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# Inoculation of Filamentous Fungi in Manufactured Gas Plant Site Soils and PAH Transformation

Rachel Rama[1$], Jean-Claude Sigoillot[3], Véronique Chaplain[2], Marcel Asther[3], Claude Jolivalt[2] and Christian Mougin[2*]

[1]KREBS SA, 1 Rue des Hérons, 78180 Montigny le Bretonneux, France

[2]Institut National de la Recherche Agronomique (INRA), Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, 78026 Versailles Cedex, France

[3]Institut National de la Recherche Agronomique (INRA), Unité de Biotechnologie des Champignons Filamenteux, Faculté des Sciences de Luminy, Centre d'Enseignement Supérieur en Biotechnologies/ESIL, Parc Scientifique et Technologique, CP 925, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France

Short title: Fungal growth and PAH transformation in soils

[$]Present address: Aventis Crop Science, 14-20 Rue P. Baizet, BP 9163, 69263 Lyon Cedex 09, France

*Corresponding author:

E-mail address: mougin@versailles.inra.fr

Tel: +33-(0)1-30-83-31-02

Fax: +33-(0)1-30-83-31-19

## ABSTRACT

Solid carriers have been developed to inoculate *Trametes versicolor* and *Cunninghamella elegans* into manufactured gas plant site soils. Pelleted wheat bran carriers were very efficient in stimulating the growth of fungi in an industrial soil containing about 2800 mg $kg^{-1}$ PAHs. Fungal biomass and activity of extracellular laccases, enzymes produced by *T. versicolor* as markers of metabolic activity in the contaminated soil, both decreased after 2 weeks of incubation. Supplementing the soil with a mixture of carbon, nitrogen and phosphorus enhanced the fungal activity period. A 38% decrease of solvent extractable PAHs was observed in manufactured gas plant site soils when supplemented with *T. versicolor*, Glucidex 19$^{TM}$, ammonium nitrate, lime phosphate and Montanox 80$^{TM}$, after 20 weeks. Then, the degradation proceeded more slowly during the following 30 weeks, and reached 43% of initial extractable PAHs. Some factors governing a limited PAH biotransformation in the soil are discussed.


Acronyms: ABTS: 2,2'-azinobis-(3-ethylbenz-thiazoline-6-sulfonic acid), CEC: cation exchange capacity, HPLC: high pressure liquid chromatography, MHC: moisture holding capacity, MGP: manufactured gas plant, PAH: polycyclic aromatic hydrocarbon, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, U: unit

Keywords: polycyclic aromatic hydrocarbons, bioremediation, immobilization, microcosms, manufactured gas plants, fungus

# INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants resulting from fossil fuel combustion and from by-products of industrial activities. They are widely dispersed in the environment [1,2]. Many of these chemicals are carcinogens, mutagens, or both [3]. For these reasons, and because of their high persistence (estimated to reach several years in soils), polluted sites represent a health risk to human populations. The high cost of classical remediation processes requiring, for example, soil excavation, incineration and/or secure landfilling, set bioremediation as a promising cost-effective technology for cleaning up soils contaminated with PAHs [4]. In that context, the bioaugmentation approach allows the development of biological processes in either land farming or composting technologies [5]. This implies to identify efficient PAH-degrading strains among microorganisms, and to develop tools ensuring good soil colonization after inoculation.

For many years, filamentous fungi have demonstrated their ability to transform hazardous organic contaminants in liquid cultures [6]. Among PAH-degrading strains were the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor*, as well as the zygomycete *Cunninghamella elegans* [7,8,9,10]. Solid-phase degradation experiments concerning PAHs have also been reviewed [4]. Nevertheless, microbial and fungal efficiencies for PAH transformation have been demonstrated only in soils from wood preservation plants [11,12,13]. On the other hand, PAHs in soils from coking or Manufactured Gas Plant sites (MGPs) were shown recalcitrant to biological degradation [14,15].

The success of a bioremediation procedure depends on the development of the microorganisms inoculated into the soil, and on the expression of their degradative enzymes. Both abiotic factors (soil moisture, pH, chemical toxicity,...) and biological factors (competition, predation,...) play critical roles in determining microbial or fungal colonization. The presence of a dense and active inoculum has been reported to overcome some of these problems for extended periods of time [16,17]. A great potential is recognized for carriers to introduce biomass into soils. Carriers provide protection, nutrients and also a more stable microenvironment for the organisms, important for survival and growth of the inoculated fungus. Recently, Lestan and Lamar [18] showed that pelleted lignocellulosic substrates were efficient tools to introduce large amounts of filamentous fungi into soils, and to degrade hazardous organic compounds. The present paper describes (i) the development of tools to assess a bioaugmentation approach using fungi for PAH degradation in MGP site soils, (ii) the assay for PAH degradation in such conditions.

## MATERIALS AND METHODS

### Chemicals

We purchased Glucidex 19$^{TM}$ (industrial source of maltose) from Roquette Frères (Lestrem, France) and Montanox 80$^{TM}$ (industrial preparation of Tween 80) from Seppic (Paris, France). Solvents were obtained from Carlo Erba (Val de Reuil, France). All other chemicals, reagents and fertilizers were commercial products.

### Fungi

We obtained the white rot strains *Trametes versicolor* (Mic 209) and *Phanerochaete chrysosporium* (BKM-F-1767, ATCC 24725) from the culture collection of UBCF (INRA Marseille-Luminy, France). *Cunninghamella elegans* (ATCC 26269) was purchased from the American Type Culture Collections (Manassas, VA, USA). The strains were maintained at 37°C on solid medium comprising malt (20 g $L^{-1}$), agar (16 g $L^{-1}$) and yeast extract (1 g $L^{-1}$).

