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Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates

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Abstract

A study was conducted to determine the location and distribution of PAH and PAH-degrading bacteria in different aggregate size fractions of an industrially polluted soil. The estimation of PAH-degrading bacteria using an MPN microplate technique indicated that these bacteria are most numerous in the aggregate size fractions corresponding to fine silt (2–20 μm) and clay (<2 μm) compared to larger fractions or unfractionated soil. PAH concentrations were also highest in the aggregate size fraction corresponding to fine silt. Similar results were found in a spiked soil (incubated for 6 months) with similar carbonated minerals. Transmission electron microscopy observations showed that the autochtonous PAH-degrading bacteria were embedded in the aggregates where PAHs were abundant. In spite of this extensive co-localisation PAH degradation was limited during 6 months incubation. This indicates that factors other than spatial distribution and PAH degrading ability control degradation rates. The fine silt fraction of the industrial soil had an elevated C/N ratio (35) compared to the clay fraction (C/N: 16). Thus the fraction which assumably had the highest specific surface area contained less PAH but similar numbers of PAH-degraders. N thus seem to play an important role in the long term, but as PAH degradation was low in fine size fractions, other sources/factors were probably limiting (easily degradable C, P org, O_2 etc.). Based on these findings, soil particle organization and structure of soil aggregates appear to be important for the characterization of a polluted soil (localization and sequestration). Manipulations that modify aggregation in polluted soils could thus potentially influence the accessibility and biodegradability of PAHs.

Introduction

Biodegradation and natural attenuation of organic pollutants in terrestrial environments are governed by several physical/chemical factors. These factors appear to control microbial energetic and nutritional requirements as well as the availability of pollutants to the microbial populations (Lion 1990; Ball & Roberts 1991, Pignatello & Xing 1996). Indigenous microflora and introduced microorganisms have both been used for bioremediation purposes (Sherriland & Sayler 1980; Madsen 1991; Cerniglia 1992; Vogel 1996). Several studies have demonstrated the ability of various micro-organisms to degrade polycyclic aromatic hydrocarbons (PAHs) (Kiyohara et al. 1983; Keuth & Rehm 1991; Walter et al. 1991; Tiehm & Fritzsche 1995), but adding laboratory grown bacteria with appropriate degradative abilities to polluted field soils are rarely successful. Such introduced bacteria may fail to degrade pollutants due to preferential use of other organic compounds, or because the pollutants are not physically accessible (Devare & Alexander 1995). Even the addition of oxygen, water and nutrients to stimulate microbial activity for *in situ* biotreatment is often inefficient (Manilal & Alexander 1991; Morgan & Watkinson 1992). The distribution of organic matter, humic substances, xenobiotics and microorganisms is affected by soil structure (Jocteur-Monrozier et al. 1991; Christensen 1992; Kukkonen & Landrum 1996). The location of PAHs is likely to have important implications for understanding and predicting their fate. Furthermore, investigations have concluded that both the release of organic pollutants (Connaughton et al. 1993) and their microbial degradation (Harms & Zehnder 1995; Hatzinger & Alexander 1995) may be inversely proportional to the duration of contact between the compounds and the surrounding soil matrix. This suggests strong interactions between these compounds and the soil constituents. The present study examined the distribution and location of PAHs and PAH-degrading bacteria in a spiked and an industrially contaminated soils to determine the influence of soil structure and aggregate size distribution on the accessibility and bioavailability of PAHs.

Table 1. Characteristics of the industrial soil (IND) and spiked soil (SPI)

	Soil	
	IND	SPI
Granulometry		
Sand	80.7	29.5
Silt	11.5	34.8
Clay	7.2	27.4
Total C	13.0	10.1
Total N	0.34	0.90
$CaCO_3$	26.3	30.0
PH (H_2O)	7.9	7.9

