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PAH biodegradation by telluric saprotrophic fungi isolated from aged PAH-contaminated soils in mineral medium and historically contaminated soil microcosms

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Abstract

Purpose Remediation of contaminated soils is of high relevance considering losses of this limited resource in most countries through erosion or through destruction for societal purposes. Most physicochemical remediation techniques lead to soil destruction avoiding reuse of the sites and loss of soil functions including ecological services like water retention/filtration, plant needs, carbon sequestration, or atmosphere restoration. This study aims to find out efficient telluric PAH-degrading fungi to both remediate soils and preserve their functioning.

Materials and methods Fifty telluric saprotrophic fungi were thus isolated from different aged PAH-contaminated soils sampled from four brownfields in the North of France. Thirty of these isolates were screened in a mineral medium for their ability to degrade benzo[a]pyrene (BaP) used as a model of HMW PAH. After 9 days of incubation, the remaining BaP was quantified through HPLC analysis. Then, a set of microcosms was performed with an aged PAH-contaminated non-sterile soil encompassing different approaches with bioaugmentation using mycelia of three strains pre-established on expanded clay particles and, for one of these strains, biostimulation strategies including nitrogen or nitrogen/phosphorous supplementations or aeration by stirring. After an incubation time of 30 days, remaining PAH were quantified through GC-MS and evolution of fungal populations evaluated through qPCR.

Results and discussion Isolated *Penicillium canescens*, *Cladosporium cladosporioides*, *Fusarium solani*, and *Talaromyces helicus* degraded more than 30% of the initial 500 μg of BaP after 9 days of incubation. The three latest strains were thus inoculated in aged PAH-contaminated soil microcosms. After 30 days, PAH quantitative analyses showed that the highest degradation was obtained by bioaugmentation with *T. helicus* (26% of the initial total PAH content: 321.7 mg kg^{-1}), which seems high considering an aged industrial contamination. Biostimulation approaches coupled to the inoculation of this strain did not improve the degradation. DNA quantification of the inoculated species confirmed their enrichment in the soil revealing the interest of the used inoculation strategy. We discuss bioaugmentation and biostimulation approaches in the case of the considered pollutants and soil.

Conclusions A strain identified as *T. helicus* appears interesting for its HMW PAH degradation capacities both in mineral medium with pure BaP and in industrial non-sterile soil microcosms contaminated with a hydrocarbon complex mixture. This study also confirms the efficiency of a well-studied BaP-degrading strain of *F. solani*. These results indicate the potentialities of the used bioaugmentation approach and underline the necessity of scale-up strategies to apply this kind of technique on site.

Keywords Polycyclic aromatic hydrocarbons (PAH) · Soil bioremediation · Soil conservation · *Talaromyces helicus* · Telluric saprotrophic fungi

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1 Introduction

Characteristic times of formation or resilience of many ecologically and agriculturally soil properties correspond with much larger timescales than human lives or even than some civilizations. Indeed, natural ecosystems are too slow to produce modifications required because of their exploitation by modern society to provide expected products and services leading to temporal limitations. Besides, with the world human population growth, space limitations of pedosphere with required thickness and area are also being reached (Targulian and Arnold 2008). Soil pollution belongs to soil chemical deteriorations, which are one of the main causes of global soil degradation with erosion and physical deteriorations (Oldeman et al. 1991).

Soil contaminations represent a wide range of problems in Europe. The data collected by the European Soil Data Centre (ESDAC) through the European Environment Information and Observation Network for Soil (EIONET SOIL) showed that there would be 342,000 contaminated sites and more 2.5 million potentially contaminated sites over 38 European countries (Panagos et al. 2013). As a result, requalification of brownfields is one of the priorities in the European Programme for Sustainable Urban Development (URBACT) since 2002. Polycyclic Aromatic Hydrocarbons (PAH) represent a bit less than 11% of soil contaminations in Europe (Panagos et al. 2013). The persistence of these molecules in the environment can be explained through the chemical stability of conjugated aromatic rings and the low water solubility of the non-polar structure decreasing consequently their bioavailability (Couto et al. 2010). The PAH recalcitrance to biodegradation increases with the aromatic ring numbers (Harmsen and Rietra, 2018) which led to distinguish low molecular weight PAH (LMW PAH with less than 4 rings) to high molecular weight ones (HMW PAH with 4 rings or more). The remediation of PAH-contaminated sites is a real challenge because of the persistence and the toxicity of such contaminants in the environment.

