Recently, naphthalene was also used in ^{13}C-labeled form to confirm biodegradation in sediment–groundwater microcosms (Morasch et al., 2007).

All techniques mentioned above can provide evidence for natural attenuation of contaminants according to the three lines of evidence established by the National Research Council (2000). By definition, conditions are met if (I) the loss of contaminant is documented at the field scale, (II) the presence of degrading microorganisms is confirmed by means of incubation experiments using field material, and (III) the direct evidence for microbial activity *in situ* can be provided. Techniques one (CSIA) and two (signature metabolite analysis) meet criterion (III). They have already been applied in combination, e.g. at a former gasworks site with a long-standing contamination with mono- and polyaromatic hydrocarbons and in a controlled release experiment of benzene, toluene, and *o*-xylene at a US Air Force base (Griebler et al., 2004; Beller et al., 2008). However, the combination of CSIA and signature metabolite analysis has its limitations. CSIA has the potential to quantify the biodegradation of smaller contaminants, while its sensitivity decreases with increasing molecular size of the compounds of interest. Since frequently the biodegradation rates also get lower with molecular size the method is not applicable for larger contaminants. Signature metabolite analysis can – in a qualitative way – provide information on the biodegradation of these larger compounds. Hence, degradation kinetics of larger contaminant molecules remains unrevealed. In this study we use a third technique (microcosms with ^{13}C-labeled compounds) to overcome these limitations and to provide additional quantitative information on *in situ* biodegradation independent of molecular size. Results of all three techniques are compared and their potentials and limits evaluated.

2. Materials and methods

2.1. *Field site*

The study site was a former coke and gas factory that was dismantled in 1984. The property of 400 m × 250 m was

situated in an industrial environment at 25 m distance from the Meuse River upstream of the city of Liège, Belgium (Fig. 1; geographical location: +50° 36′ 19.76″, +5° 29′ 13.52″, www.google.com/maps). The topmost 4 m below the surface consisted of backfill material followed by silt, sand, and clay deposits of about 2 m thickness, and 8 m of fine gravel above the carboniferous shale bedrock. The water table was located in the alluvial gravel layer at 5.5–7 m depth. Groundwater flow was in eastern direction. Previous field characterization evidenced a hydrological gradient of 0.3% and a saturated hydraulic conductivity that varied between 10^{-5} and 10^{-3} m/s (Batlle-Aguilar et al., 2009). Severe contamination with heavy metals, cyanides, mineral oils, as well as with mono- and polyaromatic hydrocarbons reached down to 11 m depth. The contamination was of unknown horizontal extent according to various measurements by the Walloon Environmental Protection Agency. In the north-western part of the site (Fig. 1), Eh values of the groundwater were at −300 mV and nitrate was almost depleted. Strictly reducing conditions prevailed up to 100 m in southeastern direction toward the Meuse River before the Eh rose to +100 mV and nitrate concentrations of up to 15 mg L^{-1} were observed. Sulfate concentrations in groundwater were between 500 and 2100 mg/L, hence sulfate was assumed to be the major electron acceptor in the degradation of organic contaminants after O_2 had been depleted (For a redox zonation map, see Batlle-Aguilar et al., 2009).

2.2. *Sampling*

Sampling campaigns took place in March 2005 and July 2006 where 17 and 23 groundwater wells were sampled, respectively (Table S1). Water was pumped with submersible pumps at a rate of 1–5 L/min. Groundwater table, temperature, conductivity, pH, and dissolved O_2 were recorded using specific field probes (WTW; Weilheim, Germany). Subsequently, water was sampled to determine alkalinity, Mn^{2+}, Fe^{2+}, HS^-, and methane. Additional samples were taken for the quantification of aromatic contaminants, CSIA, and the identification of signature metabolites. Water samples were conserved immediately on site by adding 0.1% (vol/vol) of NaOH (5 M). Upgradient from the contamination, groundwater wells E6p and F4 served as references (Fig. 1). In 2005, freshly drilled core material from location *U13* adjacent to the source zone was sampled every 0.5 m for microcosm experiments.

2.3. *Microcosm set-up and sampling*

Aquifer material was collected during drillings, filled into brown-glass bottles of 120 mL with Teflon-sealed caps, cooled immediately, and stored at 5 °C until microcosms were prepared. Sediments of core *U13* were pooled to four different depth layers (5–6, 7–8, 9, and 12–13 m). Working under an atmosphere of N_2 in a glove bag (Sigma–Aldrich), 53 microcosms were set up in culture bottles of 50 mL volume. Every microcosm consisted of 25 g of sediment and 15 mL of O_2-free groundwater that had been sterile-filtered and diluted fivefold with autoclaved nanopure water, to achieve nitrate concentrations that better resembled groundwater concentrations of the strictly reducing zone. The diluted groundwater contained 6 mg/L of nitrate and 140 mg/L of sulfate. Benzene, naphthalene, or acenaphthene labeled with ^{13}C at six positions (99% purity, Cambridge Isotope Laboratories) was dissolved in the anoxic groundwater that was added to the culture bottles (Morasch et al., 2007). The headspace either was air for sediments from the unsaturated zone or N_2 for microcosms with sediments from the saturated zone. Microcosms were closed with non-absorptive Viton rubber stoppers. Controls were

