Fate of phenanthrene, pyrene and benzo[a]pyrene during biodegradation of crude oil added to two soils

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Abstract

The release of $^{14}$CO$_2$ from 9-$^{14}$C]phenanthrene, 4,5,9,10-$^{14}$C]pyrene and 7-$^{14}$C]benzo[a]pyrene, added to Brent/Forres crude oil and mixed into a pristine sand soil (0.40% organic C) and a pristine organic soil (22.9% organic C), was determined. After 244 days at 25°C, 11.1 ± 3.5% (sand) and 17.1 ± 0.30% (organic) phenanthrene-$^{14}$C and 9.77 ± 2.8% (sand) and 5.86 ± 1.4% (organic) benzo[a]pyrene-$^{14}$C was released. After 210 days, 3.65 ± 0.5% (sand) and 4.43 ± 0.33% (organic) pyrene-$^{14}$C was released. Inoculation of these two soils with DC1 and PD2 (bacteria capable of accelerating the phenanthrene and pyrene mineralisation in soil in the absence of crude oil) either at day 0 or after release as $^{14}$CO$_2$ by indigenous degraders had ceased, failed to increase or initiate further mineralisation. Thus, aged PAH residues were non-bioavailable to these metabolically competent degrading microorganisms. At the end of the first period of incubation (210 days or 244 days), the total aromatic hydrocarbons recovered using Soxhlet extraction was 0.18% (sand) and 42.8% (organic) compared with approximately 100% from bio-inhibited soils. This confirmed that the indigenous microbiological activity not only caused a limited amount of PAH mineralisation but also reduced the extractability of residues, possibly due to the generation of metabolites which were chemisorbed and bound (and non extractable) in ‘aged’ soils. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Polycyclic aromatic hydrocarbons; Bioavailability; Ageing; Chemisorption

1. Introduction

Significant amounts of polycyclic aromatic hydrocarbons (PAHs) are contained in crude oil and some of its derivatives (e.g. diesel oil, bitumen, lubricating oils) and in coal products (e.g. coal tar, creosote, anthracene oil). Accidental spillages and improper disposal of these materials has given rise to soil contamination. Common causes of localised contamination include leakage from underground storage tanks and pipelines (e.g. during the refining and distribution of oil) and the occurrence of residues at manufactured gas plant sites or wood treatment facilities [1]. Contaminated soils are of concern since many PAHs are harmful and recalcitrant, resulting in their classification as priority pollutants [1]. Some PAHs are readily degraded by microorganisms in growth media under laboratory conditions [2,3], suggesting that microbial inoculation may
have potential in remediating contaminated soils. However, biodegradation of PAHs in soil is usually very slow [1] because the bioavailability is limited by a poor mass transfer due to strong or irreversible sorption [4,5]. Under some circumstances, microorganisms degrade PAHs but generate metabolites that may be rendered non-bioavailable by chemisorption and humification [6,7]. Evidence for coupling to soil organic matter components (i.e. humin, humic acid and fulvic acid) comes from findings that PAH metabolites may be recovered from humic material by selective chemical degradation techniques and that some PAH metabolites become covalently linked to humic material by enzyme-catalysed ester bonding [8].

We have reported previously that, despite strong sorption, the mineralisation of phenanthrene and pyrene, added as pure compounds to two non-contaminated soils, was accelerated after inoculating with PAH-degrading bacteria [9]. In this paper, we describe the fate of phenanthrene, pyrene and benzo[a]pyrene contained in crude oil added to two soils and contrast their bioavailability with that of PAHs in the absence of oil.

2. Materials and methods

2.1. Chemicals

9-[14C]phenanthrene, 4,5,9,10-[14C]pyrene and 7-[14C]benzo[a]pyrene (specific activities 59.5, 58.7 and 26.6 mCi mmol⁻¹, respectively) were from Aldrich (Gillingham, UK). Carbosorb and Permafluor E scintillation cocktail were from Packard (Pangbourne, UK). Optiphase liquid scintillation cocktail was from Wallac (Milton Keynes, UK). All other chemicals were of analytical grade. Brent/Fortes crude oil (a gift from Shell International Oil Products) consisted of (v/v) 55% Brent, 42% Fortes and 3% Oseburg crude oils. The oil had been ‘topped’ (i.e. highly volatile components had been removed by heating at 200°C for 8 h).

