Bioavailability of benzo[*a*]pyrene during NAPL-enhanced biodegradation in soil and in liquid culture

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Abstract The high molecular weight polycyclic aromatic hydrocarbon (HMW PAH) benzo[*a*]pyrene is generally persistent in the environment and its persistence may be due to bioavailability limitations. However, the presence of degradation-capable microorganisms and a suitable cosubstrate are also necessary. This is especially the case for benzo[*a*]pyrene because it may only be degraded by fortuitous metabolism. Non-aqueous phase liquid (NAPL)-enhanced benzo[*a*]pyrene biodegradation and indicators of bioavailability were measured in soil and liquid culture. In soil, $^{14}CO_2$ from 7-[^{14}C]benzo[*a*]pyrene mineralisation and overall CO_2 production were monitored for 83 d after treatment with different types of NAPLs in biometer flasks. Monitoring was followed by soil extraction and measurement of ^{14}C residues and of the remaining NAPL by gravimetry. In liquid culture, 7-[^{14}C]benzo[*a*]pyrene mineralisation was monitored after treatment with different NAPLs and followed by a radiocarbon mass balance of ^{14}C residues. Results indicated that although benzo[*a*]pyrene may have been bioavailable in both media types, benzo[*a*]pyrene mineralisation only occurred when a suitable NAPL cosubstrate was present to facilitate biodegradation. In soil, rapid increases in the rate and onset of benzo[*a*]pyrene mineralisation were shown to occur in benzo[*a*]pyrene-contaminated soils that were treated with mineral oil, which was a relatively non-biodegradable NAPL cosolvent, plus a hexane fraction-NAPL which was biodegradable and contained suitable cosubstrate(s).
Keywords Benzo[*a*]pyrene; bioavailability; biodegradation; non-aqueous phase liquids; polycyclic aromatic hydrocarbons

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Introduction

Polycyclic aromatic hydrocarbons (PAH) are hydrophobic pollutants that are often introduced into the environment in non-aqueous phase liquid (NAPL) mixtures such as creosote and various types of petroleum-derived fuels. Such petroleum-derived NAPLs may consist of hundreds of compounds that are highly variable in structure and in susceptibility to biodegradation (Bossert and Compeau, 1995). Environmental pollution caused by the release of PAHs occurs throughout the world, and considering their potential negative effects on human health, there is much interest to determine the environmental fates of these compounds. As such, they are of direct concern to regulators, industry, and environmental and public health professionals.

PAHs with greater than three rings, HMW PAHs, are a class of PAHs that are environmentally persistent and are frequently encountered as constituents of NAPLs (Cerniglia, 1992; Shuttleworth and Cerniglia, 1995; Kanaly and Harayama, 2000). Specifically, the five-ring HMW PAH benzo[*a*]pyrene has been a compound of intense study for more than 50 years due to its potent genotoxic properties (Sutherland *et al.*, 1995). In soil, benzo[*a*]pyrene may be removed by the biodegradative actions of bacteria and fungi and such microbially catalysed biotransformations may be partial or lead to aromatic ring-opening depending upon the type of organism performing the biodegradation (Cerniglia, 1992; Juhasz and Naidu, 2000; Kanaly and Harayama, 2000). However, benzo[*a*]pyrene

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and other HMW PAHs are mostly biodegraded by fortuitous metabolism whereby an organism utilises a primary substrate for growth and in the process biotransforms a second compound from which the organism derives neither energy nor carbon. A number of studies in the last 10 years have discussed benzo[*a*]pyrene biodegradation under various conditions (Juhasz *et al.*, 1996, 1997, 2000a, 2000b; Schneider *et al.*, 1996; Aitken *et al.*, 1998; Chen and Aitken, 1999; Boonchan *et al.*, 2000; Marcoux *et al.*, 2000), and extensive biotransformation of benzo[*a*]pyrene to carbon dioxide has also been shown to occur in some instances (Kanaly *et al.*, 1997; Kanaly and Bartha, 1999; Kanaly *et al.*, 2000, 2001, 2002; Kanaly and Watanabe, 2004). Even considering these studies, however, there is still much to be learned about the mechanisms underlying benzo[*a*]pyrene biodegradation.

