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Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils

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Abstract

Seven commercial 3- to 7-ring (R) polycyclic aromatic hydrocarbons (PAH) as well as PAH derived from lignite tar were spiked into 3 soils (0.8 to 9.7% of organic carbon). The disappearance of the original PAH was determined for the freshly spiked soils, for soils incubated for up to 287 d with their indigenous microflora, and for autoclaved, unsterile and pasteurized soils inoculated with basidiomycetous and ascomycetous fungi. Three to 12 d after spiking, 22 to 38% of the PAH could no longer be recovered from the soils. At 287 d, 88.5 to 92.7%, 83.4 to 87.4%, and 22.0 to 42.1% of the 3-, 4-, and 5- to 7-R PAH, respectively, had disappeared from the unsterile, uninoculated soils. In 2 organic-rich sterile soils, the groups of wood- and straw-degrading, terricolous, and ectomycorrhizal fungi reduced the concentration of 5 PAH by 12.6, 37.9, and 9.4% in 287 d. Five- to 7-R PAH were degraded as efficiently as most of the 3- to 4-R PAH. In organic-rich unsterile soils inoculated with wood- and straw-degrading fungi, the degradation of 3- to 4-R PAH was not accelerated by the presence of fungi. The 5- to 7-R PAH, which were not attacked by bacteria, were degraded by fungi to 29 to 42% in optimum combinations of fungal species and soil type. In organic-poor unsterile soil, these same fungi delayed the net degradation of PAH possibly for 2 reasons. Mycelia of *Pleurotus* killed most of the indigenous soil bacteria expected to take part in the degradation of PAH, whereas those of *Hypholoma* and *Stropharia* promoted the development of opportunistic bacteria in the soil, which must not necessarily be PAH degraders. Contemporarily, the contribution of the fungi themselves to PAH degradation may be negligible in the absence of soil organic matter due to the lower production of ligninolytic enzymes. It is concluded that fungi degrade PAH irrespective of their molecular size in organic-rich and wood chip-amended soils which promote fungal oxidative enzyme production.

Introduction

In aerated soils, lower-condensed PAH are gradually degraded by indigenous soil microorganisms. A control of 5- to 7-R PAH, however, requires some technical efforts. PAH added to soil are increasingly immobilized by sorptive substances of the organic matter, whereby bioavailability and bacterial degradability decrease (Hatzinger & Alexander 1995). PAH and comparable aromatic structures are chemically altered by abiotic oxidants such as metal ions of Mn and Fe (Cremonesi et al. 1989; Periasmy & Vivekananda Bhatt 1978; Wilk et al. 1966), by clay minerals, oxides and oxyhydroxides of Al, Fe, Mn, and Si (Wang et al. 1986), by radicals of active oxygen species (Gierer et al. 1992), by oxidative microbial enzymes, or by the radical ions the enzymes catalyze from lignin (Felby et al. 1997), and from the unsaturated fatty acids (Bogan & Lamar 1995; Mabey et al. 1982) of plant residues in soil. Chemically activated PAH molecules may covalently bind to soil humate to become toxicologically inactive (Calderbank 1989; Field et al. 1995b).

Bacteria degrade the remaining bioavailable PAH to 4 R (Cerniglia & Heitkamp 1989), and dioxygenate the pentanuclear benzo[a]pyrene (Schneider et al. 1996). Microfungi and homobasidiomycetes monooxygenate PAH molecules by the intracellular cytochrome P-450 dependent multifunctional ox-

idase (MFO) system (Cerniglia 1992; Masaphy et al. 1995). In homobasidiomycetes, a contemporary one-electron oxidation of the PAH molecule by extracellular radical-forming oxidative enzymes may also occur. In dependence on their ionization potential (IP), PAH and substituted PAH are oxidized by plant peroxidase (PO, for substances with an IP $\leq$ 7.35 eV; Cavalieri et al. 1983), a peroxidase of *Agrocybe aegerita* (Brig.)Sing. (IP at least 8.19 eV; Sack 1996), laccase of *Trametes versicolor* (L.: Fr.)Pilat (Lacc, IP $\leq$ 7.45 eV; Collins et al. 1996), lignin peroxidase of *Phanerochaete chrysosporium* Burdsall (LiP, IP $\leq$ 7.55 eV; Hammel et al. 1986), and by several enzyme/mediator systems such as enzymatically or chemically generated Mn(III) chelate (IP $\leq$ 7.8 eV; Cavalieri & Rogan 1985), manganese peroxidase (MnP)/lipid peroxidation (IP $\leq$ 8.19 eV; Bogan & Lamar 1995), Lacc/2.2′-azinobis(ethylbenzothiazoline-6-sulfonate) (ABTS; Collins et al. 1996), and MnP/reduced glutathione couples (IP at least 8.19 eV; Sack et al. 1997).

Apart from the highly carcinogenic benzo[a] pyrene, the degradation of 5- to 7-R PAH has been poorly studied. Most but not all of these compounds in the suitable IP range are oxidized by plant PO (Cavalieri et al. 1983), LiP (Hammel et al. 1986), MnP/lipid peroxidation (Bogan & Lamar 1995), and Mn(III)acetate-acetic acid systems (Cavalieri & Rogan 1985) *in vitro*. Several 4- to 6-R PAH were degraded by *Pleurotus* sp. in sterile wheat straw (Wolter et al. 1997). In two field demonstrations using creosote-contaminated soil samples of about 2 metric tons, the ligninolytic fungus, *Phanerochaete sordida* (Karst.)Erikss. & Ryv. increased (Davis et al. 1993) and reduced (Lamar et al. 1994), respectively, the degradation of 3-R PAH by +29 and −10%, increased the degradation of 4-R PAH by 32 and 10%, and was unable to deplete 5- to 7-R PAH.