2.2. Soil collection, storage and analysis

Collection, storage and analysis of the sand soil (0.40% organic carbon) and the organic soil (22.9% organic carbon) were as previously described [9]. Both soils were pristine (i.e. no history of previous PAH contamination).

2.3. Microorganisms

A Gram-negative bacterium (Pseudomonas PD2) capable of degrading phenanthrene and a bacterial consortium composed of four Gram-negative species (DC1) capable of degrading phenanthrene and pyrene were isolated (following enrichment) and grown in a basal salts medium plus the appropriate PAH [9]. Soil inocula were grown for 12 h on tryptone NaCl medium (15 g tryptone, 5 g NaCl l⁻¹). Cultures were harvested by centrifuging (8000×g, 10 min), washed twice and resuspended in sterile distilled water (dw). The bacterial suspension was applied in droplets to the soil surface and then mixed thoroughly with a sterile spatula.

2.4. Analytical methods

Radioactive 14CO₂ was measured by flushing out flask (250 ml) headspace gases for 1.5 h with nitrogen (sufficient to expel at least five flask volumes). Exhaust gas was bubbled through Carbosorb (5 ml) to trap 14CO₂. Carbosorb was added to Permafluor E (12.5 ml), quenched for 12 h and counted (Beckman LS6000 liquid scintillation counter). When two traps were arranged in series, radioactivity was not detected in the second trap confirming the trapping efficiency. When experiments ceased, the mass balance was determined by mixing soil (100–200 mg) with an equal amount of cellulose powder and pelleting with tissue paper. Pellets were combusted (Environmental Metabolism Dept, Huntingdon Life Sciences, P.O. Box 2, Huntingdon, Cambs PE18 6ES, UK) using a Harvey Oxidiser (model OX500, Laboratory Implex, Teddington, UK). Combustion products were absorbed directly into 13-ml Oxysolve C-400 scintillator (Zinsser Analytic, Maidenhead, UK) and counted. The mass balance averaged 103.0% in bio-inhibited and 95.4% in ‘live’ soils.

PAHs were determined by gas chromatography (model GC94, Ai Cambridge, UK) fitted with a 30 m×0.32 mm i.d.×0.25 µm film thickness capillary column (SE-54, BP-5 Alltech Econo-Cap). Helium was the carrier gas (split ratio 17:1, flow rate 1.8
ml min\(^{-1}\)) and the injector and detector temperature was 280°C. Detection was done by FID using the programme: 80°C for 5 min, then ramp from 80°C to 160°C at 5°C min\(^{-1}\), then ramp from 160°C to 300°C at 10°C min\(^{-1}\) and finally hold at 300°C for 17 min. PAHs were determined by reference to standards dissolved in benzene (10 µg ml\(^{-1}\) each): naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoroanthene, pyrene, chrysene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[ah]anthracene and dibenz[ghi]perylene.

2.5. Extracting and analysing crude oil residues

Soil (10 g dry weight equivalent) was mixed with anhydrous sodium sulfate (40 g), placed in a Soxhlet apparatus and refluxed for 14 h with solvent (150 ml acetone+150 ml hexane). After cooling, the solvent was shaken for 30 s with dw (300 ml). After 5 min, the lower aqueous layer was discarded and the extract washed twice with dw (300 ml). The washed organic phase was dried using anhydrous sodium sulfate (40 g), placed in a Soxhlet apparatus and reﬂuxed for 14 h with solvent (150 ml) and the mass balance were determined as described above.

2.6. Measuring the mineralisation of PAHs

Soil (10 g dry weight equivalent) was placed in 300-ml glass bottles and benzo[a]pyrene was added in 0.5 ml DCM to give 0.5 mg g\(^{-1}\) benzo[a]pyrene and 1.0×10\(^4\) dpm g\(^{-1}\) [\(^{14}\)C]benzo[a]pyrene. DCM was vented off using compressed air. Soil was amended with N and P (N as NH\(_4\)NO\(_3\) and P as KH\(_2\)PO\(_4\)) giving a C:N:P ratio of 100:1:0.1, adjusted to a 60% water holding capacity (whc) (0.155 g H\(_2\)O g\(^{-1}\) sand soil, 0.624 g H\(_2\)O g\(^{-1}\) organic soil) using sterile dw and mixed thoroughly using a sterile spatula. The bottles were sealed with rubber subaseals and incubated at 25°C in the dark. Headspace \(^{14}\)CO\(_2\) and the mass balance were determined as described above.