Generally, bioavailability limitations are considered to be responsible for the environmental recalcitrance of benzo[*a*]pyrene, but it may not be the case in all situations as has been shown recently by Cornelissen *et al.* (1998) and Huesemann *et al.* (2004). Both groups concluded that microbial factors, not bioavailability of benzo[*a*]pyrene, were responsible for its lack of degradation in soil. Interestingly, in some cases benzo[*a*]pyrene slurry biotreatments were carried out for 270 days with no biodegradation of benzo[*a*]pyrene even though it was readily bioavailable (Huesemann *et al.*, 2004). Benzo[*a*]pyrene bioavailability and biodegradation in soil and liquid culture and the role of NAPLs as bioavailability enhancers and as biodegradation cosubstrates were explored herein using radiorespirometry and radiolabel tracking techniques.

Materials and methods

[7-^{14}C]Benzo[*a*]pyrene (49.2 mCi/mmol) and (26.6 mCi/mmol) were purchased from Chemsyn Science Laboratories (Lenexa, KS, USA) and Sigma Chemical (St Louis, MO, USA) respectively. [^{14}C]Sodium bicarbonate (9.2 mCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). All had a radiochemical purity of $\geq$98% as determined by the manufacturer. Benzo[*a*]pyrene (99+% purity) and *n*-hexadecane were purchased from Aldrich Chemical (Milwaukee, WI, USA), and mineral oil was purchased from Squibb (Princeton, NJ, USA). Diesel fuel was originally obtained from Exxon Corp. (Houston, TX, USA) and a diesel fuel distillation product (high-boiling distillate; HBD) was prepared from diesel fuel by heat distillation (Kanaly *et al.*, 2001). The hexane fraction from a crude oil was prepared as described previously (Kanaly and Bartha, 1999).

Biodegradation assays in soil

Soil biodegradation experiments were carried out in duplicate in 250-mL biometer flasks (Bellco Glass, Vineland, NJ, USA; Bartha and Pramer, 1965). Total soil mass was 12.5 g (dry weight) and all samples were incubated for 83 days at 25 °C in the dark. Uncontaminated soil (Kanaly *et al.*, 1997) was sieved and mixed with $CaCO_3$ (2.5 mg/g soil), stored and aerated for three days for pH equilibration at approximately 7.2. A radiolabelled benzo[*a*]pyrene-NAPL stock solution was prepared by mixing labelled and unlabelled benzo[*a*]pyrene plus NAPL in diethyl ether. To each biometer flask 1.0 g of air-dried soil was added, the radiolabelled benzo[*a*]pyrene-NAPL stock solution was dispensed onto the soil via microsyringe, and nitrogen gas was used to evaporate the ether. NAPL was added to facilitate a total concentration in soil of 0.5% w/w (soil dry weight). Three NAPL treatments were tested as follows: (i) hexane fraction–NAPL, (ii) mineral oil–NAPL and (iii) hexane fraction–NAPL plus mineral oil–NAPL (0.25 + 0.25%, w/w). The negative control consisted of benzo[*a*]pyrene contaminated soil without NAPL addition. Finally, remaining fresh soil was added to the flask and gently mixed with the air-dried soil. Water and 1.0% (w/v) $(NH_4)_2HPO_4$ were added to minimise nutrient

imbalance and maintain a moisture level in soil of 50% of the water-holding capacity. Stock solutions were prepared such that the total benzo[*a*]pyrene and [7-^{14}C]benzo[*a*]-pyrene treatment in soil was 80 μg/g and approximately 150,000 dpm per gram dry soil, respectively. Measurements of $^{14}CO_2$ and CO_2 were performed at each sampling point by removing 10 mL of KOH from the biometer flask sidearm via a syringe and splitting the sample. For $^{14}CO_2$ measurements, two 1-mL samples of KOH were added to 10 mL each of ScintiVerse BD and analysed by liquid scintillation counting in a Beta-Trac Model 6895 counter (TM Analytic, Elk Grove Village, IL, USA). Counts were corrected for background and for efficiency by the external standard ratio method. For CO_2 analysis, the remaining 8 mL of KOH was treated with $BaCl_2$ to precipitate carbonate and the supernatant liquid was titrated with HCl.

