Microbial in situ degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation

Hans H. Richnowa,*, Eva Annweilerb, Walter Michaelisc, Rainer U. Meckenstockc

a Department of Remediation Research, UFZ-Centre for Environmental Research Leipzig-Halle GmbH, Permoserstrasse 15, D-04318 Leipzig, Germany
b Institute of Biogeochemistry and Marine Chemistry, University of Hamburg, Bundesstraße 55, D-20146 Hamburg, Germany
c Centre for Applied Geoscience, Eberhard-Karls University of Tübingen, Sigwartstr. 10, D-72076 Tübingen, Germany

Received 16 March 2001; received in revised form 15 August 2002; accepted 23 October 2002

Abstract

We present an approach for characterizing in situ microbial degradation using the $^{13}$C/$^{12}$C isotope fractionation of contaminants as an indicator of biodegradation. The $^{13}$C/$^{12}$C isotope fractionation of aromatic hydrocarbons was studied in anoxic laboratory soil percolation columns with toluene or $o$-xylene as the sole carbon and electron source, and sulfate as electron acceptor. After approximately 2 months' incubation, the soil microbial community degraded 32 mg toluene l$^{-1}$ and 44 mg $o$-xylene l$^{-1}$ to less than 0.05 mg l$^{-1}$, generating a stable concentration gradient in the column. The $^{13}$C/$^{12}$C isotope ratio in the residual non-degraded fraction of toluene and $o$-xylene increased significantly, corresponding to isotope fractionation factors ($\alpha$C) of 1.0015 and 1.0011, respectively. When the extent of biodegradation in the soil column was calculated based on the measured isotope ratios ($R_i$) and an isotope fractionation factor ($\alpha$C $\approx$ 1.0017) obtained from a sulfate-reducing batch culture the theoretical residual substrate concentrations ($C_i$) matched the measured toluene concentrations in the column. This indicated that a calculation of biodegradation based on isotope fractionation could work in systems like soil columns.

In a field study, a polluted, anoxic aquifer was analyzed for BTEX and PAH contaminants. These compounds were found to exhibit a significant concentration gradient along an 800-m groundwater flow path downstream of the source of contamination. A distinct increase in the carbon isotope ratio ($\delta^{13}$C) was observed for the residual non-degraded toluene (7.2‰), $o$-xylene (8.1‰) and...
naphthalene fractions (1.2%e). Based on the isotope values and the laboratory-derived isotope fractionation factors for toluene and o-xylene, the extent to which the residual substrate fraction in the monitoring wells had been degraded by microorganisms was calculated. The results revealed significant biodegradation along the groundwater flow path. In the wells at the end of the plume, the bioavailable toluene and o-xylene fractions had been almost completely reduced by in situ microbial degradation.

Although indane and indene showed decreasing concentrations downstream of the groundwater flow path, suggesting microbial degradation, their carbon isotope ratios remained constant. As the physical properties of these compounds are similar to those of BTEX compounds, the constant isotope values of indane and indene indicated that microbial degradation did not lead to isotope fractionation of all aromatic hydrocarbons. In addition, physical interaction with the aquifer material during the groundwater passage did not significantly alter the carbon isotope composition of aromatic hydrocarbons.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Biodegradation; Natural attenuation; Aromatic hydrocarbons; BTEX; PAH; $^{13}$C/$^{12}$C isotope fractionation

1. Introduction

To evaluate natural attenuation processes at contaminated sites, the intrinsic biodegradation rates need to be assessed. However, there is no reliable method of directly quantifying the in situ biodegradation of specific compounds in contaminated aquifers. Monitoring contaminant concentrations over time may be a technique to assess the biodegradation of organic compounds in soil or aquifer systems. However, other processes such as sorption to soil (Efroymson and Alexander, 1995; Hatzinger and Alexander, 1995), dilution and evaporation may reduce the concentration of contaminants in the environment as well and thus, it is not easy to assess the intrinsic biodegradation precisely. Microcosm studies with labelled aromatic compounds and aquifer material may be well used to provide evidence for the intrinsic microbial degradation potential (Williams et al., 2001). However, it is not clear if the mineralisation rate of labelled compounds in the laboratory are representative to quantify the biodegradation in the field. The characterization of redox conditions in ground water is very useful for understanding microbial processes (for a review, see Christensen et al., 2000), but, the in situ degradation of a particular contaminant is difficult to assess using electron donor acceptor balances because a complete mass balance of all possible electron sources and sinks is a very complicated task.

Several biochemical reactions result in carbon isotope fractionation and molecules containing the lighter $^{12}$C-isotope are used preferentially. Consequently, the $^{13}$C/$^{12}$C isotope ratio of the substrate’s residual fraction is enriched in $^{13}$C. For example, isotope fractionation is a well accepted indicator of anaerobic and aerobic microbial methane oxidation in sediments (Blair and Aller, 1995; Whiticar, 1999).

