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

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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 depeletion 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.

Keywords: Polycyclic aromatic hydrocarbon (PAH); creosote; *Trametes versicolor;* 16SrRNA; ITS; mycoremediation

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 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 analyze 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 ; Lladó 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).

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 4ºC until use.

2.3. Fungal inoculum for bioaugmentation

Trametes 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 25ºC 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 final concentration of 50 mg · L^{-1}. The erlenmeyer flasks were spiked by adding 200 µL of stock solution of a mixture of PAHs dissolved in dichloromethane which was airdried for 24 h at room temperature in a laminar flow cabinet sheltered from light. After solvent evaporation, the MEG medium was poured into the flasks. After one day, the mycelia suspension was added to the MEG medium. Triplicate flasks were sampled at the beginning of the experiment and after 10 days of incubation, to quantify PAH degradation. The residual PAHs from cultures and controls were extracted with dichloromethane (5x10 ml) from triplicate flasks at the beginning of the experiment and after 10 days of incubation, to quantify PAH degradation. Before extraction, ortho-terphenyl was added to each flask as a surrogate internal standard. The extracts were dried over Na_2SO_4 and concentrated in a rotary evaporator and subsequently analyzed by means of gas chromatography with a flame ionization detector (GC-FID). An abiotic control inoculated with 5% (v/v) of autoclaved mycelia suspension was performed to adjust for abiotic loses of PAHs after the incubation period.

2.5. *Soil slurry experiments*

In the present study, six sets of slurry experiments, designated 1S to 6S, were performed (Table 1). Each treatment was carried out in triplicate flasks for chemical and microbial analysis. Cotton-stoppered flasks (100 mL) were pre-incubated without agitation for 7 days and then covered with sterile aluminium foil and protected from light, and incubated under horizontal agitation at 25ºC for 30 days at 50 rpm, in order to minimize myceliar rupture due to friction with soil particles. Each slurry contained 5 g of aged

creosote-polluted soil resuspended in 20 mL of MEG medium or BMTM, depending on the treatment (Hareland et al., 1975). Controls with sterilized soil were also performed in triplicate flasks and were incubated in the same experimental conditions.

The fungal and bacterial microbial communities were studied by means of ergosterol quantification, a culture-dependent technique such as determining the most probable number (MPN), and culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE) and quantitative real time polymerase chain reaction (qPCR). As glucose is necessary for the survival of *T. versicolor*, a mineral medium (BMTM) was used as a control to analyze the effect of glucose on the soil communities and PAH degradation.

Each treatment was performed on three replicates from three different flasks. The abiotic controls with sterilized soil were incubated in the same conditions. In those treatments where fungal bioaugmentation by *T. versicolor* was assayed, the fungus was inoculated with mycelia suspension (5% v/v). All the analyses were carried out after the pre-incubation step; at 0, 15 and 30 days of incubation.

2.5.1. PAH extraction and quantification

The soil samples from the slurry flasks were filtered (Whatman nº3) and air-dried for 16 hours at room temperature before being extracted overnight (O/N) by Soxhlet extraction with 200 mL of acetone:dichloromethane (1:1) mixture. Ortho-terphenyl was added to each sample as a surrogate internal standard before each chemical extraction. Previously, the discarded liquid medium was also extracted, to confirm that it did not contain detectable concentrations of PAHs (limit of detection: 0.1 mg PAH $\cdot$ L^{-1} slurry). The total organic extracts (TOE) obtained were dried over Na_2SO_4 and concentrated in a rotary evaporator to dryness. To obtain the total

petroleum hydrocarbon (TPH) fraction, the TOE were resuspended in dichloromethane and cleaned up by column chromatography using the EPA 3611 method (US Environmental Protection Agency). The PAH concentrations were analyzed by gas chromatography with flame-ionization detection (GC-FID) using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) fitted with a DB-5 (30 m x 25 mm i.d. x 0.25 μm film) capillary column (J&W Scientific Products Gmbh, Köln, Germany). The column temperature was held at 50ºC for 1 min, then increased to 320ºC at 7ºC · min^{-1} and held there for 10 min. The concentrations of the most abundant PAHs in the contaminated soil at the beginning and end of the slurry set-up are shown in Table 2, together with the initial and final concentrations from the previous dynamic biopile.

2.5.2. Ergosterol extraction and quantification

Fungal growth was estimated by the ergosterol method. The soil slurries were first homogenized with an Ultra-Turrax homogenizer before being extracted with n-hexane/10% KOH in methanol (1:4) according to Davis & Lamar, 1992. 7-dehydrocolesterol dissolved in dichloromethane was added to each sample as a surrogate internal standard. No ergosterol extraction was performed on the autoclaved soil control (treatment 4S). Furthermore, due to experimental problems, no ergosterol extraction was carried out on treatment 6S.

Finally, the samples were analyzed in an Agilent Technologies 6890N gas chromatograph coupled to an Agilent Technologies 5975 inert mass spectrometer (Palo Alto, CA, United States). The GC chromatographs were equipped with DB5 capillary columns (30 m x 25 mm i.d. x 0.25 μm film). The column temperature was held at 50ºC for 1 min and then increased to 320ºC at 7ºC · min^{-1}. This final temperature was then held for 10 min. Injector and detector temperatures were set to 290ºC and 320ºC

230 respectively. The samples were injected in splitless mode using helium as the carrier
231 gas, at a flow rate of 1.1 mL · min^{-1}.

232

233 ***2.5.3. Heterotrophic and hydrocarbon-degrading microbial population quantification***

234 Soil bacterial counts were performed using a miniaturized MPN method in 96-
235 well microtiter plates, with eight replicate wells per dilution (Wrenn & Venosa, 1996).
236 Total heterotrophs were counted in tryptone soy broth (TSB) and aromatic hydrocarbon-
237 degraders were counted in BMTM (Abalos et al., 2004) containing a mixture of
238 phenanthrene (0.5 g · L^{-1}), fluorene, anthracene, and dibenzothiophene (each at a final
239 concentration of 0.05 g · L^{-1}). Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate
240 was added as a fungicide at 20 mg · kg^{-1} to avoid fungal growth in the MPN plates.

