



Technical Note

Development of permeable reactive biobarrier for the removal of PAHs by *Trichoderma longibrachiatum*



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HIGHLIGHTS

- ▶ Innovative permeable reactive barrier using fungus biofilm was developed.
- ▶ *Trichoderma longibrachiatum* over nylon sponge was selected as the bioreactive medium.
- ▶ The ability for PAHs removal of the developed bioreactive medium was demonstrated.

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ABSTRACT

In this work, the formation of permeable reactive biobarriers (PRBBs) using *Trichoderma longibrachiatum* over nylon sponge as bioreactive medium for removal of polycyclic aromatic hydrocarbons (PAHs) was studied. Colony formation was pretested without PAH presence by inoculation of fungus into nylon sponge. The fungus formed a large quantity of strongly adhesive biofilm among nylon sponge. Afterwards, the ability of the developed bioreactive medium was tested to remediate phenanthrene in aqueous medium and in soil. In aqueous medium, a 90% of phenanthrene concentration reduction was observed after 14 d. However, the pollutant removal in soil requires previous fungus colonization and the attained level was around 70% after 28 d. Subsequently, the formed bioreactive material was used in a glass column reactor to evaluate its application as PRBBs. Mixtures of phenanthrene, benzo[a]anthracene and pyrene at several concentrations, from 100 to 400 μM, were treated. In all cases, the performance of the PRBB was satisfactory and total PAH removals were achieved. These results suggest that PRBBs of *T. longibrachiatum* supported on nylon sponge can be an effective method for the treatment of PAHs.

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1. Introduction

Water is a limited natural resource that may be considered as one of the most important components of the environment. There are several pollution sources of water and groundwater as a result of anthropogenic activities including industrial spills, septic tanks, agricultural areas, underground storage tanks, wastewater treatment works, and unauthorized dump sites (Manoli and Samara, 1999; Tredoux et al., 2004). Because of this, the polluted water becomes harmful to vegetation, animals and humans. At the present time, there are a wide variety inorganic, organic and synthetic compounds that are discharged to the environment and become water contaminants. Amongst them, the presence of emerged organic pollutant such as polycyclic aromatic hydrocarbons (PAHs)

is taking the attention of scientific community (Zein et al., 2006; Gibert et al., 2007).

PAHs are spread to the environment mainly as a result of anthropogenic activities and in spite of their low solubility they can be present in surface water and groundwater (Manoli and Samara, 1999). PAHs are organic compounds that consist of more than two bonded aromatic rings and are formed in the incomplete decomposition of organic molecules. They are important environmental contaminants and clear mutagenic/carcinogenic compounds, which are included in a list of pollutants by United States Environmental Protection Agency and EU Water Framework Directives (2000/60/EC) (EU, 2000; Haritash and Kaushik, 2009). In this study several PAHs, such as phenanthrene (PHE), benzo[a]anthracene (BAA) and pyrene (PYR) were selected because they are representative PAHs for monitoring and control PAHs emissions of in the environment (Inengite et al., 2012).

Currently, permeable reactive barriers technology is considered an innovative, cost-effective and environmentally sound method

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for the *in situ* groundwater remediation (Warner, 2011). In this technology, a reactive medium is placed in the subsurface across the flow a plume of contaminated groundwater, which must move under its natural gradient, thereby creating a passive treatment system (Thiruvengkatachari et al., 2008).

The permeable reactive barriers technology can also benefit from other types of remediation, such as bioremediation. Several researchers have determined the biodegradative capacity of PAHs of both bacteria and fungi in contaminated land and waters (Haritash and Kaushik, 2009; Moscoso et al., 2012). PAH-degrading microorganisms can act by anaerobic or aerobic metabolism. The basis of aerobic metabolisms is the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide. On the other hand the anaerobic metabolism of PAHs occurs via the hydrogenation of the aromatic ring (Bamforth and Singleton, 2005).