The idea of "soil recycling" instead of disposal has been included in official regulations such as the Directive 2008/1/EC concerning integrated pollution prevention and control (EU (European Union), "Directive 2008/1/EC of the European Parliament and of the Council of 15 January 2008 concerning integrated pollution prevention and control," (29-01-2008), 2008). Bioremediation is a sustainable soil-conserving remediation alternative to the physicochemical techniques, which is based on the capacity of microorganisms to degrade organic molecules and has been relied upon since the late 1980s. As part of a research project conducted in the Région Hauts-de-France, we investigated the development of such a suitable bioremediation technology according to criteria such as environmental friendliness, pre-existing scientific knowledge available in the literature and obtained during our previous researches. A short incubation time, comparable with those used in physicochemical treatments, was also an important criterion selected in this study.

Fungal degradation processes have been of interest for over 30 years since they were discovered to cause decay in wooden utility poles despite their creosote treatment. Concerning HMW PAH, numerous studies focused on the capacity of white rot fungi (WRF) to unspecifically degrade aromatic rings through extracellular enzyme production and activity. Despite the high PAH degradation capacities under controlled conditions, these ligninolytic strains' abilities were shown to be limited in soils due to their lack of adaptation to this environment (Marco-Urrea et al. 2015). Moreover, the use of WRF in soils requires the concomitant addition of lignocellulosic materials in order to improve their competition ability with the resident microbiota. Our team focused on telluric saprotrophic fungal strains, which could be another alternative solution especially for HMW PAH. These fungi were already shown to degrade efficiently PAH (Guiraud et al. 2003; Potin et al. 2004a; Atagana et al. 2006; Rafin et al. 2013; Marco-Urrea et al. 2015) with levels comparable to some ligninolytic strains (Colombo et al. 1996; Veignie et al. 2002). However, supplementary studies are required to confirm the degradation capacities of these telluric saprotrophic fungi in soils in particular in non-sterile ones provided by historical polluted sites. Aged contaminations, generally composed of a mixture of contaminants, were already shown to better resist biodegradation than spiked soils (Semple et al. 2007) in particular due to the adsorption of aged compounds to organic matter and clay, which restrains the pollutant bioavailability for microorganisms living in the aqueous phase (Mihelcic et al. 1993).

The objectives of this study were firstly to build a collection of telluric saprotrophic fungal strains isolated from different industrial aged PAH-contaminated soil samples collected in the Région Hauts-de-France, France. Secondly, the strains were tested in glucose supplemented mineral medium for their ability to degrade Benzo[a]pyrene (BaP) as a high molecular weight (HMW) PAH model in order to select the highly efficient strains for BaP degradation. The best previously screened fungi were then introduced in an aged gasworks polluted soil in a microcosms assay at the laboratory scale. Under these conditions, the introduced strain competes with indigenous organisms and the ability to reduce pollution might be reduced with time. Thus, finding out an appropriate way to increase the competitiveness and improve the activity of the screened strains in reality is very essential. Two basic approaches were tested in parallel: biostimulation (introducing suitable amounts of water, nutrients, and oxygen into the soil in order to enhance the activity of indigenous degraders) and bioaugmentation (introduction of PAH-degrading microorganisms) into the soil. Inoculation of either *Cladosporium cladosporioides*, or *Talaromyces helicus*, or *Fusarium solani* S19 in the presence of active native microbiota allowed to

investigate the impact of the bioaugmentation with telluric saprotrophic fungi coupled or not to biostimulation strategies on the PAH biodegradation in an aged polluted industrial site during 2 months of incubation.

2 Materials and methods

2.1 Chemicals

Dichloromethane (DCM) and methanol (MeOH) were reagent grade of the highest purity available from Merck (Darmstadt, Germany). BaP was purchased from Acros Organics (Noisy-Le-Grand, France). A 16 EPA-PAH standard kit was purchased from Restek (Evry, France). Rose Bengal was obtained from Sigma-Aldrich (St Louis, USA).

2.2 Media

The mineral salt medium (MM) consisted of (g l^{-1}): KCl, 0.25; $NaH_2PO_4{\cdot}2H_2O$, 1.544; $Na_2HPO_4{\cdot}2H_2O$, 0.008; $MgSO_4$, 0.244; NH_4NO_3, 1; and trace-element solution consisting of (mg l^{-1}): $ZnSO_4{\cdot}7H_2O$, 1; $MnCl_2{\cdot}H_2O$, 0.1; $FeSO_4{\cdot}7H_2O$, 1; $CuSO_4{\cdot}5H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.1; and MoO_3, 0.2. The culture medium was about pH 5.5. The solid medium A consisted of (g l^{-1}) the following: glucose, 10; nutrient broth, 5; NaH_2PO_4 $2H_2O$, 1.015; KCl, 0.544; $MgSO_4$, 0.5; agar, 20; rose Bengal, 66.67. Distilled deionized water was used throughout this work. Malt yeast extract agar (MYEA) medium was used for routine growth.

