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An efficient PAH-degrading *Lentinus* (*Panus*) *tigrinus* strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices

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ABSTRACT

This study comparatively investigated the PAH degradation ability of *Lentinus tigrinus* and *Irpex lacteus* in a historically polluted soil and creosote-impregnated shavings. With this regard, the effect of type of inoculum carrier (*i.e.*, wheat straw, corn cobs and commercial pellets) and contaminant bioavailability was thoroughly determined. Although degradation performances of *L. tigrinus* were not significantly affected by the type of the support, they were invariably better than those of *I. lacteus* on both the polluted soil and the creosote-impregnated shavings. Although degradation efficiencies of all fungal microcosms were highly and significantly correlated with bioavailability, certain PAHs, such as chrysene and benzo[a]pyrene, were removed by *L. tigrinus* from the polluted soil at amounts that exceeded about 2.3-fold their respective bioavailabilities. Degradation of PAHs was negatively correlated with their organic carbon sorption coefficients (K_{oc}) and hydrophobicity ($\log P$). The strength of linear association with the latter parameter, however, was not affected by the type of contaminated matrix in *L. tigrinus*-based microcosms while it was significantly larger in the historically polluted soil than in the creosote-impregnated shavings in *I. lacteus* ones.

1. Introduction

In the last decades, the biological degradation of PAHs has aroused significant interest since these ubiquitous contaminants have been included among priority pollutants owing to their toxic, mutagenic and, in some cases, carcinogenic properties [1]. PAHs arise from the incomplete combustion of organic matter and are found as major components of creosote and coal tar. From a structural viewpoint, these contaminants exhibit two or more fused benzene rings arranged either in a linear, angular or cluster mode. It has been suggested that the persistence of PAHs in the environment might be due to their low susceptibility to nucleophilic attack owing to the presence of dense clouds of π-electrons on both sides of the ring structures [2]. In addition, some physical properties of PAHs, such as low aqueous solubility and high solid/water distribution ratios, hamper microbial degradation of these compounds thereby resulting in their accumulation in soils and sediments [1].

Due to these objective constraints, there is the need to develop bioremediation protocols as environmentally sustainable approaches to the remediation of PAH-contaminated matrices in alternative to physico-chemical treatments. Although several bacterial and actinomycetes species have been shown to degrade PAHs in soil [2], some peculiarities exhibited by white rot fungi (WRF) make them particularly attractive for this purpose [3]. In this respect, WRF are endowed with an extracellular, non-specific and radical-based ligninolytic machinery that confers them the ability to degrade a wide range of contaminants, including high molecular mass PAHs which are seldom prone to bacterial attack [4,5]. Moreover, the filamentous growth mode of WRFs provides them with the ability to diffusely penetrate into soil aggregates and to act as dispersion vectors of resident pollutant-degrading bacteria [6].

The colonization ability of WRF on a given contaminated matrix, however, might be hampered by both competition with the resident microbiota and adverse physico-chemical characteristics of the matrix itself [7] thus resulting in reduced degradation performances [5,8,9]. With this regard, the use of lignocellulosic wastes in inocula formulation [10,11] increased the antagonistic potential and, therefore, the growth capacity of WRF in remediation applications [12–15]. The large variability of results, however, highlights the importance of lignocellulosic waste/fungus interactions thus suggesting the need for further investigations.

Although the PAH degradation ability of WRF has been suggested to be less affected than that of bacteria and actinomycetes by the mass transfer rate of these contaminants from the solid to the

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liquid phase, the low bioavailability of these contaminants, which tends to increase with aging [16], remains a key-constraint to the fungal clean-up of PAH-contaminated matrices [17]. Consequently, it is of paramount importance to relate fungal degradation results to the extent of the bioavailable fraction in order to devise appropriate mycoaugmentation protocols.

Among WRF, *Lentinus* (*Panus*) *tigrinus* has recently attracted research interest due to its ability to clean-up PAH-contaminated liquid and solid matrices even under saline conditions [18]. Recently, the degradation ability of *L. tigrinus* CBS 577.79 towards PAHs has been shown in N-rich and N-limited standard liquid media under axenic conditions [19].

Therefore, main objective of the present study was to assess the PAH-biodegradation potential of *L. tigrinus* CBS 577.79 on real solid matrices derived from a wood treatment plant in view of its possible use in mycoremediation applications. In particular, degradation performances of the strain under study were investigated in both a historically polluted soil (HPS) and creosote-impregnated shavings (CIS) and compared with those of *Irpex lacteus* CCBAS 238/617, the degradation capacity of which had been extensively demonstrated [20,21]. In order to gain insights into the matrix effect, attempts were done to establish a relationship between the biodegradation efficiency and the contaminant bioavailability estimated with sequential supercritical fluid extraction. Moreover, both growth ability on the two contaminated matrices and PAH degradation performances were evaluated as a function of three carriers (*i.e.*, wheat straw, corn cobs and commercial pellets) employed to formulate fungal inocula. The thorough determination of the impacts of both bioavailability and inoculum formulation on fungal degradation addressed in this study might represent a significant and positive contribution to the mycoremediation general understanding.

2. Materials and methods

2.1. Materials

Wheat straw (WS), corn cobs (CC), chopped into approx. 1.5 cm pieces, and commercial pellets (0.8 mm Ø) (CP, *ATEA Praha*, Prague, Czech Republic) were used as carriers for fungal inocula. The last carrier is a wheat straw-based composite material employed for domestic-heating purposes with a density of 1.34 kg dm^{-3}, a moisture of 6.4% (w/w) and a C/N ratio of 45:1.