An earlier study already suggested that microbial degradation may also lead to the $^{13}$C/$^{12}$C isotope fractionation of compounds with higher molecular masses such as alkanes in marine environments (Stahl, 1980). Recent studies on the biodegradation of
chlorinated hydrocarbons with laboratory cultures revealed carbon isotope fractionation of these compounds (Sherwood Lollar et al., 1999; Bloom et al., 2000; Hunkeler et al., 1999). Studies on contaminated field sites showed that carbon isotope fractionation also takes place in aquifers (Hunkeler et al., 1999), and isotope fractionation factors for trichloroethene from laboratory experiments have been applied to estimate the extent of in situ biodegradation in the field (Sherwood Lollar et al., 2001). Isotopic fractionation was also obtained for toluene degradation under methanogenic and sulfate-reducing conditions with laboratory cultures (Ahad et al., 2000). Isotope fractionation is suggested to have implications for the assessment of intrinsic bioremediation of petroleum hydrocarbon and phenols in contaminated aquifer plumes (Ahad et al., 2000; Ward et al., 2000; Spence et al., 2001). Moreover, isotope fractionation factors of trichloroethene from laboratory experiments were applied to estimate the extent of in situ biodegradation in the field (Sherwood Lollar et al., 2001).

Previous experiments on toluene degradation by aerobic (Pseudomonas putida strain mt-2) and anaerobic bacteria (Thauera aromatica strain K172, Geobacter metallireducens, and a sulfate-reducing isolate, strain TRM1), showed carbon isotope fractionation (Meckenstock et al., 1999). With decreasing toluene concentrations, the carbon isotope ratio in the residual non-degraded toluene fraction increased. The carbon isotope fractionation factors ($\alpha_C$) of toluene calculated using the Rayleigh equation (Rayleigh, 1896; Hoefs, 1997) were 1.0027 for P. putida, 1.0017 for T. aromatica, 1.0018 for G. metallireducens, and 1.0017 for the sulfate-reducing strain TRM1. Although the organisms tested used different electron acceptors for toluene oxidation, and at least two different biochemical toluene degradation pathways were involved, the kinetic isotope fractionation factors $\alpha_C$ were in the same order of magnitude. Non-biological isotope effects, which may be caused in a multiphase system by transitions between the liquid and the gas phase or isotope effects due to sorption to soil in percolation experiments, were not observed (Meckenstock et al., 1999; Slater et al., 2000).

In this study, laboratory-derived carbon isotope fractionation factors were used to estimate the extent of in situ biodegradation of toluene and $o$-xylene in the plume of a contaminated aquifer. The Rayleigh equation was applied to calculate the relative percentage of biodegradation of the residual substrate fraction which may be used as an indicator to characterize biodegradation of contaminants in field studies in the future.

2. Materials and methods

2.1. Aquifer

The contaminated aquifer investigated was located in an industrial area near the city of Hamburg, Germany. The contamination dated back more than 30 years and was a result of leaking storage tanks containing an aromatic oil rich in naphthalene. The area was equipped with a number of monitoring wells and the groundwater flowed from SW to NE (Fig. 1A). The groundwater flow generated a contamination plume more than 800 m long. The Pleistocene aquifer is composed of Quaternary sands and gravel.
Fig. 1. (A) Diagram of the field site in Hamburg showing the location of the observation wells. The arrow depicts the main direction of groundwater flow. The line illustrates the monitoring profile along the groundwater flow path with the sampling wells A to F. Numbers indicate the monitoring wells upstream of the source area (see Table 1). (B) Schematic SW–NE cross-section with monitoring wells (A–F). Source = source of contaminants; qD(1) = erratic marls Drente I; qD(2) = erratic marls Drente II; qs = Quaternary sand and gravel. a.s.l. = above sea level, ♣ = water table.
with a thickness ranging between 7 and 15 m overlying erratic marls (Drente I) (Fig. 1B). Large parts of the aquifer were covered by erratic marls (Drente II) leading to confined groundwater conditions with a minor infiltration of surface water along the contamination plume. The piezometric surface was 3–10 m below the surface and the monitoring wells had filters through the entire depth of the aquifer layer. Estimates of the groundwater flux ranged between 0.2 and 0.4 m per day (internal report by the Environmental Agency of Hamburg). Water samples were taken with submersible pumps in 1998 by a professional consulting company during a routine monitoring survey on behalf of the Environmental Agency of Hamburg. Concentrations of inorganic constituents (dissolved oxygen, nitrate, sulfate, total dissolved iron) and physical data (temperature, pH, conductivity) were obtained during a groundwater monitoring program by the Environmental Agency of Hamburg using standard procedures. Sampling was performed after the wells had been purged with at least one well volume. While pumping, the redox potential was continuously measured and samples were taken when the potential reached constant values. For isotope analysis, glass flasks sealed with Teflon-lined caps were filled without headspace to avoid evaporation. The flasks were stored at 4 °C and extracted with n-pentane within 10 h after sampling.

2.2. Soil percolation columns

Soil column studies were performed as described earlier (Meckenstock et al., 1999). Glass columns (4.5 × 40 cm) equipped with five sampling ports 2.5, 7, 16, 26 and 36 cm away from the inlet were filled with contaminated aquifer material obtained from a former gas plant site near Stuttgart. The bottom-to-top water flow was adjusted with peristaltic pumps to one bed volume per day. The columns were operated in the absence of light at constant temperature (16 °C) for several months with a mineral medium containing 32 mg toluene l⁻¹ or 44 mg o-xylene l⁻¹ and sulfate as electron acceptor.