2.3 Soil sampling

Historically PAH-contaminated soils were sampled on four former industrial sites in the North of France in 2012, labeled sites 1 to 4. These sites differed from their former industrial activity. Sites 1 and 3 were localized on the workshop Union site located at Roubaix, Tourcoing, and Wattrelos and belong to the French network SAFIR (http//www.safir-network.com/site-de-lunion) in northern France and were respectively a former bus depot (site 1, Roubaix) and an old manufacturing gasworks site (site 3, Roubaix). Site 4 was a wood treatment plant (site 4, Chambly). For confidential reasons, the origin of site 2 was not indicated. Samples were taken either using mechanical techniques (Plexiglas liners driving in or auger boring) or manual auger at superficial depths (0–2 m). A composite sample of the surface horizon (0–15 cm) made of at least five random samplings (approximately 5 kg) was constituted. Soil samples were air-dried, sieved with a 2-mm test sieve, homogenized, and stored aerobically at 4 °C in the dark until use.

2.4 Soil physicochemical properties

The soils' physicochemical properties were determined by the Laboratory of Soil Analysis INRA (Arras, France) according to French standards NF X 31-107 (granulometry), NF ISO 10694 (organic carbon), NF ISO 13878 (total nitrogen), NF ISO 10390 (pH) and NF X 31-130 (cationic exchange capacity). Table 1 shows the main physicochemical characteristics of the soil material from site 3, used for microcosm essays. The soils showed a total PAH contamination of about 1.5, 289.0, 321.7, and 1401.8 mg kg^{-1} soil respectively for soils 1, 2, 3 and 4 (Table 2). Site 1 has been selected as uncontaminated soil material obtained from a bus depot in Roubaix. Site 3 was used in microcosm experiments. For this purpose, water content toward moistening was determined and adjusted using a kaolin box with an underpressure of − 150 hPa as described by Schroll et al. (2006). It was recognized as optimal water content for xenobiotics degradation according to Schroll et al. (2006) previous results. A portion of the soil was prepared according to the standard NF ISO 11464 (sieving at 2 mm or milling) and 20 g were submitted to PAH extraction using Soxhlet extractor with dichloromethane as the solvent over 17 h. PAH detection occurred through GC/MS measurements (Triple Quadrupole GC/MS – GC: Varian 3800/MS: Varian 1200).

2.5 Isolation and identification of autochthonous fungi

Two different isolation techniques were selected in order to improve the diversity of the isolated fungal strains: (i) the direct inoculation technique (DIT) described by Waksman (1916), known for preferentially selecting metabolically

Table 1 Physicochemical characteristics of soil material from site 3

	Site 3 Value	Classification
pH[a]	8.9	Strongly alkaline
Organic matter content (g kg^{-1})[b]	107.0	High
Carbon (g kg^{-1})[b]	61.8	High
Nitrogen (g kg^{-1})[b]	1.3	Low
C/N[c]	49.2	Carbon excess
CEC (cmol kg^{-1})[b]	10.2	Low
Texture (%)	31.6	Fine silt
	25.0	Coarse silt
Optimal water content (%)[c]	25.5	Moderate
Total PAHs content (mg.kg^{-1})[c]	321.7	High

[a] Soil Survey Division Staff of U.S. Department of Agriculture (1993)

[b] Martin and Nolin (1991)

[c] Internal scales

Table 2 PAH and carbon contents of soils of origin and phylogenetic classification of the isolated and morphologically/biomolecularly identified fungal strains

Site	PAH content (mg kg^{-1})	Carbon (g kg^{-1})	C/N	No. of strain	Order	Family	Identification
1	1.5	14.8	26.6	4	Eurotiales	Trichocomaceae	*Penicillium canescens* Sopp
				5	Capnodiales	Davidiellaceae	*Cladosporium cladosporioides* aggr.
				7	Eurotiales	Trichocomaceae	*Penicillium roqueforti* Thom
2	289.0	78.6	61.5	12	Hypocreales	Nectriaceae	*Fusarium solani* (Martius) Saccardo
3	321.7	61.8	49.2	18	Eurotiales	Trichocomaceae	*Talaromyces helicus* (Raper & Fennell) C.R. Benjamin
				19	Hypocreales	Nectriaceae	*Fusarium oxysporum* aggr.
				20	Sordariales	Chaetomiaceae	*Humicola sp.*
				21	Hypocreales	Bionectriaceae	*Bionectria ochroleuca* (Schweinitz) Schroers & Samuels Anam. *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams
				22	Capnodiales	Davidiellaceae	*Cladosporium bruhnei* Linder
				24	Capnodiales	Davidiellaceae	*Cladosporium sp.*
				26	Hypocreales	Hypocreaceae	*Hypocrea lixii* Patouillard Anam. *Trichoderma harzianum* Rifai
				27	Eurotiales	Trichocomaceae	*Penicillium chrysogenum* Thom
4	1401.8	14.4	32.7	30	Mortierellales	Mortierellaceae	*Mortierella alpina* Peyronel

