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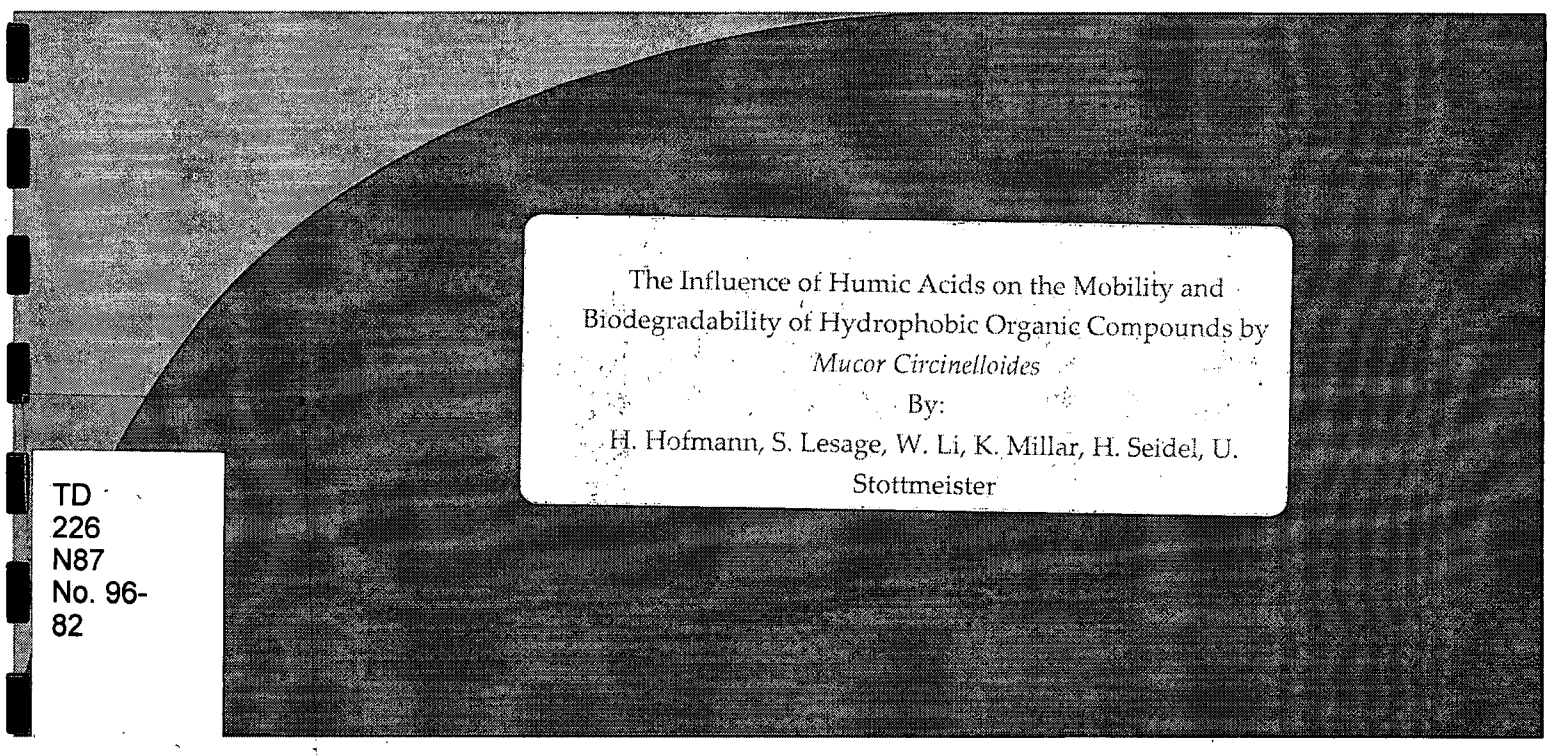

The Influence of Humic Acids on the Mobility and Biodegradability of Hydrophobic Organic Compounds by *Mucor Circinelloides*

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THE INFLUENCE OF HUMIC ACIDS ON THE MOBILITY AND BIODEGRADABILITY OF HYDROPHOBIC ORGANIC COMPOUNDS BY *MUCOR CIRCINELLOIDES*

Key words: Polycyclic Aromatic Hydrocarbons (PAHs); Bioavailability; Humic Acid; *Mucor circinelloides*; Sorption

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MANAGEMENT PERSPECTIVE

Title: The Influence of Humic Acids on the Mobility and Biodegradability of Hydrophobic Organic Compounds by *Mucor Circinelloides*

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EC Priority/Issue: This work is done under the general mandate of groundwater remediation, addressing toxics. The project was funded in part by the government of Germany under the Canada-Germany agreement and partly by GASReP (Groundwater and Soil Remediation Program- Natural Resources Canada). The objective of the project is to provide scientific data for the development of a new sustainable groundwater and soil remediation method for PAHs, which are on the CEPA priority substance list

Current Status: This is in the second year of a three year project funded by GASReP conducted at NWRI on the use of humic acids as a sustainable alternative to surfactant for the remediation of soils and groundwater contaminated by heavy petroleum products. This paper describes work done with fungi in Germany and in Canada. Results show that even if the aqueous concentrations of PAHs are significantly increased by the addition of humic acid, the biodegradation rate is not for most compounds. The humic acids are not toxic to fungi. The work with bacteria is ongoing in Canada and a comparison of the two will be published next year.

Next Steps: This information will formed the science base for the use of humic acids as a new remediation technology. It is only the beginning in understanding the long term fate of PAH metabolites in the process of humification. The current studies were conducted in the lab. It is intended that fungi and bacteria will be used at the larger scale in AQUEREF, before eventually going into field trials.

