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Title: Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil

Article Type: Research Paper

Keywords: mycoaugmentation, Pseudoallescheria sp., oil-contaminated soil, bioavailability, biostimulation, bioremediation, aliphatic hydrocarbons

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Abstract: The present work was aimed at isolating, identifying the main members of the mycobiota of a clay soil historically contaminated by mid- and long-chain aliphatic hydrocarbons (AH) and to subsequently assess their hydrocarbon-degrading ability. All the isolates were Ascomycetes and, among them, most interesting was Pseudoallescheria sp. 18A, which displayed both the ability to use AH as the sole carbon source and to profusely colonize a wheat straw: poplar wood chips (70:30, w/w) lignocellulosic mixture (LM) selected as the amendment for subsequent soil remediation microcosms. After 60 d mycoaugmentation with Pseudoallescheria sp. of the aforementioned soil, mixed with the sterile LM (5:1 mass ratio), a 79.7% AH reduction and a significant detoxification, inferred by a drop in mortality of Folsomia candida from 90 to 24%, were observed. However, similar degradation and detoxification outcomes were found in the non-inoculated incubation control soil that had been amended with the sterile LM. This was due to the biostimulation exerted by the amendment on the resident microbiota, fungi in particular, the activity and density of which were low, instead, in the non-amended incubation control soil.

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species. I therefore recommend a profound rewriting/refocusing of the manuscript, with the eventual inclusion of new data from the microbial (fungal and bacterial) community structure and dynamics.

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Dear Editor,
I am sending you the Ms. titled "Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil" (Ms. n°. STOTEN-D-14-02904) which has been revised according to the Editor's and Referees' remarks/suggestions. Following your indications, responses to Reviewers' suggestions/criticism have been done on a point by point basis. A clear identification of the added modifications can be retrieved from an additional Ms. text file where changes have been tracked (i.e., Covino_text_track_changes.doc).

Kind regards
Alessandro D'Annibale

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Highlights

- ✓ All fungal isolates from an oil-polluted soil were Ascomycetes
- ✓ High clay content and historical contamination made the soil untreatable
- ✓ 79% AH reduction was observed in soil augmented with *Pseudoallescheria sp.*
- ✓ Contaminant bioavailability was estimated by a dynamic method relying on SFE with CO2.
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Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil

Stefano Covino[b], Alessandro D'Annibale[a*], Silvia Rita Stazi[a], Tomas Cajthaml [b,c], Monika Čvančarová[b], Tatiana Stella[b], Maurizio Petruccioli[a]

[a)]Department of Innovation in Agroforestry and Biological Systems (DIBAF) University of Tuscia, Viterbo, Italy)

[b)]Laboratory of Environmental Biotechnology Academy of Sciences of the Czech Republic (Prague, CZ)

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(*) Correspondence should be addressed to:

Alessandro D'Annibale

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ABSTRACT

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1. Introduction

Crude oil extraction and transportation through pipelines on a mining claim zone can cause accidental oil spills and leaks in soil. Environmental hazards arising from this type of contamination might become even more serious in cases of reiterative spills and aging of the pollution (Brassington et al., 2007).

Bioremediation has been suggested to be a valuable soil clean-up option due to its cost-effectiveness and sustainability. However, the outcome of a given bioremediation intervention has been found to largely depend on the nature, source, concentration and bioavailability of hydrocarbon contaminants as well as on soil physico-chemical and microbiological properties (Brassington et al., 2007; Stroud et al., 2007). ~~It is noteworthy that hydrocarbons in soil are mainly~~ Although hydrocarbon degradation in soil is generally ascribed to ~~degraded by~~ bacteria, ~~, and, to a lesser extent, by yeasts and fungi. F~~filamentous fungi ~~, however,~~ exhibit peculiar characteristics which make them suitable candidates for the clean-up of soils historically ~~oil impacted~~ contaminated by crude oil (Chiu et al., 2009) ~~soils~~. With this regard, their apical growth mode enable them to reach inaccessible soil regions and their hyphal network confer them the ability of acting as spreading vectors of pollutant-degrading bacteria (Banitz et al., 2013). These favourable properties are often associated with their ability of growing in environments with low nutrient concentrations, low humidity and acidic pH (Mancera-López et al., 2008). Moreover, rather widespread among fungi is the production of unspecific lignin-modifying enzymes acting in the extracellular environment *via* radical-based reactions and able to reach poorly bioavailable organopollutants; under certain conditions, they are capable of generating hydroxyl radicals thus acting as Fenton's like reagents (Guillén et al., 2000).

At an Italian site, located along the Southern Sicilian shoreline, a widespread crude oil contamination was first detected several decades ago in areas located around the oil extraction wells and along the pipelines and the large majority of mid- and long-chain hydrocarbons tended to

accumulate over time due to the high content of clay in the vadose zone. The predominance of the clay fraction in the contaminated soil precluded the application of *in situ* treatments.

On the basis of the numerous reports claiming the higher hydrocarbon-degrading efficacy of resident fungi than allochthonous ones (April et al., 1998; Garon et al., 2004; Potin et al., 2004), the present work was aimed at isolating, identifying and assessing the hydrocarbon-degrading ability of the main members of the mycobiota of a historically contaminated soil collected from the aforementioned area. To this aim, liquid cultures of identified isolates were conducted by using hydrocarbons extracted from the same soil as the sole carbon source. The best isolates were then tested for their abilities to colonize a lignocellulose mixture, to be used as the amendmentant for remediation purposes, prior to the preparation of mycoaugmentation treatments. This led to the selection of a fungal strain which was used in the augmentation of the soil from which it had been isolated. The present study compares the degradation and detoxification efficiencies of this mycoaugmentation treatment with those observed in either amended or non-amended non-inoculated incubation controls. All these treatments were compared for their abilities to (i) affect densities of heterotrophic and hydrocarbonoclastic bacteria, (ii) enable fungal growth, (iii) modify the community structure of soil, (iv) remove aliphatic hydrocarbons with reference to their bioavailabilities and, finally, (v) detoxify the soil.

2. Materials and Methods

2.1. Materials

Soil samples were collected nearby an oil-refinery site (Gela, Italia), air-dried and sieved (< 2 mm). The soil had real and potential acidity of 7.96 and 7.50 in water and 1 N KCl, respectively. Soil texture was as follows (w/w): sand, 15.7%; silt, 40.2% and clay, 44.1%, thus, according to the USDA textural classification, it was silty clay soil with an estimated bulk density of 1.24 g cm^{-3}. The water-holding capacity (WHC) was 37.2% (w/w). Total organic carbon (TOC) and total

nitrogen (TN) and assimilable phosphorous contents were 1.48, 0.06 and 0.014%, respectively. Total aliphatic hydrocarbons (TAH) content~~, extracted and quantified according to protocols DIN EN 14039 and DIN ISO 16703,~~ was 10200±456 mg kg^{-1} soil. BTEX and total polycyclic aromatic hydrocarbons contents in soil, analyzed by Theolab spa (Turin, Italy), were low and amounted to 324.2 and 4.7 µg kg^{-1}, respectively. TOC, TN and ash contents in wheat straw (53.0, 0.48 and 3.9%, respectively), poplar wood chips (48.5, 0.48 and 2.2%, respectively), and millet seeds (49.8, 1.6 and 2.7%, respectively) were determined as previously described (Sampedro et al., 2009).

2.2. Extraction and analysis of contaminants

Aliphatic hydrocarbons from either pristine soil or non-inoculated and inoculated microcosms were extracted and quantified according to protocols DIN EN 14039 and DIN ISO 16703. In particular, samples (3 g) were suspended in 15 ml of *n*-hexane: acetone mixture (1:2, v/v) and extracted for 30 min in ultrasonic bath. The supernatant after centrifugation (3000 rpm, 10 min) was then extracted three times with Milli-Q water (20 ml) to remove polar compounds. The resulting *n*-hexane extracts were passed through mini-columns containing anhydrous Na_2SO_4 (2 g) and Florisil (2 g)~~, to remove water and aromatic compounds, respectively.~~ Quantitative analysis of this fraction, from here onwards referred to as total aliphatic hydrocarbons (TAH), was carried out using a GC-FID HP 5890 equipped with a DB-5MS column (0.25 mm × 30 m, 0.25 µm film thickness). Injector and flame ionization detector were operated at 300 ºC and helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. An initial isocratic step of 1 min at 50 ºC was followed by a temperature ramp up to 300 ºC (at a rate of 20 ºC min^{-1}), the latter temperature being held for additional 20 min. Mineral Oil Standard Mixture (Fluka Analytical) was used for calibration. Quantification of TAH was achieved by integrating the area of the aliphatic "unresolved complex mixture" (UCM) between the retention times of decane (5.585 min) and tetratriacontane (29.755 min). Percent degradation was calculated by referring residual TAH contents in amended microcosms to those ~~found~~ in coeval non-amended microcosm; in particular, actual TAH concentrations in amended

microcosms were multiplied by a correction factor ~~which took into account both the dilution of soil with the amendant and the ash content after incineration at 600 °C~~ as described by Šašek et al. (2003). Moreover, in order to quantify various sub-fractions of the UCM, the aliphatic TAH "hump" of each chromatogram was split into retention time windows (RTW)~~, each of which encompassing distinct boiling point ranges/number of carbon atoms in the UCM~~ as described in the 8015D method (EPA, 2003). Integration marks for each RTW were established by injecting *n*-alkane standards as markers (C8-C20 and C21-C40, Fluka Analytics).

2.3. Determination of contaminants bioavailability.

The overall bioavailability of either TAHs or their respective fractions pertinent to each RTW were estimated using sequential supercritical fluid extraction (SFE) with CO_2 as reported elsewhere (Covino et al., 2010). The extractions were performed using a PrepMaster extractor (Suprex, Pittsburgh, PA) equipped with VaryFlow restrictor operating at 40 °C, a refrigerated (-20 °C) hydrocarbon trap filled with octadecyl-bonded silica (Merck, Darmstadt, Germany) as the sorbent and a downward stream of CO_2 (5.5 SFE/SFC, Messer Technogas, Prague, Czech Republic). Four soil aliquots (1.0 g each) were extracted at 50 °C, 200 bar at a CO_2 flow rate of 1 mL min^{-1} and the desorbed hydrocarbons were collected after 5, 10, 20, 40, 60, 80, 120, 160 and 200 min. Sequential ~~supercritical fluid extraction~~SFE ~~represents~~ can be fitted by a desorption model presuming ~~generally~~ that the extraction is controlled by the two rate constants differing by orders of magnitude (Williamson et al., 1998). The chemical release data can be modelled by an empirical two-site model, consisting of the two first-order equations (1):

$$S_t = F \cdot S_o e^{-k1t} + (1 - F) \cdot S_o \cdot e^{-k2t} \quad (1)$$

where S_o and S_t are the initial and residual pollutant concentrations in the soil after time t, respectively, F is the fraction of chemical rapidly released; 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 while 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 (Hawthorne et al., 2002; Cajthaml and Šašek, 2005). ~~The Prism software package version 4.0 (GraphPad, La Jolla, CA) was used for calculating the F values.~~

2.4. Isolation of autochthonous fungi

Autochthonous filamentous fungi were isolated using two different approaches: *i)* direct spread plating of soil suspensions and of relative serial dilutions and *ii)* spread plating of soil suspensions after enrichment. As for the former approach, 10 g of soil were added to 90 mL of sterile deionized water and the suspension was magnetically stirred for 30 min prior to preparing dilution series (up to 10^{-5}). Enrichment cultures were prepared by adding 5 g of soil to 250-mL Erlenmeyer flasks containing the MM liquid mineral medium (45 mL) described by Prenafeta- Boldú~~Boldù~~ et al. (2001) and added with chloramphenicol (0.1 g L^{-1}) to prevent bacterial growth. After 7 d incubation on a rotary shaker (180 rpm, 28 °C), serial dilutions were prepared as described above.

Aliquots of the suspensions from both approaches and their respective dilution series were spread onto Petri dishes containing Rose Bengal Chloramphenicol Agar (RBCA; Himedia, Mumbai, India). In the attempt of isolating basidiomycetes, RBCA was supplemented with benomyl (15 mg L^{-1}). ~~All plates were incubated at 28 °C for variable periods ranging from 5 to 15 d. At the end~~~~During the time course of~~Throughout the ~~the RBCA plate~~ incubation in RBCA plates (from 5 to 15 d at 28 °C), pure cultures were isolated by the streak plate method and sub-cultured onto malt extract agar plates (MEA, Oxoid, Basingstoke, UK).

2.5. Inoculum preparation and screening of isolates

For preliminary screening experiments of fungal isolates, the TAH fraction, extracted and purified as above, was dried under vacuum at 40 °C in a R-120 rotary evaporator (Büchi, Switzerland) and, finally, suspended in acetone to a final concentration of 500 mg mL^{-1}.

The hydrocarbon-degrading ability of each pure fungal isolate was inferred by its capacity to grow on the MM medium in the presence of TAH as the sole C source. Inocula were prepared from 7-d-old cultures grown on the MEG medium (Covino et al., 2010) ~~at 28 °C under orbital shaking (150 rpm)~~. At the end of the incubation, cultures were centrifuged (6000 g, 15 min), the pellets were washed with sterile distilled water and centrifuged again as above to avoid the entrainment of residual medium components. Finally, the pellet was suspended ~~again~~ in distilled water to yield an initial biomass concentration of 2.5 g L^{-1}. The screening was carried out in Erlenmeyer flasks (250 mL) containing 40 mL sterile MM ~~which were~~ added with 400 µL TAH suspension to yield an initial hydrocarbon concentration of 200 mg per flask (5.0 g TAH L^{-1}). Each flask was added with 2 mL inoculum and then incubated at 28 °C under orbital shaking (150 rpm) for 25 d. Inoculated cultures grown on MM and added with 400 µL acetone were used as the controls. At the end of incubation, liquid cultures were filtered on pre-weighed Whatman GF/C discs ~~(diameter, 47 mm)~~ and the harvested biomass was washed once with dichloromethane (100 mL) and twice with equal volumes of distilled water. The filter was ~~then~~ dried at 105 °C for 24 h, cooled in a desiccator, and ~~finally~~ weighed. ~~For each of the isolates, t~~The fold increase in growth, due to the utilization of TAH, was calculated from the W_{TAH} /W_{CONT} ratio, where the former and the latter are the fungal biomass produced in the presence and in the absence of TAH, respectively.

Isolates were also compared for their respective abilities to grow under solid-state conditions on the wheat straw: poplar wood chips lignocellulose mixture (LM, 70:30, w/w), intended to be used as the amendant for subsequent bioremediation microcosms. To prepare inoculants, 2 g millet seeds (*Panicum miliaceum* L.) were rinsed with water for 24 h, sterilized twice in autoclave (121 °C, 20 min) and transferred to 16×3.5 cm test-tubes. Then, two 10-d-old colonized MEA agar plugs (1.0 cm Ø) were added and the mixture incubated for 5 d at 28 °C under static conditions. In particular,

the LM (8.0 g) was moistened with water to reach 65% humidity, sterilized in autoclave (121 °C, 30 min) and, after cooling, added over the pre-colonized millet seeds. ~~Solid state c~~Cultures were incubated for 30 d at 28 °C ~~under static conditions~~ and the length of the ascending mycelial front measured. Growth was assessed subjectively on a 0-10 scale ~~, with 0 meaning absence of growth and 10 complete hyphal colonization of the vertical transect of the solid substrate~~as described by ~~(~~Boyle~~,~~ (1998).

2.6. Identification of fungal isolates

Isolates showing remarkable hydrocarbon-utilizing capabilities were identified through a molecular approach associated with colony morphological features. The DNA was extracted from pure fungal cultures *via* the phenol:chloroform protocol and the Internal Transcribed Spacer Region (ITS) of the ITS1-5.8S-ITS2 rRNA ~~18 S rRNA~~ gene was amplified by PCR using the universal fungal forward and reverse ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, respectively. The composition of PCR reaction mixture and amplification conditions were reported elsewhere (D'Annibale et al., 2006). The purified PCR products were used in sequencing reactions with the same set of primers, using a BigDye Terminator cycle sequencing ready reaction kit, version 3.0 (Applied Biosystems, Foster City, CA). Sequencing was performed on an ABI 3730 DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) and compared with those of all known fungal species available from the same database.