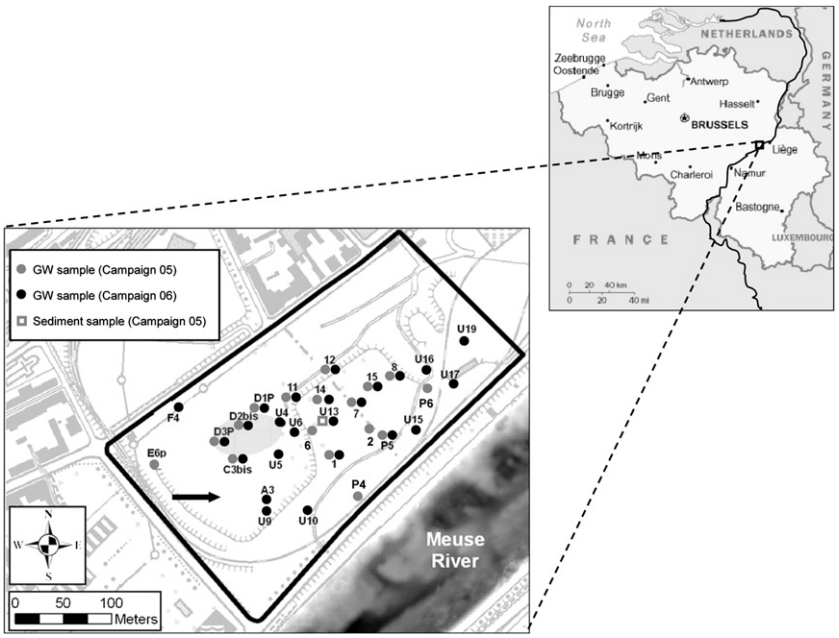

Fig. 1 – Site map of the former cokery of Flémalle, Belgium, located in the direct vicinity of the river Meuse. Locations of groundwater (GW) samples are shown as circles, the sediment sampling location U13 is depicted as square. The arrow roughly describes the groundwater flow direction. The map has been modified from Batlle-Aguilar et al. (2009).

prepared without addition of substrate or microcosms were autoclaved twice at days 1 and 4 of the experiment. Incubations took place in the dark at 16 °C.

2.4. Analysis of organic contaminants

Concentrations of benzene, toluene, ethylbenzene, and *m*-xylene (BTEX), and of low-molecular weight PAHs were analyzed using a gas chromatograph (Varian 3800) with a CP8410 autoinjector for solid phase microextraction (SPME). The aromatic compounds were extracted from the headspace of 2 mL vials filled with 0.5 mL of groundwater and 0.3 g of NaCl using polydimethylsiloxane fibers (100 μm film thickness, Supelco). Extraction and analysis were performed as described previously (Morasch et al., 2007).

2.5. Stable carbon isotope analysis

Groundwater for $^{13}C/^{12}C$ isotope analysis was sampled in volumes of 1–2 L. CSIA of aromatic hydrocarbons was performed using a Trace GC coupled to a Delta Plus XP isotope ratio mass spectrometer (IRMS) via a GC combustion III interface (Thermo Finnigan). Complete protocols of sample preparation and analysis are provided in Supplementary material.

For stable carbon isotope analysis of CO_2, 2 mL headspace samples were taken from microcosms through the Viton stoppers with a gas tight syringe working under an atmosphere of N_2. Sampling intervals were between three and seven days at the beginning of the incubation and three-monthly toward the end of the experiment (Table S2). Concentration measurements of CO_2 and stable carbon isotope measurements were performed connecting a headspace autosampler (Tekmar Dohrmann 7000) to the GC-IRMS using a previously described protocol (Morasch et al., 2007). CO_2 concentrations were quantified based on five-point calibration curves with an average correlation of $r = 0.98$. Stable isotope ratios were determined relative to an external CO_2 reference gas and reported as δ [‰] deviation to the VPDB standard

$$\delta^{13}C\ [‰] = \left(\frac{R_{sample}}{R_{std}} - 1\right) \times 1000 \quad (1)$$

where R_{sample} and R_{std} are the carbon stable isotope ratios of the sample and of the standard, respectively.

2.6. Metabolite extraction, analysis, and identification

Putative degradation intermediates of aromatic or heterocyclic contaminants were extracted with dichloromethane from 1 L groundwater samples acidified to pH 1–2 with HCl (37%). The extraction was repeated and dichloromethane fractions were pooled. The complete protocol is provided in Supplementary material.

After the completion of degradation experiments, potential metabolites were extracted from the water phase and the sediment of microcosms. For metabolite extraction from the sediment fraction, 20 mL of acetone was added into the microcosm bottles and placed into an ultrasonic bath for 10 min. The acetone phase was decanted from the microcosm bottle and the procedure was repeated with 20 mL of dichloromethane. Then, extracts from the water phase and the sediment fraction were combined. The complete extraction protocol may be found in Supplementary material.

Analysis by GC–MS was performed using a Trace GC coupled to a Polaris Q Ion Trap Mass spectrometer (Finnigan). Identity of substances was confirmed by co-elution with reference compounds and by comparison of mass spectra with published data.