ABSTRACT

The addition of humic acid to a Polycyclic Aromatic Hydrocarbon (PAH) containing malt extract broth of *Mucor circinelloides* increases the amount of the hydrocarbon in the aqueous phase significantly. However, this process does not correlate with an enhanced bioavailability of the PAHs. The hydrophobic compounds are apparently bound to the humic acid and are therefore not available to microorganisms. For 9-methyl anthracene, the amount of detected metabolites decreased from 30% in the absence of humic acid after two days to 12% or less in the presence of humic acid. In the absence of humic acid and with 50mg/l humic acid, phenanthrene is transformed to several metabolites, typical for fungal transformation reactions of this substrate. These metabolites were liberated into the aqueous phase. The main part of the non-converted phenanthrene was bound adsorptively at the biomass. Higher humic acid concentrations significantly inhibit the fungal activity. Higher PAHs (pyrene, benzo[a]pyrene) are fixed much more strongly to the biomass and the transformation process is slower. These substrates are also forming complexes with the humic matrix. The extraction of the PAHs from the aqueous phase by solid phase extraction depends on the number of condensed rings and decreases significantly for benzo[a]pyrene to phenanthrene.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread organic pollutants. PAHs are mainly formed by forest and agricultural fires or by combustion of fossil fuels / 1-3 / and are found in all parts of the environment (soils, sediments, water and groundwater) / 4-7 /. Many PAHs are known to be hazardous for humans and animals because of their high mutagenic and cancerogenic potential / 8-11 /. The oxygen containing metabolites induce cancerogenic reactions in organisms and lead to genetic damages / 12,13 /. For instance, a dihydrodiol metabolite of benzo[a]pyrene, a tobacco smoke ingredient, has been linked human lung cancer / 14 /.

Because of their potential to be highly toxic to humans and the ecosystem, many PAH contaminated areas have to be remediated. In addition to chemical and physicochemical methods, like pyrolysis / 15 /, soil washing / 16 /, or solvent extraction / 17 /, microorganisms are often used for the degradation of these compounds / 18,19 /. The biodegradability decreases generally from lower to higher molecular weight PAHs. Whereas several bacteria are known to use PAHs as their sole carbon and energy source and degrade them completely / 20,21 /, most of the fungi are unable to use PAHs as their sole substrate / 22-25 /. Normally, fungi cometabolize PAHs to partial degradation products in the presence of an additional carbon source / 26 /. In preliminary studies it was found that, amongst all yeasts and fungi isolated from a PAH-contaminated rot mixture, a *Mucor* species had the highest transformation potential for low molecular weight PAHs / 27 /. To our knowledge, no transformation of PAHs with *Mucor circinelloides* species has been previously described in literature.

Because of their low aqueous solubility, PAHs are mainly fixed adsorptively at solid matrices (soil, sediment, biomass) / 28-30 /. Remediation processes under natural conditions are therefore much slower than under idealized laboratory conditions, where interaction of the substrate with additional matrices can be minimized / 31-33 /. The limiting factor for a degradation process is often the low effective concentration of the pollutant in the aqueous phase as a requirement for microbial uptake / 34-36 /.

Surfactants are often used to enhance the concentration of the PAH in the aqueous phase / 37-39 /. The use of artificial surfactants to enhance

biodegradation have met mitigated success, because while the solubility of the target compound is increased it is accompanied by negative side effects such as toxicity / 40 /, competing carbon source or high persistence in the environment. Also, a decreased bioavailability of phenanthrene because of a lower concentration of freely dissolved substrate in the aqueous phase was observed with non-ionic surfactants / 41 /. In that context, humic acids which can be considered "natural surfactants", were considered as a potential alternative / 42,43 /.

This study was aimed at investigating the possible enhancement of the mobility of PAHs by humic acids in the presence of fungi, the differentiation between "freely dissolved" and matrix-bound PAHs, and the biodegradability of these compounds by the soil fungus *Mucor circinelloides*. Initial experiments were conducted at the UFZ, Germany, with ^{14}C-labeled 9-methylanthracene and with free humic acid, whereas in the second part of the studies, made at the NWRI, Canada, labeled phenanthrene, pyrene and benzo[a]pyrene were transformed in the presence of different concentrations of Na-humate. The amount of substrate and metabolites was determined by a sequential fractionation of the transformation mixture using solid phase extraction of the aqueous phase on Sep-Pak C18 cartridges / 44 /. The nature of the bonds formed between the substrate and the matrices were inferred from the results. Some of the fractions were analysed by HPLC to detect metabolites.

MATERIAL AND METHODS

Chemicals

The [CH_3-^{14}C]-9-methyl anthracene was synthesized from anthrone and [^{14}C]-methyl iodide. The compound has a specific activity of 670 nCi/mg and a purity of ~85% (radiochemical purity). The impurities are polymers, that cannot be analysed by GC or HPLC. The unlabeled 9-methyl anthracene was obtained from Merck (>97%,GC). The [9-^{14}C]-phenanthrene and the unlabeled phenanthrene were obtained from Sigma chemical Co. (St. Louis, MO) with a purity of >98% and a specific activity of 13.3 mCi/mmol (HPLC with radioactivity and UV detector at 220 nm). The unlabeled pyrene was obtained from Aldrich

Chemical Co., Inc. (Milwaukee, WIS) and has a purity of 99% (GC). The [4,5,9,10-^{14}C]-pyrene was from Sigma chemical Co. (St. Louis, MO) and has a purity of >98% and a specific activity of 32.2 mCi/mmol (HPLC with radioactivity and UV detector at 240 nm). The unlabeled Benzo[a]pyrene was from Sigma Chemical Co. (St. Louis, MO) and has a purity of >98% (HPLC). The [7,10-^{14}C]-benzo[a]pyrene was obtained from Amersham corporation (Oakville, Ont.) with a purity of >98% and a specific activity of 61.0 mCi/mmol (HPLC with radioactivity and UV detector at 240 nm).