The sandy-loamy HPS, collected from the same wood treatment plant in Soběslav (southern Bohemia, CZ) where CIS had been impregnated, was air-dried for 7 d at room temperature and then passed through a 2 mm-sieve. Its main properties were as follows: water-holding capacity 20%, pH 5.1, total organic carbon 0.5%, total organic matter 0.9%. Organic carbon was determined with hot chromic acid digestion and the total organic estimation was based on combustion at 650 °C. It contained several metals (mg kg^{-1}): As 17.5, Cd 0.4, Co 1.74, Cr 9.41, Cu 6.8, Fe 322, Hg 5.7, Pb 4.5 and Zn 75.6. Granulometric composition was: sand 58.2%, fine sand 27.7%, silt 8.1%, clay 6.0%. CIS from oak wood sleepers, provided by the company Eko-Bio Vysočina, Ltd., Czech Republic, were air-dried for 7 d at room temperature, mechanically ground and then passed through a 2 mm-sieve. The sandy-loamy soil (MBU) was used as the non-contaminated control. Its main properties were as follows: total organic carbon 0.8%, total organics 1.4%, pH 5.3, water-holding capacity 31% and granulometric composition was: sand 50.9%, fine sand 31.2%, silt 10.8%, clay 7.1%. All solvents of p.a. quality, trace analysis quality or gradient-grade were purchased from Merck (Darmstadt,
Germany).

2.2. Microorganisms and inocula preparation

L. tigrinus 577.79 and *I. lacteus* 238/617 were from the CBS (Baarn, NL) and CCBAS (Institute of Microbiology AS CR, Prague, CZ) culture collections, respectively. The strains were maintained at 4 °C and periodically sub-cultured on MEG agar plates containing (g l^{-1}): malt extract, 5; glucose, 10; agar, 15 g; pH 5.0.

Fungal pre-inocula were grown under stationary conditions for 7 d at 28 °C in 250 ml Erlenmeyer flasks containing 20 ml MEG liquid medium. Cultures were then homogenized with the Ultraturrax-T25 (IKA-Labortechnik, Staufen, Germany) and 1.0 ml aliquots of the mycelial suspension were added to 16 cm × 3.5 cm test-tubes either containing WS (2 g) or CC (6 g) or CP (10 g), the moisture contents of which had been previously adjusted to 70% (w/w) and subsequently sterilized by autoclaving (121 °C, 45 min). After inoculation, the cultures were covered with cotton-wool stoppers and then grown for 2 weeks at 28 °C under stationary conditions.

2.3. Fungal treatment of polluted matrices

The moisture contents of the two soils (*i.e.*, HPS and MBU) and of the CIS were previously adjusted to 15 and 25% (w/w) respectively, with sterile deionized water. Then, the test-tubes containing immobilized inocula were added with a layer of either PAH-polluted matrices (25.0 and 5.0 g of HPS and CIS, respectively) or MBU control soil (25 g). Due to the different apparent densities of the colonized carriers and the contaminated matrices, different weights of them were overlaid in order to ensure uniform heights of the resulting bed. Incubation controls were prepared by adding the polluted matrices to heat-inactivated (121 °C, 30 min) immobilized inocula. Each microcosm, namely the combination of inoculum carrier, fungal strain and target-matrix, was prepared in triplicate (controls included) and incubated for 60 d at 28 °C. Throughout the incubation, sterile deionized water was periodically added in order to keep constant the moisture content of each microcosm.

2.4. Extraction and analyses of ergosterol and aromatic pollutants

Total ergosterol was extracted and analyzed as described by Šnajdr et al. [22]. Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml of a methanolic solution of KOH (10%, w/v). After the addition of 1 ml distilled water, each sample was extracted for three times with 2 ml of cyclohexane. The solvent was evaporated under a nitrogen stream and the solid residue dissolved in 1 ml methanol. These samples (20 μl) were then analyzed isocratically by reversed-phase high performance liquid chromatography (RP-HPLC) equipped with a LiChroCart column filled with LiChrospher® 100 RP-18 (250 mm × 4.0 mm; particle size 5 μm; pore size 100 Å) equilibrated with 100% methanol at a flow rate of 1 mL min^{-1}. The elution profile was monitored at 282 nm.

Extraction of aromatic pollutants was performed with an ASE 200 System (Dionex, Voisins-le-Bretonneux, France). Either 10 g of HPS or 2 g of CIS were loaded into the extraction cell (11 ml) and subsequently extracted with hexane–acetone (3:1, v/v) mixture (HAM). Static heating was applied to the vessel (150 °C, 5 min) and subsequent extraction was performed for 7 min at the same temperature under 103.4 bar. The cell was then flushed with 7 ml HAM and finally the solvent was purged from the cell by nitrogen for 60 s. For each sample, this extraction cycle was performed twice. From here onwards, the resulting organic extracts, air-dried under vacuum at room temperature and finally dissolved in acetonitrile (5 and 20 ml for HPS and CTS, respectively) are referred to as contaminants extract. RP-HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a LiChroCart column filled with LiChrospher® PAH (250 mm × 5 mm; particle size 5 μm; pore

Table 1
Molecular weight, organic carbon sorption coefficient ($\log K_{oc}$), hydrophobicity ($\log P$), water-solubility, ionization potential (IP) and percent abundances of the bioavailable fraction of each PAHs detected in the historically polluted soil (HPS) and creosote-impregnated shavings (CIS) incubation controls.