2.7. Calculations

In situ biodegradation of BTEX was calculated using the approach of Richnow et al. (2003b):

$$B[\%] = \left(1 - \frac{c_t}{c_0}\right) \times 100 \quad (2)$$

where B is the percentage of biodegradation of the substrate; c_0 and c_t are substrate concentrations at the source and at a downgradient monitoring point. Independently from concentration measurements, c_t was obtained from c_0 in combination with the stable isotope ratios R_0 at the source and R_t at a downgradient monitoring point, and substrate-specific stable isotope enrichment factors (ε) derived from laboratory studies:

$$c_t = c_0 \times \left(\frac{R_t}{R_0}\right)^{\left(\frac{1000}{\varepsilon}\right)} \quad (3)$$

First-order biodegradation rates λ were calculated from field isotope data according to

$$\lambda = -\Delta\delta^{13}C/(\varepsilon \times t_c) \quad (4)$$

where $\Delta\delta^{13}C$ is the shift in the carbon isotope ratio between the source and a downgradient monitoring point, and t_c is the travel time of the contaminant (Hunkeler et al., 2002; Blum et al., 2009). Travel times of the contaminants were estimated based on an intermediate groundwater flow velocity of 0.29 m d^{-1} (Batlle-Aguilar et al., 2009) and compound-specific retardation factors

$$F_R = 1 + \left(\rho\frac{1-\phi}{\phi}\right) \times K_d \quad (5)$$

that were obtained assuming a sediment density of $\rho = 2500$ kg/m^3 and a mobile porosity of $\phi = 0.2$. Solid–water distribution coefficients $K_d = f_{OC} \times K_{OC}$ were calculated assuming a fraction of organic carbon in the sediments of $f_{OC} = 0.001$. Organic carbon-normalized distribution coefficients K_{OC} were predicted from compound class-specific $\log K_{OC} - \log K_{OW}$ relationships (Schwarzenbach et al., 2003) taking octanol–water coefficients K_{OW} from the Physical Properties Database (SRC Inc., 2009). Half-life times $t_{1/2}$ and half-concentration distances $x_{1/2}$ were defined as follows:

$$t_{1/2} = \frac{\ln(2)}{\lambda} \quad (6)$$

$$x_{1/2} = \frac{v}{F_R} \times t_{1/2} \quad (7)$$

where v is the intermediate groundwater flow velocity in the aquifer.

2.8. Inorganic carbon mass balance

To assess the intrinsic biodegradation potential of the ^{13}C-labeled contaminants, an inorganic carbon mass balance was applied to sediment–groundwater microcosms (a detailed description is provided in the Supplementary material). The initial amount of inorganic carbon M_0 was approximated from the sum of CO_2 (g) as determined by GC-IRMS at the beginning of the microcosm experiment, H_2CO_3 (aq), and the concentration of HCO_3^- (aq) of 2 mM determined by alkalinity titration for the groundwater added to microcosm bottles at the beginning of the experiment (see Supplementary material for determination of alkalinity). CO_2 dissolved in the water phase was calculated from CO_2 concentrations in the gas phase using Henry's law according to $[H_2CO_3(aq)] = [CO_2(g)] \times K_h$ with a Henry coefficient of $K_h = 0.77$ (SRC Inc., 2009). The amount of inorganic carbon produced upon the biodegradation of ^{13}C substrates at time t, is depicted M_S. Compared to M_S, the isotope signature of ^{13}C-CO_2 originating from the degradation of undefined background carbon sources (M_{BG}) was considered equal to the isotope signature M_0 at the beginning of the experiment.

The biodegradation rate was calculated using a first-order type equation:

$$M_{S(0)} - M_S = M_{S(0)} \times e^{\frac{-\lambda \cdot t}{F_R}} \quad (8)$$

with $M_{S(0)}$ being the amount of ^{13}C [μmol] added to the bottle at $t = 0$ d, the first-order rate constant λ, and the reciprocal value of the retardation factor $1/F_R$ designating the fraction of contaminant present in the water phase of the microcosms (Schwarzenbach et al., 2003). For hydrophobic compounds such as BTEX and PAH, the correction by F_R is necessary to account for sorption to the sediment matrix since only the dissolved compound fraction is readily available for biodegradation. ^{13}C-CO_2-based biodegradation rates were validated previously by comparing the changes in the ^{13}C-aniline concentrations in the water phase to the concomitant evolution of ^{13}C-CO_2 in the gas phase (Morasch et al., 2011).

3. Results and discussion

3.1. Abundance of aromatic hydrocarbons in groundwater

Based on a systematic assessment of contaminant concentrations in the aquifer of the Flémalle site, one major source zone was identified in the north-western part around piezometers *D2bis*, *D1p*, and *D3p* (Fig. 1). In that zone, groundwater concentrations of benzene, toluene, and *m*-xylene were in the mg/L range; ethylbenzene was in the μg/L range (Table S3). Also the highest concentrations of naphthalene (up to 25 mg/L) were detected in *D2bis*, *D1p*, and *D3p* and decreased along the groundwater flow path toward the Meuse River in eastern direction (Fig. 2a). Concentrations of low-molecular weight PAHs were elevated in piezometer 14 located 39 m downgradient of well *D2bis*. At well 15, 133 m east of the source zone, there was an additional point of increased contamination with the three-ring compounds acenaphthene and fluorene (Table S3).

3.2. Qualitative assessment of in situ biodegradation using CSIA

$^{13}C/^{12}C$ isotope signatures of residual groundwater contaminants were measured using CSIA in 2005 and 2006. Improved protocols (Purge&Trap for BTEX, SPME for PAHs) resulted in a higher number of isotope signatures that could be determined in 2006 (Table S4). Most negative δ-values of BTEX were found in the major source zone (*D2bis*, *D3p*, *D1p*). Then, ^{13}C became progressively enriched in the residual BTEX along the groundwater flow path in the methanogenic to sulfate-reducing aquifer section between the source and well *U13*. Less reducing groundwater sampled further toward the river showed lower $^{13}C/^{12}C$ ratios in the residual contaminants. Representative for all aromatic hydrocarbons detected at the Flémalle site, stable carbon isotope ratios at the Flémalle site are displayed for benzene, naphthalene, and acenaphthene (Fig. 2b–d). According to CSIA, no naphthalene degradation took place within the first 90 m of the contaminant plume. However, ^{13}C-enriched naphthalene (with a signature of $-19.7‰$) in the groundwater of well *15*, suggested biodegradation beyond the strictly reducing zone in direction toward the river. No conclusive stable isotope shift was obtained for acenaphthene.