Little attention has been paid to the production of PAH-converting oxidative enzymes by fungi growing in soil. *Bjerkandera* sp. produced MnP (Field et al. 1995a), and *P. chrysosporium* produced MnP and LiP (Bogan et al. 1996a; b) as shown on the level of gene transcription. The field-grown mycelia of fairy-ring and ectomycorrhizal fungi released free extractable phenoloxidases, PO, MnP, mono- and dioxygenases, as well as oxidases that generate hydrogen peroxide, the cosubstrate of peroxidases (Gramss 1997). Many of the PAH oxidizing enzymes were also released by mycelia of those wood- and straw-degrading and terricolous basidiomycetes that form durable and extensive mycelia in layers of soil and mineral debris around inoculated wood blocks (Gramss 1979).

The present study was thus conducted to compare the degradation dynamics of 3- to 7-R PAH in 3 soils in the presence of the indigenous soil microbia and several fungi which are known for the production of persistent soil mycelia and efficient oxidative enzymes.

Materials and methods

Fungal isolates

Pure cultures of wood-degrading, terricolous, and ectomycorrhizal fungi were derived from fruitbody plectenchyma and multispore prints. The isolates of wood-degrading fungi were stored at + 2 °C on wood dust of European beech (*Fagus sylvatica* L.) fertilized with 10 and 5% by dry wt, respectively, of beet sugar and wheat flour to avoid strain debilitation. Other fungi were maintained on cellulose fibre soaked with a watery decoction of 6% sugar, 2.5% wheat flour, and 1.5% pea flour (w/v). The following fungal species were used: Wood-degrading basidiomycetes: *Bjerkandera adusta* (Willd.: Fr.)Karst.; *Gymnopilus sapineus* (Fr.)Mre.; *Hypholoma fasciculare* (Huds.: Fr.)Kumm.; *Kuehneromyces mutabilis* (Schaeff.: Fr.)Sing. & Smith; *Lenzites betulina* (L.: Fr.)Fr.; *Pleurotus* sp. (Argentina); *Pleurotus ostreatus* (Jacq.: Fr.)Kumm. Wood- and straw-degrading basidiomycetes: *Agrocybe praecox* (Pers.: Fr.)Fayod; *Stropharia rugoso-annulata* Farlow em. Murrill.

Terricolous basidiomycetes: *Agaricus bisporus* (Lge.)Imbach; *Coprinus comatus* (Müll.: Fr.)S.F.Gray. Ectomycorrhizal ascomycete: *Morchella conica* Pers.

Soil inoculum of wood- and straw-degrading fungi

Timber cubes (3 cm) of European beech were filled into 2-l glasses with metal screw lids, moistened with 0.2 l of water, and autoclaved at 121 °C for 30 min. The glasses were aseptically inoculated with 3 to 5 agar plug inocula of the test fungus and incubated at 23 °C with the screw lid loosened to allow for gas exchange. Spawn inoculum was prepared by mixing 1 kg of air-dry beech wood dust with 0.470 kg of wheat straw chaff (5 cm long), 75 g beet sugar, 35 g wheat flour, and 3 l water.

Soils

Five soils differing in pH, organic carbon content (C_{org}), and the nature of their mineral components were obtained from 3 locations in Thuringia (Germany). Soil parameters were determined using the methods proposed by Schinner et al. (1993). The pH ($CaCl_2$), C_{org}(%, w/w), and total N (%, w/w, Kjeldahl) values, respectively, are given in brackets for soil 3.3, a builder's sand with 10% podzolic subsoil (pH 7.4; 0.8; 0.02); soil 3.5, a black farmyard soil on siluric iron ochre (pH 5.4; 9.1; 0.50); soil 3.6 from a coal tar processing facility containing builder's sand, a podzolic subsoil, and 35% by volume of tar particles (pH 7.5; 13.0; 0.24); soil 5.1, a farmyard soil on mottled sandstone (pH 7.4; 2.1; 0.12) and soil 5.2, a black calcareous forest mull soil on shell lime (pH 7.4; 9.7; 0.92).

Soil treatments

The air-dry soils were sieved through 2 mm and spiked with 10 to 44 mg kg^{-1}each of the PAH compounds phenanthrene (PHEN, 3 R), anthracene (ANTH, 3 R), fluoranthene (FLUA, 4 R), pyrene (PYR, 4 R), perylene (PER, 5 R), benzo[g,h,i]perylene (BENZ, 6 R), and coronene (COR, 7 R). PAH were dissolved in acetone overnight, and 100 ml of solution were employed to spike 1 l of the soil. After the evaporation of the solvent, the soils were moistened to about 60% of their water-holding capacity, used directly in this unsterile state, amended with 25% (v/v) of large beech wood chips (0.4 × 1 × 2 cm), pasteurized in dry heat at 70 °C for 3 h, or autoclaved twice at 121 °C for 30 min and spiked subsequently. Culture vessels were 1-l glasses whose metal lids were loosened to allow for gas exchange. Soil 5.2 was also spiked with a solution of 5% (w/w) lignite tar dissolved in acetone (0.5 l kg^{-1} of soil) to examine the conversion of tar-derived PAH in the presence of further tar constituents simulating the conditions in the soil of coal-processing facilities. From the freshly spiked soil, 53 mg kg^{-1} of 3- to 4-R, and 6.3 mg kg^{-1} of 5- to 6-R PAH could be recovered.

