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1 **Inoculation of Filamentous Fungi in Manufactured Gas Plant Site Soils**

2 **and PAH Transformation**

3

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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™, ammonium nitrate, lime phosphate and Montanox 80™, 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™ (industrial source of maltose) from Roquette Frères (Lestrem, France) and Montanox 80™ (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

1 We obtained the white rot strains *Trametes versicolor* (Mic 209) and
2 *Phanerochaete chrysosporium* (BKM-F-1767, ATCC 24725) from the culture collection
3 of UBCF (INRA Marseille-Luminy, France). *Cunninghamella elegans* (ATCC 26269)
4 was purchased from the American Type Culture Collections (Manassas, VA, USA). The
5 strains were maintained at 37°C on solid medium comprising malt (20 g L^{-1}), agar (16 g
6 L^{-1}) and yeast extract (1 g L^{-1}).
7

8 **Soil sampling**

9 Chemical and physical properties of the experimental soils are summarized
10 Table I. They were determined by using AFNOR [19] normalized protocols. We collected
11 agricultural soil (used as non-contaminated soil) in the 10-20 cm layer in a field of the
12 experimental farm of INRA at Dijon (France). PAH-contaminated industrial soil was
13 taken from a pile in a MGP site at Rouen (France). We assessed PAH concentrations by
14 using analytical methods described below.
15

16 **Medium and fungal biomass production**

17 The culture medium [10] used for biomass production contained: KH_2PO_4 (2 g L^{-1}
18), $CaCl_2.2\ H_2O$ (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
19 $SO_4.5\ H_2O$ (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}),
20 diammonium tartrate (1.842 g L^{-1}), yeast extract (1 g L^{-1}), 1 mL $L^{-1}$of a vitamin solution
21 [20], glucose (5 g L^{-1}) for *T. versicolor* and *C. elegans*, glycerol (10 g L^{-1}) for *P.*
22 *chrysosporium*. We buffered the culture medium at pH 6.5 with disodium tartrate (2.3 g
23 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.

1 **Coating of carriers and mycelium growth**

2 We used a modification of a described protocol [18] to inoculate pellets for fungal
3 growth studies. The mycelial suspension (20 mL) was mixed with 30 mL sodium
4 alginate solution at 2% (w/v). Then, pellets (100 g) were coated by consecutive spraying
5 of the previous mixture and of small amounts of 5% (w/v) sterile $CaCl_2$.
6 Carriers used for degradation studies were coated by mixing a mycelial
7 suspension (200 mL) of *T. versicolor* with sterilized wheat bran pellets (2 kg) in the
8 presence of guar gum (1-L solution at 1%, w/v) in sterile polyethylene bags.
9 We incubated all coated carriers at 27°C until they were coated with mycelium
10 (7-10 days for fungal growth studies, 14 days for degradation studies). After that period,
11 we considered the carriers ready for use as a fungal inoculum for soil treatment.
12
13 **Soil inoculation and incubation for fungal growth studies**

14 These studies were a series of small-scale experiments to investigate
15 optimization variables. Before use for fungal growth studies, each soil (agricultural or
16 industrial) was air-dried and passed through a 2-mm sieve, then mixed and stored in
17 polyethylene bags in darkness at room temperature (about 20°C) less than one week. We
18 conducted the incubations by using 30-g dry soil in 150-mL Erlenmeyer flasks, usually
19 inoculated with 3-g pellets coated with fungal mycelium, during 1 to 6 weeks. We added
20 an aliquot of deionised sterile water to achieve 80% MHC at the beginning of the
21 experiments.
22 We sealed the Erlenmeyer flasks with cotton plugs and incubated them in 1-L
23 sealed flasks with a vial containing 10-mL water to keep moisture constant [21]. The
24 headspace of the flasks was flushed with air at the beginning of the experiments, and

1 then every 7 days. We incubated samples in darkness at 25°C for 1 to 6 weeks. In some
2 cases, nutrients (C/N/P) were supplemented as an aqueous solution of sterile maltose,
3 $(NH_4)_2SO_4$ and KH_2PO_4 without any significant modification of soil pH. Carbon (1%
4 dry soil, w/w) was first added, then sufficient N and P were added to bring the C/N/P
5 ratio to 100/5/1. We completely used each soil sample for fungal biomass determination.
6 For sterile experiments, portions of the soil were autoclaved two-times at 121°C
7 for 20 min in 1-L Erlenmeyer flasks closed by air-permeable cotton stoppers.
8

