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Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site

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Abstract

The effects of nutrient addition on the *in situ* biodegradation of polycyclic aromatic hydrocarbons in creosote contaminated soil were studied in soil columns taken from various soil strata at a wood preserving plant in Norway. Three samples were used: one from the topsoil (0–0.5 m), one from an organic rich layer (2–2.5 m) and one from the sandy aquifer (4.5–5 m). The addition of inorganic nitrogen and phosphorous stimulated the degradation of polycyclic aromatic hydrocarbons (PAHs) in the top soil and the aquifer sand. These two soils, which differed strongly in contamination levels, responded similarly to nutrient addition with the corresponding degradation of 4-ring PAHs. The ratio between available nitrogen (N) and phosphorous (P) might explain the degree of degradation observed for the 4-ring PAHs. However, the degree of degradation of 3-ring PAHs did not significantly increase after nutrient addition. An increase in the respiration rate, after nutrient addition, could only be observed in the topsoil. In the aquifer sand, 4-ring PAH degradation was not accompanied by an increase in the respiration rate or the number of heterotrophic micro-organisms. PAH degradation in the organic layer did not respond to nutrient addition. This was probably due to the low availability of the contaminants for micro-organisms, as a result of sorption to the soil organic matter. Our data illustrate the need for a better understanding of the role of nutrients in the degradation of high molecular weight hydrocarbons for the successful application of bioremediation at PAH contaminated sites.

Introduction

Many sites have been polluted by creosote waste as a result of wood-preserving activities. Polycyclic aromatic hydrocarbons (PAHs) represent approximately 85–90% of the contaminants at these sites (Sundström et al. 1986). Micro-organisms which are able to biodegrade 2- to 5-ring PAH compounds have been isolated from creosote contaminated sites (Cerniglia 1992). However, little natural degradation is observed in the field (Mueller et al. 1989). This has been attributed to the limited availability of appropriate electron acceptors, microbial toxicity of the contaminants, or limited substrate availability (Dyreborg 1996; Mihelcic et al. 1993; Millette et al. 1998).

Under aerobic conditions, the supply of macro-nutrients, nitrogen (N) and phosphorous (P), has been shown to have a beneficial effect on the biodegradation of mineral oil spills in the marine and terrestrial environment (Bragg et al. 1994; Breedveld et al. 1995). This is believed to be the result of an improved carbon:nitrogen:phosphorous (C:N:P) ratio, resulting in an increase in the number of heterotrophic micro-organisms (Alexander 1994). However, inorganic nitrogen and phosphorous additions have been shown to increase transformation rates of hydrocarbons, without apparent increase in microbial biomass (Lewis et al. 1986). Swindoll and co-workers (1988) observed highly variable effects of nutrient addition on hydrocarbon degradation in different samples from

the same aquifer. Braddock et al. (1997) observed the greatest stimulation of microbial activity in a fuel oil contaminated aquifer at low nutrient addition levels, rather than at high nutrient addition levels.

Little attention has been given to the effect of macro-nutrients on the biodegradation of PAH. Cleland et al. (1997) observed an increased phenanthrene mineralization upon addition of N and P in combination. High nutrient levels had a larger effect than low nutrient levels. In a coal tar contaminated soil, higher oxygen consumption rates were observed at low macro nutrient levels than at high macro nutrient levels (Liebeg & Cutright 1999). However, mineralization of phenanthrene and pyrene decreased upon the addition of N and P in five soils with a different pollution history (Carmichael & Pfaender 1997). Johnson & Scow (1999) observed no stimulatory effect of nutrient addition on phenanthrene degradation in four soils with no known pollution history.

In the present study, the effect of inorganic N and P addition on the *in situ* biodegradation of PAH was assessed using columns containing soil material from various soil strata at a wood preserving plant in Norway.

Materials and methods

Experimental set-up

At the Lilleström site, Norway, there has been creosote wood-preserving activity for more than 50 years. The site is situated on top of sedimentary deposits of clay, sands and peat. A trial pit was excavated near the source zone of the creosote contamination. Samples were taken from 0.5 m deep in the sandy upper layer 2–2.5 m deep in an organic layer, and at 4.5–5 m deep in the underlying aquifer. Samples were homogenised, sieved to <4 mm and packed in 4-liter columns. For the top soil glass columns were used (10 cm diameter $\times$ 50 cm long). The organic layer and aquifer sand were packed in stainless steel columns with the same dimensions.