active strains in soils, and (ii) the suspension dilution one (SDT) derived from Waksman (1927) rather favoring the isolation of sporulating strains. For DIT, soil particles with a dimension between 1 and 2 mm were deposited on agar plates of solid medium A (derived from Chesters and Thornton, 1956). After 24 h of incubation in the dark at 22 °C, hyphal tips emerging from soil particles were firstly spotted under a binocular (Motic Microscopes, VWR, Pessac, France), and after 48 h of incubation, spotted hyphal tips were transferred on MYEA using a microscalpel. Concerning the SDT, 5 g of dry weight soil was suspended in 100 ml of 0.05% agar-containing sterile deionized water in a 500-ml Erlenmeyer flask and then shaken for 20 min on a reciprocating shaker (Infors, 90 rpm). After letting solid particles settle down for 30 min, serial dilutions were prepared and 0.1 ml of each dilution was spread homogeneously on the surface of MYEA agar plates containing 200 mg l^{-1} of chloramphenicol. After 6 days of incubation at 22 °C under a photoperiod of 12 h/12 h, hyphal tips of representative isolates were transferred to MYEA and replated until pure cultures were obtained. Identification of the isolated fungal strains occurred through morphological and molecular characterization. The description of the morphology was carried out through macroscopic observations based on the appearance and the growth velocity of pure cultures on MYEA and on microscopic observations of mycelia and sporulation using stereomicroscopy and contrast-phase microscopy. These morphological observations were used to have a first insight of the diversity of the obtained strain collection on the basis of the literature data (Index Fungorum, 2018; Mycobank, 2018; Seifert et al. 2011) in order to select strains with the highest phylogenetic diversity along the screening steps. For 13 selected isolates, molecular identification was carried out by BCCM™/MUCL (Louvain-la-Neuve, Belgium) based on sequencing ITS region, elongation factor gene, or β-tubulin gene, in complement of the macro- and micro-morphological features of pure cultures.

2.6 Benzo[a]pyrene degradation in MM

Thirteen isolated strains were tested for their ability to degrade BaP used as a model of HMW PAH in MM. The model strain S19 *Fusarium solani* (Rafin et al. 2000) was previously isolated from petroleum-contaminated soil in 1999 and investigated in previous studies by Rafin et al. (2000, 2006, 2013) and Veignie et al. (2002, 2004). Stock cultures were maintained on MYEA slants at 18 ± 1 °C in our collection and transferred to MYEA plates at 25 °C for use as inocula. An appropriate volume of an acetone-based BaP stock solution was transferred into each 250-ml Erlenmeyer flasks to get a final amount of 500 μg per flask. After solvent evaporation, 25 ml of MM were added per flask and then sterilized (121 °C for 20 min). Glucose was added after sterilization as a carbon source at a final concentration of 20 g l^{-1}. To assess biological degradation by each isolate, inoculation was performed by either a spore suspension (final concentration of 10^4 spores ml^{-1}) or 5 agar plugs of 7 mm in diameter for the non-sporulating

strains. All treatments were incubated at room temperature in the dark for 9 days on a reciprocating shaker. In order to evaluate adsorbed BaP on fungal hyphae and abiotic degradation (extraction controls), Erlenmeyer flasks containing 10 ml of MM without BaP were inoculated with each isolate. At the end of the experiment, these obtained cultures (mycelia + filtrate) were suspended in 15 ml MM with BaP (500 μg per flask) and were stirred for 4 h on a reciprocating shaker at 4 °C (Potin et al. 2004a). These treatments allowed us to determine the adsorption processes on hyphae and abiotic degradation (Barclay et al. 1995) used for further calculations of BaP degradation. All treatments were undertaken in triplicates.