The radioactivity analyses were made with the following scintillation cocktails: for organic extracts - Ultima Gold^{Tm} XR (Fa. Packard) or PCS^{R} (Amersham Corp., Arlington Heights, IL) and for the NaOH samples (for $^{14}CO_2$ detection) Hionic-Fluor^{TM} (Fa. Packard) or ScintiVerse II (Fisher Scientific, Ottawa, Ont.). For the radioactivity determination a TRI-CARB Liquid Scintillation Spectrometer 3255 or 1900 TR (Fa. Packard) were used.

The organic solvents used for the extraction procedure and for HPLC, were obtained as follows: methanol - Merck (Germany) or J.T. Baker, Inc. (Phillipsburg, NJ); acetone, hexane, ethyl acetate - Merck (Germany) or Caledon Laboratories Ltd. (Georgetown, Ont.); acetonitrile - Merck (Germany) or Fisher Scientific (Ottawa, Ont.). For the microbial and chemical purposes distilled water (for the experiments with 9-methyl anthracene) or water, purified by reverse osmosis and filtration (for the other substrates) was used.

The malt extract was obtained from Merck, Germany (17g/L) or from Oxoid (20g/L malt extract and 5g/L peptone from soya). The malt extract agar was obtained from Merck, Germany (30g/L malt extract, 3g/L peptone from soya, 15g/L agar-agar, pH 5.6) or from Difco (12.75g/L maltose, 2.75g/L dextrin, 2.35g/L glycerol, 0.78g/L peptone, 15g/L agar, pH 4.7).

The solid phase extraction (spe) was made with SepPak VacRC C18 cartridges (500mg adsorbent) Fa. Waters (Milford, MA). A comparison of extraction efficiency was also made with C8 cartridges (Waters) and C8 and C18 Empore disks 47mm diam. (Analytical Chem. International, Harbor City, CA).

Cultures and growth conditions

Two *Mucor circinelloides* species were tested. One of them was isolated from a rot mixture, consisting of straw and river sediment, which was contaminated artificially with 9-methyl anthracene. The other culture was obtained from the Dutch culture collection (CBS 27743). Both species were found to have very similar transformation potential.

The fungi were grown in malt extract solution under sterile conditions at a temperature of 28-30°C. For the experiments with 9-methyl anthracene, the fungi were incubated on malt extract agar plates (10mL agar per plate) and each shaking flask (500mL erlenmeyer flask with 100mL malt extract broth) was inoculated with 3 pieces (5mm diam.) from the rim of the mycelium. For the other PAH transformations, the fungi were cultivated on malt extract agar plates (10 mL Difco-agar per plate) and, after 2 days of incubation, 250ml shaking flasks (50mL Oxoid-malt extract broth, pH adjusted to 5.5) were inoculated with 3 pieces each (5mm diam.) from the rim of the mycelium. The flasks were shaken at 30°C on a horizontal shaker (100-150 r.p.m.). All agar mixtures and malt extract solutions were autoclaved for 15 min at 121°C before inoculation under sterile conditions.

Transformation procedure

After a 2-3 day incubation period, PAHs and humic acid were added under sterile conditions. The PAHs were dissolved in nonsterile DMF (N,N-dimethylformamide) and added to the culture broth (1%, v/v).

Concentrations and activities of the different PAHs: 9-methyl anthracene 10mg (=100mg/L) with 1.51μCi; phenanthrene 2.5mg (=50mg/L) with 0.5μCi; pyrene and benzo[a]pyrene 0.25mg (=5mg/l) with 0.5μCi.

For 9-methyl anthracene, free humic acid (Aldrich) was added as a sterilized powder (1500 and 3000mg/l) to each 100mL of the culture broth. For the other PAHs, sodium humate (Aldrich) was used. The nonsterilized humate powder was dissolved in a sterile malt extract solution and 10mL aliquots of the mixture were added to each flask (50, 500 and 2000mg/l). For the flasks without humic

acid, the volume was made up to 60mL by the addition of 10mL of the pure malt extract.

Extraction procedure

After the transformation was stopped, the biomass was separated by filtration through a glass fiber filter (type A, 47mm, Gelman Instrument group, Ann Arbor, MI), the wet weight of the mycelium was determined and the biomass then placed into acetone. The mixture was allowed to stand overnight at room temperature, followed by a 15min. sonication in an ultrasound bath. The solvent was then filtered off and the mycelium washed twice with a small amount of acetone. A subsample of the acetone solution was taken for radioactivity measurement. The acetone extract was evaporated to dryness redissolved in 15mL of acetonitrile for HPLC analysis.

The biomass was allowed to dry for several hours at room temperature, then 50ml of methanolic potassium hydroxide solution were added (5g of KOH, 5ml of water, 45ml of methanol). The hydrolysis mixture was allowed to stand overnight at room temperature, followed by an addition of 30ml of water. The liquid phase was then separated by filtration. After sampling for the radioactivity the solution was neutralized with HCl and the organic solvent was evaporated under mild conditions using a rotary evaporator. Especially for lower molecular weight PAHs (phenanthrene, 9-methyl anthracene) the evaporation with a rotary evaporator led to significant losses of radioactivity. Therefore subsequently, the solvent was allowed to evaporate in a fume hood at room temperature, thus reducing substrate losses. The residual aqueous phase was then extracted with ethyl acetate or by solid phase extraction (see below).