All columns were operated under unsaturated conditions with forced aeration (3–6 l/d) using, continuous air extraction at the bottom of the columns. Evaporation from the top of the columns was minimised by using a small diameter air inlet (6 mm) at a slightly positive pressure (approx. 1 mbar). In addition to aeration, water was infiltrated from the top of the columns, with or without nutrient addition (200 mg NH_4^+-N/l, and 50 mg PO_4^{3-}-P/l). The nutrient application was adjusted based on the mass of soil in the columns and the PAH concentration (16 PAH) in the column material (Table 1). The soil columns from the topsoil received continuous nutrient addition (0.8 l/d), whereas the two other soils types received weekly doses of 60 ml (organic layer) and 125 ml (aquifer sand). After 6 months of nutrient application a PAH/N/P ratio of 5/4/1 (topsoil), 3/4/1 (organic layer) and 8/4/1 (aquifer sand) was reached. Based on TOC the C/N/P ratio was 34/4/1 for the topsoil, 2600/4/1 for the organic layer and 90/4/1 for the aquifer sand. However soil humic material is highly resistant against microbial biodegradation and therefore not available as a carbon source. Leaching of PAH was monitored at weekly intervals in columns receiving continuous nutrient addition (topsoil). Evaporation was monitored in the same columns at the start of the experiment (2 weeks). Each soil type and treatment was run in duplicate columns at 10–15 °C during a six-months period. Untreated duplicate samples were frozen at −18 °C.

Chemical analyses

Treated and untreated soil samples were analyzed for PAH content (16 USEPA) using gas chromatography and flame ionization detection (GC-FID). Soil samples were dried with sodium sulphate/talcum and extracted using acetone and sonication. *o*-Terphenyl was added as an internal standard. The solvent was changed to cyclohexane and cleaned on a silica column, deactivated with 15% water. PAH levels in the cyclohexane extract were determined using external standard calibration. Selected soil samples were analyzed for heterocyclic nitrogen, sulphur and oxygen (NSO)-compounds with GC/MS. Water samples were extracted with cyclohexane and analyzed by GC-FID without further clean-up.

Total extractable hydrocarbons (TEH) levels in soil were obtained using Soxhlet extraction with dichloromethane and methanol. Fractions of aliphatic, aromatic and polar hydrocarbons were determined using thin-layer chromatography on silica rods and quantified with thin-layer chromotography and flame ionization detection (TLC-FID, Iatroscan labs, Tokyo).

Total organic carbon (TOC) and total carbon (TC) levels in soil were determined after thermal oxidation using a Dohrmann carbon analyzer (Rosemount Analytical, Santa Clara, CA).

Table 1. Characteristic parameters of the soil strata used in the column study

Parameter	Top soil	Organic layer	Aquifer sand
Depth (m)	0–0.5	2–2.5	4.5–5
Sand (%)	84	60	85
Silt (%)	5	31	12
Clay (%)	n.d.[b]	3	3
Dry bulk density (kg/l)[a]	1.5	0.6	1.6
TOC (%)	4.17	16.6	0.38
TC (%)	4.47	16.9	0.45
TN (%)	0.10	0.44	0.06
Available NO_3-N (mg/kg)	5.0	135	1.4
Available NH_4-N (mg/kg)	0.2	<0.1	0.1
TP (mg/kg)	443	914	379
Available PO_4-P (mg/kg)	0.5	1.1	0.2
pH ($CaCl_2$)	6.6	5.3	6.2
2-ring PAH (mg/kg)	300	20.1	71.5
3-ring PAH (mg/kg)	3430	22.7	186
4-ring PAH (mg/kg)	2130	94.9	62.5
5 + 6-ring PAH (mg/kg)	396	62.5	4.1
Total PAH (mg/kg)[c]	6260	200	324
Total heterotrophs (log CFU/g)	7.3	7.2	7.3
Naphthalene degraders (log MPN/g)	n.d.[b]	4.9	7.2
Creosote degraders (log MPN/g)	7.0	5.1	7.4

[a]After repacking in the columns.
[b]Not determined.
[c]Sum of 16 PAH according to USEPA.