2.3. Extraction of groundwater for isotope analysis

One-liter groundwater samples were extracted with 3–20 ml of n-pentane containing an internal standard (10 µg ml⁻¹ phenanthrene-D₁₀) for the quantification of target compounds. The pentane phase was dried with anhydrous sodium sulfate to remove traces of water and stored at −20 °C before GC and GC-C-IRMS analysis.

2.4. GC and GC-MS analysis

Gas chromatographic analyses of solvent extracts were performed on a GC-6000 Vega 2 instrument (CARLO ERBA INSTRUMENTS, Milan, Italy) equipped with a fused silica capillary column (DB-5, 60 m × 0.25 mm i.d. × 0.25 µm film, J&W SCIENTIFIC, USA) and a flame ionization detector. The carrier gas was H₂. The oven temperature was initially set at 35 °C and held for 4 min. The temperature then was increased at a rate of 3 °C
min⁻¹ to 300 °C and was held for 1 min. The injection mode was on column. A GC-MS (Quattro II, MICROMASS, UK) was applied to identify specific contaminants using the same GC conditions. The carrier gas was He.

2.5. GC-C-IRMS analysis

The carbon isotope composition of aromatic hydrocarbons was measured with a GC-C-IRMS (gas chromatography/combustion/isotope-ratio-monitoring mass spectrometry) system (FINNIGAN MAT, Bremen, Germany). Aliquots (0.2–4 μl) of n-pentane extracts were injected on column and separated on a capillary column (DB1 60 m × 0.32 mm, 1 μm film, J&W SCIENTIFIC) with the same temperature program used for GC analysis. The GC-C-IRMS system consisted of a GC unit connected to a FINNIGAN MAT combustion device with a water removal assembly coupled to a FINNIGAN MAT 252 mass spectrometer. Organic substances in the GC effluent stream were oxidized to CO₂ in the combustion interface and were transferred on line to the mass spectrometer to determine ¹³CO₂/¹²CO₂ ratios (Hayes et al., 1990; Merritt et al., 1994). The instrument was calibrated using a reference gas (CO₂) with an isotopic composition of 41.95 ‰ PDB.

The linearity and precision of the mass spectrometer was checked with the reference gas (CO₂) and toluene using the same analytical conditions which are applied for the measurement of the aromatic hydrocarbons (Fig. 2A). The isotopic composition of different amounts of reference gas (CO₂, −41.95 ‰ PDB) within a concentration interval resulting in a response of mass 44 from 10 to 2750 mV were measured in three to nine repetitions per concentration step. The response of a single injection of the reference gas (CO₂) with a response of 707 mV was used for calibration. Variation of δ¹³C due to non-linearity of the instrument is less than the analytical error of the measurement at low concentration with a response between 25 and 100 mV. However, at this concentration interval the standard deviations increase at low concentration but the isotope composition was determined with a low systematic error of 0.04 delta units per decade (Fig. 2A). The linearity was tested with toluene as a model compound for aromatic hydrocarbons in a signal range between 20 and 3500 mV (Fig. 2B). The slope of the curve indicates that the systematic error due to non-linearity of the instrument is 0.1 delta units per decade in the signal internal between 45 and 3500 mV. The standard deviation (1σ) at low concentrations increases and was greater than the systematic error due to the linearity of the instrument. For the determination of the isotope composition of aromatic hydrocarbons described in this paper the response was always above 45 mV corresponding to about 0.9 ng hydrocarbon. The δ¹³C values reported in this paper were not corrected for non-linearity. However, the likely systematic error is small compared to the difference in isotope composition encountered in this study.

All samples were measured in at least five replicates. When 10 ng phenanthrene-D₁₀ was injected on column, a signal intensity for mass 44 of about 500 mV was observed, yielding δ¹³C values with a standard deviation of ±0.3 ‰. The mixture of constituents in the environmental samples typically showed a large concentration range between individual compounds which did not allow appropriate concentrations
to be adjusted for the optimal precision of the isotope measurement of all constituents in a single analysis and resulted in larger standard deviations for small concentrations.

3. Calculations

All carbon isotope ratios are given in delta notation as $\delta^{13}C$ values [%o] which are related to PDB (Pee Dee Belemnite) as standard. $R$ is the isotope ratio $^{13}C/^{12}C$ calculated in Eq. (1).

$$\delta_{t}^{13}C[\%o] = \left( \frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1 \right) \times 1000 = \left( R_t/R_{Std} - 1 \right) \times 1000$$  \hspace{1cm} (1)

$$R_t/R_0 = (\delta_t + 1000)/(\delta_0 + 1000)$$  \hspace{1cm} (2)
\[ R_t/R_0 = f_t^{(1/\alpha C - 1)} \]  \hspace{1cm} (3)

\[ \ln(R_t/R_0) = (1/\alpha C - 1) \times \ln f_t \]  \hspace{1cm} (4)

\[ f_t = (R_t/R_0)^{(1/(1/\alpha C - 1))} \]  \hspace{1cm} (5)

\[ C_t = C_0 \times f_t \]  \hspace{1cm} (6)

\[ Bf_t[\%] = (1 - f_t) \times 100 \]  \hspace{1cm} (7)

Kinetic isotope fractionation factors \( \alpha C \) were calculated using Eq. (4), which is derived from the Rayleigh equation for a closed system (Eq. (3)) \((\text{Rayleigh, } 1896; \text{Hoefs, } 1997)\). \( \delta_t \) is the carbon isotope composition at time \( t \), \( \delta_0 \) is the initial carbon isotope composition of the substrate, and \( f_t \) is the fraction of substrate remaining in the sample at time \( t \) \((f_t = C_t/C_0)\). The slope of the linear regression curve gives the kinetic isotope fractionation factor \( \alpha C \) as \((1/\alpha C - 1)\) \((\text{Eq. (4)})\) when \( \ln(R_t/R_0) \) is plotted over \( \ln(C_t/C_0) \) for the time intervals \( t \). The remaining substrate fraction \( f_t = C_t/C_0 \) can be calculated using the isotope fractionation factor \( \alpha C \) obtained in laboratory experiments (Eq. (5)). The percentage of biodegradation of the residual substrate fraction \( \text{(Bf}_t) \) is calculated with Eq. (7).

