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Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus *Anthracophyllum discolor*

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ABSTRACT

The degradation of three- and four-ring polycyclic aromatic hydrocarbons (PAHs) in Kirk medium by *Anthracophyllum discolor*, a white-rot fungus isolated from the forest of southern Chile, was evaluated. In addition, the removal efficiency of three-, four- and five-ring PAHs in contaminated soil bioaugmented with *A. discolor* in the absence and presence of indigenous soil microorganisms was investigated. Production of lignin-degrading enzymes and PAH mineralization in the soil were also determined. *A. discolor* was able to degrade PAHs in Kirk medium with the highest removal occurring in a PAH mixture, suggesting synergistic effects between PAHs or possible cometabolism. A high removal capability for phenanthrene (62%), anthracene (73%), fluoranthene (54%), pyrene (60%) and benzo(*a*)pyrene (75%) was observed in autoclaved soil inoculated with *A. discolor* in the absence of indigenous microorganisms, associated with the production of manganese peroxidase (MnP). The metabolites found in the PAH degradation were anthraquinone, phthalic acid, 4-hydroxy-9-fluorenone, 9-fluorenone and 4,5-dihydropyrene. *A. discolor* was able to mineralize 9% of the phenanthrene. In non-autoclaved soil, the inoculation with *A. discolor* did not improve the removal efficiency of PAHs. Suitable conditions must be found to promote a successful fungal bioaugmentation in non-autoclaved soils.

1. Introduction

Xenobiotic chemicals are continuously released into the biosphere, posting a significant risk to human health due to their toxicity and persistence in the environment. Polycyclic aromatic hydrocarbons (PAHs) from natural and/or anthropogenic sources are characterized by their teratogenic, mutagenic and carcinogenic properties [1], and their persistence in the environment is related to their low aqueous solubility, vapor pressures and high octanol/water partitioning coefficients [2–5]. As a consequence, PAHs have a high affinity for association with organic carbon material (humus) in soil [6].

Microbial degradation is one of the major processes in cleaning up PAH-contaminated environments [7]. Among degradation microorganisms, white-rot fungi have demonstrated the ability to degrade a wide range of pollutants, including PAHs [8–10]. The extracellular ligninolytic enzyme system of the white-rot fungi, consisting of peroxidases and laccases, has been directly linked to biodegradation of PAHs [10–13]. Fungal extracellular enzymes catalyze PAH oxidation, generating more polar and soluble metabolites such as quinones, phtalate or diphenic acid [8]. Increased polarity and water solubility are important factors for enhancement of the bioavailability of these metabolites and their mineralization by native microorganisms present in contaminated sites [14].

The feasibility of biodegrading or mineralizing a PAH depends on several factors, such as the pollutant properties, the characteristics of the degrading microorganism, environmental factors and the properties of the contaminated soil, among others [15,16]. Several white-rot fungi are capable of transforming PAH in soil, with high metabolic versatility and evolved biodegradative pathways. Among the recent studies, Anastasi et al. [17] evaluated the potential of a consortium of three basidiomycetes isolated from compost for pyrene degradation in sterile soil microcosms; the basidiomycetes were able to efficiently colonize soil and remove about 56% of the pyrene in 28 days. The results indicate that ligninolytic enzymes such as laccase and manganese-independent peroxidase play an important role in the degradation process. Byss et al. [18] determined the efficacy of *Pleurotus ostreatus* and *Irpex lacteus* for the removal of PAH from a creosote-contaminated soil containing PAHs in the range of 50–200 mg kg^{-1}. *P. ostreatus* was found to be a more

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efficient PAH-degrading organism compared to *I. lacteus*, favoring PAH degradation through a possible synergistic interaction with actinobacteria. Bisnoi and co-workers [19] evaluated the degradation of three- and four-ring PAHs in sterile and non-sterile soil inoculated by *Phanerochaete chrysosporium*. They found that PAH degradation was lower in non-sterile soil, probably due to the effect of competition with the native microflora.

In particular, the Chilean white-rot fungus *Anthracophyllum discolor* produces high levels of manganese peroxidase (MnP) in the presence of wheat grains as lignocellulosic support [20] and to a lesser extent laccase (L) and lignin peroxidase (LiP), and is efficient in the degradation of organic pollutants such as chlorophenols and dyes [21,22]. Recently, bioremediation of soil contaminated with pentachlorophenol (PCP) was investigated using *A. discolor* [23]. The application of *A. discolor* immobilized in wheat grains to the contaminated soil favored the spread of the fungus and a high PCP removal rate (70–85%) was found in comparison to that measured with the fungus as free mycelium (30–45%) [23].

Considering that the potential of *A. discolor* to degrade PAHs has not been explored yet, the first purpose of this study was to evaluate the degradation by *A. discolor* of three- and four-ring PAHs individually and in mixtures in Kirk medium. The second objective was to investigate the removal efficiency of three-, four- and five-ring PAH removal efficiency in a contaminated soil bioaugmented with *A. discolor* in the absence or presence of indigenous soil microorganisms, as well as the production of lignin-degrading enzymes and PAH mineralization.