241

242 ***2.5.4. DGGE molecular profiling***

243 Samples for DNA extraction were collected both from the homogenized slurry
244 cultures, and the highest positive PAH-degrading dilutions from the microtiter plates
245 (MPN). These were then placed in sterile Eppendorf tubes stored at –20ºC until
246 analysis. Thus, a sample of 2 mL of each slurry culture was centrifuged at 14,000 x *g*
247 for 10 minutes and the pellets were extracted following a bead beating protocol, using
248 the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, CA, USA),
249 according to the manufacturer's instructions. To obtain DNA from the PAH-degrading
250 populations from the microtiter plates, a composite sample of 1.6 mL containing 200 µL
251 of each replicate (n=8) belonging to the last dilution with eight positives was
252 centrifuged and treated as described above.

253 Two primer sets were used to selectively amplify bacterial and fungal rRNA gene
254 fragments. Universal eubacterial forward F341GC and reverse R907 primers were used to

amplify the hypervariable V3–V5 region of the 16SrRNA gene, as previously reported (Yu & Morrison, 2004). The fungal first internal transcriber spacer (ITS1) from the ribosomal RNA was amplified with the primer pair ITS5 and ITS2 (White et al., 1990). The forward primer ITS5 and F341 contained the GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3'). All the PCR reactions were performed on a Mastercycler personal thermocycler (Eppendorff, Hamburg, Germany). 25 mL of PCR mixture contained 1.25 U Takara ExTaq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 12.5 mM dNTPs, 0.25 µM of each primer and 100 ng of DNA.

The PCR amplicons obtained were loaded into two 8% (w/v) polyacrylamide gels with a chemical denaturing gradient ranging from 40% to 60% (100% denaturant contained 7 M urea and 40% formamide (w/v)) and electrophoretically resolved in a DGGE-4001 (CBS Scientific Company, Del Mar, CA, USA). Electrophoresis was carried out at 60ºC and at 100 V for 16 h in a 1×TAE buffer solution (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). The DGGE gels were stained for 45 min in 1×TAE buffer solution containing SybrGold™ (Molecular Probes, Inc., Eugene, OR, USA) and then scanned under blue light by means of a blue converter plate (UV Products Ltd, Cambridge, UK). Images of DGGE gels were digitalized and DGGE bands were processed using the Gene Tools software v. 4.0 (SynGene Synoptics, Cambridge, UK) and manually corrected. Predominant DGGE bands were excised with a sterile filter tip, poured into 50 µL molecular grade water and stored at 4ºC overnight. A 1:50 dilution of the supernatants was subsequently amplified by PCR as described above and sequenced using R907 and ITS2 primers, for eubacterial and fungal sequences, respectively. The sequencing was performed using the ABI Prism Big Dye Terminator Cycle-Sequencing Reaction Kit v. 3.1 and an ABI 3700 DNA sequencer

(both Perkin–Elmer Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. Sequences were edited using the BioEdit software package v. 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA) and inspected for the presence of ambiguous base assignments and subjected to the Chimera check with Bellerophon version 3 (Huber et al., 2004). The sequences were then aligned with the NCBI genomic database using the BLAST search alignment tool.

The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers JQ079783-JQ079808 and JQ079809-JQ079815, for the 16SrRNA gene and ITS sequences, respectively.

2.5.5. Quantitative PCR assay

Gene copy numbers of eubacterial 16SrRNA and fungal ITS1 rRNA fragments were quantified using quantitative real-time PCR (qPCR). Each sample was analyzed in triplicate by means of three independent DNA extracts. The analysis was carried out using Brilliant II SYBRGreen® qPCR Master Mix (Stratagene, La Jolla, CA, USA) in qPCR equipment MX3000-P (Stratagene) operating under the following protocol: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 50ºC and 55ºC (for the *16SrRNA* gene and *rRNA* ITS1 region, respectively), extension at 72°C for 45 s and fluorescence measurement at 80°C. The specificity of the PCR amplification was determined by observations of melting curves and gel electrophoresis profiles. Melting curve analysis was performed to detect the presence of primer dimers after the final extension step, by increasing the temperature from 55ºC to 95°C at a heating rate of 0.05°C per cycle. Each reaction was performed in a 25 µL volume containing 2 µL of DNA template (approx 100 ng of DNA), 200 nM of each primer, 12.5 µL of the ready reaction mix and 30 nM of ROX reference dye. The

specific primer pairs used for the eubacterial and fungal populations were 519F–907R and ITS5–ITS2 respectively (all primers were purified by means of HPLC). The standard curves were produced with the following reference genes: 16SrRNA gene from *Desulfovibrio vulgaris* subsp. vulgaris ATCC 29579, inserted in a TOPO TA vector (Invitrogen, Merelbeke, Belgium); and an ITS1 gene fragment obtained from a single DGGE band (GenBank accession number JN982550) cloned onto the pGEM plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI, USA). All reference genes were quantified by Quant-iT™ PicoGreen® dsDNA Reagent using MX3000P (Stratagene) as a detector system. Ten-fold serial dilutions of known copy numbers of the plasmid DNA in the range from 10^1 to 10^8 copies were subjected to a qPCR assay in duplicate to generate both standard curves. The qPCR amplification efficiencies were greater than 96%; the Pearson correlation coefficients (R) of the standard curves were between 0.997 and 0.994; and the slopes were between -3.353 and -3.416 for *16SrRNA* and *ITS rRNA* genes, respectively. All the results were processed by means of MxPro™ QPCR Software (Agilent Technologies).

2.5.6. Statistical analysis

Multiple pairwise comparisons were performed using the Tukey test with a significance level of 0.05. Eubacterial DGGE data were also subjected to principal component analysis (PCA). For both ANOVA and PCA analysis, the Statgraphics Plus package (version 5.1; Statistical Graphics Corp., Manguistics Inc., Rockville, MD, United States) was used. Prior to ANOVA and PCA analyses, the normality of the data was assessed through the Shapiro-Wilk normality test, using the SigmaPlot package (version 11; Systat Software, San José, CA, United States).

