Tracing the transformation of labelled [1-13C]phenanthrene in a soil bioreactor

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“Capsule”: Detailed experiments are described in which the transformation of 13C-labelled phenanthrene is tracked in a soil bioreactor.

Abstract

[1-13C]-labelled phenanthrene was incubated in a closed bioreactor to study the flux and biotransformation of polycyclic aromatic hydrocarbon (PAH) in contaminated soils on a bulk and molecular level. The degradation of extractable phenanthrene was observed by GC–MS measurements and the mineralisation was monitored by 13CO2 production. The transformation of the 13C-label into non-extractable soil-bound residues was determined by carbon isotopic measurements. With these data we were able to calculate a carbon budget of the 13C-label. Moreover, the chemical structure of non-extractable bound residues was characterised by applying selective chemical degradation reactions to cleave xenobiotic subunits from the macromolecular organic soil matrix. The obtained low molecular weight products yielded 13C-labelled compounds which were identified using IRM (isotope ratio monitoring)–GC–MS and structurally characterised with GC–MS. Most of the 13C-labelled products obtained by chemical degradation of non-extractable bound residues are well-known metabolites of phenanthrene. Thus, metabolites of [1-13C]phenanthrene formed during biodegradation appear to be reactive components which are subsequently involved in the bound residue formation. Hydrolysable amino acids of the soil residues were significantly labelled with 13C as confirmed by IRM–GC–MS measurements. Therefore, phenanthrene-derived carbon was transformed by anabolic microbial processes into typical biologically derived compounds. These substances are likely to be incorporated into humic-like material after cell death. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Transformation; 13C-label; Phenanthrene; Soil bioreactor

1. Introduction

The formation of non-extractable bound residues is a significant sink of organic pollutants in soils (Bollag and Loll, 1983; Calderbank, 1989; Richnow et al., 1999a). In the case of hydrophobic pollutants like polycyclic aromatic hydrocarbon (PAH) recent advances in bound residue research could clearly assign microbial processes to be a key factor catalysing bound residue formation. A metabolic activation is required to transform relatively inert parent compounds into reactive microbial metabolites which may subsequently interact with natural soil organic matter to form bound residues (Richnow et al., 1994). Progress in search of the chemical structure of bound residues has been made applying 13C-labelled model compounds to trace the transformation of pollutants in soils (Richnow et al., 1998). Experiments with 13C-labelled anthracene revealed that microbial metabolites of PAH are structural subunits of bound residues partly linked by covalent bonds to soil organic matter. Enzyme-catalysed oxidative cross-coupling reactions which form covalent chemical bonds may be involved in the residue formation reaction (Berry and Boyde, 1985; Hatcher et al., 1993; Dawel et al., 1997). Moreover, ageing processes in soils have been shown to be responsible for limited bioavailability and biodegradability of PAH (Hatzinger and Alexander, 1995). Sequestering within non-aqueous phase liquids, micropores, clay minerals and in the soil organic matter are considered to reduce the mineralisation of anthropogenic chemicals in soils.
According to the IUPAC definition, “non-extractable residue (sometimes also referred to as bound residues, or non-extracted residues) in plants and soils are defined as chemical species originating from pesticides, that remain unextracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products.” (Roberts, 1984). The chemical species in this context refer either to parent material or metabolites of it.

Considering biological transformation processes in soils, the former pollutants may be mineralised in catabolic processes or transformed in the microbial anabolism to chemical structures identical to those of natural origin, such as carbohydrates, amino acids, and fatty acids. These latter compounds do not belong to the bound residue fraction, even if parts of their carbon originate from anthropogenic chemicals. In practice, bound residues are very difficult to distinguish from compounds transformed into biological fractions, especially when radioactively labelled tracer substances are used to quantify the bound residue fraction without any structural assignments. In this study we apply $^{13}$C-labelled contaminants to elucidate the transformation of xenobiotics in soils. We analysed the anabolic conversion of xenobiotic carbon into typical chemical structures of the microbial biomass. In particular, we discuss the transformation of $[^{1-13}C]$phenanthrene in soil on a bulk and on a molecular level emphasising: (1) mineralisation; (2) non-extractable bound residue formation; and (3) transformation into the biomass.

Moreover, ageing effects were investigated by spiking a soil with $^{13}$C-labelled phenanthrene which was contaminated at least 53 years ago and yield significant amounts of non-aqueous phase liquids (tar). In a soil bioreactor the biodegradation of labelled and tar oil-derived phenanthrene was traced to obtain information about their bioavailability.