The aqueous phase of the transformation mixture was extracted in case of 9-methyl anthracene with ethyl acetate, or, in case of the other PAHs, by solid phase extraction. Before extraction, a sample for radioactivity measurement was taken. Radioactivity was also measured from the residual aqueous phase and from the organic phase.

In case of 9-methyl anthracene, the biomass was also combusted after hydrolysis to determine irreversibly bound compounds. Additionally, a constant flow of sterile air was used to flush the flasks for the determination of evaporated

substrate by adsorption on an organic carrier material (Octadecyl) and to collect formed $^{14}CO_2$ in 5N NaOH.

Solid phase extraction (SPE)

It was decided to change from liquid-liquid extraction to solid phase extraction because the latter method is much faster, easier and cheaper with a comparable effectiveness, at least for non- and semipolar compounds. SPE was done mainly with SepPak C18 cartridges. The cartridges were pretreated with 5mL of acetonitrile and with 10mL of distilled water and used according to manufacturer's specification. After finishing the extraction, the cartridge was not dried with vacuum to avoid losses of activity by evaporation of the PAH. The organic compounds were eluted first with 5ml of acetonitrile and then 2x with 4ml of the same solvent. The fractions were collected in a graduated 15ml sample tube with glass stopper. The extract was made up to 15mL with acetonitrile and stored in a refrigerator until analysis. C8 Cartridges (Waters), were tested using the same method. In addition, C8 and C18 Empore disks were tried. The disks were placed into a sintered glass filter funnel system with a 1 L washing bottle. The disks were pretreated with sequential elution of 5mL acetonitrile, 5mL methanol and 10mL water. The aqueous phase was passed completely through the disk with a weak vacuum and collected in a 60ml tube, placed in the bottle. The disk was extracted with 3x 5ml of acetonitrile collected in a 15mL graduated tube, and stored in a refrigerator until analysis.

HPLC analysis

To detect metabolites, an HPLC Class-LC10 Fa. Shimadzu with a Photo Diode Array detector (wavelength range 200-400nm) was used. The best results for the separation of 9-methyl anthracene and phenanthrene metabolites were obtained with a narrowbore column (Fa. Vydac, 2.1mm i.d., length 150mm, particle size 5µm) and the following gradient (% acetonitrile : % bidistilled water with a flow rate of 0.2ml/min): 0-1min 10:90, 1-50min linear change to 90:10, 50-70min 90:10, 90-110min linear change to 10:90.

The LC-MS analyses were made with an instrument Fa. Hewlett-Packard (methanol-water 80:20 isocratic, thermospray interface, 50-300 amu).

RESULTS

9-Methyl anthracene

The initial experiments were made with unlabeled 9-methyl anthracene / 26 /. This substrate was transformed in the absence of humic acid and with 1500 and 3000mg/L of humic acid, respectively. The extracts were analyzed by HPLC and the metabolites were identified by comparison with literature data / 45 /. The primary metabolites were identified as 9-hydroxymethyl anthracene and 9-methyl anthracene dihydrodiol (1,2- and 3,4- regioisomers, respectively). Both compounds were further transformed into the trihydroxy metabolite (see fig. 1). Other metabolites were not identified.

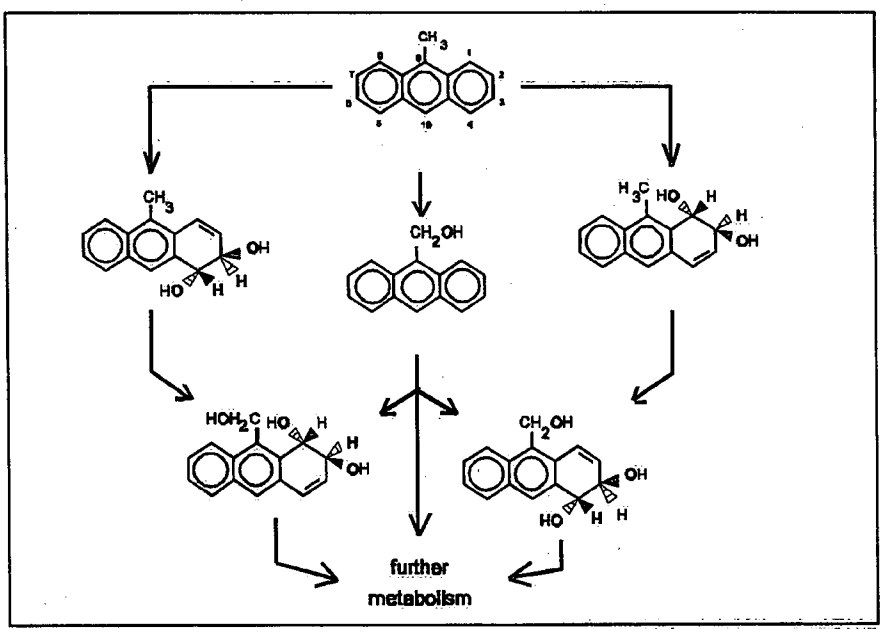

fig. 1: metabolic pathway of 9-methyl anthracene with *Cunninghamella elegans* redrawn from / 45 /

The metabolites were identified by HPLC, by their UV spectra and by LC-MS. For the diol, two peaks were found in the chromatogram, whereas for the triol only one of the two possible peaks was present. The methods used did not permit to distinguish the two isomers. We were unable to reproduce the separation results of Cerniglia /45/.

