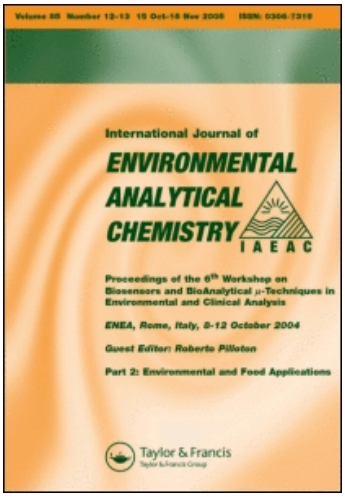

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Wischmann, H. , Steinhart, H. , Hupe, K. , Montresori, G. and Stegmann, R.(1996) 'Degradation of Selected PAHs in Soil/Compost and Identification of Intermediates', International Journal of Environmental Analytical Chemistry, 64: 4, 247 — 255
To link to this Article: DOI: 10.1080/03067319608028346
URL: http://dx.doi.org/10.1080/03067319608028346

Intern. J. Environ. Anal. Chem., Vol. 64, pp. 247–255
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DEGRADATION OF SELECTED PAHs IN SOIL/COMPOST AND IDENTIFICATION OF INTERMEDIATES

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(Received, 29 January 1996)

Solvent-extractable degradation products of polycyclic aromatic hydrocarbons formed in a soil/compost mixture were analysed using a clean-up procedure including solid phase extraction. From glass columns, containing silica gel deactivated with 6% water, two fractions were eluted: a moderately polar fraction of ketones and quinones and a more polar fraction of phenolic compounds and organic acids. Such oxidation products were identified using reversed-phase high performance liquid chromatography and gas chromatography-mass spectrometry. Applying this method to a degradation study on four selected PAHs in soil/compost mixtures, 1-hydroxy-2-naphthoic acid could be found in the system fortified with phenanthrene, and 9-fluorenol and 9-fluorenone were identified as degradation products of fluorene. The occurrence of the latter in sterilized controls shows that PAH metabolism is accompanied by abiotic degradation mechanisms.

KEY WORDS: Polycyclic aromatic hydrocarbons, oxidation products, soil/compost mixture, analytical method, HPLC, GC-MS.

INTRODUCTION

Biological treatment is a modern and widespread technology for remediating soils contaminated with mineral oil residues. At present many companies offer methods that utilize special microorganisms able to degrade defined contaminant classes. Others stimulate the authochthonous population by supplying necessary additional nutrients in the form of complex supplements such as straw, bark, or compost. Although microorganisms have been proved to degrade one or more PAHs, the fate of the contaminant class PAHs in the complex soil matrix is relatively unknown. In this context it has been indicated that the formation of more polar and more bioavailable metabolites and their possible accumulation have to be taken into account[1]. From studies in culture media the main pathways of the microbiological degradation have been elucidated; relevant reviews have been published by Cerniglia and by Gibson and Subramanian[2,3]. However, it is doubtful whether the processes in soils can be described by these mechanisms alone. Other influences on the environmental behaviour of organic contaminants may accompany biological transformations: abiotic/chemical and photooxidation as well as sorption onto organic or inorganic soil particles.

As a contribution to balancing the conversion of PAHs in soils, a method was developed on the basis of PAH metabolites, previously identified in vitro, which ensures the identification of solvent-extractable degradation products. The analytical procedure includes an ultrasonic extraction and a subsequent fractionation of different polar structural classes. This was achieved by a modified solid phase extraction (SPE) over silica gel that had been previously established for the structure-type separation of mineral oils[4]. The method was applied to the treatment of four PAHs in soil/compost mixtures as discussed for soil remediation techniques including complex organic supplementations[5–7]. In the course of this experiment the PAHs were analysed separately using a second extraction method that combines the advantages of a fast ultrasonic extraction in glass centrifugation-tubes[8] with those of an effective solvent mixture of cyclohexane-acetone[9].