9 **Soil inoculation and incubation for degradation studies**

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

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

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

8

9 **Fungal biomass determination**

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

16

17 **PAH extraction and analysis**

18 PAHs were Soxhlet-extracted from each soil subsample (30 g dry weight) in the
19 presence of acetonitrile (100 ml) for 8 hours. Pellets coated with fungi were removed
20 from the soil before extraction. After cooling, the extracts were brought to 100 ml and
21 filtered on a Whatman 0.2 µm nylon membrane. We obtained separation and
22 quantification of PAHs by injections of a 20-µl sample in the HPLC through a 7125
23 Rheodyne valve. PAHs were then eluted on the analytical column Supelcosil LC-PAH
24 (15 cm x 4.6 mm *id*; Supelco, Saint-Quentin Fallavier, France) with a Varian 9010

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

6

7 **Enzymatic assays**

8 We used laccase activity as an indirect method to assess fungal activity in the
9 soil, in order to acquire data on the physiological state of the fungus. Enzymatic activity
10 was measured in buffered extracts of the industrial soil. The 30-g subsamples of soils
11 were shaken with 100 mL of 50 mM citrate phosphate buffer (pH 6.0) for 15 min and
12 then the slurry was centrifuged at 4000 g for 10 min. After 30-min incubations with
13 catalase (10U) to eliminate residual H_2O_2 and thus prevent any activity of the
14 peroxidases, this of laccase was determined by oxidation of 2,2'-azinobis-(3-ethylbenz-
15 thiazoline-6-sulfonic acid) (ABTS) in spectrophotometer cells [23]. The reaction mixture
16 (1 mL final volume) contained 5 mM ABTS in 100 mM citrate phosphate buffer (pH
17 3.0), and 100 µL of soil extract. We followed enzymatic oxidation of ABTS (E_{420} =
18 36000 M^{-1} cm^{-1}) by an absorbance increase at 420 nm at 30°C against a cell without
19 enzyme.

20

21 **Experimental design**

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

24

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

1 inoculated with carriers not coated with the fungi corresponded to biomass amounts
2 lower than 0.3 g kg^{-1} soil (on a dry weight basis) during a 6-week period. After fungal
3 inoculation, Fig. 2A and 2B showed similar growth profiles for *T. versicolor* and *C.*
4 *elegans* in the industrial soil supplemented with pure water. Although initial biomass
5 values were lower in the presence of *T. versicolor* than *C. elegans*, fungal development
6 proceeded in a first time by a rapid growing phase. Proliferation of mycelium in the soil
7 was also observed through the glass flasks. Maximal dry fungal biomass amounted in
8 the industrial soil to 13.2 g kg^{-1} for *T. versicolor* after 2 weeks, and 15.2 g kg^{-1} for *C.*
9 *elegans* after one week.
10
11 Biomass of *T. versicolor* and *C. elegans* was then checked during 6-week studies
12 in the non-sterilized industrial soil inoculated with each fungus and supplemented with
13 water or a nutrient solution (Fig. 2). On the one hand, a decrease of fungal biomass
14 occurred in the industrial soil supplemented with pure water after 2-3 weeks of
15 incubation. It was more pronounced in the case of *T. versicolor* than in the case of *C.*
16 *elegans*. *T. versicolor* biomass declined to 3.7 g kg^{-1} after 6 weeks of incubation (Fig.
17 2A), whereas growth profiles obtained with *C. elegans* in a similar experiment
18 demonstrated, after the 1-week increasing phase, a regular decrease of the biomass to
19 the final values of 10.9 g kg^{-1} dry weight after 6 weeks (Fig. 2B).
20 On the other hand, in another set of incubations, the industrial soil was
21 supplemented with a mixture of carbon, nitrogen and phosphorus to give a final ratio of
22 C/N/P: 100/5/1 to assess the effects of nutrients on fungal growth. *T. versicolor* growth
23 exhibited a 1-week lag-phase followed by an increase during 2 weeks. Then, the
24 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

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

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

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

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

1 after 20 and 50 weeks of incubations are reported Table II. In both periods of time, they
2 are negligible or low in the jars 5-8 (non inoculated by *T. versicolor*). No laccase
3 activity was detected between 20 and 50 weeks of incubation.
4