2. Materials and methods

2.1. Soil

The soil material used in this experiment was collected from a military base in Achim near Bremen (Germany). The soil was derived from the upper soil column and consisted of 1.2 wt.% clay, 2.2 wt.% silt, 92.0 wt.% sand and 5.2 wt.% gravel. The total organic carbon content was 1.5 wt.%. The soil was heavily contaminated by mineral-oil derived aliphatic hydrocarbons and PAHs. The acidic soil (pH 4.8) was free of carbonates, and the molecular ratio of C:N:P (100:3:0.2) revealed nitrogen and phosphorous deficiencies. The historical investigation indicates that the soil was contaminated during the destruction of a fuel depot in World War II and is at least more than 53 years old.

Soil material (40 kg) was sieved to a particle size of < 1 mm to remove gravel material and carefully homogenised prior to any further treatment. The pH was adjusted to 6.3 by adding fine Ca(OH)$_2$ powder to the soil. The water content of the soil was adjusted with distilled water to 50% of the maximal water-holding capacity before incubation in the bioreactor experiments.

To stimulate the microbial activity the soil was supplemented by the addition of compost (5 wt.% of dry compost). The compost added was produced at a windrow composting plant in Hamburg which processed yard waste and separately collected vegetable kitchen waste. The mature biocompost had a grain size of less than 4 mm, a maturity degree of V (German systematic for highly matured compost) and was at least more than 6 months old. Prior to spiking the $[^{1-13}C]$phenanthrene, the soil was mixed with dry compost in a stainless steel pastry blending machine.

Soil material (4 kg) was loaded with the isotopic tracer by adding dropwise (drop size < 10 μl) ethylacetate solutions containing $[^{1-13}C]$phenanthrene (50.03 mg ml$^{-1}$). For a batch experiment with non-labelled phenanthrene 3.2 kg soil material was contaminated likewise. The soil material was carefully homogenised during the contamination procedure. Then the solvent (ethyl acetate) was evaporated at ambient temperature for 1.5 h during continuous mixing of the soil material.

2.2. Chemicals

Several grams of $^{13}$C-labelled phenanthrene were synthesised (Scheme 1). The synthesis of small amounts of $[^{1-13}C]$phenanthrene was described earlier (Soderquist et al., 1992) but some modifications were essential. The commercially available 1-methylnaphthalene (I, Scheme 1) was deprotonated with butyl-lithium in a mixture of diethyl ether and $N,N,N',N'$-tetramethylethylendiamine (TMEDA). The reaction with racemic 2-(2-bromoethoxy)-tetrahydropyran gave compound 2 (Scheme 1) (Mallan and Bebb, 1969). In the next step the tetrahydropyran group (THP) was removed with $p$-toluenesulphonic acid in methanol (Marshall, 1969) and then the obtained alcohol was treated with triphenylphosphonium dibromide to yield the corresponding bromide 3 (Scheme 1) according to Wiley et al. (1969). The bromide 3 (Scheme 1) was converted to its Grignard reagent with $^{13}$C carbon dioxide, generated from $[^{13}C]$sodium carbonate with concentrated sulphuric acid, to obtain the labelled carboxylic acid 4 (Scheme 1). Compound 4 was transformed to its acid chloride with oxalyl chloride and then cyclised with tin tetrachloride in benzene to give the ketone (5, Scheme 1) (Bachmann et al., 1940).
After reduction of 5 with lithium-aluminium-tetrahydride using a description of Brown and Krishna-murthy (1979), dehydratisation, and oxidation with 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) (Fu and Harvey, 1978) the [1-13C]phenanthrene (6) was obtained. [1-13C]Phenanthrene was finally purified by column chromatography with silica gel as stationary phase (Merck, 230–400 mesh) using n-hexane as developer. The chemical and isotopic purity of the [1-13C]phenanthrene (99%) were verified by 1H-NMR, 13C-NMR and GC–MS.

All other chemicals used for synthesis, extraction and preparation were obtained from Merck (Darmstadt, Germany). The organic solvents (p.A.-quality) were distilled before use.

2.3. pH determination

The pH of the soil was measured according to DIN 38404-65. Briefly, 10 g soil was mixed with 75 ml 0.01 M CaCl₂ and the pH was measured after 24 h.

2.4. Extraction procedures

Prior to the extraction, soil samples were dried in a freeze dryer (Christ Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH; Osterode, Germany). The water content was calculated by weight difference of the soil before and after drying. Soil samples were extracted with an organic solvent sequence of decreasing polarity [I, acetone; II, acetone:dichloromethane (1:1, v:v); III, dichloromethane, twice]. For extraction, the soil material (10–20 g soil) was mixed with an excess of organic solvent (50–100 ml). The extraction procedure was repeated four times using ultrasonification (RK100H, Sonorex, Bandelin, Berlin, Germany) to enhance the efficiency of the extraction. Aliquots of the extracts were analysed for the phenanthrene concentration by GC and GC–MS.