MATERIALS AND METHODS

Reagents

PAHs and derivatives were obtained from the following suppliers: acenaphthene-1, 2-dione, anthracene-9, 10-dione, fluorene, 9-fluorenone, 1-hydroxy-2-naphthoic acid, 2-naphthol from E. Merck, Darmstadt, FRG; benz[a]anthracene, fluoranthene, 9-fluorenol, 1-indanone, 2-methylanthracene-9,10-dione, 1,8-naphthalic anhydride and benz[a]anthracene-7,12-dione from Aldrich, Steinheim, FRG; phenanthrene from Serva, Heidelberg, FRG. The solvents used were of analytical grade (chloroform, cyclohexane, ethyl acetate, toluene, hydrochloric acid (30%), acetic acid (100%), *ortho*-phosphoric acid (85%); E. Merck, Darmstadt, FRG) or of HPLC grade (acetone, acetonitrile, *n*-hexane, methanol; Biomol, Hamburg, FRG). Dichloromethane (E. Merck, Darmstadt, FRG) was of synthetic grade and distilled over potassium carbonate before use. Sodium sulfate and the derivatization reagents—N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS)—were of analytical grade and purchased from E. Merck, Darmstadt, FRG. Mercury(II)chloride was supplied by Baker, Groß-Gerau, FRG. The SPE-materials—8-ml borosilicate glass columns, PTFE frits (20 μm pores), stainless brass taps with PTFE cones, and silica gel (particle size 40 μm, particle size distribution 30–60 μm)—were purchased from Baker, Gross-Gerau, FRG.

Soil/compost mixture

An AhA1 soil (1.1% organic carbon, 6.5% kaolin, maximum water capacity 35.9% (w:w)) was sieved at 2 mm mesh size and mixed with biocompost (degree of maturity at V, maximum water capacity 213% (w:w)) resulting in a ratio of 9 : 1, relating to dry weight. 60% of maximum water capacity was adjusted for extraction and degradation studies.

Fortification procedure

129.5 g of this mixture (100 g dry weight) was filled into 1.5–1 jars. In each case seven jars were spiked with a solution of one polyaromatic compound in 2 ml ethyl acetate resulting in concentrations of 100 mg/kg dry weight for fluorene, phenanthrene, and fluoranthene and 28 mg/kg dry weight for benz[a]anthracene. A beaker with 20 ml 1 M

sodium hydroxide solution for measuring the amount of CO_2 produced was placed in each jar. The jars were closed airtight by clamped fit rubber rings and glass lids and stored at 20°C in a room that was illuminated for 12 h each day. The glass vessels were opened only after 0, 3, 6, 8, 10, 15, 23, 31, 42, 59, 77 and 189 days for estimation of the CO_2 formation or when withdrawing the whole content for analysis. Additionally, unfortified controls were prepared and analysed to demonstrate freedom from interference.

To estimate the influence of abiotic processes on the decrease of the organic contaminants and the formation of intermediates, seven portions of the soil/compost mixture were treated with a chloroform-fumigation-method which has been developed for the estimation of biomass[10,11]. The mixtures and a beaker filled with chloroform were placed into a desiccator and the system was evacuated. Subsequently the cock was closed. After 2 d these prepared materials were put into autoclaved jars and contaminated with a mixture of all four PAHs in 2 ml ethyl acetate. Additionally, tubes filled with calcium hydroxide, which had been prepared with an acid-base-indicator and a fixed millimeter graph paper for scaling, were autoclaved and placed into these jars. The zone colouring is proportionate to the formed and absorbed CO_2. These jars were opened only once for analysis.

An alternative method of suppressing biotic degradation was performed with a fortified mixture of both fluorene and phenanthrene. In this case the soil/compost mixtures were autoclaved and humidity was adjusted with a solution of mercury(II) chloride in water, resulting in a final concentration of 1 g/kg wet soil. These jars were closed as described above and stored at ambient temperature in the dark.

For establishing recoveries, 100 g of soil/compost mixture were fortified with a 2-ml aliquot of an ethyl acetate solution containing the four PAHs and known PAH metabolites resulting in concentrations of 2.2–10.6 mg/kg per PAH and 1 mg/kg per metabolite. These mixtures were stored overnight and then extracted in quadruplicate with both of the methods described below.