2. Materials and methods

2.1. Microorganism and inoculum

The fungal strain used was *A. discolor* (culture collection of the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile) isolated from decayed wood in the rainforest of southern Chile. This fungus was maintained at 4 °C in glucose malt extract agar (G-MEA) slant tubes containing: malt extract 30 g l^{-1}; agar 15 g l^{-1} and glucose 10 g l^{-1}. The fungus was transferred from slant culture tubes to Petri dishes with G-MEA medium and incubated at 30 °C for 7 days.

2.2. Chemicals

MBTH (3-methyl-2-benzothiazolinone) and DMAB (3-(dimethylamino) benzoic acid) were supplied by Aldrich Chemical Co., Germany. 3,4-Dimethoxy-benzyl alcohol (>95.0%) was supplied by Fluka.

Anthracene (>96.0%) and phenanthrene (>97.0%) were purchased from Merck (Hohenbrunn, Germany); pyrene (98%) and fluoranthene (98%) were purchased from Aldrich. Benzo(*a*)pyrene was supplied by Fluka.

Labeled benzo(*a*)pyrene ([7,10-^{14}C]benzo(*a*)pyrene, specific activity, 61.0 mCi $mmol^{-1}$; radiochemical purity, 98.9%) was supplied by Amersham Biosciences (UK). Labeled phenanthrene ([9-^{14}C]phenanthrene, specific activity, 55.7 mCi $mmol^{-1}$; radiochemical purity, 98.9%) and [4,5,9,10-^{14}C]pyrene (specific activity, 55.0 mCi $mmol^{-1}$; radiochemical purity, 97.8%) were supplied by Sigma (USA). Acetone (HPLC grade) and toluene (HPLC grade) were provided by Merck. Insta-Gel plus was supplied by Perkin-Elmer.

All other chemicals were supplied by Merck, Oxoid, Sigma and Duchefa (Netherlands).

2.3. Soil

An agricultural topsoil containing 14% clay, 3% organic matter, 1.8% organic carbon, 0.11% nitrogen and with a pH in water of 6.6 was used [24]. It was collected in Uppsala, Sweden, sieved (<2 mm) and stored at 4 °C until use.

2.4. PAH removal in liquid culture

Briefly, 20 g of glass beads (5 mm diameter) were added to 50-ml tubes and autoclaved for 20 min at 120 °C [24]. Solutions of individual PAHs (anthracene, phenanthrene, fluoranthene and pyrene) or a mixture of the four PAHs in acetone were added aseptically onto the glass beads to give a final concentration in the Kirk medium [25] of 50 mg l^{-1} of each PAH. The acetone was allowed to evaporate and 10 ml Kirk medium supplemented with Tween 80 (0.05% v/v) was added to the tube and inoculated with an agar plug of *A. discolor*. Incubation was carried out at 30 °C for 28 days. Each experiment was carried out in duplicate under destructive sampling mode. The tubes were maintained at −20 °C until PAH concentration in all the treatments (individual and mixture) and ligninolytic enzyme activity (in treatments with PAH mixture) were determined. A set of tubes with the medium inoculated with *A. discolor* without PAHs was run in parallel to evaluate the enzymatic activities. Also, the PAH extraction yield in each treatment without *A. discolor* was evaluated.

2.5. PAH removal in soil

The degradation of PAHs in soil by *A. discolor* was determined in the presence or absence of indigenous microorganisms. Ligninolytic enzyme activity in soil was also determined in the absence of indigenous microorganisms. Autoclaved (30 min at 121 °C, 1 bar, three times with 24 h of interval) soil (1 kg) or non-autoclaved soil was contaminated with the mixture of 50 mg kg^{-1} of each of the following PAHs: phenanthrene, anthracene, pyrene, fluoranthene and 40 mg kg^{-1} of benzo(*a*)pyrene. The soil spiking was carried out according to Brinch et al. [26] by treating a subsample of the soil (25%) with the PAH mixture in acetone. After evaporating the acetone, the contaminated soil was mixed with the remaining soil. Contaminated autoclaved and non-autoclaved soil (10 g) was weighed into 30 ml-tubes, supplemented with Tween 80 0.05% (v/w soil), and 0.5 g of sterile wheat grains were placed above the soil. An agar plug with active mycelia of *A. discolor* was placed on top of the wheat grains as inoculum. The tubes were incubated at 30 °C. The soil water content was kept at 60% of the water-holding capacity. Contaminated soil (either autoclaved or non-autoclaved soil) supplemented with Tween 80 0.05% (v/w soil) and 0.5 g wheat grains, without the addition of fungus, was used as control for determination of PAH content. Autoclaved soil without PAHs, supplemented with Tween 80 0.05% (v/w soil) and 0.5 g wheat grains and inoculated by *A. discolor* was used as control for determining ligninolytic enzyme activity. Also, the PAH extraction yield in autoclaved soil supplemented with Tween 80 0.05% (v/w soil) and 0.5 g wheat grains without *A. discolor* was evaluated. Each experiment was carried out in triplicate under destructive sampling mode. Sampling in autoclaved and non-autoclaved soil was done periodically for 60 days to determine PAH content and the enzymatic activities were evaluated in autoclaved soil.

