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Fungal and bacterial microbial community assessment during bioremediation assays in an aged creosote-polluted soil

S. Lladó [a], E. Gràcia [b], A.M. Solanas [a], M. Viñas [c,*]

[a] Department of Microbiology, University of Barcelona, Diagonal 643, E-08028 Barcelona, Spain
[b] Department of Botanics, University of Barcelona, Diagonal 643, E-08028 Barcelona, Spain
[c] GIRO Joint Research Unit IRTA-UPC, IRTA, Torre Marimon, Caldes de Montbui, E-08140 Barcelona, Spain

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ABSTRACT

The application of bioremediation technologies to polycyclic aromatic hydrocarbon (PAH)-contaminated soils does not remove the excess of the high-molecular-weight fraction (HMW-PAHs), as has been widely reported. Taking into account the metabolic capacities of white-rot fungi, their bioaugmentation has been extensively assayed on polluted soils, but with controversial results.

The aim of this study is to gain insight into how fungal bioaugmentation assays affect both PAH degradation and autochthonous microbial populations in a previously biotreated aged creosote-polluted soil contaminated with HMW-PAHs. To this end, we performed a set of slurry bioassays encompassing different biostimulation and bioaugmentation strategies.

The results show that the autochthonous microbial populations degraded PAHs the most; specifically, the 4-ring PAHs under carbon-limiting conditions (26% and 28% degradation for benzo(*a*)anthracene and chrysene respectively). Although *Trametes versicolor* amendment produced the highest depletion of benzo($b + k$)fluoranthene and benzo(*a*)pyrene concentrations in an autoclaved soil, it did not improve either the 4-ring or the 5-ring PAH degradation, when active native PAH-degrading microbiota was present. Microbial community analysis of fungal and eubacterial populations, based on the *16SrRNA* gene and ITS1 region respectively, revealed that the ribotypes closely related to the eubacterial genera *Chryseobacterium*, *Pusillimonas* and *Sphingobium*, that are concomitant with the autochthonous fungal genus *Fusarium*, could be important in HMW-PAH degradation processes in polluted soils.

Antagonistic effects or resource competition resulting from the effects of active native soil microbiota on augmented white-rot fungi should be evaluated in polluted soil before scaling up the remediation process to field scale.

1. Introduction

As a consequence of the widespread presence of polycyclic aromatic hydrocarbons (PAHs) in soils, mainly due to the increase in industrial activity over the last decade, their remediation through the field application of biological treatments has been growing, due to both their reduced environmental impact and their relatively low cost compared with other technologies (Singh and Tripathi, 2007).

It is well known that autochthonous microbial populations can remove PAHs from polluted soils. Furthermore, it has been widely reported that soil characteristics such as moisture content, aeration conditions and nutrients can affect removal rates (Chaîneau et al., 2003). When such autochthonous populations are present, biostimulation is recommendable to counter natural attenuation. However, on aged historically contaminated soils, where the bioavailability of the most recalcitrant compounds, such as high-molecular-weight PAHs (HMW-PAHs), may be extremely low, or in those soils where there is no active microbial population capable of degrading PAH compounds, the success of biostimulation may be greatly restricted (Chung and Alexander, 1999). In difficult cases such as these, amelioration of the bioavailability of the contaminants together with microbial or, in particular, fungal bioaugmentation can yield better biodegradation (Juhasz and Naidu, 2000).

A wide variety of fungi have been shown to metabolize PAHs. Among them, white-rot basidiomycetes are one of the most important groups used in soil bioremediation treatments. This is due to their enzymatic system, which includes intracellular cytochrome

* Corresponding author. Tel.: +34 93 467 4040; fax: +34 93 467 4042.
E-mail addresses: vadorllado21@ub.edu (S. Lladó), egracia@ub.edu (E. Gràcia), asolanas@ub.edu (A.M. Solanas), marc.vinas@irta.cat (M. Viñas).

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P450 and extracellular lignin peroxidase, manganese peroxidase and laccase. As a consequence, some have the ability to cleave the aromatic rings and mineralize the PAHs (Harms et al., 2011).

One of the major obstacles in the implementation of field-scale mycoremediation is that most laboratory-scale research is carried out using artificially polluted soils (spiked or sterilized). For this reason, it is important to increase the number of studies using non-sterile soils from actual polluted sites. In recent work (Llado et al., 2009), in order to ascertain the reasons for the lack of further degradation of HMW-PAHs in a previously bioremediated creosote-contaminated soil, and to analyse the microbial population related to PAH degradation, a strategy involving slurry incubation with a liquid mineral medium was assessed. Slurry conditions may increase the access of microorganisms to hydrophobic contaminants such as PAHs. Indeed, slurry technology has been shown to significantly enhance mineralization extents of a low molecular weight PAH as phenanthrene (Semple et al., 2006), In previous studies (Sabaté et al., 2006; Llado et al., 2009), it was concluded that bioavailability was a key factor in the lack of degradation of 4-ring PAHs, and that the slurry approach, coupled with molecular ecology techniques, was a suitable method for increasing biodegradation and to better understand the chemical and microbial aspects of aged hydrocarbon-polluted sites subjected to a bioremediation process. However, the same slurry strategy, when examined in 5-ring PAH experiments, failed to enhance degradation rates.