## Soil sampling

Chemical and physical properties of the experimental soils are summarized Table I. They were determined by using AFNOR [19] normalized protocols. We collected agricultural soil (used as non-contaminated soil) in the 10-20 cm layer in a field of the experimental farm of INRA at Dijon (France). PAH-contaminated industrial soil was taken from a pile in a MGP site at Rouen (France). We assessed PAH concentrations by using analytical methods described below.

## Medium and fungal biomass production

The culture medium [10] used for biomass production contained: $KH_2PO_4$ (2 g $L^{-1}$), $CaCl_2.2\ H_2O$ (0.14 g $L^{-1}$), $Mg\ SO_4.7\ H_2O$ (0.7 g $L^{-1}$), $Fe\ SO_4.7\ H_2O$ (0.07 g $L^{-1}$), $Zn\ SO_4.5\ H_2O$ (0.046 g $L^{-1}$), $Mn\ SO_4.H_2O$ (0.035 g $L^{-1}$), $Cu\ SO_4.5\ H_2O$ (0.007 g $L^{-1}$), diammonium tartrate (1.842 g $L^{-1}$), yeast extract (1 g $L^{-1}$), 1 mL $L^{-1}$of a vitamin solution [20], glucose (5 g $L^{-1}$) for *T. versicolor* and *C. elegans*, glycerol (10 g $L^{-1}$) for *P. chrysosporium*. We buffered the culture medium at pH 6.5 with disodium tartrate (2.3 g $L^{-1}$).

Biomass production was achieved in Roux flasks containing 200 mL culture medium, developed from five agar plugs (5 mm) of mycelium taken from the solid medium plates, and cultured for 7 (*C. elegans*) or 10 days (other strains) at 27°C in darkness.

## Fragmentation of fungal mycelia

We filtered the mycelia from the growth media, and the biomass (0.7-0.8 g dry weight for the white rot fungi and 0.9-1.0 g dry weight for *C. elegans*, *per* Roux flask) was suspended in sterile, deionized water. The resulting mycelial suspensions were fragmented for 1 min with an Ultra Turrax blender (10000 rpm). For fungal growth studies, the fragmented suspension contained 2 mats (about 2 g dry biomass) in a final volume of 100 mL. It contained 20 mats (about 20 g dry biomass) in 400 mL for degradation studies.

## Pelleting and sterilization of carriers

The carriers used for soil inoculation were composed of low-cost agricultural by-products, such as wheat bran, wheat straw and sugar beet pulp. Each substrate was ground to 3 mm cross section and passed through an industrial thermic pellet press (Unité de Préparation des Aliments Experimentaux, INRA, Jouy en Josas, France). Final pelleted substrate was 4.8 mm in diameter and averaged 8 mm in length. Then, we sterilized the pellets by two successive autoclavings for 20 min at 121°C in 1-L Erlenmeyers flasks closed by air-permeable cotton stoppers. Carrier sterility was then checked by culture on the solid medium described above.

## Coating of carriers and mycelium growth

We used a modification of a described protocol [18] to inoculate pellets for fungal growth studies. The mycelial suspension (20 mL) was mixed with 30 mL sodium alginate solution at 2% (w/v). Then, pellets (100 g) were coated by consecutive spraying of the previous mixture and of small amounts of 5% (w/v) sterile $CaCl_2$.

Carriers used for degradation studies were coated by mixing a mycelial suspension (200 mL) of *T. versicolor* with sterilized wheat bran pellets (2 kg) in the presence of guar gum (1-L solution at 1%, w/v) in sterile polyethylene bags.

We incubated all coated carriers at 27°C until they were coated with mycelium (7-10 days for fungal growth studies, 14 days for degradation studies). After that period, we considered the carriers ready for use as a fungal inoculum for soil treatment.

## Soil inoculation and incubation for fungal growth studies

These studies were a series of small-scale experiments to investigate optimization variables. Before use for fungal growth studies, each soil (agricultural or industrial) was air-dried and passed through a 2-mm sieve, then mixed and stored in polyethylene bags in darkness at room temperature (about 20°C) less than one week. We conducted the incubations by using 30-g dry soil in 150-mL Erlenmeyer flasks, usually inoculated with 3-g pellets coated with fungal mycelium, during 1 to 6 weeks. We added an aliquot of deionised sterile water to achieve 80% MHC at the beginning of the experiments.

We sealed the Erlenmeyer flasks with cotton plugs and incubated them in 1-L sealed flasks with a vial containing 10-mL water to keep moisture constant [21]. The headspace of the flasks was flushed with air at the beginning of the experiments, and

then every 7 days. We incubated samples in darkness at 25°C for 1 to 6 weeks. In some cases, nutrients (C/N/P) were supplemented as an aqueous solution of sterile maltose, $(NH_4)_2SO_4$ and $KH_2PO_4$ without any significant modification of soil pH. Carbon (1% dry soil, w/w) was first added, then sufficient N and P were added to bring the C/N/P ratio to 100/5/1. We completely used each soil sample for fungal biomass determination.

For sterile experiments, portions of the soil were autoclaved two-times at 121°C for 20 min in 1-L Erlenmeyer flasks closed by air-permeable cotton stoppers.

**Soil inoculation and incubation for degradation studies**

These studies were larger-scale bench studies to investigate fungal effectiveness in soils from a contaminated site. For degradation studies, the industrial soil was mixed, sieved at 15 mm and used without more storage. This size was related to soil preparation performed in industrial bioremediation procedures. Experiments were conducted in polyethylene jars refilled with 6.0-kg dry industrial soil that had not been sterilized, during 50 weeks. We submitted the soil to several treatments (Table II), each corresponding to one jar. In four of them, it was mixed with 0.6 kg of pellets coated with *T. versicolor*. In all cases, we inserted an inlet of compressed air in a 4-cm layer of gravel (1 cm diameter) in the bottom of the jars. Soil and pellets were mixed, and stacked above. Soil moisture was checked every week. It was reduced to 55-65% of the MHC every week in the soil and consequently brought to 80% of the MHC by adding water. The jars were continuously air-flushed, and incubated in the dark at room temperature (about 20°C). According to the treatment scheme, nutrient solutions (C: Glucidex 19$^{TM}$, N: ammonium nitrate, P: lime phosphate, ratio 100/5/1) and neutral surfactant (Montanox 80$^{TM}$, 5% dry soil, w/v) were added in some jars at the beginning

of the studies. Then, only nutrients were provided every 10 weeks of incubation. Carbon content of Glucidex 19$^{TM}$ was used to determine N and P addition, because soil carbon was considered unavailable for fungi.

