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# Effects of carbon substrate enrichment and DOC concentration on biodegradation of PAHs in soil

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## ABSTRACT

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**Aims:** Two common reasons to explain slow environmental biodegradation of polycyclic aromatic hydrocarbons (PAHs), namely lack of appropriate carbon sources for microbial growth and limited bioavailability of PAHs, were tested in a laboratory bioassay using a creosote-contaminated soil.

**Methods and Results:** The soil, containing a total of 8 mg g<sup>-1</sup> of 16 PAHs, was sieved and incubated in bottles for 45 days. The first explanation was tested by enrichment with the analogue anthracene and the non-analogue myristic acid, and both failed to stimulate degradation of all PAHs except anthracene. The second explanation was tested by addition of different concentrations of dissolved organic carbon (DOC), with effects depending on the DOC concentration and the molecular size of the PAH. The degradation was enhanced from 10 to 35% for 12 PAHs when the soil was saturated. The degraded amounts of individual PAHs were proportional to their concentration in the soil.

**Conclusions:** The slow *in situ* degradation of PAHs was enhanced by more than three times by adding water as a solvent. Addition of DOC facilitated the degradation of four- to six-ring PAHs.

**Significance and Impact of Study:** Bioremediation of PAH-contaminated sites may be facilitated by creating water-saturated conditions but retarded by addition of other carbon substrates, such as analogue compounds.

**Keywords:** Analogue substrate enrichment, bioavailability, bioremediation, sorption, surfactant.

## INTRODUCTION

Creosote, used to impregnate wood, consists of up to 85% of polycyclic aromatic hydrocarbons (PAHs) (Cerniglia 1992), and both wood preservation and gas work sites are often contaminated with PAHs (Wilson and Jones 1993). Owing to the detrimental effects of the compounds, whether toxic (Darville and Wilhm 1984), mutagenic or carcinogenic (White 1986), the United States Environmental Protection Agency (US EPA) listed 16 PAHs as priority pollutants (Keith and Telliard 1979). Microbiological degradation is the chief process for natural elimination of PAHs from soils and sediments (Cerniglia 1984), but *in situ* degradation is a slow process (Durant *et al.* 1995). Nonetheless, it is often

possible to demonstrate high degradation rates in laboratory experiments by strains or consortia isolated from PAH contaminated soils (Kästner *et al.* 1994; Carmichael and Pfaender 1997). Such differences in activity between laboratory and field studies are related to constraints imposed in the field by limiting abiotic and biotic factors, such as temperature, pH, redox conditions, concentration of inorganic nutrients and metals, predation and parasitism (Thomas *et al.* 1989; Wilson and Jones 1993).

The aim of this study was to shed light on two other potential reasons for the slow degradation of PAHs in soil: (i) lack of substrates for microbial growth and (ii) low bioavailability of PAHs. The first of the two reasons implies that a complementary substrate is needed to promote degradation of PAHs in the soil. Other studies have shown that addition of certain carbon substrates, like yeast extract, peptone, glucose, and oil refinery and creosote wood

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preserving wastes (Keck *et al.* 1989; Keuth and Rehm 1991), can stimulate PAH degradation. A substrate that is structurally analogous to the pollutants may stimulate the growth of the degrading population(s) specifically and induce the expression of broad substrate degradative enzyme systems (Ogunseitan *et al.* 1991). A successful application of this approach enhanced biodegradation of polychlorinated biphenyls (PCBs) in soil treated with biphenyl (Brunner *et al.* 1985).

This study tested enrichment in a PAH-polluted soil using two substrates with the same number of carbon atoms, a non-analogue fatty acid, myristic acid, and anthracene (ANT). ANT is more readily degraded in soil under laboratory conditions than its closest congener phenanthrene (PHE). Sims *et al.* (1988) used laboratory incubations to calculate half-lives of 16 days for 200 mg kg<sup>-1</sup> of ANT and 134 days for 900 mg kg<sup>-1</sup> of PHE applied to sandy loam soils. ANT is cometabolized by some PAH-degrading strains isolated from gas manufacturing plant soils but not by others (Bouchez *et al.* 1995).

Based on these earlier observations, it was not unreasonable to expect degradation of PAHs to be faster in a polluted soil enriched with ANT than in the same non-enriched soil. It was also expected that the addition of myristic acid would stimulate growth and metabolism of all microorganisms, including the PAH degraders. As a result of their increased metabolic activity, the degradation of PAHs in the enriched soil would be faster than, or at least equal to, the degradation in the same non-enriched soil.

The other issue addressed in the study was the low bioavailability of PAHs in soil, which is considered to be a main challenge to the bioremediation of contaminated industrial sites. PAHs are sparingly water soluble, and sorption (Onken and Traina 1997) and other associations with the soil (Soheila *et al.* 1996) make them unavailable for bacterial degradation. The fraction that is unavailable for biodegradation and even for extraction by organic solvents (Eschenbach *et al.* 1994) becomes larger as the contact time between the PAHs and the soil increases. As a result of this aging, desorption becomes very slow and may limit the rate of degradation (Hatzinger and Alexander 1995; Carmichael and Pfaender 1997; Madsen and Kristensen 1997).