For comparison, at a former gas manufacturing plant in Southern Germany $\delta^{13}C$ shifts of 3.3 and 3.6‰ provided evidence for intrinsic anaerobic biodegradation of benzene and naphthalene (Griebler et al., 2004). At that site, acenaphthene formed long contaminant plumes with almost constant concentrations and insignificant stable carbon isotope shifts over a distance of more than 135 m (Zamfirescu and Grathwohl, 2001; Steinbach et al., 2004). This lack of evidence for *in situ* biodegradation of acenaphthene is in agreement with our findings (Fig. 2d).

3.3. Quantitative assessment of in situ biodegradation

The BTEX plume originating from the source zone around piezometer *D2b* was delineated using the piezometric lines (as established by Batlle-Aguilar et al., 2009) in combination with ^{13}C ratios of the residual BTEX of the campaign 2006 (Fig. S1). Due to the continuous increase in the $\delta^{13}C$ values of residual benzene, toluene, ethylbenzene, and *m*-xylene with distance in the strongly reducing part of the aquifer, piezometers *D2bis*, *D1p*, *U4*, *11*, and *U13* were attributed to the same contaminant plume. Significantly more negative δ-values in wells *U6* and *12* (partially even below the isotope signatures at the source *D2bis*) suggested local secondary sources of contaminants with BTEX concentrations that were orders of magnitude below the concentration in the major source zone (well *D1p*). Wells *U6* and *12* were consequently excluded from the quantitative assessment of *in situ* biodegradation. Mixing of contaminants originating from secondary sources with the main contaminant plume that is more enriched in ^{13}C, would lead to slight underestimations of the *in situ* degradation. In the following moderately reducing to oxic aquifer section 90–160 m from the major source zone, no further ^{13}C-enrichment in residual BTEX was observed. Occasionally, the residual monoaromatic contaminants showed more negative $\delta^{13}C$ ratios (Fig. S1). Well *15* was not considered for quantitative evaluation, since it was surrounded by a less reducing to oxic zone (Batlle-Aguilar et al., 2009).

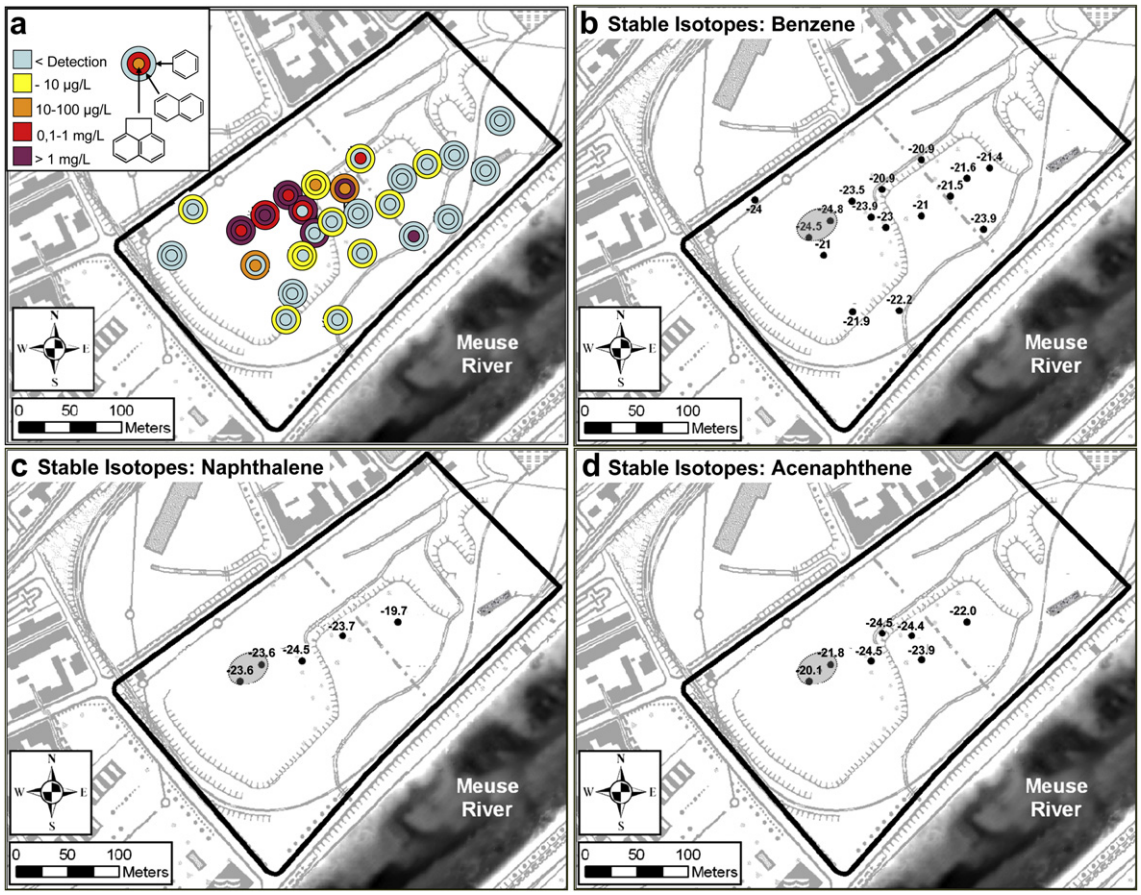

Fig. 2 – a–d: Concentrations of benzene, naphthalene, and acenaphthene and corresponding stable carbon isotope ratios determined in groundwater of the Flémalle site in 2006. Benzene (outer circle), naphthalene (middle circle), and acenaphthene (inner circle) were chosen as representatives of mono-, di-, and tri-ring aromatic compounds. b was modified from Batlle-Aguilar et al. (2009).