Every 10 weeks, we analyzed a soil fraction from each bag. It was obtained by mixing 10 samples of 10 g withdrawn within the soil layer, and then divided in 3 subsamples (each of 30 g) for fungal biomass determination, PAH analysis and laccase assay.

## Fungal biomass determination

We estimated fungal biomass in soils in 30-g soil subsamples, according to the modified method of Seitz *et al*. [21], as recently described [22]. Briefly, the protocol involves sample homogenization in methanol, saponification, partitioning of non-polar extractives to an organic solvent (pentane), and quantitation by HPLC. A calibration curve was made with standard ergosterol and a correlation between dry fungal biomass and ergosterol content of pure cultures was performed for each fungal strain.

## PAH extraction and analysis

PAHs were Soxhlet-extracted from each soil subsample (30 g dry weight) in the presence of acetonitrile (100 ml) for 8 hours. Pellets coated with fungi were removed from the soil before extraction. After cooling, the extracts were brought to 100 ml and filtered on a Whatman 0.2 µm nylon membrane. We obtained separation and quantification of PAHs by injections of a 20-µl sample in the HPLC through a 7125 Rheodyne valve. PAHs were then eluted on the analytical column Supelcosil LC-PAH (15 cm x 4.6 mm *id*; Supelco, Saint-Quentin Fallavier, France) with a Varian 9010

pump delivering a mixture of acetonitrile and water. Elution began with 45% acetonitrile, followed by a linear increase to 55% acetonitrile over 5 min, another increase to 100% acetonitrile over 30 min, and a stationary phase of 10 min, before a return to the initial conditions. The flow-rate was 1.5 mL $min^{-1}$. We monitored absorbance at 254 nm with a variable wavelength detector (Varian 9050).

## Enzymatic assays

We used laccase activity as an indirect method to assess fungal activity in the soil, in order to acquire data on the physiological state of the fungus. Enzymatic activity was measured in buffered extracts of the industrial soil. The 30-g subsamples of soils were shaken with 100 mL of 50 mM citrate phosphate buffer (pH 6.0) for 15 min and then the slurry was centrifuged at 4000 g for 10 min. After 30-min incubations with catalase (10U) to eliminate residual $H_2O_2$ and thus prevent any activity of the peroxidases, this of laccase was determined by oxidation of 2,2'-azinobis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in spectrophotometer cells [23]. The reaction mixture (1 mL final volume) contained 5 mM ABTS in 100 mM citrate phosphate buffer (pH 3.0), and 100 µL of soil extract. We followed enzymatic oxidation of ABTS ($E_{420}$ = 36000 $M^{-1}$ $cm^{-1}$) by an absorbance increase at 420 nm at 30°C against a cell without enzyme.

## Experimental design

Experiments were conducted in duplicates, each analyzed two times. Results are expressed as means (4 values for each point) ± standard deviation (error bars).

# RESULTS

## Selection of pellet carriers for soil inoculation

Correlation between dry fungal biomass and ergosterol content of pure fungal cultures have shown that 1 mg ergosterol corresponded to 105 mg dry fungal biomass for *T. versicolor,* 101 for *C. elegans*, and 191 for *P. chrysosporium* [22]. The latter strain was used in the present study for comparison purposes, although it was not an efficient PAH-degrader when screened on wet sand [10]. The ergosterol-to-biomass ratios calculated were in the range of values previously reported for filamentous fungi [24]. The fungal biomass in soils where mixture of strains were detected, or in soils that have not been inoculated by the fungi, was calculated using a mean correlation factor: 1 mg ergosterol corresponded to 132 mg dry fungal biomass. Fig. 1 shows the dry fungal biomass amounts obtained after inoculation of the sterilized agricultural soil with carriers coated with *T. versicolor*, *C. elegans* and *P. chrysosporium*. Initial fungal biomass, inoculated into the soil, was 3-4 g $kg^{-1}$ soil (on a dry weight basis). Fungal biomass amounts in the uninoculated soils were below 0.3 g $kg^{-1}$ soil, over a 2-week period. It increased in the soils when the fungi were grown on the pellets, whatever their composition. Maximal amounts were obtained for each strain with carriers composed of wheat bran. Levels were 10.5 g $kg^{-1}$ soil for *T. versicolor,* 17.0 for *C. elegans*, and 23.7 for *P. chrysosporium*, after 2 weeks.

## Efficiency of wheat bran carriers for fungal inoculation in industrial soils

Fig. 2 shows the fungal biomass in the non-sterilized industrial soil, following *T. versicolor* or *C. elegans* inoculation. Levels of ergosterol measured in the soils

inoculated with carriers not coated with the fungi corresponded to biomass amounts lower than 0.3 g $kg^{-1}$ soil (on a dry weight basis) during a 6-week period. After fungal inoculation, Fig. 2A and 2B showed similar growth profiles for *T. versicolor* and *C. elegans* in the industrial soil supplemented with pure water. Although initial biomass values were lower in the presence of *T. versicolor* than *C. elegans*, fungal development proceeded in a first time by a rapid growing phase. Proliferation of mycelium in the soil was also observed through the glass flasks. Maximal dry fungal biomass amounted in the industrial soil to 13.2 g $kg^{-1}$ for *T. versicolor* after 2 weeks, and 15.2 g $kg^{-1}$ for *C. elegans* after one week.