Inoculation of spiked soils

Fungal degradation of PAH in sterile soils was tested in Erlenmeyer flasks (50 ml) containing 10 g (dry wt) of spiked soil 5.2 and a beech wood chip 0.3 × 1 × 1 cm. The flasks were autoclaved at 121 °C for 30 min, inoculated with agar plugs of the test fungi in a 6-fold replication, weighed, and incubated at 20 °C for up to 287 d. Control samples were not inoculated. Losses in moisture, expressed as losses in weight, were compensated by adding autoclaved, deionized water. Comparable tests were performed with 1-l samples of spiked soil in the absence of additional wood chips. The test series comprising pasteurized and unsterile soils were carried out in 1-l glasses in quadruplicate. Soils were inoculated with one fungus-colonized timber cube or a piece of spawn (about 15 cm^3), and incubated at 20 °C in the dark. The density of the mycelia, an estimated value describing the number of parallel hyphae in soil pores as seen through the glass wall of the vessel, was determined by a visual comparison with mycelial density standards.

PAH extraction from soil

Free soluble PAH of inoculated and uninoculated soils were extracted in 2 steps. Samples of air-dry soils (3 g as dry wt) were filled into glass tubes 10 ml and infused in 3 ml toluene. The tubes were closed by screw caps with teflon-coated rubber membranes, sonicated at 225 W for 30 min, and centrifuged at 3,000 rev min^{-1} for 15 min. The toluene phase was collected with a syringe after storing the tubes with the screw cap down for 1 h. In a subsequent alkaline extraction step, the soil sample was re-infused in 2.8 ml methanol (HPLC grade) and 0.2 ml of 2 N KOH, stored at 80 °C for 1 h, and sonicated for 15 min. After a renewed centrifugation at 3,000 rev min^{-1} for 15 min, the methanol extract was collected as described above.

HPLC examination

Soluble PAH in the toluene and methanol phase were separately quantified by High Pressure Liquid Chromatography (HPLC) using a Kontron system (model 322) with fluorescence detection (Kontron Instruments, Zurich), and the columns SCC 8/4 LiChrospher 100 RP18 e.c., and ET 150/4 Nucleosil 100-5C 18 PAH (Macherey-Nagel, Düren) arranged in series. The wavelengths of excitation and emission (nm) were 265/350 from 0 to 16.5 min, 265/430 from 16.5 to 27.5 min, and 295/460 from 27.5 to 55 min. The mobile phase (1 ml min^{-1}) consisted of methanol/water in proportions (v/v) of: 0 to 5 min, 70: 30; 5 to 16.5 min, linearly to 100: 0; 16.5 to 40 min, 100: 0; 40 to 55 min, 70: 30. The device was calibrated with a 16 EPA-PAH kit (Ehrenstorfer GmbH, Augsburg) completed by the higher-condensed PAH species.

Number of colony-forming bacteria in fungus-colonized soils

Glass beakers 100 ml were filled with 70 ml of freshly collected unsterile soil (sieve 2 mm) in quadruplicate, moistened to about 60% of its water-holding capacity, and inoculated with 1 timber cube each of 3 wood-degrading fungi. The beakers were partially covered with parafilm to limit desiccation and incubated at 20 °C for 2 to 3.5 wk. From the freshly overgrown soil, samples of 5 g (dry wt) were removed, suspended in 50 ml of 0.9% (w/v) NaCl solution and agitated vigorously for 3 h. The number of colony-forming bacteria was determined by dispersing 0.1-ml aliquots of serially diluted soil suspensions on 90-mm Standard I agar plates (Merck) in quadruplicate. The colonies were counted after 4 d of incubation at 28 °C.

Extraction of enzymes from fungus-colonized inocula and soils

Fungus-colonized timber and spawn inoculum was shreddered and suspended in deionized water for 24 h at room temperature. Glass vessels containing overgrown unsterile or pasteurized soil and the original fungal inoculum were flooded with deionized water until 10 to 20 ml of water remained free decantable, and were also kept for 24 h. The nonadsorbed aqueous extract was then decanted under avoidance of mycelial disruption, and portions of the extract were pre-buffered to pH 4.5 and 7.0, respectively, by adding potassium phosphate-citric acid crystals to a final concentration of about 0.1 M in order to avoid disturbing precipitations during the spectrophotometric enzyme assays. Contemporarily, the quantities of the extracting solvent and the dry wt of soil and inoculum were determined.

Colorimetric enzyme assays

Enzyme activities were monitored in a Pharmacia Ultrospec Plus spectrophotometer for the following groups of oxidoreductase enzymes.

Heme-containing peroxidases: Peroxidase (EC 1.11.1.7) activity was measured in 0.4 mM pyrogallol and 0.44 mM H_2O_2 at pH 4.5 as increase in absorbance at 430 nm (A_{430}). The activity of manganese peroxidase (EC 1.11.1.13) was measured in 0.2 mM $MnSO_4 \times H_2O$, 0.2 mM H_2O_2 and 50 mM Na-malonate at pH 4.5 (increase in A_{270}), whilst that of lignin peroxidase (EC 1.11.1.14) was measured in 3.3 mM veratryl alcohol and 0.44 mM H_2O_2 at pH 3.0 (increase in A_{310}).