Materials and methods

Soils

Two soils with similar chemical characteristics (carbonated soils) (Table 1) but differing in particle size distribution were selected: the industrially polluted soil (IND) was from an abandoned gas and coke Site in France and the spiked soil (SPI) was from an uncontaminated forest (Bellefontaine, France). The surface horizons (0–20 cm depth) were collected, air dried and sieved to 2 mm. Particle dispersion was done using a saturated sodium Amberlite resin (IR-120; 500 μm) in a soil suspension agitated end-over-end (16 h, 40 rpm). Sand, silt and clay particles were separated by sieving and sedimentation using the Robinson pipette method (Rouiller et al. 1972). PAH content (8 PAHs) in the IND soil was 1.3 g PAH kg^{-1}. The spiked soil received 1 g Kg^{-1} of the same eight PAHs by dissolving 2 g of a PAH mixture in hexane, mixing it with 100 g soil, evaporating off the hexane and mixing it with 1.9 kg of uncontaminated soil. The respective initial contents of 8 PAHs in soil were (mg Kg^{-1}) phenanthrene (200); anthracene (200); fluoranthene (200); benz(a)anthracene (50); chrysene (200); benzo(k)fluoranthene (50); Dibenz(a,h)anthracene (50); and benzo(g,h,)perylene (50). All compounds could be extracted quantitatively shortly (1 day) after their addition to the soil.

Experimental design

Biodegradation assays were performed with the industrial soil incubated under two different conditions (soil in suspension and soil in microcosms) and followed by measurements of PAH-degrading bacteria and PAH concentrations. Incubation times varied from 1 to 4 months for both incubations. For batch incubation, soil samples (25 g) were incubated with 200 ml of water in continuously shaken (200 rpm) flasks under aerobic conditions at 28 °C in the dark. Soil in microcosms was incubated at water saturation, outdoor in the dark (temperature range 5–25 °C during the incubation). Water loss was determined by periodic weighing and compensated by water addition. Incubation in microcosms was also performed with spiked soil.

Bacterial enumeration

Total heterotrophic bacteria and PAH-degrading bacteria were quantified with a most probable-number (MPN) procedure using 96-well microtiter plates (Haines et al. 1996; Wrenn & Venosa 1996). Total microbial populations were estimated using 200 μl per well of a 1 g l^{-1} nutrient broth medium (Difco) inoculated with 25 μl from serial dilutions of total soil or aggregate fractions (40 wells per dilution). The plates were incubated for up to 3 days at 28 °C and growth monitored regularly as change in OD at 620 nm using a MicroStation plate reader (CERES UV 900C, OSI). Data were converted to binary values for estimation of MPN by a computer program using standard Mac Grady tables (Hugues & Plantat 1983).

PAH-degraders were enumerated after inoculating the microtiter plates as above, using a mineral medium containing PAHs as a carbon source. The microplates for the two categories of PAH-degraders were prepared by adding 10 μl hexane containing only phenanthrene (500 μg ml^{-1}) or a mixture of the three PAHs: anthracene (50 μg ml^{-1}), phenanthrene (500 μg ml^{-1}) and fluorene (50 μg ml^{-1}) per well. Hexane was evaporated for 10 min before adding 200 μl per well of mineral liquid medium (Bushnell-Haas, Difco) supplemented with 0.85% NaCl and adjusted to pH 7.4. Plates were incubated at 28 °C for 20 days in the dark. PAH biodegradation was assessed by disappearance of PAH crystals, color changes, and increases in optical density of the medium. Positive wells turned orange or yellow indicating formation of degradation intermediates and accumulation of metabolic compounds corresponding to 1-hydroxy-2-naphthoic acid or 9-hydroxy-9-fluorenone (Boldrin et al. 1993) and confirmed the biodegradation of phenanthrene or fluorene respectively. Growth was monitored by measuring colored product formation and increase in bacterial biomass as the difference in OD between 405 and 620 nm.

Soil fractioning

The distribution of PAHs and PAH-degrading bacteria within different aggregate size fractions of both soils were investigated after physical separation. Serial wet sieving and sedimentation in water was used to obtain aggregates corresponding to four classes: sand, coarse and fine silt, and clay (SA: 2000-50 μm, CS 50-20 μm, FS: 20-2 μm and CL: 2-0 μm fractions, respectively). Bacterial quantification and PAH analyses were then performed on these aggregate size classes. The industrial and spiked soils were sampled (two replicates per treatment) for fractioning and analyses.