Biomass of *T. versicolor* and *C. elegans* was then checked during 6-week studies in the non-sterilized industrial soil inoculated with each fungus and supplemented with water or a nutrient solution (Fig. 2). On the one hand, a decrease of fungal biomass occurred in the industrial soil supplemented with pure water after 2-3 weeks of incubation. It was more pronounced in the case of *T. versicolor* than in the case of *C. elegans*. *T. versicolor* biomass declined to 3.7 g $kg^{-1}$ after 6 weeks of incubation (Fig. 2A), whereas growth profiles obtained with *C. elegans* in a similar experiment demonstrated, after the 1-week increasing phase, a regular decrease of the biomass to the final values of 10.9 g $kg^{-1}$ dry weight after 6 weeks (Fig. 2B).

On the other hand, in another set of incubations, the industrial soil was supplemented with a mixture of carbon, nitrogen and phosphorus to give a final ratio of C/N/P: 100/5/1 to assess the effects of nutrients on fungal growth. *T. versicolor* growth exhibited a 1-week lag-phase followed by an increase during 2 weeks. Then, the biomass decreased to reach its final level of 8.2 g $kg^{-1}$ after 6 weeks (Fig. 2A). In these

incubations conditions, *C. elegans* biomass reached the highest levels, with a 20% increase when compared to values obtained in watered soils, to a final amount of 14.4 g $kg^{-1}$ (Fig. 2B).

## Assessment of fungal activity in the soil

The laccase activity was measured during 6 weeks after inoculation of the non-sterilized industrial soil with *T. versicolor* (Fig. 3). For comparison, a biomass production curve was also shown for that period. The two curves showed a similar profile. Laccase activity was the highest between 1 to 2 weeks after inoculation (450 nmol $min^{-1}$ $g^{-1}$), and then gradually decreased. At this moment, laccase identity was checked by SDS-PAGE by comparison with purified enzyme from *T. versicolor* (data not shown). ABTS oxidation was not modified after preincubation of the extracts with catalase, excluding the possible involvement of peroxidases in the reaction. ABTS oxidation did not occur in extracts from control soils inoculated with sterile pellets. Yet, laccase production seemed closely related to fungal growth. Both became negligible after 4 weeks of incubation.

## Assay for PAH degradation in industrial soil using fungi

Before treatment, total PAHs in soil were 2880 mg $kg^{-1}$ dry soil (Fig. 4). The fate of PAH concentrations during 50 weeks differed according to the treatment. When the soil was moistened only with pure water (jar 8), a slight PAH increase (9% versus initial values) was observed. Consequently, no PAH loss occurred by volatilization. Moreover, the soil appeared to have a very high bulk density. Supplementing the soil with uninoculated pellets and nutrients (jar 5) resulted in a decrease of extracted PAHs

observed at week 20, and in a decrease of soil bulk density. After that period, PAH amounts were similar to initial values. Nutrients (jar 6) and surfactant (jar 7), when applied alone, did not modify the degrading activity of the indigenous microflora.

When comparing the concentrations of PAHs in the different extracts resulting from soils inoculated with *T. versicolor* (jars 1-4) or non-inoculated (jars 5-8), it is clear that long-term decrease of the hydrocarbon levels occurred only in the presence of the fungus. The decreasing rates depended on both the presence of nutrients (C/N/P) and surfactant (Montanox 80$^{TM}$). The more rapid degradation was obtained in the soil when the fungus was grown with nutrients and surfactant in mixture (jar 1). PAH concentrations were only 62% of initial values after 20 weeks. Then, degradation proceeded very slowly until the end of the study. Similar final concentrations were obtained without nutrients (jar 3), but in the presence of the surfactant. In soil not supplemented with the surfactant, with (jar 2) or without (jar 4) nutrients, the degradation efficiency was lower than in the presence of surfactant, with no clear effect of the nutrients on the transformation.

All these results seemed to indicate that, in the presence of the fungus, the highest PAH degradation could be achieved by supplementing the soil with surfactant alone.

We measured also fungal biomass and laccase production. Initial biomass of *T. versicolor* was 6.0 g kg$^{-1}$ soil (dry weight). After 20 weeks, the morphology of the fungi coating the carriers was different from that of the initial *T. versicolor*. Cultures of isolates on solid media evidenced a mixture of several fungal strains. Consequently, we calculated biomass amounts on the basis of the mean ergosterol-to-biomass correlation factor (1 mg ergosterol corresponded to 132 mg dry biomass). Fungal biomass measured

after 20 and 50 weeks of incubations are reported Table II. In both periods of time, they are negligible or low in the jars 5-8 (non inoculated by *T. versicolor*). No laccase activity was detected between 20 and 50 weeks of incubation.

## DISCUSSION

This paper describes a simple procedure to introduce large amounts of fungal biomass to soil, in order to improve biological degradation of PAHs in a soil from a manufactured gas plant (MGP) site. Results show the efficiency of our inoculation method to enhance the propagation of filamentous fungi in PAH-polluted soils, and confirm the ability of these organisms in transforming the contaminants when adequate treatment conditions are followed.

High inoculum potential determines the success of a bioremediation procedure. As reported by other studies [24,25], our experiments confirm that low-cost lignocellulosic materials can be used for the inoculation of large amounts of fungal biomass in soils. Our data agree with those reported by Boyle [26] and Morgan *et al*. [27], who reported that agricultural materials were effective at stimulating the growth of white-rot fungi in soils. In our case, wheat bran pellets were the most efficient carriers. Nevertheless, the fungi were unable to develop in alfalfa pellets in our study (data not shown), whereas *T. versicolor* grew in soils amended with alfalfa [26]. In accordance with the work of Lestan and Lamar [18], the potential of the pelleted substrates for industrial application was thus confirmed. They are practical tools to handle biomass during transportation from the inoculum production unit to the contaminated site, and to apply it on site. Our pellets

supported the growth of fungi including ligninolytic (*T. versicolor*) and non-ligninolytic (*C. elegans*) strains. Studies need to be extended to other strains such as soil fungi.