4. Results

4.1. Laboratory studies

Experiments with non-sterile soil percolation columns were conducted in order to study the sulfate-dependent anaerobic degradation of toluene and \( \text{o-xylene} \) under conditions similar to those prevailing in aquifers. A microbial community was established which was able to degrade toluene or \( \text{o-xylene} \) to a concentration below 0.05 mg l\(^{-1}\), generating a stable concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations. To obtain the isotope fractionation factor \( \alpha C \), the logarithm of concentration gradient of the respective aromatic hydrocarbon along a flow path of 26 cm in the percolation column (Fig. 3A). Analysis of the carbon isotope ratios of the residual toluene and \( \text{o-xylene} \) fraction revealed a significant increase in \( \delta^{13}C \) with decreasing concentrations.
Eq. (4). The $^{13}$C/$^{12}$C isotope fractionation factors $\alpha_{C} = 1.0015$ for toluene and $\alpha_{C} = 1.0011$ for $o$-xylene were calculated from the linear regression of the curve (Fig. 3B).

The isotope fractionation factor of toluene degradation under sulfate-reducing conditions from batch cultures ($\alpha_{C} = 1.0017$) (Meckenstock et al., 1999) was applied to calculate the extent of biodegradation in the soil percolation column experiment using Eqs. (5) and (6). Based on the enrichment of $^{13}$C in the residual toluene fractions ($R_t$) the theoretical residual concentrations ($C_t$) of toluene were calculated and matched the measured concentrations of toluene in the soil percolation column (Fig. 3C).

4.2. Field studies

Dissolved electron acceptors for microbial degradation such as nitrate, iron and sulfate were monitored in all the groundwater wells. Oxygen concentrations were below the detection limit of 0.1 mg l$^{-1}$ in contaminated and non-contaminated areas, indicating that anoxic conditions prevailed in the aquifer. The average concentration of 1 mg l$^{-1}$ dissolved total iron in non-contaminated zones of the aquifer upgradient of the contamination source increased to iron concentrations between 3.0 and 24 mg l$^{-1}$ in the plume (Tables 1 and 2). Nitrate was found at average concentrations of 19 mg l$^{-1}$ in the non-polluted zone. In contaminated areas of the aquifer, a significant nitrate depletion to concentrations between <0.5 and 6.5 mg l$^{-1}$ suggested that nitrate was used as a terminal electron acceptor for microbial respiration (Tables 1 and 2). In wells C, D and E, the nitrate concentration increased slightly compared to the center of contamination in wells A and B. Sulfate was depleted from an average concentration of about 281 mg l$^{-1}$ in non-contaminated areas to 132 mg l$^{-1}$ near the contamination source. Further downstream,
the sulfate content increased in wells C and D, at wells E and F almost reaching concentrations found upstream of the contaminant source. Overall, the depletion of electron acceptors suggests that anaerobic degradation processes are prevailing in this particular aquifer.

Fig. 4. Concentrations (squares) and $^{13}$C/$^{12}$C isotope ratios (triangles) of toluene (A), $o$-xylene (B) and naphthalene (C) along the monitoring profile of a contaminated aquifer.
The carbon isotope ratios and concentrations of aromatic contaminants such as BTEX, naphthalene, indane and indene were analyzed along the 800 m long profile downstream of the contamination source. All the aromatic hydrocarbons analyzed showed a significant concentration gradient in the plume (Figs. 4 and 5; Table 3). Toluene concentrations decreased from 160 to 1.9 μg l\(^{-1}\) along the profile and dropped below the detection limit of 0.5 μg l\(^{-1}\) in observation well F (Fig. 4A; Table 3). With decreasing concentrations, the \(^{13}\)C/\(^{12}\)C isotope ratio in the residual toluene fraction was enriched on average by 7.2 \(\%\) with an uncertainty of \(\pm 1.2 \%\) between wells A and E. \(o\)-Xylene concentrations decreased between wells A and D from 450 to 1.2 μg l\(^{-1}\) and dropped below the

![Graph A](image-url)  
**A**  
Indane [μg L\(^{-1}\)]  
\(\delta^{13}\)C [%]  
Distance from source [m]  

![Graph B](image-url)  
**B**  
Indene [μg L\(^{-1}\)]  
\(\delta^{13}\)C [%]  
Distance from source [m]  

Fig. 5. Concentrations (squares) and \(^{13}\)C/\(^{12}\)C isotope ratios (triangles) of indane (A) and indene (B) with constant \(^{13}\)C/\(^{12}\)C isotope composition along the monitoring transect of the aquifer.
Table 3
The concentrations and isotope composition of contaminants along the monitoring profile