Estimating colony-forming units

At the end of the experimental time the number of colony-forming units was estimated: A suspension of 1 g soil/compost mixture in 10 ml physiological sodium hydroxide solution was prepared and decimal dilutions were incubated on inhibitor-free plate-count-agar for 48 h at 30°C.

Extraction of PAHs

Approximately 3 g soil and 2 g sodium sulfate were filled with 7 ml cyclohexane-acetone (1:1; v:v) into a glass centrifuge tube which was then tightly closed using Teflon/silicone septa and Teflon tape. The tube was shaken by hand, sonicated for 15 min (Elma Digital Ultrasonic Cleaner T 480 H-2, Singen, FRG), and then centrifuged (850 × g, 10 min). An aliquot of the supernatant was removed with a graduated pipette, evaporated almost to dryness and diluted with methanol.

Extraction of PAH degradation products

For the extraction of intermediates 1 ml 4M hydrochloric acid was added to 20 g of contaminated soil. After 20 min incubation the soil was dried with 20 g of sodium sulfate

and sonicated with 40 ml dichloromethane. The solution was centrifuged, decanted into 50-ml round flasks and evaporated to ca. 5 ml. The extraction was repeated twice, the solutions combined and again evaporated. The concentrated extracts were then decanted into modified Kuderna-Danish tubes and further evaporated under nitrogen almost to dryness.

Solid phase extraction

The 8-ml borosilicate SPE columns were prepared by filling 2 g of silica gel, deactivated with 6% water, between 2 PTFE frits. Before transferring the sample these columns were solvated with 8 ml hexane. The PAHs were eluted with 12 ml hexane-dichloromethane (90:10; v:v). Subsequently, a less polar fraction II of ketones was eluted with 6 ml dichloromethane and a polar fraction III of alcohols and acids was eluted with 5 ml methanol-acetic acid (99:1; v:v).

The polar fractions were evaporated under nitrogen to ca. 0.3 ml and diluted with methanol in a 1-ml graduated flask. The polar fraction II was trimethylsilylated before CGC-MSD analysis: A 100 µl aliquot was evaporated to dryness and derivatized in 20 µl toluene with 20 µl BSTFA/TMCS (99:1, v:v; 60°C, 45 min).

High performance liquid chromatography

The HPLC system consisted of a Merck-Hitachi (Darmstadt, FRG) L-6200 low pressure gradient system fitted with a 150 mm × 4 mm i.d. ET 150/8/4 Nucleosil 5 C18 PAH column packed with 5 µm C18 (Macherey and Nagel, Düren, FRG). For detection a Millipore-Waters (Eschborn, FRG) 994 photodiode-array detector and a Merck-Hitachi D-2000 integrator were connected. The separations were performed at 30°C with a flow rate of 1 ml/min. PAH analysis was performed with a mobile phase gradient consisting of methanol (M) and water. The gradient elution program started with 70% M and was changed linearly to 100% M between 6 and 15 min which was held for 10 min. The detector was operated at 251 nm. Analysis of the degradation products was performed with a ternary gradient starting with 25% acetonitrile (A), 10% M, and 65% water-phosphoric acid (pH 2), changing linearly to 20% A and 50% M between 10 and 30 min, then to 100% M between 30 and 40 min which was held for 5 min. The detection was performed simultaneously at 227 and 251 nm (Table 1).

Gas chromatography—mass spectrometry

CGC-MSD analysis was performed on a Hewlett-Packard (Palo Alto, USA). Model 5890 gas chromatograph connected to a HP 5971 mass-selective detector equipped with the HP evaluation unit HP G1043B MS ChemStation. Separations were achieved on a fused-silica capillary column (30 m × 0.32 mm i.d.) coated with a 0.10-µm film of DB-1 (J and W Scientific, Folsom, USA). Injection port temperature was 250°C, MSD interface temperature was 280°C. Injections were conducted in the splitless mode with the inlet liner purged 1.0 min after injection, the splitting ratio was then about 1:30. Helium carrier gas flowrate was 1.3 ml/min. The MSD was operated in the SCAN-mode, the electron ionization energy was 70 eV. For fraction II initial oven temperature was 70°C

Table 1 Recoveries of PAH oxidation products from soil/compost mixtures after ultrasonic extraction (n = 4).