2.7. Soil remediation conditions

The 18A isolate, that had been selected through the screening and identified as *Pseudallescheria* ~~boydii~~sp., was maintained on malt extract agar (MEA) plates and periodically sub-cultured on a monthly basis. Its inocula on millet seeds were prepared as described above. Aliquots of contaminated soil (150 g) were moistened with sterile distilled water to reach 50% of the WHC,

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mixed with the LM (30 g) and then with ~~*P. boydii*~~*Pseudoallescheria* sp. pre-grown millet seeds (15 g). The amended incubation control (AIC) was prepared by mixing the contaminated soil with both sterile LM and non-colonized milled seeds at the same ratios. ~~It is noteworthy that b~~Both LM and millet seeds underwent two sterilization cycles (each at 121 °C for 30 min) in order to avoid the risk of adding foreign microorganisms to AIC. As a further control, the pristine soil was incubated as described below after adjusting its moisture content to 50% of its WHC and this treatment was referred to as non-amended incubation control (NAIC). All microcosms were incubated on 1.7-L cylindrical glass jars under non-axenic conditions for 0, 30 and 60 d at 28±2 °C in the dark. Moisture was maintained constant by periodical additions of sterile distilled water ~~with the aid of a nebulizer.~~ For each sampling time, triplicate microcosms were totally sacrificed.

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2.8. Bacterial counts, fungal growth and phospholipid fatty acids (PLFA) analysis

Cultivable heterotrophic and hydrocarbon-degrading bacteria were enumerated in soil according to the most probable number (MPN) counting technique (Wrenn and Venosa, 1996). As for the heterotrophs, soil serial dilutions were incubated in Tryptic Soy Broth (TSB, 30 g L^{-1}), while specialized bacteria were enumerated using *n*-hexadecane as the sole carbon and energy source in a mineral medium (Wrenn and Venosa, 1996). Cycloheximide (500 mg L^{-1}) was added in both culture media in order to repress fungal growth. Ergosterol was used as a specific indicator of fungal growth~~. Extraction and HPLC determination of this fungal sterol were carried out~~ as reported elsewhere (Covino et al., 2010).

The microbial community structure was investigated using phospholipid fatty acid (PLFA) analysis according to Frostegård et al. (1993). Briefly, each microcosm (5 g) was extracted for 2 h with a mixture of chloroform, methanol and citrate buffer pH 4.0 (1:2:0.8, v:v:v). ~~After dividing the extract into two phases by adding chloroform and buffer, t~~The lipid-containing phase was collected, ~~and~~ evaporated under N_2. ~~The lipid material was~~and fractionated by solid phase extraction (SPE) columns (3 ml/500 mg silica Sep-pak VacTM, Waters)~~on columns containing silicic acid~~ into

neutral, glycolipids and ~~polar~~ phospholipids by elution with chloroform (5 ml), acetone (5 ml) and methanol (5 ml), respectively. Each of the lipid fractions was dried under a stream of N_2, ~~re~~suspended in hexane and the purity was checked by GCMS analysis. A derivatization of p~~P~~hospholipids into their corresponding methyl esters~~contained in the last fraction were derivatized into their corresponding methyl esters~~ was performed by mild alkaline methylation before ~~being analyzed~~analysis by GC-MS. Mass spectra were recorded by the use of a QP-5050 (Shimadzu, Japan) spectrometer equipped with an AT 20 capillary column (0.25 mm i.d., 25 m) (Alltech, Deerfield) at 80–280 °C with a splitless injection and an isothermal program at 80 °C for 2 min, then 6 °C•min^{-1} up to 280 °C and finally isothermal at 280 °C for 5 min. Methylated fatty acids were identified according to their mass spectra and using BAME 24 and 37-Component FAME Mix (47080-U 47885-U, respectively, Sigma–Aldrich, Milan, I) as chemical standards. ~~Under the chromatographic conditions, it was not possible to separate *cis-trans* isomers of unsaturated fatty acids.~~ Individual PLFAs were used as signature markers of microorganisms and~~are~~ named ~~in the text according to standard conventions~~ as reported by ~~(~~Tunlid and White (1992). ~~Straight chain saturated fatty acids were used as general markers of bacteria. Terminally branched (a, i) and mid-branched (e.g., 10Me18:0) fatty acids were used as markers for Gram-positive bacteria while the cyclopropyl branched fatty acids (i.e., cy17 and cy19) and their respective monoenoic precursors (i.e., 16:1ω7 and 18:1 ω7) for Gram-negative bacteria. The polyunsaturated fatty acid 18:2ω6,9 was used as signature marker for fungi while the monoenoic fatty acid 18:1ω9 was omitted from calculations since it can be found in both fungi and Gram-positive bacteria (Frostegård et al., 2011).~~

2.9. Enzyme ~~assays~~ and ecotoxicity assays

Extracellular enzymes were extracted from soil microcosms as previously reported (D'Annibale et al., 2006). Laccase and Mn-dependent peroxidase and glycosyl hydrolase (i.e., endo-β-1,4-glucanase, cellobiohydrolase and endo-β-1,4-xylanase) activities were determined as reported elsewhere (D'Annibale et al., 2006; Sampedro et al., 2009). Non-bioaugmented and bioaugmented

soil microcosms were analyzed for their residual toxicity using two contact tests based on either the higher plant watercress (*Lepidium sativum* L.) or the Collembola *Folsomia candida* (Willem) (D'Annibale et al., 2006). ~~For sake of comparison, immediately prior to toxicity testing, the NAIC microcosms were mixed with the LM and the inoculum carrier at the same ratios employed for AIC and mycoaugmented microcosms.~~

3. Results

3.1. Isolation and screening of indigenous strains

Direct plating of soil suspensions on selective agar media (i.e., RBCA) and plating of serial soil dilutions after selective enrichment enabled the isolation of several autochthonous fungal strains. As a whole, 64 pure cultures were obtained by the streak plate technique onto MEA plates. However, owing to the fact that several colonies appeared to belong to the same morphotypes, only 11 isolates were screened for both their hydrocarbon-degrading ability and capacity to grow on the lignocellulosic amendment~~ant~~ to be used in bioremediation microcosms (Table S1). According to the macro- and micro-morphological features of pure cultures, all the isolates were putatively ascribable to the phylum Ascomycota and, in particular, to the genera *Fusarium*, *Pseudallescheria* and, to a lesser extent, *Penicillium* and *Aspergillus*. No yeast or yeast-like microorganisms were observed; in addition, none of the filamentous fungi were basidiomycetes, as inferred by the lack of distinctive anatomical features (*e.g.* clamp connections at septa). ~~although some isolates exhibited the ability to grow in benomyl supplemented media.~~

Among these, the isolate 53B was by far the most effective hydrocarbon-utilizing fungus, since its biomass production in liquid media containing TAH as the sole C source was about 8.7-fold higher than in TAH-lacking control cultures. Remarkable results were also observed with isolates 50B, 1A, 35B and 18A, the respective biomasses being increased by 2.8-, 3.6-, 4.4- and 5-fold in TAH-containing media as compared with those in the respective control cultures (~~Table 1~~Table S1).

However, solid-state experiments on the LM showed that the isolate 53B had a scarce capacity to grow therein while the isolate 18A profusely colonized the solid matrix (~~Table 1~~Table S1).

3.2. Molecular identification of selected isolates

The five isolates (i.e., 1A, 18A, 35B, 50B and 53B) that had clearly shown the ability to grow on a mineral medium containing TAH as the sole carbon source (~~Table 2~~Table S2) were identified at the genus level by means of molecular techniques. The sequences obtained *via* amplification of the ITS1-5.8S-ITS2 region with the universal fungal primers ITS1-ITS4 had a length of approx. 600 bp, except for the isolate 18A the fragment's length of which was 650 bp. The ITS partial sequences of three isolates, namely 1A, 35B and 50B, matched at rates higher than 99% those of various species belonging to the genus *Fusarium* (Table S2). However, the aforementioned isolates were deposited in the GenBank as *Fusarium* spp. since the identification at the species level for this genus is currently achieved *via* multilocus sequence typing (MLST), where ITS1-5.8S-ITS2 is just one of the phylogenetic markers used (Deburgogne et al., 2012; O'Donnell et al., 2012)~~based solely on the ITS1-5.8S-ITS2 sequence for this genus is difficult.~~

The ITS partial sequences of the isolates 18A and 53B showed high~~99%~~ similarity (>99%) with ~~ITS partial sequence~~those of *Pseudoallescheria* ~~*boydii* and its anamorph *Scedosporium apiospermum*, while the isolate 53B belonged to the~~*boydii* and *Metarhizium* *anisopliae*, respectively. ~~*anisopliae* complex~~ (Table S2). Nevertheless, the isolates were deposited in GenB~~b~~ank database as *Pseudoallescheria* sp. and *Metarhizium* sp. due to the poor resolving power of ITS sequences within these two genera (Lackner et al., 2012; Bischoff et al., 2009).

3.3. Microbial growth and community structure in remediation microcosms

To assess growth of both cultivable heterotrophic and specialized bacteria (CHB and CSB, respectively) in selected soil remediation microcosms, microbial counts, based on most probable numbers, were performed at the beginning of the experiment and after 30 and 60 d incubation (Fig.

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1A and 1B, respectively) while fungal growth was indirectly inferred by changes in soil's ergosterol content (Fig. 1C). Although in the pristine soil, namely in the non-amended incubation control (NAIC) at the zero time point, densities of both CHB and CSB were not negligible (i.e., $6.6 \cdot 10^4$ and $1.8 \cdot 10^3$ MPN g^{-1}, respectively), they did not significantly change over the incubation time with the only exception of the former at the first harvest (Fig. 1 A). In the amended incubation control (AIC), conversely, both CHB and CSB densities increased by more than two orders of magnitude after 30 d incubation reaching levels which did not significantly change in the subsequent harvest. The same trend was observed in *Pseudoallescheria* sp. ~~*boydii*~~ augmented soil (*P*~~*b*~~A), the CHB and CSB densities of which did not differ from those of coeval AIC microcosms (Fig. 1A and 1B, respectively).

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Ergosterol was also detected in NAIC at the zero time point, albeit at low levels (i.e., 0.02 mg kg^{-1} soil) (Fig. 1C). However, upon incubation, its content did not significantly change in this microcosm thus suggesting that no fungal growth had occurred therein. Conversely, the ergosterol content increased by around 10- and 17-fold in 30- and 60-d-old AIC microcosms, respectively, with respect to the zero time-point (Fig. 1C). In *P*~~*b*~~A microcosm, instead, an almost linear increase in fungal growth was evident along the whole incubation time, although fungal growth was lower than in AIC ~~regardless of the incubation time~~at both harvests. With this regard, *Pseudoallescheria* sp. ~~*boydii*~~ tended to develop an aerial mycelium and to colonize the upper layer of the contaminated soil in the early weeks of incubation rather than growing throughout the whole matrix (data not shown). However, cultivable bacteria only represent a minor fraction of the whole microbial community. Thus, in order to gain insights into the community structure of the pristine soil and into the effects of treatments, a culture-independent method, based on phospholipid fatty acid (PLFA) profiling, was used. ~~Table 3~~Table 1 shows identities and concentrations of PLFAs and indices thereof derived related to the pristine soil and to 60-d-old microcosms. In the pristine soil and its respective incubation control (NAIC), no specific markers of Gram-negative and Gram-positive bacteria were found with the exception of the mid-branched fatty acid 10Me 18:0.

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~~boydii augmented (*Pb*~~Conversely, in the same microcosms, even-numbered saturated straight chain PLFAs, non specific markers of bacteria, were found and the detection of the 18:2ω6,9 indicated the presence of fungi; the monoenoic PLFA 18:1ω9 was also detected but, since it is not a specific marker of microbial groups (Frostegård et al., 2011), it was not used in calculations. No significant differences in total bacterial and fungal biomass were observed between the pristine soil and its non-inoculated and non-amended incubation control, namely the 60-d-old NAIC microcosm (~~Table 3~~Table 1).

Conversely, both in AIC and *P~~b~~*A microcosms, in addition to a marked increase in the number of PLFAs detected (richness) and to the onset of signature markers of Gram-positive and Gram-negative bacteria, a high stimulation of bacterial (247.4 and 95.5 pmoles g^{-1}, respectively) and fungal (95.7 and 44.7 pmoles g^{-1}, respectively) communities was observed (~~Table 3~~Table 1). The fungal/bacterial ratios found in AIC and *P~~b~~*A (i.e., 0.39 and 0.47, respectively) were significantly higher than that of the 60-d-old NAIC microcosm (i.e., 0.13). The values of the Shannon-Weaver index in both NAIC and *P~~b~~*A (2.19 and 1.94, respectively), were higher than that found in AIC thus indicating increased biodiversity (~~Table 3~~Table 1).

The activities of some extracellular enzymes, including glycosyl hydrolases and lignin-modifying enzymes, were determined as an index of both microbial activity and utilization of the lignocellulosic amendment~~ant~~. ~~Table 4~~Table 2 shows that endo-β-1,4-glucanase, cellobiohydrolase and endo-β-1,4-xylanase activities were found in both AIC and *P~~b~~*A microcosms while they were not detected in NAIC. Among lignin-modifying enzymes, only laccase activity was detected in the same microcosms.

3.4. Hydrocarbon degradation and detoxification of the contaminated soil

Due to aforementioned low concentrations of both BTEX and polycyclic aromatic hydrocarbons in the soil of concern, the attention of this study was focused on its aliphatic fraction.

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~~**Table 3.** *boydii* sp. augmented (*Pbb* **Table 4***P. boydiiP. boydii*~~By applying sequential ~~supercritical fluid extraction~~SFE with carbon dioxide under mild conditions to the pristine soil, TAH desorption data were found to strongly fit the Williamson's two-site model shown in Equation (1), as indicated by the high value of the coefficient of determination (R^2_{adj} =0.997) and the significance level of the regression (P<0.001) (Figure 2).

Thus, from the so called fast fraction (F), it was possible to estimate the percent bioavailability of total aliphatic hydrocarbons (C_{10}-C_{34}) which was found to amount to 72.4±3.4% with reference to the total content. Figure 3A, 3B and 3C show the time courses of total aliphatic hydrocarbon contents in NAIC, AIC and *P~~b~~*A microcosms, respectively, and the amounts of the non-bioavailable fraction at start. In NAIC, no significant changes in hydrocarbon concentrations were observed along the incubation. Conversely, in AIC microcosms, TAH concentration dropped from an initial content of around 9995 to 2500 mg kg^{-1} after 30 d incubation and a further decline was observed after 60 d (1954 mg kg^{-1}). A similar time-dependent decline in contaminant concentration was observed in *P~~b~~*A microcosms albeit TAH residual contents after 30 d were significantly higher than those found in AIC (Fig. 3C vs. Fig. 3B). Noteworthy, the residual TAH concentrations found in 60-d-old NAIC and *P~~b~~*A microcosms were significantly lower than their respective non-bioavailable fractions (*P* equal to 0.021 and 0.018, respectively) (Fig. 3B and 3C). The use of the retention time window (RTW) approach (EPA, 2003), showed that the large majority of residual TAH in 60-d-old NAIC microcosm was within the C10-C27 range with the C15-C20 fraction being the most abundant one (~~Table 4~~Table 3). Coeval 60-d-old AIC and *P~~b~~*A microcosms did not significantly differ each other in their abilities to remove the hydrocarbon fractions pertinent to each RTW (~~Table 4~~Table 3); in the former and latter coeval microcosms, degradation proceeded beyond the bioavailable thresholds only for C10-C14 and C21-C27 fractions. It was not possible, instead, to estimate the F fraction for the RTW related to C28-C34 since desorption data did not reliably fit the

aforementioned two-site model with large standard errors of the estimate for both F and the two first order desorption constants (k_1. and k_2).

The contaminated soil exerted a high phytotoxicity towards *L. sativum*. On average, and regardless of the microcosm type at start, percent inhibition of germinability and radical elongation amounted to 73.3 and 87%, respectively, with respect to the distilled water control. The phytotoxicity of 0- and 60-d-old NAIC microcosms did not significantly differ (~~Table 5~~Table 4).