Nutrient levels were determined spectrophotometrically. Total nitrogen and total phosphorous levels were determined after sulphuric acid extraction. Available NO_3^-, NH_4^+ and PO_4^{3-} levels were determined in 0.01 M $CaCl_2$ extracts (1:5 by weight).

Carbon dioxide content in the gas phase was monitored using an alkali trap (topsoil) or a direct reading CO_2/O_2 analyzer (Edinburgh Sensors, Livingston, UK) for the other two soils.

Microbial characterization

Soil samples were extracted with Bushnell-Haas medium (1:10) using a Waring blender (3 × 1 min). The total number of colony forming units (CFU) was determined by plate counts on nutrient agar (Difco, Detroit, MI). Mineral agar (Bushnell-Haas/Bacto agar, Difco) with creosote as the sole carbon source was used to determine the number of creosote degrading micro-organisms in the topsoil samples. In the two other soils, the most probable number (MPN) technique was used to quantify the naphthalene and creosote degraders using Bushnell-Haas medium and naphthalene and creosote respectively, as the sole carbon source. The carbon source was added as three droplets or crystals of the pure compound. The creosote used in these experiments (MT 90, Tarconord, DK) was artificially aged by N_2 purging of volatile hydrocarbons over night and washing with de-ionized water (1:10 by volume).

Results

Soil characteristics

The samples from the soil strata used in this study showed distinct differences in physical and chemical composition as well as levels of contamination (Table 1). The topsoil was composed of a coarse, sandy material with a very high PAH content. The organic layer showed a high organic carbon content with a relatively low PAH level, whereas the fine textured aquifer sand

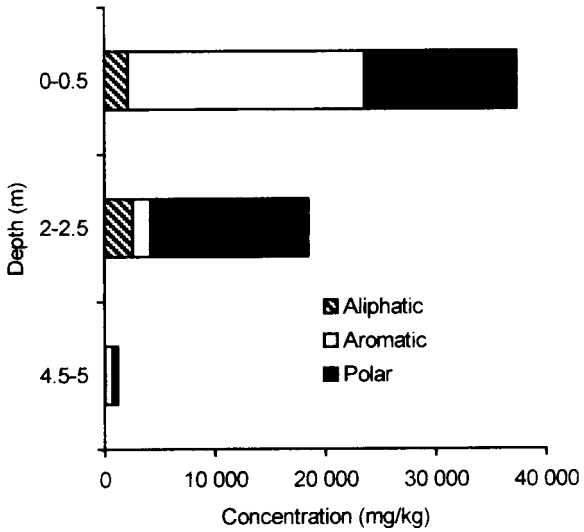

Figure 1. Total extractable hydrocarbons characterized with TLC-FID in samples from different soil strata at the creosote contaminated site.

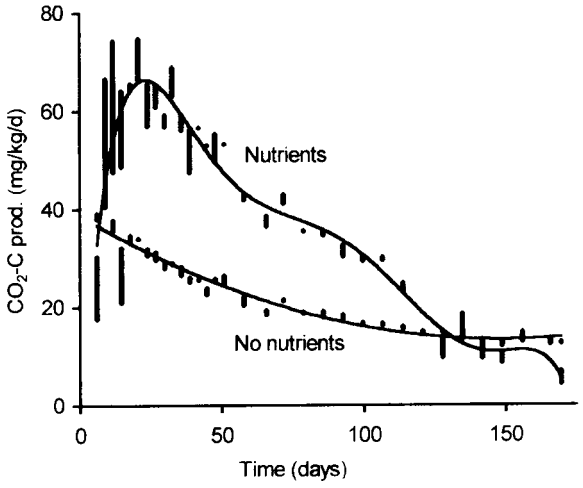

Figure 2. Mineralization rate in columns from the top soil, with and without nutrient addition (error bars indicate the range for 2 parallel columns).

had a low organic carbon content and displayed a PAH level of approximately 5% of the top soil.