Compound	*Detection, nm*	*Recovery, %*	*S.D.*[a]	*Recovery, %*	*S.D.*[a]
		with acidification		*without acidification*	
1-Indanone	251	40	6.7	68	1.7
Acenaphthene-1,2-dione	227	53	3.6	66	4.3
1, 8-Naphthalic anhydride	227	60	5.6	52	3.8
2-Naphthol	227	49	2.3	76	4.7
9-Fluorenol	227	89	4.1	91	6.6
1-Hydroxy-2-naphthoic acid	251	63	3.4	17	3.1
9-Fluorenone	251	88	7.6	92	4.8
Anthracene-9,10-dione	251	85	2.2	92	4.8
1-Methylanthracene-9,10-dione	251	69	4.8	86	6.4
Benz[a]anthracene-7,12-dione	251	60	2.2	71	5.8

[a] Standard deviation.

and was held for 3 min. The oven was heated at 6°C/min to a final temperature of 270°C and was held for 10 min. For the trimethylsilylated derivatives of fraction III initial oven temperature was 80°C and was held for 3 min. The oven was heated at 10°C/min to a final temperature of 270°C which was held for 8 min.

RESULTS AND DISCUSSION

Recoveries

The average percent recoveries of the polar PAH derivatives after ultrasonic extraction with dichloromethane and clean-up using SPE are listed in Table 1. Particularly the ketonic and quinonic derivatives of the higher molecular PAHs showed recoveries higher than 70% when extraction was performed after acidification of the soil matrix. Though these results could be further enhanced by omitting this acidification step, the described method was used in the following degradation study because of the improved extraction results with regard to the more polar aromatic alcohols and 1-hydroxy-2-naphthoic acid.

A crucial step during clean-up of the soil extracts is the solid phase extraction procedure. Silica deactivated with 6% water was used since studies with the activated stationary phase as used for the separation of mineral oils into structure types[4,9] have shown that highly polar compounds such as phenolcarboxylic acids and dihydroxyphenols either could not or could only scarcely be eluted. A similar phase (10% water) was used in the column chromatography scale by Bodzek *et al.* for the fractionation of oxygen derivatives of PAHs in airborne particulate matter[12].

The PAH degradation was estimated with a fast and reproducible ultrasonic extraction method using a solvent mixture of cyclohexane-acetone (v:v; 1:1) that has been proved effective in a previous study[9]. The average percent recoveries and standard deviations of the four PAHs after 1 d incubation are listed in Table 2. The given values of 95 to 107% demonstrate the quantitative yield in one extraction step.

Table 2 Recoveries of PAHs from soil/compost mixtures after ultrasonic extraction (n = 4).

PAH	*Fortification, mg/kg*	*Recovery, %*	*S.D.*[a]
Fluorene	9.04	95	1.3
Phenanthrene	8.17	104	1.6
Fluoranthene	10.58	106	1.3
Benz[a]anthracene	2.23	107	4.9

[a] Standard deviation.

Degradation study

Using this developed method a degradation study with four PAHs was conducted. Of these only phenanthrene was almost completely degraded during the eleven weeks of the experiment. After a lag-phase of two weeks the amount decreased rapidly within two weeks to about 7% of the initial amount and further to 3% during the following eight weeks (Figure 1). Fluorene was degraded slowly; 33% of the initial concentration remained after eleven weeks. The concentrations of fluoranthene and benz[a]anthracene decreased only insignificantly.

After 4 weeks in SPE fraction III 1-hydroxy-2-naphthoic acid was identified as a phenanthrene metabolite (Figure 2). The identification was confirmed by its UV-spectrum and its retention time during HPLC analysis. Though phenanthrene was degraded almost completely in the two weeks before, only small amounts could be detected and were not recoverable another two weeks later. This ring cleavage product has been shown to be a major intermediate during phenanthrene degradation in culture media[13-15].