If desorption is the rate-limiting step in biodegradation, addition of excess of water to a soil as a solvent should enhance biodegradation by facilitating the desorption rate of the non-aged fraction of PAHs. Water addition may also increase degradation rates by stimulating microbial metabolism and proliferation. Tests were developed to study the effect of soil moisture by comparing the degradation of PAHs in the soil at 50% of water holding capacity (WHC, moist soil) and at water saturation.

The presence of surfactants facilitates desorption of PAHs and may also enhance their degradation (Aronstein and Alexander 1992; Madsen and Kristensen 1997; Tiehm *et al.* 1997; Marcoux *et al.* 2000). However, some studies have shown synthetic surfactants to be biodegraded (Loser *et al.* 2000). An alternative to persistent synthetic compounds would be natural surfactants, e.g. biosurfactants from bacteria (Cerniglia 1984; Willumsen and Karlson 1997) and dissolved organic carbon (DOC). The latter has not received as much attention as surfactants, but the addition of humic acids has been found to increase the degradation of PHE (Thomas *et al.* 1989). Instead, the focus has been on characteristics of DOC that increase the apparent solubility of organic pollutants, including PAHs, in water (Chiou *et al.* 1986) and soil (Raber *et al.* 1998) systems and subsequently facilitate long-range transport of the pollutants in, e.g. aquifers (Enfield *et al.* 1989). The effect of natural DOC on the degradation of PAHs by varying the concentration of DOC added to the polluted soil was tested. It was expected that high DOC concentrations would promote the degradation of PAHs by increasing their desorption rates and hence the bioavailability before sorption mechanisms may come to dominate.

While it is the practice to include all the 16 PAHs on US EPA's list in chemical analysis of field samples of soil, sediment, and water, only one or a few of them are usually examined in degradation bioassays. This study is unusual in that all 16 PAHs were assayed, as a primary aim of bioremediation must be to reduce the total load of PAHs in a soil.

## MATERIALS AND METHODS

### Soil

A sandy, silty soil from a former wood preservation area in Hässleholm, southern Sweden, was used. At this site, railroad sleepers were impregnated with creosote for many years. Samples from 1.0-m depth of the preservation area were brought to the laboratory and kept for less than a month at 4°C in tightly sealed plastic containers until used in microcosms and for isolation and enumeration of PAH degraders. Replicate soil samples were analysed for PAHs by Hedeselskabet, Viborg, Denmark, using US EPA's method 3540C. The total concentration of the 16 priority PAHs was 8 mg g<sup>-1</sup> soil. The organic content of the soil (TOC) was 0.67%, pH 7.2, and the water content 14%. The WHC was estimated to be 30% of the saturated weight.

### Microcosms

The soil was sieved through a 2-mm mesh, thoroughly mixed, and air dried at room temperature for 65 h.

Autoclaved 100-ml serum bottles received 15 g each of the dried soil. Abiotic losses, i.e. because of evaporation, were measured in sterile microcosms, in which the soil was autoclaved twice at 121°C with 3 days in between before any further treatment. All microcosms were incubated in the dark for 45 days at 23°C on a rotating table (70 rev min<sup>-1</sup>) with free gas exchange through cotton plugs.

## Experiments

**Analogue enrichment.** Anthracene (ANT, 14 carbon atoms, EGA, Germany) was added as an analogue carbon source at a concentration of 500 µg g<sup>-1</sup> soil. In a parallel treatment, the same amount of carbon in the form of the non-analogue, saturated fatty acid myristic acid (Fluka, Switzerland) was added to the soil. Both compounds were dissolved in acetone (p.a., Labasco, Sweden), and 2-ml solutions were mixed well into the soil. In a separate treatment, acetone alone was added to the soil. The acetone was allowed to evaporate before any water was added to the microcosms.

Two different water regimes (moist and saturated) were used. In the moist regime, 3-ml sterile groundwater (GW) per microcosm was added at the beginning of the experiment, and thereafter approximately 1 ml every 6 days to maintain the water content. The saturated microcosms were provided with 50 ml of sterile GW at the start of the incubation; no corrections for water losses were made during the incubation. The average water volume was 35 ml at the end of the incubation period. The GW was collected from a well at Vomb water works, southern Sweden, and first filtered through GF/A glass microfibre filter (Whatman, UK) and then filter sterilized through 0.22-µm GV filters (Millipore, Ireland) under helium pressure. GW was used to wet and saturate the soil, as it was a natural soil solution with low carbon and nutrient content and much more easily collected than the soil solution from the unsaturated soil samples.

**DOC treatment.** Four solutions with different DOC concentrations were prepared to test the effect of DOC on PAH degradation. Fifty millilitres of each solution were added to the soils. The treatments were designated DOC1–4. Fifty millilitres of filter-sterilized GW were added to the soils in a parallel treatment. There were sterile controls for DOC3, DOC4, and GW, with autoclaved soil and 0.2% w/v sodium azide (AnaR, BDH, UK) added to the aqueous solution. Treatment DOC4 was started and terminated 15 days after the other treatments.