Accordingly, permeable reactive barriers can be coupled with bioremediation forming the named permeable reactive biobarriers technology (PRBBs). In this technology, the microorganisms, retained on the soil surface or on a porous support, are used as reactive medium to degrade or stabilize the contaminants in the groundwater (Choi et al., 2007; Seo and Bishop, 2008; Tiehm et al., 2008). In addition to the microbial activity, biofilm formation plays important role in the performance of the PRBB (Harding et al., 2009). In spite that the biofilm ability is mainly related to bacteria and yeast, researchers have reported that some filamentous fungi as *Aspergillus* and *Trichoderma* are able to grow on hard surfaces forming biofilm (Te Biesebeke et al., 2002; Villena and Gutierrez-Correa, 2003b).

The genera *Trichoderma*, isolated from oil polluted sites, have been reported as benzo(a)pyrene-degrading microorganisms, converting it into intermediate metabolites, such as benzo(a)pyrene trans, 4,5-dihydrodiol, benzo(a)pyrene trans 8,9 dihydrodiol, benzo(a)pyrene trans, 9,10 dihydrodiol, and benzo(a)pyrene 1,6 quinone (Mueller et al., 1998). In addition our previous studies have determined the enormous potential of the isolated fungus *Trichoderma longibrachiatum* in the degradation of high-molecular weight PAHs such as BAA (Rosales et al., 2012b).

Several materials can be used as support to biofilm formation; however the use of low cost supports such as agro-industrial wastes (orange peel and potato peel), solid organic materials (mulch and peat moss) or inorganic materials (nylon sponge) is attracting the interest of scientific community (Yerushalmi et al., 1999; Rosales et al., 2012a). Among them, the biofilm formation over nylon sponge seems to be adequate low cost support to form PRBBs. The immobilization in nylon sponge offers a physical retention, without reaction with the microorganisms or pollutants and providing a homogeneous solid support.

Based on the above, the aim of this work is to evaluate the formation of bioreactive material for PRBBs using *T. longibrachiatum* over nylon sponge. Afterwards, the developed bioreactive material will be tested to PHE remediation in aqueous medium and soil. Finally, the performance PRBB using the developed material will be examined in column reactor to treat a solution of PAH mixture.

2. Materials and methods

2.1. Microorganism

T. longibrachiatum was isolated from waste containers, polluted with PAHs and heavy metals (Rosales et al., 2012b). The genetic identification of the strain was done using PCR AmpliTaq DNA polymerase and was compared with sequences in public databases of GeneBank (Rosales et al., 2012b). The isolated strain was maintained in Potato Dextrose Agar (PDA) plates at 4 °C.

2.2. Growth medium (GM)

The GM was composed of (g L⁻¹) Na₂HPO₄·2H₂O 8.5, KH₂PO₄ 3.0, NaCl 0.5, NH₄Cl 1.0, MgSO₄·7H₂O 0.5, CaCl₂ 0.0147, mycological peptone 1, glucose 10. The pH was initially adjusted to five with HCl and was autoclaved at 121 °C for 20 min. After that trace elements were added by 0.22 µm filtration MgSO₄ 0.24 g L⁻¹, CaCl₂ 0.555 µg L⁻¹ and micronutrients solution (in g L⁻¹) was composed of CuSO₄ 4 × 10⁻⁴, KI 1 × 10⁻³, MnSO₄·H₂O 4 × 10⁻³, ZnSO₄·7H₂O 4 × 10⁻³, H₃BO₃ 5 × 10⁻³, H₂MoO₄·2H₂O 1.6 × 10⁻³ and FeCl₃·6H₂O 2 × 10⁻³.

2.3. Column assays medium (RM)

The removal medium (RM) used in the column assays was composed of aqueous solution of PHE, BAA and PYR in Tween 80 1%, acetone 2% and minimal medium of (g L⁻¹) Na₂HPO₄·2H₂O 8.5, KH₂PO₄ 3.0, NaCl 0.5, NH₄Cl 1.0, MgSO₄·7H₂O 0.5, CaCl₂ 0.0147. The pH was initially adjusted to five with HCl and was autoclaved at 121 °C for 20 min. After that trace elements were added by 0.22 µm filtration MgSO₄ 0.24 g L⁻¹, CaCl₂ 0.555 µg L⁻¹ and micronutrients solution was composed of (g L⁻¹) CuSO₄ 4 × 10⁻⁴, KI 1 × 10⁻³, MnSO₄·H₂O 4 × 10⁻³, ZnSO₄·7H₂O 4 × 10⁻³, H₃BO₃ 5 × 10⁻³, H₂MoO₄·2H₂O 1.6 × 10⁻³ and FeCl₃·6H₂O 2 × 10⁻³.