Characterization of the soil contaminants using TLC-FID allowed quantification of the total extractable hydrocarbon fraction, including the high molecular weight compounds which cannot be characterized using gas chromatography (Karlsen & Larter 1991). Distinct differences in the chemical composition along the soil profile were observed (Figure 1). In the upper 0.5 m, a high level of total extractable hydrocarbons (TEH) was observed (37,400 mg/kg which equals 3.74% by weight), which was dominated by the fraction of aromatic hydrocarbons. The observed TEH level was similar to the TOC level (3.74% and 4.17% respectively). This indicates that this soil stratum was contaminated by free-product creosote, with little, extraction resistant, soil organic matter (SOM). In the organic layer at 2 to 2.5 m depth the TEH level was 18,500 mg/kg (1.85%), dominated by polar hydrocarbons. In the aquifer at 4.5–5 m depth, the TEH level was low (1300 mg/kg = 0.13%) and aromatic hydrocarbons dominated. In the organic layer and the aquifer sand, the extractable hydrocarbons comprised only a small portion of the observed TOC levels (11% and 34%, respectively), indicating the presence of SOM. Breedveld and Karlsen (2000) presented a detailed discussion of the contaminant composition.

Microbial parameters showed little differences between the three soil strata. High numbers of both heterotrophic micro-organisms and creosote degraders were found in most samples (Table 1). The organic layer contained lower numbers of PAH degrading organisms than the two other soil strata.

Respiration

Carbon dioxide production in the columns was monitored continuously. Organic carbon mineralization was extensive in the column containing the topsoil (Figure 2). Nutrient addition resulted in a significant increase in mineralization levels, as well as a decrease in TOC levels in the soil (Table 2). In the organic layer and the aquifer sand, no increase in mineralization rate was observed, when nutrients were supplied (data not shown). In these soil strata, total mineralization levels after six months were comparable in the columns with or without nutrient addition (Table 2).

PAH degradation

GC-FID analyses of the soil samples after treatment showed that the degradation of PAHs in the columns receiving water and aeration was limited to 2- and 3-ring compounds for the topsoil (0–0.5 m) and the aquifer sand (4.5–5 m). Analyses of column leachate and gas phase composition in the topsoil showed that leaching and stripping contributed by less than 1% to the observed removal of 2- and 3-ring PAH. Nutrient addition resulted in the additional removal of 4-ring PAH compounds in these soil strata (Figure 3). In the organic layer columns with nutrient addition, some PAH removal was observed. However, in one of the

Table 2. Total mineralization and soil characteristics after the 6 months column study (mean values and range for 2 parallel columns)

Parameter	Top soil		Organic layer		Aquifer sand	
	−N/P	+N/P	−N/P	+N/P	−N/P	+N/P
CO_2-C production[a]	3416 ± 80	5501 ± 181	642 ± 10	532 ± 19	531	508 ± 65
TOC (%)	3.95 ± 0.06	3.40 ± 0.10	n.d.	17.0	n.d.	0.43
Total heterotrophs[b]	6.5 ± 0.3	7.9 ± 0.6	6.2 ± 0.2	6.5 ± 0.0	6.7 ± 0.1	6.7 ± 0.1
Creosote degraders[b]	5.8 ± 0.3	6.5 ± 0.3	n.d.	n.d.	n.d.	n.d.

[a]Cumulative CO_2-C production in mg/kg soil during the entire experiment.
[b]log CFU/g.