3. Results and discussion

3.1. Assessment of PAH degradation capacity of T. versicolor

T. versicolor degraded all the 3–5-ring PAHs assayed (Fig. 1) with degradation percentages that varied from 50% for benzo(a)pyrene to 95% for phenanthrene, while growing in liquid MEG medium for 10 days. These results confirm the capacity of this white-rot fungus to degrade PAHs in liquid media, as reported previously. For example, Vyas et al. (1994) described an anthracene degradation of 60% at 21 days of treatment. The high rates of degradation of 5-ring PAHs in just 10 days of incubation are worthy of note.

3.2. PAH removal from soil slurry

Table 2 reports the final HMW-PAH concentrations in all the biostimulation and bioaugmentation treatments, compared to the initial soil slurry. Considerable differences were observed between the final PAHs content in the soil biopile and in the initial soil slurries. This fact could be explained due to the long period of air-dried soil storage at 4ºC (6 months). In addition, as benzo(*a*)pyrene concentration was maintained both in the endpoint of soil biopile and in the initial slurries, this would also confirm that no additional PAH loses have been carried out during the chemical extraction procedure.

The highest 4-ring PAH degradation was achieved by the native microbiota while growing in carbon limiting conditions (6S). However, depletion of the 5-ring PAHs was only enhanced by *T. versicolor* bioaugmentation when no native microbiota was present in the soil (2S)..

After 30 days of treatment, the autochthonous microbial populations degraded between 51% and 54% of the fluoranthene and pyrene, and between 26% and 28% of

355 the benzo(*a*)anthrance and chrysene, only when incubation was performed in BMTM
356 (6S), as shown in Table 2. However, there was no degradation of the 5-ring PAHs
357 within 30 days.

358 It should be noted that the carbon-rich MEG medium resulted in lower
359 degradation of the 4-ring PAHs by the autochthonous microbial population (3S
360 treatment, Table 1) than was achieved solely with the mineral medium (6S treatment).
361 This phenomenon was also reported in a previous study focused on bioremediation of a
362 mineral oil-contaminated soil (Sabaté et al., 2004), and might be the result of utilization
363 of glucose and other carbon sources such as malt extract, as a preferred source of carbon
364 and energy by autochthonous degrading populations. That would cause depletion of the
365 degradation kinetics in the short-term, throughout the biostimulation experiments. The
366 lower 4-ring PAH concentration decline carried out by the autochthonous microbiota
367 with MEG media (3S) was not significantly enhanced by *T. versicolor* when inoculated
368 onto the non-autoclaved slurry (1S), even after a seven-day pre-incubation. This pre-
369 incubation period was performed in order to obtain higher allochthonous biomass and
370 to reduce the glucose concentration of the medium before addition of the soil based on
371 the results described by Rodriguez-Rodriguez et al., 2010.

372 In contrast, when autoclaved soil was utilized (2S), fungal bioaugmentation
373 significantly promoted fluoranthene and pyrene removal(29% and 31%, respectively).
374 Furthermore, the depletion observed in the 5-ring PAHs was significantly greater than
375 in the other treatments ($P < 0.05$), but with lower depletion of total PAH (19%) in
376 comparison to biostimulated (6S) native microbiota (31% of PAH depeletion) (Table 2).
377 The observed improvement of PAH depletion when autoclaved soil-slurry is further
378 inoculated with *T. versicolor* could be explained, either by the lack of native microbiota
379 competition, or due to the biostimulation processes associated with the release of

available sources of carbon or enzymatic cofactors such as manganese (Berns et al., 2008; Wolf and Skipper, 1994). Mechanisms such as resource competition for some growing factors or enzymatic cofactors among microbial populations cannot be ruled out as an explanation of the lack of 5-ring PAH degradation when *T. versicolor* is concomitant with active native microbiota in the polluted soil. It should also be taken into account that the autoclaving process (treatment 2S) could have led to physicochemical changes in the polluted soil that promote 5-ring PAH accessibility by *T. versicolor*.

Degradation of benzo*(a)*anthracene and chrysene (4-ring PAHs) was not enhanced by *T. versicolor* when no active native microbiota was present (condition 2S) in comparison to treatments 1S and 3S where active autochthonous microbial populations were present. Other studies have reported low percentages of degradation of HMW-PAHs in soil slurry systems where a fungal inoculation was performed (Zappi et al., 1996), and a *T. versicolor* preference has even been reported for degradation of certain HMW-PAHs such as benzo*(a)*pyrene, to the detriment of others such as chrysene during soil bioremediation (Borràs et al., 2010).

3.3. Microbial community characterization during soil slurry experiments

3.3.1. Mycelial growth in biostimulation and bioaugmentation treatments

To evaluate the viability of *T. versicolor* in the slurry system, it is necessary to confirm the growth and biological activity of the fungus. The ergosterol concentration was monitored as a biomass indicator due to its importance in the membrane of the fungal cells (Fig. 2A). The initial ergosterol values of those treatments where *T. versicolor* was inoculated (1S, 2S and 5S) were higher than those where no

bioaugmentation was performed (3S), due to the fungal seven-day pre-incubation step. During the 30 days of incubation, a slight decrease in the *T. versicolor* ergosterol concentration was observed while growing without the polluted soil (5S). Autochthonous fungal soil microbiota (3S) experienced only a small increase during the first 15 days and still remained at low concentrations (6.4 ± 2.2 mg $\cdot$ kg^{-1}) in comparison to the other bioaugmented treatments where concentrations were one order of magnitude higher.

Moreover, in the bioaugmentation treatments (1S and 2S) there was an increase in ergosterol after 15 days of incubation that was independent of the autochthonous microbial population activity. Furthermore, the capacity to generate new *T. versicolor* biomass in the autoclaved soil (treatment 2S) indicates that soil components (without alive native microbiota) did not hamper *T. versicolor* colonization in the short-term, and that soil slurry agitation was not a drawback for fungal growth. In contrast, the ergosterol concentration dropped in both bioaugmentation treatments (1S and 2S) after 30 days of incubation, reaching similar ergosterol concentrations to those described for *T. versicolor* without soil amendment (5S), but higher than those obtained without allochthonous fungal inoculation (3S).