~~*P. boydii*~~**~~Table 5.~~** ~~*boydii*~~*pseudoallescheria* ~~sp. augmented (*Pbb*~~ Toxicity in 60-d-old AIC and *P~~b~~*A microcosms was reduced, as inferred by the 3.5- and 3.9-fold increases in radical length, respectively. In the same microcosms, germinability also increased from 24.9 and 20.9%, respectively, to 36.6 and 35.3%, respectively (~~Table 5~~Table 4). The time-dependent detoxification in 60-d-old AIC and *P~~b~~*A microcosms was confirmed by 5.1 and 6.6 increase, respectively, in the values of the cumulative IG parameter which, conversely, was not affected in NAIC.

The soil also turned out to be highly toxic towards *Folsomia candida*; in fact, an average mortality of around 90% was observed in all microcosms at start (~~Table 5~~Table 4). In NAIC, toxicity did not decrease over time; by contrast, a high extent of detoxification was found in both AIC and *P~~b~~*A microcosms where mortality after 60 d incubation dropped to 11.5 and 24.5%, respectively (~~Table 5~~Table 4).

4. Discussion

One of the main technical constraints to *in situ* remediation techniques, such as bioventing, is the clayey soil texture which negatively affects intrinsic soil's permeability in the unsaturated zone (vadose) and thus the rate at which oxygen is provided to degrading organisms (EPA, 2004). In such a case, *ex situ* techniques, requiring the excavation of the contaminated soil, have to be used and the application of soil conditioners, such as lignocellulosic wastes, is often required to improve porosity and thus gas exchange rates. *Ex situ* techniques might rely either on the biostimulation of resident microbiota or on the addition of microbial inoculants; in the latter case, such an approach is

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termed bioaugmentation. Within this frame, there is an ever increasing recognition that microbial inoculants, based on indigenous microorganisms, are often superior to those composed of allochthonous ones (Potin et al., 2004; Mancera-Lopez et al., 2008). These findings have been often reported in augmentation treatments of soils when using resident fungi in comparison to culture collection strains belonging to the same species (April et al., 1998; Garon et al., 2004). In fact, the selective pressure exerted by historical contamination leads to the supremacy of species able to either tolerate or resist the contaminant load; it has been suggested that the use of resident species adapted to the target contaminated environment might enable better survival probabilities and competitive abilities to the added inoculum (Potin et al., 2004).

Based on this expectation, this study was aimed at isolating and identifying ~~the~~ resident ~~mycobiota~~ fungi of a clayey soil heavily and historically contaminated by aliphatic hydrocarbons. The isolates of concern were first tested in liquid cultures for their capacity of using TAH as the sole carbon source and, then, for their ability to colonize the lignocellulosic mixture selected as the soil ~~conditioner~~ amendment for subsequent remediation microcosms. No yeasts and basidiomycetes were found and all the isolated strains were ascomycetes mainly belonging to the genera *Fusarium*, *Pseudoallescheria* and *Metarhizium*. Interestingly, in another study conducted on the isolation of fungi from soil around flare pit sites, where petroleum wastes are burned, the large majority of isolates belonged to the phylum Ascomycota and no basidiomycetes were found (April et al., 2000). Although the isolate 53B, tentatively identified as *M.~~ anisopliae~~etarhizium* sp., was the most efficient one in using TAH, it showed a limited ability to grow on the lignocellulosic mixture. The high efficiency of TAH use by this species is not surprising. In this respect, *Metarhizium* spp.~~ *anisopliae* is~~ are entomopathogenic and haves the ability to use long chained alkanes located in the insect epicuticle (Pedrini et al., 2007) and this degradation trait is also found in other entomopathogenic fungi, such as *Beauveria bassiana* (Davies and Westlake, 1979; April et al., 2000). The 18A isolate, conversely, identified as *P.~~ boydii~~seudoallescheria* sp., fulfilled both selection criteria, since, besides being able to use TAH as the sole C source, turned out to be

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capable of fast growth on the LM. Interestingly, four *P. boydii* strains were isolated from oil-soaked soils in British Columbia and compared with other strains of the same species isolated from hydrocarbon-free matrices; such a comparison showed that only the strains isolated from the petroleum-contaminated soil exhibited alkane-degrading ability (April et al., 1998). *P. boydii* strains were also isolated from flare pit soils and liquid culture experiments conducted with ^{14}C-hexadecane as the carbon source showed that the alkane was mineralized by this species at a greater extent than other fungi (April et al., 2000). For all these reasons, in the present study, *P. ~~boydii~~seudoallescheria* sp. 18A was selected as the best isolate to be augmented to the soil from which it had been isolated and its performance compared with those observed in the non-amended and amended incubation controls.

In the present study, *Pseudoallescheria* sp. ~~*P. boydii*~~ did not profusely colonize the contaminated soil in the early weeks of incubation and its growth was initially limited to the formation of an aerial mycelium which tended to form dark brown cleistothecia. As a matter of fact, both ergosterol and 18:2 ω6,9 concentrations in 30-d-old *P~~b~~*A microcosms were significantly lower than those found in coeval amended incubation control. This might be concomitantly due to both the limited growth of the inoculant and to the widely known ~~*P. boydii*~~ ability of *Pseudoallescheria* spp. to produce fungistatic substances which also hampered growth of resident fungi (Ko et al., 2010). On the one hand, and with regard to the impact of *Pseudoallescheria* sp. ~~*P. boydii*~~ on bacterial biota, the absence of significant differences in densities of cultivable heterotrophic and hydrocarbonoclastic bacteria with the non-inoculated and amended incubation control, suggested that the augmented fungus did not negatively affect the bacterial biota. On the other hand, the use of a cultivation-independent approach, based on PLFA profiling, showed that the growth of the resident bacterial community and relative richness and diversity were even stimulated by augmentation with *Pseudoallescheria* sp. ~~*P. boydii*~~ albeit to a lesser extent than in the amended incubation control. As a matter of fact, the observed increase of specific signature markers of both Gram-positive and Gram-negative bacteria in these amended microcosms was in agreement with

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Shi and collaborators (2002) who claimed that concentrations of these markers were negatively correlated with hydrocarbon load. In this respect, the mere addition of the LM to the contaminated soil succeeded in biostimulating the resident microbial community, the mycobiota in particular, promoting a faster and more abundant proliferation of fungi during the early phases of incubation than that observed in *Pseudoallescheria* sp.~~*P. boydii*~~-augmented microcosms. With this regard, Callaham et al. (2002) attributed enhanced biological activity in response to the application of wheat straw to a hydrocarbon-contaminated soil to increased soil porosity with ensuing increase in oxygen transfer. Regardless of the alternative degradation mechanisms (i.e., mono-, di- or sub-terminal oxidation) by which aliphatic hydrocarbons are processed, the initial step of microbial attack on these compounds relies on oxygen-dependent alkane-monoxygenase systems (Stroud et al., 2007). In addition, it has been shown that decomposition of either wheat straw or other lignocellulosic wastes (e.g., maize stalks, wood bark) provides a valuable supply of readily available carbon and energy source for resident microorganisms (Lang et al., 2000) with positive impact on remediation efficiency (Steffen et al., 2007; Federici et al., 2012).

The bioavailability of organic contaminants in soil is a matter of great relevance for remediation (especially when dealing with historically contaminated soils) since this parameter has been proposed as the underlying basis for risk-assessment and setting of clean-up goals at contaminated sites (Latawiec et al., 2011; Naidu et al., 2013). Methods to evaluate pollutants release and availability include chemical and biological approaches (Stroud et al., 2007).: A~~a~~mong these, chemical-based methods, e.g. mild extractions with organic solvents, resins (Tenax, XAD) and cyclodextrins, appear to be more rapid, precise and cheap compared to biological assays (Latawiec et al., 2011). In this respect, supercritical fluid extraction (SFE) has also been used to predict the availability of hydrophobic aromatic contaminants in soil (Hawthorne et al., 2002; Cajthaml and Sasek, 2005; Covino et al., 2010). SFE is a dynamic (sequential) desorption method unaffected by contaminant mass transfer to the water phase and it relies on the use of CO_2, which has polarity values close to that of biological lipids under supercritical conditions (Hawthorne et al., 2002). To

the best of our knowledge, the present paper reports for the first time an estimation of the availability of complex aliphatic hydrocarbon mixtures in a historically contaminated soil by means of SFE. As opposed to the method 3560 (EPA, 1996), relying on the use SFE to extract petroleum hydrocarbons from soils under harsh extraction conditions (single step desorption after 30 min at 80 °C, 345 bars and CO_2 flow rate of 500-1000 ml min^{-1}), a sequential SFE process was used in the this study to determine the F fraction under "mild" conditions which were previously adopted for the same purpose for lipophilic aromatic contaminants (Hawthorne et al., 2002; Cajthaml and Sasek, 2005; Covino et al., 2010). The goodness of fit of TAH desorption data with the two-site model enabled an accurate determination of the bioavailable fraction. Noteworthy, the residual TAH amounts found in both 60-d-old AIC and *P~~b~~*A microcosms were lower than their respective initial non-bioavailable fractions thus indicating that contaminant degradation exceeded the bioavailable threshold. These results are in line with those of Huesemann and collaborators (2003, 2004) showing that the easily desorbed fraction of alkanes was much less than that biodegraded thus suggesting that the degradation of these contaminants in soil did not require their previous mobilization from the solid to the aqueous phase.

Thus, this study shows that the biostimulation of the resident microbiota of a historically contaminated soil gave similar degradation outcomes to those observed with a bioaugmentation treatment relying on a fungal strain that had been isolated from the same soil. In this respect, our results contrast with those reported by Mancera-Lopez and collaborators (2008) showing the superiority of bioaugmentation with three indigenous fungal isolates over a biostimulation approach. This discrepancy might depend on several factors, such as the matrices under study, characterized by both diverse contamination profiles and initial microbial densities as well as on the different experimental setup. For instance, although the C:N:P ratio of the present soil (100:4.1: 1.1) was imbalanced with respect to the widely accepted 100: 10: 1 optimal ratio for hydrocarbons degradation (Ward and Cutright, 1999), it was not deliberately modified by the addition of N-containing mineral solutions as it was done by Mancera-Lopez et al. (2008). In addition, the values

of the same ratio in both wheat straw and poplar wood chips (100:0.9:0.3 and 100:1:0.4, respectively) were even more unbalanced with respect to the above mentioned C:N:P reference ratio. However, their addition in sterile form resulted in the aforementioned large stimulation of the resident microbiota and in tangible TAH degradation in 30-d-old AIC microcosms. With this regard, the addition of wheat straw as a bulking agent improved petroleum hydrocarbon removal in biopile experiments from a soil belonging to the same textural class of that used in the present study (Rojas-Avelizapa et al., 2007); a further similarity of the same study with the present one was the generalized increase in bacterial populations that also included hydrocarbonoclastic bacteria. In this respect, similar findings in relation to the activation of the resident microbiota in oil-~~impacted~~ contaminated soils upon the addition of lignocellulosic wastes were reported in other studies (Jorgensen et al., 2000; Zhang et al., 2008).

In the present study, a phytotoxicity test with the highly sensitive species *L. sativum* was used to assess the impact of ~~both~~ biostimulation and mycoaugmentation treatments on soil detoxification; ~~since~~ in fact, petroleum hydrocarbons are known to adversely affect the germination and growth of plants and re-vegetation of soils contaminated by fuel or oil spills is one of the goals of bioremediation. Crude oil in soil can disturb germination and emergence through the formation of a persistent film of hydrocarbons around the seeds producing a physical barrier to water and oxygen uptake (Adam and Duncan 2002). Hydrocarbons, entering seeds through the micropyle and coleorhizae, can negatively affect the assimilation of starch by inhibiting either amylase or starch phosphorylase activities (Achuba, 2006). The extent of phytotoxicity is strongly species-dependent and some higher plants, such as *Vigna unguiculata* (Isikhuemhen et al., 2003) and *Zea mays* (Ogbo et al., 2010), retained significant germination and development capacity in soils spiked with high loadings of petroleum hydrocarbons. In the present study, the pristine soil and its incubation controls proved to be highly phytotoxic to *L. sativum* and both germination and radical elongation were severely inhibited. Such phytotoxic effects were only partially removed by both the AIC and P~~b~~A treatments with the most remarkable effect being observed on radical elongation. In this

respect, these findings are in agreement with those reported by Adenipekun et al. (2013) reporting an 84% TPH reduction in soil after 60 d colonization with *Pleurotus pulmonarius* at a similar level of contaminants to those of the soil under study; despite these degradation extents, the phytotoxicity reduction of soil towards the semi woody herb *Corchorus olitorius* was only partial (Adenipekun et al., 2013). Similarly, only a partial removal of phytotoxicity towards *Amaranthus hybridus* was obtained upon mycoaugmentation of a crude oil polluted soil with *P. pulmonarius* (Olusola and Anslem, 2010). In agreement with the present study, the most evident effect related to phytotoxicity reduction, due to mycoaugmentation of a petroleum-contaminated soil with *Pleurotus tuberregium*, was the recovery of the radical elongation capacity in *Z. mays* seedlings (Isikhuemhen et al., 2003).

F. candida, a common and widespread arthropod that occurs in soils throughout the world, was even more susceptible to the toxic effects of the contaminated soil which led to a 90% mortality. In this case, the detoxification due to either mycoaugmentation or biostimulation was made evident by the dramatic drop in mortality. In this respect, another study showed that contaminants characterized by high lipophilicity were most effective in interfering with fundamental processes, such as osmo-regulation, involved in the survival of *F. candida* (Skovlund et al., 2006). Thus, the high removals of alkanes, extremely hydrophobic compounds (Stroud et al., 2007), which were attained in biostimulated and augmented microcosms, might be the determinant for the significant drop in mortality of this test organism.

5. Conclusions

This study shows that bioremediation can be a viable approach to the clean-up and detoxification of a clay soil historically contaminated by petroleum hydrocarbons. This investigation compared site-specific bioaugmentation with a resident fungal isolate with its respective amended control. The latter treatment ~~turned out to~~ markedly ~~stimulate~~ increased density and activity of the soil microbiota leading to similar degradation and detoxification outcomes to those observed upon myco augmentation. In this respect, the lignocellulosic mixture might be regarded as a slow-release

fertilizer which can be used in alternative to rapid-release inorganic fertilizers, the use of which can lead to a variety of inconveniences, such as leaching and toxicity to resident microbiota (Zhou and Crawford, 1995; Sarkar et al., 2005). An additional consideration, that may not be omitted, stems from the potential biohazard of the isolate 18A. It belongs to the genus *Pseudoallescheria* encompassing some species, which like other hydrocarbon-degrading fungi (Prenafeta-Boldú et al., 2006), might act as opportunistic pathogens in immunocompromised hosts.

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References

Achuba FI. The effect of sublethal concentrations of crude oil on the growth and metabolism of Cowpea (*Vigna unguiculata*) seedlings. Environmentalist 2006; 26:17-20.

Adam G, Duncan HJ. Influence of Diesel fuel on seed germination. Environ Pollut 2002; 120:363-70.

Adenipekun CO, Ayanleye OO, Oyetunji OJ. Bioremediation of soil contaminated by spent diesel oil using *Pleurotus pulmonarius* Fries (Quelet) and its effects on the growth of *Corchorus olitorius* (L.) J Appl Biosci 2013; 68:5366-73.

April TM, Abbott SP, Foght JM, Currah RS. Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae). Can J Microbiol 1998; 44:270-8.

April TM, Foght JM, Currah RS. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. Can J Microbiol 2000; 46:38-49.

Banitz T, Johst K, Wick LY, Schamfuß S, Harms H, Frank K. Highways versus pipelines: contributions of two fungal transport mechanisms to efficient bioremediation Environ Microbiol Rep 2013; 5:211-18.

Bischoff JF, Rehner SA, Humber RA. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 2009; 101:512-30.

Boyle D. Nutritional factors limiting the growth of *Lentinula edodes* and other white-rot fungi in wood. Soil Biol Biochem 1998; 30:817–23.

Brassington KJ, Hough RL, Paton GI, Semple KT, Risdon GC, Crossley J, Hay I, Askari K, Pollard SJT. Weathered Hydrocarbon Wastes: a Risk Management Primer. Crit Rev Env Sci Technol 2007; 37:199–232.

Cajthaml T, Šašek V. Application of supercritical fluid extraction (SFE) to predict bioremediation efficacy of long-term composting of PAH-contaminated soil. Environ Sci Technol 2005; 39:8448–52.