The total amount recovered and the amount of detected metabolites decreased over time probably because of the formation of nonextractable complexes of substrate and humic acid. Whereas in the absence of humic acid ca. 30% of the substrate were transformed into metabolites within the first 2 days, this amount decreased significantly in the presence of humic acid. Lower recoveries were observed in the experiment without humic acid because of the formation of more polar metabolites, which have a higher aqueous solubility and are not extractable (see below the results with labeled substrate). The relatively low contrast between the different experiments might be due to the use of free humic acid instead of the water soluble salt. Over the incubation period under these conditions the powder didn't dissolve completely. The contact between the PAH and the humic acid was not as intensive as with dissolved humic acid. In the absence of biomass, a strong sorption of the PAH to the free humic acid was also found, but mainly after 2-3 weeks. The extraction of the mixture led to a recovery of less than 70% (data not shown). The percent recovery and the amounts of different metabolites are shown in figure 2.

Because of the incomplete mass balance, labeled 9-methyl anthracene was used for further experiments in the absence of humic acid. It was found that no $^{14}CO_2$ was produced by the fungus *Mucor circinelloides*. As it was expected, the fungus is unable to mineralize the PAH completely. Evaporation of the substrate and irreversible binding (resistant to hydrolysis) of the PAH or metabolites to the biomass, were also not observed. The amount of radioactivity in the different fractions of the transformation mixture are shown in figure 3. The most significant

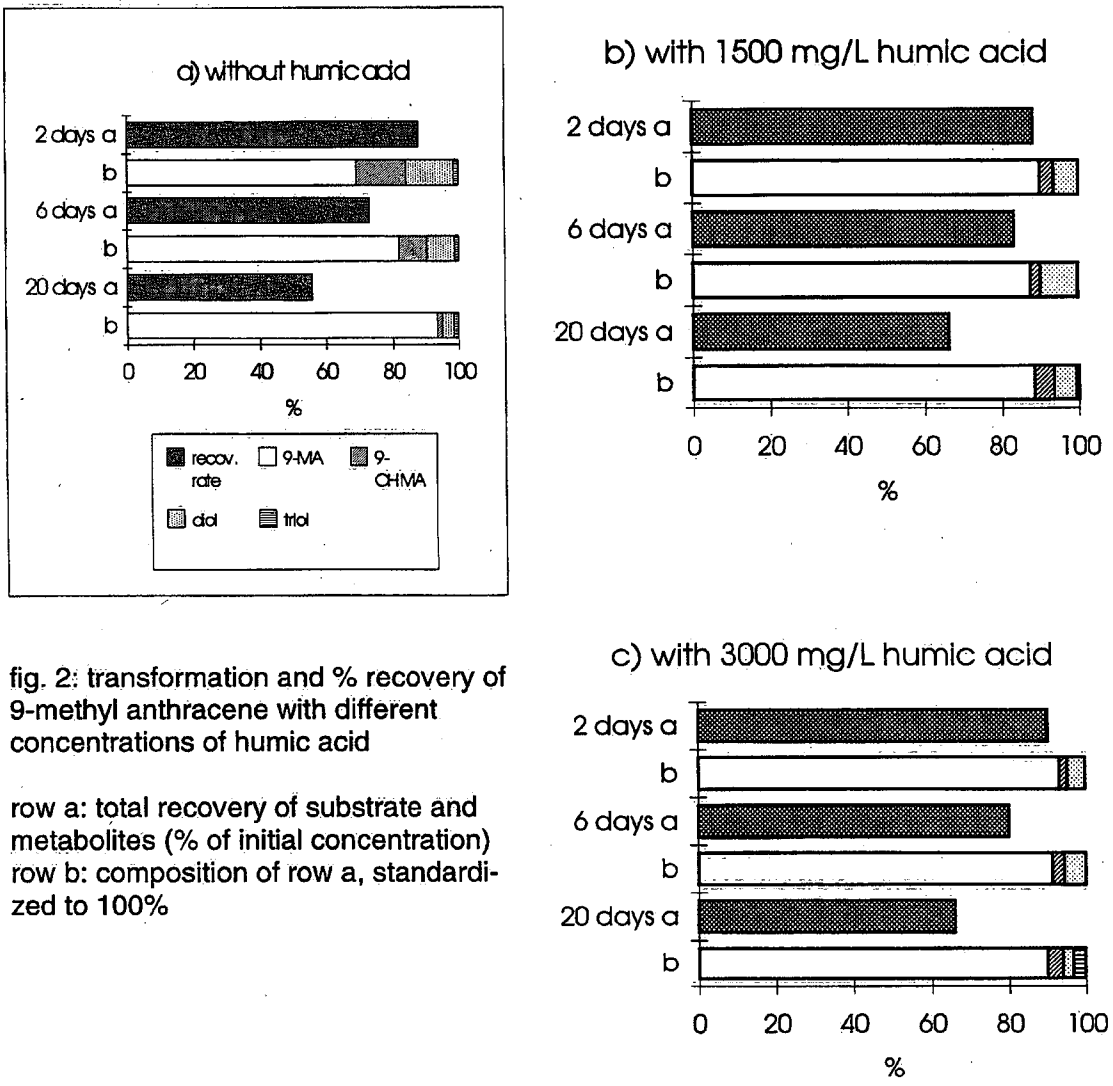

fig. 2: transformation and % recovery of 9-methyl anthracene with different concentrations of humic acid

row a: total recovery of substrate and metabolites (% of initial concentration)
row b: composition of row a, standardized to 100%

change was found in the aqueous phase where the activity increased from 11% after 3 days to 35% after 20 days (total extractable and non-extractable).

Whereas after 3 days 90% of the radioactivity in the aqueous phase could be extracted with ethyl acetate and analyzed by HPLC, this amount decreased to 40% after 20 days. These values correspond to the formation of metabolites.