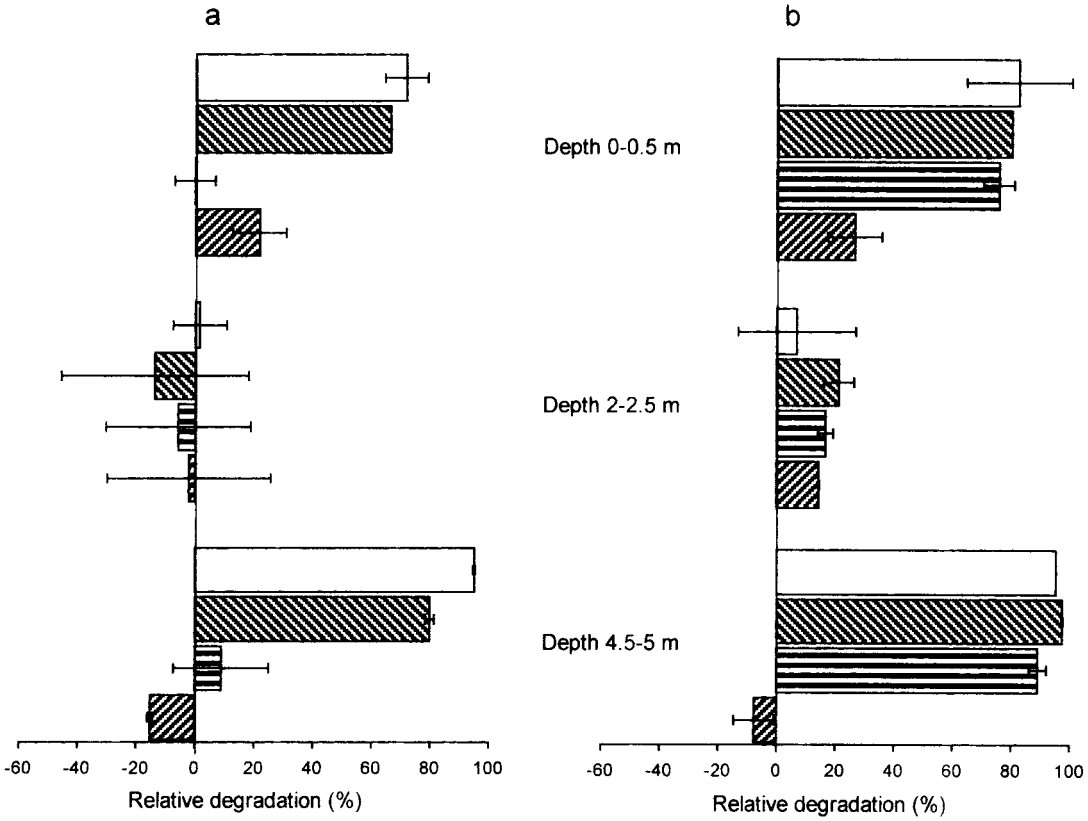

Figure 3. Relative degree of PAH degradation (mean values and range for 2 parallel columns) in laboratory columns with (b) or without nutrient addition (a), analyzed by GC-FID. (□ 2-ring PAH, ▨ 3-ring PAH, ▤ 4-ring PAH and ▨ 5+6-ring PAH)

two organic layer columns, without additional nutrients, a similar PAH removal was observed, resulting in a large deviation in the data (Figure 3). Examination of the complete GC-FID chromatograms of aquifer sand samples (including unidentified peaks) showed a sharp boundary at approximately 17 min (Figure 4). This is the approximate retention time of fluoranthene, the 4-ring PAH which elutes first in GC-analyses. After six months of aeration, no change in the part of the chromatogram where 4-, 5- and 6-ring PAH elute (17–40 min) could be observed. After nutrient addition clear changes are observed in the retention time window of 17–25 min where 4-ring PAH elute. A similar response was registered for samples from the topsoil (data not shown).

The significant difference in 4-ring PAH removal upon nutrient addition was comparable for all 4-ring PAH compounds analyzed in the top soil and the aquifer sand (Figure 5). No significant reduction in the levels of 5 + 6-ring PAH was observed in any of