DOC1 was prepared as a 1 : 1 mixture of DOC2 and GW. DOC2 was prepared as a 1 : 1 mixture of DOC3 and GW. DOC3 was prepared by mixing 1700 ml deionized water (Millipore MilliQ 185) with 150 g of commercial planting

soil (Hasselfors Gardens) for 100 h on a rotary shaker (KS 500, Janke & Kunkel, Germany). The soil was a mixture of 60 vol.% low humified peat, 30 vol.% highly humified peat, 50 vol.% composted bark and 5 vol.% sand, with pH 5.5–6.5. The slurry was centrifuged in 250-ml aliquots in screw-capped plastic tubes for 30 min at 6000 rpm (Sorvall RC5B Plus, Du Pont, CT, USA), and the supernatant first filtered with suction through a GF/A filter and then through a 0.45-µm filter (Gelman Sciences, MI, USA) to remove particulate carbon. The filtered solution was divided into four open glass beakers and UV sterilized on a shaker in a sterile bench for 24 h. DOC4 was prepared as DOC3, but after the sterilization step it was frozen to –80°C and freeze dried (Lyovac GT2, Leybold-Heraeus, Germany). The volume was reduced from around 8 to 1.1 l for 12 days. The residue was dissolved by ultrasonic treatment (Transsonic 570, Elma, Germany) for 10 min.

The sterility of the DOC3 and DOC4 solutions was examined by plating aliquots of 0.5 ml of each on PYG medium plates (see section 'Isolation and enumeration of degraders and other microorganisms') and incubating the plates at room temperature for a week. No growth was recorded. The DOC concentrations of DOC1–DOC4 and GW were 20, 34, 52, 255 and 10 mg l<sup>-1</sup>, respectively (Shimadzu TOC 5000).

Altogether, 11 treatments and one abiotic control were used in the assays (Table 1).

## PAH analysis

A detailed description of the analysis of PAHs in soil and water is reported elsewhere (Zerhouni and Bengtsson 2001). Briefly, the soil samples were extracted with a combination of dichloromethane and alkaline methanol, and the extract purified on aluminium oxide. Water samples were extracted with hexane. The three largest PAHs in the soil extracts (indeno[1,2,3-*c,d*]pyrene, dibenzo[*a,h*]anthracene and benzo[*g,h,i*]perylene) were separated and quantified by gas

**Table 1** Summary of the design of the microcosm assays

Experiment	Treatment	Water regime	Sterile control
Control	Dry	Dry	x
Analogue-enrichment experiment	Analogue	Moist/saturated	x
	Non-analogue	Moist/saturated	x
	Solvent	Moist/saturated	x
DOC-enrichment experiment	GW	Saturated	x
	DOC1	Saturated	
	DOC2	Saturated	
	DOC3	Saturated	x
	DOC4	Saturated	x

chromatography and the other PAHs by a combination of gas chromatography and mass spectrometry.

### Calculations

When the evaporation losses during autoclaving were subtracted from the dry soil controls, it was found that only the three smallest PAHs with the lowest molecular weight had evaporated during the incubation period in the moist treatment (Zerhouni and Bengtsson 2001). However, the saturated sterile controls were not useful in calculating the abiotic loss as they were affected by both evaporation and an extraction efficiency that increased during the incubation, especially for the low molecular size PAHs. The problem was circumvented by making the following assumptions: (i) the soil extraction efficiency of the method was constant over time for dry and moist treatments; (ii) the relative evaporation loss was the same for all treatments; (iii) the soil extraction efficiency of the method was the same for non-autoclaved as for the autoclaved microcosms with the corresponding treatment; and (iv) the aqueous extraction efficiency was the same for all aqueous samples irrespective of the DOC concentration.

The correction factor for evaporation loss of a compound,  $ELF_i = C_{s_i}(T)/C_{s_i}(T_0)$ , was averaged from data for the sterile dry and moist treatments and applied to correct for evaporation in all treatments.  $C_{s_i}$  ( $\mu\text{g g}^{-1}$  of soil) is the concentration of compound  $i$  in the soil at the beginning ( $T_0$ ) and end ( $T$ ) of the incubation. The extraction efficiency correction factors ( $EEF_i$ ) for each saturated treatment were calculated using sterile GW, DOC3 and DOC4 data, respectively. For DOC1 and DOC2, the average  $EEF_i$  of sterile GW and DOC3 was used. The  $EEF_i(\text{GW})$  was also applied to the saturated treatments in the analogue-enrichment experiment. By using the ELF<sub>s</sub>, the EEF<sub>s</sub> could be calculated and the two effects separated:  $EEF_i = C_{s_i}(T)/[ELF_i \times C_{s_i}(T_0) - Ca_{q_i}(T)]$ , where  $Ca_{q_i}(T)$  is the concentration of compound  $i$  in the aqueous phase ( $\mu\text{g g}^{-1}$  of soil). The correction factors were important almost exclusively for the smaller sized PAHs (Table 2). The absolute biological degradation, in  $\mu\text{g g}^{-1}$  soil, of a compound, was calculated by subtracting the total compound concentration at the end of the incubation, corrected for the extraction efficiency, from the initial concentration, corrected for evaporation:  $ELF_i \times C_{s_i}(T_0) - [(C_{s_i}(T)/EEF_i) + Ca_{q_i}(T)]$ . The relative degradation, in percentage of the initial concentration, was also calculated:  $100 \times \{ELF_i \times C_{s_i}(T_0) - [(C_{s_i}(T)/EEF_i) + Ca_{q_i}(T)]\}/C_{s_i}(T_0)$ . The relative extraction efficiency of DOC for the PAHs was determined by averaging the ratios of their concentrations in the aqueous phase and the total concentrations at the end of incubation in the sterile microcosms:  $(1/n) \times \sum [Ca_{q_i}/(Ca_{q_i} + C_{s_i}/EEF_i)]$ , where  $n$  is the number of PAHs considered.