The percentage of biodegradation was based on the ^{13}C-enrichment in residual groundwater contaminants with distance from the source (Eqs. (2) and (3)). The results confirmed that a considerable part of benzene, toluene, and *m*-xylene was already biodegraded within the strictly reducing zone (Fig. 3). According to CSIA, the percentage of biodegradation was highest for *m*-xylene (>90% relative to *D2bis*); 80% of the initial benzene and toluene, and 40% of the initial ethylbenzene were removed by biodegradation. Due to decreasing δ-values in the residual BTEX at 90–160 m distance from the source zone, it was not possible to quantify the biodegradation in this aquifer section using the stable isotope-based approach (Fig. 3). This is comparable to another anoxic aquifer, where ≥99% of the decrease in toluene and *o*-xylene could be attributed to intrinsic biodegradation based on the observed stable isotope shifts (Richnow et al., 2003a).

Apart from the percentage of biodegradation, the kinetics of the anaerobic *in situ* biodegradation was determined. First-order rate constants (λ) were calculated based on the continuous ^{13}C-enrichment downgradient the source zone (Eq. (4)). For this, distances were converted into travel times using an intermediate groundwater flow velocity of 0.26 m/d. For the single stretches from *D2bis* to the individual piezometers

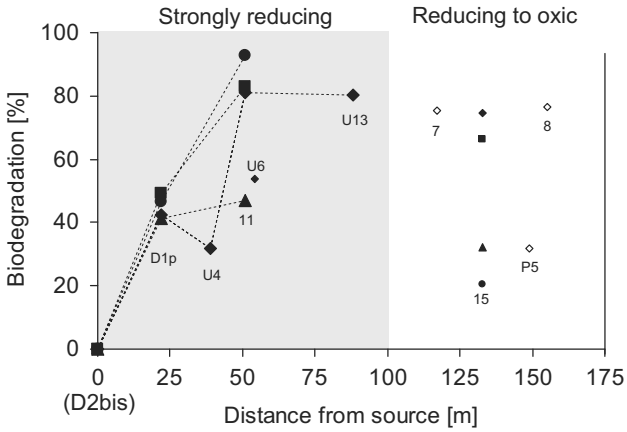

Fig. 3 – Percentage of biodegradation of benzene (◆), toluene (●), ethylbenzene (▲), and *m*-xylene (■) along the groundwater flow path. Filled symbols indicate strictly reducing—, open symbols mildly reducing to oxic groundwater samples. Large symbols represent samples, which were incorporated in the first-order rate calculations (see text for details).

downgradient of the source, anaerobic *in situ* biodegradation rate constants were in the range of 1.4×10^{-3}/d for ethylbenzene and 4.0×10^{-3}/d for toluene (Table 1). Assessing first-order biodegradation between *D2bis* and *U13* via linear regression resulted in comparable rate constants.

Recently, the stable isotope-based model was applied to determine first-order rate constants for *o*-xylene and naphthalene degradation at a former wood preservation plant (Blum et al., 2009). Under strictly reducing conditions, biodegradation of *o*-xylene proceeded at a rate of 2×10^{-3}/d which was in the same range as the rate constants for monoaromatic compounds at the Flémalle site. At the other site, it was possible to determine first-order rate constants of naphthalene of 4×10^{-3}/d and 3×10^{-3}/d for the anoxic and oxic sections of the plume, respectively (Blum et al., 2009).

3.4. *Screening for signature metabolites of contaminant biodegradation in groundwater*

Screening campaigns for metabolites in groundwater samples were performed in 2005 and 2006. In the first year, a series of carboxylic acid and alcohol derivatives of aromatic compounds were detected in groundwater samples of wells *D2bis* and *C3bis*, however, no signature metabolites in the strict sense could be identified. Groundwater of more than 94 m distance from the source mostly was free of contamination with aromatic hydrocarbons (wells *1*, *2*, *7*, *8*, *P4*, and *P6*), and no potential metabolites of BTEX or PAHs were detected. In order to improve the sensitivity, larger volumes of groundwater were extracted in the second campaign. Methylsuccinyl-adducts of five different aromatic hydrocarbons and carboxylic acids of twelve different aromatic and heterocyclic compounds were identified in form of their methyl esters in groundwater samples of ten different wells (Table S5). The fumarate adduct of toluene was identified by comparison with an authentic benzylsuccinate standard and had a GC-retention time of 28.0 min. Methyl esters of the fumarate adducts of xylene, methyl- and dimethylnaphthalene, were tentatively identified based on published reference spectra without distinguishing between the different isomers (Table S5). Fumarate adducts of BTEX were only detected in samples of well *D1p* (source zone), whereas methylsuccinates of naphthalene and methylnaphthalene were extracted from several wells in the strictly reduced zone of the aquifer. Putative acenaphthyl methylsuccinate was present in groundwater from *D2bis*, *D1p*, and well *14* (for the identification of this compound, see paragraph below). Carboxylic acids that were potentially related to the degradation of BTEX, biphenyl, or PAHs occurred in groundwater of the source zone and close by, as well as in piezometers *7* and *15*, both in the reducing section of the aquifer in 117 and 133 m distance from the source, respectively. Carboxylic acids of the heterocyclic compounds benzothiophene, benzofuran, indane, and indene were equally present in the source zone and in the strictly reducing groundwater sampled further downgradient (Table S5).