*T. versicolor* and *C. elegans* well grew in a soil from a MGP site (containing high levels of contaminants: PAHs, cyanides and other inorganic compounds) which was apparently not or poorly toxic for fungi growing within the pellets and around them in the soil. It is also noteworthy that the industrial soil offered a high soil pore size, thus ensuring a better airing and proliferation of the mycelium. In the soil that was not sterilized, we showed that the competition of the fungi with the indigenous microflora did not prevent fungal development. Among methods available to estimate fungal growth in solid substrates, ergosterol content has been shown to be a more sensitive and reliable indicator of fungal growth than other indirect methods [24], because ergosterol is endogenous only to fungi and some green algae. Ergosterol measurement is also adapted to routine, multiple analyses of a great number of samples. But ergosterol content, as an indicator of total fungal biomass, does not allow an estimate of the growth of a single strain among a mixture.

Although the carriers contained nutrients that supported in part the fungal growth in the soil, and despite a high content of organic carbon of the MGP soil, adding a nutritive solution of carbon, nitrogen and phosphorus increased the lifetime of the fungi. Soil carbon may have been in great part unavailable for microorganisms, as often reported in the literature [26]. It is also noteworthy that indigenous fungi colonize the pellets during long-term experiments.

Laccases are enzymes able to oxidize a wide range of PAHs (at the least during *in vitro* assays), directly or in the presence of exogenous mediators [28,29,30]. They are mainly used in the present study to assess fungal activity in the soil. The knowledge of

enzyme production kinetics in a soil may reflect the metabolic activity of fungi. In our experiments as well as in studies previously reported [26], laccase production was closely related to the fungal growing phase in short-term incubations, and was easy to quantify by spectrophotometric measurements. However, we never detected laccase activity in the industrial soil during long-term experiments. In that case, environmental conditions may repress enzyme production or the enzyme could be inactivated by chemicals or soil-like constituents, incorporated or adsorbed on soil products, thus reducing the concentration of active forms of enzymes [31]. On the other hand, we know that inorganic compounds, such as metals, are strong inactivators of ABTS enzymatic oxidation (unpublished data). Consequently, the spectrophotometric measurement of laccase activity is not an efficient tool to assess fungal activity in contaminated soils during long-term experiments. In such experiments, laccase production could be more efficiently checked by other methods involving for example molecular probes [32].

PAH transformation has been studied in soils from wood preservation plants and MGPs by several laboratories. All reported the recalcitrance of PAHs to microbial transformation in the soils from MGP sites. Erikson *et al.* [14] and Weissenfels *et al.* [15] suggested that contaminant bioavailability was the main limiting factor for biotransformation in MGP soils contaminated for a long time. Kotterman *et al.* [33] reported that neutral surfactants such as Tween 80 increased the PAH oxidation rates by the ligninolytic system of the white-rot fungus *Bjerkandera sp*. strain BOS55 in liquid cultures. This effect was attributed to an increase of contaminant aqueous solubility by partition, and occurred also in soil [34]. Our results obtained from jars 1 and 2, as well as jars 3 and 4, agree with these hypotheses, *as T. versicolor* and *C. elegans* were able to transform PAHs in liquid and wet sand cultures [10], and in the sterilized agricultural soil

spiked with free labeled phenanthrene and benzo[a]pyrene prior to fungal inoculation (unpublished data). It resulted a rapid loss of both chemicals in the presence of the fungi with a high conversion of phenanthrene to labeled carbon dioxide. By contrast, we were unable to evidence a clear effect of nutrients on PAH transformation. Another hypothesis to explain the recalcitrance of PAHs to biodegradation can be the inhibition of degrading enzymatic systems already reported above.

Our results show that carriers formed from agricultural by-products and covered with filamentous fungi may provide an economical and practical method to induce fungal growth in polluted soils. Nevertheless, the efficiency of fungal PAH biotransformation is modest, as 57% of the contaminants were still present in the soil after 50 weeks. *T. versicolor* may initiate some transformation mechanisms, before carrier colonization by other fungal strains. Our results also suggest that MGP site PAHs may be bound to soil particles or organic matter in a way that makes them poorly available for biological degradation. Bioavailability can be improved by using surfactant, in order to enhance fungal transformation, without inducing losses due to abiotic leaching. Physico-chemical studies are under progress to define the most efficient surfactants in the soil, as well as their application conditions [35].

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# REFERENCES


1. Aamot, E., Steinnes, E. and Schmid, R., *Environ. Pollut.* **92,** 275 (1996).

2. Meador, J. P., Stein, J. E., Reichert, W. L. and Varanasi, U., *Rev. Environ. Contamin. Toxicol.* **143**, 79 (1995).

3. Shaw, G. R. and Connell, D. W., *Rev. Environ. Contamin. Toxicol*. **135**, 1 (1994).

4. Wilson, S. C. and Jones, K. C., *Environ. Pollut*. **81**, 229 (1993).

5. Holroyd, M. L. and Caunt, P., *Land Contamin. Reclam*. **2**, 183 (1994).

6. Barr, D. P. and Aust, S. D., *Environ. Sci. Technol*. **28**, 78A (1994).

7. Cerniglia, C. E. and Gibson, D. T., *J. Biol. Chem*. **254**, 12174 (1979).

8. Collins, P. J. and Dobson, A. D. W., *Biotechnol. Letters* **18**, 801 (1996).

9. Haemmerli, S. D., Leisola, M. S. A., Sanglard, D. and Fiechter, A., *J. Biol. Chem.* **261**, 6900 (1986).

10. Rama-Mercier, R., Mougin, C., Sigoillot, J.-C., Sohier, L., Chaplain, V. and Asther, M., *Biotechnol. Tech*. **12**, 725 (1998).