**Table 2** Correction factors used to account for evaporation losses and extraction efficiencies of DOC during the bioassays

Correction factor	Compound				
	NAP	ACY	ACE	FLU	All others
ELF*	0.52	0.81	0.86	1	1
EEF <sub>GW</sub> <sup>†</sup>	1	2.67	2.6	1.24	1
EEF <sub>DOC1-2</sub>	1	2.11	2.27	1.2	1
EEF <sub>DOC3</sub>	1	1.54	1.95	1.17	1
EEF <sub>DOC4</sub>	1	1	0.86	0.76	0.82

\*Evaporation loss factor.

<sup>†</sup>Extraction efficiency factor.

### Statistics

The absolute and relative degradations were statistically analysed with Statview 4.5 (Abacus Concepts, CA, USA). The absolute degradation data were heteroscedastic and the data were log transformed,  $\log(\text{abs deg} + 100)$  (Zar 1984). The relative values were normalized by arcsine transformation,  $\arcsine[(\text{rel deg}/100)^{1/2}]$  (Zar 1984).

The transformed data were analysed with equal replication two-factor ANOVA, treatment and compound being the factors. This is a rather robust test that accepts some deviations from normality and homoscedasticity (Zar 1984). More detailed analyses of differences between compounds and treatments were made by post-hoc testing or multiple comparisons with Fisher's protected least significant difference. The analogue experiments were compared with and without data on ANT degradation. When ANT was included, the degradation was corrected for the added amount. Also, PHE was left out in some comparisons because its degradation dominated quantitatively over that of the other compounds.

### Isolation and enumeration of degraders and other microorganisms

Isolation and enumeration of bacteria in the soil were made at the beginning of the assay. Two grams (wet weight) of sieved (2-mm mesh) soil was mixed with 20 ml of autoclaved sodium hexametaphosphate (0.2% w/v), homogenized in an omnimixer at high speed for 2 min, and then left to settle for 1 h. Dilutions of the original extracts were prepared with sodium hexametaphosphate.

The innate PAH-degradation capacity of the soils was estimated by growing microorganisms on minimal medium with PAH as the sole source of carbon (Kiyohara *et al.* 1982). Four PAHs (PHE, ANT, fluoranthene and pyrene) were separately dissolved in acetone to a concentration of  $4 \text{ mg ml}^{-1}$ . One millilitre of one of these PAH solutions was spread on the surface of an M9 (Sambrook *et al.* 1989)

mineral medium agar plate. The acetone was allowed to evaporate under sterile conditions and left an opaque white layer of PAH on the surface. Aliquots of 0.1 ml of the original extracts and a 1 : 10 dilution were spread on minimal medium plates with or without (controls) PAH coating. The plates were incubated for 35 days at 23°C in plastic bags. At the end of the incubation, there were clear zones around some colonies, called zone-forming units (ZFU), which were considered to represent degraders of the particular PAH they had grown on (Heitkamp *et al.* 1988; Kästner *et al.* 1994). ZFUs were transferred to fresh PAH-coated plates. They grew and continued to clear zones.

The total numbers of culturable cells in the PAH-contaminated soil were obtained from 0.1-ml aliquots of dilutions spread on PYG plates. PYG was of composition ( $l^{-1}$ ): 250 mg peptone (Difco, MI, USA), 500 mg yeast extract (Difco), 500 mg glucose (Baker, Holland), 30 mg  $MgSO_4 \cdot 7H_2O$ , 3 mg  $CaCl_2 \cdot 2H_2O$  and 15 g Bacto-agar. The plates were incubated in a constant room at 23°C and the colonies counted after 2 days.

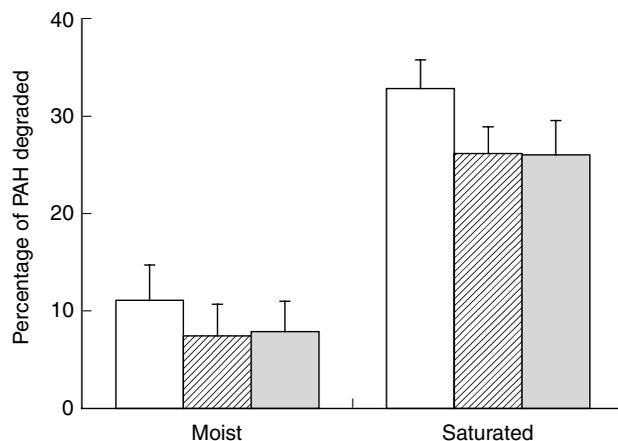
## RESULTS

### Treatment effects

The treatments had significantly different effects on the extent of absolute ( $F = 14.73$ ,  $P < 0.0001$ ) and relative degradation ( $F = 15.72$ ,  $P < 0.0001$ ), regardless of whether ANT was excluded from the comparison or included. There were also significant differences in degradation of individual PAHs, regardless of the effects of the treatments ( $F = 65.31$ ,  $P < 0.0001$ , and  $F = 24.64$ ,  $P < 0.0001$ , respectively, for absolute and relative degradations). The interaction effect of treatments and individual PAHs on the degradation was significant ( $F = 12.41$ ,  $P < 0.0001$  and  $F = 1.58$ ,  $P < 0.0007$ , respectively, for absolute and relative degradations). The interaction effect was interpreted such that the degradation of the different compounds was affected in different ways by different treatments.