Unspecific copper-containing oxidase: The activity of laccase (EC 1.10.3.2) was determined using either 0.54 mM 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) at pH 4.5 (increase in A_{420}), or 0.1 mM syringaldazine at pH 6.0 (increase in A_{525}) (for references and details compare Gramss 1997). All test substances were dissolved in 0.1 M citrate-phosphate buffer, whilst those for the manganese peroxidase test were dissolved in deionized water. In dependence on the activity of the enzyme solution, portions of 0.1 to 0.5 ml were used to make up a total of 1 ml reaction mixture. The enzyme reactions were followed for 3 to 10 (to 30) min at 29 °C. Blanks preferentially consisted of enzyme solution inactivated by boiling for 10 s. The nature of the products formed by the enzyme reactions was examined by scanning spectroscopy.

Statistical treatments

Standard deviations to the results of quadruplicate cultures and correlation coefficients were calculated by means of the common computer programmes.

Results

Revovery of PAH from uninoculated soils

Recoveries determined 3 to 12 d after spiking were in a similar order for soils whose organic carbon content ranged as wide as from 0.8 to 9.7% (Table 1). Upon the further incubation for up to 280 d, the PAH recoveries from sterile soils widely ceased to decline, whereas those from unsterile soils with their unimpaired indigenous microflora declined progressively to as little as 7.3 to 16.6% for 3- to 4-R PAH. The limited reductions in the concentration of 5- to 7-R PAH in the sterile, pasteurized, and unsterile treatments of the soils 3.5 and 5.2 were not significantly different, and were thus caused by abiotic mechanisms. For the pasteurized soils whose microbial activity was permanently reduced to 64 to 73% (data not shown), the PAH recoveries remained slightly higher, but they were basically congruent with those in unsterile soils.

Table 1. Recoveries of 7 PAH spiked into uninoculated control soils

Soil No.	Recovery at	Treatment	Recovery [%] of the groups of PAH (No. of aromatic rings)		
			PHEN/ANTH (3 R)	FLUA/PYR (4 R)	PER/BENZ/COR (5/6/7 R)
3.3	12 d	U	62.3	65.5	74.8[1]
	102 d	U	17.3	25.0	58.1[1]
	280 d	U	11.5	16.6	38.5[1]
3.5	3 d	U	65.7	67.2	78.1[2]
	85 d	U	16.0	32.5	73.2[2]
	245 d	U	7.3	12.6	69.4[2]
5.2	3 d	U	68.1	70.0	68.3[2]
	85 d	U	11.7	35.2	60.1[2]
	245 d	U	7.8	13.4	62.5[2]
3.5	3 d	P	65.2	67.4	77.6[2]
	85d	P	17.1	33.8	74.1[2]
	245 d	P	9.1	14.2	72.7[2]
5.2	3 d	P	69.0	68.9	68.4[2]
	85d	P	41.9	59.9	57.9[2]
	245 d	P	12.8	16.6	59.4[2]
3.5	3 d	S	59.7	64.9	65.1[2]
	85 d	S	58.8	65.1	63.1[2]
	245 d	S	52.7	64.6	59.4[2]
5.2	3 d	S	56.7	62.9	58.4[2]
	85 d	S	50.3	60.4	53.9[2]
	245d	S	49.4	61.9	53.2[2]

Initial PAH net wt 100%. Soil treatment U, unsterile; P, pasteurized at 70°C for 3 h; S, sterile.
Average SD for the PAH recoveries between ±1.2 to 6.6%.
[1]Data refer only to PER.
[2]Decreases in the recoveries of unsterile and pasteurized samples are not significantly ($\alpha = 0.05$) different from those of the sterile samples, as determined by Turkey's multiple comparison test.

Disappearance of PAH in sterile soils inoculated with fungi

In autoclaved 10-g samples of the spiked soil 5.2, the groups of wood-degrading, wood- and straw-degrading, terricolous, and ectomycorrhizal fungi reduced the sum of 5 PAH to an extent of 12.3, 12.6, 37.9, and 9.4%, respectively, over 287 d (Table 2). Of this quantity of PAH, 71 to 96% disappeared within the first 100 d of incubation. The combined amounts of the sorptive immobilization, the abiotic, and the most efficient fungal PAH transformation resulted in the disappearance of 98% of the free extractable 3- to 5-R PAH in the soil. The comparatively poor correlation between the mycelial densities and the recoveries ($r = 0.728$) indicates qualitative differences in the specific activities of fungal hyphae to PAH. Increases in the mycelial density of *H. fasciculare* and *S. rugoso-annulata* by soil amendment with keratine also correlated poorly ($r = 0.695$) with the associated increases in the disappearance of PAH. Unlike the conditions in bacteria, several fungi degraded 5- to 7-R PAH as efficiently as the lower-condensed compounds, thereby depending on the type of the soil matrix (Table 3).

Disappearance of PAH in fungus-colonized unsterile soils rich in organic carbon

The disappearance of PAH was recorded for 1-l samples of unsterile and pasteurized soils which had been amended, in part, with 25% beech wood chips. The chips were rapidly colonized by mycelia of *H. fasciculare* and *S. rugoso-annulata*, but poorly by those of *K. mutabilis*. Wood chips increased the mycelial density in some, but not all, cases (Table 4). The disappearance of PAH was again influenced by the type of the soil matrix, and accelerated in the presence of well colonized wood chips. Nevertheless, the extent of PAH degradation did not correlate ($r = 0.183$) with the densities of the soil mycelia. Soil pasteurization damaged the indigenous microflora and reduced its degradative capacity. According to Table 1, the microflora of the