The pre-extracted soil was dried for 15 h under reduced pressure (40 mbar) at 40°C to remove the organic solvent. The extracted soil sample was defined as the non-extractable soil-bound residue fraction and then subjected to further analyses. The total organic carbon content of the total soil and bound residue fraction was determined with a LECO CHN 100 elementary analyser (Leco Instruments, Kirchheim, Germany).

2.5. Chemical degradation

Alkaline hydrolysis was applied to cleave ester-linked bound residues from the non-extractable macromolecular soil matrix (Richnow et al., 1994). Extracted soil (1–2 g) was transferred into a glass ampoule and mixed with 10 ml 2 M NaOH. The ampoule was closed under N₂ atmosphere and the mixture was hydrolysed for 2 h at 100°C. The reaction mixture was separated by centrifugation followed by two extractions with distilled water. Then the alkaline solution was acidified with 6 M HCl to pH 1–2 and extracted with dichloromethane. The dichloromethane extract was dried with anhydrous Na₂SO₄. Organic acids and aromatic alcohols were
derivatised with diazomethane to methyl esters and methyl ethers.

2.6. Hydrolysable amino acids

Soil material (1–2 g) was hydrolysed with 5 ml distilled 6 M HCl for 22 h at 110°C. The acidic supernatant of the reaction mixture was used for further analysis. An aliquot of the reaction mixture was used for the quantification of amino acids using ortho-phthalaldehyde reagent for derivatisation prior to HPLC analysis (Dawson and Liebezeit, 1983). Another aliquot was derivatised with 2-propanol to obtain isopropyl esters and trifluoroacetic acid to obtain corresponding trifluoroacetates (Silfer et al., 1991). The amino acids were identified using GC–MS and authentic reference compounds before determination of their isotopic composition by isotope ratio monitoring–gas chromatography–mass spectroscopy (IRM–GC–MS).

2.7. GC and GC–MS analyses

High-resolution gas chromatographic analyses were performed on a gas chromatograph (GC-6000 Vega 2, Carlo Erba Instruments, Milan, Italy) equipped with a fused silica capillary column (DB-5, 30 m × 0.25 mm, J&W Scientific, USA). The temperature program for the analysis of aromatic hydrocarbons and metabolites was: 80°C, 3 min isothermal; 80–300°C, 4°C min⁻¹, 20 min isothermal 300°C; injection mode: on column; carrier gas: H₂. GC–MS measurements were performed with a Varian CH7A (Finnigan, Bremen, Germany) mass spectrometer (ionisation mode: EI; 70 eV ionisation energy; 250°C source temperature; mass range: m/z 50–600; resolution 1000) coupled with a Carlo Erba gas chromatograph (carrier gas: He) using the same conditions as for the GC measurements. Phenanthrene-d₁₀ was used as internal standard for quantification of PAH. Ion chromatograms of m/z 188 (internal standard), m/z 178 and m/z 179 were integrated and used to quantify the concentration of tar oil-related phenanthrene and [1-¹³C]phenanthrene, respectively.

2.8. Isotope ratio monitoring GC–MS

The isotopic composition of amino acids and chemical degradation products was measured with IRM–GC–MS (Finnigan Mat, Bremen, Germany). The system consisted of a GC-unit, interfaced to a Finnigan Mat combustion device with a water removal assembly and coupled to a Finnigan Mat 252 mass spectrometer. The organic substances in the effluent stream from the GC were oxidised to CO₂ in the combustion interface. The CO₂ produced was transferred continuously to the mass spectrometer to determine the ¹³CO₂/¹²CO₂ ratios. Methodical details are described in Hayes et al. (1990).

2.9. Stable isotope analyses

For the determination of the mineralisation of the ¹³C-tracer, the ¹³CO₂ was trapped in 20 ml 1 M NaOH solution. Dissolved carbonate in the NaOH solution was precipitated with a BaCl₂ solution. The BaCO₃ precipitate was washed several times with distilled water and dried before determination of the weight to calculate the amount of CO₂.