The [\(^{14}\)C]PAHs were added individually to samples of crude oil at 1000 dpm µl\(^{-1}\). The PAH-containing oil was applied to duplicate 10 g (dry weight equivalent) soil, placed in 250-ml glass conical ﬂasks (giving 1% (w/w) PAH and 1.15×10\(^4\) dpm g\(^{-1}\)). Soil was amended with N and P (as NH\(_4\)NO\(_3\) and KH\(_2\)PO\(_4\)) giving a C:N:P ratio of 100:1:0.1 and adjusted to 60% whc (appropriate for aerobic activity) using sterile dw. Flasks were enclosed using rubber subaseals and incubated at 25°C in the dark. The inoculum (DC1) was prepared as described above and applied to give 2×10\(^6\) cells g\(^{-1}\) soil. After 244 days, soils were divided into two equal (5.0 g dry weight equivalent) parts and placed in 250-ml conical ﬂasks. One set was re-inoculated with DC1 (7.3×10\(^7\) cells g\(^{-1}\)) and the second set (controls) received a heat-killed autoclaved inoculum. After 326 days, the first set was inoculated again but this time with PD2 (3.3×10\(^5\) cells g\(^{-1}\)). Heat-killed cells were again added to the second set. Further sets of controls were microbiobially inhibited (5% (w/w) HgCl\(_2\)) at time zero and used to determine any PAH degradation due to abiotic processes as well as losses due to extraction inefficiencies and volatilisation. Headspace \(^{14}\)CO\(_2\) and the mass balance were determined as described above. Crude oil (1 ml 0.872 g\(^{-1}\)) was fractionated by scale up the method. The aromatic fraction of the oil (27.6±1.2%) was dissolved in DCM (1 ml) and [\(^{14}\)C]phenanthrene was added. This preparation was added to soils (10 g dry weight equivalent) to give 2.760 mg kg\(^{-1}\) aromatic hydrocarbons and 1.5×10\(^4\) dpm g\(^{-1}\) [\(^{14}\)C]phenanthrene and placed in 250-ml quickﬁt conical ﬂasks. Soil was moistened to 60% whc, N and P added, incubated and analysed at the same time as those treated with crude oil.

2.7. Sorption and desorption

Benzo[a]pyrene in acetone (1.5 µg ml\(^{-1}\)) was amended with [\(^{14}\)C]benzo[a]pyrene (1.4×10\(^6\) dpm ml\(^{-1}\)) and crystallised onto the inner walls of dup-licate teflon centrifuge tubes to give five concentra-
tions ranging from 600–3000 ng per tube (sand soil) and 360–1800 ng per tube (organic soil). Soil (187 mg dry weight equivalent sand soil or 12.9 mg dry weight equivalent organic soil) and 40 ml 0.01 M CaCl$_2$ containing 20 mM sodium azide were added. Contents were mixed (orbital shaking at 150 rpm) and equilibrated at 25°C for 24 h. After centrifuging (10,000 g, 10 min, Beckman J2-21 High Speed Centrifuge fitted with JA20 rotor), the aqueous phase (1 ml) was added to the Optiphase scintillation cocktail (4 ml) and counted. The desorption was measured by decanting and discarding a total volume of 30 ml from each tube and replacing with fresh 0.01 M CaCl$_2$ solution. After mixing and equilibrating contents for a further 24 h, the tubes were centrifuged and analysed as before. The sorption and desorption were measured by decreases and increases in the aqueous phase concentration, respectively.

2.8. Survival of inocula in soil

Soil (20 g dry weight equivalent) was placed in duplicate 300-ml glass bottles and N and P were added as above. Soil was adjusted to 60% w/c with dw and one set was amended with crude oil (10,000 mg kg$^{-1}$). Bacterial consortium DC1 was added to give 8.6×10$^8$ cells g$^{-1}$ soil. Soils were mixed thoroughly. Sub-samples (0.5 g) were removed and vortexed (1 min, full speed) with 3 ml phosphate buffer (21 mM, pH 7.1) and 0.1 ml of the suspension was spread (after dilution in phosphate buffer) on plates pre-coated with pyrene as described previously [9].