<table>
<thead>
<tr>
<th>Monitoring well</th>
<th>Distance from source [m]</th>
<th>Toluene [µg l⁻¹]</th>
<th>STDV [µg l⁻¹]</th>
<th>δ¹³C [%e]</th>
<th>STDV</th>
<th>δ¹³C [%e]</th>
<th>STDV</th>
<th>Naphthalene [µg l⁻¹]</th>
<th>STDV</th>
<th>Indane [µg l⁻¹]</th>
<th>STDV</th>
<th>Indene [µg l⁻¹]</th>
<th>STDV</th>
<th>δ¹³C [%e]</th>
<th>STDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>160</td>
<td>450</td>
<td>-23.0</td>
<td>0.5</td>
<td>-23.0</td>
<td>0.5</td>
<td>7720</td>
<td></td>
<td>-23.3</td>
<td>0.1</td>
<td>807</td>
<td></td>
<td>-22.6</td>
<td>0.1</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>186</td>
<td>304</td>
<td>-22.8</td>
<td>0.2</td>
<td>-24.0</td>
<td>0.2</td>
<td>3879</td>
<td></td>
<td>-23.7</td>
<td>0.5</td>
<td>1060</td>
<td></td>
<td>-22.6</td>
<td>0.1</td>
</tr>
<tr>
<td>C</td>
<td>387</td>
<td>4.3</td>
<td>11.2</td>
<td>-21.9</td>
<td>0.1</td>
<td>-18.0</td>
<td>1.9</td>
<td>1570</td>
<td></td>
<td>-23.7</td>
<td>0.4</td>
<td>55.8</td>
<td></td>
<td>-22.5</td>
<td>0.2</td>
</tr>
<tr>
<td>D</td>
<td>542</td>
<td>0.8</td>
<td>1.2</td>
<td>-16.8</td>
<td>2.3</td>
<td>-13.6</td>
<td>0.4</td>
<td>215</td>
<td></td>
<td>-22.4</td>
<td>0.1</td>
<td>145</td>
<td></td>
<td>-22.7</td>
<td>0.1</td>
</tr>
<tr>
<td>E</td>
<td>698</td>
<td>1.9</td>
<td>2.5</td>
<td>-15.8</td>
<td>0.7</td>
<td>-14.9</td>
<td>1.7</td>
<td>102</td>
<td></td>
<td>-22.1</td>
<td>0.4</td>
<td>62.0</td>
<td></td>
<td>-22.5</td>
<td>0.1</td>
</tr>
<tr>
<td>F</td>
<td>925</td>
<td>&lt;0.5</td>
<td>n.d.</td>
<td>&lt;0.5</td>
<td>n.d.</td>
<td>&lt;0.5</td>
<td>n.d.</td>
<td>&lt;0.5</td>
<td></td>
<td>n.d.</td>
<td></td>
<td>n.d.</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
detection limit of 0.5 \( \mu g \) l\(^{-1}\) in well F. Concomitantly, the carbon isotope ratio of \( o \)-xylene increased on average by 8.1 \( \pm \) 2.2 \%e (Fig. 4B; Table 3).

Naphthalene concentrations decreased from 7720 to 102 \( \mu g \) l\(^{-1}\) between wells A and E (Fig. 4C; Table 3). The corresponding \( ^{13}C/^{12}C \) isotope ratio of the residual naphthalene fraction increased on average by 1.2 \( \pm \) 0.5 \%e. Although residual toluene, \( o \)-xylene and naphthalene fractions became clearly enriched in \( \delta^{13}C \) along the groundwater flow path, the extent of isotope fractionation differed significantly among the hydrocarbons.

Concentrations of other soluble aromatic hydrocarbons such as indene and indane also decreased by one to two orders of magnitude during the groundwater passage between wells A and E (Fig. 5, Table 3). Although the concentration gradient was similar to that of toluene, \( o \)-xylene and naphthalene, the carbon isotope ratio of the residual indane and indane fraction remained constant along the groundwater flow path (Fig. 5).

### 4.3. Estimation of the extent of biodegradation

To estimate the extent of in situ biodegradation, the carbon isotope composition and concentration of contaminants determined along the plume and the isotope fractionation factor (\( \alpha C \)) were applied using derivatives of the Rayleigh equation (Table 4).

The percentage of biodegradation (\( Bf_t \)) were assessed with Eq. (6) using the isotopic composition of the respective compound of well A as the initial isotopic composition of the source (\( R_0 \)). \( R_t \) is the isotope composition of contaminants down gradient of well A. The uncertainty of the calculations were estimated by adding the standard deviations (1\( \sigma \)) of the isotope values. \( Bf_t \) is an index characterizing the extent of biodegradation, which is necessary to alter the isotopic composition of a compound, but it is not quantitative in terms of the contaminants concentrations.

The carbon isotope fractionation factor for anaerobic toluene degradation in the soil column (\( \alpha C = 1.0015 \)) was applied for calculation. The percentages of biodegradation increase down gradient well A and indicate that the residual toluene fraction was to 95 \( \pm \) 4.7\% to 99 \( \pm \) 0.6\% relative to the source (\( R_0 \)) in wells D and E, respectively (Table 4).