Precipitated barium carbonate and pre-extracted soils were analysed for the composition of stable carbon isotopes using a MAT 251 (Finnigan, Bremen, Germany) mass spectrometer connected to an elemental analyser. The isotopic composition of carbon is expressed in standard differences to the Pee Dee Belemnite standard (PDB) in terms of δ values: (δ¹³C [%] = ([¹³C/¹²C]sample/ [¹³C/¹²C]std – 1)×1000) (Hoefs, 1997). To calibrate the mass spectrometer for the determination of ¹³C-enriched carbon, reference material IAEA-309 UL-Glucose A 93.9 (%) PDB and IAEA-309 UL-Glucose B 535.3 (%) PDB was purchased from Analytical Quality Control Service (Vienna, Austria).

2.10. Fixed-bed bioreactor with continuous oxygen supply

The bioreactor consisted of a 5-l glass vessel which was loaded with 3 kg contaminated soil material (Fig. 1). A continuous flow of air was maintained at a flux of

![Fig. 1. Fixed-bed bioreactor.](image-url)
11 h\(^{-1}\) kg\(^{-1}\) dry soil. The humidity of the air was achieved by leading the gas stream through a gas-washing flask filled with water. A detector array including a flow controller was used for permanent registration of gas flow, CO\(_2\), volatile organic carbon (VOC). The data set was stored on a computer for further analysis. CO\(_2\) was measured with an IR-spectrometer (Gas Analyzer ADC 7000, Analytical Development Co. Ltd, Hoddesdon, UK), gas flow with a mass flow controller (Bronkhorst, AK Ruurlo, Netherlands) and VOC with a gas analyser (Hydrocarbon Analyzer Mod. 3002, Bernath Atomic, Wennigsen, Germany). In the case of the experiment with labelled phenanthrene, the air was purged to a gas-washing flask (600 ml) filled with 4 M NaOH (400 ml) to remove CO\(_2\) from the air supply. A similar gas-washing flask (600 ml) with 400 ml 2 M NaOH was used to trap CO\(_2\) before the gas stream was sent to the detector array. More information about the bioreactor system is given by Hupe et al. (1996) and Koning et al. (1998).

3. Results and discussion

3.1. Carbon budget traced with the \(^{13}\text{C}\)-labelled compounds

To elucidate the transformation of PAH in soil during microbial degradation \([1-^{13}\text{C}]\)phenanthrene with a theoretical \(\delta^{13}\text{C}\) of 5845\% (PDB) was applied as a model compound. The distribution of the \(^{13}\text{C}\)-label between CO\(_2\), solvent extractable and non-extractable bound residue fraction was used to calculate a carbon budget and to obtain information about the fate and transformation processes of PAH in soils (Fig. 2; Tables 1 and 2). The mineralisation of \(^{13}\text{C}\)-labelled phenanthrene was determined by the amount and isotopic composition of the CO\(_2\) fraction. The concentration of organic carbon and its isotopic composition was used to quantify the immobilisation of the \(^{13}\text{C}\)-label within the non-extractable soil-bound residues according to Richnow et al. (1999b). Both phenanthrene species in the solvent-extractable fraction was measured with conventional GC and GC–MS to quantify residual parent material.

The concentration of extractable \([1-^{13}\text{C}]\)phenanthrene decreased from 354.3 to 1.5 \(\mu\)g g\(^{-1}\) within the first 50 days and then slightly declined until the end of the experiment (0.11 \(\mu\)g g\(^{-1}\)). \(^{13}\text{CO}_2\) was already formed after 5 days characterising the short adaptation period necessary for the microflora to develop the ability to mineralise phenanthrene. The \(^{13}\text{CO}_2\) evolution was most pronounced between days 0 and 14, indicating mineralisation as the main elimination process of phenanthrene. Between days 15 and 40, bound residue formation was an important sink of phenanthrene in the soil. The residue formation reached the highest concentration of 35.5 \(\mu\)g g\(^{-1}\) phenanthrene equivalents at day 35. Parts of the bound residues were then slowly degraded in the next 47 days to a final concentration of 21.3 \(\mu\)g g\(^{-1}\) phenanthrene equivalents. The bound residue formation takes place during the period of intensive biodegradation, indicating a clear microbial contribution in the formation process.