That unsuccessful enhancement led us to consider a similar slurry method but including optimization of the fungal bioaugmentation strategy with a view to enhancing the degradation of the residual PAHs remaining in the soil. The white-rot fungus *Trametes versicolor* was chosen because its laccase has been widely reported to be optimal for degrading HMW-PAHs (Collins et al., 1996). Taking into account that several failures have been reported in bioremediation processes using white-rot fungi (Radtke et al., 1994; Wiesche et al., 2003; Borràs et al., 2010; Gao et al., 2010; Mikesková et al., 2012), studying the interactions between microbial populations has become essential in order to improve mycoremediation technologies.

The aim of this work is to study the PAH-degrading capability of autochthonous and allochthonous microbial soil populations subjected to different conditions in an aged creosote-polluted soil. In addition, inoculation of *T. versicolor* in the presence of either active or inhibited native microbiota allowed us to assay the influence on degradation capabilities of the interaction between them. The study focuses on the degradation of HMW-PAHs remaining in a soil after the application of a dynamic biopile, for which the degradation rates were close to those achieved in laboratory-scale studies (Sabaté et al., 2006; Viñas et al., 2005).

In order to assess the microbial interactions and to characterize the effect of the exogenous fungal inoculum, on both the autochthonous eubacterial and the autochthonous fungal microbial community structure and dynamics during the different soil bioremediation strategies, DGGE molecular profiling of both populations was performed.

2. Materials and methods

2.1. Chemicals

Phenanthrene, fluorene, anthracene, dibenzothiophene, benzo(*a*)anthracene, chrysene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, ergosterol, 7-dehydrocholesterol, o-terphenyl and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate were purchased from Sigma–Aldrich (Madrid, Spain). Solvents were purchased from Scharlab S.L. (Barcelona, Spain). All solvents, chemicals and reagents were of the highest purity available. PAH standards for gas chromatography (GC-FID) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Table 1
Description of the soil treatment strategies performed.

Code	Treatment	Description
1S	MEG + soil + fungal bioaugmentation	5 g of soil[a] in 20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% (*v*/*v*).
2S	MEG + autoclaved soil + fungal bioaugmentation	5 g of autoclaved soil[b] in 20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% (*v*/*v*).
3S	MEG + soil	5 g of soil[a] in 20 mL of malt extract glucose medium.
4S	MEG + autoclaved soil	5 g of autoclaved soil[b] in 20 mL of malt extract glucose medium.
5S	MEG + fungal bioaugmentation	20 mL of malt extract glucose medium with a seven-day pre-grown mycelium of *T. versicolor*, inoculated at 5% (*v*/*v*).
6S	BMTM + soil	5 g of soil[a] in 20 mL of mineral medium BMTM

[a] Soil was previously ground in order to avoid very large particles that could damage the mycelium.
[b] Soil was previously ground and then autoclaved three times at 121 °C for 21 min on consecutive days in an attempt to eliminate the growth of sporulated microorganisms.

2.2. Soil

A composite sample of an aged creosote-contaminated soil (20 kg) that had previously undergone bioremediation by biostimulation in a pilot-scale biopile, as described elsewhere (Realp et al., 2008), was obtained, and sieved (<6 mm) and stored at 4 °C until use.

2.3. Fungal inoculum for bioaugmentation

T. versicolor (ATCC#42530) was utilized as the exogenous fungal inoculum for bioaugmentation purposes. The fungus was maintained on 2% malt agar slants at 25 °C until use. Subcultures were routinely made, as described elsewhere (Borràs et al., 2010). A mycelial suspension of *T. versicolor* was obtained by inoculation of four 1 cm diameter plugs, from the fungus growing zone on 2% malt agar, in 150 mL of 2% (w/v) malt extract medium in a 500 mL Erlenmeyer flask. This was incubated at 25 °C with a constant horizontal rotary agitation (135 rpm, $r = 25$ mm). After 4–5 days, a dense mycelial mass was formed that was separated from the culture medium, resuspended in an equal volume of a sterile saline solution (0.8% (w/v) NaCl) and then disrupted with an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany). The resulting mycelial suspension was stored at 4 °C until use.

2.4. PAH-degrading capability of T. versicolor

The PAH-degrading capability of *T. versicolor* was assessed in liquid malt extract glucose medium (MEG) in 250 mL cotton-stoppered Erlenmeyer flasks, shielded from light, for 10 days at 25 °C and 200 rpm in an orbital shaker. Each flask contained 50 mL of MEG medium (Novotny et al., 2000) and the mycelia suspension was utilized to inoculate the experiments (5% v/v). A mixture of six PAHs (phenanthrene, anthracene, benzo(a)anthracene, chrysene, benzo(k)fluoranthene and benzo(a)pyrene) was utilized for the degradation experiments in the MEG medium at a

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