Reextraction of the acidified residual aqueous phase at 20 days, resulted in the extraction of ca. 7% of very polar, yet to be identified compounds.

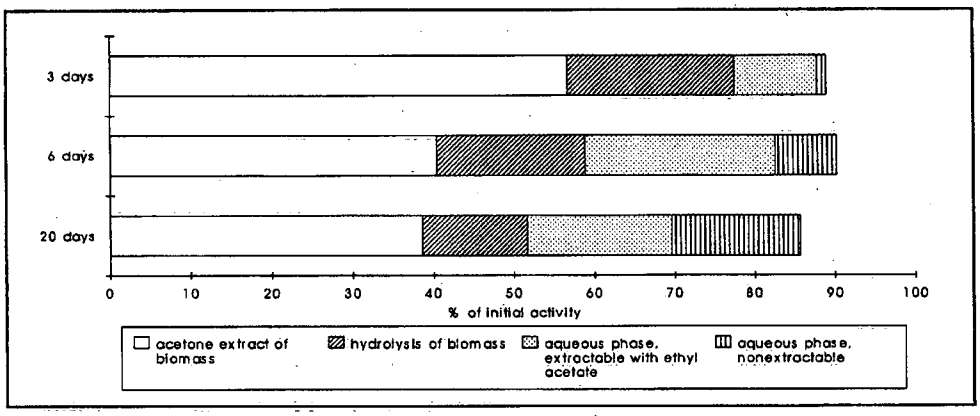

fig. 3: ^{14}C Activity distribution in different fractions with increasing incubation times.

Phenanthrene

Phenanthrene is a substrate that is relatively biodegradable. Many fungi are described in the literature, producing various metabolites, including three monohydroxylated phenolic derivatives and two dihydrodiols / 46,47 /.

^{14}C labeled phenanthrene was incubated with fungi without and with an addition of 50, 500, and 2000 mg/L of humic acid in form of the sodium salt. Because of the use of sodium humate adjusted to a pH of 5.0-5.5 instead of the free acid, no precipitation occured.

The distribution of radioactivity in the different fractions is shown in fig. 4. The main portion of the ^{14}C activity is clearly associated with the biomass. HPLC analysis of the acetone extracts showed that they consisted of unchanged phenanthrene. The fraction liberated by hydrolysis of the biomass also almost only consisted of the parent hydrocarbon. The hydrolysis step releases not only chemically bound metabolites, but also compounds which are strongly sorbed to the biomass or incorporated into the mycelium without changes in their chemical

structure. The proportion of activity released by hydrolysis did not change over time and did not seem to be influenced by the presence of increasing humic acid concentrations.

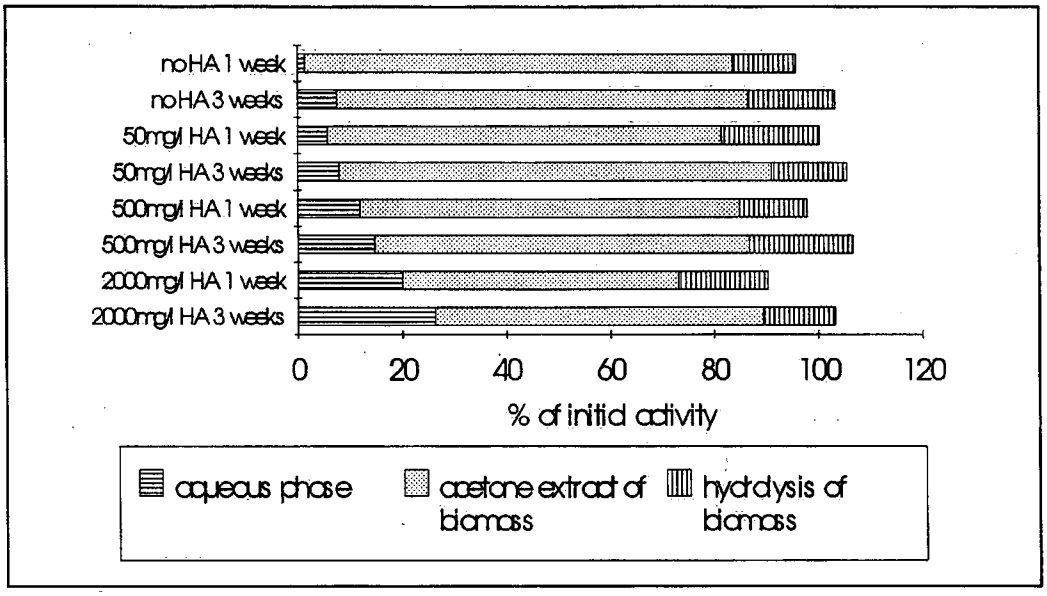

fig. 4: effect of different concentrations of humic acid on the distribution of radioactivity.

The main effect of humic acid is the significant increase of the amount of activity in the aqueous phase. Up to 25% of the initial activity was found in the aqueous phase in the presence of 2000mg/l humic acid (~15 mg/l phenanthrene, tenfold its aqueous solubility). More than 90% of this activity was extractable by s.p.e. on C18 cartridges and eluted with acetonitrile. The organic extracts were analyzed by HPLC. Metabolites were only found in the microcosms containing 50 mg/L or no humic acid. Whereas after one week only traces of oxygenated compounds were found in both experiments, a prolongation of the incubation time resulted in significantly higher amount of metabolites. In the experiments with higher humic acid concentrations only unchanged substrate was found in the aqueous phase. The humic matrix appears to sorb the PAH, forming a water soluble complex, in equilibrium with the dissolved PAH. The PAHs bound to the

humic acid are not bioavailable because the microbial membrane must compete with humic acid for the sorption of the PAH.