The total recovery of \(^{13}\text{C}\) was 83\% of the initially applied amount after 82 days. The carbon budget revealed a continuous loss of about 17\% over time which may be the result of CO\(_2\) leakages or incomplete CO\(_2\) sorption in the NaOH traps of the bioreactor system. From a number of similar bioreactor experiments with \(^{14}\text{C}\)-labelled substances, we know that volatile...
metabolic products are quantitatively insignificant but a volatilisation of small amounts of the parent phenanthrene in the gas stream of the oxygen supply cannot be excluded. Nevertheless, the mean recovery of applied $^{13}$C was 83% and may be sufficient for a carbon budget. The partition of the $^{13}$C-label among various fractions normalised to the applied amount was analysed at day 82. Seventy-three per cent of the $^{13}$C-label was found as CO$_2$. Four per cent was extractable of which very minor amounts were positively identified as the parent $[1-^{13}$C]phenanthrene (0.03%) and 6% was transformed into bound residues. Compared to bound residue formation of $[9-^{13}$C]anthracene (11%) and $[9-^{14}$C]-anthracene (20%) in pristine soils (Richnow et al., 1999b) and bioremediation of $[9-^{14}$C]phenanthrene (36%) with white rot fungi in a contaminated soil (Brotkorb and Legge, 1992) the residue formation in our experiment was significantly lower. The relatively low content of bound residues might be a good indication for the ability of the soil microflora to degrade phenanthrene efficiently. In earlier studies we observed a low tendency to form bound residues in the case of a high mineralisation capacity of the soil microflora (Richnow et al., 1999b). In this experiment a short lag phase for the phenanthrene degradation and a high mineralisation capacity point to a microflora already adapted to degrade the contamination.

### 3.2. Bioavailability of phenanthrene

In order to elucidate the bioavailability of the tar oil-related phenanthrene in the, at least 53-year-old, soil contamination, the soil material was spiked with $[1-^{13}$C]phenanthrene and the concentration of both phenanthrene species was monitored in the bioreactor. The tar oil contamination consists of typical tar oil-related PAH like phenanthrene, anthracene, chrysene, benzo(a)anthracene and methylated homologues, whereas fluoranthene and pyrene are predominant compounds (Fig. 3). Large amounts of extractable tar oil (15–25 mg g$^{-1}$) characterise a significant non-aqueous liquid phase in this soil contamination. The initial concentration of tar oil-borne phenanthrene in the soil was 88.6 mg g$^{-1}$ which was spiked with 354.3 mg g$^{-1}$ labelled phenanthrene (Table 2). During the time course of the degradation experiment, phenanthrene species were degraded efficiently compared to 0.23 mg g$^{-1}$ unlabelled and 0.11 mg g$^{-1}$ labelled phenanthrene, respectively. The degradation rates were calculated as the percentage of decreasing phenanthrene concentrations per day in the extractable fraction (Table 2). During the first 14 days, mean degradation rates of about 5.4% day$^{-1}$ were observed for both phenanthrene species (Fig. 4). Between 35 and 63 days tar oil-related phenanthrene of the old contamination was degraded in the same order.

### Table 1

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Soil (g dry wt)</th>
<th>CO$_2$ ($\mu$g g$^{-1}$ soil)</th>
<th>CO$_2$ cumulative ($\mu$g g$^{-1}$ soil)</th>
<th>Total extract ($\mu$g g$^{-1}$ soil)</th>
<th>Bound residue ($\mu$g g$^{-1}$ soil)</th>
<th>Budget ($\mu$g g$^{-1}$ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2699.1</td>
<td>0.0</td>
<td>0.0</td>
<td>350.1</td>
<td>0.8</td>
<td>350.9</td>
</tr>
<tr>
<td>5</td>
<td>2613.0</td>
<td>112.3</td>
<td>112.3</td>
<td>186.6</td>
<td>14.4</td>
<td>313.3</td>
</tr>
<tr>
<td>14</td>
<td>2527.0</td>
<td>124.5</td>
<td>236.8</td>
<td>62.7</td>
<td>21.5</td>
<td>321.0</td>
</tr>
<tr>
<td>35</td>
<td>2440.2</td>
<td>13.6</td>
<td>250.4</td>
<td>22.7</td>
<td>35.5</td>
<td>308.7</td>
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<td>50</td>
<td>2353.3</td>
<td>3.6</td>
<td>254.0</td>
<td>12.8</td>
<td>26.9</td>
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</tr>
<tr>
<td>63</td>
<td>2266.2</td>
<td>0.3</td>
<td>254.3</td>
<td>17.2</td>
<td>22.4</td>
<td>294.0</td>
</tr>
<tr>
<td>82</td>
<td>2178.1</td>
<td>0.0</td>
<td>254.3</td>
<td>15.0</td>
<td>21.5</td>
<td>290.8</td>
</tr>
</tbody>
</table>

*The concentration of $^{13}$CO$_2$, total extracts, bound residues and the budget are given in $^{13}$C-phenanthrene equivalents. Phenanthrene equivalents are recalculated based on the $^{13}$C composition of $[1-^{13}$C]phenanthrene, assuming that all carbon atoms of the model substance behave similarly in the transformation process.*