2.4. Support

Fibrous nylon sponge was used for the immobilization of fungus (Scotch brite TB, 3M Company, Spain). This supporting matrix allows the exchange of nutrients and/or the flow of the medium through it. The nylon sponge was pretreated according to Moldes et al. (2003) by boiling for 10 min and washing thoroughly 3 times with distilled water. After that, the supports were dried at room temperature overnight and autoclaved at 121 °C for 20 min.

2.5. Soil

Soil samples were collected from the northwest of Spain (Galicia) (Pazos et al., 2011). Using an Eijkelkamp sampler, the soil samples were taken of the top 30 cm in different points. Soil mineralogical analysis, pH and texture are shown in Table 1. The collected samples were mixed and sieved and the fraction containing particles of sizes <2 mm was selected. After that, the soil samples were polluted with PHE (98% Aldrich) by adding the PAH dissolved in hexane. The polluted soil was placed in a ventilation hood for 9 d until the hexane completely evaporated. The initial concentration of PHE in the soil was approximately 285 mg kg⁻¹ of dried soil.

2.6. Experimental set-up

2.6.1. Developed of bioreactive material

To obtain *T. longibrachiatum* biofilm over fibrous nylon sponge, bioreactive material, submerged aerobic cultures were prepared in 250 mL-Erlenmeyer flasks with 15 mL of GM and 1 g nylon sponge (0.5 × 0.5 cm). The flasks were inoculated with 4-agar portion (diameter, 3 mm), extracted from a fungal colony growing on PDA plate. The flasks were incubated for 7 d at 30 °C in darkness with dynamic incubation at 50 rpm with a passive aeration permitted by cellulose stoppers.

2.6.2. Bioreactive material performance to PHE removal in aqueous medium

Once the biofilm was formed on the support, henceforth bioreactive material, the GM was removed from the Erlenmeyer flasks and changed by 15 mL of aqueous solution of PHE. The aqueous

Table 1
Soil mineralogical analysis, pH and texture.

Characteristics		Mineralogical analysis (%)	
Origin	Northwest Spain	Kaolinite	3.6
Type	Loamy sand	Mica	7.7
pH	4.5	Quartz	48.9
		Calcite	ND
		Dolomite	3.4
		Albite	ND
		Chlorite	<1
		Illite	ND
		Orthoclase	18.2

solution was prepared dissolving PHE (98% Aldrich) at a concentration of 1000 μM in aqueous medium using 1% Tween 80 and 2% acetone. The flasks containing PHE solution and biofilm over nylon sponge were maintained at darkness in an environmental chamber at 30 °C and 90% humidity, to prevent evaporation. The experiments were performed in duplicated for each time (0, 7, 14, 21 and 28 d). Each experimental set was stopped at desired contact time in order to not disturb the system. Abiotic controls were carried out in parallel in order to evaluate the pollutant volatilization or the pollutant adsorption onto nylon sponge.

2.6.3. Bioreactive material performance to PHE removal in soil

For the PAH soil removal assays, 10 g of PHE polluted soil, prepared according to Section 2.5, were placed in 250 mL-Erlenmeyer flasks and were autoclaved. After that, 6 mL of GM and the biofilm over nylon sponge (7 d old) were transferred to the flask with the polluted soil. The flasks containing PHE polluted soil and biofilm over nylon sponge were maintained at the same conditions as previous experiments. The experiments were performed in duplicated for each time (0, 7, 14, 21 and 28 d). Each experimental set was stopped at desired contact time in order to not disturb the system. Abiotic controls were carried out in parallel in order to evaluate the pollutant volatilization.