Table 2. Disappearance of PAH [%] at 287 d in 10-g samples of sterilized soil 5.2, exclusively caused by fungi. Values of uninoculated control samples have been subtracted

Fungus	MD	PHEN	ANTH	FLUA	PYR	PER	Mean of 5 PAH
Wood-degrading basidiomycetes that form persistent mycelia in soil							
B. adusta	22	1.2	8.7	7.1	2.8	1.2	4.2
G. sapineus	16	0	4.2	5.8	7.4	0	3.5
H. fasciculare	63	0	13.0	8.2	5.3	4.6	6.2
H. fasciculare + K	104	6.2	18.1	13.7	10.7	9.5	11.6
K. mutabilis	104	27.7	20.6	20.5	17.5	9.0	19.2
L. betulina	18	13.6	18.9	9.1	7.6	4.3	10.7
Pleurotus sp.	88	35.5	26.0	24.7	40.0	24.2	30.1
Wood- and straw-degrading basidiomycetes that colonize soil							
A. praecox	52	41.0	1.9	0.5	22.1	0.8	13.3
S. rugoso-annulata	88	3.8	12.8	27.6	7.4	7.3	11.9
S. rugoso-annulata + K	104	28.9	20.5	52.3	22.9	12.8	27.5
Terricolous basidiomycetes							
A. bisporus	73	38.0	25.9	36.1	47.1	41.9	37.7
C. comatus	125	32.2	26.3	44.2	48.0	39.9	38.1
Ectomycorrhizal ascomycete							
M. conica	52	11.5	14.1	11.2	6.0	4.0	9.4
Average net PAH degradation for all fungi together							
		18.6	15.7	17.7	19.2	12.5	16.7

Initial net wt of each PAH compound 10 mg kg^{-1}.
MD, mycelial density (number of parallel hyphae mm^{-1}). K, soil amended with keratine (chicken feather meal) at 10% (v/v).
Average SD of the PAH recoveries between ±1.4 to 4.4% in 67% of all cases, but never larger than ± 6.6%.

unsterile soil accounted for a rapid degradation of 3- to 4-R PAH, but had little effect on higher-condensed PAH (see also Figure 1). Whereas in the case of the rapidly depleted lower-condensed PAH the fungal degradative potential was not expressed, in the case of the persistent higher-condensed PAH the fungal activity was apparent and pretended a preference of fungi to 5- to 7-R PAH (Figure 1).

Disappearance of PAH in fungus-colonized unsterile soil 3.3 poor in organic carbon

In the soil 3.3 which preferentially consisted of builder's sand, *H. fasciculare* and *S. rugoso-annulata* formed mycelia with densities of 88 to 125 hyphae mm^{-1} and were thus expected to dominate the degradation of PAH. Surprisingly, the preferential disappearance of tri- to tetranuclear, and the high persistence of the pentanuclear PAH in uninoculated control soil indicated the presence of a highly efficient PAH degrading bacterial flora in spite of the negligible C_{org} content of the soil (Figure 2). An introduction of fungi

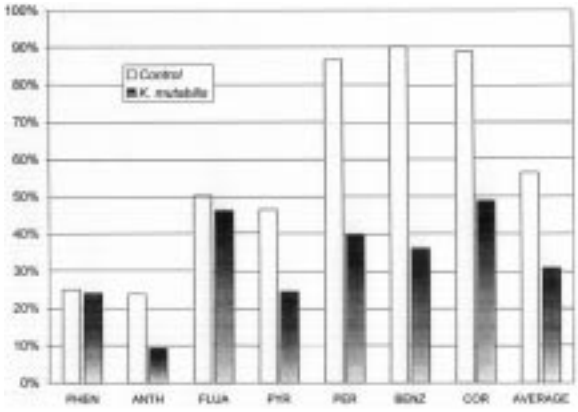

Figure 1. PAH revovery [%] from unsterile, nonamended control soil 3.5 and from soil inoculated with *K. mutabilis* at 85 d of incubation (samples of 1 l). PAH recovery from freshly spiked soil = 100%.

reduced the extent of PAH degradation significantly by 6.0 ± 1.7% in unsterile soil, and by 15.0 ± 1.5% in pasteurized soil within 102 d of incubation. These

Table 3. Disappearance of PAH [%] at 245 d in 2 types of sterilized soil samples of 1 l, exclusively caused by fungi. Values of uninoculated control samples have been subtracted

Fungus	PHEN	ANTH	FLUA	PYR	PER	BENZ	COR	Mean of 7 PAH
Soil 5.2								
H. fasciculare	1.6	13.0	1.2	1.0	2.2	0.4	1.5	3.0
S. rugoso-annulata	2.9	11.2	4.3	2.4	1.5	3.1	5.3	4.4
Soil 3.5								
H. fasciculare	10.6	32.9	7.4	16.2	20.8	5.6	1.6	13.6
K. mutabilis	13.4	30.4	4.7	8.4	7.9	3.8	7.0	10.8

Initial net wt 42 mg kg^{-1} each for 3- to 4-R PAH, 20 mg kg^{-1} each for 5- to 7-R PAH (= 100%). Average SD of the PAH recoveries between ±1.2 to 5.4%.