Ultrastructural examination of the silt aggregate size fraction was performed by transmission electron microscopy (TEM). Ultrathin sections (80 nm) were prepared from agar cubes (1 mm^3) containing this aggregate size class from both soils. These had been dehydrated by progressive acetone-water exchange at 4 °C, embedded in an Epon 812 resin, and stained with lead citrate and uranyl acetate solutions (Villemin & Toutain 1987). The narrower part of the moulded, impregnated aggregates was pyramidally shaped with a Reichert model TM6O ultramill and finally cut with a diamond knife (model OM U2, Reichert ultramicrotome). Ultrathin sections of each sample were covered with ultrathin carbon layers, and examined in a Zeiss EM9 S2 transmission electron microscope.

PAH analysis

Soil samples were dried at 50 °C, and PAHs extracted from 25 g soil using soxhlet extraction (4 h) with chloroform. Extracted PAHs were analyzed by GC-MS analysis (3400 CX Varian, ION TRAP Saturn III) (for details see Binet et al. 2000).

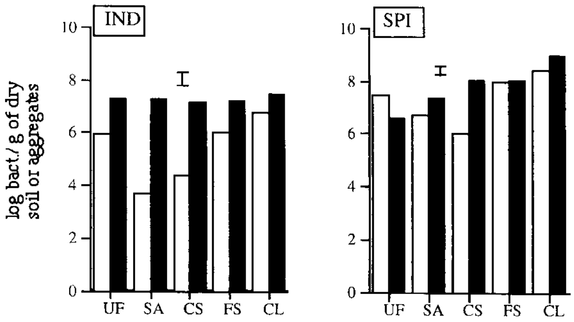

Figure 1. Density of autochtonous bacteria (total (■) and PAH-degraders (□)) in unfractionated soil (UF) and aggregate Size fractions (sand: SA, coarse silt: CS, fine silt: FS, and clay: CL) of industrial soil (IND) and spiked soil (SPI) after 6 months incubation. Vertical bars represent limit standard deviation values at $P < 0.05$.

Results

Bacterial distribution

The microbial communities able to degrade PAH in the industrial and spiked soils were not evenly distributed among the soil aggregate size classes although distribution of total heterotrophs was fairly homogenous for all fractions (Figure 1). The number of bacteria able to grow on the PAH mixture as sole C and energy source was higher in the fine silt and clay aggregate size fractions than in the other fractions of the industrial soil (IND). PAH-degraders represented 6.5 and 19.7% of the total microflora in these two fractions, respectively. In sand and coarse silt, the PAH-degraders were less abundant, representing 4.7×10^3 or 2.5×10^4 bact. g^{-1} dry soil, respectively, corresponding to 0.02 and 0.13% of the total microbial counts.

In the spiked soil, distribution and location of bacteria, in particular the PAH-degrading bacteria, were strongly affected by the presence of PAH. A ten-fold increase in bacterial density from the large (sand size) to the fine (silt and clay size) aggregates was found (Figure 1). Six months after spiking, total and PAH-degrading bacterial counts reached similar numbers in the fine aggregate fractions. The percentage of PAH-degrading bacteria was lower in the larger aggregates (ca. 25 and 1% of total microflora in sand and coarse silt, respectively), but was still higher than in the industrial soil. The number of bacteria measured in the