2.6.4. Column reactor assays

A glass reactor, consisting of a column (diameter 4 cm; height 50 cm; working volume 0.6 L) filled with the bioreactive material, was employed to perform the present study. Initially, the biofilm formation was carried out in a 1 L-Fernbach flask with 100 mL of GM and 13 discs (3.5 cm diameter and 0.5 cm thickness) and 27-agar portion (diameter, 3 mm) extracted from a fungal colony growing on PDA plate. The Fernbach flask was incubated for 1 week at 30 °C in darkness with dynamic incubation at 50 rpm and a passive aeration permitted by cotton plugs. After the biofilm formation, the discs were fixed in a steel rod with a plastic spacer of 0.3 cm and this rod was inserted in the glass column reactor. The bioreactor was kept at 30 °C in a thermostatic chamber and the air supply was maintained at 5 mL min^{-1} . RM medium was used to fill the reactor. Several concentrations were assayed for each PAHs ranged from 100 to 400 μM at successive batches and peristaltic pumps were used to simulate the natural horizontal flow through PRBB. PAHs solutions were prepared dissolving the PHE (98% Aldrich), BAA (99% Aldrich) and PYR (99% Aldrich) at a desired concentration in aqueous medium using 1% Tween 80 and 2% acetone. Samples were taken periodically in the middle of the column to evaluate PAH removal.

2.7. Sample treatment and analysis

2.7.1. Scanning electron microscopy analyses

A series of scanning electron microscopy (SEM) images were taken to provide a visual characterization of the fungus grown over

the support. The samples were dehydrated, critical point dried, coated with gold and then photographed. Images were collected on a FE-SEM JSM6700 F (JEOL) scanning electron microscope using an accelerating voltage of 10 kV (Electron Microscopy Service, C.A.C.T.I., University of Vigo).

2.7.2. PAH analysis in aqueous samples

PAH concentration in aqueous samples was determined by HPLC (Agilent 1100) equipped with an XDB-C8 reverse-phase column (150 \times 4.6 mm id, 5 μm). Prior to injection, the samples were centrifuged (10000 rpm, 5 min) and filtered through a 0.45 μm Teflon filter. The PAH concentration was measured following the standard method UNE-EN ISO 17993. The injection volume was set at 5 μL , and the isocratic eluent (60:40 acetonitrile:water) was pumped at a rate of 1 mL min^{-1} for 10 min. Detection was performed with a diode array detector from 100 to 400 nm, and the column temperature was maintained at 20 °C. Duplicated measures were done and the deviation standards were lower than 5%.

2.7.3. PAH analysis in biofilm

To determine the PAHs adsorbed by the biofilm a solid-liquid extraction was done. Samples of biofilm were crushed prior solid-liquid extraction to eliminate the interstitial fluid. Then, 10 mL of methanol were added to crushed biofilm and shaken at 150 rpm for 24 h. The residual PAHs in methanol solution were determined by HPLC.

2.7.4. PHE analysis in soil samples

The PHE was extracted from the soil by fast high-pressure extraction in a PSEOne (Pressurized solvent extraction) (EPA Method 3545). Dry samples, after 1 week in 30 °C oven, were thoroughly mixed with pelletized diatomaceous earth. The resulting free-flowing powder was placed into an extraction vessel in the instrument. The extraction was realized by 4 cycles of 5 min each at 110 °C and 10 MPa, the solvent employed was hexane:acetone 1:1 (v/v). The collected sample was used to determine the PHE concentration by HPLC as it was described in previous section. Duplicated measures were done and the deviation standards were lower than 8%.

2.7.5. pH

The pH in liquid samples was measured directly with an IQ Scientific Instruments pH metre (model Stainless Steel ISFET pH Probes). The soil pH was measured by adding a distilled H_2O to the dry sample in a ratio of 2.5 mL g^{-1} . After 1 h of contact time, the pH was measured with an IQ Scientific Instruments pH metre (model Stainless Steel ISFET pH Probes).