3.3.2. Effect of augmentation and carbon availability on cultivable bacteria

The evolution of heterotrophic microbial populations and PAH degraders during the 30 days of incubation is illustrated in Figure 2B. The heterotrophic population from the polluted soil increased by almost 2.5 orders of magnitude during the first 15 days in those treatments where MEG medium was used (1S and 3S). In contrast, in BMTM, only a single order of magnitude increase was observed. Nevertheless, in biostimulated

soil with the mineral medium (treatment 6S), the hydrocarbon-degrading microbial populations showed higher levels, reaching 10^7 MPN · g^{-1}, as well as higher ratios of PAH degraders (65%-81%) with respect to the total heterotrophic population. This is in agreement with the fact that the autochthonous microbial soil population growing in the mineral medium degraded more 4-ring PAHs in all the treatments. This is an important point to be highlighted, and is concomitant with our previous results (Viñas et al., 2005). The presence in the soil of easily assimilable substrates, such as those present in the MEG medium, might favour those fast-growing microorganisms to the detriment of the hydrocarbon-degrading microbial population, which encompasses organisms with slower growth rates. Therefore, when a mineral medium such as BMTM is utilized, the level of heterotrophic microbial populations is lower, but it is enriched with the specialized PAH-degrading microorganisms, which are able to achieve the higher values and ratios. This is an interesting point to be taken into account for further field-scale soil bioremediation when implemented at later stages that are enriched with HMW-PAHs.

3.3.3. Fungal/bacterial ratio quantified by qPCR

Most studies use this methodology to quantify specific bacterial degradative genes; but qPCR can also be used to quantify the whole microbial population in soils of interest using 16SrRNA and ITS primers, for bacterial and fungal communities respectively.

Our results show that, in those treatments where *T. versicolor* was previously inoculated (1S, 2S and 5S), approximately 10^9 ITS gene copies were quantified by qPCR (Fig. 3A), whereas approximately 10^6 gene copies were found in treatments where only the native microbial community was present (3S and 6S), according to

455 ergosterol quantification. In contrast, the initial amount of the 16SrRNA gene in the soil
456 was between 10^7 and 10^8 gene copies, depending on the treatment (Fig. 3B). As a
457 consequence, the fungal/bacterial ratio (Fig. 3C) was three-fold higher in those flasks
458 where *T. versicolor* was inoculated (1S), compared to those treatments where only the
459 autochthonous population was present (3S and 6S).

460 Moreover, where native populations grew with an easily assimilable carbon
461 source but without *T. versicolor* bioaugmentation (3S), the number of ITS copies
462 experienced an increase of three orders of magnitude during the first 15 days of
463 incubation. This was probably due to the large amounts of glucose present in the
464 medium producing an increase in two orders of magnitude in the fungal/bacterial ratio;
465 in the presence of the white-rot fungus (1S), however, the growth of heterotrophic
466 bacteria, combined with a slightly but statistically significant loss of ITS gene copies,
467 produced a reduction of two orders of magnitude in the ratio. This process was
468 accentuated after 30 days of incubation (Fig. 3B). However, during the last 15 days, the
469 number of ITS gene copies remained stable where *T. versicolor* was not present (3S),
470 but a one-fold increase was observed in the fungal/bacterial ratio due to the significant
471 reduction in the 16SrRNA gene copies, which reverted to its initial levels. The
472 maintenance of fungal populations in long-term incubation could be explained on the
473 basis of fungal populations surviving by using the reserves of glycogen produced (Rúa
474 et al., 1993).

475 It is highly remarkable that such a considerable ITS increment in the
476 autochthonous fungal soil population growing in rich carbon conditions (3S), observed
477 during the first 15 days of incubation, was not determined when membrane ergosterol
478 was quantified. This would appear to be contrary to the large mycelial growth visually
479 observed in the flasks. The results show that it is difficult to correlate ITS-based qPCR

fungal biomass and ergosterol content in complex matrices such as soils. Our results are coincident with the controversial results reported by Lang-Yona et al. (2012). There, the correlation between the two analytical methods was low and difficult to follow on the basis of the heterogeneity of ergosterol spore content and stability, as well as the variable genomic number of copies encompassed within the spores and mycelia. Therefore, further research is needed in order to gain an understanding of which methods, or combination thereof, can more faithfully reveal the evolution of fungal biomass in real systems. In this sense, qPCR seems to be a more sensitive technique that should be studied further. However, although qPCR averts the bias inherent in culture-based methods, remaining uncertainties, such as the effects of amplification of DNA from dead cells, might mask the real dynamics and need to be taken into account during data analysis (Tay et al., 2001). Also, significant differences between *T. versicolor* when growing with and without the autochthonous bacterial population (1S and 2S) were revealed by the qPCR results, while the ergosterol concentration showed very similar evolution in both treatments during the first 15 days of incubation, with a significant increase in the fungal biomass. Interestingly, higher levels of fungal biomass, as detected by both techniques at the end of incubation in treatment 2S, may be related to the slightly higher PAH degradation observed when the soil had previously been sterilized.

Finally, it is important to highlight that the qPCR results for treatment 6S indicate the existence of an active native fungal population. This community, dominated by the Ascomycota phylum, could play a role in the high 4-ring PAH degradation that was detected.

3.3.4. Effect of bioaugmentation and carbon availability on autochthonous microbial community structure

3.3.4.1. Eubacterial community analysis

To analyze the eubacterial population initially present in the soil and its response to the different treatments, DGGE analysis with DNA from two different origins was performed. The total DNA present in the slurries was compared to the DNA obtained from the more diluted wells used to enumerate the PAH degraders in non-sterile treatments, in order to better understand the metabolic functions of the microbial community. A total of twenty-seven dominant bands were excised (Figure S1) and sequenced (Table 4).