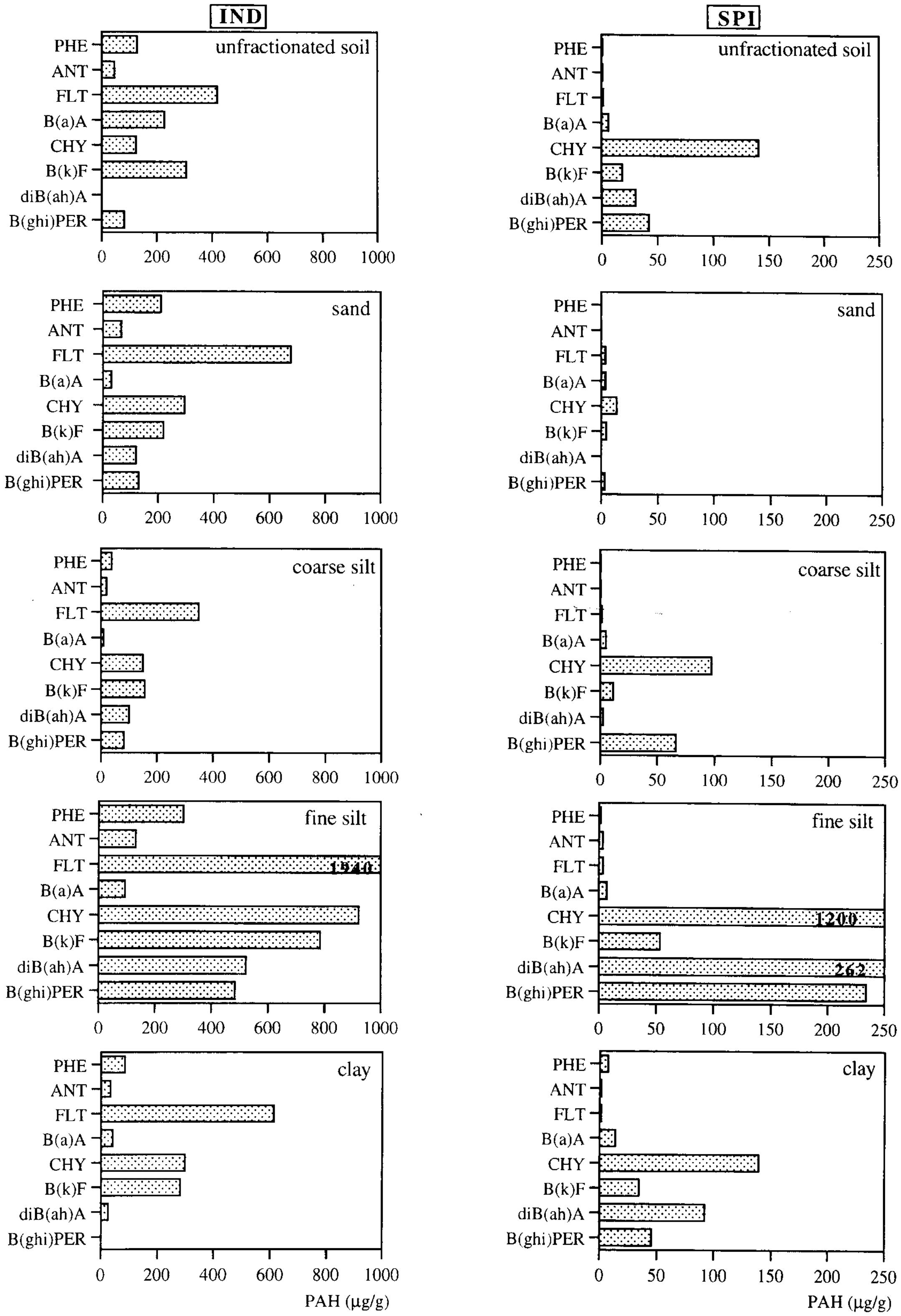

Figure 2. PAH concentration in aggregate size fractions obtained from the industrial soil (IND) and the spiked soil (SPI) after 6 months incubation.

Table 2. Number of culturable heterotrophs, and bacteria able to grow on only phenanthrene or a mixture of 3 PAHs as sole C source in unfractionated soil prior to or 6 months after spiking 8 PAHs

	C source in growth medium		
	Complex medium	3 PAHs	Phenanthrene
Treatment	MPN (log bact. g^{-1} soil)	MPN (log bact. g^{-1} soil)	MPN (log bact. g^{-1} soil)
Uncontaminated soil	6.6 ± 0.24^{a}	2.3 ± 0.3^{b}	1 ± 0.4^{c}
Spiked soil	6.9 ± 0.18^{a}	7.5 ± 0.28^{a}	7.1 ± 0.33^{a}

Each value correspond to the mean of three replicates with standard deviation. Values followed by the same letter are not significantly different ($P < 0.05$, $n = 3$).

Table 3. Characteristics of aggregate fractions from industrial soil

Aggregate size fractions	Fraction weight (%)	C/N
Sand (2000-50 μm)	81.4	70
Coarse silt (50-20 μm)	8.0	48
Fine silt (20-2 μm)	7.6	35
Clay (<2 μm)	3.0	16

unfractionated soil was lower than those in the sum of soil fractions. The recovery of bacteria was thus enhanced by the aggregate separation procedure, which seemingly acted as a pre-conditioning treatment.