3. Results and discussion

3.1. Bioreactive material characterization

One of the most important points to biobarrier formation is to get a proper retention of the microorganism over the selected support. The microorganism selected for this study, *T. longibrachiatum*, belongs to the filamentous fungi species that are naturally adapted to growth on surfaces. Filamentous fungi require close contact with the substrate due to heterotrophic nutrition, secretion of extracellular enzymes, absorption of nutrients through the cell wall and apical growth of the hyphae (Jones, 1994). Thus, it can be inferred that these fungi have a tendency to the formation of biofilms, which can develop even on synthetic materials.

Nylon sponge had been successfully used for white-rot fungi retention to industrial applications as enzyme production or effluent treatment (Rosales et al., 2002). Accordingly, this support was chosen as a result of its adequate characteristics because it offers

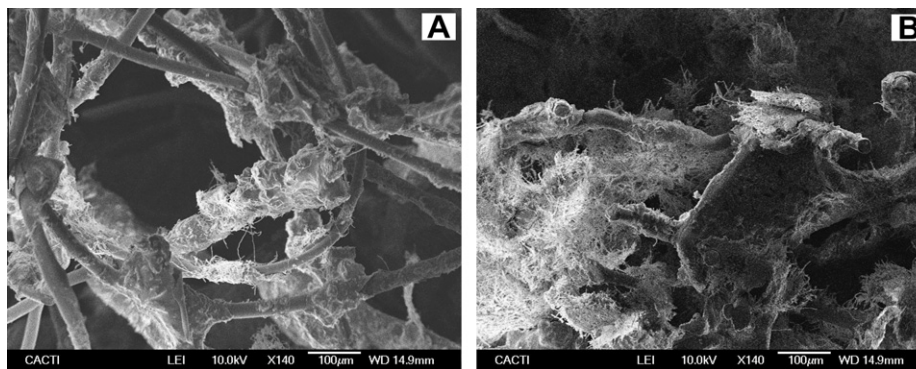


Fig. 1. Scanning electron microscopy microphotographs of *T. longibrachiatum* grown over nylon sponge after 2 (A) and 7 (B) days.

an inert physical retention providing a homogeneous solid support. In addition, it is expected that the nylon porous structure allows a good contact between the polluted flow and the microorganisms.

T. longibrachiatum biofilm developed on nylon sponge was evaluated considering two aspects related to the biobarrier formation: structure and physiological behavior focused on PAH removal (studied in the next section). The biofilm structure was assessed by using SEM microphotographs. The biofilm formation is a process which can be distinguished in three phases which include the initial cell adhesion, growth and development and maturation (Villena and Gutierrez-Correa, 2003a). The limiting step of the process is the accession of spores to a surface, which is influenced by physical, chemical and environment. In Fig. 1A, the fungal growth among nylon fibres can be appreciated since the first days. An effective adhesion and growth from the spore germination was demonstrated. After 7 d almost all available nylon fibres surface has been colonized (Fig. 1B). In this figure, it can be clearly appreciated that the biomass density highly increased in comparison to initial days (Fig. 1A). Nevertheless, there are internal channels that guarantee the flow through biofilm. Hence, the biofilm structure examined by SEM analysis clearly shows that the nylon sponge is an adequate support for the *T. longibrachiatum* growth.

3.2. Bioreactive material performance to PHE removal

As it was stated in previous section, once the bioreactive material was formed, it is necessary to evaluate its physiological behavior focused on PAH removal. To overcome this issue, different experimental sets were realized in order to evaluate the bioremoval ability of developed biofilm in aqueous medium and in soil.

3.2.1. PHE removal in aqueous medium

Our previous studies (Rosales et al., 2012b) have determined the enormous potential of *T. longibrachiatum* in the degradation of BAA in liquid cultures, however the performance of the *T. longibrachiatum* biofilm for PAH removal had not reported yet. Therefore, an important step of this study was to demonstrate the ability of *T. longibrachiatum* biofilm to PAH removal. Initially, the removal of PHE, a molecule composed of three fused benzene and widely used as an indicator for monitoring PAH-contaminated sites (Sack et al., 1997), was assayed. The experiment using fungus biofilm over nylon sponge was monitored for 4 weeks. The system pH was constantly increased during the experiments from 5 at 0 d to 6.3 at 28 d of experiment while there were no observable changes in pH of the controls. Thus, the changes on pH were attributed to microbial activity. After 1 week, the removal of PHE in aqueous medium was higher than 60% (Fig. 2A). Similar results were reported by Raghukumar et al. (2006) who used the marine fungus NIOCC# 312 immobilized in polyurethane foam for PHE removal/

