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

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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 method relying on SFE with CO_2.
- Biodegradation extents of AH exceeded their respective bioavailable fraction.

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ABSTRACT

The present work was aimed at isolating and 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, the 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 chip (70:30, w/w) lignocellulosic mixture (LM) selected as the amendment for subsequent soil remediation microcosms. After a 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.

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 the 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 enables them to reach inaccessible soil regions and their hyphal network confers them the ability of acting as spreading vectors of pollutant-degrading bacteria (Banitz et al., 2013). These favorable

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properties are often associated with their ability to grow 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-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 the remediation purposes, prior to the preparation of the 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 acidities 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 hydrocarbon (TAH) content was 10,200 ± 456 mg kg^{-1} soil. BTEX and total polycyclic aromatic hydrocarbon 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 an 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 (TAHs), 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). The injector and flame ionization detector were operated at 300 °C 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 °C was followed by a temperature ramp of up to 300 °C (at a rate of 20 °C min^{-1}), the latter temperature being held for an 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, the 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 contaminant 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 a 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 and 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 modeled by an empirical two-site model, consisting of the two first-order Eq. (1):

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

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 was 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 of incubation on a