Contaminant	Abbreviation	Molecular weight	$\log K_{oc}$	$\log P$	WS ($mg\,l^{-1}$)	IP (eV)*	Percent abundance of the bioavailable faction in:[a] HPS	CIS
Fluorene	FLU	166	3.88	4.18	1.90	7.88	96.0 ± 4.5	97.8 ± 5.5
Phenanthrene	PHE	178	4.15	4.46	1.29	8.03	96.6 ± 12.3	40.3 ± 4.7
Anthracene	ANT	178	4.47	4.54	0.045	7.43	94.1 ± 14.8	59.6 ± 5.4
Fluoranthene	FLT	202	4.69	5.20	0.26	7.90	92.5 ± 6.8	96.7 ± 7.2
Pyrene	PYR	202	4.58	5.18	0.135	7.53	88.3 ± 5.8	93.1 ± 7.0
Benz[a]anthracene	BaA	228	5.55	5.76	0.009	7.56	37.7 ± 9.1	90.0 ± 9.0
Chrysene	CHR	228	5.49	5.81	0.002	7.59	41.1 ± 12.6	73.4 ± 5.8
Benzo[b]fluoranthene	BbF	252	6.08	5.80	0.0015	7.65	33.7 ± 8.6	67.2 ± 2.3
Benzo[k]fluoranthene	BkF	252	6.09	6.00	0.0008	7.48	21.7 ± 14.1	69.0 ± 6.4
Benzo[a]pyrene	BaP	252	5.98	6.13	0.0016	7.12	21.9 ± 9.9	54.4 ± 2.4
Dibenzo[a,h]anthracene	DBA	278	6.28	6.75	0.0006	7.38	n.r.[b]	n.r[b]
Benzo[g.h.i]perylene	BghiP	276	6.20	6.63	0.0003	7.15	n.r.[b]	n.r.[b]
indeno[1.2.3-cd]pyrene	IPY	276	6.54	6.70	0.0002	8.02	n.r.[b]	n.r.[b]

* From either [3,26,27].
[a] Estimated from the *F* fraction of supercritical fluid extraction (SFE). Data are the mean ± standard deviation of three extractions.
[b] n.r. not reported: desorption data of these compounds upon SFE were not fitted by Eq. (1); thus it was not possible to gain a reliable estimate of their bioavailability.

size 150 Å; Merck, Darmstadt, Germany) and a 2996 diode-array detector and 2475 fluorescent detector (Waters). Separation of the PAHs was achieved with a gradient programme, using (A) a mixture of methanol:acetonitrile (1:1, v/v) and (B) Milli-Q water. The elution program was as follows: isocratic elution with 70% (A) for 5 min, gradient to 100% (A) in 15 min, isocratic elution at 100% A for 20 min. PAHs were identified on the basis of both UV spectra and match of retention times with commercially available standards (Dr. Ehrenstorfer, Augsburg, Germany). Concentrations of 13 PAHs out of the 16 compounds according to the U.S. EPA method 610 were determined. Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due to volatilization. The detected compounds were quantified with the fluorescent detector under following excitation/emission conditions: phenanthrene (PHE), anthracene (ANT) indeno[1,2,3-cd]pyrene (IPY) – 250/390 nm; fluorene (FLU), fluoranthene (FLT), pyrene (PYR), benz[a]anthracene (BaA), chrysene (CHR) – 280/340 nm; benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBA), benzo[g,h,i]perylene (BghiP) – 305/430 nm. Calibration curves with the standards were determined over a 0.1–10 $\mu g\,ml^{-1}$ concentration range from for each compound.

2.5. Estimation of PAH bioavailability

Bioavailable fractions of PAHs were estimated using sequential supercritical fluid extraction (SFE) [23]. The extraction was performed with a PrepMaster extractor (Suprex, Pittsburgh, PA) equipped with VaryFlow restrictor operating at 40 °C and with a downward stream of carbon dioxide (5.5 SFE/SFC, Messer Technogas, Prague, CZ). The samples (1 g of either HPS or 0.5 g of CIS) were extracted at 50 °C and 200 bar. Each extraction was carried out in triplicate and the compounds were collected after 5, 10, 20, 40, 60, 80, 120, 160 and 200 min. Sequential supercritical fluid extraction represents a desorption model presuming generally that the extraction is controlled by the two rate constants differing by orders of magnitude [24]. The chemical release data can be modelled by an empirical two-site model, consisting of the two first-order equations (1):

$$S_t = F \cdot S_o \cdot e^{-k_1 t} + (1 - F) \cdot S_o \cdot e^{-k_2 t} \quad (1)$$

where S_t is the pollutant concentration remaining in the soil after time t; F is the fraction of chemical rapidly released; S_o is the original concentration of the pollutant in soil; k_1 and k_2 are the first-order rate constants. The so-called "*F* fraction" is usually assumed to be representative of equilibrium release conditions, and the remaining, slowly released portion is considered to be kinetically rate limited. Therefore, *F* fraction represents the portion of the target chemical that is bioavailable in soil [17,25]. The fractions were analyzed separately to complete desorption-kinetic profiles. Prism version 4.0 (GraphPad, La Jolla, CA) was used for calculating the *F* values. To relate the degradation performances of each microcosm to the actual PAH bioavailability, the degradation fold with respect to the bioavailable fraction (DFBF) was calculated for each contaminant by the following expression:

$$\mathrm{DFBF} = \frac{C_{ic} - C_r}{\mathrm{BF}} \quad (2)$$

where C_{ic} and C_r are the residual concentrations in the incubation control and fungal-treated material, respectively, and BF the mass amount of the bioavailable fraction referred to unit weight of the contaminated material. The relative percent abundances of the bioavailable fraction for each PAH compound detected in the matrices under study and some salient physico-chemical properties are reported in Table 1.

2.6. Phytotoxicity assay

Static-type germinability assays with barley (*Hordeum vulgare* L.) seeds were conducted for 3 d at room temperature in 90-mm Petri dishes containing Whatman GF/C filters soaked with 2.0 ml of the contaminants extract derived from both fungal-treated matrices and relative incubation controls. After the extract addition, the filters were held at room temperature for 12 h to allow solvent evaporation; then 2.0 ml distilled water were added on a daily basis. A randomized complete blocks experimental design with 3 replicates and 50 seeds per Petri dish was used. Germinability tests conducted in the presence of distilled water were also run in parallel and served as the control. Percent inhibition (I%) of germinability was calculated from Eq. (3).

$$I\ (\%) = \left(1 - \frac{G}{G_c}\right) \times 100 \quad (3)$$

where G is the number of germinated seeds in the presence of the contaminants extract from either fungal-treated matrices or their incubation controls and G_c the same parameter in the absence of the extract.