2.6. Mineralization studies of PAHs in soil

The degree of mineralization of ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo(*a*)pyrene by *A. discolor* in autoclaved soil contaminated previously with 50 mg kg^{-1} of each of the following PAHs: phenanthrene, anthracene, pyrene, fluoranthene and 40 mg kg^{-1} of benzo(*a*)pyrene was monitored for 60 days. Non-labeled CO_2 (microbial respiration) was measured during 60 days at 30 °C in autoclaved and non-autoclaved soils contaminated with PAHs and inoculated by *A. discolor*.

A portion (10 g) of contaminated autoclaved or non-autoclaved soil was weighed into 100 ml plastic vials, supplemented with Tween 80 (0.05% v/w soil) and 0.5 g sterile wheat grains, and inoculated with an agar plug of *A. discolor*. The water content was adjusted to 60% of the water-holding capacity. ^{14}C-phenanthrene, ^{14}C-pyrene or ^{14}C-benzo(*a*)pyrene was added to the samples to give a total radioactivity in the soil material of about 60,000 dpm. These 100-ml plastic vials were placed in a hermetic glass jar together with two 20-ml plastic vials containing 4 ml of a 0.2 M NaOH solution and incubated at 30 °C for 60 days. NaOH solution was periodically removed and respiration and mineralization determined in the respective vial. Non-inoculated soil was used as the control. The experiment was carried out in triplicate.

Mineralization was determined by collecting $^{14}CO_2$ from the degradation of the labeled PAHs in the NaOH solution and measuring it in a liquid scintillation counter (Beckmann LS 600 series, USA), after mixing it with 4 ml of Insta-gel Plus. Mineralization was expressed as accumulated $^{14}CO_2$ as a percentage of the initial radioactivity.

In the respiration tests, the CO_2 captured in the NaOH solution was determined by titrating the remaining alkali with 0.1 M HCl (TIM850 titration manager, Tritalab®, Radiometer Ananlytical SAS) after precipitation of the carbonate with 0.1 M $BaCl_2$. The respiration was expressed as accumulated mg CO_2 g soil^{-1}.

2.7. Analyses

2.7.1. Enzyme extraction

In liquid medium, 2 ml of culture was centrifuged for 10 min at 5000 rpm, and ligninolytic enzyme activity was determined in the supernatant.

In soil, 10 g of sample was weighed in an Erlenmeyer flask and 20 ml succinate/lactate buffer 0.1 M at pH 4.5 was added. The flask was shaken at 100 rpm for 1 h. After 10 min of sedimentation, 5 ml of the liquid phase were centrifuged (4000 rpm) and filtered through a 0.45 μm filter unit [27]. MnP, L and LiP activities were determined in the filtrate.

2.7.2. Enzymatic assay

MnP activity was determined through the MBTH-DMAB assay [28]. The reaction mixture (2 ml) contained 1460 μl of 100 mM succinate–lactate buffer (pH 4.5), 300 μl of 6.6 mM DMAB, 100 μl of 1.4 mM MBTH, 30 μl of 20 mM $MnSO_4$, and 100 μl of supernatant. The reaction was initiated by adding 10 μl of 10 mM H_2O_2 and the absorbance of the deep purple compound that formed was measured at 590 nm. One unit was defined as the amount of enzyme needed to form 1 μmol of product in 1 min [28].

Laccase activity was also measured using the MBTH-DMAB method but H_2O_2 was omitted [28].

LiP activity was determined according to Tien and Kirk [25] and modified by Castillo et al. [29]. The reaction mixture (2 ml) contained 1420 μl of 100 mM sodium-tartrate buffer pH 3.0, 400 μl of 20 mM veratryl alcohol as substrate and 100 μl of supernatant. The reaction was initiated by adding 80 μl of 10 mM H_2O_2 and the increase of absorbance was followed at 310 nm. The extinction coefficient is 0.0093 μM^{-1} cm^{-1}. One unit representing 1 μmol veratryl alcohol oxidized to veratraldehyde per minute, at pH 3.0 and 30 °C.

2.7.3. PAH extraction and quantification

PAHs in the liquid medium were extracted by adding 10 ml toluene to each 50 ml-tube and shaking it vigorously for 1 h. After 10 min centrifugation at 492 × *g* an aliquot of the supernatant was analyzed directly with gas chromatography-flame ionization detector (GC-FID). Metabolites were identified with gas chromatography–mass spectrometry (GC–MS).

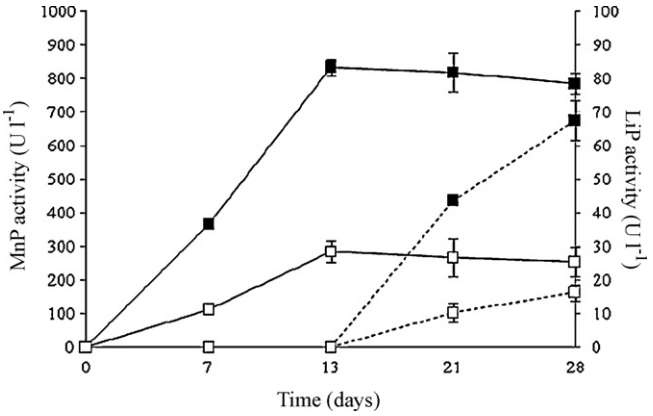

Fig. 1. Manganese peroxidase (solid line) and lignin peroxidase (dashed line) activity in *A. discolor* cultures growing in Kirk medium contaminated with PAH mixture (■) and non-contaminated (□), for 28 days. The values are means ± CI.