degradation. The removal increased up to 90% after second week, reaching the maximum removal around 97% after 21 d of treatment. After experiments, the residual PHE retained by the biofilm was lower than 2% and negligible PHE reduction of concentration was observed in abiotic control. This means that PAH removal was only due to biological action. According to our previous study (Rosales et al., 2012b) the removal of PAHs by *T. longibrachiatum* can be adjusted to a logistic Eq. (1) in which D is the PAH removal degree (%) at a specific moment of the culture time t (d), D_0 and D_{max} are the initial and maximum removal percentage (%), respectively, and μ_D is the maximum specific degradation rate (d^{-1}).

$$D = \frac{D_{max}}{1 + e^{\left[\ln \left(\frac{D_{max}-1}{D_0} \right) - \mu_D t \right]}} \quad (1)$$

Sigma Plot 8.0 software was utilized to adjust the experimental data to model using an iterative procedure, based on the Marquardt–Levenberg algorithm, which seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable.

The logistic model fitted well to data achieving a high determination coefficient, $r^2 = 0.995$. The maximum specific removal rate, $1.13 d^{-1}$, was similar to that obtained for the degradation of ben[a]anthracene in free cultures of *T. longibrachiatum*, $1.6 d^{-1}$ (Rosales et al., 2012b). Therefore, it can be stated that working with the fungus biofilm does not significantly reduce the removal efficiency.

3.2.2. PHE removal in soil

In this work the treatment of PHE polluted soil with fungus biofilm was studied. Once the bioreactive material was transferred to flask containing PHE polluted soil, the fungus colonization was efficiently performed and after 5 d and homogenous fungus wrap over the soil was observed. As it is shown in Fig. 2B, for rate was not constant and there was a rise in the last weeks of treatment from 37% to 63%. The significant increase of PHE removal is possibly related with the time of exposition and adaptation of the fungi to PHE polluted soil. This fact is in agreement with the performance reported by Rivera-Cruz et al. (2002). They found that genera *Trichoderma* have mechanisms of physiological adaptation in soil, probably of genetic origin, which permit to use PAH carbon in their cellular multiplication. Furthermore, the metabolic activity of fungus for PAHs is widely influence by medium pH (Haritash and Kaushik, 2009), thus soil pH was monitoring. In comparison with control assays, where pH was kept around pH = 4.5, a continuous increase of soil pH due to microbial activity was development for 4 weeks of treatment (Fig. 2B). Gaanappriya et al. (2011) stated that the fungus metabolic has its optimum pH for the growth and xylan-degrading enzyme production between 5.5 and 5.7.