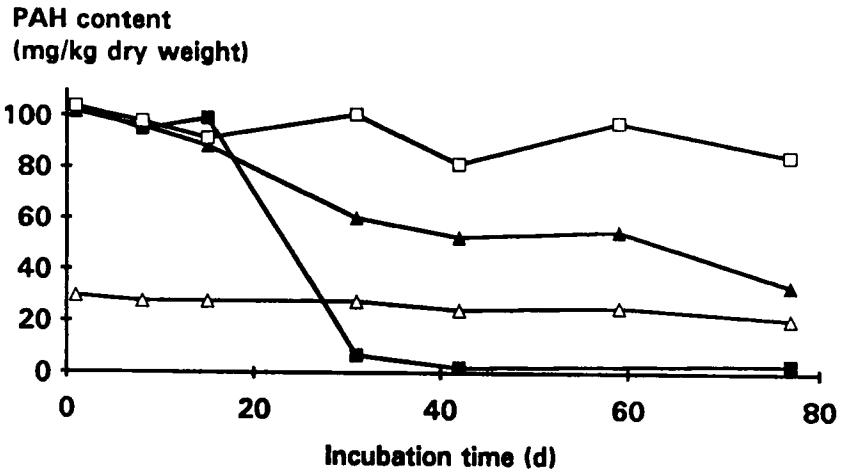

Figure 1 Course of concentrations for PAHs in soil/compost mixtures during degradation study. Flu = fluorene, phe = phenanthrene, fln = fluoranthene, b[a]an = benz[a]anthracene.

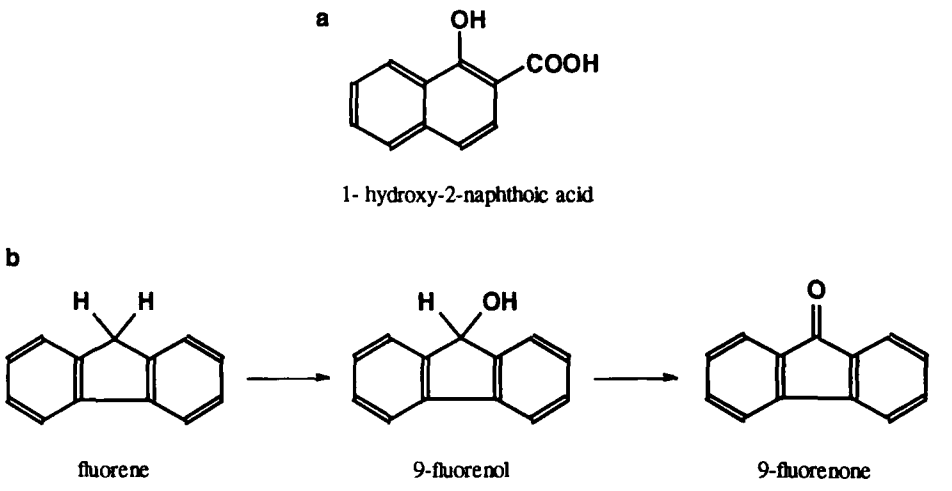

Figure 2 Identified oxidation products of a) phenanthrene and b) fluorene.

From the soil/compost mixtures contaminated with fluorene two metabolites were identified (Figure 2). Fraction II eluted with dichloromethane contained 9-fluorenone, which was identified from its UV-spectrum with absorption maxima at λ of 258 and 250 nm and its mass spectrum with a molecular ion (M^+) at m/z 180 and fragment ions at m/z 152 (M^+-CO) and m/z 76, and also by comparing its retention time to that of the authentic standard. Analogously, 9-fluorenol could be identified in fraction III. The substance has UV absorption maxima at λ of 228, 235 and 272 nm; for GC-MS analysis an aliquot of the polar fraction was trimethylsilylated with BSTFA: the corresponding derivative of 9-fluorenol exhibited a molecular ion (M^+) with an *m/z* of 254 and fragment ions at m/z 165 (M^+-$OSi(CH_3)_3$), m/z 239 (M^+-CH_3), and m/z 73 ($Si(CH_3)_3^+$). This metabolite was detected from the first up to the eighth week with the highest amount after one week (0.2 mg/kg). 9-fluorenone was determined over the whole experimental period with higher amounts in the first (0.7 mg/kg) and the second week (1.1 mg/kg) and then constant small concentrations from the fourth week (Figure 3).