The DGGE band B1, corresponding to the genus *Bacillus*, was highly prevalent in the initial soil. However, after the addition of soil to the liquid mediums, the profile diversity shifted dramatically, increasing in both MEG and BMTM, but differently according to the carbon content. The shift produced by the presence of either glucose or the white-rot fungus was different when compared to the shift produced by only activating the soil with water and mineral nutrients (6S).

Moreover, *T. versicolor* inoculation and growth in the MEG medium did not noticeably change the detectable biodiversity for treatment 1S compared to treatment 3S (Figure S2). The same behaviour was also observed in DGGE profiles of PAH-degrading populations, where almost all the sequenced bands corresponding to the treatment 1S were shared by the DGGE profiles corresponding to treatment 3S (Figure S1).

Among all the genotypes (Table 4), band B5 (identical on the basis of DNA sequencing to DGGE bands B7; B12; B13; B17; B18; B19; and B22), which is very similar to the *Chryseobacterium* genus, seems to be playing an important role, since it

was the only band that was also found in the slurry profiles after 15 and 30 days of incubation. Chryseobacteria, belonging to the Bacteroidetes phylum, are ubiquitous bacteria that have previously been related to hydrocarbon-polluted sites (Martinez-Pascual et al., 2010). The presence of *Chryseobacterium sp.* in the PAH-degraders MPN plates in treatment 6S (6S 15d MPN), on the fifteenth day, coinciding with the higher degradation rates of 4-ring PAHs suggests that this genus could be related to the degradation of the more bioavailable fraction of the HMW-PAHs present in the soil. Furthermore, the high intensity of the bands corresponding to *Pusillimonas* sp. (DGGE band B10) and *Sphingobium* sp. (B11) in the same DGGE lane (6S 15d MPN), also suggests that both genera could also be playing an important role in the degradation of 4-ring PAHs..

It is worth noting that the slurry set-up conditions produced a huge increment (Fig. 2) and shift (Figure S1) in PAH-degrading microbial populations when BMTM was used, which indicates the importance of the availability of N and P sources, macro and micronutrients and neutral pH (Hareland et al., 1975), to PAH degraders. However, independently of the treatment carried out, the *Proteobacteria* phyla constituted practically all the microbiota detected, with α and β families the most represented in the DGGE profiles.

3.3.4.2. Fungal community analysis

With the aim of studying the autochthonous fungal population of the creosote-polluted soil and the effect of bioaugmentation of the allochthonous fungus *T. versicolor* on it, as well as the use of different culture media during a slurry bioremediation process, a DGGE analysis of the ITS region was carried out (Fig. 6). To the best of our knowledge, no similar studies have been reported which explore

554 autochthonous fungal diversity by means of a DNA-based approach in historically
555 polluted sites during a bioremediation process.
556 With respect to the effect of the medium (MEG or BMTM) on the indigenous
557 fungal soil populations, it can be observed in the DGGE profiles that *Fusarium solani*,
558 which was not one of the most important fungi in the initial soil, (Table 3) was the only
559 fungus consistently present in those treatments where MEG or BMTM medium was
560 used, in spite of the presence of *T. versicolor*. In contrast, other sequences closely
561 related to fungal genera such as *Peziza* sp. and *Chromelosporium* sp. harboured in
562 creosote-polluted soils were not detected from the DGGE profiles during the slurry
563 incubation. This would suggest that the MEG medium probably promotes the growth of
564 *F. solani* compared to other microorganisms that are more acclimated to the initial
565 polluted soil conditions, probably because of faster growth kinetics (Wu et al., 2010).
566 Moreover, *Fusarium* spp. were also detected after 30 days of biostimulation with
567 BMTM as the mineral media, which could indicate that this genus is playing a role
568 throughout the degradation process in the polluted soil. In a previous study (Viñas et al.,
569 2005b) we isolated a PAH-degrading *Fusarium* strain concomitant with *Sphingomonas*
570 *sp* as the main 3- and 4-ring PAH-degrading strains in a stable non-defined microbial
571 consortium with the ability to grow in a mineral medium with a mixture of five 3- and
572 4-ring PAHs as the sole carbon and energy source. It is noteworthy that our results
573 reveal the ability of *Fusarium* to maintain stable interactions with other PAH-degrading
574 eubacterial communities encompassing the Proteobacteria (alpha, beta and gamma) and
575 Bacteroidetes groups. The capacity of *Fusarium* spp. to degrade HMW-PAHs has been
576 demonstrated elsewhere (Chulalaksananukul et al., 2006). Furthermore, Wu et al.
577 (2010) reported how the metabolic pathways of non-white-rot fungi are in part similar
578 to those of white-rot fungi, and also that laccase is involved in the transformation of

PAHs. In the present study, the presence of *F. solani* in the presence of both MEG and mineral medium (treatments 1S, 3S, and 6S) and *F. oxysporium* in the slurry incubated in the mineral medium (6S), (Table 3 and Figure S2) could be related with 4-ring PAH degradation processes. In fact, it is important to highlight the findings in Furuno et al. (2009) that report that fungal mycelia are able to promote both PAH and microbial translocation in water-unsaturated environments, thereby improving the accessibility and bioavailability of contaminants in polluted soils. Therefore, strategies that promote the growth of fungal biomass in the presence of native PAH-degrading microbial communities should be studied further, to better understand interactions of fungi such as *Fusarium*, with other PAH-degrading eubacterial populations in polluted soils.

Again, it is noteworthy that fungal population diversity seemed to be higher in treatment 6S (mineral medium) after 30 days of incubation (Figure S2), probably due to the lack of glucose and *T. versicolor* in the treatment 6S (BMTM medium). Unfortunately, no DNA signal was found in the lane corresponding to the fifteenth day of incubation, where the presence of certain fungal species could be associated with higher degradation rates of fluoranthene and pyrene. However, four DGGE bands were sequenced after 30 days, all belonging to the Ascomycota phylum.