### Table 2

Concentration and degradation rates of extractable tar oil-borne phenanthrene and $[1-^{13}$C]phenanthrene in the bioreactor experiment

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>$^{12}$C ($\mu$g g$^{-1}$)</th>
<th>$^{13}$C ($\mu$g g$^{-1}$)</th>
<th>Time interval (days)</th>
<th>Degradation (% day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>88.6</td>
<td>354.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>21.3</td>
<td>84.8</td>
<td>0–14</td>
<td>5.42</td>
</tr>
<tr>
<td>35</td>
<td>3.69</td>
<td>5.09</td>
<td>14–35</td>
<td>3.94</td>
</tr>
<tr>
<td>50</td>
<td>1.48</td>
<td>1.47</td>
<td>35–50</td>
<td>4.00</td>
</tr>
<tr>
<td>63</td>
<td>0.23</td>
<td>0.27</td>
<td>50–63</td>
<td>6.49</td>
</tr>
<tr>
<td>82</td>
<td>0.23</td>
<td>0.11</td>
<td>63–82</td>
<td>0.03</td>
</tr>
</tbody>
</table>
of magnitude between 3.9 and 6.5% day\(^{-1}\) as the spiked [1-\(^{13}\)C]-phenanthrene. Thus, during the degradation of the bulk material, both compounds were degraded at quite similar rates. Only at minor concentrations, e.g. lower than 0.2 \(\mu\)g g\(^{-1}\), the tar oil-related phenanthrene was degraded at a very small rate (0.03% day\(^{-1}\)) and remained as a relatively stable species in the soil. The spiked [1-\(^{13}\)C]phenanthrene was degraded further but the degradation rates also slowed down at very low concentrations between day 63 and 82 (Table 2). Thus, the degradation rates of spiked \(^{13}\)C-phenanthrene and the tar oil-borne phenanthrene were in the same order of magnitude in the concentration range between 350 and 0.3 \(\mu\)g g\(^{-1}\). Only at very low concentrations we did observe a lower degradation rate of tar oil-related phenanthrene. This result was striking as the old tar oil contamination was aged at least for about 53 years, and ageing is believed to reduce the bioavailability of contaminants (Hatzinger and Alexander, 1995). In model experiments with aged \(^{14}\)C-phenanthrene, a reduced mineralisation of phenanthrene was observed in the presence of non-aqueous phase liquids, clay minerals and natural soil organic matter. The reduced mineralisation was interpreted as a slow desorption of the aged pollutants into the water phase of soil, which is suggested to be necessary to make the pollutants bioavailable (Efroymson and Alexander, 1995). Compared to our approach, these authors have used much lower concentrations of phenanthrene in their experiments. At higher concentrations, which may be more realistic for many tar oil-polluted soils, ageing is less pronounced. Soils may have a limited capacity to sequester hydrophobic pollutants, and in the case of high concentrations free sorption sites are not available. Ageing processes are likely to be dependent on the concentration of the pollutant and the soil properties.

Our results suggest that bioavailability in heavy tar oil contaminated soils may be less affected by ageing as previously expected and a limited biodegradation of pollutants on bioremediation sites is not necessarily a soil matrix problem. Furthermore, the addition of

Fig. 3. [1-\(^{13}\)C]Phenanthrene degradation in a tar oil-contaminated soil matrix. Gas chromatograms of the solvent-extractable fraction. Ant, anthracene; Ph, phenanthrene.

Fig. 4. Phenanthrene degradation rates. (Dotted bars = tar oil-borne phenanthrene; white bars = [1-\(^{13}\)C]phenanthrene.)
the $^{13}$C-labelled substances is a very useful method to measure bioavailability of contaminants to elucidate the remediation capacity of a soil.

In our case the microflora in the contaminated soil was already adapted to degrade the contaminants as the lag-phase was relatively short compared to other degradation experiments with PAH (Kästner et al., 1995; Richnow et al., 1999b). The supplementation with compost, aeration, and the increase of the pH from 4.8 to 6.3 have stimulated the microbial capacity to degrade bulk phenanthrene that was already aged for more than 53 years.

3.3. Chemical structure of bound residues

To investigate the nature of bound residues the pre-extracted soil was hydrolysed and the products analysed by IRM–GC–MS to screen the content of $^{13}$C-labelled metabolites. The chemical structure of products was subsequently investigated by conventional GC–MS comparing mass spectra, and additionally by co-injection of authentic reference compounds.