Both 9-fluorenol and 9-fluorenone have been described several times before as the main fluorene metabolites. Different bacterial strains from coal gasification sites, oil refinery wastewater sludge, and also from soil not contaminated by PAHs can oxygenate the C-9 carbon by monooxygenases to form 9-fluorenol and 9-fluorenone[15-17]. Though these have been found as dead end products, a further degradation via angular 1,1a-dioxygenation has been discussed[17].

Both these oxidation products were also found in the systems fumigated with chloroform. In contrast to the non-fumigated mixtures no decrease of concentrations was observed after the relatively high amounts in the beginning: the concentration of 9-fluorenol remained constant during the whole experimental time (0.3 mg/kg), whereas the concentration of 9-fluorenone increased continuously from 0.1 mg/kg in the beginning up to 3.6 mg/kg after eleven weeks (Figure 2). The amounts of fluoranthene (85%) and phenanthrene (88%) remained almost constant, only fluorene showed a significant decrease (68%). Though no CO_2-production was detected with the calcium hydroxide-indicator method during experimental time, 2.5×10^4 colony-forming units per g of soil were counted after eleven weeks, indicating non-sterile conditions.

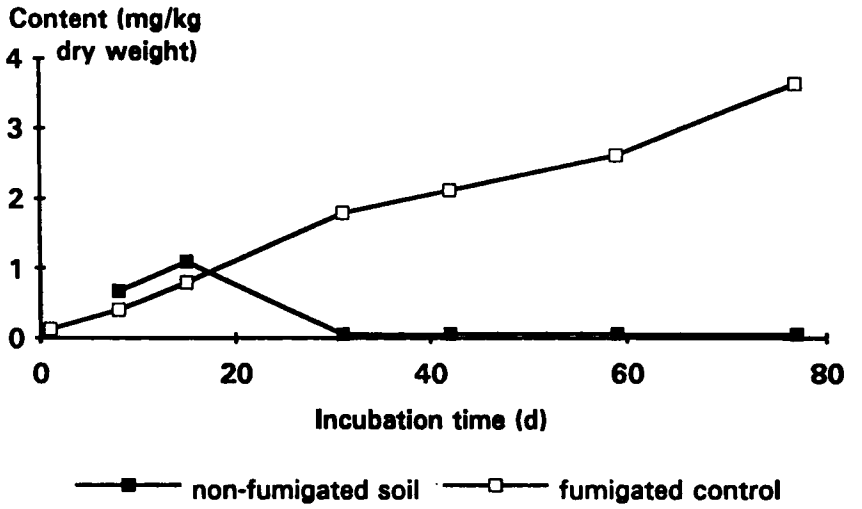

Figure 3 Course of 9-fluorenone formation in soil/compost mixtures fortified with fluorene.

To find out whether this formation of small amounts of oxidation products is a result of remaining biological degradation, an alternative sterilization method including mercury(II)chloride, which has been proved to suppress any biotic reactions, was performed with a fortified mixture of both fluorene and phenanthrene. In this case also the same products could be estimated in similar amounts while both PAHs disappeared in a comparable manner: after seven weeks 94% of the initial amount of phenanthrene were recovered whereas only 81% of fluorene remained. The count of < 10 colony-forming units per g of soil confirmed sterile conditions.

The appearance of oxygenated derivatives in soil/compost mixtures free from germs show that other than microbiological mechanisms accompany the PAH degradation. In the literature abiotic/chemical oxidation catalysed by inorganic soil components (e.g. iron and manganese oxides) is discussed[18]. However, the higher degradation rates in the non-germ-reduced systems and the lack of metabolite accumulation in contrast to the sterilized soils prove that biotic transformations are the main paths.

Acknowledgements

The authors thank the "Deutsche Forschungsgemeinschaft" for financial support of "Sonderforschungsbereich 188: Reinigung kontaminierter Böden". H. Wischmann gratefully acknowledges K. Macht for conducting extractions and hplc analysis during the degradation study and G. Schaefer for providing know-how and technical assistance in preparation of the fumigated controls.

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