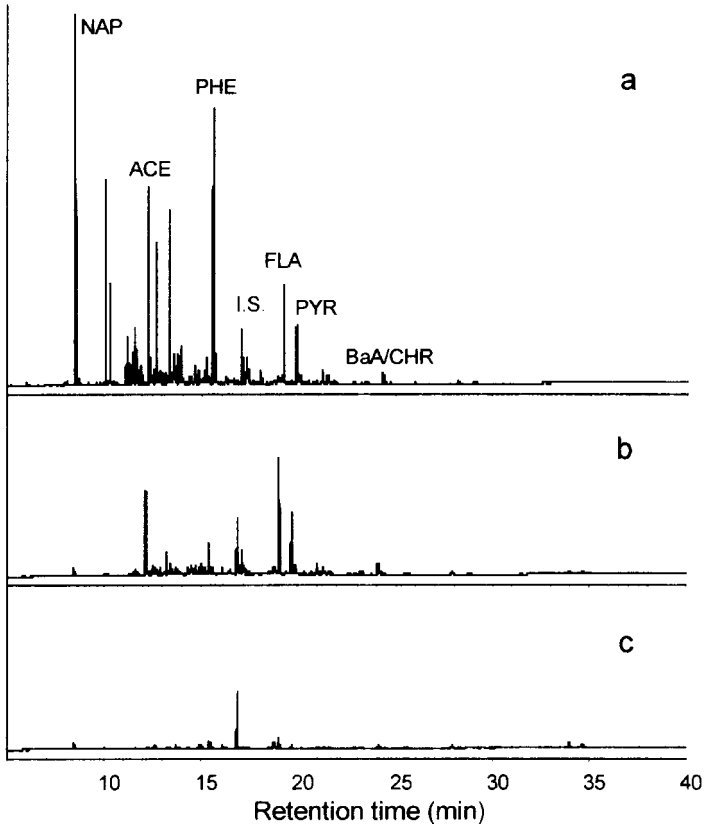

Figure 4. Chromatograms of aquifer sand before treatment (a), after 6 months treatment in laboratory columns with aeration (b), and aeration and nutrient addition (c) analyzed by GC-FID (NAP, naphthalene; ACE, acenaphthene; PHE, phenanthrene; I.S. internal standard; FLA, fluoranthene; PYR, pyrene; BaA, benzo(a)anthracene; CHR, chrysene).

the samples. In addition, analysis of selected samples with GC-MS showed that nutrient addition also resulted in removal of high molecular weight NSO-compounds (Table 3). On the other hand, removal of 3-ring NSO-compounds (carbazole, dibenzofuran and dibenzothiophene) was not influenced by nutrient addition.

Available nutrients

The levels of available nutrients varied considerably in the initial samples from the soil strata (Table 1). Nutrient addition resulted in an increased level of available nutrients in all soil samples. However, clear differences between the three soil strata were observed. Where the levels of available NH_4-N increased in the topsoil and the aquifer sand (100–200 fold increase), the organic layer showed a small increase in NO_3-N levels and no apparent increase in ammonium levels, indicating a complete nitrification of the ammonium added (data not shown). Subsamples of the columns showed marked differences in available nutrients although no direct relationship with depth in the soil column could be observed (data not shown).

Microbial population

The total number of heterotrophic micro-organisms was nearly identical before and after treatment in the organic layer and the aquifer sand (Tables 1 and 2). In the topsoil, a small decrease in the number of heterotrophs was observed without nutrient addition. A similar reduction was observed in the number of creosote degraders.

Discussion

Samples taken from three different soil strata at a site contaminated with creosote contained micro-organisms that were able to use creosote as sole carbon source. Supply of an electron acceptor (oxygen) by

Table 3. Heterocyclic NSO-compounds in the soil strata, at the beginning and end of the 6 months column study (mg/kg)

Compound	Organic layer			Aquifer sand		
	Initial	−N/P	+N/P	Initial	−N/P	+N/P
Dibenzofuran	4.42	4.31	3.59	22.39	1.44	1.50
Dibenzothiophene	1.46	1.69	0.91	6.48	1.00	0.30
Carbazole	4.46	4.55	2.71	3.24	0.34	0.39
Benzo(d,e,f)dib.thioph.	1.50	1.61	1.03	1.06	1.00	0.23
Benzothionaphthene	3.64	3.65	2.57	1.26	1.20	0.23
Benzophenanthridine	0.75	0.82	0.53	0.25	0.24	0.03