11. Aust, S.D., *Experimental bioremediation of a creosote site using white rot fungus,* 82nd Annual Meeting and Exhibition, Anaheim, California, 1989, Chap. 9:89, pp.165-173.

12. Davis, M. W., Glaser, J. A., Evans, J. W. and Lamar, R. T., *Environ. Sci. Technol.* **27,** 2572 (1993).

13. George, E. J. and Neufeld, R. D., *Biotechnol. Bioeng*. **33**, 1306 (1989).

14. Erickson, D. C., Loehr, R. C. and Neuhauser, E. F., *Water Res.* **27**, 911 (1993).

15. Weissenfels, W. D., Kleber, H.-J., Langhoff, J., *Appl. Microbiol. Biotechnol*. **36**, 689 (1992).

16. Cassidy, M. B., Lee, H. and Trevors, J. T., *J. Indust. Microbiol*. **16**, 79 (1996).

17. Trevors, J. T., van Elsas, J. D., Lee, H. and van Overbeek, L. S., *Microb. Releases* **1**, 61 (1992).

18. Lestan, D. and Lamar, R. T., *Appl. Environ. Microbiol.* **62**, 2045 (1996).

19. AFNOR, Qualité des sols. AFNOR Ed., Paris, 1996, ISSN 0335-3931.

20. Tatum, E. L., Barrat, R. W., Fries, N. and Bonner, D., *Am. J. Bot*. **37**, 38 (1950).

21. Seitz, L. M., Mohr, H. E., Burroughs, R. and Sauer D. B., *Cereal Chem*. **54**, 1207 (1977).

22. Mougin, C., Pericaud, C., Dubroca, J. and Asther, M., *Soil Biol. Biochem*. **29**, 1321 (1997).

23. Wolfenden, B. S. and Wilson, R. F., *J. Chem. Soc. Perkin Trans.* **II**, 805 (1982).

24. Davis, M. W. and Lamar, R. T., *Soil Biol. Biochem*. **24**, 189 (1992).

25. Lamar, R. T., Evans, J. W. and. Glaser, J.A., *Environ. Sci. Technol*. **27**, 2566 (1993).

26. Boyle, C. D., *Can. J. Microbiol*. **41**, 345 (1995).

27. Morgan, P., Lee, S. A., Sheppard, A. N. and Watkinson, R. J., *Soil Biol. Biochem*. **25**, 279 (1993).

28. Johannes, C., Majcherczyk, A. and Hüttermann, A., *Appl. Microbiol. Biotechnol.* **46**, 313 (1996).

29. Majcherczyk, A., Johannes, C. and Hüttermann, A., *Enzyme Microbiol. Technol*. **22**, 335 (1998).

30. Rama, R., Mougin, C., Boyer, F.-D., Kollmann, A., Malosse, C. and Sigoillot, J.-C., *Biotechnol. Letters* **20**, 1101 (1998).

31. Filazolla,M. T., Sannino, F., Rao, M.A. and Gianfreda, L. *J. Environ. Qual.* **28**, 1929 (1999).

32. Collins, P. J. and Dobson, A. D. W. *Appl. Environ. Microb.* **63**, 3444 (1997).

33. Kotterman, M. J. J., Rietberg, H. J., Hage, A. and Field, J.A., *Biotechnol. Bioeng*. **57**, 220 (1998).

34. Yeom, I. T., Ghosh, M. M. and Cox, C. D., *Environ. Sci. Technol*. **30**, 1589 (1996).

35. Dur, J.-C., Rama, R., Mougin, C. and Chaplain V. *Polycycl. Aromat. Comp.* (in press, 2000).

Fig 1. Biomass (g $kg^{-1}$ on a dry weight basis) of *T. versicolor* (■), *C. elegans* (■), *P. chrysosporium* (■) in the agricultural soil 2 weeks after inoculation with carriers coated by the fungi.

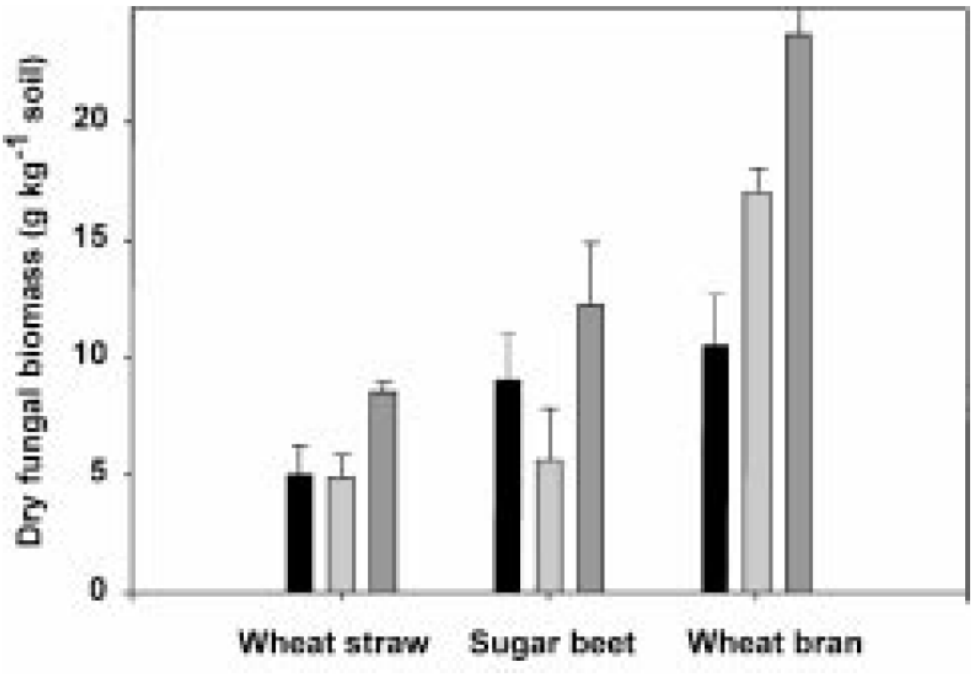

Fig. 2. Biomass (g $kg^{-1}$ on a dry weight basis) of *T. versicolor* (A) and *C. elegans* (B) in the industrial soil inoculated with fungi and supplemented with water (●) or a nutrient solution (■), during 6-week studies.