3.5. *Tentative identification of acenaphthene methylsuccinate*

Acenaphthyl methylsuccinate was not commercially available as reference compound; therefore, the potential first

Table 1 – First-order rate constants λ and corresponding half-life times $t_{1/2}$ of anaerobic *in situ* biodegradation of monoaromatic groundwater contaminants at the site of the former cokery of Flémalle. Values were calculated between isotope signatures in groundwater of *D2bis* (source) and individual piezometers located along the groundwater flow path in the strongly reducing part of the aquifer. An intermediate groundwater flow velocity of 0.26 m/d was assumed (Batlle-Aguilar et al., 2009). Calculations were based on average isotope enrichment factors taken from the literature (Hunkeler and Morasch, 2010). For comparison, rate constants from linear regression analysis retrieved from the stable isotope-based first-order biodegradation model are shown.

Well	Benzene			Toluene			Ethylbenzene			m-Xylene		
	Δδ [‰]	λ [/d]	$t_{1/2}$ [d]	Δδ [‰]	λ [/d]	$t_{1/2}$ [d]	Δδ [‰]	λ [/d]	$t_{1/2}$ [d]	Δδ [‰]	λ [/d]	$t_{1/2}$ [d]
D2bis	0.0			0.0			0.0			0.0		
D1p	1.3	4.6×10^{-3}	152	1.0	3.7×10^{-3}	185	1.1	1.9×10^{-3}	371	1.1	2.1×10^{-3}	337
U4	0.9	1.8×10^{-3}	389									
11	3.9	5.9×10^{-3}	117	2.6	4.2×10^{-3}	165	1.3	1.0×10^{-3}	727	4.6	3.7×10^{-3}	187
U13	3.8	3.3×10^{-3}	208									
Average		3.9×10^{-3}	178		4.0×10^{-3}	175		1.4×10^{-3}	491		2.9×10^{-3}	240
Linear regression		3.7×10^{-3}	187		4.3×10^{-3}	161		0.9×10^{-3}	770		3.9×10^{-3}	178
		$R^2 = 0.74$			$R^2 = 0.99$			$R^2 = 0.80$			$R^2 = 0.96$	

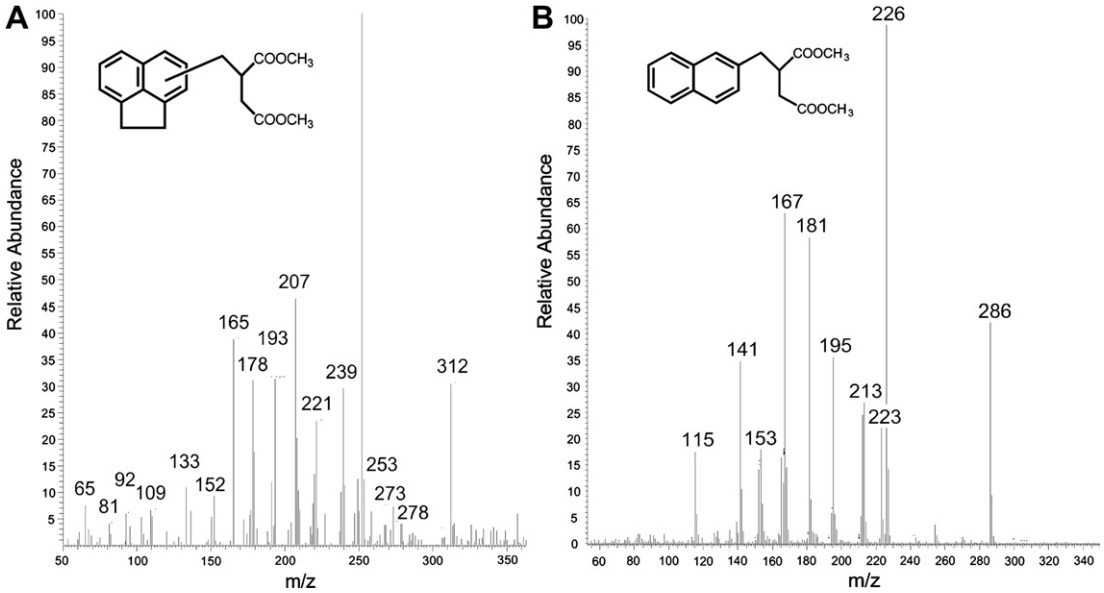

Fig. 4 – a and b: Mass spectra of dimethyl esters of putative acenaphthyl methylsuccinate (A), and naphthyl methylsuccinate (B) tentatively identified by comparison with a reference spectrum from Annweiler et al. (2000).