It is interesting to mention that all of the phenanthrene metabolites were liberated into the aqueous phase (at least up to 3 weeks). No chemical reaction with the humic matrix or with the biomass was observed. Initially metabolites were found only in the aqueous phase, indicating that no fixation at the biomass or other solid particles had occured. When the aqueous phase was extracted on cartridges, an almost complete retention of the activity was observed at the stationary phase, which was subsequently eluted with acetonitrile. No (or low amounts) of activity was found in the residual aqueous phase after extraction.

Extraction efficiency using solid phase

The solid phase extraction leads to an almost complete retention of the phenanthrene at the stationary phase. Comparative studies with the C8 cartridges and the C8 and C18 extraction disks led to similar extraction results. While the effectiveness was similar, the procedure using C8 material was slower because of the stronger interactions between the stationary phase and the humic matrix. The extraction cartridges were preferred because the apparatus allows for the simultaneous extraction of three samples, whereas the filters extraction apparatus allowed for the processing of only one sample at a time. For larger volumes without a high macromolecular content, the disks second method might have some advantages.

Pyrene and Benzo[a]pyrene

Because higher m.wt. PAHs are less water soluble and are transformed more slowly, 5mg/L pyrene and benzo[a]pyrene were used (compare to 9-methyl anthracene: 100mg/l, phenanthrene: 50mg/l) and the microcosms were extracted for the first time after 3 weeks of incubation. Only one level of humic acid was used (500mg/L). The activity distribution of the different PAHs in comparison to that of phenanthrene under similar conditions are shown on figure 5. There is a significant difference in the strength of the fixation of different PAHs. Whereas

the main part of the biomass bound phenanthrene is acetone extractable, higher PAHs were only partially extracted with acetone. Thirty to forty percent of the initial activity was found in the basic hydrolysis solutions. Similar to phenanthrene, the addition of humic acid leads to an increase in the amount of substrate in the aqueous phase. In both cases, the amount of PAH in presence of sodium humate is much higher than the solubility of these compounds in pure water (benzo[a]pyrene: ~1mg/L in the aqueous phase, aqueous solubility <5µg/L / 48 /).

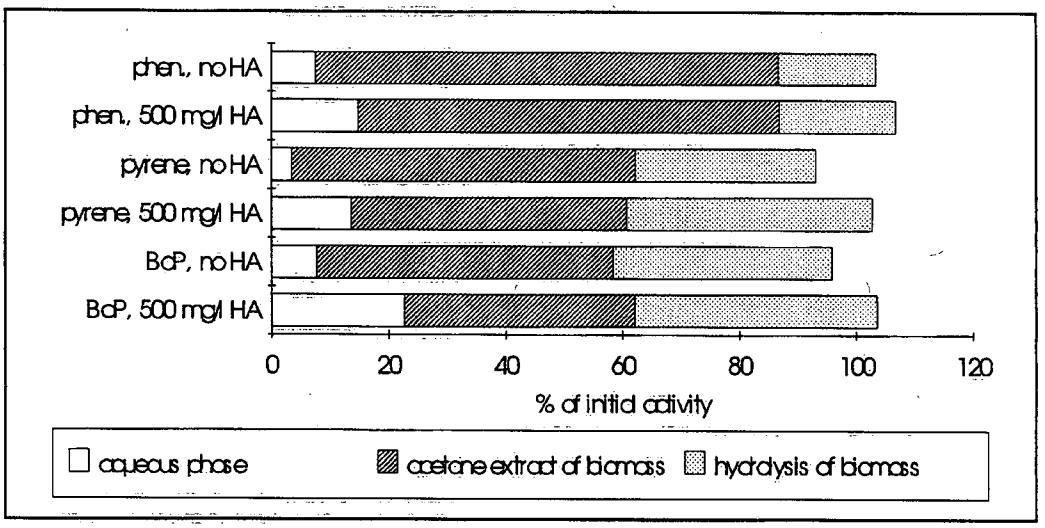

fig 5: distribution of PAHs in different fractions of the transformation mixture after 3 weeks

It was determined by HPLC, that all of the activity of the acetone extract and of the hydrolysis solution of the biomass consisted completely of unchanged substrate. For the analysis of the aqueous phase, the liquids were extracted by solid phase extraction on C18 cartridges. Figure 6 shows the effectiveness of the extraction. Compared to the respective experiment with phenanthrene, an increasing number of condensed rings leads to a much less complete extraction of the PAHs from the aqueous phase, especially in the presence of humic acid Whereas with phenanthrene the solid phase extraction from the aqueous phase is nearly complete without humic acid, and in a range of 80-90%, even in the presence of humic acid, in case of pyrene, 80% of the PAH are recovered

without humic acid and two third with an addition of humic acid, respectively. The recovery of benzo[a]pyrene was in the range of 50% at the stationary phase, when no humic acid was added, and of ~35% in the presence of humic acid. This phenomenon can be explained by the partitioning equilibrium between the humic acid bound PAH, the "freely dissolved" PAH and the stationary phase of the extraction cartridge for the investigated PAHs. If the aqueous solubility is high enough (e.g. for phenanthrene), the extraction time is sufficient for a complete extraction. In case of higher m.wt. PAHs, the aqueous solubility of the pure compound is significantly lower. The equilibrium is on the side of the humic acid complex, and therefore the extraction would need a much longer time to go to completion. Interactions between PAHs and components of the malt extract broth and / or the presence of extracellular fungal surfactants might be the reason for the relatively high amount of activity, compared to the solubility of the compounds in pure water, in the aqueous phase of experiments without humic acid.