2.7 BaP extraction and analytical procedure

At the end of the incubation, cultures were lyophilized for 4 days (Alpha 2-4 LD plus, Bioblock Scientific). Flasks containing total lyophilized cultures were introduced into a Soxhlet apparatus and extracted for 17 h with DCM. Organic fractions were concentrated in 20 ml DCM/MeOH (50:50, *v*/*v*). BaP concentrations were determined using a HPLC Waters 600 control system fitted with a Waters XTerra®, RP18, 5 μm, and a Waters 996 Photo Diode Array Detector. The separation was achieved with a 10-min isocratic condition of acetonitrile/water (90:10, *v*/*v*) at a solvent flow rate of 1.0 ml min^{-1}. Concentrations were determined by UV absorbance at 254 nm. The percentage of BaP degradation was given by the formula: $[(m_{Ec} - m_T / m_{Ec}] \times 100$, in which m_{Ec} was the quantity of BaP recovered in extraction controls (conducted to detect adsorbed BaP on fungal hyphae) and m_T was the quantity of BaP obtained in each treatment. Results were expressed as mean value ± SE for three replicates. Statistical analysis was performed by a two-sample it test comparing treatments between them (at 99% confidence).

2.8 Soil microcosms

All the microcosm treatments were conducted with soil samples provided from site 3. Each microcosm was set up in 250-ml brown glass flasks containing 120 g of dry mass equivalent of soil. Three scenarii were studied.

1. For bioaugmentation experiments, microcosms were inoculated with one fungal strain: either *Cladosporium cladosporioides* 5 isolated from uncontaminated soil collected in site 1, or *Talaromyces helicus* 18 isolated from historically contaminated soils collected in site 3, or *Fusarium solani* S19 as our model strain. Each treatment was called according to the identification number of the inoculated strain: T5, T18, and T19. Cultures were maintained on MYEA slants at 18 °C and subcultured every 3 months before inoculum preparation. Inoculum preparation started by the establishment of the mycelium of each strain on a carrier material through 10 days culture in 25 ml MM with 20 g l^{-1} of glucose in the presence of expanded clay particles (Seramis®) on a reciprocating shaker (110 rpm). Inoculation of each pre-culture was performed by adding a spore suspension at a final concentration of 10^4 spores ml^{-1}. After 10 days of incubation, mycelial mats were observable surrounding the clay particles. Each microcosm was inoculated by introducing 20 granules of the carrier material surrounded by mycelium and homogenized manually. The soil was moistened at its optimal water content and densified at 1.3 g cm^{-3}.
2. For biostimulation with nutriments, a solution of ammonium nitrate and sodium phosphate was introduced in soil samples to establish a C/N ratio of 17 in the soil. The N/P ratio was set at 15 in the solution. Then, microcosms were inoculated by *T. helicus* 18 prepared as described previously.
3. For biostimulation by aeration, soil microcosm was mixed using a lab spatula and densified again to 1.3 g cm^{-3} one time a week. Then, microcosms were inoculated by *T. helicus* 18 prepared as described previously.

In the control microcosm (endogenous biotic degradation), non-inoculated carrier material impregnated by MM with 20 g l^{-1} of glucose over 24 h was introduced in soil microcosm. All treatments were conducted in triplicate at room temperature in the dark over a period of 30 days. In each microscom, air renewal occurred through a needle inserted in the stopper and an opening of 3 min three times a week under a chemical hood. After 30 days of incubation, triplicate soil samples from each microcosm were collected in order to analyze PAH concentrations and the fungal populations as described below. Degradation percentages were calculated using PAH contents of the non-inoculated controls after 30 days to consider only the effects of the different treatments on PAH biodegradation. The average and the standard deviation for each triplicate were calculated. Statistical analysis was performed by a two-sample *t* test comparing treatments between them (at 99% confidence).

2.9 DNA extraction

At the end of the incubation, total DNA extraction was performed on soil samples from microcosm incubations (essays 5, S19, 18, 18+NP, 18+O_2) using Mobio PowerSoil® Total DNA/RNA Isolation Kit (Mobio, Carlsbad, CA, USA). For each sample, total DNA extraction was performed using 1 g of soil. DNA extracts were eluted in a final volume of 100 μl in DEPC-treated water.

DNA extraction yields ranged from 10 to 100 ng/μl of DNA. DNA extracts were stored at − 20 °C until use.