2.9. Mobility of non-degraded PAH residues in contaminated soil

After 244 days, soil was removed from non-inoculated flasks amended with crude oil and [14C]PAHs. Sand soil (10 g wet weight) or organic soil (6 g wet weight) was packed into glass columns (6 mm i.d.×30 cm length) giving bulk densities of 1.42 g cm$^{-3}$ (sand) or 0.30 g cm$^{-3}$ (organic). Calcium chloride (500 ml 0.01 M, pH 6.0) was pumped down the columns at a flow rate of 17–25 ml h$^{-1}$ (sand) and 31–71 ml h$^{-1}$ (organic). Aqueous eluate (250 ml) was extracted by shaking (1 min) three times with hexane:acetone (30 ml:30 ml). The hexane fraction was collected, evaporated under nitrogen, dissolved in 2 ml Carbosorb and 3 ml Permafluor E and the radioactivity was counted. Soil (100 mg) was removed from the top, middle and bottom sections of the columns and the radioactivity was determined by sample oxidation as described above.

2.10. Statistical analysis

Values presented are means of either two or three replicates (±1 S.E.M.). Student’s $t$-test and regression analyses were performed using Sigmamplot for Windows 2.01 (Jandel Scientific, Erkrath, Germany).

3. Results and discussion

3.1. Sorption and desorption of benzo[a]pyrene

Sorption and desorption-fitted linear isotherms and constants were obtained by regression of the Freundlich equation (95% confidence interval). Sorption constants (1/nsrp and K$_a$) were 0.997 and 18,200 ($r^2=0.976$) on sand soil and 0.784 and 276,000 ($r^2=0.926$) on organic soil, K$_oc$ values ($K_{oc}=(K_a\times100)/%\text{ organic carbon}$) were 4.54×10$^6$ on sand soil and 1.20×10$^6$ on organic soil. Desorption constants (1/n$_{des}$ and K$_d$) were 1.18 and 32,200 ($r^2=0.985$) on sand soil and 1.27 and 942,000 ($r^2=0.896$) on organic soil, confirming a marked sorption hysteresis. The sorption capacity of both soils for benzo[a]pyrene was much greater than for naphthalene, phenanthrene and pyrene [9].

3.2. Survival of inocula added to soils

When DC1 was added (to give 8.6×10$^8$ cells g$^{-1}$ soil) to non-contaminated soils and to soils freshly contaminated with crude oil, the numbers of cultivable (i.e. recoverable) pyrene degraders detected ranged between 4.7×10$^6$ and 2.95×10$^7$ g$^{-1}$, 1 h after inoculation, and declined to 5.31×10$^4$–2.48×10$^5$ g$^{-1}$ between 1 and 8 days in both soils whether contaminated or non-contaminated. This confirmed that a significant number of bacteria that were capable of degrading pyrene survived when added to oil-contaminated soils.
Fig. 1. Release of $^{14}$C as $^{14}$CO$_2$ from 9-$[^{14}$C$]$phenanthrene added to (a) sand soil or (b) organic soil, from 4,5,9,10-$[^{14}$C$]$pyrene added to (c) sand soil or (d) organic soil and from 7-$[^{14}$C$]$benzo[a]pyrene added to (e) sand soil or (f) organic soil in Brent/Fortes crude oil: (●) non-inoculated soil, (□) DC1-inoculated soil, (▲) bio-inhibited soil.
3.3. Carbon dioxide release from benzo[a]pyrene, added to soils

Release as $^{14}\text{CO}_2$ from $[^{14}\text{C}]$benzo[a]pyrene, added to the soils, did not take place during 349 days (sand) and 242 days (organic). Benzo[a]pyrene is co-oxidised by microorganisms in soil [10] and the absence of suitable co-substrates may have limited the biodegradation. In addition, strong and irreversible sorption may have reduced the aqueous phase concentrations of benzo[a]pyrene to levels below those required for the induction of degradative enzymes or to sustain microbial growth. The extrapolation of sorption isotherms indicated initial aqueous phase concentrations in soil of 3.5 ng ml$^{-1}$ (sand) and therefore at saturation and 1.4 ng ml$^{-1}$ (organic) equivalent to 13.9 nM and 5.56 nM, respectively.