The presence of two Fusarium species previously related to HMW-PAH degradation, *F. solani* and *F. oxysporum* (Silvaa et al., 2009, Viñas et al., 2005b), as well as bacterial community shifts concomitant with the degradation process, indicates a potential synergistic role of fungal biomass for 4-ring PAH degradation.

4. Conclusions

Slurry incubation is a feasible strategy for increasing our knowledge of fungal-bacterial interactions in real historically polluted soils.

Although *Trametes versicolor* degraded HMW-PAH under in-vitro conditions, it did not promote HMW-PAH depletion when native soil microbiota was present. In fact, the native microbial populations in the soil were found to be able to degrade 4-ring PAHs at high rates, but only under carbon limiting conditions. The microbial genera *Chryseobacterium*, *Pusillimonas*, *Sphingobium* and *Fusarium* could play an important role in PAH depletion.

Further research is needed to gain insight into potential mechanisms of interaction between the autochthonous fungal and eubacterial communities able to promote degradation of HMW-PAHs in polluted soils. The capacity of the autochthonous fungal community to promote PAH translocation from soil to fungal biomass should be address. Furthermore, the application of new-generation sequencing tools (NGS) such as pyrosequencing would lead to a better understand of the complex potential interactions between eubacterial and fungal communities in polluted soils.

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766 **Figure Captions**

767 **Fig. 1.** PAH concentrations in synthetic PAH biodegradation experiments with *T.*
768 *versicolor* in MEG medium. 0 days (black); 10 days abiotic control (grey); 10 days
769 (dark grey).Same lowercase letters indicate lack of statistically significant difference
770 within treatments for each PAH compound ($P \geq 0.05$). Values represent the means of
771 three independent experiments and error bars indicate the standard deviations.

772 **Fig. 2. (A)** Ergosterol concentration at 0 days (black); 15 days (grey); 30 days (dark
773 grey). **(B)** Heterotrophic (black) and PAH-degrading population (white pattern) in soil
774 slurry treatments over the course of 30 days of incubation.Same lowercase letters
775 indicate lack of statistically significant difference within the same treatment at different
776 incubation times ($P \geq 0.05$). Same uppercase letters indicate lack of statistically
777 significant difference between treatments at same incubation time ($P \geq 0.05$).The
778 percentage (%) of the heterotrophic population that was represented by the PAH-
779 degrading population is also indicated (B). Values represent the means of three
780 independent experiments and error bars indicate the standard deviations.

781 **Fig. 3.** ITS region gene copies quantified by qPCR **(A);** 16SrRNA gene copies
782 quantified by qPCR **(B);** and fungi/bacteria ratio between ITS and 16SrRNA gene
783 copies **(C)** in soil slurry treatments over the course of 30 days of incubation. 0 days
784 (black); 15 days (grey); 30 days (dark grey). Same lowercase letters indicate lack of
785 statistically significant difference within the same treatment at different incubation
786 times ($P \geq 0.05$). Same uppercase letters indicate lack of statistically significant
787 difference between treatments at same incubation time ($P \geq 0.05$). Values represent the
788 means of three independent replicates per experiment and error bars indicate the
789 standard deviations.

Table 1: Description of the soil treatment strategies performed.

Code	Treatment	Description
1S	MEG + Soil + Fungal bioaugmentation	5g 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	5g 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	5g of soil[a] in 20 mL of malt extract glucose medium.
4S	MEG + Autoclaved Soil	5g 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	5g 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 121ºC for 21min on consecutive days in an attempt to eliminate the growth of sporulated microorganisms.

Table2

Table 2: PAH residual concentrations of treated soil.

Compound[a]	Initial biopile (mg ·kg^{-1} of soil)	Final biopile (mg · kg^{-1} of soil)	Slurry 0 days (mg · kg^{-1} of soil)	MEG/Soil/Fungi (1S 30d) (mg · kg^{-1} of soil)	MEG/est.soil/Fungi (2S 30d) (mg · kg^{-1} of soil)	MEG/Soil (3S 30d) (mg · kg^{-1} of soil)	MEG/est.soil (4S 30d) (mg · kg^{-1} of soil)	BMTM/Soil (6S 30d) (mg · kg^{-1} of soil)
Fluoranthene	562±39	133±12	70.3±1.0A	58.4±2.9B[c]	50.4±3.1C	60.7±3.4B	69.9±1.2A	33.2±0.7D
Pyrene	343±22	134±7	75.7±1.6A	58.2±3.2B	52.3±2.2C	60.0±4.6B	77.2±3.6A	36.7±1.8D
Benzo(*a*)anthracene	106±3	47±3	32.5±0.2A	33.0±3.2A	30.8±2.0A	35.8±1.7A	34.2±0.8A	24.0±2.9B
Chrysene	117±3	61±27	48.8±0.9A	51.6±0.9A	46.3±2.4A	51.2±0.3A	51.6±2.8A	35.3±4.8B
Benzo(*b*+*k*)fluoranthene	105±6	94±13	72.7±2.5A	65.9±2.7B	60.9±0.6C	67.6±0.4B	71.5±1.7A	70.2±1.1A
Benzo(*a*)pyrene	40±1	31±2	30.2±0.2A	29.6±0.2A	25.6±1.2B	29.6±0.1A	29.8±1.1A	29.1±1.2A
Total PAHs[b]	**2270**	**500**	**330**	**296**	**266**	**305**	**334**	**228**

[a]Phenanthrene and anthracene (3-ring PAHs) were not detected after the biopiling process. Before the dynamic biopile, corresponding concentrations were 814±62 and 183±13 mg · kg^{-1} of soil).

[b]Total PAHs determined from the total target PAHs analysed.

[c]Data are the mean ± standard deviation of three independent experiments. Statistical pairwise multiple comparisons of homogenous data was carried out by the Tukey test: column means followed by the same uppercase letters were not significantly different ($P \geq 0.05$).