The products of the alkaline hydrolysis of the bound residue fraction at day 50 were analysed by IRM–GC–MS (Fig. 5). $^{13}$C-labelled products produce a very strong signal in the chromatogram depicting the mass ratio $m/z$ 45/44 ($^{13}$CO$_2$/$^{12}$CO$_2$) due to the relatively high abundance of $^{13}$C in labelled products. Although the absolute concentration of labelled products may be low, the mass ratio $m/z$ 45/44 clearly indicates the presence of labelled metabolites. Major compounds in the hydrolysis fraction are long-chain carboxylic acids deriving from the natural soil organic matter without any enrichment of $^{13}$C. Possible precursors are fatty acids from plants which have been incorporated into the soil organic matter during humification processes. Methoxylated derivatives of benzoic acids may be derived from lignin degradation products which contribute to the aromatic domain of soil humic substances.

A number of $^{13}$C-labelled compounds were detected in the hydrolysates. The chemical structure of most of the major constituents could be identified. This includes the parent $[1-^{13}$C]phenanthrene, typical primary oxidation products such as hydroxy- and dihydroxyphenanthrenes, a number of ring cleavage products including 1,2-dicarboxynaphthalene and hydroxynaphthoic acid. Moreover, phthalic acid and salicylic acid represent typical metabolites of the second ring cleavage. Beside these major metabolites, a significant number of non-identified labelled metabolites were present and contribute to the total amount of bound residues. The major metabolites in the hydrolysates of the residues are well-known metabolites of the aerobic phenanthrene degradation described in liquid cultures with _Pseudomonas_ strains (Evans et al., 1965). Therefore, metabolites of the aerobic phenanthrene degradation might react with soil organic matter and may be incorporated into humic material during natural humification processes in soil. Some constituents such as the parent phenanthrene might be sequestered within micropores leading to a limited extractability. Functionalized metabolites such as acids and hydroxylated compounds might be bound to a significant extent via ester bonds to the macromolecular organic matrix in the soil which has been demonstrated to be a significant mode of binding in bound residues formation during biodegradation of selected PAH in pristine soil (Richnow et al., 1994). Moreover, aromatic alcohols such as phenanthenols and phenanthrediols might also be linked by oxidative coupling via ether and carbon–carbon bonds and contribute to bound residues. These linkages are not amenable to the alkaline degradation technique and the characterisation of ether and carbon–carbon bonds was not covered by our analytical approach.
3.4. Transformation into biological compartments

The soil microorganisms may use organic xenobiotica as a source of carbon and transform the xenobiotic carbon in anabolic processes into typical chemical structures of the biomass. Up to 80% of the bacterial biomass consists of protein, which are built up by proteinaceous amino acids (Gottschalk, 1986). To elucidate the microbial transformation of the $^{13}$C-label of phenanthrene into amino acids in soil, we hydrolysed the solvent-extracted soil and analysed the isotopic composition of hydrolysable amino acids with IRM–GC–MS (Table 3). In the background experiment with non-labelled phenanthrene, the isotopic signature of amino acids was between $-14$ and $-26\%$ (PDB) which is similar to the isotopic composition of the total organic carbon ($-23.5\%$ PDB) and the non extractable organic carbon in the soil ($-25.7\%$ PDB). The hydrolysable amino acids of the bound residue fraction revealed an isotopic composition between $-3.1$ and $-10.4\%$ (PDB) which implies a significant enrichment in $^{13}$C. The only source of $^{13}$C-enriched carbon in the bioreactor was the labelled [1-$^{13}$C]phenanthrene, which was incorporated into amino acids of the soil biomass via anabolic processes. The total amount of $^{13}$C derived from $^{13}$C-phenanthrene transferred into hydrolysable amino acids accounted for $3.1 \mu$g g$^{-1}$ phenanthrene equivalents in the extracted soil. This amount is equivalent to 11% of the total bound residues present in the soil at day 50. Thus, the soil-bound amino acids receive a significant amount of $^{13}$C. However, we have analysed only hydrolysable amino acids in the soil. The total contribution of $^{13}$C derived from phenanthrene bound within amino acid derivatives may be larger because amino acids undergo humification reactions in the soil forming non-hydrolysable condensation products which are not amenable to our method. Moreover, our results imply, that bacterial anabolic metabolism may incorporate contaminant derived carbon into fatty acids, carbohydrates, glycerols and other cell constituents. Although we have not analysed these cell constituents, we assume that a significant amount of contaminant-derived carbon was bound within these fractions.