3.4. Carbon dioxide release from PAHs in crude oil, added to soil

The release of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$PAHs contained in crude oil was rapid initially. The rapid phase was the same for phenanthrene and pyrene but more than twice as long in the sand than in the organic soil for benzo[a]pyrene. After 27 days, 9.7 ± 3.3% (sand) and 14.1 ± 0.2% (organic) of the added $[^{14}\text{C}]$phenanthrene was evolved, after 48 days, 1.67 ± 0.29% (sand) and 3.04 ± 0.5% (organic) of the $[^{14}\text{C}]$pyrene was evolved and after 111 days, 9.5 ± 3.1% (sand) and after 70 days, 3.2 ± 0.1% (organic) $[^{14}\text{C}]$benzo[a]pyrene was evolved (Fig. 1.). During a second much slower phase (27–244 days ranged from 2.65% (benzo[a]pyrene, organic soil) to 0.307% (benzo[a]pyrene, sand soil) phenanthrene, 70–244 days, benzo[a]pyrene, 48–210 days pyrene), a further release as $^{14}\text{CO}_2$ was and may be a result of the slow desorption of PAH and its metabolites. Extensive catabolism of $[^{14}\text{C}]$phenanthrene and $[^{14}\text{C}]$pyrene is required for the release as $^{14}\text{CO}_2$. However, since $[^{14}\text{C}]$benzo[a]pyrene is labelled at C-7 (and during bacterial catabolism it is predominantly attacked by bacteria at the 7, 8 or 9, 10 positions [2]), the release as $^{14}\text{CO}_2$ may exceed the mineralisation (i.e. conversion to $\text{CO}_2$ and $\text{H}_2\text{O}$) of benzo[a]pyrene. This would explain why the total release from $[^{14}\text{C}]$benzo[a]pyrene after 244 days (9.77 ± 2.78% sand and 5.86 ± 1.37% organic) exceeded the total release from $[^{14}\text{C}]$pyrene after 210 days (3.65 ± 0.81% sand and 4.43 ± 0.33% organic) despite a stronger sorption of benzo[a]pyrene (see above) than pyrene ($K_{oc}$ 1.92 × 10$^5$ sand, 1.58 × 10$^5$ organic [9]). The provision of suitable co-substrates and/or the enrichment of a hydrocarbon-degrading population in the oil-contaminated soils, no doubt, stimulated the release as $^{14}\text{CO}_2$ from $[^{14}\text{C}]$benzo[a]pyrene, which contrasted with the absence of $^{14}\text{CO}_2$ evolution when benzo[a]pyrene was added individually (i.e. not in oil) to the soils (see above). Inoculation of soils with DC1 did not increase the release of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$phenanthrene or $[^{14}\text{C}]$pyrene added in crude oil (Fig. 1.). This contrasts with the accelerated mineralisation recorded when DC1 was added to PAH-contaminated soils in the absence of oil [9]. Nonetheless, PAH degraders in the DC1 consortium survived beyond 8 days oil-contaminated soils. Possibly, DC1 was constrained by low bioavailability of phenanthrene and pyrene, inhibited by oil components or preferentially metabolised more easily degradable substrates contained in the oil (e.g. straight chain or branched alkanes).

Table 1
Recovery of aromatic oil hydrocarbon residues from Brent/Forbes-contaminated soil after 244 days (quantified by GC-FID after Soxhlet extraction)

<table>
<thead>
<tr>
<th></th>
<th>Top section</th>
<th>Middle section</th>
<th>Bottom section</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sand soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>103.2 ± 12.1</td>
<td>92.00 ± 7.94</td>
<td>103.1 ± 6.15</td>
</tr>
<tr>
<td>Pyrene</td>
<td>88.78 ± 4.17</td>
<td>88.29 ± 3.39</td>
<td>87.39 ± 4.27</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>85.16 ± 4.81</td>
<td>84.95 ± 5.92</td>
<td>84.80 ± 5.96</td>
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<tr>
<td><strong>Organic soil</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>76.00 ± 9.04</td>
<td>78.70 ± 5.98</td>
<td>79.10 ± 6.11</td>
</tr>
<tr>
<td>Pyrene</td>
<td>110.0 ± 10.9</td>
<td>95.91 ± 9.63</td>
<td>111.3 ± 13.4</td>
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<tr>
<td>Benzo[a]pyrene</td>
<td>82.42 ± 7.47</td>
<td>84.96 ± 9.40</td>
<td>78.91 ± 6.33</td>
</tr>
</tbody>
</table>
3.5. Carbon dioxide release from phenanthrene in the crude oil aromatic fraction added to soil

When \(^{14}\text{C}\)phenanthrene contained in the separated aromatic fraction from crude oil was added to soil (Fig. 2.), the release as \(^{14}\text{CO}_2\) after 27 days was \(25.0 \pm 1.9\%\) (sand) and \(22.9 \pm 0.4\%\) (organic) and was more rapid and extensive compared with \(^{14}\text{C}\)phenanthrene added to soils in crude oil (see above). This degradation was enhanced in both soils by removing the aliphatic hydrocarbons, suggesting that aliphatics either inhibited or were toxic to the indigenous PAH-degrading microorganisms or were utilised as preferred substrates.