5 DISCUSSION

6

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

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

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

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

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

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

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

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

1 spiked with free labeled phenanthrene and benzo[a]pyrene prior to fungal inoculation
2 (unpublished data). It resulted a rapid loss of both chemicals in the presence of the fungi
3 with a high conversion of phenanthrene to labeled carbon dioxide. By contrast, we were
4 unable to evidence a clear effect of nutrients on PAH transformation. Another
5 hypothesis to explain the recalcitrance of PAHs to biodegradation can be the inhibition
6 of degrading enzymatic systems already reported above.
7
8 Our results show that carriers formed from agricultural by-products and covered
9 with filamentous fungi may provide an economical and practical method to induce
10 fungal growth in polluted soils. Nevertheless, the efficiency of fungal PAH
11 biotransformation is modest, as 57% of the contaminants were still present in the soil
12 after 50 weeks. *T. versicolor* may initiate some transformation mechanisms, before
13 carrier colonization by other fungal strains. Our results also suggest that MGP site PAHs
14 may be bound to soil particles or organic matter in a way that makes them poorly
15 available for biological degradation. Bioavailability can be improved by using
16 surfactant, in order to enhance fungal transformation, without inducing losses due to
17 abiotic leaching. Physico-chemical studies are under progress to define the most
18 efficient surfactants in the soil, as well as their application conditions [35].
19

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23

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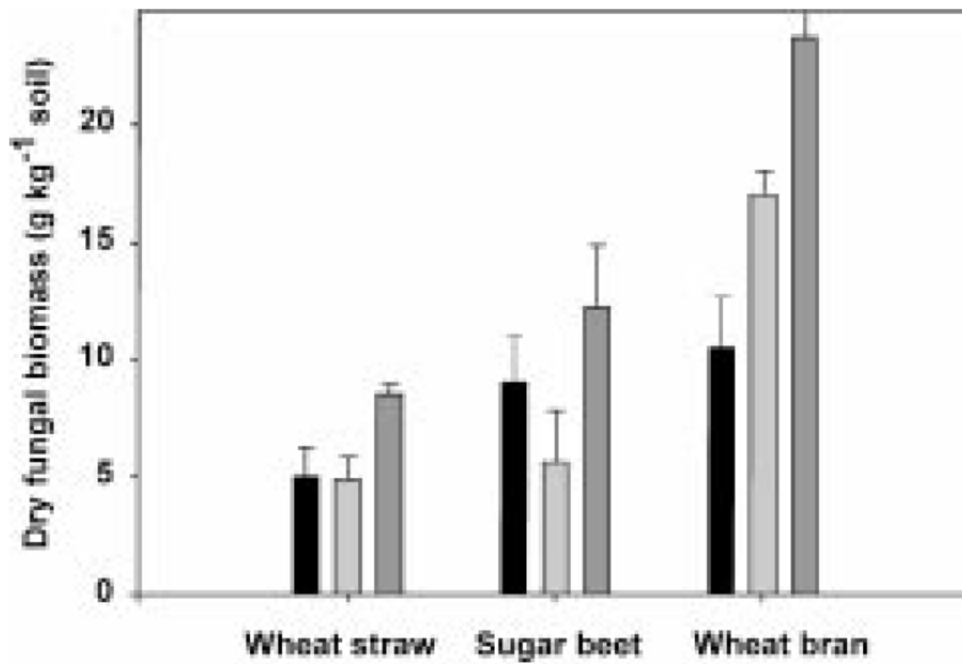
2 Fig 1. Biomass (g kg^{-1} on a dry weight basis) of *T. versicolor* (■), *C. elegans* (■), *P.*

3 *chrysosporium* (■) in the agricultural soil 2 weeks after inoculation with carriers coated

4 by the fungi.