PAHs in soil were extracted by adding 10 ml of toluene and 10 ml of 0.05 M sodium pyrophosphate to tubes containing 10 g soil and shaking them vigorously for 16 h on a shaker. The extracts were centrifuged for 10 min at 492 × *g* and an aliquot of the supernatant was analyzed directly by GC-FID and GC–MS.

GC-FID analysis was performed using an HP 6890 Series GC-system equipped with a flame ionization detector and an Agilent 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 μm thickness). The oven programme was 80 °C for 3 min followed by ramping at 10 °C min^{-1} up to 310 °C maintained for 2 min. Splitless mode was used and the injector temperature was 250 °C. Quantification was performed using external standards.

The extraction yield for phenanthrene, anthracene, fluoranthene and pyrene in Kirk medium contaminated individually or simultaneously was >95%. The extraction yield for phenanthrene, anthracene, fluoranthene, pyrene and benzo(*a*)pyrene in autoclaved soil was >91%.

The analysis of metabolites from PAH degradation was performed using an HP 6890 Series GC-system equipped with an HP 5971 mass selective detector and an HP 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 μm thickness). The oven programme was 80 °C for 4 min followed by ramping at 7 °C min^{-1} up to 310 °C maintained for 4 min. The injector temperature was 250 °C. Quantification was performed using external standards. Metabolite identification was carried out using the Wiley 275 mass spectral library.

3. Results

3.1. PAH removal in liquid culture

The removal efficiency of phenanthrene, anthracene, fluoranthene and pyrene by *A. discolor* in liquid medium after 7, 14, 21 and 28 days of incubation is shown in Table 1. When added individually (alone) and after 28 days of incubation, the removal efficiency was in the following order: phenanthrene had the highest removal (22.6%) followed by fluoranthene (19.5%), pyrene (8.5%) and anthracene (7.0%) (Table 1). The same order but with a higher removal efficiency was observed when a mixture of PAHs was studied: phenanthrene (26.5%), fluoranthene (23.5%), pyrene (17.5%) and anthracene (11.3%).

The PAH removal by *A. discolor* occurred mainly during the first 14 days, as shown in Table 1, when the ligninolytic enzymes reached their maximal activity (Fig. 1). In fact, these results showed a relationship with the increase in MnP activity which reached a maximal value (832 U l^{-1}) at day 13 in the presence of PAH mix-

Table 1
Removal efficiency of PAHs for 28 days in Kirk medium inoculated with *A. discolor*. The values are means ± CI.

Compound	Removal efficiency (%)							
	Individual				Mixture			
	7 days	14 days	21 days	28 days	7 days	14 days	21 days	28 days
Phenanthrene	5.7 ± 0.6	17.5 ± 0.2	17.8 ± 2.9	22.6 ± 1.9	13.2 ± 2.6	23.5 ± 5.2	24.4 ± 1.3	26.5 ± 2.1
Anthracene	4.2 ± 1.2	5.3 ± 1.3	6.8 ± 2.4	7.0 ± 1.7	5.3 ± 3.7	6.3 ± 4.2	10.1 ± 1.0	11.3 ± 0.3
Fluoranthene	10.1 ± 4.9	18.0 ± 3.1	18.3 ± 3.7	19.5 ± 4.0	4.4 ± 4.0	7.1 ± 4.4	18.8 ± 1.3	23.5 ± 1.1
Pyrene	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 1.1	8.5 ± 0.2	2.7 ± 4.0	6.0 ± 4.8	12.8 ± 1.8	17.5 ± 0.1

ture, and was being three times higher than the control (without PAHs) (Fig. 1). The enhanced degradation of pyrene after 14 days may be associated with the increase of LiP, together with the simultaneous production of MnP (Fig. 1). Only negligible laccase activity was detected.

3.2. PAH removal in soil

A. discolor was inoculated in autoclaved and non-autoclaved soil contaminated with a mixture of phenanthrene, anthracene, fluoranthene, pyrene and benzo(*a*)pyrene.

Wheat grains were utilized as a support for the inoculum and as a vehicle for the soil colonization. *A. discolor* effectively colonized the contaminated autoclaved soil with a dense mycelial growth over the whole period of incubation, reaching the bottom of the tubes.

The content of the individual PAHs during the 60-day incubation period in inoculated and non-inoculated autoclaved soil is shown in Fig. 2. The soil, bioaugmented with *A. discolor,* showed an enhanced PAH removal efficiency compared to the control (non-inoculated), suggesting that *A. discolor* was able to degrade the studied PAHs. An initial decrease in concentration in both inoculated and non-inoculated autoclaved soil was observed until the 14th day for phenanthrene, anthracene, fluoranthene and benzo(*a*)pyrene (Fig. 2a–c and e, respectively). A sharp decrease in all PAH concentrations was observed between 14 and 21 days,