3.6. Carbon dioxide release from aged PAH residues in crude oil-contaminated soil

Inoculation of soils containing crude oil with DC1 after \(^{14}\text{CO}_2\) release had ceased (244 days, phenanthrene; 210 days, pyrene) did not initiate further mineralisation. Similarly, inoculation of the soils containing \(^{14}\text{C}\)phenanthrene residues with PD2 after 322 days failed to stimulate a further mineralisation. This confirmed that ‘aged’ residues were non-bioavailable even to high densities of metabolically competent degrading microorganisms.

3.7. Disappearance of aromatic oil components from soil

Residual aromatic hydrocarbons in the soils were quantified by GC-FID analysis. After 244 days, aromatic hydrocarbons (as a percent of recovery at time zero) were reduced to \(0.18 \pm 0.18\%\) (sand) and \(42.8 \pm 11.6\%\) (organic) by the indigenous microorganisms in non-inoculated soils and to \(1.42 \pm 0.25\%\) (sand) and \(34.8 \pm 5.8\%\) (organic) in soils initially inoculated with DC1. There was no reduction in aromatic hydrocarbons in bio-inhibited soils (Table 1.). This confirmed the almost complete disappearance of aromatics from the ‘live’ sand soil and extensive dis-

Table 2

<table>
<thead>
<tr>
<th>Recovery of aromatics (% of time 0)</th>
<th>Aromatics</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand soil</td>
<td></td>
<td></td>
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<tr>
<td>Non-inoculated</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>DC1-inoculated</td>
<td>1.42</td>
<td>0.25</td>
</tr>
<tr>
<td>Bio-inhibited</td>
<td>131.2</td>
<td>24.5</td>
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<tr>
<td>Organic soil</td>
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<td></td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>42.8</td>
<td>11.6</td>
</tr>
<tr>
<td>DC1-inoculated</td>
<td>34.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Bio-inhibited</td>
<td>92.65</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Fig. 2. Release of \(^{14}\text{C}\) as \(^{14}\text{CO}_2\) from \(9\text{[}\(^{14}\text{C}\)phenanthrene contained in (○) Brent/Forties crude oil and (▲) Brent/Forties crude oil aromatics and added to (a) sand soil or (b) organic soil.}
sappearance from ‘live’ organic soil, despite a limited release of $^{14}$CO$_2$ from the $[^{14}C]$PAHs. The microbiological activity may have generated metabolites, such as quinones and hydroxylated or carboxylated intermediates [3] which are more reactive than unsubstituted molecules. These metabolites are more likely to chemisorb and become incorporated or bound into the soil organic matter [8,11] and this may explain the disparities between the reduction in PAH levels and the release of $^{14}$CO$_2$.

3.8. Mobility of non-degraded PAH residues in soil columns

An experiment was conducted to demonstrate the mobility of the bound residues.

Hexane extractable $[^{14}C]$phenanthrene, $[^{14}C]$pyrene or $[^{14}C]$benzo[a]pyrene residues were not eluted from soil columns packed with soils, homogenously contaminated with crude oil and perfused with a CaCl$_2$ solution. Combustion analysis of soil from the upper, middle and bottom sections of the columns showed that residues had not moved significantly ($P < 0.05$) downwards through the soil after 244 days (Table 2). This suggests that neither bound residues nor the soil colloids with which they are associated migrate downwards through soil and the risks of bound PAH residues polluting to groundwater is small.

4. Conclusions

In this study, bacterial inocula did not increase the mineralisation of phenanthrene and pyrene contained in crude oil added to two pristine soils. Suggested reasons for this include the preferred substrate utilisation, direct inhibition of the competent bacteria and the decreased bioavailability of the two PAHs with time. Inoculation may under certain circumstances be beneficial to soil bioremediation processes, but most laboratory and field studies fail to demonstrate clear advantages [12]. From our data and other observations it is clear that increasing the number of microorganisms in soil by inoculation does not accelerate the degradation of aged PAH contaminants since mass transfer severely limits their bioavailability and biodegradation.

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