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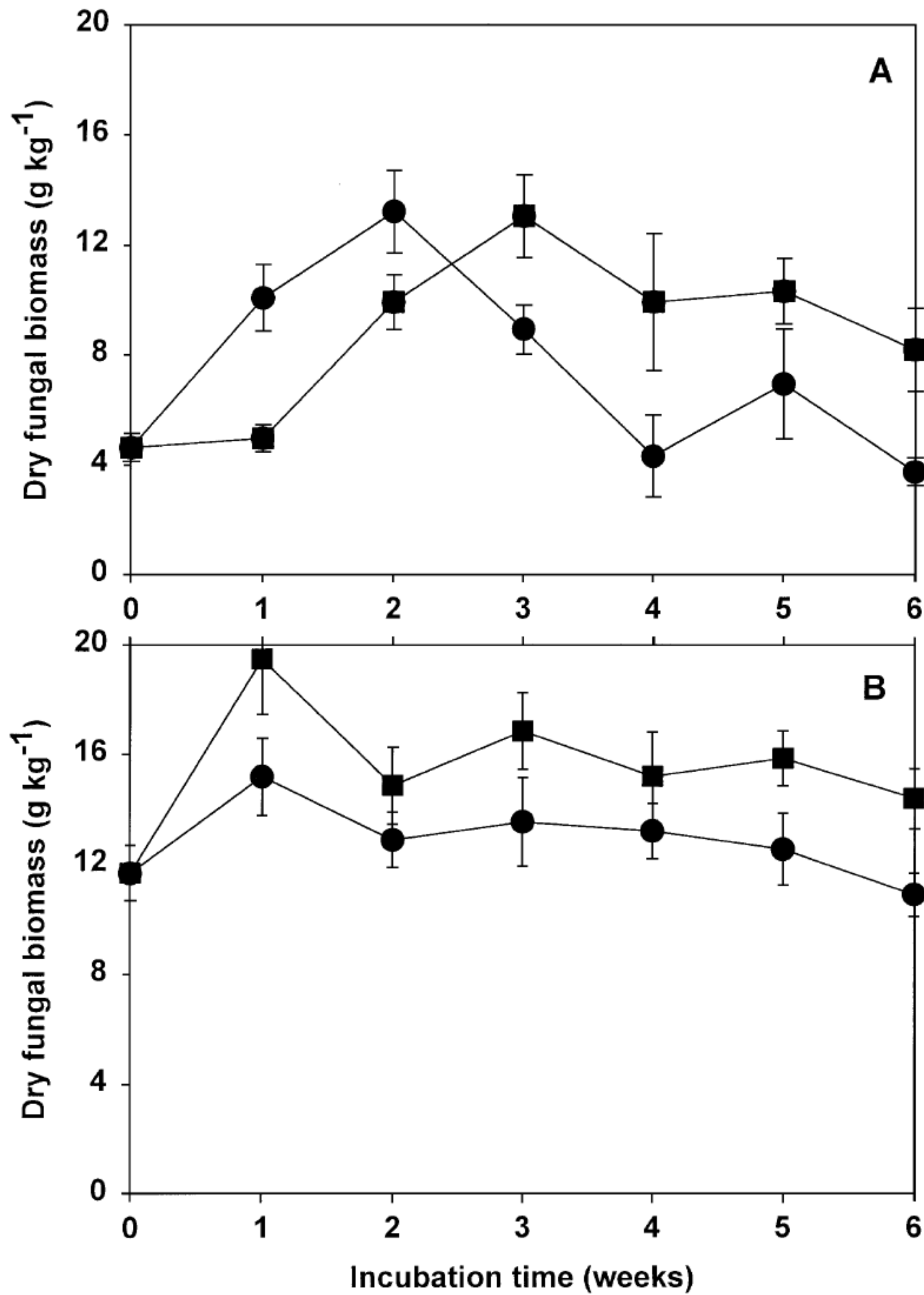
1 Fig. 2. Biomass (g kg^{-1} on a dry weight basis) of *T. versicolor* (A) and *C. elegans* (B) in
2 the industrial soil inoculated with fungi and supplemented with water (●) or a nutrient
3 solution (■), during 6-week studies.