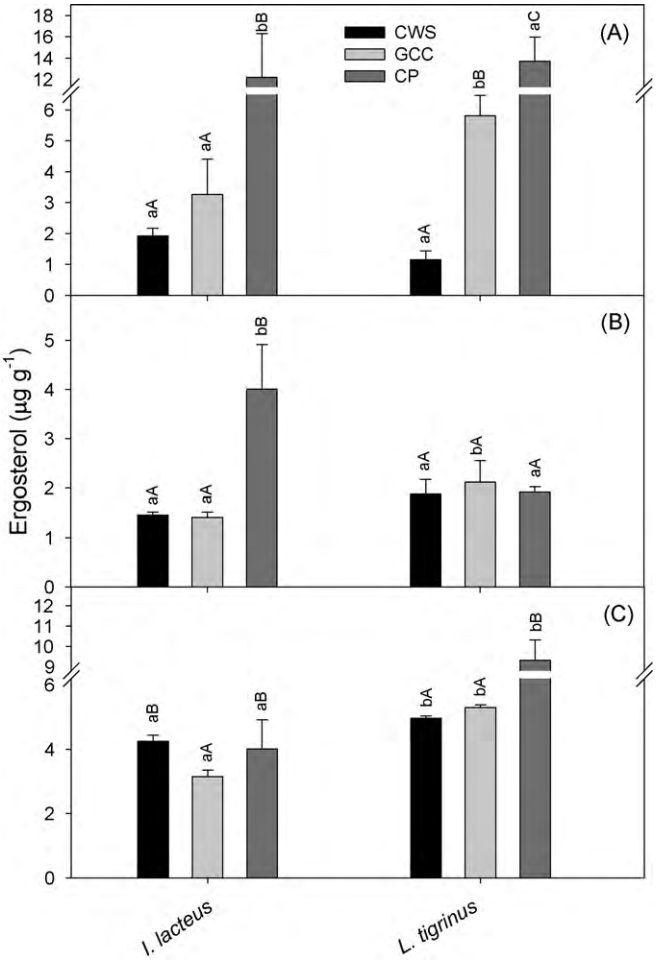

Fig. 1. Ergosterol concentrations in non-contaminated MBU soil (A), historically polluted soil (B) and creosote-impregnated shavings (C) incubated for 60 d at 28 °C with *I. lacteus* CCBAS 238/617 and *L. tigrinus* CBS 577.79 previously supported on either wheat straw (WS). Corn cobs (CC) or commercial pellets (CP).

3. Results and discussion

3.1. Mycelial growth on contaminated matrices

Fig. 1 summarizes growth responses of *I. lacteus* and *L. tigrinus* after 60 d incubation on the MBU, HPS and CIS; both CP-immobilized fungi best grew on the MBU soil (12.2 and 13.7 μg ergosterol g^{-1}, respectively). The type of the inoculum carrier did not significantly affect *L. tigrinus* growth on the HPS where *I. lacteus*, instead, exhibited best growth (4.0 μg ergosterol g^{-1}) when immobilized on CP. The situation was reversed when determining fungal growth on CIS where the type of carrier did not affect *I. lacteus* growth and CP significantly stimulated that of *L. tigrinus*. In fact, the latter fungus grew better on CP than on WS and CC (9.3 *vs.* 5.0 and 5.3 μg ergosterol g^{-1}, respectively). Regardless of the carrier, *L. tigrinus* invariably exhibited better growth than *I. lacteus* on CIS (Fig. 1). Both fungi grew better on CIS than on the HPS, despite the former had a markedly higher PAH concentration than the latter; with this regard, the high content of potential growth substrates (*i.e.*, cellulose and hemicelluloses) in oak wood, the predominant component of CIS, might explain this effect [28]. Several studies emphasize the importance of inoculum formulation on mycoremediation and suggest that lignocellulosic carriers might either confer to fungi an initial competitive advantage over resident microbiota [12,14] or an increased tolerance to inhibitory effects exerted by contaminants [29]. An additional rationale is that an external nutritional supply is provided by lignocellulose-based inocula carriers to basidiomycetes which, unlike bacteria, are unable to use the large majority of organopollutants as C and N sources [10]. Although the use of inocula carriers promoted a satisfactory growth of the strains under study on both HPS and CIS, their matrix-dependent opposite responses clearly suggest the necessity of previous laboratory testing prior to field-scale experiments.

3.2. Fungal PAH degradation

The PAH degradation abilities of *L. tigrinus* and *I. lacteus* were comparatively examined on both the HPS taken from the Sobeslav wood treatment plant and on CIS that had been produced there. For both matrices, the PAH residual contents of non-inoculated incubation controls carried out in the presence of the three carriers did not significantly differ each one another ($P < 0.05$; data not shown). Consequently, Tables 2 and 3 show for each matrix a single incubation control, which, irrespective of the added inoculum carrier, report the averaged PAH contents of all incubation controls.

The most abundant contaminants in the HPS were FLT, PYR, PHE, CHR and FLU the relative contents of which amounted to 28.8, 18.8, 15.8, 15.5 and 12.2%, respectively, with respect to the total PAH content (Table 2). On the one hand, and regardless of both the fungus and the inoculum carrier, the most degraded contaminants were FLU, PHE and ANT the removal extents of which ranged from 75 to 91% with respect to the incubation control. On the other hand, CHR was the least degraded with percent removals ranging from 1 to 9%, thus confirming the results of other studies conducted with different fungi [11,18,30]. This might be due to the fact that CHR has a lower water-solubility (0.002 mg l^{-1}) and higher hydrophobicity ($\log P = 5.81$) than the other 4-ring PAHs; consistently, and with the exception of BaA, the relative bioavailable fraction of CHR was markedly lower than those of FLT and PYR (41.1 *vs.* 92.5 and 88.3%, respectively). The degradation activity of *L. tigrinus* towards FLT, PYR and BaP was significantly ($P \leq 0.05$) higher than that of *I. lacteus* with the largest differences observed for the last compound. Although degradation performances of *L. tigrinus* were not significantly affected by the type of the support, they were invariably better than those of *I. lacteus*. The worst inoculant was WS-immobilized *I. lacteus*, the use of which led to the highest residual PAH sum (1408 mg kg^{-1}) corresponding to an overall removal of 38.4%.