Soil extraction was performed at the end of the incubation period. The entire contents of flasks from three treatments were dried with anhydrous sodium sulphate and extracted with dichloromethane in a Soxhlet apparatus for 8 h (greater than 60 cycles). The extracts were concentrated and measured for ^{14}C activity by liquid scintillation counting and total extractable residue by gravimetric analyses. Total extractable residue was calculated by subtracting the total mass extracted from each soil from the mass of residue extracted from the negative control.

Biodegradation assays in liquid culture

Biodegradation assays in liquid culture consisted of a bacterial consortium recovered from soil (Kanaly *et al.*, 2000) incubated with 10 mg/L benzo[*a*]pyrene plus NAPL. [7-^{14}C]Benzo[*a*]pyrene mineralisation was monitored in 300-mL Erlenmeyer flasks with stoppers fitted with two stainless steel syringe needles. Experimental treatments were prepared by the addition of [7-^{14}C]benzo[*a*]pyrene plus NAPL to flasks by a technique similar to that as described above for the soil assays. In this case, the stock solution was applied to the flask bottom, however, rather than to dried soil. After solvent evaporation, Staniers Basal Medium (30 mL) and inocula were added. Flasks were incubated in duplicate at 28 °C in the dark with rotary shaking at 150 rpm with the following treatments (percentages are units of w/v): HBD (0.1%; positive control), *n*-hexadecane (0.1, 0.5 and 1.5%) and no NAPL addition (negative control). Radiolabelled carbon dioxide released during [7-^{14}C]benzo[*a*]pyrene mineralisation was trapped by flushing the flasks with air through a series of vials which contained Oxosol C^{14} (National Diagnostics, Atlanta, GA, USA). Radioactivity was measured using a Tri Carb model 1900CA liquid scintillation analyser (Packard Instrument Co., Meriden, CT, USA) and counts were corrected for background against pure Oxosol C^{14}. The efficiency of the CO_2-trapping apparatus was checked routinely by transferring aliquots of an aqueous Na$H^{14}CO_3$ (pH 10) solution to sealed Erlenmeyer flasks followed by addition of HCl and the resulting $^{14}CO_2$ was measured. After 18 days, cultures were extracted and radiocarbon mass balance analyses were conducted similarly as described previously (Kanaly *et al.*, 2000).

Results and discussion

Effects of NAPLs on benzo[*a*]pyrene mineralisation in soil

Three compositionally different NAPLs were applied to soil and the differences in the manner of benzo[*a*]pyrene mineralisation were monitored for almost three months. Three soil treatments, (i) hexane fraction–NAPL (0.5%, w/w), (ii) mineral oil–NAPL (0.5%, w/w) and (iii) hexane fraction–NAPL plus mineral oil–NAPL (0.25 + 0.25%, w/w; hereafter referred to as the combination–NAPL) each stimulated benzo[*a*]pyrene mineralisation in soil differently.

As shown in Figure 1, the hexane fraction–NAPL stimulated rapid benzo[*a*]pyrene mineralisation, but only after incurring a lag period of 30–40 days.

After the lag period, the rate of benzo[*a*]pyrene mineralisation increased and resulted in approximately 35% total mineralisation of benzo[*a*]pyrene by day 83. The mineral oil–NAPL minimally stimulated mineralisation and resulted in approximately 8% mineralisation after 83 days. In contrast, the combination–NAPL stimulated benzo[*a*]pyrene mineralisation faster than in soils treated with either the hexane fraction–NAPL or the mineral oil–NAPL. When the combination–NAPL was applied, mineralisation began after a lag period of 10 d and continued relatively linearly through to at least day 70. Although the combination–NAPL facilitated an earlier and rapid start to mineralisation, the final extent of mineralisation by day 83 was similar to the soil treated with hexane fraction–NAPL (35–40% mineralisation). In negative control soils (no NAPL addition), benzo[*a*]pyrene mineralisation was less than 5%.