2.10 qPCR amplification

The oligonucleotide primer couples amplifying the Internal Transcribed Spacers ITS1 and ITS2 were defined specifically for each fungus from sequences available in the database (NCBI). These primer couples were validated by experimental runs on soil samples in a preliminary study and allowed the detection of each of the three Ascomycota of interest: *F. solani*, *T. helicus* and *C. cladosporioides*.

qPCR amplification of the ITS 1 and ITS 2 regions was performed using oligonucleotide primer couples F5′-CGCCGCAGCTTCCATT-3′ and R5′-GATTCGAGGTCAACATTCAGAAGT-3′, F5′-AGCGTCATTTCTGCCCTCAA-3′ and R5′-CAAGTGACAAAGCCCCATGC-3′, and F5′-CATTACAAGTGACCCCGGTCTAAC-3′ and R5′-CCCCGGAGGCAACAGAG-3′ for the detection of *F. solani*, *T. helicus*, and *C. cladosporioides*, respectively. Expected amplification product lengths are 98 bp, 118 bp, and 73 bp for *F. solani*, *T. helicus*, and *C. cladosporioides*, respectively. qPCR reactions were carried out in a Biometra T-Professional thermocycler (Biometra GmbH, Göttingen, Germany) using a final volume of 50 μl and 0.2 μM of each primer. PCR reactions were performed using the KAPA Taq Hot Start PCR Kit (Kapa Biosystems, Boston, USA) using 10 μl of 5X KAPA Taq Hot Start Buffer, 3 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP Mix, 0.2 μl of 5 U/μl KAPA Taq Hot Start DNA Polymerase, 2 μl of DNA template, and 31.8 μl of PCR-grade water. The cycling protocol used was as follows: an initial denaturation step of 2 min at 95 °C followed by 35 cycles of 30 s at 95 °C (denaturation), 30 s at 60 °C (annealing) and 30 s at 72 °C (extension). Cycles were followed by a final extension at 72 °C for 5 min and on hold at 10 °C. DNA fragment amplification was confirmed by visualizing amplified PCR products on a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) using Agilent DNA 1000 microfluidic chips. Quantitative PCR amplifications were carried out in a Qiagen real-time PCR cycler Rotor-Gene Q 2plex system (Qiagen, Valencia, CA, USA) using The SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK) and a final volume of 20 μl, 0.3 μM of each primer and 2 μl of DNA template. The cycling protocol used was as follows: an initial denaturation step of 3 min at 95 °C followed by 40 cycles of 5 s at 95 °C (denaturation), 10 s at 60 °C (annealing), and 10 s at 72 °C (extension). Data were analyzed using the Rotor-Gene 6000 Application Software. Copy numbers averages were calculated per triplicate as well as corresponding standard deviations.

3 Results and discussion

3.1 Fungal isolation from contaminated soils

The direct inoculation technique and the suspension dilution one enabled the isolation of more than 50 pure cultures of autochthonous fungal strains. By morphological characteristics observed on MYEA (mycelium shape, form, elevation, surface, color, and growth velocity), several colonies appeared to belong to the same morphotypes. Thus, just 13 different isolates were selected and further identified by morphology data and by molecular techniques (Table 2). The purpose of this first step was to isolate by cultural approach some of the dominant saprotrophic telluric fungi present in historically contaminated soils (from various origins in the North of France), and to purify them before screening their ability to degrade BaP as a model of HMW PAH. These isolation techniques did not obviously intend to recover the whole diversity of cultivable fungal species even less so the total fungal biodiversity present in soils. We just could underline that the identifications, conducted by morphological and molecular technics, reveal a large predominance of the Ascomycota phylum among selected isolates. Indeed, only strain 30 belongs to the Zygomycota phylum and the other to the Ascomycota phylum. This tendency correlated well with other studies resting on fungal isolation from industrial aged PAH-contaminated soils. In fact, using culture-based techniques, many common native indigenous telluric fungi have been identified as belonging to the Ascomycota phylum (Czaplicki et al. 2016), due to the fact that meiosporic and mitosporic ascomycetes represent the largest fungal group in terrestrial and aquatic environments, accounting for roughly 64% of all described fungi (Harms et al. 2011). Indeed Potin et al. (2004a) isolated 19 Deuteromycetes (anamorphic forms of Ascomycetes), 1 Zygomycota and 1 Mastigomycota, as well as Zafra et al. (2014) who isolated 20 Ascomycota and 1 Zygomycota. The most retrieved genera were *Penicillium*, *Cladosporium*, and *Fusarium,* respectively. The *Penicillium* genus was already reported as prevalent in PAH-impacted soils (Oudot et al. 1987; Saraswathy and Hallberg 2002) and sediments (Ravelet et al. 2000; da Silva et al. 2003; Salvo et al. 2005). Strains belonging to the *Cladosporium* genus were also commonly isolated from PAH-contaminated substrates (Ravelet et al. 2000; Salvo et al. 2005; Potin et al. 2004a, b; Atagana et al. 2006) as well as *Fusarium* strains (Rafin et al. 2000; Potin et al. 2004a; Atagana et al. 2006; Thion et al. 2012). Among the other genera, *Trichoderma harzianum* was already isolated from a gasworks impacted soil (Saraswathy and Hallberg 2002) and a *Mortierella* strain from aliphatic hydrocarbon-contaminated soil (Hughes et al. 2007).