Although the same PAHs were detected on CIS incubation controls, an approx. 21-fold higher concentration than that of the HPS was found in incubation controls of this matrix; moreover, the relative abundances of single contaminants greatly differed from those found in the contaminated soil (Table 3). In particular, the most abundant PAHs on CIS were ANT (41%), FLT (18%), PHE (14%) and PYR (11%). Although PHE, FLU and ANT exhibited the highest susceptibility to fungal degradation, *P. tigrinus* was much more efficient than *I. lacteus* in the degradation of these compounds. Only exception was CP-immobilized *I. lacteus* the PHE degradation capacity of which did not differ from that of *L. tigrinus*-based inoculants. Regardless of the type of inoculum support, the largest differences between the two fungi were observed for the degradation of FLU, and, to a lesser extent, of PHE and ANT (Table 3). As already observed for the HPS, CHR was scarcely degraded. No differences in BaP degradation were observed between the inoculants under study. On an overall basis, best degradation results were observed with CP-immobilized *L. tigrinus* leading to a 68% reduction in the PAH sum with respect to the incubation control (15,494 mg kg^{-1} *vs.* 47,731 mg kg^{-1}, respectively).

To assess whether and which PAH might be degraded by fungal microcosms beyond its respective bioavailable amount, degradation fold with respect to the bioavailable fraction (DFBF) was calculated for each contaminant in both HPS and CIS (Fig. 2). DBA,

Table 2
PAH residual concentrations observed in averaged incubation control and after incubation for 60 d at 28 °C with *Irpex lacteus* 617/93 and *Lentinus tigrinus* CBS 577.79 on historically polluted soil (HPS) using either wheat straw (WS), corn cobs (CC) and commercial pellets (CP) as the inoculums carrier.

PAH	Residual concentrations ($\mu g\,g^{-1}$ soil)*						
	Control	*I. lacteus*			*L. tigrinus*		
		WS	CC	CP	WS	CC	CP
FLU	278.0 ± 18.1	48.3 ± 6.7^{b}	41.3 ± 0.4^{b}	31.9 ± 1.3^{a}	33.2 ± 0.2^{a}	30.0 ± 2.2^{a}	29.1 ± 3.3^{a}
PHE	353.9 ± 36.1	87.1 ± 7.7^{b}	48.1 ± 1.9^{a}	56.5 ± 5.1^{a}	69.0 ± 1.6^{ab}	67.2 ± 9.5^{ab}	48.9 ± 13.2^{a}
ANT	104.6 ± 11.2	20.6 ± 2.2^{b}	19.0 ± 1.2^{b}	16.5 ± 0.6^{b}	9.7 ± 1.9^{a}	12.9 ± 1.3^{ab}	10.4 ± 1.5^{a}
FLT	659.0 ± 51.8	466.8 ± 1.5^{b}	426.5 ± 32.8^{b}	496.9 ± 49.0^{b}	373.5 ± 12.4^{a}	368.8 ± 45.3^{a}	388.6 ± 36.5^{ab}
PYR	429.0 ± 42.2	345.1 ± 31.3^{b}	309.2 ± 56.9^{b}	283.9 ± 20.4^{b}	252.5 ± 4.4^{a}	254.4 ± 29.4^{a}	267.3 ± 2.5^{a}
BaA	77.0 ± 6.9	75.9 ± 9.7^{b}	66.7 ± 12.0^{ab}	60.4 ± 0.6^{ab}	55.9 ± 0.7^{a}	56.8 ± 6.7^{a}	59.0 ± 0.2^{ab}
CHR	353.9 ± 18.7	337.7 ± 14.2^{a}	314.4 ± 10.7^{a}	349.9 ± 3.3^{a}	322.0 ± 11.6^{a}	319.5 ± 31.4^{a}	322.3 ± 9.2^{a}
BbF	12.7 ± 1.2	11.8 ± 1.7^{b}	9.7 ± 1.6^{ab}	10.9 ± 0.1^{ab}	8.6 ± 0.1^{a}	8.6 ± 0.9^{a}	9.0 ± 0.2^{ab}
BkF	6.4 ± 0.7	6.1 ± 0.8^{a}	4.8 ± 0.8^{a}	5.0 ± 0.3^{a}	4.3 ± 0.1^{a}	4.4 ± 0.5^{a}	4.5 ± 0.1^{a}
BaP	6.1 ± 0.5	5.8 ± 1.0^{b}	5.4 ± 0.8^{b}	5.1 ± 0.5^{b}	2.8 ± 0.2^{a}	3.0 ± 0.3^{a}	3.1 ± 0.3^{a}
DBA	0.6 ± 0.1	0.16 ± 0.0^{c}	0.51 ± 0.0^{b}	0.58 ± 0.1^{b}	0.40 ± 0.1^{a}	0.31 ± 0.1^{a}	0.32 ± 0.1^{a}
BghiP	1.0 ± 0.2	1.0 ± 0.3^{a}	0.6 ± 0.1^{a}	0.8 ± 0.1^{a}	0.8 ± 0.2^{a}	0.8 ± 0.1^{a}	0.8 ± 0.2^{a}
IPY	1.9 ± 0.3	1.6 ± 0.2^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	1.1 ± 0.2^{b}	0.6 ± 0.1^{ab}	0.4 ± 0.1^{a}
PAH Sum	2284.1 ± 34.3	1408.0 ± 12.9^{d}	1245.8 ± 28.8^{b}	1318.5 ± 24.0^{c}	1133.3 ± 8.6^{a}	1127.3 ± 31.3^{a}	1143.7 ± 16.3^{a}

* Data are the mean ± standard deviation of three independent experiments. Statistical multiple pair-wise comparisons were carried on row means by the Tukey test: means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

BghiP and IPY were omitted from this analysis since their desorption did not fit the model described by Eq. (1) [24] thus hindering the determination of their *F* fraction.