To calculate the microbial \( o \)-xylene degradation, the \( ^{13}C/^{12}C \) isotope fractionation factor \( \alpha C = 1.0011 \) obtained in laboratory experiments and the carbon isotope ratio at well A (\( R_0 = –23 \%e \)) were used. The isotope ratio in well A was enriched in \( ^{13}C \) compared to

<table>
<thead>
<tr>
<th>Monitoring well</th>
<th>Tol. [( \mu g ) l(^{-1})]</th>
<th>Calc. [( \mu g ) l(^{-1})]</th>
<th>STDV</th>
<th>( Bf_t )</th>
<th>STDV</th>
<th>( o )-Xylene [( \mu g ) l(^{-1})]</th>
<th>Calc. [( \mu g ) l(^{-1})]</th>
<th>STDV</th>
<th>( Bf_t )</th>
<th>STDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>160</td>
<td>160</td>
<td>–</td>
<td>0.0</td>
<td>–</td>
<td>450</td>
<td>450</td>
<td>–</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>186</td>
<td>157</td>
<td>74.0</td>
<td>1.9</td>
<td>46.3</td>
<td>304</td>
<td>(1442)</td>
<td>(845)</td>
<td>(– 220)</td>
<td>(188)</td>
</tr>
<tr>
<td>C</td>
<td>4.3</td>
<td>117</td>
<td>91.3</td>
<td>26.7</td>
<td>57.1</td>
<td>11.2</td>
<td>20.1</td>
<td>20</td>
<td>95.4</td>
<td>4.5</td>
</tr>
<tr>
<td>D</td>
<td>0.8</td>
<td>8.9</td>
<td>7.7</td>
<td>95.0</td>
<td>4.7</td>
<td>1.2</td>
<td>0.1</td>
<td>0.1</td>
<td>99.9</td>
<td>0.01</td>
</tr>
<tr>
<td>E</td>
<td>1.9</td>
<td>1.6</td>
<td>1.1</td>
<td>99.0</td>
<td>0.6</td>
<td>2.5</td>
<td>0.9</td>
<td>0.9</td>
<td>99.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\( Bf_t \) = percentage of biodegradation. STDV = standard uncertainty (1\( \sigma \)) calculated according the propagating uncertainty of both isotope measurements (1\( \sigma \), \( R_0 \) and 1\( \sigma \), \( R_t \)) (see text for further explanation).

\( ^a \) Measured concentration.

\( ^b \) Calculated concentration.
the isotopic composition $\delta^{13}C$ of o-xylene in well B (−24‰) and consequently, the calculation resulted in a negative value of o-xylene biodegradation in well B. Nevertheless, we use the isotopic composition of well A as $R_0$ because this well is located near the source of contaminants. Further downstream the calculated percentage of biodegradation (Bf) increased from 95.4 to 99.9% with an uncertainty between 4.5% and 0.01% at wells C to E, respectively (Table 4).

The theoretical residual concentration to which extent contaminants fractions were degraded by microorganisms were calculated with Eq. (7). In this case the isotopic composition of contaminants in well A was used as initial isotopic composition ($R_0$) and initial concentration ($C_0$). The uncertainty of the calculations were estimated by adding the standard deviations ($1\sigma$) of the isotope values as above.

The extent of biodegradation of the residual substrate fraction can be quantified with Eq. (6), assuming that (I) all monitoring wells (A–F) were located directly downstream of the source of contamination, (II) the contaminants were bioavailable, and (III) dilution and sorption could be neglected. Based on these assumptions, the isotopic compositions and concentrations monitored in well A could be employed as $R_0$ and $C_0$ to calculate the absolute amount of biodegraded toluene and o-xylene in wells B–E using the Rayleigh equation (Eqs. (5) and (6)) and the laboratory-derived fractionation factors. The residual toluene concentrations calculated showed a slow decrease for wells B and C associated with a relatively large uncertainty of 74–91 µg l$^{-1}$ (Table 4). However, further downstream of the groundwater flow path the calculated concentrations decreased strongly, showing the order of the measured toluene concentrations in the wells D and E. In the case of o-xylene, the calculated concentration for monitoring well B differed drastically from the concentration measured in the field, because the isotopic composition of xylene was more negative in well B than in well A. This showed that the isotopic composition of o-xylene in well A was not the representative initial isotopic composition ($R_0$) for well B. However, further down the water flow path from wells C to E, the calculated concentrations showed the same order of magnitude as the o-xylene concentrations measured in the field (Table 4).

5. Discussion and conclusion

Previous studies have shown that isotope fractionation processes can be used to describe in situ biodegradation of chlorinated hydrocarbons in both qualitative and quantitative terms (Hunkeler et al., 1999; Sherwood Lollar et al., 2001). Here we report the use of laboratory-derived isotope fractionation factors $\alpha C$ to calculate the extent of the in situ biodegradation of individual aromatic hydrocarbons in contaminated aquifers.

5.1. Isotope fractionation factors

Anaerobic toluene degradation in pure cultures with different electron acceptors result in fractionation factors of about $\alpha C$ 1.0017 which are similar to the fractionation factor ($\alpha C = 1.0015$) obtained in degradation experiments in a non-sterile soil percolation column operated under sulfidogenic conditions with toluene carbon sources (Meckenstock et al.,
Morasch et al. (2001) showed that carbon isotope fractionation of toluene under sulfidogenic conditions did not vary significantly at different temperatures. The similarity of fractionation factors may provide evidence to apply laboratory derived fractionation factors to characterize in situ biodegradation in anoxic aquifer plumes. However, a more systematic survey testing a large number of microbial cultures will be needed to allow a final evaluation of the variability of compound specific isotope fractionation factors.