Callaham MA Jr, Stewart AJ, Alarcón C, McMillen SJ. Effects of earthworm (*Eisenia fetida*) and wheat (*Triticum aestivum*) straw additions on selected properties of petroleum-contaminated soils. Environ Toxicol Chem 2002; 21:1658-63.

Chiu SW, Gao T, Chan CS, Ho CK. Removal of spilled petroleum in industrial soils by spent compost of mushroom *Pleurotus pulmonarius*. Chemosphere 2009; 75:837-42.

Covino S, Čvančarová M, Muzikář M, Svobodová K, D'Annibale A, Petruccioli M, Federici F, Křesinová Z, Cajthaml T. An efficient PAH-degrading *Lentinus* (*Panus*) *tigrinus* strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J Hazard Mater 2010; 183: 669–76.

Davies JS, Westlake DW 1979. Crude oil utilization by fungi. Can J Microbiol 25:146-56.

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Debourgogne A, Gueidan C, de Hoog S, Lozniewsky A, Machouart M. Comparison of two DNA sequence-based typing schemes for the *Fusarium solani* Species Complex and proposal of a new consensus method. J Microbiol Meth 2012; 91:65-72.

D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl Environ Microbiol 2006;72:28-36.

EPA, Environmental Protection Agency. Supercritical fluid extraction of total recoverable petroleum hydrocarbons- Method 3560 1996; Revision 0. Available from: http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3560.pdf.

EPA, Environmental Protection Agency. Non-halogenated organics using GC-FID. Method 8015D; Revision 4 June 2003. Available from: http://www.epa.gov/osw/hazard/testmethods/pdfs/8015d_r4.pdf.

EPA, Environmental Protection Agency. How to Evaluate Alternative Cleanup Technologies for Underground Storage Tank Sites. A Guide for Corrective Action Plan Reviewers. EPA 510-R-04-002 2004. Available from: http://www.epa.gov/oust/pubs/tums.htm.

Federici E, Giubilei MA, Covino S, Zanaroli G, Fava F, D'Annibale A, Petruccioli M. Addition of maize stalks and soybean oil to a historically PCB-contaminated soil: effect on degradation performance and indigenous microbiota. New Biotechnol 2012; 30:69-79.

Frostegård Å, Tunlid A., Bååth E. Phospholipid fatty acid composition, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol 1993; 59:3605-17.

Frostegård Å, Tunlid A, Bååth E. Use and misuse of PLFA measurements in soils. Soil Biol. Biochem 2011; 43:1621-25.

Garon D, Sage L, Wouessidjewe D, Seigle-Murandi F. Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere 2004; 56:159–66.

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Guillén F, Gómez-Toribio V, Martínez MJ, Martínez AT. Production of hydroxyl radical by the synergistic action of fungal laccase and aryl alcohol oxidase. Arch Biochem Biophys 2000; 383:142-7.

Hawthorne SB, Poppendieck DG, Grabanski CB, Loehr RC. Comparing PAH availability from manufactured gas plant soils and sediments with chemical and biological tests. 1. PAH release during water desorption and supercritical carbon dioxide extraction. Environ Sci Technol 2002; 36:4795–803.

Huesemann MH, Hausmann TS, Fortman TJ. Assessment of bioavailability limitations during slurry biodegradation of petroleum hydrocarbons in aged soils. Environ Toxicol Chem 2003; 22:2853–60.

Huesemann MH, Hausmann TS, Fortman TJ. Does bioavailability limit biodegradation? A comparison of hydrocarbon biodegradation and desorption rates in aged soils Biodegradation 2004; 15:261–74.

Isikhuemhen OS, Anoliefo GO, Oghale OI. Bioremediation of crude oil polluted soil by the white rot fungus, *Pleurotus tuberregium* (Fr.) Sing. Environ Sci Pollut Res 2003; 10:108–12.

Jorgensen KS, Puutinen J, Suortti AM. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 2000; 107:245–54.

Ko WH, Tsou YJ, Ju YM, Hsieh HM, Ann PJ. Production of a fungistatic substance by *Pseudallescheria boydii* isolated from soil amended with vegetable tissues and its significance. Mycopathologia 2010; 169:125-31.

Lackner M, Najafzadeh MJ, Sun J, Lu Q, G. Sybren de Hoog GS. Rapid identification of *Pseudallescheria* and *Scedosporium* strains by using rolling circle amplification. Appl Environ Microbiol 2012; 78:126–33.

Lang E, Kleeberg I, Zadrazil F. Extractable organic carbon and counts of bacteria near the lignocellulose-soil interface during the interaction of soil microbiota and white rot fungi. Bioresour Technol 2000; 75:57-65.

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Latawiec AE, Swindell AL, Simmons P, Reid BJ. Bringing Bioavailability into Contaminated Land Decision Making: The Way Forward? Crit. Rev. Environ Sci Technol 2011; 41:52-77.

Mancera-López ME, Esparza-García F, Chávez-Gómez B, Rodríguez-Vázquez R, Saucedo-Castañeda G, Barrera-Cortés J. Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation–bioaugmentation with filamentous fungi. Int Biodeterior Biodegr 2008; 61:151–60

Naidu R, Channey R, McConnell S, Johnston N, Semple KT, McGrath S et al. Towards bioavailability-based soil criteria: past, present and future perspectives. Environ Sci Pollut Res 2013; doi: 10.1007/s11356-013-1617-x.

O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and Fusarium MLST. Mycologia 2012; 104:427-45.

Ogbo EM, Tabuanu A, Ubebe R. Phytotoxicity assay of diesel fuel-spiked substrates remediated with *Pleurotus tuberregium* using *Zea mays*. Int J Appl Res Nat Prod 2010; 3:12-6.

Olusola SA, Anslem EE. Bioremediation of a crude oil polluted soil with *Pleurotus pulmonarius* and *Glomus mosseae* using *Amaranthus hybridus* as a test plant. J Bioremed Biodegrad 2010; 1:113.

Pedrini N, Crespo R, Juárez MP. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comp Biochem Physiol C Toxicol Pharmacol 2007; 146:124-37.

Potin O, Rafin C, Veignie E. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. Int Biodeterior Biodegr 2004; 54:45–52.

Prenafeta-Boldú FX, Kuhn A, Luykx DMAM, Anke H, Van Groenestijn JW, De Bont JAM. Isolation and characterization of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. Mycol Res 2001; 105: 477–84.

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Prenafeta- Boldú FX, Summerbell R, de Hoog GS. Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? FEMS Microbiol Rev 2006; 30: 109-130.

Rojas-Avelizapa NG, Roldán-Carrillo T, Zegarra-Martínez H, Muñoz-Colunga AM, Fernández-Linares LC. A field trial and ex-situ bioremediation of a drilling mud-polluted site. Chemosphere 2007; 66:1595–1600.

Sampedro I, Cajthaml T, Marinari S, Petruccioli M, Grego S, D'Annibale A. Organic matter transformation and detoxification in dry olive mill residue by the saprophytic fungus *Paecilomyces farinosus*. Process Biochem 2009; 44:216–25.

Sarkar D, Ferguson M, Datta R, Birnbaum S. Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ Pollut 2005; 136: 187-95.

Šašek V, Bhatt M, Cajthaml T, Malachová K, Lednická D. Compost-mediated removal of polycyclic aromatic hydrocarbons from contaminated soil. Arch Environ Contam Toxicol 2003; 44:336–42.

Shi W, Bischoff M, Turco R, Konopka A. Association of microbial community composition and activity with Pb, Cr and hydrocarbon contamination. Appl Environ Microbiol 2002; 68:3859-66.

Steffen K, Schubert S, Tuomela M, Hatakka A, Hofrichter M. Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi. Biodegradation 2007; 18:359–369.

Stroud JL, Paton GI, Semple K. Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. J Appl Microbiol 2007; 102:1239-53.

Skovlund G, Damgaard C, Bayley M, Holmstrup M. Does lipophilicity of toxic compounds determine effects on drought tolerance of the soil collembolan *Folsomia candida*? Environ Pollut 2006; 144:808-15.

Tunlid A, White DC. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Stotzky G, Bollag JM, editors. Soil Biochemistry. New York: Marcel Dekker; 1992. p. 229-62.

Ward E, Cutright JT, The investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil. Int Biodeterior Biodegr 1999; 44:55–64.

Williamson DG, Loehr RC, Kiumura Y. Release of chemicals from contaminated soils. J Soil Contam 1998; 7:543–58.

Wrenn BA, Venosa AD. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a Most-Probable-Number procedure. Can J Microbiol 1996; 42:252-8.

Zhang K, Hua X-F, Han H-L, Wang J, Miao C-C, Xu Y-Y, Huang Z-D, Zhang H, Yang J-M, Jin W-B, Liu Y-M, Liu Z. Enhanced bioaugmentation of petroleum- and salt-contaminated soil using wheat straw. Chemosphere 2008; 73:1387–92.

Zhou E, Crawford R. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. Biodegradation 1995; 6:127-40.

Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil

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ABSTRACT

The present work was aimed at isolating, identifying the main members of the mycobiota of a clay soil historically contaminated by mid- and long-chain aliphatic hydrocarbons (AH) and to subsequently assess their hydrocarbon-degrading ability. All the isolates were Ascomycetes and, among them, most interesting was *Pseudoallescheria* sp. 18A, which displayed both the ability to use AH as the sole carbon source and to profusely colonize a wheat straw: poplar wood chips (70:30, w/w) lignocellulosic mixture (LM) selected as the amendment for subsequent soil remediation microcosms. After 60 d mycoaugmentation with *Pseudoallescheria* sp. of the aforementioned soil, mixed with the sterile LM (5:1 mass ratio), a 79.7% AH reduction and a significant detoxification, inferred by a drop in mortality of *Folsomia candida* from 90 to 24%, were observed. However, similar degradation and detoxification outcomes were found in the non-inoculated incubation control soil that had been amended with the sterile LM. This was due to the biostimulation exerted by the amendment on the resident microbiota, fungi in particular, the activity and density of which were low, instead, in the non-amended incubation control soil.

Keywords: mycoaugmentation, *Pseudoallescheria sp.*, oil-contaminated soil, bioavailability, biostimulation, bioremediation, aliphatic hydrocarbons

1. Introduction

Crude oil extraction and transportation through pipelines on a mining claim zone can cause accidental oil spills and leaks in soil. Environmental hazards arising from this type of contamination might become even more serious in cases of reiterative spills and aging of the pollution (Brassington et al., 2007).

Bioremediation has been suggested to be a valuable soil clean-up option due to its cost-effectiveness and sustainability. However, the outcome of a given bioremediation intervention has been found to largely depend on the nature, source, concentration and bioavailability of hydrocarbon contaminants as well as on soil physico-chemical and microbiological properties (Brassington et al., 2007; Stroud et al., 2007). Although hydrocarbon degradation in soil is generally ascribed to bacteria, filamentous fungi exhibit peculiar characteristics which make them suitable candidates for the clean-up of soils historically contaminated by crude oil (Chiu et al., 2009). With this regard, their apical growth mode enable them to reach inaccessible soil regions and their hyphal network confer them the ability of acting as spreading vectors of pollutant-degrading bacteria (Banitz et al., 2013). These favourable properties are often associated with their ability of growing in environments with low nutrient concentrations, low humidity and acidic pH (Mancera-López et al., 2008). Moreover, rather widespread among fungi is the production of unspecific lignin-modifying enzymes acting in the extracellular environment *via* radical-based reactions and able to reach poorly bioavailable organopollutants; under certain conditions, they are capable of generating hydroxyl radicals thus acting as Fenton's like reagents (Guillén et al., 2000).

At an Italian site, located along the Southern Sicilian shoreline, a widespread crude oil contamination was first detected several decades ago in areas located around the oil extraction wells and along the pipelines and the large majority of mid- and long-chain hydrocarbons tended to accumulate over time due to the high content of clay in the vadose zone. The predominance of the clay fraction in the contaminated soil precluded the application of *in situ* treatments.

On the basis of the numerous reports claiming the higher hydrocarbon-degrading efficacy of resident fungi than allochthonous ones (April et al., 1998; Garon et al., 2004; Potin et al., 2004), the present work was aimed at isolating, identifying and assessing the hydrocarbon-degrading ability of the main members of the mycobiota of a historically contaminated soil collected from the aforementioned area. To this aim, liquid cultures of identified isolates were conducted by using hydrocarbons extracted from the same soil as the sole carbon source. The best isolates were then tested for their abilities to colonize a lignocellulose mixture, to be used as the amendment for remediation purposes, prior to the preparation of mycoaugmentation treatments. This led to the selection of a fungal strain which was used in the augmentation of the soil from which it had been isolated. The present study compares the degradation and detoxification efficiencies of this mycoaugmentation treatment with those observed in either amended or non-amended non-inoculated incubation controls. All these treatments were compared for their abilities to (i) affect densities of heterotrophic and hydrocarbonoclastic bacteria, (ii) enable fungal growth, (iii) modify the community structure of soil, (iv) remove aliphatic hydrocarbons with reference to their bioavailabilities and, finally, (v) detoxify the soil.

2. Materials and Methods

2.1. Materials

Soil samples were collected nearby an oil-refinery site (Gela, Italia), air-dried and sieved (< 2 mm). The soil had real and potential acidity of 7.96 and 7.50 in water and 1 N KCl, respectively. Soil texture was as follows (w/w): sand, 15.7%; silt, 40.2% and clay, 44.1%, thus, according to the USDA textural classification, it was silty clay soil with an estimated bulk density of 1.24 g cm^{-3}. The water-holding capacity (WHC) was 37.2% (w/w). Total organic carbon (TOC) and total nitrogen (TN) and assimilable phosphorous contents were 1.48, 0.06 and 0.014%, respectively. Total aliphatic hydrocarbons (TAH) content was 10200±456 mg kg^{-1} soil. BTEX and total

polycyclic aromatic hydrocarbons contents in soil, analyzed by Theolab spa (Turin, Italy), were low and amounted to 324.2 and 4.7 μg kg^{-1}, respectively. TOC, TN and ash contents in wheat straw (53.0, 0.48 and 3.9%, respectively), poplar wood chips (48.5, 0.48 and 2.2%, respectively), and millet seeds (49.8, 1.6 and 2.7%, respectively) were determined as previously described (Sampedro et al., 2009).

2.2. Extraction and analysis of contaminants

Aliphatic hydrocarbons from either pristine soil or non-inoculated and inoculated microcosms were extracted and quantified according to protocols DIN EN 14039 and DIN ISO 16703. In particular, samples (3 g) were suspended in 15 ml of *n*-hexane: acetone mixture (1:2, v/v) and extracted for 30 min in ultrasonic bath. The supernatant after centrifugation (3000 rpm, 10 min) was then extracted three times with Milli-Q water (20 ml) to remove polar compounds. The resulting *n*-hexane extracts were passed through mini-columns containing anhydrous Na_2SO_4 (2 g) and Florisil (2 g). Quantitative analysis of this fraction, from here onwards referred to as total aliphatic hydrocarbons (TAH), was carried out using a GC-FID HP 5890 equipped with a DB-5MS column (0.25 mm × 30 m, 0.25 μm film thickness). Injector and flame ionization detector were operated at 300 ºC and helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. An initial isocratic step of 1 min at 50 ºC was followed by a temperature ramp up to 300 ºC (at a rate of 20 ºC min^{-1}), the latter temperature being held for additional 20 min. Mineral Oil Standard Mixture (Fluka Analytical) was used for calibration. Quantification of TAH was achieved by integrating the area of the aliphatic "unresolved complex mixture" (UCM) between the retention times of decane (5.585 min) and tetratriacontane (29.755 min). Percent degradation was calculated by referring residual TAH contents in amended microcosms to those in coeval non-amended microcosm; in particular, actual TAH concentrations in amended microcosms were multiplied by a correction factor as described by Šašek et al. (2003). Moreover, in order to quantify various sub-fractions of the UCM, the aliphatic TAH "hump" of each chromatogram was split into retention time windows (RTW) as described in

the 8015D method (EPA, 2003). Integration marks for each RTW were established by injecting *n*-alkane standards as markers (C8-C20 and C21-C40, Fluka Analytics).