Regardless of the inoculum carrier, FLU, PHE and ANT in HPS were degraded to the limit of their respective bioavailable portion by both *L. tigrinus* and *I. lacteus* (Fig. 2B and A, respectively). By contrast, *L. tigrinus*-based inoculants degraded CHR, BkF and BaP far beyond their respective bioavailable fractions (Fig. 2B); in particular, the first and the third one had the highest DFBF values regardless of the inoculum carrier (approx. 2.25 and 2.35, respectively). Conversely, only BkF was degraded by CC-immobilized *I. lacteus* slightly above its bioavailable portion (*i.e.*, DFBF equal to about 1.2) (Fig. 2A).

On an overall basis, on CIS the highest DFBF values were obtained for PHE with both fungi (Fig. 2C and D). In this respect, PHE degradation by all *P. tigrinus* inoculants exceeded its bioavailable portion at similar extents (DFBF values of about 2.4) (Fig. 2D); a similar DFBF for PHE was only observed with CP-immobilized *I. lacteus* (Fig. 2C). As for the other PAHs investigated, ANT degradation by *P. tigrinus* also exceeded its respective bioavailable portion, thus yielding DFBF values (about 1.60) which were not affected by the inoculum carrier (Fig. 2D).

To evaluate whether and to which extent bioavailability correlated with PAH degradation, linear regression analysis was performed for each fungal microcosm and the significance of the resulting correlation and the differences among regression slopes were statistically determined. Table 4 shows that degradation was highly correlated with bioavailability in all microcosms ($P < 0.001$) with very similar correlation coefficients with the only exception of that relative to WS-immobilized *I. lacteus* on CIS. Interestingly, regression slopes of all *L. tigrinus* inoculants on CIS were significantly higher than the respective counterparts on HPS while this result was only observed with CC-immobilized *I. lacteus*. This suggests that same variations in bioavailability promote a larger PAH degradation in CIS than in HPS.

It is known that the scarce bioavailability of PAHs depends on their low water-solubility, high hydrophobicity ($\log P$ ranging from 3.30 to 6.75) and tendency to strongly associate with surface particles ($\log K_{oc}$ ranging from 3.3 to 6.54) [3,16]. A general overview on the relationships between these salient physico-chemical properties of PAHs and respective percent removals in fungal microcosms is provided in Table 5. As expected, regardless of both the fungus and the contaminated matrix, PAH degradation was negatively correlated with both $\log P$ and $\log K_{oc}$ and positively correlated

Table 3
PAH residual concentrations observed in averaged incubation control and after incubation for 60 d at 28 °C with *Irpex lacteus* 617/93 and *Lentinus tigrinus* CBS 577.79 on creosote-impregnated shavings (CIS) using either wheat straw (WS). Corn cobs (CC) and commercial pellets (CP) as the inoculums carrier.

PAH	Residual concentrations ($\mu g\,g^{-1}$ soil)*						
	Control	*I. lacteus*			*L. tigrinus*		
		WS	CC	CP	WS	CC	CP
FLU	2647 ± 163	1562 ± 365^{b}	1193 ± 92^{b}	1228 ± 151^{b}	36 ± 9^{a}	26 ± 1^{a}	31 ± 0^{a}
PHE	6698 ± 400	3021 ± 406^{d}	2388 ± 185^{c}	424 ± 121^{ab}	1218 ± 215^{a}	916 ± 161^{a}	847 ± 100^{a}
ANT	19,502 ± 131	16,869 ± 903^{c}	7445 ± 1756^{b}	8388 ± 1792^{b}	3855 ± 545^{a}	4042 ± 184^{a}	4663 ± 610^{a}
FLT	8510 ± 529	6525 ± 331^{b}	5090 ± 460^{ab}	5159 ± 1384^{ab}	4533 ± 193^{ab}	5125 ± 662^{ab}	3268 ± 747^{a}
PYR	5308 ± 372	4439 ± 189^{ab}	3495 ± 284^{a}	3823 ± 646^{a}	4544 ± 265^{ab}	3346 ± 249^{a}	3374 ± 174^{a}
BaA	1923 ± 119	895 ± 62^{a}	970 ± 60^{ab}	1060 ± 142^{ab}	1106 ± 15^{ab}	1212 ± 132^{b}	953 ± 159^{ab}
CHR	1324 ± 105	1327 ± 225^{b}	1022 ± 73^{ab}	1222 ± 189^{ab}	1046 ± 35^{ab}	1078 ± 70^{ab}	930 ± 54^{a}
BbF	651 ± 30	551 ± 14^{b}	435 ± 32^{a}	494 ± 22^{ab}	623 ± 21^{b}	587 ± 47^{b}	560 ± 11^{ab}
BkF	385 ± 67	288 ± 12^{b}	215 ± 14^{a}	276 ± 23^{b}	346 ± 9^{bc}	319 ± 15^{b}	292 ± 11^{b}
BaP	481 ± 111	438 ± 47^{a}	335 ± 44^{a}	414 ± 74^{a}	451 ± 41^{a}	367 ± 30^{a}	363 ± 47^{a}
DBA	55 ± 4	23 ± 1^{a}	17 ± 1^{a}	21 ± 4^{a}	48 ± 3^{b}	20 ± 6^{a}	19 ± 5^{a}
BghiP	88 ± 19	83 ± 10^{b}	52 ± 5^{a}	76 ± 9^{b}	84 ± 3^{b}	75 ± 2^{b}	75 ± 7^{b}
IPY	156 ± 19	124 ± 6^{ab}	102 ± 6^{a}	125 ± 10^{ab}	141 ± 13^{b}	131 ± 6^{ab}	119 ± 9^{ab}
PAH Sum	47,731 ± 264	36,144 ± 565^{d}	22,759 ± 752^{c}	22,710 ± 1114^{c}	18,031 ± 251^{b}	17,244 ± 313^{ab}	15,494 ± 399^{a}