Table3

Table 3: Properties of ITS DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	1S	3S	5S	6S	Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[a]	Phylogenetic group
ITS B1[b]	x		x		208	*Trametes versicolor* FP1022316sp (JN164984)	100%	*Polyporaceae* (Basidiomycota)
ITS B2		x			201	*Peziza pseudoviolacea* 16504 (JF908564)	97%	*Pezizaceae* (Ascomycota)
ITS B3		x			164	*Chromelosporium* sp. CID601 (EF89890)	96%	*Pezizaceae* (Ascomycota)
ITS B6[c]	x	x		x	171	*Fusarium solani* isolate 177 (JN232143)	100%	*Nectriaceae* (Ascomycota)
ITS B39				x	152	*Scedosporium prolificans* strain 776497 (GU594770)	90%	*Microascaceae* (Ascomycota)
ITS B41				x	161	*Fusarium oxysporum* isolate 1 (JN558555)	93%	*Nectriaceae* (Ascomycota)
ITS B42				x	161	*Cosmopora* sp. strain GJS96186 (JN995635)	100%	*Nectriaceae* (Ascomycota)

[a]Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[b]Band ITS: B1=B4=B9=B16=B17=B18=B19=B20=B26=B37
[c]Band ITS: B5=B6=B7=B8=B10=B11=B12=B13=B14=B15=B21=B22=B23=B24=B25=B27=B28=B29=B30=B31=B32=B33=B34=B35=B36=B40

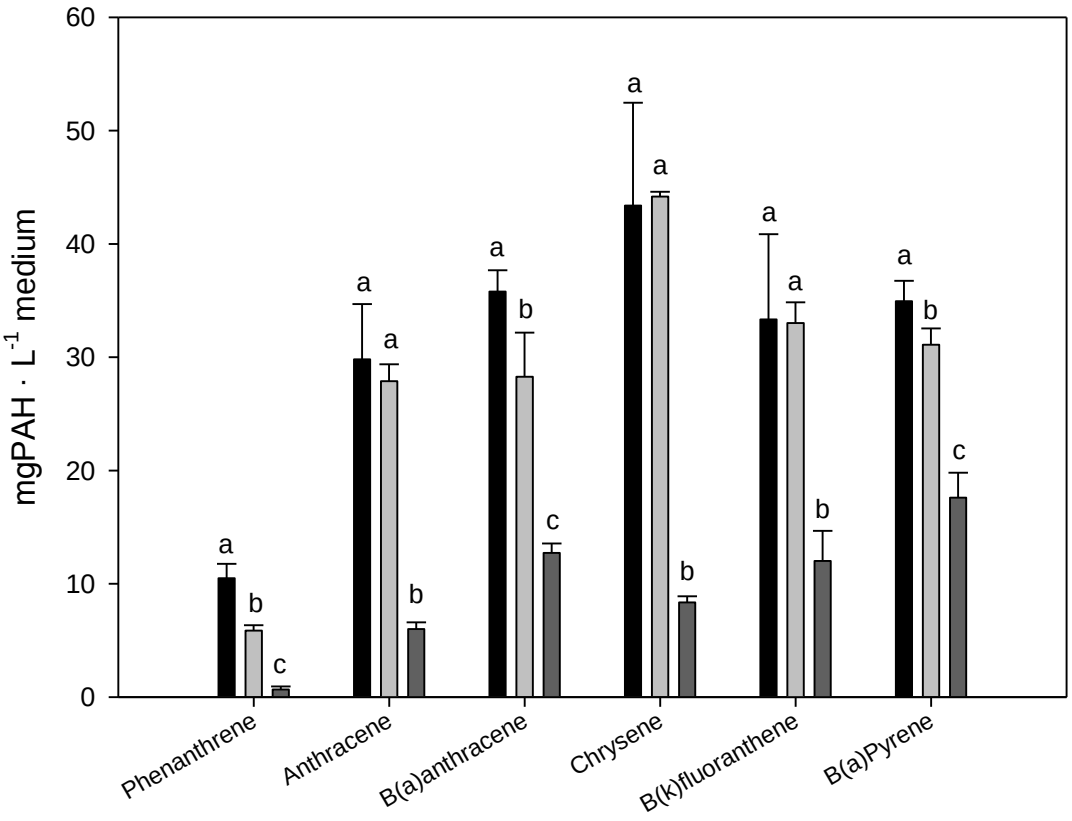
Table4

Table 4: Properties of *16SrRNA* gene DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Band detection[a]								Length (bp)	Closest organism in GenBank database (accession no.)	% similarity[b]	Phylogenetic group[c]
	0d	0dMPN	1S	1SMPN	3S	3SMPN	6S	6SMPN				
16S B1	X	-	-	-	-	-	-	-	505	*Bacillus niacin* strain Y2S4 (EU221374)	99%	*Bacillaceae (*Firmicutes)
16S B2	-	X	-	-	-	-	-	-	400	*Bordetella sp.* (EU082149)	100%	*Burkholderiaceae* (β)
16S B3	-	X	-	-	-	-	-	-	389	*Rhizobium sp.* NJUST18 (JN106368)	99%	*Rhizobiaceae* (α)
16S B4	-	-	-	X	-	-	-	-	419	*Sphingomonas sp.* FI301 (GQ829498)	100%	*Sphingomonadaceae* (α)
16S B5[d]	-	-	x	x	x	x	-	-	439	*Chryseobacterium sp.* LKL10 (HQ331141)	100%	*Flavobacteriaceae* (CFB group)
16S B6	-	-	-	-	x	-	-	-	381	*Enterobacter pulveris*strain E443 (EF614996)	95%	*Enterobacteriaceae* (γ)
16S B8	-	-	-	-	-	X	-	-	445	*Herbaspirillum sp.* Os45 (HQ728575)	93%	*Oxalobacteriaceae* (β)
16S B9	-	-	-	-	-	x	-	-	441	*Sphingomonas sp.* FO416 (GQ849286)	98%	*Sphingomonadaceae* (α)
16S B10	-	-	-	-	-	-	-	X	483	*Pusillimonas sp.* PB3-7B (FJ948170)	100%	*Alcaligenaceae* (β)
16S B11	-	-	-	-	-	-	-	X	422	*Sphingobium sp.* F2 (EF534725)	99%	*Sphingomonadaceae* (α)
16S B14	-	-	-	X	-	-	-	-	477	*Achromobacter sp.* HPABA02 (HQ257212)	98%	*Alcaligenaceae* (β)
16S B15	-	-	-	X	-	-	-	-	450	*Sphingomonas sp.* MPSS (GQ214027)	98%	*Sphingomonadaceae* (α)
16S B16	-	-	-	X	-	x	-	x	449	Uncultured bacterium clone EBL50 (GU591539)	97%	-
16S B20	-	-	-	-	X	-	-	-	250	*Enterobacter sp.* DG10 (JN208201)	94%	*Enterobacteriaceae* (γ)
16S B21	-	-	-	-	X	-	-	-	437	Uncultured bacterium DGGE band B4 (JF729189)	100%	-
16S B23	-	-	-	-	-	X	-	-	400	*Achromobacter xylosoxidans*strain E1 (JN590249)	100%	*Alcaligenaceae* (β)
16S B24	-	-	-	-	-	X	-	-	446	*Novosphingobium sp.* MN38 (JN082747)	99%	*Sphingomonadaceae* (α)
16S B25	-	-	-	-	-	X	-	-	405	*Azospirillum oryzae* strain KNUC9025 (JF505959)	99%	*Rhodospirillaceae* (α)