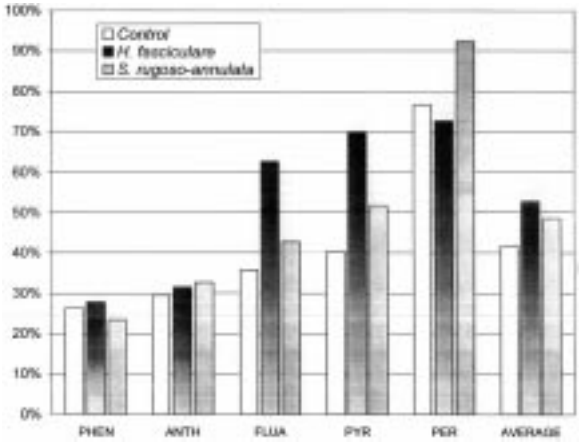

Figure 2. PAH recovery [%] from unsterile, nonamended control soil 3.3 and from soil inoculated with *H. fasciculare* and *S. rugoso-annulata* at 102 d of incubation (samples of 1 l). PAH recovery from freshly spiked soil = 100%.

differences declined to 1.4 ± 1.1% in unsterile, and to 7.4 ± 2.5% in pasteurized soil at 280 d when the fungal metabolic activity apparently no longer impaired the degradative potential of the bacteria.

Fungal effects on PAH from lignite tar

Soil 5.2 spiked with lignite tar was inhibitory to fungal growth. While 50-g samples of the unspiked and unsterile soil were overgrown by *S. rugoso-annulata* in 15 d, the spiked soil was slowly overgrown in 65 d with a 5-fold mycelial density. In comparison to uninoculated control samples, the fungus degraded 5.0% of the lower-condensed, and 27.3% of the higher-condensed PAH (benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, and benzo[g,h,i]perylene) under sterile conditions at 20 °C in 110 d. It also degraded 29.3% of the lower-condensed, and 26.3% of the higher-condensed PAH under unsterile conditions.

Influence of fungal mycelia on the number of colony-forming soil bacteria

In the unsterile soils 3.6, 5.1, and 5.2 which had been inoculated with wood-decay fungi, the number of colony-forming bacteria changed considerably (Table 5). Around the growing hyphae of *H. fasciculare* and *S. rugoso-annulata*, the bacterial count increased in the samples incubated for 2 wk, and declined sometimes to the level of the control soil in the samples incubated for 3.5 wk. By contrast, *P. ostreatus* reduced the number of colony-forming bacteria significantly and permanently.

Activities of ligninolytic enzymes in fungal inocula and overgrown soils

Table 6 comprises the activities of ligninolytic enzymes extracted from shreddered fungal inocula and from the nondisrupted inoculum/soil mycelium systems of unsterile and pasteurized soil and brickmeal samples. Natural, unpasteurized soil 5.2 contained traces of Lacc, which were destroyed by pasteurization. The fungi produced extracellular Lacc, and peroxidases such as common PO, MnP, and LiP. Most of these enzymatic activities were found in extracts of the shreddered fungal timber and straw inocula. They may indicate the presence of enzymes from inside and outside the disrupted fungal cells. About 1% of these enzymatic activities were found in extracts from mycelia growing in soil whose cells had not been injured during extraction. They may thus be based on true extracellular enzymes. Control tests assured that these enzymes were not simply effluents from the in-

Table 4. Disappearance of PAH [%] in 2 types of pasteurized and unsterile soil samples of 1 l at 85 d of incubation with fungi. Values of uninoculated control samples have been subtracted

Fungus and treatment		PHEN	ANTH	FLUA	PYR	PER	BENZ	COR	Mean of 7 PAH	MD
Soil 5.2										
H. fasciculare	P	0.0	8.9	0.2	2.1	0.0	0.0	0.0	1.6	147
		(22.1)	(24.9)	(10.5)	(11.6)	(9.0)	(2.9)	(5.1)	(12.3)	125
	U	3.1	1.9	4.6	6.1	0.0	1.1	3.7	2.9	177
		(1.4)	(1.4)	(5.8)	(9.7)	(2.3)	(2.3)	(11.4)	(4.9)	147
S. rugoso-annulata	P	6.6	18.3	9.1	1.7	4.7	0.8	3.5	6.4	104
		(26.1)	(26.1)	(13.6)	(11.3)	(9.1)	(5.9)	(20.5)	(16.1)	147
	U	4.5	1.2	8.4	10.0	2.9	5.8	6.8	5.7	88
		(5.6)	(2.9)	(14.7)	(17.6)	(10.6)	(11.2)	(17.2)	(11.4)	104
Soil 3.5										
H. fasciculare	P	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.3	73
		(0.0)	(4.8)	(0.0)	(0.0)	(5.4)	(1.6)	(0.0)	(1.7)	104
	U	3.7	4.4	0.0	0.0	1.2	1.0	2.3	1.8	104
		(5.3)	(5.6)	(12.6)	(12.1)	(12.6)	(12.4)	(9.3)	(10.0)	147
K. mutabilis	P	0.0	8.3	0.0	7.5	30.2	29.6	17.7	13.3	147
		(0.8)	(9.0)	(0.0)	(7.7)	(26.3)	(22.0)	(14.1)	(11.4)	147
	U	0.7	9.2	2.7	15.0	41.7	39.4	28.7	19.6	147
		(5.7)	(8.9)	(13.6)	(17.8)	(37.9)	(38.6)	(28.6)	(21.6)	147

Initial net wt 42 mg kg^{-1} each for 3- to 4-R PAH, 20 mg kg^{-1} each for 5-to 7-R PAH (=100%). P, soil pasteurized at 70°C for 3 h; U, soil unsterile; ( ), soil amended with 25% (v/v) beech wood chips; MD, mycelial density (number of parallel hyphae mm^{-1}).
Average SD for the PAH recoveries between ±1.2 to 6.2%.

oculum alone (Table 6). Enzymatic activities were not influenced by a use of pasteurized soils. The brick meal extracts of *Pleurotus* sp. and *S. rugoso-annulata*, however, contained not more than a mean of 15 to 23% the activities found in the organic-rich soil 5.2.