Compared to toluene the substance specific fractionation factor of o-xylene biodegradation in percolation column experiments under anoxic conditions (\(\alpha_C = 1.0011\)) is lower. This clearly shows the needs for a database of substance specific isotope fractionation factors when the in situ biodegradation of aromatic hydrocarbons should be assessed using the technique described below.

5.2. Validation of in situ biodegradation

The concentrations and isotopic compositions of contaminants in the field were used to examine in situ biodegradation at the test site by applying laboratory-derived fractionation factors and the Rayleigh equation (Eq. (5)). The concept is based on a mathematical description of isotope fractionation by the Rayleigh equation for closed systems (Rayleigh, 1896; Hoefs, 1997). Closed system conditions are certainly realized in biodegradation experiments with pure cultures in closed serum bottles where the only loss of substrate is caused by microbial degradation. Although a soil percolation column is not a closed system in the strict sense, under the steady state conditions described herein, the removal of contaminants is only a result of biodegradation. Dilution and adsorption can be neglected and the concentration and isotopic composition of the substrate at the inlet of the column remains constant along the water flow path if no biodegradation occurs. In this case, the concentration and isotopic composition vary over the distance from the inlet instead of time as within batch cultures, and the Rayleigh equation for closed systems can be applied to calculate the isotope fractionation factor \(\alpha\). Therefore, the isotope fractionation factor can be used to calculate the extent of biodegradation. In the soil percolation column experiment, the calculated amounts of the residual toluene fractions matched the measured concentrations when a carbon isotope fractionation factor (\(\alpha_C = 1.0017\)) of a toluene degrading, sulfate-reducing batch culture was used. This showed that the Rayleigh equation could be used to quantify biodegradation in the soil column where dilution and sorption could be neglected as a relevant sink for contaminants.

This theoretical concept was applied to estimate the amount of biodegraded fraction of contaminants in the aquifer. As an aquifer is not a closed system, the Rayleigh approach to calculate the extent of biodegradation runs up against some limitations because other sinks of contaminants have to be taken into consideration. The concentrations of the contaminants could be reduced along the groundwater flow path (1) by mixing with pristine groundwater, (2) by adsorption to the aquifer matrix, and by (3) evaporation, respectively. Evaporation was considered to be of minor influence because this aquifer was widely covered by erratic marls and the geological situation prevented a significant loss of contaminants to the vadose zone. Sorption and dilution may influence the concentration of the contaminants but did not affect the isotope composition to a significant extent.
In contrast to toluene, xylene and naphthalene, which exhibited significant isotope fractionation in the aquifer, the carbon isotope signatures of indane and indene remained constant along the groundwater flow path. As the concentrations of all compounds decreased drastically over a distance of 700 m, it was evident that neither transport related processes like adsorption or dilution during the groundwater passage nor biodegradation influenced the carbon isotope composition of indane and indene. As indene and indane have similar chemical and physical properties to BTEX and PAH, the constant isotopic compositions demonstrated at least that transport-related processes such as adsorption and dilution did not influence the isotopic signatures of aromatic hydrocarbons in this particular aquifer. This suggests that shifts in the carbon isotope ratio of toluene, $o$-xylene and naphthalene during the groundwater passage were exclusively the result of biodegradation. The extent of biodegradation of the residual substrate fraction can therefore be calculated with Eqs. (5) and (7) using the difference in the isotopic compositions of the contaminants for any point along the ground water flow path. The calculation gives the percentage of biodegradation (Bf) which was necessary to change the isotopic composition of the residual fraction (see Eq. (7)).

An increasing percentage of biodegradation (Bf) along the groundwater flow path for toluene and $o$-xylene based on the increasing isotope ratios was calculated. In the groundwater wells D and E, the remaining toluene and xylene fractions had been degraded by more than 95% and 99%, respectively. A similar approach was used by Sherwood Lollar et al. (2001) who estimated the in situ biodegradation of tetrachlororethene and obtained comparable results.

Similarly, percentage of biodegradation (Bf) only refers to the hydrocarbon fraction which is available to the microorganisms (C0) and thus can be biodegraded potentially. This fraction might have been reduced by dilution and adsorption before this fraction becomes bioavailable to the organisms and therefore the percentage of biodegradation is not quantitative in terms of absolute concentration.

However, the index “percentage of biodegradation” can be influenced by the fact that the sampling of groundwater covers the whole water column of the aquifer. Water from different preferential flow paths may be mixed in the wells. In a worst case situation, contaminants are almost completely degraded in one flow path, whereas no degradation occurs in the other flow path. After both water bodies are mixed in the well, the isotope ratios of the contaminants show the initial isotopic composition of the source because the substrate from the flow path which does not exhibit biodegradation has a much higher concentration and influences the isotope ratio of the mixed sample to a higher extent. Thus, although significant amounts of substrate have been degraded, calculation will only indicate minor biodegradation, underestimating its true extent. However, presuming that biochemical degradation conditions such as temperature, redox and electron acceptors are similar in different parts of the contaminated aquifer and an appropriate isotope fractionation factor is assessable, the calculation will not overestimate the extent of biodegradation regardless of mixing processes in the aquifer or sorption to the aquifer’s matrix.