The distribution of total heterotrophs among aggregate fractions was not significantly affected by spiking with PAH, and the highest bacterial density (9×10^{8} bact. g^{-1}) was already observed in the clay fraction before spiking (results not shown). Spiking did however increase the number of PAH-degraders (Table 2). The initial values for PAH-degrading bacteria was approx. 10^{2} bact. g^{-1} (<0.01% of total microflora), but reached 3×10^{7} bact. g^{-1} after six months exposure to PAH. Initial number of phenanthrene degrading bacteria in spiked soil (data not shown) was lower than those able to grow in the PAH mixture, but their numbers were similar after 6 months incubation. Unlike the spiked soil, the industrial soil initially had high numbers of competent bacteria, but a low density of phenanthrene degraders (Figure 5). This specific population, phenanthrene degraders, reached the same level as the PAH-degraders after one month incubation of IND soil in slurry and microcosms. No difference in bacterial numbers was observed between soil in suspensions and soil in microcosms after 4 months incubation. The ratio of PAH-degraders to total heterotrophs differed between the two soils: In the spiked soil, the bacterial numbers of PAH degraders and total microflora were not statistically different (Table 2), whereas in the industrial soil the PAH-degraders comprised less than 10% of the total microflora (Figure 5).

PAHs in size fractions

PAH concentrations were highest in the fine silt aggregate fraction (20- 2 μm) and lowest in the largest aggregate size fractions for both soils (Figure 2). Comparing the concentrations of individual PAH in unfractionated spiked soil with the sum of those in all aggregate size fractions showed that higher molecular weight PAHs comprised a lower proportion in the former.

TEM observations of 20-2 μm aggregate size fraction showed that bacteria were frequently trapped inside aggregates, or embedded in soil organic materials or pollutants (Figure 3). This demonstrates the proximity of organic compounds and bacteria within aggregates, and suggests that bacteria present in the fine silt aggregate would break down the PAH if they were bioavailable. In spiked soil, the 6 months incubation had increased the amount of aggregates in the silt size fraction from 3.6% (initial value) to 11.6% of total soil weight.

PAHs in incubated industrial soil

Incubations of the industrial soil either as stirred batch suspensions or as microcosms with intact soil led to different PAH dissipation patterns compared to spiked soil. Total PAH concentration remained unchanged after four months irrespective of incubation regime (Figure 4). The concentration of the 3-ring PAHs (PHE, ANT, FLT) in soil suspensions had however decreased significantly after 4 months incubation.

The fine aggregate fractions (fine silt and clay) obtained by wet-sieving represent a low proportion in IND only 7.6 and 3% of total soil weight, respectively