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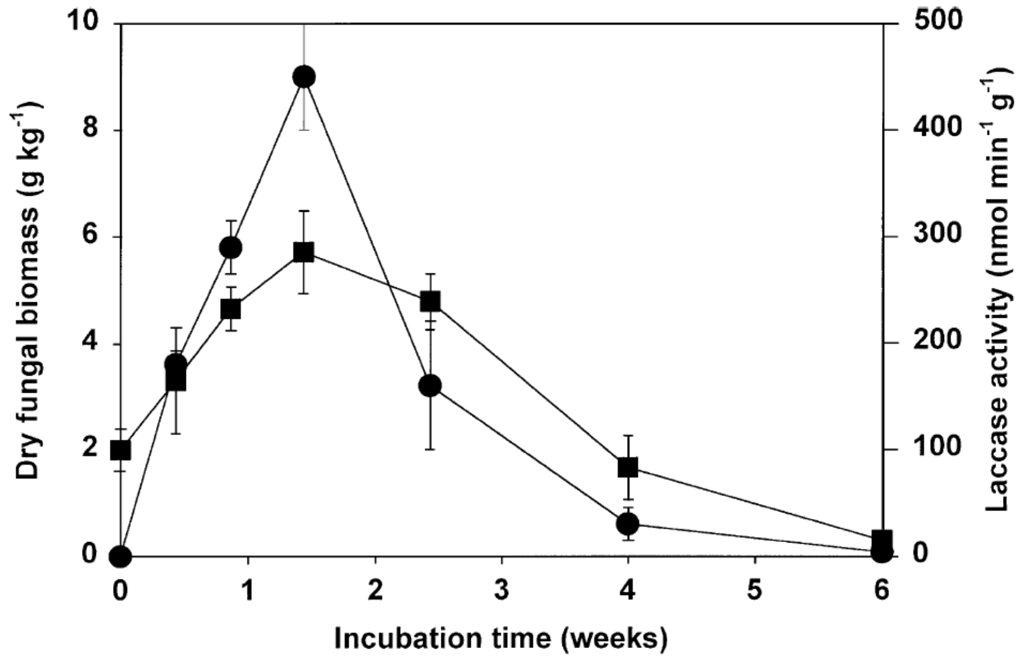
1 Fig. 3. Fungal biomass (■, g kg^{-1} on a dry weight basis) and laccase activity (●, nmol

2 min^{-1} g^{-1} dry soil) in industrial soil inoculated *with T. versicolor*, during 6-week studies.



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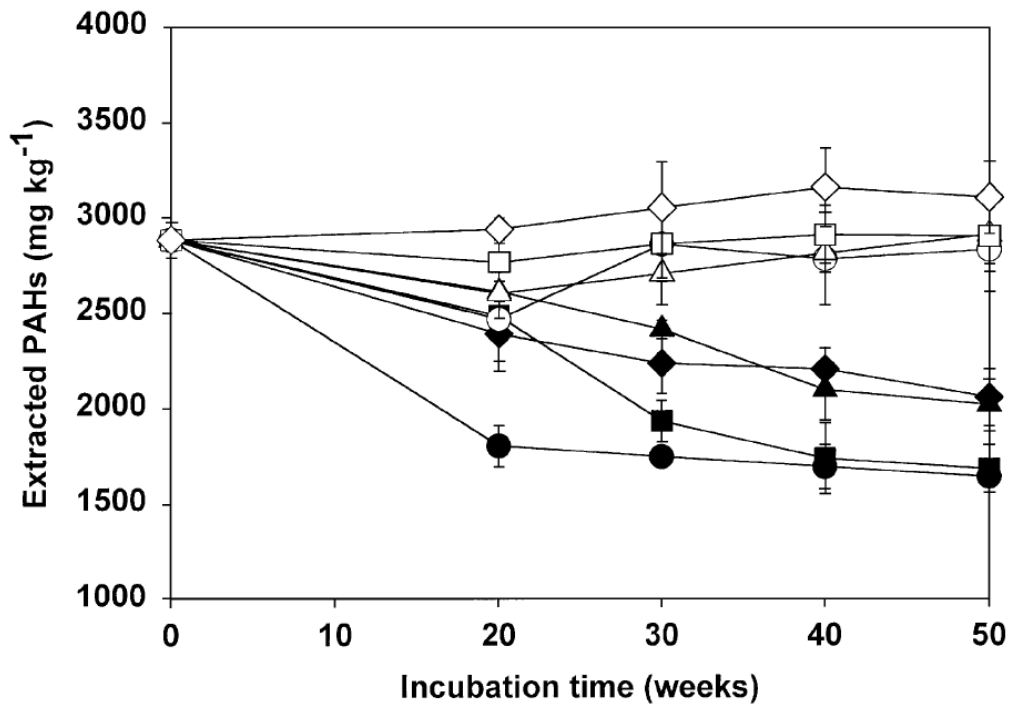
1 Fig. 4. PAHs (mg kg^{-1} dry soil) extracted from the industrial soil submitted to various
2 treatments, during 50-week incubations. Symbols refer to: ●, jar 1; σ, jar 2; ■, jar 3;
3 ◆, jar 4; O, jar 5; Δ, jar 6; □, jar 7 and ◇, jar 8.



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

2

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

3

4 Values are based on dry soils.

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6

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