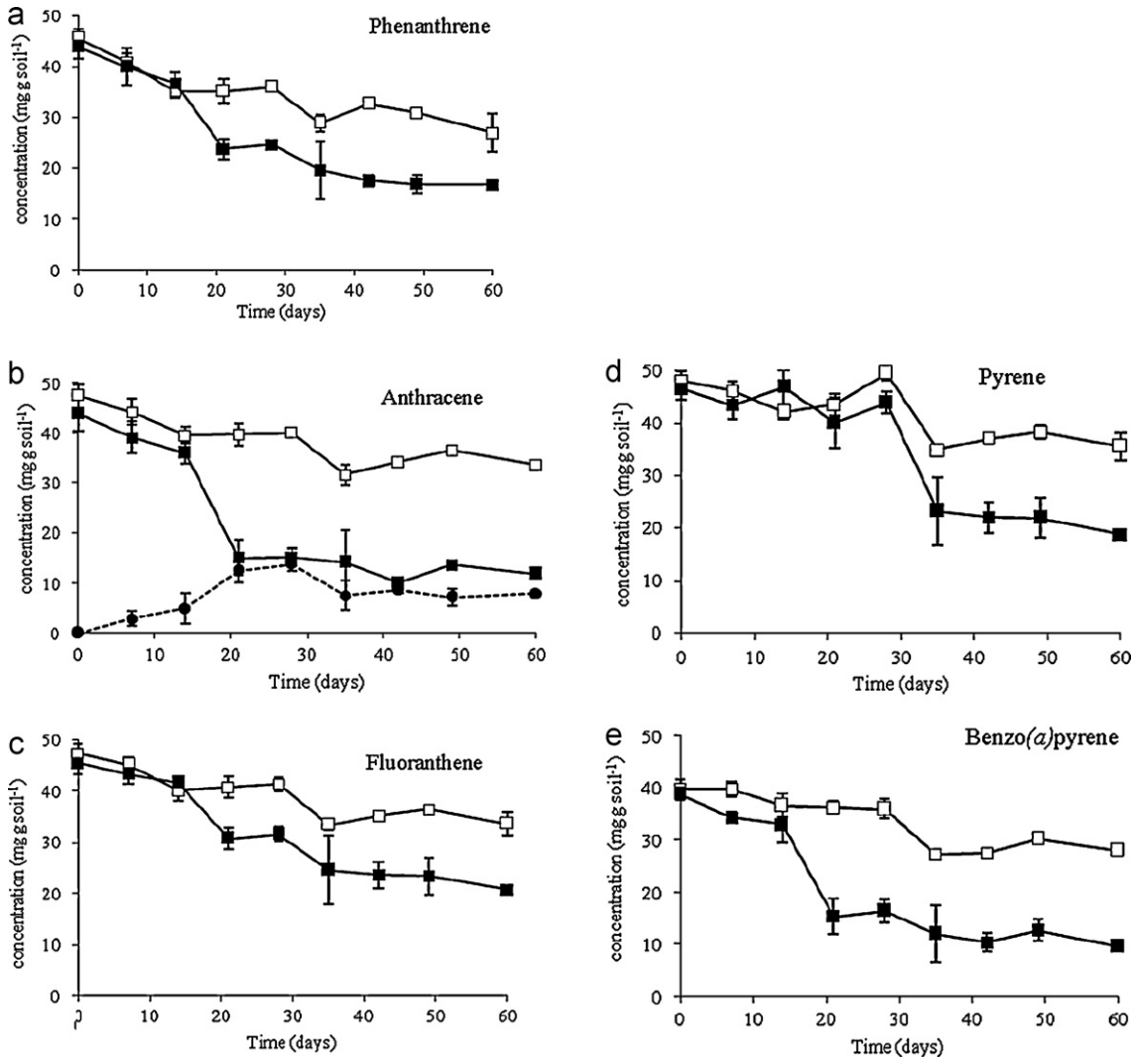

Fig. 2. (a) Phenanthrene (b) anthracene (c) fluoranthene (d) pyrene (e) benzo(*a*)pyrene concentration in autoclaved soil incubated at 30 °C, inoculated with *A. discolor* (■) and non-inoculated (□). Anthraquinone concentration (dashed line, ●) is represented in (b). The values are means ± CI.

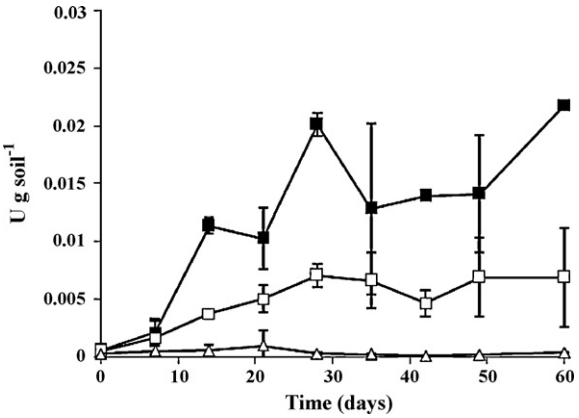

Fig. 3. Production of MnP in autoclaved soil inoculated with *A. discolor*, in presence of PAH mixture (■), inoculated with *A. discolor* without PAHs (□), non-inoculated, in presence of PAHs mixture (△). The values are means ± CI.