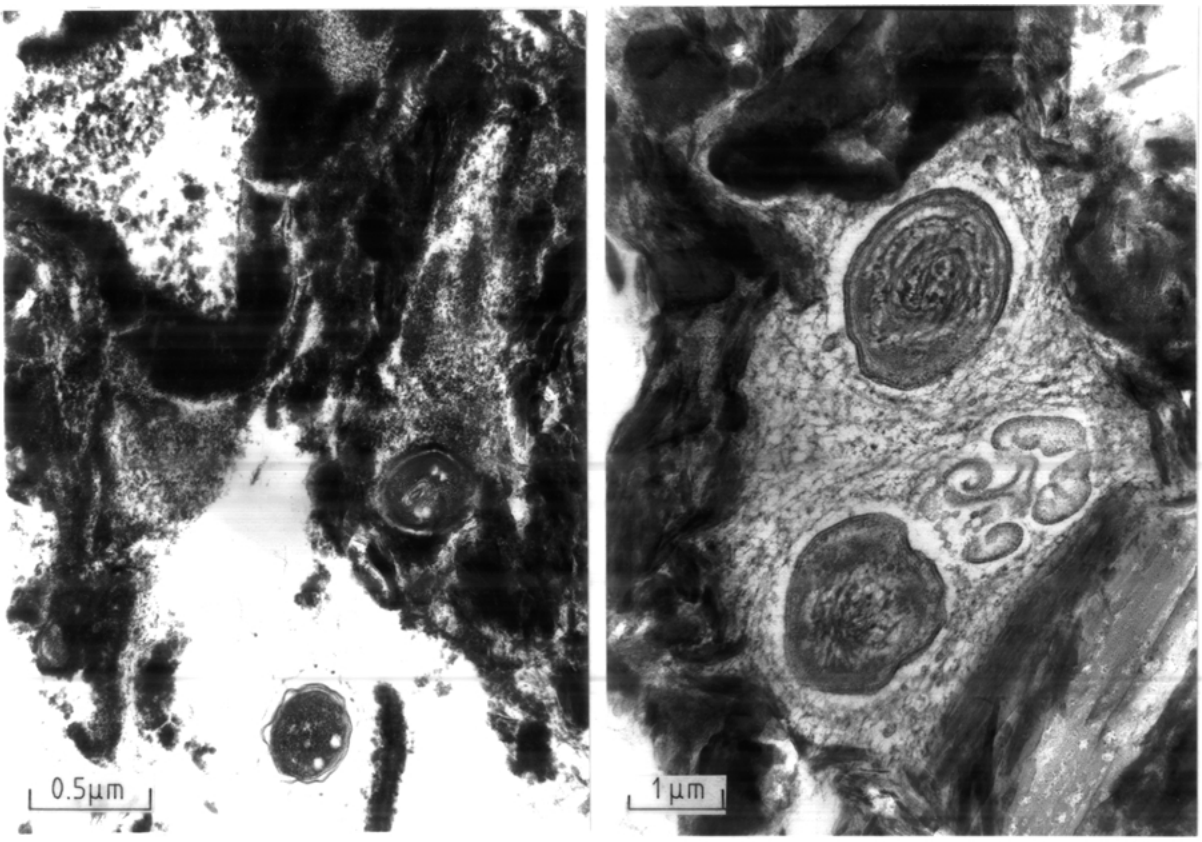

Figure 3. Ultra-thin section of the fine silt aggregate size fraction (20-2 μm) from the industrial soil (a), and the spiked soil (b).

(Table 3). But the fine silt fraction had an elevated C/N ratio (35) compared to the clay fraction (C/N: 16).

Discussion

The major findings of the present report are that PAHs are unevenly distributed between different aggregate size classes and that within a size class there is a close correlation between PAH concentration and the number of PAH degrading bacteria. Surprisingly, the highest values for PAHs and PAH degrading bacteria were found in an intermediate aggregate size class, rather than in one of the extremes. This may be explained by the relatively high proportion of primary particles within the two largest aggregate size classes (sand and coarse silt) which render their internal surface area comparatively low relative to the smaller aggregates, coupled with an increase in C/N ratio with increasing aggregate size. While the former would lead to low sequestration of PAHs within aggregates (spatially protected) for the two largest size classes, the latter would render the smallest aggregates a more favorable environment for microbial metabolism. Our findings are thus consistent with those of Stella et al. (1990) who showed that the influence of aggregate size on oil waste degradation is a question of available surface area that renders the pollutant exposed and available for degradation. While this concerns PAH located on the outside of aggregates, PAH within the aggregates are physically protected and poorly accessible to microorganisms and their enzymes (Steinberg et al. 1987; Rijnaarts et al. 1990). Soil organic matter, inorganic soil components, and mineral surfaces were probably implicated in PAH sequestration (Means et al. 1980; Chiou & Shoup 1985; Knaebel et al. 1996).

Agitated batch incubations may potentially break up soil aggregates and render physically protected PAH available to microbes in a soil slurry. When we compared such incubation (which also featured higher temperature and O_2 availability) to outdoor incubation of microcosms, effects on PAH dissipation was only observed for 3-ring PAHs and benz(a)anthracene.