metabolite of methyl acenaphthene was tentatively identified based on three independent lines of evidence. Firstly, mass spectra of putative acenaphthyl methylsuccinate were compared to those of naphthyl methylsuccinate; both converted to their dimethyl esters (Fig. 4a and b). Due to the additional ethylene bridge of the acenaphthene skeleton, both molecules had an absolute difference of 26 *m/z* in their respective mass peaks (312 and 286 *m/z*). This shift of 26 *m/z* was reflected in all major peaks of both MS-fractionation patterns. Comparing the relative abundances of the major fragments of naphthyl methylsuccinate- and putative acenaphthyl methylsuccinate dimethyl ester, a correlation coefficient of $R = 0.879$ was obtained (Fig. 5). Secondly, the MS-fragmentation pattern of acenaphthyl methylsuccinate was compared to commercially available acenaphthene succinate. An absolute difference of mass fragments in 14 *m/z* was observed between the two respective dimethyl esters (Fig. 4a, Fig. S2a). Their GC-retention times were 41.7 and 40.5 min. Thirdly, several microcosms that had been incubated with ^{13}C-acenaphthene were solvent-extracted at the end of the incubation period in search of the metabolite additionally bearing six ^{13}C-atoms. Operating the GC–MS in single ion mode for higher sensitivity, putative $^{13}C_6$-acenaphthyl methylsuccinyl dimethyl ester was detected at 42.2 min (Fig. S2b). Although, several studies reported on the anaerobic degradation of acenaphthene in microcosm experiments, the degradation pathway has remained unknown (Mihelcic and Luthy, 1988; Rothermich et al., 2002; Chang et al., 2003; Yuan and Chang, 2007). The metabolite that was tentatively identified in this study suggested the introduction of a methyl group in the acenaphthene skeleton and a subsequent fumarate addition in analogy to the anaerobic degradation of naphthalene (Safinofski and Meckenstock, 2006; Foght, 2008).

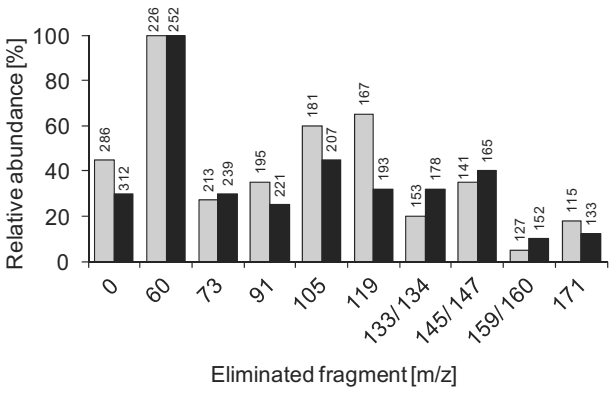

Fig. 5 – Comparison of mass fragmentation patterns of naphthyl methylsuccinate (gray bars) and putative acenaphthyl methylsuccinate (black bars). On the *x*-axis displayed is the size of the eliminated fragment (*m/z*), in case of deviating fragment size, the first value refers to naphthyl methylsuccinate and the second to acenaphthyl methylsuccinate. Values on top of the bars are major mass fragments of the original GC–MS spectra.

3.6. *Microcosms under* in situ*-like conditions with ^{13}C-labeled substrates*

The intrinsic biodegradation potential was studied in sediment–groundwater microcosms that were spiked with benzene, naphthalene, or acenaphthene in ^{13}C-labeled form and incubated under *in situ*-like conditions. Sediments originated from four different depth layers of drilling location U13 in the strictly reducing part of the aquifer (Fig. 1). The content in δ^{13}C-CO_2 in the headspace of all alive and dead controls stayed constant over the whole incubation experiment (Table S2). The aerobic degradation of benzene and naphthalene started within the first day, and the aerobic degradation of acenaphthene within less than one week after microcosm set-

Table 2 – Intrinsic first-order rate constants λ derived from sediment–groundwater microcosms at the site of the former cokery of Flémalle. Drilling material from different depth layers of location U13 was used. The table shows mean values of duplicate incubations, except for ^{13}C-benzene at 5 m, where one microcosm did not grow. n.d.: not determined.

Depth [m]	Conditions	^{13}C-Benzene		^{13}C-Naphthalene		^{13}C-Acenaphthene	
		λ [/d]	$t_{1/2}$ [d]	λ [/d]	$t_{1/2}$ [d]	λ [/d]	$t_{1/2}$ [d]
5	Oxic	2.1×10^{-2}	33	1.5×10^{-3}	457	8.7×10^{-3}	80
7	Anoxic	1.2×10^{-3}	581	0.3×10^{-4}	28,250	3.4×10^{-4}	2056
9	Anoxic	1.6×10^{-3}	422	0.7×10^{-4}	9412	n.d.	n.d.
12	Anoxic	0.9×10^{-3}	771	1.1×10^{-4}	6104	n.d.	n.d.

up. Under anoxic conditions, the mineralization of ^{13}C-benzene started within five days and reached a plateau after 48 days (158 days for the less permeable sediment from 7 to 8 m depth). Comparably, the anaerobic mineralization of naphthalene started within the first week of the incubation period but proceeded much slower than under oxic conditions. In sediments from two of the three examined anoxic depth layers, acenaphthene biodegradation started after a lag phase of more than 100 days. In anoxic sediment from 12 to 13 m depth, no intrinsic biodegradation potential for acenaphthene was detected over the whole duration of the microcosm experiment of 327 days (Table S2).