Our approach did not allow the distinction between living and dead biomass. The contribution of the bacterial-derived living biomass is considered to make up very small parts of the total organic matter in soils (1–5%) although bacteria transform major amounts of organic carbon in soils (Oades, 1995). Therefore, large parts of bacteria-derived organic substances may belong to the dead biomass. A good indication that parts of the amino acids belong to non-living organic matter in soils is the relatively high concentration of labelled $\gamma$-amino butyric acid. $\gamma$-Amino butyric acid is a non-proteinaceous amino acid which is formed during the degradation of proteinous organic matter in sediments (Lee and Cronin, 1982; Ittekott et al., 1984). The microbial decarboxylation of glutamic acid leads to the formation of $\gamma$-amino butyric acid (Meister, 1965). Therefore, labelled $\gamma$-amino butyric acid may well indicate the decay of dead cells which had have transformed phenanthrene related carbon into cell constituents. The dead biomass may provide a food source for other soil organisms. During this process, parts of the dead microbial biomass are incorporated into soil organic matter during natural humification processes in the natural carbon cycle.

In the light of the IUPAC definition, non-extractable bound residues of highly metabolised xenobiotic carbon in a chemical structure identical to biological compounds is not considered as bound residues. In most classical transformation studies with $^{14}$C-labelled PAH, the structural assignments of non-extractable bound residues in soils and sediments were not carried out. Therefore, limited information about the nature of soil-bound residues was obtained. Our results clearly indicate that xenobiotic carbon transferred into

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Background $\delta^{13}$C (% PDB)</th>
<th>50 days (SL3) $\delta^{13}$C (% PDB)</th>
<th>Phenanthrene (eq) (ng g$^{-1}$ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>$-17.1$</td>
<td>2.38</td>
<td>$-6.3$</td>
</tr>
<tr>
<td>Glycine</td>
<td>$-18.5$</td>
<td>3.22</td>
<td>$-6.0$</td>
</tr>
<tr>
<td>$\gamma$-Amino butyric acid</td>
<td>$-17.0$</td>
<td>10.00</td>
<td>$-6.7$</td>
</tr>
<tr>
<td>Valine</td>
<td>$-20.6$</td>
<td>1.26</td>
<td>$-8.0$</td>
</tr>
<tr>
<td>Leucine</td>
<td>$-24.4$</td>
<td>1.65</td>
<td>$-9.2$</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>$-23.9$</td>
<td>0.86</td>
<td>$-10.4$</td>
</tr>
<tr>
<td>Proline</td>
<td>$-17.2$</td>
<td>1.44</td>
<td>$-3.3$</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>$-14.1$</td>
<td>2.84</td>
<td>$-9.3$</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>$-14.0$</td>
<td>2.50</td>
<td>$-3.1$</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>$-26.5$</td>
<td>0.71</td>
<td>$-7.2$</td>
</tr>
<tr>
<td>Lysine</td>
<td>$-19.9$</td>
<td>0.35</td>
<td>$-3.3$</td>
</tr>
</tbody>
</table>

*a The isotopic composition is corrected for the derivative.
the biomass by anabolic processes in the course of biodegradation is significant. These types of residues clearly possess no ecological risk related to the parent compound of concern and may often mimic bound residues in 14C-studies.

4. Conclusions

13C-labelled PAH are useful tracers to elucidate the transformation of PAH in soil on a bulk and on a molecular level. They may also be applied to investigate the transformation of PAH in tar oil-contaminated soils. The carbon budget revealed a relatively fast and efficient mineralisation of the tracer substance after the adjustment of the pH and a sufficient oxygen supply. The tendency to form bound residues was lower compared to former studies, which is a result of the high intrinsic capacity of the soil microflora to degrade the contaminant.

13C-labelled PAH can be used to assess the ageing effects on the bioavailability of contaminants in old soil contaminations. We observed no significant differences in the bioavailability of the tar oil-related phenanthrene in the 53-year-old contamination compared to the spiked 13C-tracer over a large range of concentration. Only at minor concentrations were ageing effects evident. During the degradation of the bulk of phenanthrene, no significant ageing effects reducing the bioavailability of tar oil-related phenanthrene were observed. In our case, ageing processes sequestering the parent material in the soil were not relevant for phenanthrene present in a concentration range of environmental concern. The matrix effects of tar-related non-aqueous phase liquids and humic substances were negligible in our case. Further investigations with different types of aged real contaminated material will have to elucidate the effects of non-aqueous phase liquids and macromolecular natural organic matter in soil under real conditions in more detail.

Part of the xenobiotic carbon is transformed into biomass-related compounds, such as amino acids via anabolic processes. An incorporation of carbon from the pollution into other cell components is likely. This part of the non-extractable residues is not considered as bound residue in the light of the IUPAC definition. Without a precise structural investigation on a molecular level, the bound residue formation may lead to misleading conclusions.

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