2.3. Determination of contaminants bioavailability.

The overall bioavailability of either TAHs or their respective fractions pertinent to each RTW were estimated using sequential supercritical fluid extraction (SFE) with CO_2 as reported elsewhere (Covino et al., 2010). The extractions were performed using a PrepMaster extractor (Suprex, Pittsburgh, PA) equipped with VaryFlow restrictor operating at 40 °C, a refrigerated (-20 °C) hydrocarbon trap filled with octadecyl-bonded silica (Merck, Darmstadt, Germany) as the sorbent and a downward stream of CO_2 (5.5 SFE/SFC, Messer Technogas, Prague, Czech Republic). Four soil aliquots (1.0 g each) were extracted at 50 °C, 200 bar at a CO_2 flow rate of 1 mL min^{-1} and the desorbed hydrocarbons were collected after 5, 10, 20, 40, 60, 80, 120, 160 and 200 min. Sequential SFE can be fitted by a desorption model presuming that the extraction is controlled by the two rate constants differing by orders of magnitude (Williamson et al., 1998). The chemical release data can be modelled by an empirical two-site model, consisting of the two first-order equations (1):

$$S_t = F \cdot S_o e^{-k1t} + (1 - F) \cdot S_o \cdot e^{-k2t} \quad (1)$$

where S_o and S_t are the initial and residual pollutant concentrations in the soil after time t, respectively, F is the fraction of chemical rapidly released; 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 while 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 (Hawthorne et al., 2002; Cajthaml and Šašek, 2005).

2.4. Isolation of autochthonous fungi

Autochthonous filamentous fungi were isolated using two different approaches: *i)* direct spread plating of soil suspensions and of relative serial dilutions and *ii)* spread plating of soil suspensions after enrichment. As for the former approach, 10 g of soil were added to 90 mL of sterile deionized water and the suspension was magnetically stirred for 30 min prior to preparing dilution series (up to 10^{-5}). Enrichment cultures were prepared by adding 5 g of soil to 250-mL Erlenmeyer flasks containing the MM liquid mineral medium (45 mL) described by Prenafeta-Boldú et al. (2001) and added with chloramphenicol (0.1 g L^{-1}) to prevent bacterial growth. After 7 d incubation on a rotary shaker (180 rpm, 28 °C), serial dilutions were prepared as described above.

Aliquots of the suspensions from both approaches and their respective dilution series were spread onto Petri dishes containing Rose Bengal Chloramphenicol Agar (RBCA; Himedia, Mumbai, India). In the attempt of isolating basidiomycetes, RBCA was supplemented with benomyl (15 mg L^{-1}). Throughout the incubation in RBCA plates (from 5 to 15 d at 28 °C), pure cultures were isolated by the streak plate method and sub-cultured onto malt extract agar plates (MEA, Oxoid, Basingstoke, UK).

2.5. Inoculum preparation and screening of isolates

For preliminary screening experiments of fungal isolates, the TAH fraction, extracted and purified as above, was dried under vacuum at 40 °C in a R-120 rotary evaporator (Büchi, Switzerland) and, finally, suspended in acetone to a final concentration of 500 mg mL^{-1}.

The hydrocarbon-degrading ability of each pure fungal isolate was inferred by its capacity to grow on the MM medium in the presence of TAH as the sole C source. Inocula were prepared from 7-d-old cultures grown on the MEG medium (Covino et al., 2010). At the end of the incubation, cultures were centrifuged (6000 g, 15 min), the pellets were washed with sterile distilled water and centrifuged again as above to avoid the entrainment of residual medium components. Finally, the pellet was suspended in distilled water to yield an initial biomass concentration of 2.5 g L^{-1}. The screening was carried out in Erlenmeyer flasks (250 mL) containing 40 mL sterile MM added with

400 μL TAH suspension to yield an initial hydrocarbon concentration of 200 mg per flask (5.0 g TAH L^{-1}). Each flask was added with 2 mL inoculum and then incubated at 28 °C under orbital shaking (150 rpm) for 25 d. Inoculated cultures grown on MM and added with 400 μL acetone were used as the controls. At the end of incubation, liquid cultures were filtered on pre-weighed Whatman GF/C discs and the harvested biomass was washed once with dichloromethane (100 mL) and twice with equal volumes of distilled water. The filter was dried at 105 °C for 24 h, cooled in a desiccator, and weighed. The fold increase in growth, due to the utilization of TAH, was calculated from the W_{TAH} /W_{CONT} ratio, where the former and the latter are the fungal biomass produced in the presence and in the absence of TAH, respectively.

Isolates were also compared for their respective abilities to grow under solid-state conditions on the wheat straw: poplar wood chips lignocellulose mixture (LM, 70:30, w/w), intended to be used as the amendant for subsequent bioremediation microcosms. To prepare inoculants, 2 g millet seeds (*Panicum miliaceum* L.) were rinsed with water for 24 h, sterilized twice in autoclave (121 °C, 20 min) and transferred to 16×3.5 cm test-tubes. Then, two 10-d-old colonized MEA agar plugs (1.0 cm Ø) were added and the mixture incubated for 5 d at 28 °C under static conditions. In particular, the LM (8.0 g) was moistened with water to reach 65% humidity, sterilized in autoclave (121 °C, 30 min) and, after cooling, added over the pre-colonized millet seeds. Cultures were incubated for 30 d at 28 °C and the length of the ascending mycelial front measured. Growth was assessed subjectively on a 0-10 scale as described by Boyle (1998).

2.6. Identification of fungal isolates

Isolates showing remarkable hydrocarbon-utilizing capabilities were identified through a molecular approach associated with colony morphological features. The DNA was extracted from pure fungal cultures *via* the phenol:chloroform protocol and the Internal Transcribed Spacer Region (ITS) of the ITS1-5.8S-ITS2 rRNA gene was amplified by PCR using the universal fungal forward and reverse ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

primers, respectively. The composition of PCR reaction mixture and amplification conditions were reported elsewhere (D'Annibale et al., 2006). The purified PCR products were used in sequencing reactions with the same set of primers, using a BigDye Terminator cycle sequencing ready reaction kit, version 3.0 (Applied Biosystems, Foster City, CA). Sequencing was performed on an ABI 3730 DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) and compared with those of all known fungal species available from the same database.

2.7. Soil remediation conditions

The 18A isolate, that had been selected through the screening and identified as *Pseudallescheria* sp., was maintained on malt extract agar (MEA) plates and periodically sub-cultured on a monthly basis. Its inocula on millet seeds were prepared as described above. Aliquots of contaminated soil (150 g) were moistened with sterile distilled water to reach 50% of the WHC, mixed with the LM (30 g) and then with *Pseudoallescheria* sp. pre-grown millet seeds (15 g). The amended incubation control (AIC) was prepared by mixing the contaminated soil with both sterile LM and non-colonized milled seeds at the same ratios. Both LM and millet seeds underwent two sterilization cycles (each at 121 °C for 30 min) in order to avoid the risk of adding foreign microorganisms to AIC. As a further control, the pristine soil was incubated as described below after adjusting its moisture content to 50% of its WHC and this treatment was referred to as non-amended incubation control (NAIC). All microcosms were incubated on 1.7-L cylindrical glass jars under non-axenic conditions for 0, 30 and 60 d at 28±2 °C in the dark. Moisture was maintained constant by periodical additions of sterile distilled water. For each sampling time, triplicate microcosms were totally sacrificed.

2.8. Bacterial counts, fungal growth and phospholipid fatty acids (PLFA) analysis

Cultivable heterotrophic and hydrocarbon-degrading bacteria were enumerated in soil according to the most probable number (MPN) counting technique (Wrenn and Venosa, 1996). As for the heterotrophs, soil serial dilutions were incubated in Tryptic Soy Broth (TSB, 30 g L^{-1}), while specialized bacteria were enumerated using *n*-hexadecane as the sole carbon and energy source in a mineral medium (Wrenn and Venosa, 1996). Cycloheximide (500 mg L^{-1}) was added in both culture media in order to repress fungal growth. Ergosterol was used as a specific indicator of fungal growth as reported elsewhere (Covino et al., 2010).

The microbial community structure was investigated using phospholipid fatty acid (PLFA) analysis according to Frostegård et al. (1993). Briefly, each microcosm (5 g) was extracted for 2 h with a mixture of chloroform, methanol and citrate buffer pH 4.0 (1:2:0.8, v:v:v). The lipid-containing phase was collected, evaporated under N_2. and fractionated by solid phase extraction (SPE) columns (3 ml/500 mg silica Sep-pak VacTM, Waters) into neutral, glycolipids and phospholipids by elution with chloroform (5 ml), acetone (5 ml) and methanol (5 ml), respectively. Each of the lipid fractions was dried under a stream of N_2, suspended in hexane and the purity was checked by GCMS analysis. A derivatization of phospholipids into their corresponding methyl esters was performed by mild alkaline methylation before analysis by GC-MS. Mass spectra were recorded by the use of a QP-5050 (Shimadzu, Japan) spectrometer equipped with an AT 20 capillary column (0.25 mm i.d., 25 m) (Alltech, Deerfield) at 80–280 °C with a splitless injection and an isothermal program at 80 °C for 2 min, then 6 °C•min^{-1} up to 280 °C and finally isothermal at 280 °C for 5 min. Methylated fatty acids were identified according to their mass spectra and using BAME 24 and 37-Component FAME Mix (47080-U 47885-U, respectively, Sigma–Aldrich, Milan, I) as chemical standards. Individual PLFAs were used as signature markers of microorganisms and named as reported by Tunlid and White (1992).

2.9. Enzyme and ecotoxicity assays

Extracellular enzymes were extracted from soil microcosms as previously reported (D'Annibale et al., 2006). Laccase and Mn-dependent peroxidase and glycosyl hydrolase (i.e., endo-β-1,4-glucanase, cellobiohydrolase and endo-β-1,4-xylanase) activities were determined as reported elsewhere (D'Annibale et al., 2006; Sampedro et al., 2009). Non-bioaugmented and bioaugmented soil microcosms were analyzed for their residual toxicity using two contact tests based on either the higher plant watercress (*Lepidium sativum* L.) or the Collembola *Folsomia candida* (Willem) (D'Annibale et al., 2006).

3. Results

3.1. Isolation and screening of indigenous strains

Direct plating of soil suspensions on selective agar media (i.e., RBCA) and plating of serial soil dilutions after selective enrichment enabled the isolation of several autochthonous fungal strains. As a whole, 64 pure cultures were obtained by the streak plate technique onto MEA plates. However, owing to the fact that several colonies appeared to belong to the same morphotypes, only 11 isolates were screened for both their hydrocarbon-degrading ability and capacity to grow on the lignocellulosic amendment to be used in bioremediation microcosms (Table S1). According to the macro- and micro-morphological features of pure cultures, all the isolates were putatively ascribable to the phylum Ascomycota and, in particular, to the genera *Fusarium*, *Pseudoallescheria* and, to a lesser extent, *Penicillium* and *Aspergillus*. No yeast or yeast-like microorganisms were observed; in addition, none of the filamentous fungi were basidiomycetes, as inferred by the lack of distinctive anatomical features (*e.g.* clamp connections at septa).

Among these, the isolate 53B was by far the most effective hydrocarbon-utilizing fungus, since its biomass production in liquid media containing TAH as the sole C source was about 8.7-fold higher than in TAH-lacking control cultures. Remarkable results were also observed with isolates 50B, 1A,

35B and 18A, the respective biomasses being increased by 2.8-, 3.6-, 4.4- and 5-fold in TAH-containing media as compared with those in the respective control cultures (Table S1).

However, solid-state experiments on the LM showed that the isolate 53B had a scarce capacity to grow therein while the isolate 18A profusely colonized the solid matrix (Table S1).

3.2. Molecular identification of selected isolates

The five isolates (i.e., 1A, 18A, 35B, 50B and 53B) that had clearly shown the ability to grow on a mineral medium containing TAH as the sole carbon source (Table S2) were identified at the genus level by means of molecular techniques. The sequences obtained *via* amplification of the ITS1-5.8S-ITS2 region with the universal fungal primers ITS1-ITS4 had a length of approx. 600 bp, except for the isolate 18A the fragment's length of which was 650 bp. The ITS partial sequences of three isolates, namely 1A, 35B and 50B, matched at rates higher than 99% those of various species belonging to the genus *Fusarium* (Table S2). However, the aforementioned isolates were deposited in the GenBank as *Fusarium* spp. since the identification at the species level for this genus is currently achieved *via* multilocus sequence typing (MLST), where ITS1-5.8S-ITS2 is just one of the phylogenetic markers used (Debourgogne et al., 2012; O'Donnell et al., 2012)

The ITS partial sequences of the isolates 18A and 53B showed high similarity (>99%) with those of *Pseudoallescheria boydii* and *Metarhizium anisopliae*, respectively. (Table S2). Nevertheless, the isolates were deposited in GenBank database as *Pseudoallescheria* sp. and *Metarhizium* sp. due to the poor resolving power of ITS sequences within these two genera (Lackner et al., 2012; Bischoff et al., 2009).

3.3. Microbial growth and community structure in remediation microcosms

To assess growth of both cultivable heterotrophic and specialized bacteria (CHB and CSB, respectively) in selected soil remediation microcosms, microbial counts, based on most probable numbers, were performed at the beginning of the experiment and after 30 and 60 d incubation (Fig.

1A and 1B, respectively) while fungal growth was indirectly inferred by changes in soil's ergosterol content (Fig. 1C). Although in the pristine soil, namely in the non-amended incubation control (NAIC) at the zero time point, densities of both CHB and CSB were not negligible (i.e., $6.6 \bullet 10^4$ and $1.8 \bullet 10^3$ MPN g^{-1}, respectively), they did not significantly change over the incubation time with the only exception of the former at the first harvest (Fig. 1 A). In the amended incubation control (AIC), conversely, both CHB and CSB densities increased by more than two orders of magnitude after 30 d incubation reaching levels which did not significantly change in the subsequent harvest. The same trend was observed in *Pseudoallescheria* sp.-augmented soil (*P*A), the CHB and CSB densities of which did not differ from those of coeval AIC microcosms (Fig. 1A and 1B, respectively).

Ergosterol was also detected in NAIC at the zero time point, albeit at low levels (i.e., 0.02 mg kg^{-1} soil) (Fig. 1C). However, upon incubation, its content did not significantly change in this microcosm thus suggesting that no fungal growth had occurred therein. Conversely, the ergosterol content increased by around 10- and 17-fold in 30- and 60-d-old AIC microcosms, respectively, with respect to the zero time-point (Fig. 1C). In *P*A microcosm, instead, an almost linear increase in fungal growth was evident along the whole incubation time, although fungal growth was lower than in AIC at both harvests. With this regard, *Pseudoallescheria* sp tended to develop an aerial mycelium and to colonize the upper layer of the contaminated soil in the early weeks of incubation rather than growing throughout the whole matrix (data not shown). However, cultivable bacteria only represent a minor fraction of the whole microbial community. Thus, in order to gain insights into the community structure of the pristine soil and into the effects of treatments, a culture-independent method, based on phospholipid fatty acid (PLFA) profiling, was used. Table 1 shows identities and concentrations of PLFAs and indices thereof derived related to the pristine soil and to 60-d-old microcosms. In the pristine soil and its respective incubation control (NAIC), no specific markers of Gram-negative and Gram-positive bacteria were found with the exception of the mid-branched fatty acid 10Me 18:0.

Conversely, in the same microcosms, even-numbered saturated straight chain PLFAs, non specific markers of bacteria, were found and the detection of the 18:2ω6,9 indicated the presence of fungi; the monoenoic PLFA 18:1ω9 was also detected but, since it is not a specific marker of microbial groups (Frostegård et al., 2011), it was not used in calculations. No significant differences in total bacterial and fungal biomass were observed between the pristine soil and its non-inoculated and non-amended incubation control, namely the 60-d-old NAIC microcosm (Table 1).

Conversely, both in AIC and *P*A microcosms, in addition to a marked increase in the number of PLFAs detected (richness) and to the onset of signature markers of Gram-positive and Gram-negative bacteria, a high stimulation of bacterial (247.4 and 95.5 pmoles g^{-1}, respectively) and fungal (95.7 and 44.7 pmoles g^{-1}, respectively) communities was observed (Table 1). The fungal/bacterial ratios found in AIC and *P*A (i.e., 0.39 and 0.47, respectively) were significantly higher than that of the 60-d-old NAIC microcosm (i.e., 0.13). The values of the Shannon-Weaver index in both NAIC and *P*A (2.19 and 1.94, respectively), were higher than that found in AIC thus indicating increased biodiversity (Table 1).