Catalytic effects of peroxidases depend on the presence of hydrogen peroxide or several other hydroperoxides serving as electron acceptors (Schomburg et al. 1994). An unspecific peroxide test (Merck) was thus applied to extracts from inocula and overgrown soils. Traces of peroxide were found in *Pleurotus* sp. and *A. bisporus*. They were rapidly destroyed by catalase (0.1 mg ml^{-1}) and were thus most probably hydrogen peroxide. In *Pleurotus* sp., the production of H_2O_2 was possibly catalyzed by aryl-alcohol oxidase (data not shown).

Discussion

The destiny of the 3- to 7-R PAH spiked into soil once more raises the question, whether or not PAH contaminations can be biologically controlled by a sole propagation of the bacterial microflora in the contaminated soil. Treatment of soils containing 104,000 mg kg^{-1} of PAH in reactors (Wittmaier et al. 1992) and in field plots (Findlay et al. 1995) for up to 300 d, accompanied by fertilizing, aerating, and rototilling, resulted in a depletion of 2-, 3- and 4-R PAH by 97-78-41% and 99-96-82%, respectively. PAH with 5 and more rings were not depleted. Nevertheless, Schneider et al. (1996) reported the degradation of benzo[a]pyrene (5 R) by a mycobacterium. Benzo[a]pyrene was also mineralized to 50% within 100 d in unsterile soil after a lag phase of 50 d (Kanaly et al. 1997).

In the present study, the disappearance of PAH also comprised the higher-condensed compounds. Within 3 to 12 d after spiking into 3 soils, 22 to 38% of the PAH could no longer be recovered by an extraction method which even dissolved the PAH adsorbed to soil humate. Although in sterilized soil, the concentration of PAH decreased subsequently by no more than another 2 to 8% by sorptive and abiotic-oxidative mechanisms (Mahro & Kästner 1993), the extent of disappearance reached 88.5 to 92.7%, 83.4 to 87.4%,

Table 5. Changes in the number of colony-forming bacteria in 1-g samples (dry wt) of 3 soils overgrown by fungal mycelia for 2 to 3.5 wk

Soil sample	Incubation [wk]	Number (±SD) of bacterial colonies $\times 10^6$						
		Uninoculated	*H. fasciculare*	MD	*P. ostreatus*	MD	*S. rugoso-annulata*	MD
Tar soil 3.6	2	35.9 ± 1.2	104.1 ± 6.4	147	20.4 ± 8.3	88	65.6 ± 19.5	104
	3.5	29.8 ± 2.4	36.8 ± 9.4	125	13.9 ± 4.6	88	79.8 ± 11.1	88
Sandy soil 5.1	2	31.4 ± 6.5	44.6 ± 8.9	104	6.7 ± 0.3	52	40.3 ± 5.3	52
	3.5	33.7 ± 4.2	34.2 ± 6.3	125	3.1 ± 0.1	52	45.0 ± 8.5	52
Forest soil 5.2	2	40.6 ± 5.0	77.9 ± 21.3	147	30.5 ± 3.3	13–73	90.5 ± 31.5	73
	3.5	47.6 ± 4.3	45.5 ± 8.6	147	8.5 ± 0.7	147	79.2 ± 17.0	73

MD, mycelial density (No. of parallel hyphae mm^{-1}).

Table 6. Fungal enzymatic activities in timber and spawn inocula, and in soil (C_{org} = 9.7%) and brickmeal (C_{org} = 0.11%) permeated by the mycelia

Fungus	Matrix	Enzymatic activities[1] in μM min^{-1}				
		Lacc Syring-aldazine	Lacc ABTS	PO	MnP	LiP
H. fasciculare	Timber	0–0.43	0.1–52	0–44	0–44	0
	Soil	0	0.01–0.97	0–0.07	0	0
Pleurotus sp.	Timber	6.4–357	16.6–3195	3.6–142	31–82	0–0.54
	Soil	0.07–10.8	0.44–40	0.03–0.61	0	0
	Brick meal	0.16–0.44	4.1–7.3	0–0.05	0	0
S. rugoso-annulata	Spawn	0	5.6–7	0	0	0
	Soil	0	0.04–4	0–1.5	0–0.23	0
	Brick meal	0	0.47–0.82	0.04–0.36	0	0
A. bisporus	Spawn	12.3–14.6	2611–3294	0	0	0
	Soil	0.05–0.12	0.11–43	0	0	0.24–0.64
	Brick meal	0.09–0.17	12.5–30	0	0	0
Control[2] + *Pleurotus* sp.	Soil	0	0.38–0.74	0	0	0
Control[2] + *A. bisporus*	Soil	0	1.21–17.7	0	0	0
Control	Soil, non-pasteurized	0	0–0.14	0	0	0
Control	Brick meal, nonpasteurized	0	0	0	0	0

[1] Enzymatic activities valid for mycelia in 1 g (dry wt) of substrate extracted by 1 ml of water, and determined with a portion of 0.33 ml enzyme solution in a total of 1 ml reaction mixture. Range of values refers to 3 to 10 samples.
[2] Mycelia of the timber inoculum of *Pleurotus* sp. and the spawn inoculum of *A. bisporus* have not yet grown into the soil but release enzymes into the extraction fluid. Soil and brickmeal matrix pasteurized at 70° C for 3 h.

and 22.0 to 42.1% for 3-, 4-, and 5- to 7-R PAH, respectively, in unsterile soils within 245 to 280 d. Soil indigenous microorganisms contributed 42 to 52% to the degradation of 3- to 4-R PAH, but no significant amounts to the degradation of 5- to 7-R PAH in the soils 3.5 and 5.2. They degraded nevertheless PER in soil 3.3. A biological control of 5- to 7-R PAH remains thus a domain of those basidiomycete fungi that form extensive mycelia around inoculated wood blocks and spawn inocula inserted into soil (Gramss 1979).