Carbon dioxide monitoring indicated that all soils were active even though benzo[*a*]-pyrene may not have been mineralised much in some cases. Figure 2 shows the cumulative carbon dioxide recovered from the four soil treatments over 83 d. Of the NAPL-treated soils, those treated with mineral oil–NAPL produced the least amount of carbon dioxide, and indicated that the mineral oil–NAPL was minimally utilised as a carbon source by the soil microbiota. Soils treated with hexane fraction–NAPL and those treated with the combination–NAPL each produced a similar total amount of carbon dioxide. Although the combination–NAPL consisted of half as much easily biodegradable carbon as the hexane fraction–NAPL this result most likely occurred as the result of a soil priming effect (Sharabi and Bartha, 1993). As expected, the negative control resulted in the least amount of carbon dioxide detected.

In addition to the carbon dioxide monitoring data, chemical analyses of the NAPL-treated soils confirmed that mineral oil carbon was minimally utilised by the soil microorganisms. Soils from biometer flasks that contained mineral oil–NAPL and

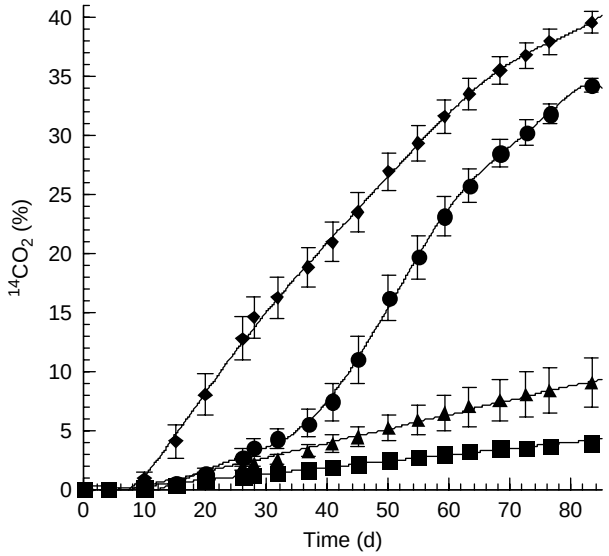

Figure 1 Mineralisation of 80 μg/g [7-^{14}C]benzo[*a*]pyrene in soil during incubation in biometer flasks for 83 d. Each point represents an average of replicates; error bars show the range between samples and are omitted when smaller than the symbol. Hexane fraction–NAPL combined with mineral oil–NAPL (0.25% w/w each) (◆), hexane fraction–NAPL (0.5% w/w) (●), mineral oil–NAPL (0.5% w/w) (σ) and without NAPL addition (■)

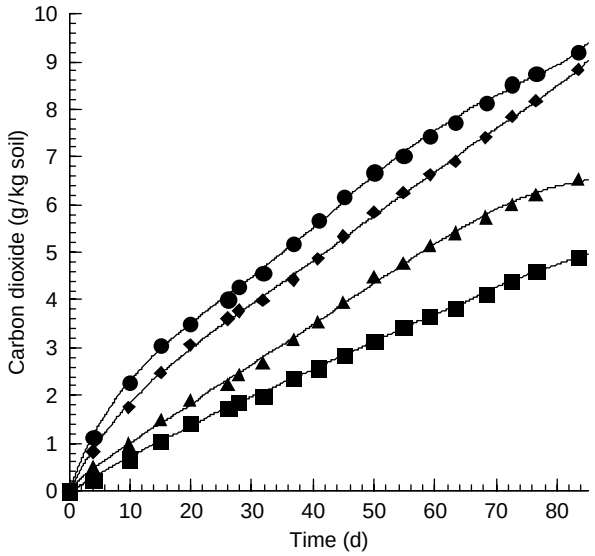

Figure 2 Carbon dioxide (g/kg dry soil) recovered from soil during 83 d of incubation. Each point represents an average of replicates; the range never exceeded ± 5.0% of the mean and error bars are omitted for clarity. Hexane fraction–NAPL combined with mineral oil–NAPL (0.25% w/w each) (◆), hexane fraction–NAPL (0.5% w/w) (●), mineral oil–NAPL (0.5% w/w)(σ) and without NAPL addition (■)