Thus, the percentage of biodegradation may be a useful index in risk assessment studies concerning groundwater contamination and represent the first step towards a quantification of the in situ biodegradation in aquifers.
5.3. Quantification of in situ biodegradation

Adsorption and dilution restrict the use of the Rayleigh equation in quantifying the absolute amount of the biodegraded substrate fraction. Assuming that (I) the aquifer is in steady state with respect to additional adsorption or desorption of BTEX, (II) the total substrate concentration is bioavailable, and (III) dilution is insignificant, the initial isotope ratio \( R_0 \) and the substrate concentration \( C_0 \) would not change along the groundwater flow path if no biodegradation occurred. In this case, the Rayleigh equation could be used to calculate the theoretical residual substrate concentration in the wells \( C_t \) at any point along the water flow path using the isotopic composition of the residual fraction \( R_t \).

At this field site the contamination was more than 30 years old. It can be assumed that the input of contaminants at the source of contaminants was relatively constant over time due to leaching of free oil phases and most likely the concentration gradient of BTEX was not affected by additional sorption to the aquifer matrix to a significant extent. As the monitoring profile from wells A to F was mainly parallel to the groundwater flow path we also assumed that a representative water flow path without significant dilution was sampled. Under these circumstances, Eqs. (5) and (6) were used to calculate the theoretical residual toluene and \( o \)-xylene concentration \( C_t \), using the hydrocarbon concentrations in well A as the source concentration \( C_0 \). Indeed, the calculation revealed that the theoretical residual hydrocarbon concentrations \( C_t \) were in the same order as the substrate concentrations measured at the edge of the plume. However, if the shift in the isotope ratios was small, the calculation is biased because of the inaccuracy of the isotope measurements, which is generally no better than \( \pm 0.3 \)‰. This is demonstrated by the toluene and \( o \)-xylene concentrations calculated in wells B and C, which deviated drastically from the actual concentrations and were associated with a high uncertainty. However, the calculation of the residual concentrations is possible when the shift in the isotope composition is high. This may demonstrate that the method can be used to assess intensive biodegradation. Vice versa low biodegradation rates resulting in minor shifts in the isotopic composition are difficult to estimate. This is reasonable because the Rayleigh equation is an exponential function. Nevertheless, in wells D and E the shift in the isotopic composition was high and regardless of the standard uncertainty of the isotope measurement, the concentration calculated based on the isotope values predicted a decrease in the hydrocarbon concentrations matching the situation in the field.

In the aquifer investigated, the sampling profile may be slightly different from the main groundwater flow direction, because increasing concentrations of the dissolved electron acceptors nitrate and sulfate in wells C to E indicated mixing with non-contaminated water along the groundwater flow path. In future, combining the isotope fractionation concept with groundwater modeling and tracer studies could certainly improve the quality and reliability of the data and lead to better interpretations by taking dilution effects into account.

In order to select an appropriate fractionation factor for the calculation, it is important to analyze the redox conditions of a contaminated aquifer. Aerobic and anaerobic bacteria use different biochemical reactions for degradation, and thus, the isotope fractionation factors might differ. In this aquifer, the microbial community used nitrate, ferric iron, and sulfate as terminal electron acceptors, as indicated by their depletion along the groundwater flow
and the absence of molecular oxygen. Therefore, experimentally derived isotope fractionation factors for the anaerobic degradation of toluene and o-xylene were applied, assuming that anaerobic degradation pathways lead to similar carbon isotopic fractionation. In the case of toluene and xylene, the pathway is quite similar because sulfate-reducing and denitrifying bacteria use the addition of fumarate to the methyl group as the initial activation step to degrade the compound (Biegert et al., 1996; Beller and Spormann, 1997; Wilkes et al., 2000; Heider et al., 1999).

To sum up, carbon isotope fractionation during biodegradation can be used to estimate the extent of microbial degradation in the contaminated aquifer. Although the calculations based on isotope fractionation could not quantitatively distinguish between dilution, adsorption and biodegradation, the results describe the extent of biodegradation in different zones of the aquifer. Tools applying isotope fractionation processes may have large potential with respect to the identification and quantification of natural attenuation processes.

Acknowledgements

We would like to thank Prof. Dr. Bernhard Schink for his helpful discussion and continuous support. We are grateful to Dr. Pierre Albrecht and Patrick Wehrung for providing facilities and technical assistance for IRM-GC-MS measurements. Dr. Czekalla (Consul Aqua) and Dipl. Geol. Bocian and Dipl. Geol. Quek (Umweltbehörde Hamburg) are gratefully acknowledged for providing the test field and for helpful discussions. We thank the Environmental Agency of Hamburg (Umweltbehörde der Freien und Hansestadt Hamburg) for using their data bank. Additional thanks go to four anonymous reviewers for their suggestions and critical comments. This paper represents publication no. 183 of the Priority Program 546 “Geochemical processes with long-term effects in anthropogenically affected seepage- and groundwater”. Financial support was provided by the Deutsche Forschungsgemeinschaft (grant Schi 187/7) and the Bundesministerium für Bildung und Forschung (grant 02WT0022).

References