Penta- to heptanuclear PAH in the suitable IP range have been repeatedly oxidized by purified plant PO (Cavalieri et al. 1983), LiP, and MnP *in vitro* (Bogan & Lamar 1995; Hammel et al. 1986). PER (7.06 eV), BENZ (7.32 eV), and COR (7.57 eV, Cavalieri et al. 1983) have low ionization potentials. They are thus substrates of fungal laccases, peroxidases, and their Mn^{2+} mediators. A comparative degradation in 2 sterile, organic-rich soils over 245 to 287 d confirmed that fungi converted higher-condensed PAH as easily as most of the lower-condensed PAH. This fact was also stated for the degradation of PAH by *Pleurotus* sp. in wheat straw substrate (Wolter et al. 1997). Thus, the disappearance of a PAH compound did not correlate well with the number of its benzene rings ($r = -0.428$). PAH were more rapidly degraded by 2 terricolous than by the wood-decay fungi. Degradation was stimulated by wood chip and keratine supplements and by the use of the acid (pH 5.4) rather than the alkaline (pH 7.4) soil. This fact is so far of interest as fungal laccases and peroxidases were most active at pH 3 to 5. The degradation of PAH in unsterile and pasteurized organic-rich soils also depended on a suitable combination of fungal species and soil type, and on soil amendment with wood chips. The rapid depletion of PAH up to 4 R by indigenous soil bacteria widely masked the lower degradative activity of the fungus to these PAH compounds. In the case of the higher-condensed PAH which were not depleted by bacteria, the remarkable degradative potential of the fungus was clearly expressed. This also applied to PAH derived from lignite tar. In the laboratory-scale tests, pasteurization of the soils did not increase the density of the fungal mycelia in all cases. The treatment reduced however the metabolic activity of those microorganisms, which take part in the degradation of PAH even in the presence of fungal mycelia.

Whereas in organic-rich soils, fungal mycelia contributed to the depletion of PAH, these same mycelia delayed the degradation of all PAH in the sandy soil 3.3. A reduced fungal production of oxidative enzymes in the absence of soil organic matter could be one of the reasons. Highest activities of Lacc, PO, and MnP, but also occasional traces of LiP were found in timber and straw materials overgrown by fungi. The activities declined to 1% in a soil C_{org} = 9.7%, and to 0.1 to 0.2% in a brick meal C_{org} = 0.11%. The presence of a dominant fungal mycelium could also change the number of those soil microorganisms that degrade the major portion of PAH. It is known that the microflora of timber blocks and sawdust are almost completely killed around basidiomycete mycelia (Gramss 1987). In grassland soil, the actively-growing fairy-ring fungus, *Marasmius oreades* (Bolt.: Fr.)Sing. also reduced the number of soil bacteria (Smith 1980). In the present study, mycelia of *P. ostreatus* reduced the number of colony-forming soil bacteria directly to 9 to 18%, whereas those of *S. rugoso-annulata* and *H. fasciculare* increased bacterial counts temporarily up to 290%. It cannot be excluded that these opportunistic bacteria replace populations of the initial soil microflora. The new bacterial populations must not necessarily be PAH degraders. Soil supplementations with bacterial nutrients, surfactants, and inducers of PAH degrading metabolic pathways revealed that the bacterial numbers readily increased, but the new populations did not incorporate ^{14}C phenanthrene (Carmichael & Pfaender 1997).

It is thus concluded that due to the inhibitory influences of fungal mycelia on indigenous microorganisms of an unsterile soil, the quantitative contribution of fungi to the degradation of 3- to 4-R PAH cannot be separated from that of the soil bacteria. Nevertheless, a control of 5- to 7-R PAH can be performed with the persistent mycelia of several wood- and straw-degrading fungi, although the presence of fungal mycelia sometimes increased und reduced the overall extent of PAH degradation. Fungi increased the degradation of PAH in soils rich in organic carbon and enriched with wood chips, i.e., in soils with organic materials that stimulated fungal ligninolytic enzyme production. Fungi inhibited the degradation of PAH in soils poor in organic carbon, in which fungal enzyme production was low, and in which PAH degrading microorganisms were possibly repressed by the direct and indirect antagonistic influences of the fungal mycelia. In these interactions between soil types, fungal enzymes, fungal antagonisms, and soil microbial activities, the role of soil pH and its vicinity to the pH optima of fungal enzymes, but also the dependence of fungal enzyme production on organic matter amendments should be further studied. The mode of action

of the fungal enzymes in organic-rich soil is also of interest. Enzymes are expected to catalyze the initial oxidation of PAH directly. The enzymatic catalysis of cation radicals from phenolic-lignin (Felby et al. 1997) and lipid constituents (Bogan & Lamar 1995) of plant residues in soil, which mediate further abiotic reactions, could also account for the increased degradation of PAH in complex substrates such as wheat straw (Sack 1996; Wolter et al. 1997) and organically enriched soils.

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