3.2 Extent of benzo[a]pyrene degradation by isolates in mineral medium

We were over careful with the BaP absorption on *F. solani* hyphae that could account for BaP dissipation in liquid culture as demonstrated by Thion et al. (2012) with phenanthrene, pyrene, and dibenz(a,h)anthracene. The cultures were totally lyophilised and extracted into a Soxhlet apparatus for 16 h with DCM. With this method, we assumed that the remaining BaP was totally extracted whatever the place it could be present: either absorbed or strongly adsorbed to the biomass. Moreover, the method used for the calculation of BaP degradation subtracted any remaining balance of BaP adsorbed.

The ability of the isolated fungi to grow and thrive in environments with high levels of pollutants as usually found in aged PAH-contaminated soils motivated further interest into the metabolism of these fungi as a possible remedy for contaminated soil treatment. Indeed, these fungi are well adapted to the soil environment and are susceptible to be resistant to PAH toxicity and could thus be suitable for PAH bioremediation in soils. Therefore, isolated strains and the model strain S19 identified as *F. solani* (Rafin et al. 2000) were studied for their ability to degrade BaP in MM over 9 days (Fig. 1). Among the 14 studied isolates, 10 could degrade BaP significantly with an average degradation rate outcome of 10% after 9 days of incubation, which is already relatively high considering the BaP recalcitrance to biodegradation and the short incubation time. The most efficient isolates were *Penicillium canescens* (isolate 4) with 44.2% BaP degradation, *C. cladosporioides* (isolate 5) with 42.6% BaP degradation, *F. solani* (the model strain S19) with 33.4% BaP degradation, and *T. helicus* (isolate 18) with 31.4% BaP degradation. The BaP degradation efficiency of the model strain S19 (*F. solani*) is in accordance with results obtained previously (Rafin et al. 2000; Veignie et al. 2004) and could be linked to the demonstrated ability of this strain to actively uptake BaP into its cells before to degrade it (Fayeulle et al. 2014). Wu et al. (2010) conducted also investigations on the biodegradation of two other PAH, anthracene and benz[a]anthracene, by *F. solani* with 40% and 60% of the initial amount degraded respectively after 40 days of incubation. Strain 18 is identified as *T. helicus*, a species already described for its ability to degrade biphenyls (Romero et al. 2005, 2006). Strain 5 corresponds to *C. cladosporioides* whose capacities for PAH degradation (Giraud et al. 2001) including BaP (Passarini et al. 2011) have been already described. Strain 4 belongs to the species *P. canescens* which is already reported in biphenyls (Schauer and Borriss 2004) and fluorene degradation (Garon et al. 2004). Under the limit of 30%, strains 26 and 30 also trigger significant degradation percentages of 21.5% and 17.7% respectively. Strain 26 is identified as *Trichoderma harzianum*, a species already studied for PAH biodegradation (Mahmood and Rama Rao 1993; Colombo et al. 1996; Saraswathy and Hallberg 2002). Strain 30 corresponds to *Mortierella alpina*, an oleaginous fungi recently used to highlight a new degradation pathway of an aromatic substance i.e. phenylalanine (Wang et al. 2013) and belonging to a genus described for its capacity to use petroleum derivatives (such as dodecane) as the sole carbon and energy sources (Davies and Westlake 1979; Hughes et al. 2007).

3.3 PAH biodegradation in soil microcosms

For the biodegradation of the 16 EPA-PAH in soil microcosms, treatments were conducted in soil samples provided from site 3. These soil samples were taken from a historical