The activities of some extracellular enzymes, including glycosyl hydrolases and lignin-modifying enzymes, were determined as an index of both microbial activity and utilization of the lignocellulosic amendment. Table 2 shows that endo-β-1,4-glucanase, cellobiohydrolase and endo-β-1,4-xylanase activities were found in both AIC and *P*A microcosms while they were not detected in NAIC. Among lignin-modifying enzymes, only laccase activity was detected in the same microcosms.

3.4. Hydrocarbon degradation and detoxification of the contaminated soil

Due to aforementioned low concentrations of both BTEX and polycyclic aromatic hydrocarbons in the soil of concern, the attention of this study was focused on its aliphatic fraction.

By applying sequential SFE with carbon dioxide under mild conditions to the pristine soil, TAH desorption data were found to strongly fit the Williamson's two-site model shown in Equation (1), as indicated by the high value of the coefficient of determination (R^2_{adj} =0.997) and the significance level of the regression (P<0.001) (Figure 2).

Thus, from the so called fast fraction (F), it was possible to estimate the percent bioavailability of total aliphatic hydrocarbons (C_{10}-C_{34}) which was found to amount to 72.4±3.4% with reference to the total content. Figure 3A, 3B and 3C show the time courses of total aliphatic hydrocarbon contents in NAIC, AIC and *P*A microcosms, respectively, and the amounts of the non-bioavailable fraction at start. In NAIC, no significant changes in hydrocarbon concentrations were observed along the incubation. Conversely, in AIC microcosms, TAH concentration dropped from an initial content of around 9995 to 2500 mg kg^{-1} after 30 d incubation and a further decline was observed after 60 d (1954 mg kg^{-1}). A similar time-dependent decline in contaminant concentration was observed in *P*A microcosms albeit TAH residual contents after 30 d were significantly higher than those found in AIC (Fig. 3C vs. Fig. 3B). Noteworthy, the residual TAH concentrations found in 60-d-old NAIC and *P*A microcosms were significantly lower than their respective non-bioavailable fractions (*P* equal to 0.021 and 0.018, respectively) (Fig. 3B and 3C). The use of the retention time window (RTW) approach (EPA, 2003), showed that the large majority of residual TAH in 60-d-old NAIC microcosm was within the C10-C27 range with the C15-C20 fraction being the most abundant one (Table 3). Coeval 60-d-old AIC and *P*A microcosms did not significantly differ each other in their abilities to remove the hydrocarbon fractions pertinent to each RTW (Table 3); in the former and latter coeval microcosms, degradation proceeded beyond the bioavailable thresholds only for C10-C14 and C21-C27 fractions. It was not possible, instead, to estimate the F fraction for the RTW related to C28-C34 since desorption data did not reliably fit the aforementioned two-site model with large standard errors of the estimate for both F and the two first order desorption constants (k_1. and k_2).

The contaminated soil exerted a high phytotoxicity towards *L. sativum*. On average, and regardless of the microcosm type at start, percent inhibition of germinability and radical elongation amounted to 73.3 and 87%, respectively, with respect to the distilled water control. The phytotoxicity of 0- and 60-d-old NAIC microcosms did not significantly differ (Table 4).

Toxicity in 60-d-old AIC and *P*A microcosms was reduced, as inferred by the 3.5- and 3.9-fold increases in radical length, respectively. In the same microcosms, germinability also increased from 24.9 and 20.9%, respectively, to 36.6 and 35.3%, respectively (Table 4). The time-dependent detoxification in 60-d-old AIC and *P*A microcosms was confirmed by 5.1 and 6.6 increase, respectively, in the values of the cumulative IG parameter which, conversely, was not affected in NAIC.

The soil also turned out to be highly toxic towards *Folsomia candida*; in fact, an average mortality of around 90% was observed in all microcosms at start (Table 4). In NAIC, toxicity did not decrease over time; by contrast, a high extent of detoxification was found in both AIC and *P*A microcosms where mortality after 60 d incubation dropped to 11.5 and 24.5%, respectively (Table 4).

4. Discussion

One of the main technical constraints to *in situ* remediation techniques, such as bioventing, is the clayey soil texture which negatively affects intrinsic soil's permeability in the unsaturated zone (vadose) and thus the rate at which oxygen is provided to degrading organisms (EPA, 2004). In such a case, *ex situ* techniques, requiring the excavation of the contaminated soil, have to be used and the application of soil conditioners, such as lignocellulosic wastes, is often required to improve porosity and thus gas exchange rates. *Ex situ* techniques might rely either on the biostimulation of resident microbiota or on the addition of microbial inoculants; in the latter case, such an approach is termed bioaugmentation. Within this frame, there is an ever increasing recognition that microbial inoculants, based on indigenous microorganisms, are often superior to those composed of

allochthonous ones (Potin et al., 2004; Mancera-López et al., 2008). These findings have been often reported in augmentation treatments of soils when using resident fungi in comparison to culture collection strains belonging to the same species (April et al., 1998; Garon et al., 2004). In fact, the selective pressure exerted by historical contamination leads to the supremacy of species able to either tolerate or resist the contaminant load; it has been suggested that the use of resident species adapted to the target contaminated environment might enable better survival probabilities and competitive abilities to the added inoculum (Potin et al., 2004).

Based on this expectation, this study was aimed at isolating and identifying resident fungi of a clayey soil heavily and historically contaminated by aliphatic hydrocarbons. The isolates of concern were first tested in liquid cultures for their capacity of using TAH as the sole carbon source and, then, for their ability to colonize the lignocellulosic mixture selected as the soil amendment for subsequent remediation microcosms. No yeasts and basidiomycetes were found and all the isolated strains were ascomycetes mainly belonging to the genera *Fusarium*, *Pseudoallescheria* and *Metarhizium*. Interestingly, in another study conducted on the isolation of fungi from soil around flare pit sites, where petroleum wastes are burned, the large majority of isolates belonged to the phylum Ascomycota and no basidiomycetes were found (April et al., 2000). Although the isolate 53B, tentatively identified as *Metarhizium* sp., was the most efficient one in using TAH, it showed a limited ability to grow on the lignocellulosic mixture. The high efficiency of TAH use by this species is not surprising. In this respect, *Metarhizium* spp. are entomopathogenic and have the ability to use long chained alkanes located in the insect epicuticle (Pedrini et al., 2007) and this degradation trait is also found in other entomopathogenic fungi, such as *Beauveria bassiana* (Davies and Westlake, 1979; April et al., 2000). The 18A isolate, conversely, identified as *Pseudoallescheria* sp., fulfilled both selection criteria, since, besides being able to use TAH as the sole C source, turned out to be capable of fast growth on the LM. Interestingly, four *P. boydii* strains were isolated from oil-soaked soils in British Columbia and compared with other strains of the same species isolated from hydrocarbon-free matrices; such a comparison showed that only the

strains isolated from the petroleum-contaminated soil exhibited alkane-degrading ability (April et al., 1998). *P. boydii* strains were also isolated from flare pit soils and liquid culture experiments conducted with ^{14}C-hexadecane as the carbon source showed that the alkane was mineralized by this species at a greater extent than other fungi (April et al., 2000). For all these reasons, in the present study, *Pseudoallescheria* sp. 18A was selected as the best isolate to be augmented to the soil from which it had been isolated and its performance compared with those observed in the non-amended and amended incubation controls.

In the present study, *Pseudoallescheria* sp. did not profusely colonize the contaminated soil in the early weeks of incubation and its growth was initially limited to the formation of an aerial mycelium which tended to form dark brown cleistothecia. As a matter of fact, both ergosterol and 18:2 ω6,9 concentrations in 30-d-old *P*A microcosms were significantly lower than those found in coeval amended incubation control. This might be concomitantly due to both the limited growth of the inoculant and to the widely known ability of *Pseudoallescheria* spp. to produce fungistatic substances which also hampered growth of resident fungi (Ko et al., 2010). On the one hand, and with regard to the impact of *Pseudoallescheria* sp. on bacterial biota, the absence of significant differences in densities of cultivable heterotrophic and hydrocarbonoclastic bacteria with the non-inoculated and amended incubation control, suggested that the augmented fungus did not negatively affect the bacterial biota. On the other hand, the use of a cultivation-independent approach, based on PLFA profiling, showed that the growth of the resident bacterial community and relative richness and diversity were even stimulated by augmentation with *Pseudoallescheria* sp. albeit to a lesser extent than in the amended incubation control. As a matter of fact, the observed increase of specific signature markers of both Gram-positive and Gram-negative bacteria in these amended microcosms was in agreement with Shi and collaborators (2002) who claimed that concentrations of these markers were negatively correlated with hydrocarbon load. In this respect, the mere addition of the LM to the contaminated soil succeeded in biostimulating the resident microbial community, the mycobiota in particular, promoting a faster and more abundant proliferation of fungi during the

early phases of incubation than that observed in *Pseudoallescheria* sp.-augmented microcosms. With this regard, Callaham et al. (2002) attributed enhanced biological activity in response to the application of wheat straw to a hydrocarbon-contaminated soil to increased soil porosity with ensuing increase in oxygen transfer. Regardless of the alternative degradation mechanisms (i.e., mono-, di- or sub-terminal oxidation) by which aliphatic hydrocarbons are processed, the initial step of microbial attack on these compounds relies on oxygen-dependent alkane-monoxygenase systems (Stroud et al., 2007). In addition, it has been shown that decomposition of either wheat straw or other lignocellulosic wastes (e.g., maize stalks, wood bark) provides a valuable supply of readily available carbon and energy source for resident microorganisms (Lang et al., 2000) with positive impact on remediation efficiency (Steffen et al., 2007; Federici et al., 2012).

The bioavailability of organic contaminants in soil is a matter of great relevance for remediation (especially when dealing with historically contaminated soils) since this parameter has been proposed as the underlying basis for risk-assessment and setting of clean-up goals at contaminated sites (Latawiec et al., 2011; Naidu et al., 2013). Methods to evaluate pollutants release and availability include chemical and biological approaches (Stroud et al., 2007). Among these, chemical-based methods, e.g. mild extractions with organic solvents, resins (Tenax, XAD) and cyclodextrins, appear to be more rapid, precise and cheap compared to biological assays (Latawiec et al., 2011). In this respect, supercritical fluid extraction (SFE) has also been used to predict the availability of hydrophobic aromatic contaminants in soil (Hawthorne et al., 2002; Cajthaml and Sasek, 2005; Covino et al., 2010). SFE is a dynamic (sequential) desorption method unaffected by contaminant mass transfer to the water phase and it relies on the use of CO_2, which has polarity values close to that of biological lipids under supercritical conditions (Hawthorne et al., 2002). To the best of our knowledge, the present paper reports for the first time an estimation of the availability of complex aliphatic hydrocarbon mixtures in a historically contaminated soil by means of SFE. As opposed to the method 3560 (EPA, 1996), relying on the use SFE to extract petroleum hydrocarbons from soils under harsh extraction conditions (single step desorption after 30 min at 80

°C, 345 bars and CO_2 flow rate of 500-1000 ml min^{-1}), a sequential SFE process was used in the this study to determine the F fraction under "mild" conditions which were previously adopted for the same purpose for lipophilic aromatic contaminants (Hawthorne et al., 2002; Cajthaml and Sasek, 2005; Covino et al., 2010). The goodness of fit of TAH desorption data with the two-site model enabled an accurate determination of the bioavailable fraction. Noteworthy, the residual TAH amounts found in both 60-d-old AIC and *P*A microcosms were lower than their respective initial non-bioavailable fractions thus indicating that contaminant degradation exceeded the bioavailable threshold. These results are in line with those of Huesemann and collaborators (2003, 2004) showing that the easily desorbed fraction of alkanes was much less than that biodegraded thus suggesting that the degradation of these contaminants in soil did not require their previous mobilization from the solid to the aqueous phase.

Thus, this study shows that the biostimulation of the resident microbiota of a historically contaminated soil gave similar degradation outcomes to those observed with a bioaugmentation treatment relying on a fungal strain that had been isolated from the same soil. In this respect, our results contrast with those reported by Mancera-López and collaborators (2008) showing the superiority of bioaugmentation with three indigenous fungal isolates over a biostimulation approach. This discrepancy might depend on several factors, such as the matrices under study, characterized by both diverse contamination profiles and initial microbial densities as well as on the different experimental setup. For instance, although the C:N:P ratio of the present soil (100:4.1: 1.1) was imbalanced with respect to the widely accepted 100: 10: 1 optimal ratio for hydrocarbons degradation (Ward and Cutright, 1999), it was not deliberately modified by the addition of N-containing mineral solutions as it was done by Mancera-López et al. (2008). In addition, the values of the same ratio in both wheat straw and poplar wood chips (100:0.9:0.3 and 100:1:0.4, respectively) were even more unbalanced with respect to the above mentioned C:N:P reference ratio. However, their addition in sterile form resulted in the aforementioned large stimulation of the resident microbiota and in tangible TAH degradation in 30-d-old AIC microcosms. With this

regard, the addition of wheat straw as a bulking agent improved petroleum hydrocarbon removal in biopile experiments from a soil belonging to the same textural class of that used in the present study (Rojas-Avelizapa et al., 2007); a further similarity of the same study with the present one was the generalized increase in bacterial populations that also included hydrocarbonoclastic bacteria. In this respect, similar findings in relation to the activation of the resident microbiota in oil-contaminated soils upon the addition of lignocellulosic wastes were reported in other studies (Jorgensen et al., 2000; Zhang et al., 2008).

In the present study, a phytotoxicity test with the highly sensitive species *L. sativum* was used to assess the impact of biostimulation and mycoaugmentation treatments on soil detoxification; in fact, petroleum hydrocarbons are known to adversely affect the germination and growth of plants and re-vegetation of soils contaminated by fuel or oil spills is one of the goals of bioremediation. Crude oil in soil can disturb germination and emergence through the formation of a persistent film of hydrocarbons around the seeds producing a physical barrier to water and oxygen uptake (Adam and Duncan 2002). Hydrocarbons, entering seeds through the micropyle and coleorhizae, can negatively affect the assimilation of starch by inhibiting either amylase or starch phosphorylase activities (Achuba, 2006). The extent of phytotoxicity is strongly species-dependent and some higher plants, such as *Vigna unguiculata* (Isikhuemhen et al., 2003) and *Zea mays* (Ogbo et al., 2010), retained significant germination and development capacity in soils spiked with high loadings of petroleum hydrocarbons. In the present study, the pristine soil and its incubation controls proved to be highly phytotoxic to *L. sativum* and both germination and radical elongation were severely inhibited. Such phytotoxic effects were only partially removed by both the AIC and *P*A treatments with the most remarkable effect being observed on radical elongation. In this respect, these findings are in agreement with those reported by Adenipekun et al. (2013) reporting an 84% TPH reduction in soil after 60 d colonization with *Pleurotus pulmonarius* at a similar level of contaminants to those of the soil under study; despite these degradation extents, the phytotoxicity reduction of soil towards the semi woody herb *Corchorus olitorius* was only partial (Adenipekun et

al., 2013). Similarly, only a partial removal of phytotoxicity towards *Amaranthus hybridus* was obtained upon mycoaugmentation of a crude oil polluted soil with *P. pulmonarius* (Olusola and Anslem, 2010). In agreement with the present study, the most evident effect related to phytotoxicity reduction, due to mycoaugmentation of a petroleum-contaminated soil with *Pleurotus tuberregium*, was the recovery of the radical elongation capacity in *Z. mays* seedlings (Isikhuemhen et al., 2003).

F. candida, a common and widespread arthropod that occurs in soils throughout the world, was even more susceptible to the toxic effects of the contaminated soil which led to a 90% mortality. In this case, the detoxification due to either mycoaugmentation or biostimulation was made evident by the dramatic drop in mortality. In this respect, another study showed that contaminants characterized by high lipophilicity were most effective in interfering with fundamental processes, such as osmo-regulation, involved in the survival of *F. candida* (Skovlund et al., 2006). Thus, the high removals of alkanes, extremely hydrophobic compounds (Stroud et al., 2007), which were attained in biostimulated and augmented microcosms, might be the determinant for the significant drop in mortality of this test organism.