Compared to Morasch et al. (2007), we applied an advanced, quantitative approach where first-order rate constants (λ) of intrinsic contaminant degradation were determined based on an inorganic carbon mass balance (Eq. (8)). For sediments of the unsaturated zone incubated in presence of air, λ values were 2.1×10^{-2}/d, 1.5×10^{-3}/d, and 8.7×10^{-3}/d for benzene, naphthalene, and acenaphthene (Table 2). These rates were equivalent to half-life times ($t_{1/2}$) of 0.1, 1.3, and 0.2 years, respectively (Eq. (6)). In anoxic microcosms that contained sediments from the saturated zone, the 15–30 times lower rate constants corresponded to mean half-life times of 1.6, 40, and 5.6 years. Comparison of microcosm-derived first-order rate constants of benzene with the field-derived rates of the first approach revealed rates that were on average three times lower.

3.7. *Implications for the field site*

Based on stable isotope generated first-order rate constants (Eq. (7)), predicted half-concentration distances ($x_{1/2}$) of BTEX under anoxic conditions were between 15 and 32 m which is

Table 3 – Comparison of the three different approaches applied in this study summarizing their potentials and limits proving the *in situ* biodegradation of benzene, naphthalene, and acenaphthene. The quantitative and qualitative assessment of the three approaches is divided by a slash; + designates appropriate and – designates inappropriate approaches, ± stands for limited applicability.

	Stable isotopes (field)	Signature metabolites	^{13}C-microcosms
	+/+	–/±[b]	+/+
	+/±[a]	–/+	+/+
	–/–	–/±[c]	+/±[d]
Assessment	• Fast • Multi compound	• Fast • Multi compound	• Slow, long term • Single compound
Restrictions	• For smaller molecules	• For compounds with signature metabolites	• Time-consuming
Needs	• Experimental ε value	• Knowledge of degradation pathway	• ^{13}C substrates
Prospects	• Determination of reaction mechanisms from isotope effect	• Identification of new metabolites	• Detection of metabolites • Microcosms can be used for SIP[e]

Gray fields mark the most suitable options.
a Method successful in another study.
b Metabolites are ambiguous.
c New metabolites postulated based on this method.
d Duration of experiment in parts too short for rate determination.
e Stable isotope probing – for a review, see Madsen, 2006.

in agreement with the flow and transport model of benzene (Batlle-Aguilar et al., 2009). In contrast, predictions based on the half-life times of benzene, naphthalene, and acenaphthene in anoxic microcosms resulted in $x_{1/2}$ of 100, 412, and 16 m, respectively. The groundwater flow path from the contaminant source *D2bis* to the river bank is approximately 220 m long and the first 100 m are under anoxic conditions. For the following oxic plume interval, shorter half-concentration distances of 6, 13, and 1 m for benzene, naphthalene, and acenaphthene were predicted based on microcosm-derived rate constants that were corrected for contaminant retardation in groundwater. Event-based infiltration of surface water supplies additional O_2 in this part of the aquifer (Batlle-Aguilar et al., 2009). Predictions on the basis of stable isotopes matched the observations from the field: neither BTEX nor PAHs were ever detected in piezometers close to the river bank (Table S3).

4. Synthesis – complementarity of approaches

In a recent review on the assessment of *in situ* biodegradation, Bombach et al. (2010) recommended using several approaches in combination according to the local conditions. In the present study, we employed three approaches in order to gain qualitative and quantitative information on the *in situ* biodegradation of BTEX and PAHs (Table 3). What distinguishes our combination from many others is its applicability to a wider variety of contaminants – independent of molecule size and hydrophobicity.

CSIA of groundwater pollutants, the first technique that we applied, is useful for collecting data on the anaerobic *in situ* biodegradation of several BTEX compounds at once. Using CSIA, biodegradation rates of BTEX can be determined and the fate of naphthalene can be assessed in a qualitative way. However, substituted naphthalenes and larger PAHs cannot be examined. In practice, the CSIA-based field approach is barely applicable to study the intrinsic biodegradation potential of polyaromatic compounds because the bulk isotope effect is below the detection limit of the method (Elsner, 2010).

Signature metabolite analysis, the second approach, bears the potential to identify new degradation intermediates and pathways, as presented in this study for the anaerobic degradation of acenaphthene and elsewhere for heterocyclic compounds (Safinofski et al., 2006). Nevertheless, its biggest potential lies in a reliable detection of the anaerobic biodegradation of (methylated) aromatic- and aliphatic hydrocarbons as well as heterocyclic compounds. The signature metabolite approach thus provides additional qualitative insights into the biodegradation of larger compounds where CSIA is not applicable.

Microcosms with ^{13}C-labeled substrates, the third approach, allow the quantitative assessment of biodegradation for any ^{13}C-labeled compound of interest (Table 3). Their substrate specificity combined with very sensitive detection, makes ^{13}C-microcosms a particularly interesting option for compounds that sorb, are rather recalcitrant, or cannot be studied by CSIA. Moreover, novel ^{13}C-labeled metabolites of the specific substrate may be extracted from the microcosms and provide new insights into degradation pathways. Even though ^{13}C-microcosms may also be used as stand-alone technique, combination with CSIA and signature metabolite analysis in the field overcomes the limitation of substrate specificity and allows conclusions on a wider spectrum of contaminants.

Acknowledgements

This work was funded by the EU integrated project Aquaterra. We thank M. Aragno for providing lab space and J. Batlle-Aguilar and S. Brouyère for coordinating work on site. F. Chatelain is acknowledged for technical assistance and D. Grandjean for assistance at the GC–MS. We thank three anonymous reviewers for their valuable comments and suggestions.

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