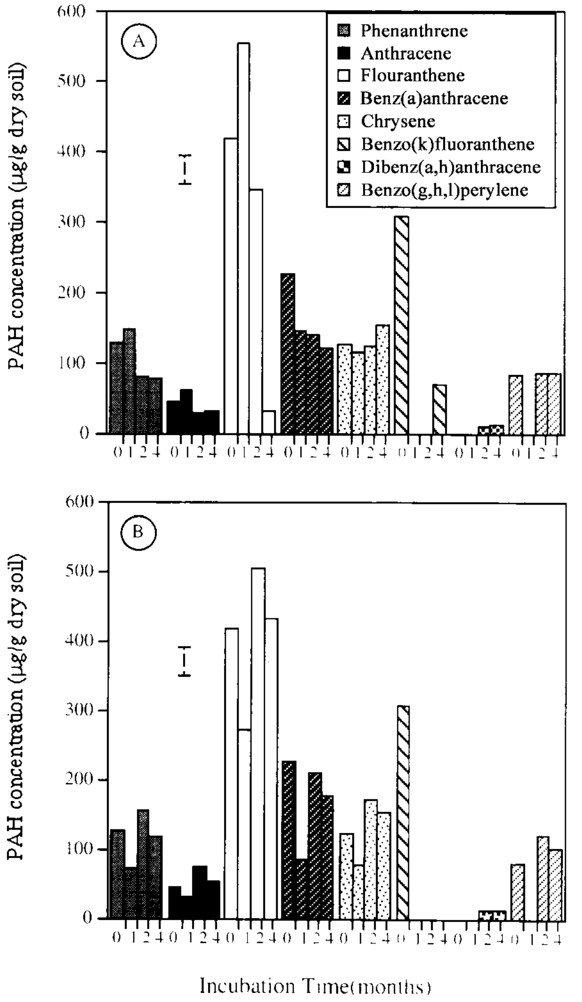

Figure 4. Evolution of the extractable PAH concentrations in industrial soil during two types of incubation.

Other 4-ring and 5–6-ring PAH were unaffected or their extractability increased. The degradation of high molecular weight compounds (four or more rings) by indigenous microorganisms is usually much slower than the degradation of low molecular weight PAHs (Shiaris 1989; Wild & Jones 1993). Sequential removal of PAHs in batch incubations where recalcitrant PAH were removed only after degradation of more labile PAH has previously been suggested (Mueller et al. 1989). Thus, the more soluble compound was preferentially entered the soil suspension compared to the less soluble compounds. Similar desorption effects of condensed PAHs were seen as a result of aggregate separation, as unfractionated soil contained less condensed PAH compared to the sum of its fractions. The aggregate fractionation renders a large area accessible to the extractability as previously observed for particle size separation (Guggenberger et al. 1996). The small differences in PAH dissipation observed between the two incubation regimes indicate that physical protection of PAH within aggregates was of minor importance for PAH availability.

Biodegradation of organic pollutants and bioremediation processes depend on the presence and activity of appropriate microorganisms (Thomas et al. 1986; Stucki & Alexander 1987), but also on environmental conditions (e.g., temperature and O_2) and the availability of the substrate. Microorganisms capable of hydrocarbon degradation is commonly present even in uncontaminated soil (Atlas & Bartha 1992). This was confirmed for PAH degraders in the spiked soil. In the IND and SPI soils, PAH degraders were, at least partially, localized in a PAH containing matrix, as revealed by TEM. Thus, the small differences in PAH dissipation in IND soil under two highly different incubation regimes, and the higher PAH degradation rates in SPI soil indicate that PAH availability was the factor limiting dissipation/degradation in the IND soil. A similar conclusion was drawn by Morgan et al. (1992) who demonstrated far higher degradation of phenanthrene added to an industrially polluted soil than of the PAHs originally present in that soil.

In conclusion, the present study demonstrated that the biodegradation of a complex mixture of PAHs by soil microbial communities is influenced by the nature of PAH and their distribution in different aggregate size fractions. Bioremediation of PAH contaminated soil has rarely been completely effective (Wilson & Jones 1993) and our results show that the persistence of PAH's is partially due to their preferential accumulation in small aggregates. Biodegradation of PAH mixtures may be further complicated by substrate interactions causing enhanced biodegradation of individual compounds and a simultaneous inhibition of other compounds (Alvarez & Vogel 1991; Kelley & Cerniglia 1995). Their biodegradation in soil is not only dependent on microbial biomass and activity, but also on their immobilization within soil aggregates. Accessibility could be improved by mechanical treatments that can increase disintegration of aggregates and particles. Further work is needed to understand the preferential accumulation of bacteria and PAHs in certain soil aggregate size classes