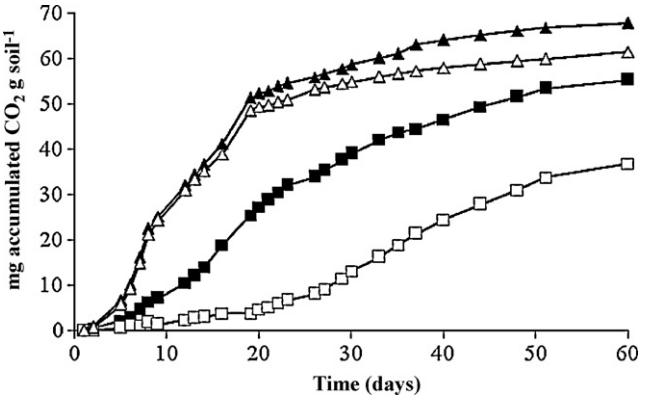

Fig. 4. Respiration (expressed as accumulated mg CO_2 g $soil^{-1}$) in non-inoculated autoclaved soil (□), autoclaved soil inoculated with *A. discolor* (■), non-inoculated non-autoclaved soil (△), non-autoclaved soil inoculated with *A. discolor* (▲).

particularly for anthracene and benzo(*a*)pyrene, and between 28 and 35 days for pyrene, in autoclaved soil inoculated with *A. discolor*, compared to the control (non-inoculated) (Fig. 2). After day 21, almost all PAHs showed only a small decrease in their concentration, except for pyrene which showed this tendency after 35 days (Fig. 2). In the case of anthracene degradation, the formation of anthraquinone was observed; the peak of anthraquinone production was correlated with the lowest concentration level of anthracene (Fig. 2b).

Table 2 summarizes the removal efficiency of PAHs after 60 days in soil inoculated with *A. discolor*, compared with the control. In the case of autoclaved soil, the removal efficiency was higher in soil bioaugmented with *A. discolor* compared with the control (non-inoculated).

Metabolites of PAH degradation were detected by GC–MS analysis. The anthracene degradation by *A. discolor* in autoclaved soil was confirmed through the detection of anthraquinone and phthalic acid. The degradation metabolites for fluoranthene were 4-hydroxy-9-fluorenone and 9-fluorenone and for pyrene it was 4,5-dihydropyrene. None of these compounds were found in the non-inoculated soil.

A high MnP activity was observed in autoclaved soil inoculated by *A. discolor* during 60 days (Fig. 3), reaching a maximum of 0.022 U g^{-1} on day 60, compared with the control (without PAHs). In non-inoculated, autoclaved soil and in the presence of PAH mixtures no significant enzymatic activity was detected. Neither laccase nor lignin peroxidase was detected at any time during the experiment.

In the case of non-autoclaved soil bioaugmented with the fungus, after 30 days the removal efficiency of PAHs was similar to the non-inoculated control (data not shown). Approximately 67% of the phenanthrene, 23% of the anthracene, 14% of the fluoranthene, 14% of the pyrene and 5% of the benzo(*a*)pyrene were removed in non-autoclaved soil, both inoculated or not inoculated with *A. discolor*. In the inoculated soil, 95.4% of the phenanthrene, 61.5% of the anthracene, 43.1% of the fluoranthene, 43.1% of the pyrene and 15.8% of the benzo(*a*)pyrene were removed after 60 days (Table 2), whereas 98.3% of the phenanthrene, 83.1% of the anthracene, 82.5% of the fluoranthene, 82.5% of the pyrene and 14.2% of the benzo(*a*)pyrene were removed in non-autoclaved contaminated soil without *A. discolor*.

3.3. Mineralization studies of PAHs in soils

The degree of mineralization of ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo(*a*)pyrene by *A. discolor* in autoclaved soil contaminated with the PAH mixture was monitored for 60 days. The mineralization of labeled PAHs was low, about 9% for ^{14}C-phenanthrene, 0.8% for ^{14}C pyrene and 0.6% for benzo(*a*)pyrene No significant amount of ^{14}C (<2%) was released from controls (non-inoculated autoclaved soil) during phenanthrene, pyrene and benzo(*a*)pyrene degradation (data not shown).

Microbial respiration was measured for 60 days at 30 °C in autoclaved and non-autoclaved contaminated soils, with and without *A. discolor*.

In the non-inoculated autoclaved soil, very low production of CO_2 was recorded at the beginning of the experiment but the values increased over time, slowly at first and markedly after day 26. Nonetheless, respiration in autoclaved, non-inoculated soil was always considerably lower than in autoclaved inoculated soil (Fig. 4). The increase in respiration in the non-inoculated soil was attributed to the recolonization by the indigenous microflora.

The highest CO_2 production was observed in the non-autoclaved soils where there was no appreciable difference between soil inoculated with *A. discolor* and non-inoculated soil during the first 19 days (Fig. 4). Subsequently, there was a slightly higher respiration in the samples inoculated with *A. discolor*.

4. Discussion

4.1. PAH removal in liquid culture

Biodegradation trials at laboratory scale using a liquid medium are normally the first approach to establishing the ability of microorganisms to degrade specific pollutants.