combination–NAPL were extracted with dichloromethane and extractable ^{14}C was measured. We used $^{14}CO_2$ mineralisation data and ^{14}C extractability from soil as indicators of benzo[*a*]pyrene bioavailability. Equal quantities of ^{14}C were bioavailable in soils containing mineral oil–NAPL and combination–NAPL even though mineral oil–NAPL-treated soil resulted in little benzo[*a*]pyrene mineralisation. In the case of the mineral oil–NAPL-treated soil, 64.0 ± 0.1% of the ^{14}C was bioavailable (9.0 ± 2.1% as $^{14}CO_2$ and 55.4 ± 1.8% as soil extractable ^{14}C). In the case of the combination–NAPL, 65.1 ± 0.4% of the ^{14}C was bioavailable (39.6 ± 0.9% as $^{14}CO_2$ and 25.5 ± 1.3% as soil extractable ^{14}C). These results indicated that soils treated with mineral oil–NAPL and combination–NAPL contained equally bioavailable amounts of benzo[*a*]pyrene; however, in the case of the mineral oil–NAPL, benzo[*a*]pyrene was not much mineralised due to the absence of a suitable cosubstrate. Additionally, results of gravimetric analyses of dichloromethane extracts provided further evidence that mineral oil was minimally utilised by the soil microbiota. In flasks treated with mineral oil, 84.0 ± 2.3% of the added mass was recovered after incubation of soils for 83 d. In soils treated with combination–NAPL, 60.0 ± 4.8% of the added mass was recovered. Although these techniques may be useful measures of bioavailability in soil, the meaning of bioavailability is taking on a broader context in light of recent results which show that pollutants associated with organic material are still available or more available depending upon the type of pollutant and the type of bacteria involved in the degradation (Laor *et al.*, 1999; Feng *et al.*, 2000; Grosser *et al.*, 2000).

In summary, the mineral oil–NAPL promoted benzo[*a*]pyrene mineralisation to a greater extent than the negative control and in a linear pattern. This pattern of stimulation most likely occurred due to an increase in the bioavailability of benzo[*a*]pyrene through a mass transfer effect of benzo[*a*]pyrene to the degrading organism(s). In such a case, the bioavailability of benzo[*a*]pyrene to the organisms was the rate-controlling step. Considering that mineral oil was not much utilised by the soil community, it is unlikely that mineral oil promoted benzo[*a*]pyrene mineralisation by fortuitous metabolism.

Table 1 Mass balance of benzo[*a*]pyrene radiocarbon following an 18-d incubation period under different conditions

Radiocarbon	0.1% HBD	0.1% *n*-hexadecane	0.5%*n*-hexadecane	1.5%*n*-hexadecane	Negative control
Filter-trapped ^{14}C*	26.4 ± 0.5	56.6 ± 4.4	86.5 ± 9.0	74.6 ± 3.4	18.5 ± 0.2
Flask-attached [^{14}C]benzo[*a*]pyrene**	2.0 ± 1.9	37.9 ± 3.7	4.5 ± 0.7	3.6 ± 0.3	71.0 ± 10.8
$^{14}CO_2$	59.1 ± 1.4	2.1 ± 0.2	0.9 ± 0.4	0.4 ± 0.1	2.5 ± 0.2
Total recovery	87.5 ± 2.8	96.6 ± 0.5	91.9 ± 7.9	78.6 ± 3.2	92.0 ± 10.4

All values in the table are expressed as percent recovery of radiolabel

*[7-^{14}C]benzo[*a*]pyrene that was detected in suspension after filtration

**Greater than 95% of flask-attached ^{14}C was previously determined to be whole [7-^{14}C]benzo[*a*]pyrene by thin layer chromatography (Kanaly *et al.*, 2000)

The hexane fraction–NAPL, however, stimulated a large amount of benzo[*a*]pyrene mineralisation (more than 25 μg/g soil) and this most likely occurred via fortuitous metabolism, but after a lengthy lag period. Lastly, when mineral oil–NAPL was combined with hexane fraction–NAPL, the lag period before onset of benzo[*a*]pyrene mineralisation was considerably shortened and the rate of initial mineralisation increased. Additionally, the pattern of mineralisation was relatively linear, indicating that mass transfer, not bacterial cell growth, may have been the rate-limiting step. These results indicate that when a combination of NAPLs that each possess different but complementary characteristics relevant to benzo[*a*]pyrene biodegradation are present, mineralisation may be greatly enhanced.