* Data are the mean ± standard deviation of three independent experiments. Statistical multiple pair-wise comparison was carried out on row means by the Tukey test: means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

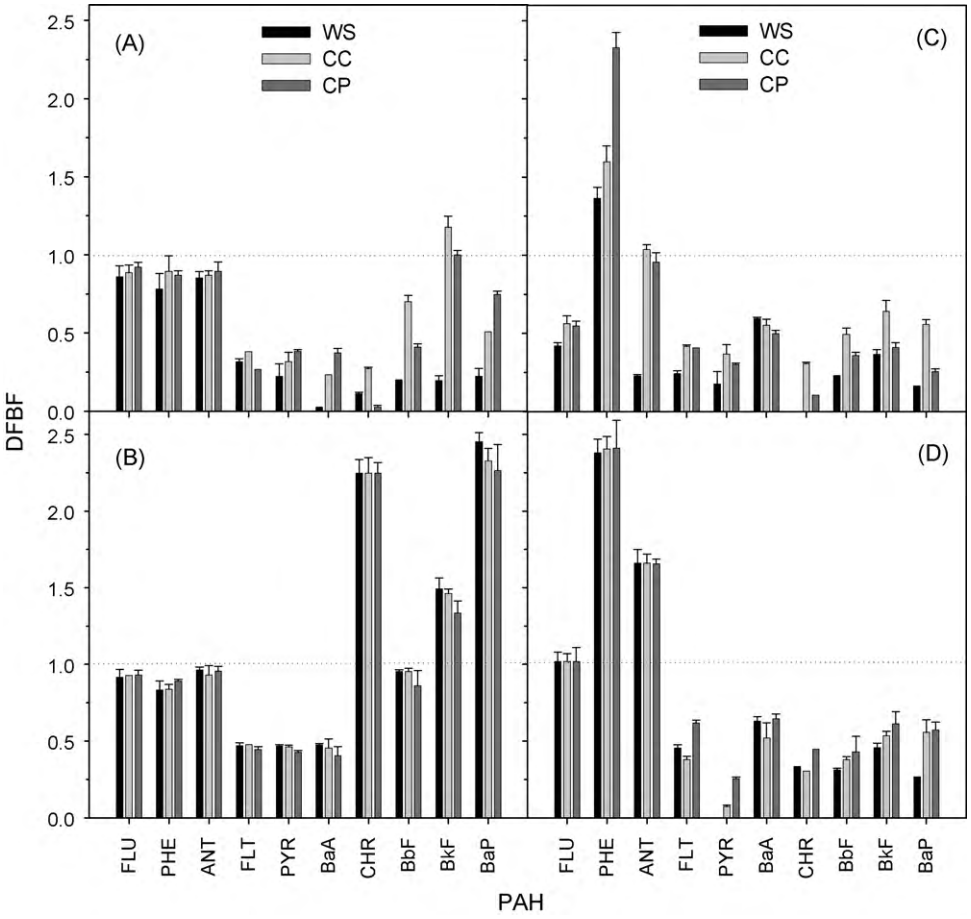

Fig. 2. Degradation fold with respect to the bioavailable fraction (DFBF) of fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF) and benzo[a]pyrene (BaP) in the historically polluted soil and the creosote-impregnated shavings after 60 d incubation at 28 °C with *I. lacteus* CCBAS 238/617 (A and C, respectively) and *L. tigrinus* CBS 577.79 (B and D, respectively) previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean ± standard deviation of three replicates.

Table 4
Regression analysis of PAH degraded *vs.* respective bioavailability in historically polluted soil (HPS) and creosote-impregnated shavings (CIS) incubated with either *L. tigrinus* or *I. lacteus* immobilized on wheat straw (WS) or corn cobs (CC) or commercial pellet (CP). The following parameters of the regression analysis are shown: correlation coefficient (R), coefficient of determination adjusted by the degrees of freedom (R^2_{adj}), slope and F values.

	R[†]	R^2_{adj}	Slope[‡]	F
I. lacteus				
WS-HPS	0.756 (<0.001)	0.556	0.386 ± 0.063[a]	32.257
WS-CIS	0.619 (<0.001)	0.361	0.207 ± 0.050[a]	17.377
CC-HPS	0.810 (<0.001)	0.643	0.461 ± 0.063[a]	53.32
CC-CIS	0.879 (<0.001)	0.764	0.847 ± 0.087[b]	94.972
CP-HPS	0.723 (<0.001)	0.506	0.396 ± 0.071[a]	30.715
CP-CIS	0.821 (<0.001)	0.663	0.785 ± 0.103[ab]	57.985
L. tigrinus				
WS-HPS	0.885 (<0.001)	0.774	0.521 ± 0.055[a]	89.926
WS-CIS	0.842 (<0.001)	0.698	1.085 ± 0.139[b]	60.973
CC-HPS	0.897 (<0.001)	0.798	0.539 ± 0.050[a]	115.466
CC-CIS	0.848 (<0.001)	0.719	1.052 ± 0.124[b]	71.775
CP-HPS	0.863 (<0.001)	0.735	0.515 ± 0.057[a]	81.564
CP-CIS	0.894 (<0.001)	0.792	1.074 ± 0.102[b]	111.324

[†] Each R value is followed by its respective significance level between round brackets.

[‡] Each value of slope is followed by the standard error of its estimate; multiple pair-wise comparisons of slope values were performed by a two-tailed t-test ($P \leq 0.05$). Same letters indicate absence of statistically significant differences.

Table 5
Product-moment Spearman coefficients of correlation between percent PAH removal efficiencies in historically polluted soil (HPS) and creosote-impregnated shavings (CIS) after 60 d incubation at 28 °C with either *I. lacteus* and *P. tigrinus* immobilized on wheat straw (WS), corn cobs (CC) or commercial pellets (CP) and respective PAH physico-chemical properties including molecular weight (MW), organic carbon adsorption coefficient ($\log K_{oc}$), hydrophobicity ($\log P$), water-solubility (WS) and ionization potential (IP).