A. discolor was able to remove phenanthrene, anthracene, fluoranthene and pyrene in Kirk medium individually and in mixtures (Table 1). The removal efficiency of anthracene (11.3%) and pyrene (17.5%) in the PAH mixture in liquid medium after 28 days was higher than that of the individual compounds (7.0% and 8.5%, respectively), suggesting synergistic effects between PAHs [30] or possible cometabolism [31]. Similar results were observed by Boldrin et al. [32]. Fluorene was cometabolically degraded with other PAHs as growth substrates [32]. As mixtures of PAHs are often present in contaminated soils, cometabolic degradation or synergistic effects between PAHs could be important for bioremediation.

On the other hand, Bouchez et al. [33] observed an inhibition phenomenon but also synergistic interactions in PAH degradation by bacterial strains. Desai et al. [34] observed that the initial degradation rates of individual components decreased in the presence of other PAHs. Results from the mixture experiments indicated competitive inhibition interactions. Therefore, potential PAH inter-

Table 2
PAH removal efficiency in soil contaminated inoculated and non-inoculated with *A. discolor*, in absence and presence of soil indigenous microorganisms, after 60 days. The values are means ± CI.

Compound	Removal efficiency (%)			
	Autoclaved soil		Non-autoclaved soil	
	Non-inoculated	Inoculated	Non-inoculated	Inoculated
Phenanthrene	39.5 ± 10.0	61.9 ± 1.8	98.3 ± 1.3	95.4 ± 2.0
Anthracene	27.3 ± 5.0	72.9 ± 2.2	83.1 ± 6.2	61.5 ± 10.7
Fluoranthene	27.7 ± 8.0	54.3 ± 2.8	82.5 ± 12.9	43.1 ± 6.5
Pyrene	24.9 ± 9.8	59.7 ± 1.9	82.5 ± 12.9	43.1 ± 6.5
Benzo(*a*)pyrene	28.1 ± 7.2	75.4 ± 0.7	14.2 ± 5.9	15.8 ± 6.4

actions are quite numerous and complex [34], and no particular relationship can be assumed.

When comparing the results of the enzymatic activity in inoculated liquid with and without PAHs (Fig. 1), the presence of PAHs stimulated MnP production. An important increase in MnP activity in liquid medium started on day 7 compared with the control (without PAHs), followed by a decrease of PAH content. These results suggest that MnP may be involved in PAH degradation. In fact, MnP can oxidize (by means of the strong oxidant Mn^{3+}) PAHs with ionization potential (IP) value up to 7.8 eV such as anthracene (7.43 eV) and pyrene (7.53 eV), whereas compounds with higher IP values such as phenanthrene (8.03 eV) and fluoranthene (7.90 eV) are oxidized in the presence of cooxidants (lipids or Tween 80) [5]. In addition, LiP produced and stimulated in presence of PAHs after day 14 would be also responsible for PAH degradation, particularly for anthracene and pyrene. PAHs with IP values of less than 7.55 eV are suitable substrates for direct one-electron oxidation by LiP [35]. Peroxidases and laccase expressed under nutrient-limiting conditions may be involved in PAH degradation by fungi as observed by Cajthaml et al. [36], but there is insufficient knowledge about their combined role and interactions [37]. High complexity biodegradation mechanisms, in addition to the ligninolytic enzymes, as well as other biochemical systems, may be responsible for PAH degradation by fungi [38,39]. The measurement of high activity levels of a specific enzyme (MnP in this case and LiP to a lesser extent), coupled with the removal of a pollutant (PAHs in our case) may be considered to be indicative of a relationship between enzyme activity and degradation yield [17,38].

4.2. PAH removal in soil

Upon visual examination, *A. discolor* showed a relevant growth, with the hyphae able to penetrate through the autoclaved soil to the bottom of the tube and simultaneously adhering to the surface of the wheat grains. Soil is a matrix presenting high porosity, which promotes adequate aeration and humidity for fungal growth. Moreover, the presence of wheat grains may stimulate *A. discolor* growth and a higher production of ligninolytic enzymes as shown by Rubilar [20]. When introduced into soil, white-rot fungi may undergo an adaptation process related to exposure to an environment which is different from wood in many respects [39]. Lignocellulosic materials have been successfully used for introducing white-rot fungi into soil [39], promoting larger inoculum biomass and faster and more successful establishment of the fungus in the soil [40,41]. In this sense, wheat grains were chosen as lignocellulosic substrate/support in this study.

During the first 14 days, the removal efficiency in non-inoculated and inoculated autoclaved soil was attributed to sorption into soil particles and wheat grains. An initially rapid, reversible sorption process was produced by the interaction of PAHs with soils, followed by a longer period characterized by increasingly strong interactions and leading to the recalcitrance of these compounds [42]. Between 14 and 28 days, PAHs were significantly removed by *A. discolor*; this removal was directly correlated to an increase in MnP activity. Finally, between 28 and 60 days, the removal of all PAHs may be also attributed to the combined action of PAH adsorption on soil particles, and degradation by *A. discolor* and by soil microflora reactivated after 26 days. As shown in the control test (Fig. 4), soil microflora was able to recolonize the autoclaved soil after 26 days. Some spores were able to survive under the autoclaving conditions and these specific microorganisms were able to colonize the soil again [43–45]. Furthermore, *A. discolor* showed biodegradation capability in autoclaved soil contaminated with PAHs; here, the degradation of benzo(*a*)pyrene was significant. In spite of the recalcitrant characteristics of benzo(*a*)pyrene and its preference for soil particles [46], a high removal efficiency (75.4%) of this compound was observed in autoclaved soil. Although a great diversity of organisms are capable of degrading low molecular weight PAHs such as anthracene and phenanthrene, relatively few genera have been observed to degrade high molecular weight PAHs such as benzo(*a*)pyrene [47], one of the most potent carcinogenic PAHs.