### Analogue and non-analogue enrichment and soil moisture experiments

The control microcosms had a higher relative degradation than the analogue- and non-analogue-enriched treatments when ANT was excluded from the analyses (Fig. 1). When the absolute degradation was compared, PHE, which represented more than 50% of the total degradation, was also excluded from the calculations to allow a comparison of the treatments for other PAHs. The difference between the control and the enrichments was significant ( $P < 0.05$ ) only in the saturated water regime (Table 3).



**Fig. 1** Percentage degradation of PAHs after incubation of a moist or water-saturated creosote-contaminated soil in darkness for 45 days at 23°C with addition of an analogue (anthracene, ▨) or a non-analogue (myristic acid, ▩) compound. The solvent of the analogue and non-analogue compounds, acetone, was added to the soil separately (control, □). Anthracene was excluded from the calculation of the degradation. Error bars represent standard error ( $n = 6$ )

When ANT was included in the comparison and adjustments made for the enrichment, the analogue-enriched treatments had the highest absolute degradation (Fig. 2). PHE was also included in this comparison as ANT dominated quantitatively. The absolute degradation under the moist regime was significantly higher ( $P < 0.05$ ) in the analogue-enriched treatment and in the control than in the non-analogue-enriched treatment (Table 3). Under the saturated regime, the absolute degradation differed significantly ( $P < 0.05$ ) between all three treatments, with the highest degradation in the analogue enrichment and the lowest in the non-analogue treatment (Fig. 2, Table 3). The relative degradation of ANT alone was highest in the analogue treatments and lowest in the non-analogue-enrichment treatments (Table 4).

The absolute and relative degradation were significantly higher ( $P < 0.001$ ) in the saturated than in the corresponding moist treatments (Table 3) regardless of the contribution of ANT to the comparison of the relative degradation and of ANT and PHE to the absolute degradation.

### DOC extraction experiment

There were few differences in absolute degradation between the treatments when all compounds were included in the comparison ( $P \geq 0.15$ ). The general trend, however, was an increased degradation with the DOC concentration from GW to DOC2 and then a decrease to a smaller degradation for DOC3 and DOC4 (Fig. 3). When PHE was excluded, the differences increased but were still not significant

**Table 3** Fischer's PLSD test on the analogue-enrichment experiment\*

Compared treatments	Abs. degradation (all compounds), <sup>†</sup> <i>P</i> -value	Rel. degradation (all compounds), <sup>‡</sup> <i>P</i> -value	Abs. degradation (10 compounds), <sup>§</sup> <i>P</i> -value	Rel. degradation (11 compounds), <sup>¶</sup> <i>P</i> -value
<b>Moist</b>				
Analogue : non-analogue	<0.0001	0.51	0.69	0.88
Analogue : control	0.09	0.52	0.19	0.37
Non-analogue : control	<0.0001	0.19	0.37	0.27
<b>Saturated</b>				
Analogue : non-analogue	<0.0001	0.57	0.97	0.99
Analogue : control	0.003	0.10	0.05	0.05
Non-analogue : control	0.05	0.03	0.05	0.05
<b>Moist vs saturated</b>				
Analogue	<0.0001	<0.0001	<0.0001	<0.0001
Non-analogue	<0.0001	<0.0001	<0.0001	<0.0001
Control	<0.0001	<0.0001	<0.0001	<0.0001

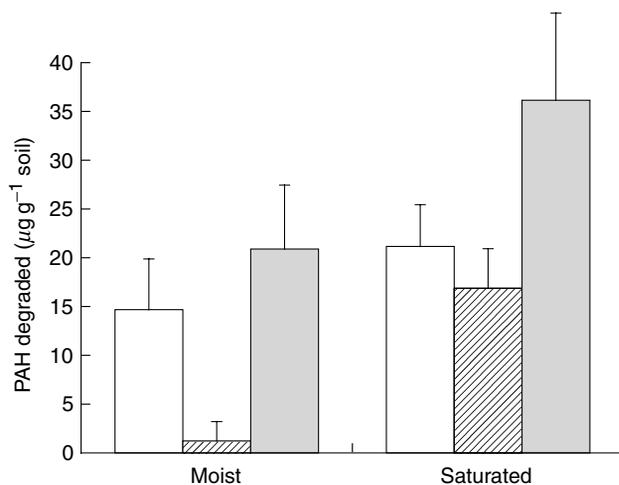
\*The test was performed on all 11 treatments but only the relevant comparisons are shown here, BbF and BkF treated as one compound.

<sup>†</sup> $\log(\text{abs. degradation} + 100)$ , 12 compounds, ANT adjusted for enrichment.

<sup>‡</sup> $\arcsin(\sqrt{\text{rel. degradation}})$ , 12 compounds, ANT adjusted for enrichment.