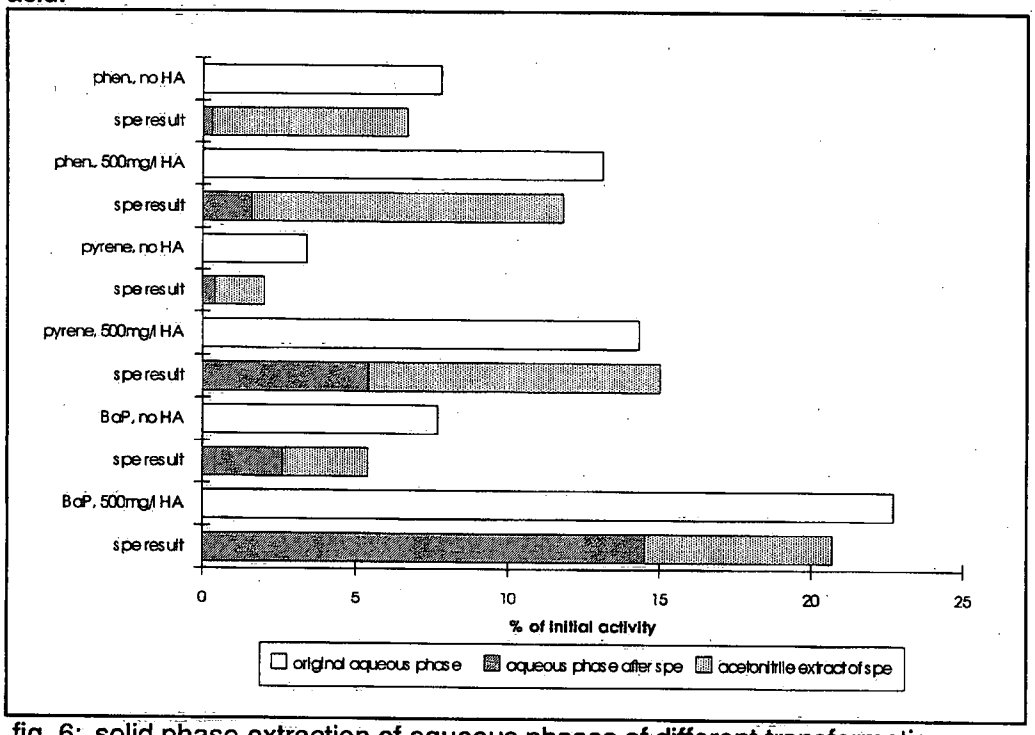

fig. 6: solid phase extraction of aqueous phases of different transformation mixtures (see fig. 5)

The acetonitrile extracts were analyzed by HPLC. In the case of pyrene, only the parent hydrocarbon was found in the microcosms with and without humic acid.

For benzo[a]pyrene, one possible metabolite was found in the acetonitrile extract of the SPE of the aqueous phase in the microcosm with 500 mg/L of humic acid. This compound elutes approx. 10 min earlier than the parent PAH and the UV spectra of both compounds are very similar. Further investigation on the structure of this compound was still impossible because of the extremely low concentration. In the experiment without humic acid this metabolite was not detected, probably because of an even lower concentration in the aqueous phase.

DISCUSSION AND CONCLUSION

The experiments have shown that the addition of humic acid to a PAH containing system increases the solubility of these hydrophobic compounds significantly. There are interactions between the hydrocarbons and the humic matrix, which lead to an adsorptive fixation of the PAH, unlike surfactants which only enhance dissolution above their critical micelle concentration. Humic acids show a strong potential to enhance dissolution, which might be useful for the extraction of hydrophobic organic pollutants from contaminated solid matrices (soil, sediment). This can be an inexpensive and easy alternative to artificial surfactants or oil-surfactant microemulsions for technical applications. The pollutant containing humic acid could be precipitated after extraction by changing the pH to concentrate the organics for further treatment.

The increase in aqueous concentrations of PAHs did not result in an enhanced rate of biodegradation by *Mucor circinelloides*. The PAHs which are transported into the aqueous phase in the presence of humic acid, must be fixed to the humic acid molecules and are not easily available, at least in the short term, to be attached to microbial membranes or enzymes. The microbial reaction can occur only with the freely dissolved substrate which must be transported through the cell membrane. The bioavailability of hydrophobic compounds is therefore limited by the actual aqueous solubility of the different substrates and not determined by the concentration in the aqueous phase.

The main difference between humic acids and surfactants rests in the mechanism of solubilization. In the case of surfactants the PAHs are enclosed in micelles, but are not bound strongly to the surfactant molecule. In the case of humic acid, the PAHs seem to form real complexes. The biological uptake is therefore subjected to a competitive binding between the membranes of the microorganisms and the humic acid molecules.

Also, as is observed with many surfactants, potential toxic effects of higher humic acid concentrations on the microorganisms were of concern. In this study, the production of the biomass did not seem to be affected by the concentrations of humic acid used.

This study did not define the nature of the humic acid complexes or prove whether humic acid can be used for the irreversible elimination of hazardous oxygen containing PAH metabolites by the formation of chemical bonds. Little is known about the long time behaviour of complexes of PAHs with humic matter, especially in complex microcosms, where species adapted to the degradation of specific compounds may have evolved. Scenarios could be envisaged where the microorganisms are able to first degrade a PAH-humic complex and then degrade the thus rereleased contaminants.

Further studies will be needed to study the ultimate fate of the fungal metabolites. In living systems, fungi coexist with bacteria, which can be envisaged as possible agents of further degradation. The behaviour of microorganisms in the presence of petroleum products is also very different than when incubated with pure compounds and will be the subject of future investigations.

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