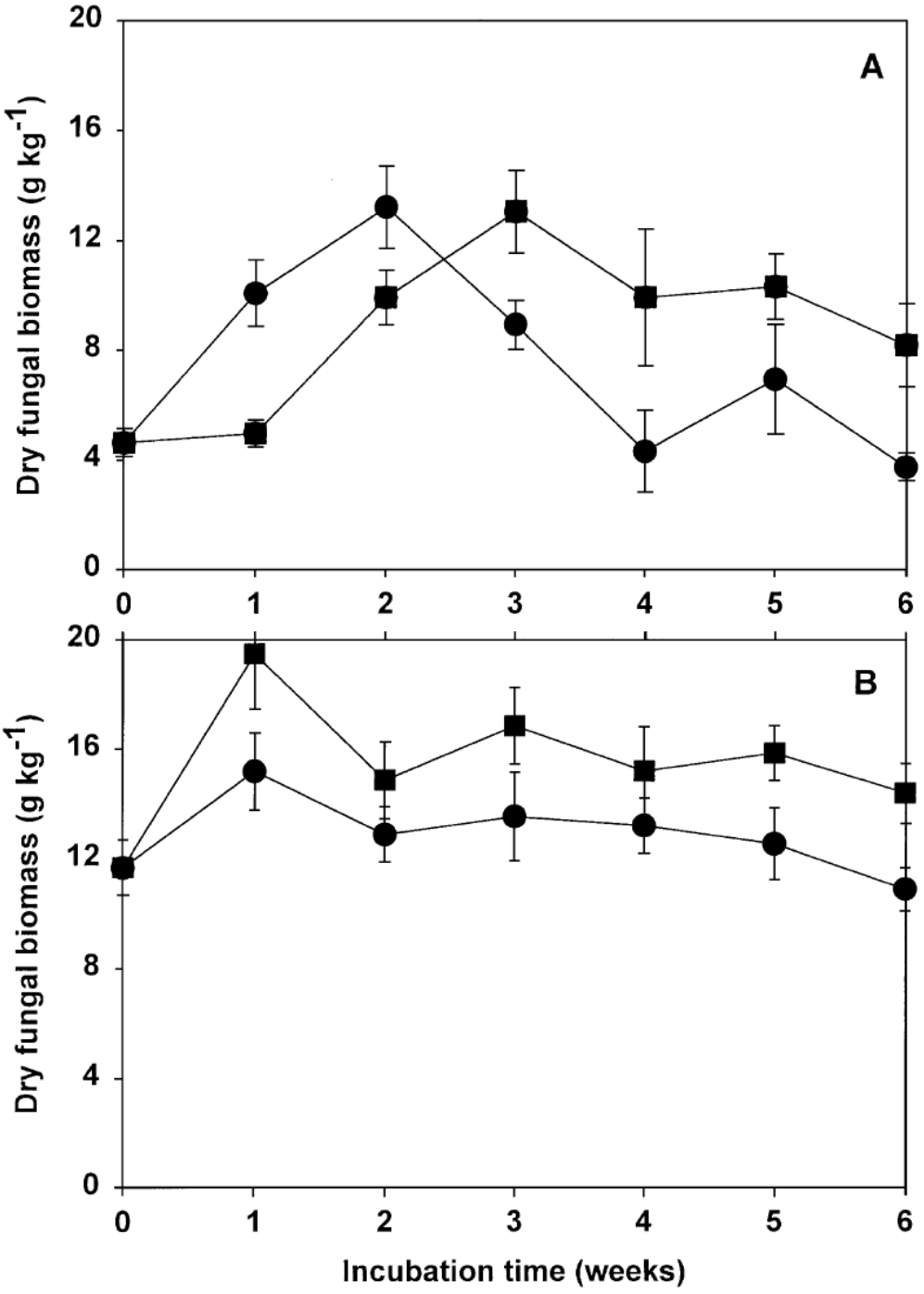

Fig. 3. Fungal biomass (■, g $kg^{-1}$ on a dry weight basis) and laccase activity (●, nmol $min^{-1}$ $g^{-1}$ dry soil) in industrial soil inoculated *with T. versicolor*, during 6-week studies.