<sup>§</sup> $\log(\text{abs. degradation} + 100)$ , ANT and PHE excluded.

<sup>¶</sup> $\arcsin(\sqrt{\text{rel. degradation}})$ , ANT excluded.



**Fig. 2** Average absolute degradation of PAHs in the analogue- and non-analogue-enrichment experiments. Anthracene was included in the calculation of degradation after adjusting its initial soil concentration with the enrichment made. Error bars represent standard error ( $n = 6$ ). Legends are as in Fig. 1

( $P \geq 0.06$ ). The relative degradation of all compounds was significantly higher with DOC2 than with GW or DOC3 ( $P = 0.001$ ).

The separation of PAHs into two groups based on molecular weight, with NAP, ACY, ACE, FLU, PHE and ANT in one group designated low molecular weight (LMW) PAH, and FLT, PYR, BaA, CHR, Bb/kF and BaP in another group designated high molecular weight (HMW)

**Table 4** Relative degradation (%) of ANT (average and standard deviation (S.D.),  $n = 6$ ) in the analogue-enrichment experiment

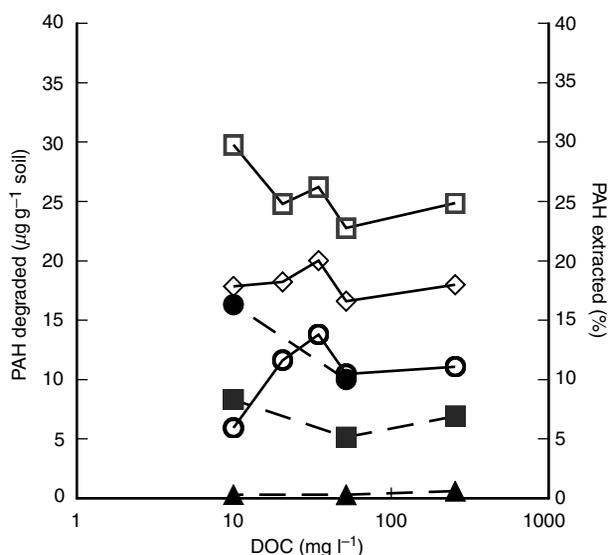
Water regime	Treatment		
	Analogue enriched	Non-analogue enriched	Control
Moist	29	8	20
S.D.	16	18	14
Saturated	47	26	39
S.D.	14	11	8

PAH, revealed some more treatment effects when the comparisons described above were repeated. The LMW PAH showed a trend of decreased absolute degradation with increasing DOC concentration (Fig. 3), whereas degradation of the HMW PAH fraction followed the same pattern as for all compounds. The trends were the same for relative degradation as for absolute degradation but more pronounced.

The relative PAH extraction efficiency was highest with GW, lower with DOC3, and then slightly higher again with DOC4 (Fig. 3). The pattern was the same for the LMW PAH group (Fig. 3). The extraction efficiency for the HMW PAH was marginal and independent of the DOC concentration (Fig. 3).

### Compound degradation

PHE had the highest initial concentration of PAH detected in the soil,  $275 \mu\text{g g}^{-1}$  (Fig. 4a). Next to PHE in



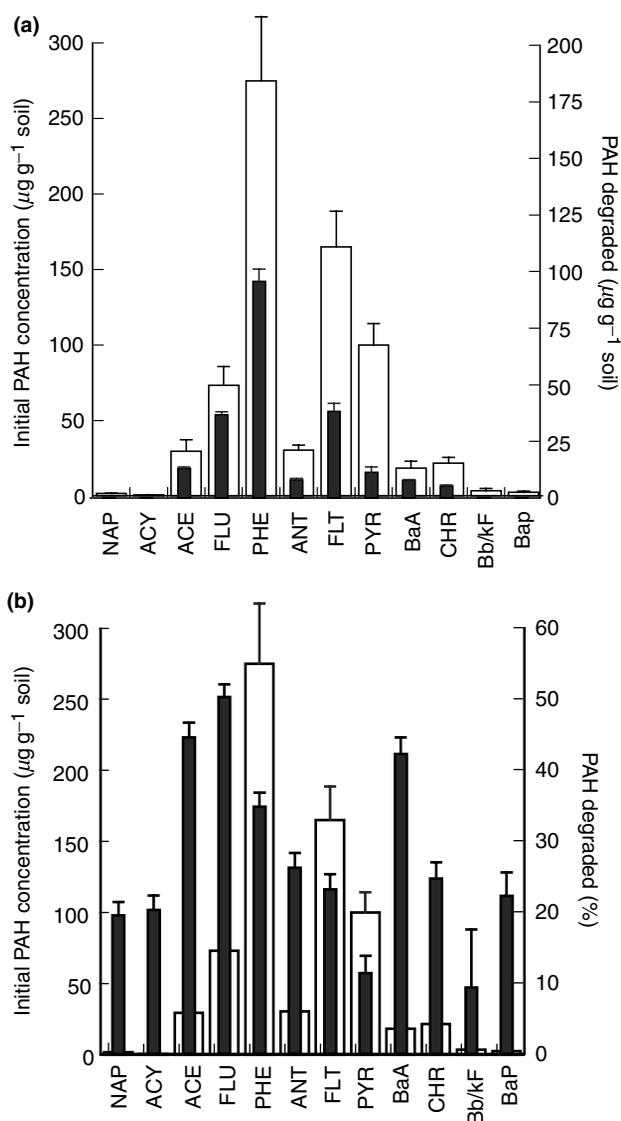
**Fig. 3** Relative extraction efficiency of PAHs (all PAHs ■, LMW PAHs ●, HMW PAHs ▲) in different concentrations of DOC (right axis) and average absolute degradation of PAHs (all PAHs ◇, LMW PAHs □, HMW PAHs ○) in the presence of different DOC concentrations (left axis). LMW PAHs were NAP, ACY, ACE, FLU, PHE and ANT. HMW PAHs were FLT, PYR, BaA, CHR, Bb/kF and BaP. The DOC concentrations ( $\text{mg l}^{-1}$ ) were 10 (GW), 20 (DOC1), 34 (DOC2), 52 (DOC3) and 255 (DOC4)