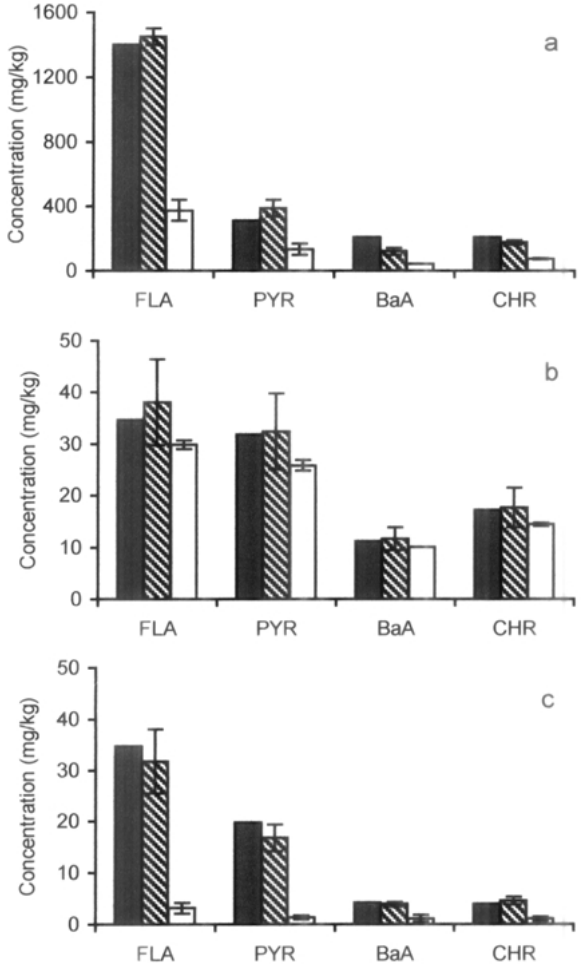

Figure 5. Concentrations of 4-ring PAH compounds in top soil (a), organic layer (b) and aquifer sand (c) before (■) and after 6 months treatment without (▨) or with (□) additional nutrients, mean values and range of two parallel columns are indicated (FLA, fluoranthene; PYR, pyrene; BaA, benzo(a)anthracene; CHR, chrysene).

aeration resulted in degradation of 2- and 3-ring PAH in two of the three soil strata, i.e., the topsoil and aquifer sand. In addition, nutrient addition of both N and P resulted in a significant reduction of 4-ring PAH compounds in the same two soil strata. In samples from the third soil stratum, an organic layer, no apparent effect of nutrient addition was observed. The soil from the organic layer had a high organic carbon content (17%) resulting in strong sorption of hydrophobic compounds like PAH (e.g., Karickhoff 1981). Strong sorption results in low availability of the contaminants for micro-organisms, and therefore in low degradation rates (Breedveld & Karlsen 2000; Mihelcic et al. 1993).

In the topsoil, with high contaminant levels, the mineralization rate increased upon nutrient addition. This supports the general theory that the major effect of nutrient addition is an increase in the total microbial activity, which results in a higher degradation activity (Alexander 1994). However, no apparent increase in the number of creosote degraders was observed. A general higher microbial activity can hardly explain the observed degradation of 4-ring PAH compounds upon nutrient addition.

In general, degradation rates of PAH compounds decrease with an increasing number of condensed rings (Trezesicka-Mlynarz & Ward 1995). This is believed to be a result of reduced availability for micro-organisms as a result of lower pore-water concentrations of high molecular weight PAH compounds. If this were the case, fluoranthene with an aqueous solubility of 240 μg/l would be expected to degrade faster than chrysene with a solubility of 2 μg/l. However, in our study all 4-ring PAHs showed a comparable degree of degradation. In a similar fashion, one would expect anthracene (3-ring PAH) with a solubility of 75 μg/l to degrade at a comparable rate as fluoranthene. In