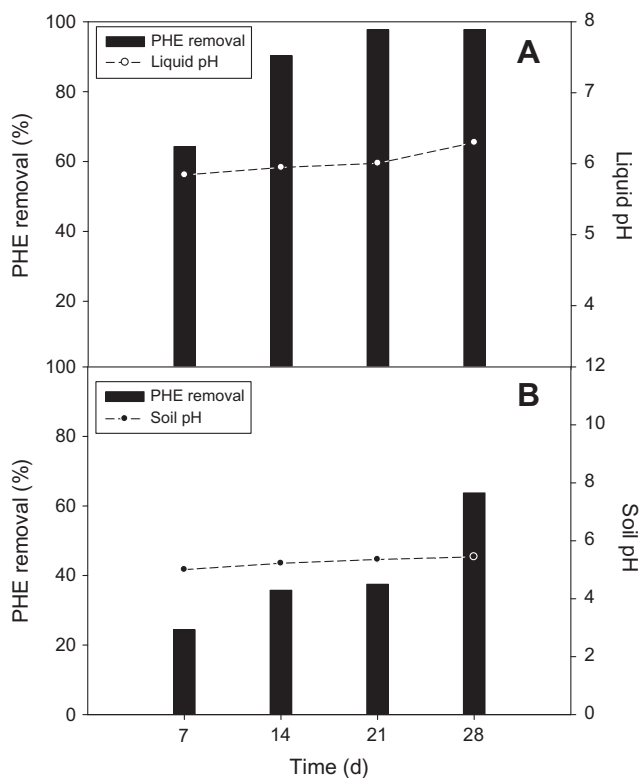


Fig. 2. PHE removal profiles and pH evolution by using *T. longibrachiatum* biofilm over nylon sponge. (A): In aqueous medium (initial PHE concentration 1000 µM at pH 5) and (B): in soil (initial PHE concentration 285 mg kg⁻¹ at pH 4.5).

And these values coincide with the soil pH developed in the last week when rate was highest.

3.3. Biobarrier column reactor assays

After demonstrating the viability of the developed bioreactive material to PHE removal in several environments (aqueous medium and soil), it was necessary to evaluate its application as PRBB. Therefore column reactor assays using the developed material were carried out. Mixture of three model PAHs, PHE, BAA and PYR were treated in the glass column reactor. In Fig. 3, the evolution of PAH concentrations in the glass column along the time is

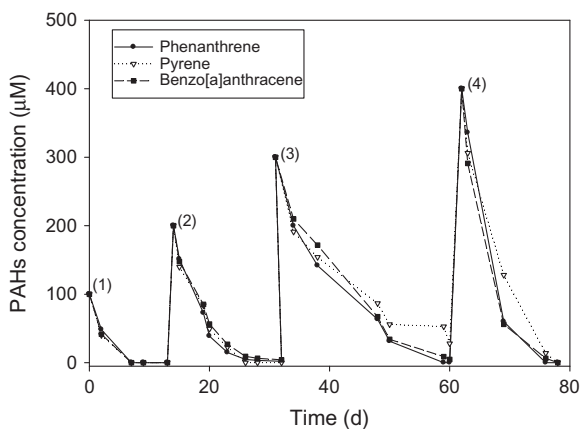


Fig. 3. PAHs removal by *T. longibrachiatum* biofilm over nylon sponge column batch reactor assays. Initial PAHs concentration: 100 µM (1), 200 µM (2), 300 µM (3) and 400 µM (4).

represented. As it is shown, in all tested concentrations a total PAH removal was achieved and the bioremoval rates do not differ significantly for the different studied PAHs. It is postulated that the rapid disappearance of PAHs from aqueous medium is due to a first step of adsorption to the fungal biomass. Many researchers have reported that hydrophobic organic compounds have a high affinity for biofilms (Ebihara and Bishop, 1999; Seo and Bishop, 2008). The hypothesis reported in the present work is also in agreement with Raghukumar et al. (2006) who determined that once the PAHs are adsorbed by the biomass they are subsequently metabolized by the microorganisms.

The developed biobarrier of *T. longibrachiatum* worked in successive batches during more than 70 d without a significant decrease of its activity. Thus, the present study demonstrates that the biobarrier activity endures along the time. In addition, the ability of *Trichoderma* species as potential biocontrol agents can be useful in a real application of groundwater treatment where there are no aseptic conditions (Rojo et al., 2007).

4. Conclusions

In this work the *T. longibrachiatum* biofilm over nylon sponge was evaluated for its use as bioreactive material to PRBBs for the treatment of PAH polluted groundwater. Initially, the capacity of the fungus to grow forming biofilm over nylon sponge was demonstrated. After that, the physiological behavior of bioreactive material focused on PAH removal was tested. The developed material was able to reduce effectively PHE in soil and aqueous medium. Subsequently, the formed reactive material was used in a column reactor to evaluate its performance as PRBB to treat PAH mixtures. The developed fungus biobarrier worked in successive batches during more than 70 d without a significant decrease of its activity. In view of the achieved results, it can be suggested that the PRBBs of *T. longibrachiatum* supported on nylon sponge could be a potentially useful method for the treatment of groundwater polluted with PAHs. However, further work is necessary in this field in order to analyze the implementation in a real polluted groundwater.

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