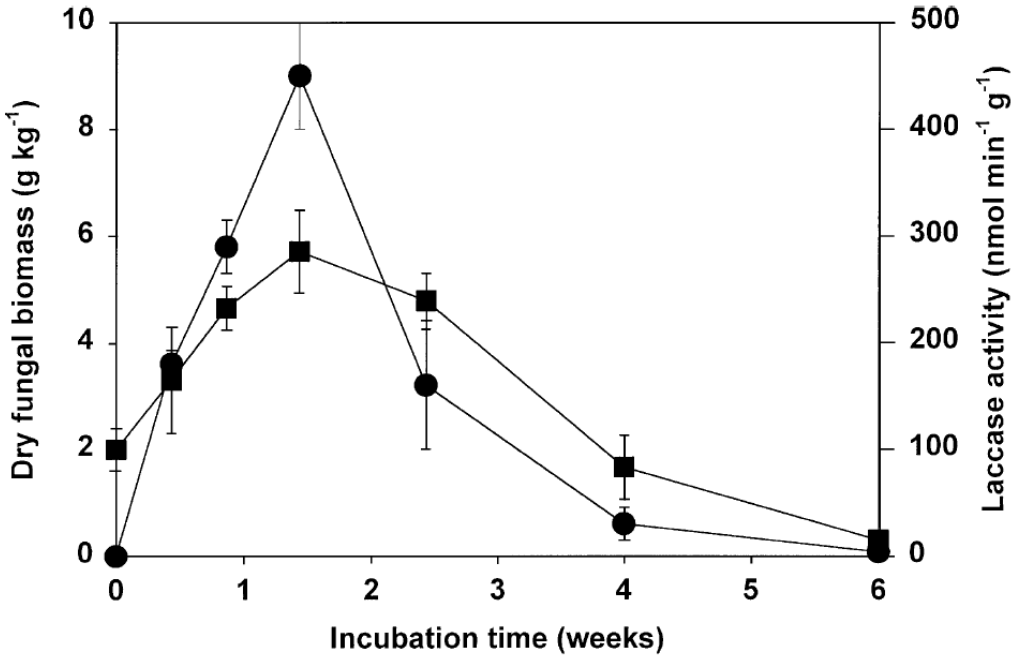

Fig. 4. PAHs (mg $kg^{-1}$ dry soil) extracted from the industrial soil submitted to various treatments, during 50-week incubations. Symbols refer to: ●, jar 1; σ, jar 2; ■, jar 3; ◆, jar 4; O, jar 5; Δ, jar 6; □, jar 7 and ◇, jar 8.

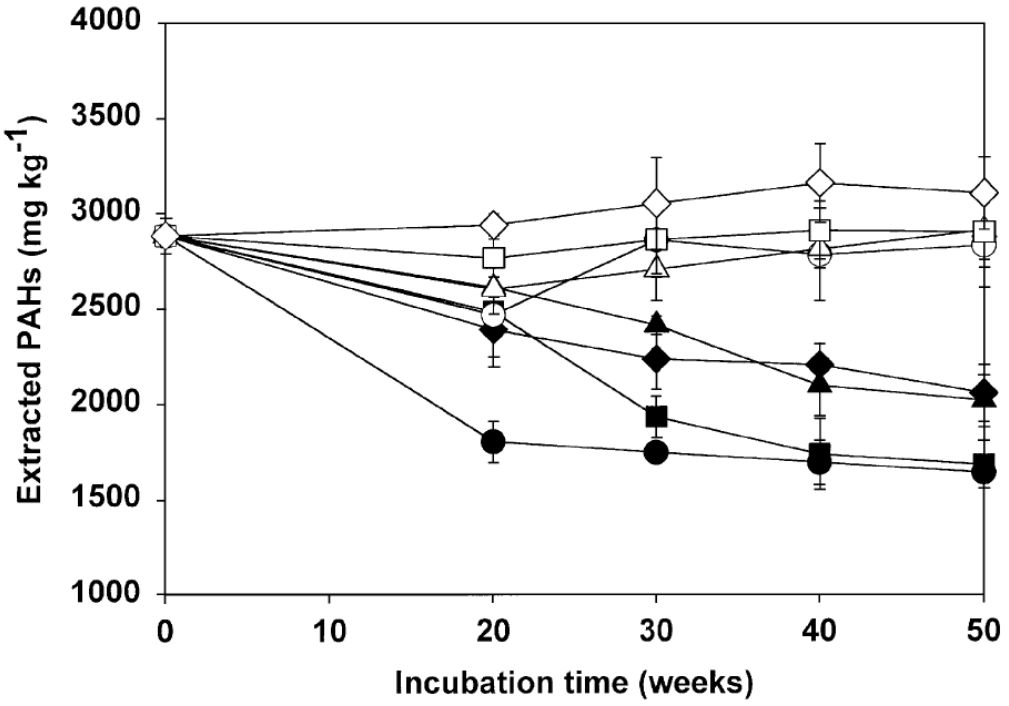

Table I. Physical and chemical properties of the agricultural and industrial soils

| Property | Agricultural soil | Industrial soil |
|---|---|---|
| Sand, % | 18 | 57 |
| Silt, % | 49 | 27 |
| Clay, % | 33 | 16 |
| MHC, % | 60 | 36 |
| pH ($H_2O$) | 7.9 | 6.9 |
| Organic C, g $kg^{-1}$ | 13.6 | 36.4 |
| Total N, g $kg^{-1}$ | 1.2 | 1.6 |
| $P_2O_5$, mg $kg^{-1}$ | 130 | 180 |
| CEC, cmol $kg^{-1}$ | 20 | / |
| Elect. Conduct., µS $m^{-1}$ | 190 | 1390 |
| Initial PAHs, mg $kg^{-1}$ | / | 2800 |
| Cyanides, mg $kg^{-1}$ | / | 220 |
| Inorganic compounds, g $kg^{-1}$ | | |
| Fe | / | 10.4 |
| Al | / | 5.6 |
| Σ Cu,Mn,Zn,Ni,Pb,Cd | / | 0.7 |

Values are based on dry soils.

Table II. Soil treatments used for PAH degradation studies and fungal biomass after 20 and 50 weeks of incubation

| Jar number | Supplementation | | | | Fungal biomass (g $kg^{-1}$ soil) | |
|---|---|---|---|---|---|---|
| | Carriers | *T. versicolor* | Nutrients | Surfactant | 20 weeks | 50 weeks |
| 1 | + | + | + | + | 18.8 ± 2.4 | 11.2 ± 2.3 |
| 2 | + | + | + | - | 13.0 ± 1.0 | 12.7 ± 1.1 |
| 3 | + | + | - | + | 9.8 ± 1.0 | 4.6 ± 0.6 |
| 4 | + | + | - | - | 26.7 ± 7.5 | 3.6 ± 0.6 |
| 5 | + | - | + | - | 4.3 ± 0.5 | 2.5 ± 0.2 |
| 6 | - | - | + | - | 0.5 ± 0.1 | 0.2 ± 0.1 |
| 7 | - | - | - | + | 0.3 ± 0.1 | 0.2 ± 0.1 |
| 8 | - | - | - | - | 0.2 ± 0.1 | 0.1 ±0.1 |

+: with, -: without