Effects of NAPLs on benzo[*a*]pyrene mineralisation in liquid culture

In liquid culture, as in soil, increased benzo[*a*]pyrene bioavailability did not necessarily result in increased benzo[*a*]pyrene mineralisation. In our assay, *n*-hexadecane was chosen as a representative NAPL because it was shown previously to enhance benzo[*a*]pyrene bioavailability by acting as a cosolvent in liquid culture, could act as a growth substrate for the consortium and has been shown to stimulate weakly benzo[*a*]pyrene mineralisation (Kanaly *et al.*, 2001). The assay consisted of five microcosms as shown in Table 1. Radiolabelled carbon dioxide was monitored for 18 d, after which the flask contents were subjected to a mass balance analysis. We used the amounts of 7-[^{14}C]benzo[*a*]pyrene remaining attached to the flask surface, vs. the amount of ^{14}C detected in suspension (in various forms), as indicators of bioavailability. The aqueous solubility of benzo[*a*]pyrene is very low (0.038 mg/L) and this factor plays a role in its environmental persistence by limiting its access to degrading organisms. In this assay, benzo[*a*]pyrene mineralisation was stimulated only in the case of the positive control, HBD, and after 18 d 2% of the originally added benzo[*a*]pyrene was detected as remaining attached to the flask surface. The addition of increasing levels of *n*-hexadecane resulted in very little $^{14}CO_2$, 2.1% or less, which barely exceeded the level of impurities present in 7-[^{14}C]benzo[*a*]pyrene. However, increased transfer of benzo[*a*]pyrene from the flask surface into suspension was measured. Although over 50% of the radiolabel was detected in suspension in the case of 0.1% *n*-hexadecane addition, and greater than 70–80% of the radiolabel was detected in suspension in the case of 1.5 and 0.5% *n*-hexadecane addition, benzo[*a*]pyrene mineralisation was not stimulated in any case. These results were similar to the results of the soil bioassay where the presence of a suitable cosubstrate was a necessary requirement in addition to bioavailability. Additionally, previous experiments which employed *n*-hexadecane as a NAPL in benzo[*a*]pyrene biodegradation experiments indicated that it stimulated benzo[*a*]pyrene mineralisation but that the mechanism was unclear based upon the pattern of mineralisation (Kanaly *et al.*, 2001). At the time, it was thought that *n*-hexadecane may have acted as a cosolvent for benzo[*a*]pyrene and increased its bioavailability to bacterial cells that were already induced to transform benzo[*a*]pyrene. In the assays performed herein we used a 50-fold lower inoculum to test our hypothesis. Mineralisation did not occur and these results provide evidence that *n*-hexadecane, indeed, acts as a cosolvent and not a cosubstrate during benzo[*a*]pyrene mineralisation.

Conclusions

In soil, microbial degradation of poorly accessible PAHs such as benzo[*a*]pyrene is thought to be controlled by mass transfer rates rather than by biological factors and it is generally accepted that the biodegradation rates of pollutants in soil mostly rely on the transfer of chemicals rather than on the movement of bacteria (Harms and Bosma, 1997).

In the case of benzo[*a*]pyrene at least, the combined effects of mass transfer and fortuitous metabolism are relevant. In soil and liquid culture, although benzo[*a*]pyrene may have been bioavailable, it was not mineralised unless a suitable cosubstrate was present.

Specifically, greatest stimulation of benzo[*a*]pyrene mineralisation in soil by a combination–NAPL was most likely due to the mineral oil component acting as a cosolvent in combination with the stimulation of fortuitous metabolism that was supported by the hexane fraction component. In liquid culture, ^{14}C derived from benzo[*a*]pyrene was detected in suspension in various forms at levels greater than 50–80% of the originally added amount; however, mineralisation did not occur without the presence of a suitable cosubstrate. The role of NAPLs in facilitating the biodegradation of benzo[*a*]pyrene and HMW PAHs under different conditions includes factors related to the presence of capable microorganisms, bioavailability and suitable cosubstrates.

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