The PAH degradation was confirmed by the appearance of known metabolites from ligninolytic enzyme action. The appearance of anthraquinone was observed at the beginning of anthracene degradation by *A. discolor*; this compound has been reported to be the main and most stable dead-end metabolite of anthracene degradation by the enzymatic system of white-rot fungi [48], such as strains from the genera *Bjerkandera* and *Phanerochaete* [49]. Phthalic acid was reported as an oxidation product of anthracene from *Bjerkandera* sp. BOS55 [12]. The degradation products for fluoranthene were 4-hydroxy-9-fluorenone and 9-fluorenone and for pyrene, it was 4,5-dihydropyrene. Other metabolites resulting from the degradation of fluoranthene by *I. lacteus*, such as 1,8-naphthalic anhydride and 2-formyl-acenaphthen-1-carboxylic acid methylester were found by Cajthaml et al. [48]. In addition, trans-4,5-dihydrodiolpyrene has been reported as a degradation product of pyrene by several white-rot fungi [50–53]. During the experiment, quinone intermediates of phenanthrene and benzo(*a*)pyrene biodegradation were not detected by GC–MS. Apparently, during the degradation in vivo of this study, quinones are rapidly metabolized even more.

The measured amounts of $^{14}CO_2$ generated from labeled phenanthrene, pyrene and benzo(*a*)pyrene in autoclaved soil were found to be less than 15%, similar to amounts usually found in other white-rot fungi [14,54], demonstrating the low ability of this fungus to mineralize PAHs. A diverse group of white-rot fungi have the ability to oxidize PAHs, but the degree of mineralization is always limited [14,47]. The PAH metabolites are more polar and soluble than the parent PAHs, and thus are likely more available for degradation by other microorganisms [14]. Due to the complexity of PAH contamination in soil, the soil microflora plays a pivotal role in minimizing the risks of accumulated PAH metabolites. Furthermore, synergistic effects between *A. discolor* and indigenous soil microorganisms for PAH mineralization are desired. During the first 30 days, the removal efficiency for all PAH was com-

parable between the non-autoclaved soil bioaugmented with *A. discolor* and non-bioaugmented soil. However, after 60 days the presence of *A. discolor* in non-autoclaved soil resulted in a less efficient removal of anthracene (83.1% vs. 61.5%) and fluoranthene and pyrene (82.5% vs. 43.1%) compared with the control, indicating possible inhibition and competition of native microbes with the fungus. *A. discolor* may trigger some defense mechanisms which could negatively affect indigenous soil microorganisms. These mechanisms are not clear and still unresolved. Ligninolytic fungi typically react to the presence of soil microorganisms with an increase in laccase activity [55]. Eggert [56] reported that this enzyme catalyzes the formation of antibacterial compounds in *Pycnoporus cinnabarinus,* but the direct effects of laccase on soil bacteria are not confirmed [57]. Some fungi produce toxic hydroxyl radicals in the presence of antagonistic bacteria as demonstrated by Tornberg and Olsson [58], while their antibiotic compound production may be another possible mechanism [39].

Most white-rot fungi utilize wheat grains, straw or wood shavings as preferred substrates [21,59,60]. However, in this study, the main factor affecting the colonization of non-autoclaved soil by *A. discolor* was clearly the presence of indigenous soil microorganisms, which may compete with the fungus for the lignocellulosic substrate as a source of carbon and nitrogen, disabling the fungal growth. In fact, substrate limitation may occur due to competition with indigenous soil microorganisms, inhibiting lignocellulosic material decomposition by the fungus [61]. Bacterial populations can decompose lignocellulosic material [62] and have the ability to use it as a carbon and nitrogen source [39]. Adequate treatment of a lignocellulosic material may enhance its selectivity to support *A. discolor* growth in non-autoclaved soil. In fact, when Castillo et al. [61] used straw treated with formic acid and hot water as lignocellulosic material for *P. chrysosporium*, the effect of competition with other microorganisms diminished.

As shown in this study, the success of fungal bioaugmentation for enhancing removal of PAHs may depend on several factors. Furthermore, suitable conditions must be found to promote the growth of *A. discolor* in soil contaminated with PAHs.

5. Conclusions

A. discolor, a white-rot fungus isolated from the Chilean forest, showed the ability to degrade PAHs in a liquid medium. The highest removal rate occurred in PAH mixtures, suggesting synergistic effects or cometabolism. In addition, *A. discolor* was able to degrade PAHs in autoclaved soil, associated with the production of ligninolytic enzymes, mainly MnP. In particular, degradation of benzo(*a*)pyrene, one of the most potent carcinogenic PAHs, was the highest (75%) using this fungus. In non-autoclaved soil, the inoculation of *A. discolor* did not improve the PAH removal efficiency, and determining suitable conditions for promoting successful fungal bioaugmentation in soils requires further study.

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