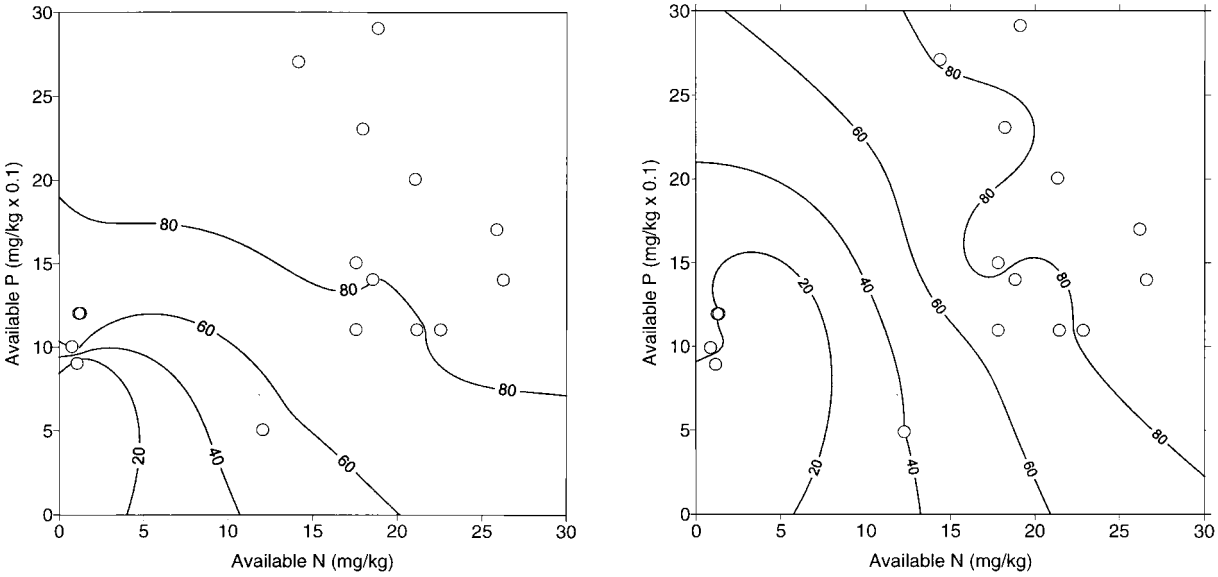

Figure 6. Contour map of the relative degree of degradation (%) of anthracene (a) and fluoranthene (b) in aquifer sand as a function of available N and P. The contours were obtained using a kriging procedure to interpolate between data points (○).

our study, anthracene was degraded to a large extent, while fluoranthene was not degraded at all without the addition of nutrients. This could suggest a stronger sorption of fluoranthene to the soil matrix.

In the aquifer sand, with low contaminant levels, the addition of nutrients did not result in changes in the mineralization rate or the total number of heterotrophic micro organisms. The absence of an increase in the microbial population could be a result of grazing by protozoa masking the microbial biomass increase (Madsen et al. 1991). This soil stratum was low in organic carbon and had therefore a low sorption capacity. However, 4-ring PAH compounds were only degraded after nutrient addition.

Degradation of 4-ring PAH compounds by the indigenous microbial population apparently requires specific nutrient levels which is not the case for 2- and 3-ring PAH degradation. The ratio between available N and P, rather than the absolute level, was found to explain differences in hexadecane and phenanthrene mineralization (Smith et al. 1998). Available N and P and the relative degree of degradation can be considered as coordinates in three dimensions. To be able to study patterns between these variables, assuming spatial continuity, a trend surface analysis can be carried out (Davis 1986). Contour maps of relative PAH degradation in the aquifer sand as a function of available N and P, using kriging, were established for anthracene and fluoranthene (Figure 6). Kriging is a geostatistical method which allows surface analysis based on scattered data points (Huijbregts & Matheron 1971). Although kriging was used on a limited data set, Figure 6b indicates that there is a strong relation between available P, available N, and the degradation of fluoranthene. Optimal degradation was observed at an available N:P ratio of approximately 10:1. A similar trend was, however, not observed for the degradation of anthracene (Figure 6a). High degrees of degradation were observed without increase in available N. Eriksson and co-workers (2000) also observed that the degradation of 3-ring PAHs was independent of nutrient addition. However, fluoranthene and other 4-ring PAHs were persistent in their experiments.

Our study shows that a better understanding of the role of nutrients in the degradation of high molecular weight hydrocarbons is needed in order, to be able to apply bioremediation successfully at PAH contaminated sites.

Acknowledgments

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