5. Conclusions

This study shows that bioremediation can be a viable approach to the clean-up and detoxification of a clay soil historically contaminated by petroleum hydrocarbons. This investigation compared site-specific bioaugmentation with a resident fungal isolate with its respective amended control. The latter treatment markedly increased density and activity of the soil microbiota leading to similar degradation and detoxification outcomes to those observed upon mycoaugmentation. In this respect, the lignocellulosic mixture might be regarded as a slow-release fertilizer which can be used in alternative to rapid-release inorganic fertilizers, the use of which can lead to a variety of inconveniences, such as leaching and toxicity to resident microbiota (Zhou and Crawford, 1995; Sarkar et al., 2005). An additional consideration, that may not be omitted, stems from the potential biohazard of the isolate 18A. It belongs to the genus *Pseudoallescheria* encompassing some

species, which like other hydrocarbon-degrading fungi (Prenafeta-Boldú et al., 2006), might act as opportunistic pathogens in immunocompromised hosts.

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References

Achuba FI. The effect of sublethal concentrations of crude oil on the growth and metabolism of Cowpea (*Vigna unguiculata*) seedlings. Environmentalist 2006; 26:17-20.

Adam G, Duncan HJ. Influence of Diesel fuel on seed germination. Environ Pollut 2002; 120:363-70.

Adenipekun CO, Ayanleye OO, Oyetunji OJ. Bioremediation of soil contaminated by spent diesel oil using *Pleurotus pulmonarius* Fries (Quelet) and its effects on the growth of *Corchorus olitorius* (L.) J Appl Biosci 2013; 68:5366-73.

April TM, Abbott SP, Foght JM, Currah RS. Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae). Can J Microbiol 1998; 44:270-8.

April TM, Foght JM, Currah RS. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. Can J Microbiol 2000; 46:38-49.

Banitz T, Johst K, Wick LY, Schamfuß S, Harms H, Frank K. Highways versus pipelines: contributions of two fungal transport mechanisms to efficient bioremediation Environ Microbiol Rep 2013; 5:211-18.

Bischoff JF, Rehner SA, Humber RA. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 2009; 101:512-30.

Boyle D. Nutritional factors limiting the growth of *Lentinula edodes* and other white-rot fungi in wood. Soil Biol Biochem 1998; 30:817–23.

Brassington KJ, Hough RL, Paton GI, Semple KT, Risdon GC, Crossley J, Hay I, Askari K, Pollard SJT. Weathered Hydrocarbon Wastes: a Risk Management Primer. Crit Rev Env Sci Technol 2007; 37:199–232.

Cajthaml T, Šašek V. Application of supercritical fluid extraction (SFE) to predict bioremediation efficacy of long-term composting of PAH-contaminated soil. Environ Sci Technol 2005; 39:8448–52.

Callaham MA Jr, Stewart AJ, Alarcón C, McMillen SJ. Effects of earthworm (*Eisenia fetida*) and wheat (*Triticum aestivum*) straw additions on selected properties of petroleum-contaminated soils. Environ Toxicol Chem 2002; 21:1658-63.

Chiu SW, Gao T, Chan CS, Ho CK. Removal of spilled petroleum in industrial soils by spent compost of mushroom *Pleurotus pulmonarius*. Chemosphere 2009; 75:837-42.

Covino S, Čvančarová M, Muzikář M, Svobodová K, D'Annibale A, Petruccioli M, Federici F, Křesinová Z, Cajthaml T. An efficient PAH-degrading *Lentinus* (*Panus*) *tigrinus* strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J Hazard Mater 2010; 183: 669–76.

Davies JS, Westlake DW 1979. Crude oil utilization by fungi. Can J Microbiol 25:146-56.

Debourgogne A, Gueidan C, de Hoog S, Lozniewsky A, Machouart M. Comparison of two DNA sequence-based typing schemes for the *Fusarium solani* Species Complex and proposal of a new consensus method. J Microbiol Meth 2012; 91:65-72.

D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl Environ Microbiol 2006;72:28-36.

EPA, Environmental Protection Agency. Supercritical fluid extraction of total recoverable petroleum hydrocarbons- Method 3560 1996; Revision 0. Available from: http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3560.pdf.

EPA, Environmental Protection Agency. Non-halogenated organics using GC-FID. Method 8015D; Revision 4 June 2003. Available from: http://www.epa.gov/osw/hazard/testmethods/pdfs/8015d_r4.pdf.

EPA, Environmental Protection Agency. How to Evaluate Alternative Cleanup Technologies for Underground Storage Tank Sites. A Guide for Corrective Action Plan Reviewers. EPA 510-R-04-002 2004. Available from: http://www.epa.gov/oust/pubs/tums.htm.

Federici E, Giubilei MA, Covino S, Zanaroli G, Fava F, D'Annibale A, Petruccioli M. Addition of maize stalks and soybean oil to a historically PCB-contaminated soil: effect on degradation performance and indigenous microbiota. New Biotechnol 2012; 30:69-79.

Frostegård Å, Tunlid A., Bååth E. Phospholipid fatty acid composition, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol 1993; 59:3605-17.

Frostegård Å, Tunlid A, Bååth E. Use and misuse of PLFA measurements in soils. Soil Biol. Biochem 2011; 43:1621-25.

Garon D, Sage L, Wouessidjewe D, Seigle-Murandi F. Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere 2004; 56:159–66.

Guillén F, Gómez-Toribio V, Martínez MJ, Martínez AT. Production of hydroxyl radical by the synergistic action of fungal laccase and aryl alcohol oxidase. Arch Biochem Biophys 2000; 383:142-7.

Hawthorne SB, Poppendieck DG, Grabanski CB, Loehr RC. Comparing PAH availability from manufactured gas plant soils and sediments with chemical and biological tests. 1. PAH release during water desorption and supercritical carbon dioxide extraction. Environ Sci Technol 2002; 36:4795–803.

Huesemann MH, Hausmann TS, Fortman TJ. Assessment of bioavailability limitations during slurry biodegradation of petroleum hydrocarbons in aged soils. Environ Toxicol Chem 2003; 22:2853–60.

Huesemann MH, Hausmann TS, Fortman TJ. Does bioavailability limit biodegradation? A comparison of hydrocarbon biodegradation and desorption rates in aged soils Biodegradation 2004; 15:261–74.

Isikhuemhen OS, Anoliefo GO, Oghale OI. Bioremediation of crude oil polluted soil by the white rot fungus, *Pleurotus tuberregium* (Fr.) Sing. Environ Sci Pollut Res 2003; 10:108–12.

Jorgensen KS, Puutinen J, Suortti AM. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 2000; 107:245–54.

Ko WH, Tsou YJ, Ju YM, Hsieh HM, Ann PJ. Production of a fungistatic substance by *Pseudallescheria boydii* isolated from soil amended with vegetable tissues and its significance. Mycopathologia 2010; 169:125-31.

Lackner M, Najafzadeh MJ, Sun J, Lu Q, G. Sybren de Hoog GS. Rapid identification of *Pseudallescheria* and *Scedosporium* strains by using rolling circle amplification. Appl Environ Microbiol 2012; 78:126–33.

Lang E, Kleeberg I, Zadrazil F. Extractable organic carbon and counts of bacteria near the lignocellulose-soil interface during the interaction of soil microbiota and white rot fungi. Bioresour Technol 2000; 75:57-65.

Latawiec AE, Swindell AL, Simmons P, Reid BJ. Bringing Bioavailability into Contaminated Land Decision Making: The Way Forward? Crit. Rev. Environ Sci Technol 2011; 41:52-77.

Mancera-López ME, Esparza-García F, Chávez-Gómez B, Rodríguez-Vázquez R, Saucedo-Castañeda G, Barrera-Cortés J. Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation–bioaugmentation with filamentous fungi. Int Biodeterior Biodegr 2008; 61:151–60

Naidu R, Channey R, McConnell S, Johnston N, Semple KT, McGrath S et al. Towards bioavailability-based soil criteria: past, present and future perspectives. Environ Sci Pollut Res 2013; doi: 10.1007/s11356-013-1617-x.

O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and Fusarium MLST. Mycologia 2012; 104:427-45.

Ogbo EM, Tabuanu A, Ubebe R. Phytotoxicity assay of diesel fuel-spiked substrates remediated with *Pleurotus tuberregium* using *Zea mays*. Int J Appl Res Nat Prod 2010; 3:12-6.

Olusola SA, Anslem EE. Bioremediation of a crude oil polluted soil with *Pleurotus pulmonarius* and *Glomus mosseae* using *Amaranthus hybridus* as a test plant. J Bioremed Biodegrad 2010; 1:113.

Pedrini N, Crespo R, Juárez MP. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comp Biochem Physiol C Toxicol Pharmacol 2007; 146:124-37.

Potin O, Rafin C, Veignie E. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. Int Biodeterior Biodegr 2004; 54:45–52.

Prenafeta-Boldú FX, Kuhn A, Luykx DMAM, Anke H, Van Groenestijn JW, De Bont JAM. Isolation and characterization of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. Mycol Res 2001; 105: 477–84.

Prenafeta- Boldú FX, Summerbell R, de Hoog GS. Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? FEMS Microbiol Rev 2006; 30: 109-130.

Rojas-Avelizapa NG, Roldán-Carrillo T, Zegarra-Martínez H, Muñoz-Colunga AM, Fernández-Linares LC. A field trial and ex-situ bioremediation of a drilling mud-polluted site. Chemosphere 2007; 66:1595–1600.

Sampedro I, Cajthaml T, Marinari S, Petruccioli M, Grego S, D'Annibale A. Organic matter transformation and detoxification in dry olive mill residue by the saprophytic fungus *Paecilomyces farinosus*. Process Biochem 2009; 44:216–25.

Sarkar D, Ferguson M, Datta R, Birnbaum S. Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ Pollut 2005; 136: 187-95.

Šašek V, Bhatt M, Cajthaml T, Malachová K, Lednická D. Compost-mediated removal of polycyclic aromatic hydrocarbons from contaminated soil. Arch Environ Contam Toxicol 2003; 44:336–42.

Shi W, Bischoff M, Turco R, Konopka A. Association of microbial community composition and activity with Pb, Cr and hydrocarbon contamination. Appl Environ Microbiol 2002; 68:3859-66.

Steffen K, Schubert S, Tuomela M, Hatakka A, Hofrichter M. Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi. Biodegradation 2007; 18:359–369.

Stroud JL, Paton GI, Semple K. Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. J Appl Microbiol 2007; 102:1239-53.

Skovlund G, Damgaard C, Bayley M, Holmstrup M. Does lipophilicity of toxic compounds determine effects on drought tolerance of the soil collembolan *Folsomia candida*? Environ Pollut 2006; 144:808-15.

Tunlid A, White DC. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Stotzky G, Bollag JM, editors. Soil Biochemistry. New York: Marcel Dekker; 1992. p. 229-62.

Ward E, Cutright JT, The investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil. Int Biodeterior Biodegr 1999; 44:55–64.

Williamson DG, Loehr RC, Kiumura Y. Release of chemicals from contaminated soils. J Soil Contam 1998; 7:543–58.

Wrenn BA, Venosa AD. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a Most-Probable-Number procedure. Can J Microbiol 1996; 42:252-8.

Zhang K, Hua X-F, Han H-L, Wang J, Miao C-C, Xu Y-Y, Huang Z-D, Zhang H, Yang J-M, Jin W-B, Liu Y-M, Liu Z. Enhanced bioaugmentation of petroleum- and salt-contaminated soil using wheat straw. Chemosphere 2008; 73:1387–92.

Zhou E, Crawford R. Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. Biodegradation 1995; 6:127-40.

Table 1. Amounts of phospholipid fatty acids (PLFA), expressed as pmoles g^{-1}, and indices thereof derived in the pristine soil at start and in 60-d-old non-amended or amended soil incubation controls (NAIC and AIC, respectively) or *Pseudoallescheria* sp.-augmented (*P*A) soil microcosms.

Parameter	Pristine soil	NAIC	AIC	*P*A
14:0	1.1±0.6a	1.3±0.1a	4.4±0.7b	0.8±0.0a
15:0	0a	0a	3.3±0.4c	1.8±0.1b
16:0	25.6±1.6a	31.2±3.4a	103.7±2.8c	54.6±1.9b
17:0	0a	0a	1.9±0.3b	0.3±0.0a
18:0	7.4±1.2a	9.8±1.3a	16.3±0.9b	9.5±0.1a
i 14:0	0a	0a	1.8±0.3b	2.1±0.3b
i 15:0	0a	0a	19.9±2.3c	4.0±0.3b
a15:0	0a	0a	27.3±2.7c	3.8±0.10b
i 16:0	0a	0a	20.9±2.0c	2.8±0.1b
i 17:0	0a	0a	3.8±0.6b	0.7±0.0a
a 17:0	0a	0a	11.2±1.8c	2.8±0.1b
10Me 18:0	2.6±0.7b	3.5±0.7b	0a	0a
16:1ω7c	0a	0a	6.7±1.0c	2.2±0.2b
Cy17:0	0a	0a	4.7±0.5c	1.6±0.0b
18:1ω7c	0a	0a	6.5±0.8c	3.4±0.1b
Cy 19:0	0a	0a	15.0±0.9c	5.14±0.3b
18:1ω9†	9.4±1.6a	18.3±1.6ab	64.4±12.2c	34.2±0.2b
18:2ω6,9	5.6±0.9a	6.1±0.8a	95.7±9.1c	44.7±0.6b
20:0	0a	0a	1.2±0.2c	0.4±0.0b
Total PLFA	51.7±8.9a	70.2±11.5a	408.8±48.6c	174.7±4.1b
Total bacteria (TB)	36.7±4.1a	45.8±6.7a	247.4±18.0c	95.5±3.3b
Gram(+)	2.6±0.7a	3.5±0.7ab	84.9±9.7c	16.1±0.6b
Gram(-)	0a	0a	32.9±3.2c	12.3±0.6b
G(+)/G(-) ratio	n.c.	n.c.	2.58	1.31
Fungi/TB	0.15±0.02a	0.13±0.02a	0.39±0.03b	0.47±0.01c
Shannon Weaver index	1.36±0.04a	1.39±0.05a	2.18±0.02c	1.94±0.01b

†This PLFA was not assigned to any specific group (Frostegård et al., 2011). Data are mean ± standard deviation of 3 biological replicates. Multiple pair-wise comparisons of row means were carried out by the post-hoc Tukey test and same lowercase letters denote lack of statistically significant differences ($P<0.05$) among means.

Table 2.

Maximal extracellular glycosyl hydrolase (*i.e.*, endo-β-1,4-glucanase, cellobiohydrolase and endo-β-1,4-xylanase) and ligninolytic (*i.e.*, laccase and MnP) activities detected in non-amended and lignocellulose-amended non-inoculated control soil (NAIC and AIC, respectively) and in *Pseudoallescheria* sp.-augmented soil. Numbers within round brackets indicate the time (days) at which maximal activity was attained.

Microcosm	Glycosyl hydrolase activities†			Ligninolytic Enzymes activities†	
	Endo-β-1,4-glucanase (mU g^{-1} soil)	Cellobiohydrolase (mU g^{-1} soil)	Endo-β-1,4-xylanase (mU g^{-1} soil)	Laccase (mU g^{-1} soil)	MnP (mU g-1 soil)
NAIC	n.d	n.d	n.d	n.d	n.d
AIC	90.9±10.6 (60)[b]	46.5±2.8 (60)[b]	49.1±11.8 (60)[b]	8.4±1.0 (30)[a]	n.d.
Pseudoallescheria sp.	27.8±2.6 (60)[a]	19.6±2.9 (60)[a]	23.8±7.9 (60)[a]	13.3± 3.8 (30)[a]	n.d.

†Data are means ± standard deviation of 6 replicates (3 parallel experiments, each one assayed twice).

Table 3.