	MW	$\log K_{oc}$	$\log P$	WS	IP[‡]
I. lacteus					
WS-HPS	−0.90 aB	−0.86 aB	−0.95 bC	0.74 aA	0.50
CC-HPS	−0.85 aB	−0.81 aB	−0.92 aC	0.74 aA	0.53
CP-HPS	−0.87 aB	−0.82 aB	−0.93 aC	0.74 aA	n.s.[†]
WS-CIS	−0.39 aA	−0.39 aA	−0.42 aA	0.56 aA	0.56
CC-CIS	−0.65 aAB	−0.59 aAB	−0.71 aAB	0.53 aA	n.s.[†]
CP-CIS	−0.72 aAB	−0.69 aAB	−0.77 aB	0.62 aA	0.65
L. tigrinus					
CWS-HPS	−0.76 aA	−0.72 aA	−0.83 aA	0.66 aA	n.s.[†]
CC-HPS	−0.76 aA	−0.73 aA	−0.84 aA	0.68 aA	n.s.[†]
CP-HPS	−0.78 aAB	−0.75 aA	−0.85 aA	0.69 aA	n.s.[†]
CWS-CIS	−0.90 bAB	−0.85 bA	−0.91 bA	0.76 aA	0.59
CC-CIS	−0.92 aB	−0.89 bA	−0.94 bA	0.80 aA	0.50

[†] n.s. not significant; difference tests for correlation coefficients were performed by pair-wise comparisons with the two-sided t-test. Same lowercase and uppercase letters denote absence of statistical significance ($P \leq 0.05$) between fungi within the same contaminated matrix and inoculum carrier and, regardless of the contaminated matrix and inoculum carrier, within the same fungus, respectively.

[‡] Correlation coefficients relating IP with percent PAH degradation were not significant in several microcosms; thus, difference tests of relative r were not performed.

Table 6
Inhibition of barley seeds germination after incubation for 72 h at room temperature with contaminants extract (see Section 2.4) derived from the historically polluted soil (HPS) and creosote-treated shavings (CIS) incubated for 60 d at 28 °C with *L. tigrinus* and *I. lacteus* previously supported on either chopped wheat straw (WS), ground corn cobs (CC) or commercial pellets (CP) and from their relative incubation controls.

Sample	Germinability inhibition[†] in:	
	HPS	CIS
Incubation controls		
WS	44.2 ± 4.2 bA	65.6 ± 10.9 bA
CC	45.0 ± 3.9 bA	66.2 ± 10.6 bA
CP	43.0 ± 3.5 bA	65.8 ± 11.5 bA
I. lacteus		
WS	34.0 ± 5.6 bA	51.0 ± 5.1 bB
CC	24.0 ± 2.8 aA	29.0 ± 4.2 aA
CP	30.0 ± 2.8 abA	30.0 ± 2.8 aA
L. tigrinus		
WS	20.7 ± 4.6 aA	29.3 ± 3.1 aA
CC	23.3 ± 2.3 aA	32.0 ± 4.2 aA
CP	20.7 ± 4.2 aA	20.0 ± 6.0 aA

[†] Data are the mean ± standard deviation of three replicates. Multiple pair-wise comparisons of column means were performed by the Tukey test ($P < 0.05$). Same lowercase letters above bars indicate that differences between microcosms (incubation control, *L. tigrinus*, *I. lacteus*) within the same inoculum carrier were not significant. Same uppercase letters indicate lack of statistically significant difference within each fungus at different inoculation carriers.

with their water-solubility. However, two-sided difference tests between Pearson product-moment correlation coefficients showed that strength of linear dependence between $\log P$ and $\log K_{OC}$ and degradation was significantly higher in *L. tigrinus* than *I. lacteus* microcosms on CIS (Table 5). By contrast, the degradation performances of both fungi were correlated to a similar extent with water-solubility.

The susceptibility of PAHs to oxidation by lignin-modifying enzymes of WRF has been shown to generally increase as the IP decreases. In this study, a positive correlation of PAH degradation with IP was found albeit such a relationship was not significant in several microcosms. Although this result might be unexpected, it has to be taken into account that besides to the extracellular degradation pathway, fungi can degrade PAHs *via* the intracellular cytochrome P-450/epoxide hydrolase complex the activity of which is not dependent on IP; the presence of the active form of this complex was recently show in *L. tigrinus* [19].

3.3. Phytotoxicity removal by P. tigrinus and I. lacteus

The contaminants extracts from both HPS and CIS incubation controls were highly phytotoxic towards barley seeds leading to germinability inhibitions ranging from 43 to 45% and from 65.8 to 66.2%, respectively (Table 6). In the HPS, phytotoxicity was significantly reduced by all *P. tigrinus* inoculants, resulting in percent inhibition values of 20.7, 23.3 and 20.7% with the WS-, CC- and CP-immobilized fungus, respectively. In the same matrix, phytotoxicity was significantly reduced only by the CC-immobilized *I. lacteus* (Table 6). Although CIS was more phytotoxic than HPS, a generalized and significant decrease of phytotoxicity was observed in the matrix that had been incubated with the majority of *P. tigrinus* and *I. lacteus* inoculants with the sole exception of the WS-immobilized *I. lacteus*.

4. Conclusions

The present study shows the ability of *L. tigrinus* to colonize and detoxify solid PAH-contaminated matrices derived from a wood treatment facility under non-sterile conditions. Its growth and degradation performances were invariably higher than those of *I. lacteus*. The opposite responses of the two strains to inocula carriers in dependence on the contaminated matrix points out that a preliminary assessment of strain/carrier combination is fundamental prior to field-scale mycoremediation. Although PAH degradation was highly and significantly correlated with bioavailability in all microcosms, the breakdown of certain PAHs proceeded well above their respective bioavailabilities and this capacity was more pronounced in *L. tigrinus* than *I. lacteus*.

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