concentration were FLT at  $165 \mu\text{g g}^{-1}$ , PYR at  $100 \mu\text{g g}^{-1}$  and FLU at  $73 \mu\text{g g}^{-1}$ . Together, these four compounds represented 85% of the total PAH content of the soil. The absolute degradation was correlated with the initial concentrations of the compounds (Fig. 4a). An average over all treatments of  $95 \mu\text{g g}^{-1}$  of PHE were degraded, followed in order by FLT and FLU, each with approximately  $38 \mu\text{g g}^{-1}$ , and ACE, PYR, ANT, BaA and CHR with  $5\text{--}13 \mu\text{g g}^{-1}$  soil. The absolute degradation of BaP, Bb/kF, NAP and ACY was negligible. The 12 PAHs together were on average degraded at a rate of  $4.8 \mu\text{g g}^{-1}$  soil per day.

The relative degradation was not dominated by any one compound and not related to the initial concentration (Fig. 4b). In relative terms, FLU was degraded the most with 50%, closely followed by ACE and BaA. Bb/kF were degraded the least, by 9%. Also, PYR showed low relative degradation, 11%. The other compounds had relative degradations between 20 and 35%.

### Enumeration and isolation of degraders

ZFUs were only isolated on plates with PHE as the carbon source. The number of PHE degraders was  $8.9 \times 10^5$  ( $n = 2$ ) ZFU  $\text{g}^{-1}$ , but there were also many colonies on



**Fig. 4** Average initial PAH concentrations in the creosote-contaminated soil (9) and the average absolute (a) and relative (b) degradation of PAHs (in all treatments in the experiments. Error bars for the degradation represents standard error ( $n = 66$ ). Abbreviations: NAP, naphthalene; ACY, acenaphthylene; ACE, acenaphthene; FLU, fluorine; PHE, phenanthrene; ANT, anthracene; FLT, fluoranthene; PYR, pyrene; BaA, benzo[*a*]anthracene; CHR, chrysene; Bb/kF, benzo[*b*]fluoranthrene; BkF, benzo[*k*]fluoranthrene; BaP, benzo[*a*]pyrene

plates coated with other PAHs that did not form clear zones after more than 35 days of incubation. There were  $6.1 \times 10^6$  cfu  $\text{g}^{-1}$  (S.D.  $1.6 \times 10^6$ ,  $n = 12$ ), which was slightly less than in some previously examined PAH-contaminated soils (Carmichael and Pfaender 1997) and slightly more than in others (Thomas *et al.* 1989). Hence, of the cultivable bacteria in the soil,  $>10\%$  were able to use PHE as a sole carbon source.

## DISCUSSION

The first of the two suggested explanations for slow *in situ* PAH degradation, namely limited availability of carbon sources, was not supported by the observations, as both analogue and non-analogue substrate enrichment reduced the degradation of all PAHs except ANT (Fig. 1). The degradation of the enriched substrate, i.e. ANT, at the expense of other PAHs (Fig. 2, Table 4) was in agreement with the general correlation between the initial soil concentration of a PAH and its degradation (Fig. 4a).

The preference for ANT degradation after ANT enrichment indicates that there were autochthonous ANT degraders in the soil and that ANT was not cometabolized. This is interesting because the only PAH for which degraders were isolated from the Hässleholm soil by the zone-formation methodology was PHE, which was the dominating PAH in the soil. In two other soils, originating from abandoned coal gasification plants in the cities of Malmö and Ystad, with FLT and PYR as the most abundant contaminants, we found the same correspondence between the dominant PAH and the specificity of isolated PAH degraders (data not shown). Other PAHs than PHE and ANT were also degraded, albeit without isolation of their degraders. This lack of correlation between the number of degraders isolated from a soil and its PAH-degrading capacity was also observed by Carmichael and Pfaender (1997). It may be a consequence of the inability of the zone-formation methodology with the pure strains to account for a requirement of more than one strain for the degradation of a specific PAH (Alexander 1994), of the requirement of solubilization of the PAH by biosurfactant excretion of the cell, or of the inadequacy of the used growth medium to support the growth of the degraders. The same limitations would hold for other studies in which PAH degraders have been isolated using a PAH as their sole carbon source and in which their activity was assayed by cleared zones (Heitkamp *et al.* 1988; Thomas *et al.* 1989; Kästner *et al.* 1994) with similar or lower densities of ZFUs compared with this study.