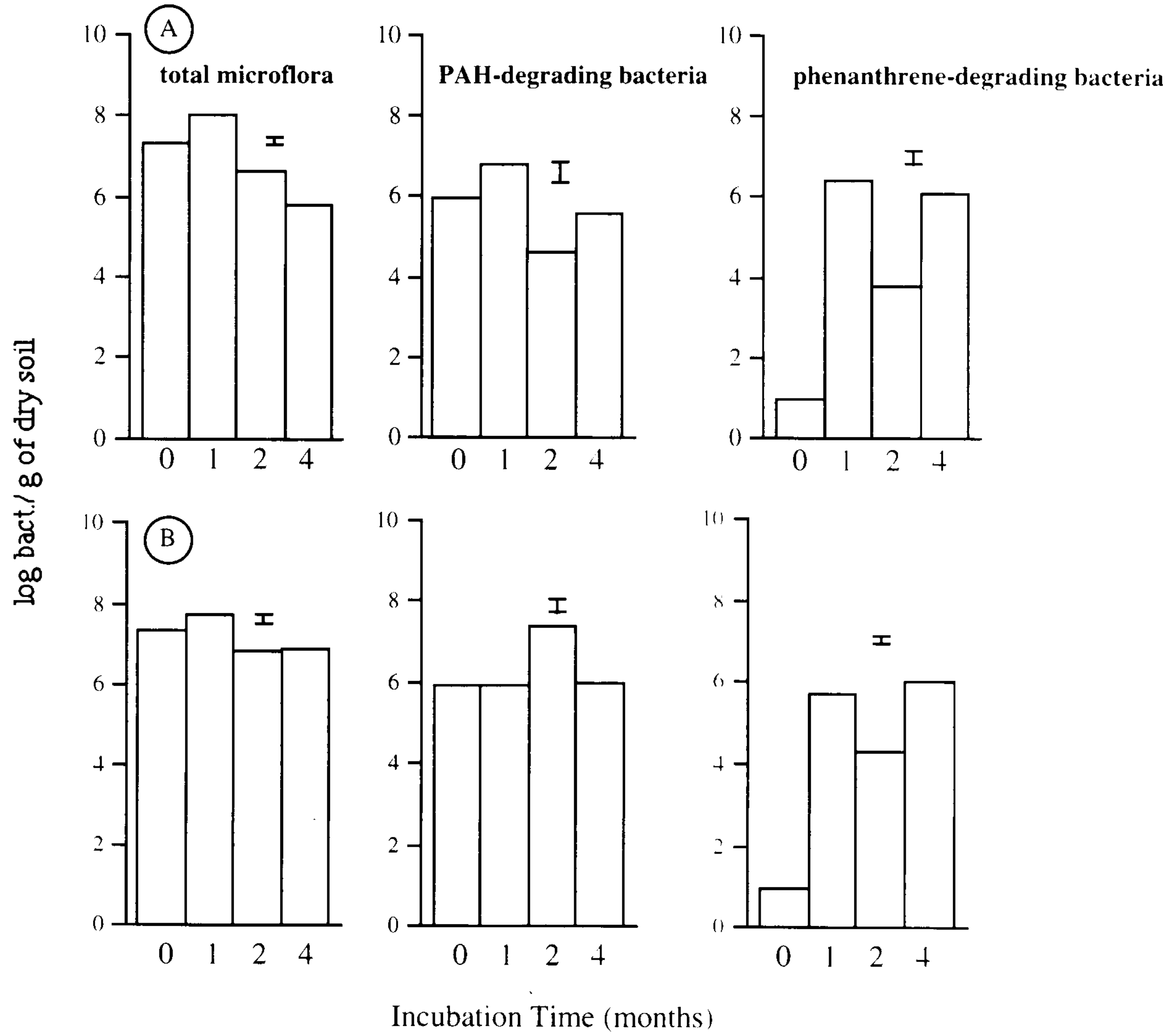

Figure 5. Effect of time and two types of incubation (a) slurry and (b) microcosms on the bacterial numbers (total microflora, PAH and phenanthrene-degraders) in industrial soil. Vertical bars represent limit standard deviation values at $P < 0.05$.

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