[a]Band detection (+) above 1% of relative intensity.
[b]Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[c]Phylogenetic groups were defined by using the Ribosomal Data Project (RDP) Naive Bayesian Classifier(Wang et al., 2007). Family is represented.α, β, γ represent α-proteobacteria, β-proteobacteriaand γ-proteobacteria,respectively.
[d]Band16S: B5=B7=B12=B13=B17=B18=B19=B22; B16=B26=B27.

Figure 1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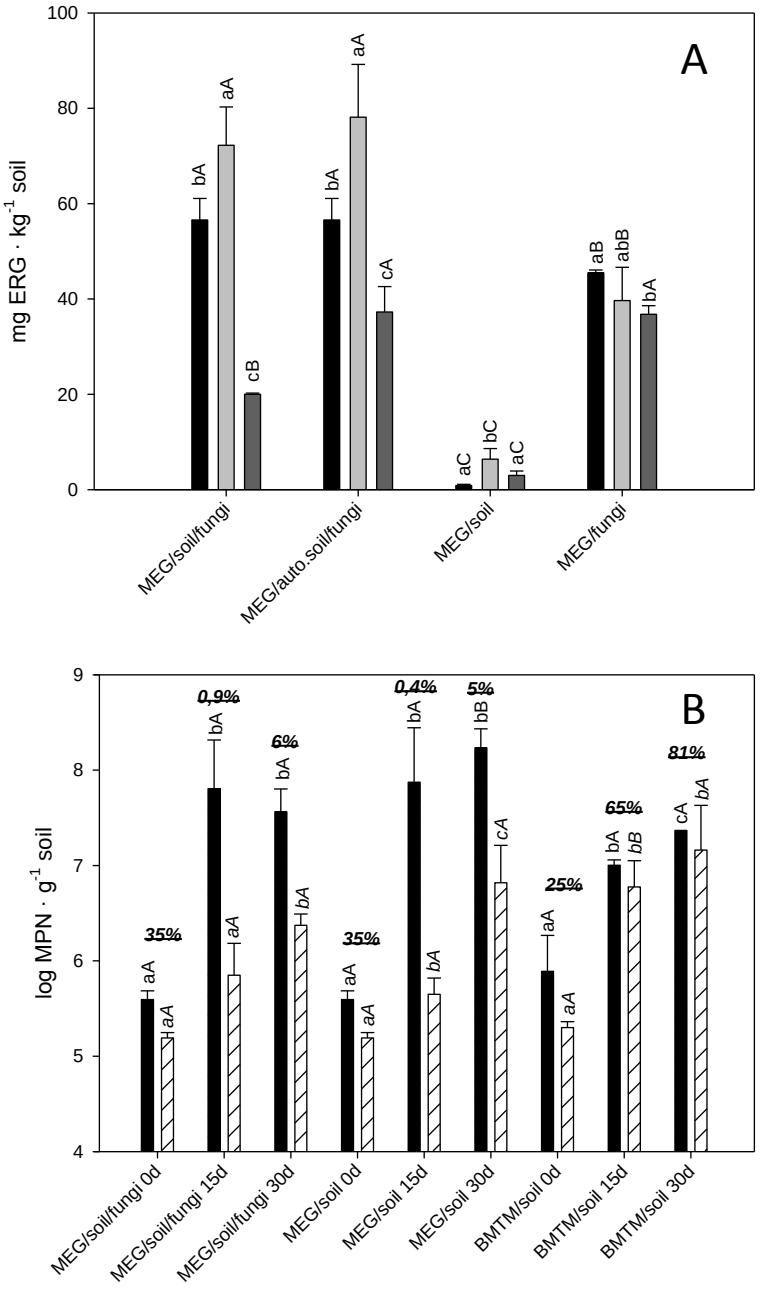
50
40
30
20
10
0
mgPAH · L-1 medium
a
b
c
a
a
b
a
b
c
a
a
b
a
a
b
a
b
c
Phenanthrene
Anthracene
B(a)anthracene
Chrysene
B(k)fluoranthene
B(a)Pyrene

Figure 2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80
60
40
20
0
bA
aA
cB
bA
aA
cA
aC
bC
aC
aB
abB
bA
MEG/soil/fungi
MEG/auto.soil/fungi
MEG/soil
MEG/fungi
B
log MPN · g-1 soil
9
8
7
6
5
4
35%
0,9%
6%
35%
0,4%
5%
25%
65%
81%
aA
aA
bA
aA
bA
aA
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bB
cA
aA
aA
bA
bB
cA
bA
MEG/soil/fungi 0d
MEG/soil/fungi 15d
MEG/soil/fungi 30d
MEG/soil 0d
MEG/soil 15d
MEG/soil 30d
BMTM/soil 0d
BMTM/soil 15d
BMTM/soil 30d

Figure3

Figure 3