Concentration (mg kg^{-1}) and percent bioavailability of aliphatic hydrocarbons pertinent to different retention time windows (RTW) in the non-amended incubation control (NAIC) after 60 d of incubation and percent degradation of the same fractions in coeval amended incubation control (AIC) and in the *Pseudoallescheria* sp.-augmented (*P*A) microcosms

Aliphatic fractions†	RTW (min)	Extractable aliphatic hydrocarbons in NAIC (mg kg^{-1})	Bioavailable fractions† (%)	Degradation of extractable aliphatic hydrocarbons § (%)	
				AIC	*P*A
C10-C14	5.585-9.765	3155.1±47.6	72.3±3.4	99.1±0.1A***	98.9±0.2A***
C15-C20	9.765-13.578	5493.6±36.9	73.5±4.1	69.0±0.6A	69.2±2.4A
C21-C27	13.578-18.040	1230.2±43.1	69.5±2.1	76.3±1.4A**	74.3±2.5A*
C28-C34	18.040-29.755	156.0±41.5	n.d.	73.4±2.6A	76.0±6.6A

†Aliphatic fractions within each relative RTW and respective bioavailable fractions were quantified as reported in Subsections 2.2 and 2.3, respectively. §Degradation percentages are mean ± standard deviation of 3 parallel experiments and same uppercase letter denote absence of statistically significant differences between row means, as assessed by the post-hoc Tukey test ($P<0.05$). Pair-wise comparisons between bioavailable fractions of each aliphatic fraction and respective percent degradation were performed by a Student's t-test and the following notation was used to indicate significant differences: *, $0.01\leq P<0.05$; **, $0.001\leq P<0.01$; ***, $P<0.001$.

Table 4.

Germinability of *Lepidium sativum* L. seeds, radicle lengths and index of germination and Collembola mortality (%) in the presence of non-amended and lignocellulose-amended non-inoculated soil controls (NAIC and AIC, respectively) and in *Pseudoallescheria* sp.-augmented soil (*P*A)at start (T_o) and after 60 d incubation (T_f).

Treatment	Germinability † (%)		Radicle length † (mm)		Index of germination, IG‡		Collembola mortality (%)	
	T_o	T_f	T_o	T_f	T_o	T_f	T_o	T_f
NAIC	22.4±2.8aA	25.1±3.1aA	2.4±0.6aA	2.7±0.9aA	0.031	0.040	92.3±4.4aA	89.7±1.7aC
AIC	24.9±2.4aA	36.6±4.2bB	2.6±0.8aA	9.1±3.7bB	0.038	0.196	91.1±3.8bA	11.5±5.4aA
*P*A	20.9±4.1aA	35.3±12.1bB	2.9±1.5aA	11.3±4.7bB	0.036	0.234	94.6±2.8bA	24.5±7.7aB

† Percent germinability of *L. sativum* L. seeds and radicle length in distilled water control were 85.0±4.2% and 20.0±6.8 mm, respectively; ‡ IG values were calculated as reported elsewhere (D'Annibale et al., 2006); Data are means ± standard deviation of 12 replicates (three parallel experiments each one assayed in quadruplicate); Same lowercase and uppercase letters above bars denote lack of statistically significant differences ($P<0.05$) among row and column means, respectively, as assessed by the post-hoc Tukey test.

Figure captions

Fig. 1.

Concentration of cultivable heterotrophic (A) and specialized (B) bacteria and ergosterol content (C) in non amended and lignocellulose-amended non-inoculated soil controls (NAIC and AIC, respectively) and in *Pseudoallescheria* sp.-augmented (*P*A) soil after 0, 30 and 60 d incubation. Data are mean ± standard deviation of three parallel experiments. Statistical pair-wise multiple comparisons of data (two way ANOVA) were carried out by the post-hoc Tukey test. Same uppercase and lowercase letters above bars denote lack of statistically significant differences ($P<0.05$) among means within the same treatment type as a function of time and among different treatments at coeval harvests, respectively.

Figure 2.

Relative contaminant fraction retained in soil after sequential supercritical fluid extraction with carbon dioxide in the 5-200 min time range: The inset shows the values of the two first order rate constants (k_1 and k_2) and the fast released F fraction. Arabic numbers within round brackets are their respective standard errors of the estimate. Some goodness of fit parameters, such as the coefficient of determination adjusted by the degrees of freedom (R^2_{adj}), the Fisher-Snedecor's coefficient (F_{reg}) and significance level (P) of the regression are also shown.

Figure 3.

Residual concentrations and respective initial non-bioavailable fraction (NBF) of total aliphatic hydrocarbons in non amended and amended soil incubation controls (A and B, respectively) and in the same soil augmented with *Pseudoallescheria* sp. (C) after 0, 30 and 60 d incubation. Residual concentrations and NBF data are means ± standard deviation of 3 and 4 replicates, respectively. Statistical pair-wise multiple comparisons of residual TAH concentrations (two way ANOVA) were

carried out by the post-hoc Tukey test. Same uppercase and lowercase letters above bars denote lack of statistically significant differences ($P<0.05$) among means within the same treatment type as a function of time and among different treatments at coeval harvests, respectively. The presence of an asterisk indicates that residual TAH concentrations were significantly ($P<0.05$) lower than NBF as assessed by the Student's t test.

Figure 1

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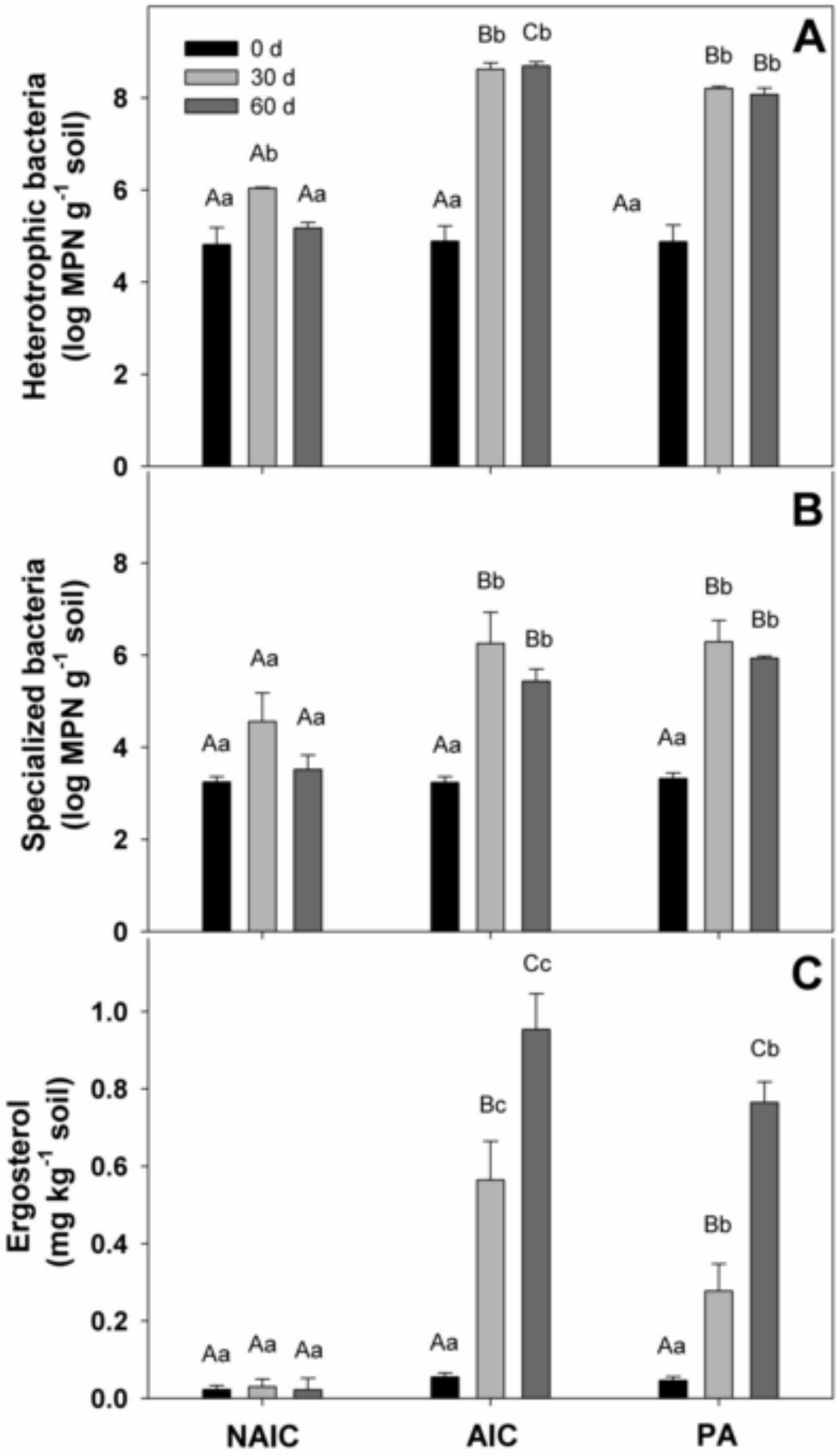

Figure 2

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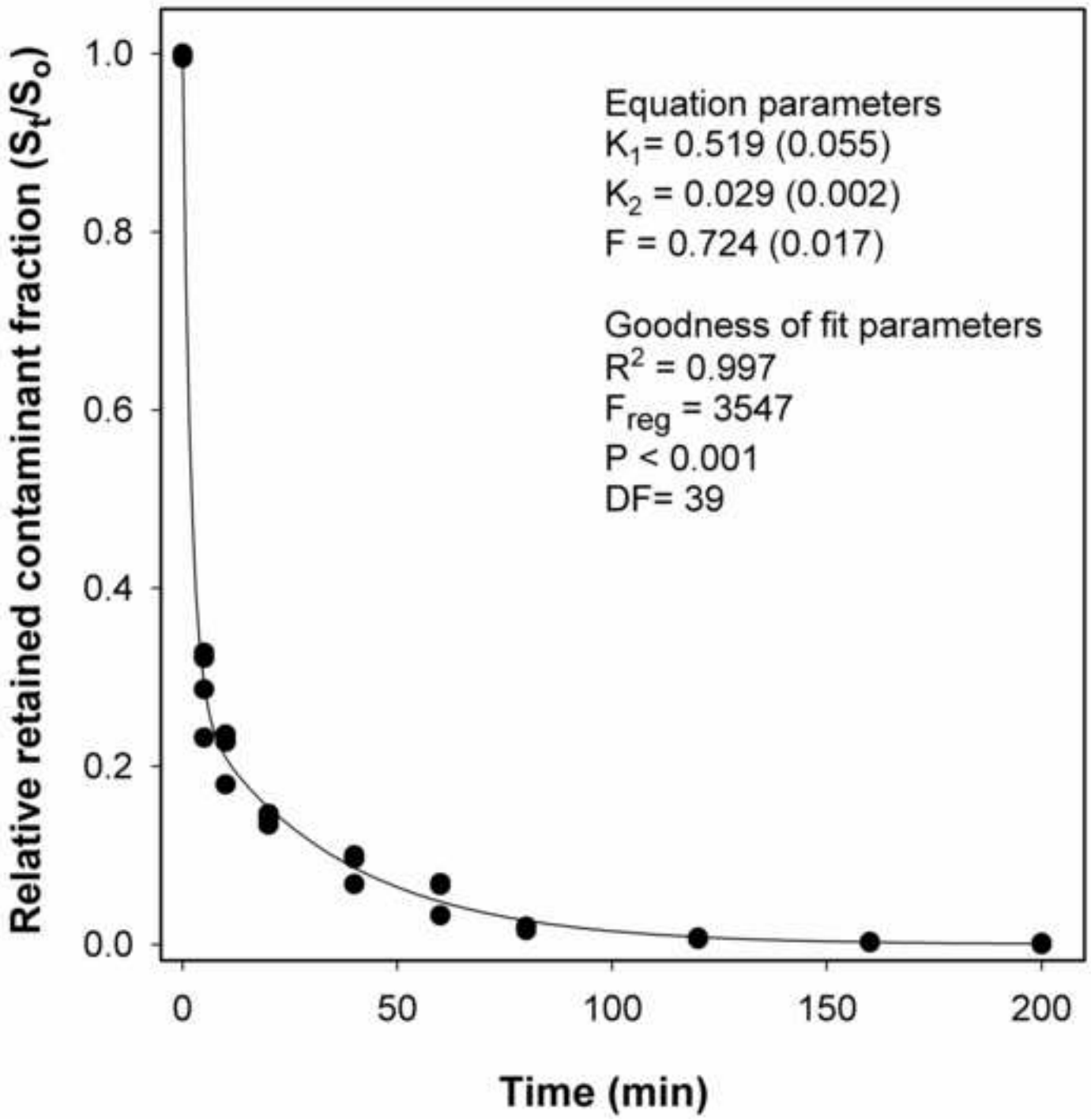

Figure 3

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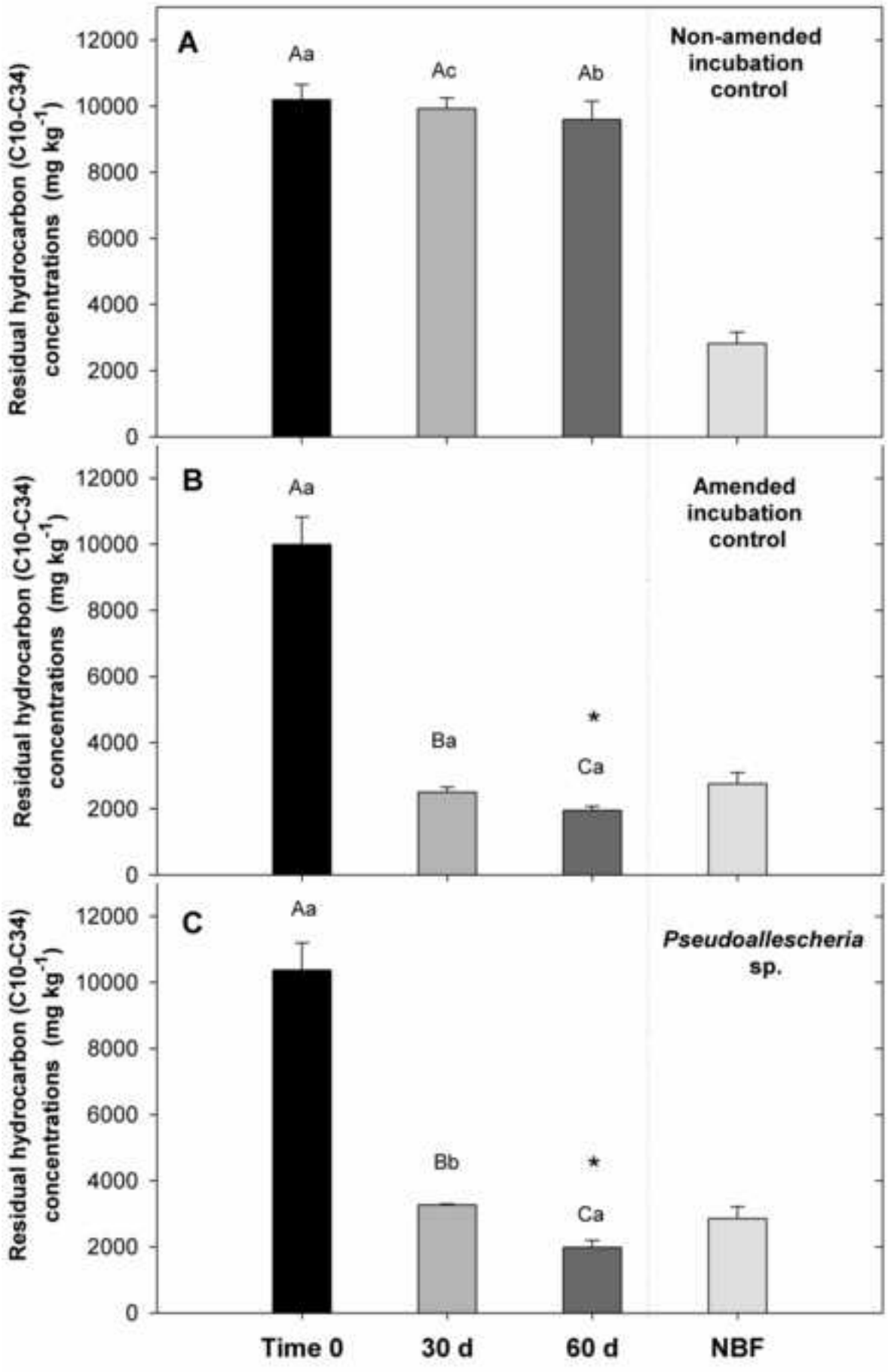

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