The failure to stimulate PAH degradation by analogue enrichment may be the compound specificity of PAH communities and/or enzymes (Wilson and Jones 1993). This compound specificity was also evident from the interaction effects of the factors in the ANOVAs, demonstrating that the degradation of different compounds was affected in different ways by different treatments, and from other studies in which strains isolated on one PAH failed to degrade other PAHs and even became inhibited by them (Kästner *et al.* 1994; Bouchez *et al.* 1995). The opposite has also been found (Heitkamp *et al.* 1988), indicating that compound specificity may be site and strain specific. The uncertainty about the extent of compound specificity among

PAH degraders in a soil makes predictions about the efficiency of analogue enrichment difficult and argues for the simultaneous inclusion of several PAHs in both *in vitro* and in field studies.

Non-analogue enrichment sometimes increases the degradation of pollutants, often attributed to a fortuitous increase in biomass of the degrading population (Alexander 1994), and sometimes, like in this case, it does not. The effect of substrate enrichment can also vary with the concentration of the added substrate (Keuth and Rehm 1991), but since not even an analogue substrate promoted the metabolism in our experiment, the prospects of finding a substrate that will stimulate PAH degradation in this soil does not seem promising.

The second suggested explanation for the slow PAH degradation, limited bioavailability, could not be rejected by the test involving addition of water to the soil, but whether that was an effect of water enhancing the bioavailability of PAHs, or just enhanced microbial activity in general, is difficult to know. Water competes with PAHs for sorption to mineral sites on soil grains and can increase desorption of PAHs in this way (Karimi-Loftabad *et al.* 1996; Soheila *et al.* 1996), as well as a solvent. The large increase in PAH degradation in response to water addition is in agreement with earlier experience of PAH degradation limited to the dissolved fraction (Volkering *et al.* 1992), but there are also studies suggesting that sorbed PAHs and other hydrophobic substances can be extracted and assimilated by bacteria directly from surfaces (Kefford *et al.* 1982; Guerin and Boyd 1992).

The second test of bioavailability as a limiting factor for PAH degradation used DOC as surfactant, with mixed success, possibly dependent on the different ways that DOC can affect degradation: (i) It can increase the apparent solubility of PAHs (McCarthy and Jimenez 1985; Chiou *et al.* 1986). Some studies support the view that DOC associated, solubilized PAHs becomes bioavailable (Thomas *et al.* 1989; Hatzinger and Alexander 1995), while others suggest the opposite (McCarthy and Jimenez 1985; Leslie *et al.* 1987), and even claim that PAHs can age on DOC (Johnsen 1987). (ii) It can sorb to the soil, i.e. increase the organic content of the soil and thus enhance the partitioning of PAHs into the organic fraction (Soheila *et al.* 1996), or co-sorb to soil organic carbon as a DOC-PAH complex (Totsche *et al.* 1997). (iii) A fraction of DOC can serve as a source of carbon for microorganisms (Egli 1995), or have an inhibitory effect on microorganisms.

The test was conclusive since desorption was either independent (HMW PAH) or negatively correlated (LMW PAH) with the DOC concentration (Fig. 3). The pattern of degradation of LMW PAH coincided with the pattern of desorption (Fig. 3), suggesting that DOC enhanced sorption of LMW PAH to the solid phase by increasing its organic

carbon fraction. The data are not sufficiently detailed to explain the increased degradation of HMW PAH as the DOC concentration increased from GW to DOC2. Although the data are missing for GW1 and GW2, it seems unlikely that the extraction efficiency would be significantly larger for any DOC concentration since the extraction efficiency in general was very low. Likewise, we have no data to demonstrate a positive correlation between the metabolic activity and the DOC concentration, but it seems unlikely that a metabolic effect of DOC would only extend to HMW PAH. Another possibility is that DOC increased the apparent solubility of the PAHs already present in the aqueous phase. That would solubilize the HMW PAHs, with their higher  $K_{ow}$  to a larger extent than the LMW PAHs and increase their bioavailability.

The average relative degradation of the compounds (Fig. 4b) followed the same pattern as in many other studies, with higher relative degradation of LMW PAHs compared to HMW PAHs (Bossert and Bartha 1986; Thomas *et al.* 1989; Carmichael and Pfaender 1997). Most compounds were degraded by between 20 and 40%, some up to 50%, and none was degraded by less than 10%.

The bioassays demonstrated that purely by adding water as a solvent to a creosote-polluted soil, the slow *in situ* degradation of PAHs can be enhanced by more than three times. Water as well as other polar solvents such as acetone and ethanol (Lee *et al.* 2001) increase the apparent solubility and bioavailability of even aged PAHs but will also enhance their mobility. DOC seems to have the opposite effect on mobility and bioavailability of two- to three-ring (LMW)PAHs, probably by facilitating their sorption to the solid phase. Addition of DOC to the soil facilitated the degradation of four- to six-ring (HMW)PAHs, probably by increasing their apparent water solubility. With DOC as well as water treatment of the soil, a correlation between *in situ* concentration and absolute degradation of the 16 PAHs was observed, but only degraders of the most abundant PAH, PHE, were isolated and found to represent 10–15% of strains that grew on nutrient medium.

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