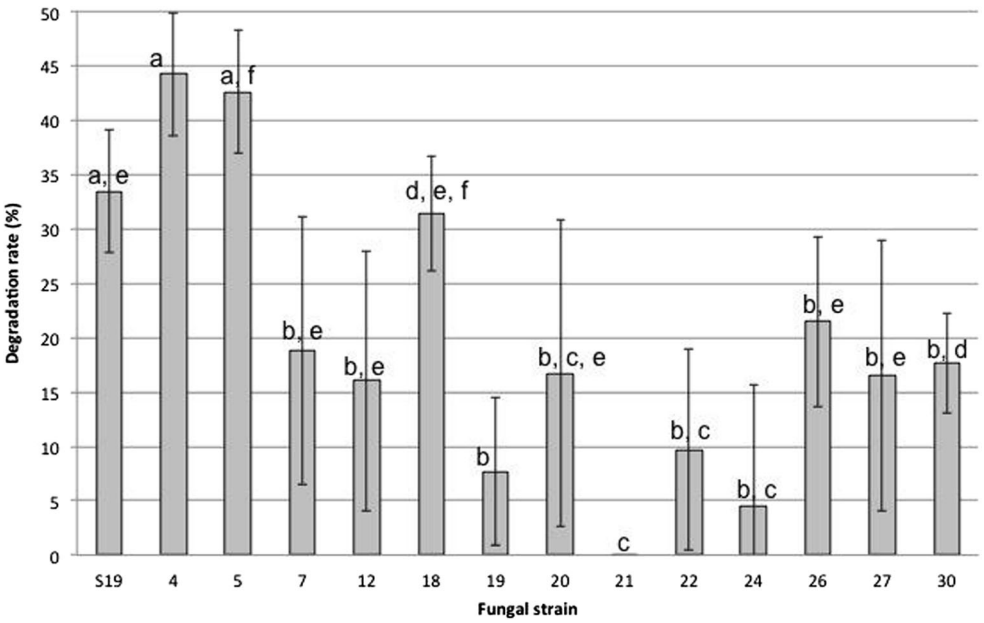


Fig. 1 Average benzo[a]pyrene degradation rates by different fungal strains after 9 days of incubation in mineral medium (% of 500 μg in 15 ml MM). Error bars represent standard deviations. Letters designate groups of results with no statistically significant differences

aged gas manufacturing plant during its requalification and contained a total PAH contamination of about 321.7 mg kg^{-1} soil. For bioaugmentation experiments, soils were inoculated with an autochtonous isolate: *T. helicus* 18, and two allochthonous strains: *C. cladosporioides* 5 (isolated from site 1) and, *F. solani* S19 used as a model strain. The selection of these strains is due to their BaP degradation abilities in MM and a rapid growth under the selected conditions of culture.

Concerning PAH quantifications, the comparison of inoculated microcosms with the non-inoculated control enabled determining the effect of the inoculated strains in the presence of the endogenous microflora after 30 days on the biodegradation (Fig. 2). A significant increase of total PAH degradation compared to controls is observed in microcosms inoculated with *F. solani* (treatment T19) and *T. helicus* (T18). However, it is not possible to conclude whether this result is directly linked to the strains themselves or to their interaction with endogenous fungal and bacterial strains. The results obtained for these two treatments were also significantly different from those obtained in the treatment inoculated with *C. cladosporioides* (T5), which triggers no significant increase of total PAH degradation in comparison to the control.

In more detail, the results show that both treatments inoculated either with *F. solani* (T19) or *T. helicus* (T18) seem to influence as much or even more efficiently the degradation of high molecular weight PAH than the degradation of the low molecular weight ones. This result is of interest considering the increasing recalcitrance of PAH to biodegradation with the number of aromatic cycles and their molecular weight. Considering all PAH classes, it is interesting to point out that degradation percentages in treatments inoculated with *T. helicus* (18) are the highest with an average of 26.3%. This result is pretty high considering the short incubation time and the considered pollutants. Incidentally, *T. helicus* (18) was isolated from the considered soil and could be more adapted to its physicochemical properties as an autochthonous strain. This positive influence on degradation for a large range of different PAH coupled to a good resistance to heavy metals (Romero et al. 2006) could make *T. helicus* (18) a good candidate for bioremediation of soils impacted with complex mixtures of pollutants in the presence of the indigenous microflora.

In the second part of the experiment, biostimulation/bioaugmentation treatments inoculated with *T. helicus* 18 were conducted in order to highlight the impact of the nutrients and the aeration on the soil functioning and its biodegradation capacity. Results show that the good degradation levels obtained with the inoculation of *T. helicus* (T18) are completely inhibited by the supplementation with nitrogen and phosphorus sources for all PAH classes (T18+NP). This observation is quite surprising considering the literature reporting the efficiency of biostimulation with these nutrients added as mineral salts (Smith et al. 1998; Llado et al. 2012) or inorganic fertilizer (Silva-Castro et al. 2013). However, the application of NH_4Cl and K_2HPO_4/KH_2PO_4 in the biostimulation experiment of Saponaro et al. (2002) led to more contrasted results with no significant effects on low molecular weight PAH in accordance with our study.

Our results with soil aeration by stirring (T18+O2) gave unexpected results as well. In comparison with the bioaugmentation treatment with *T. helicus* (T18) alone, there is no significant variation for all PAH classes. An explanation could be a negative effect on fungal development through a mechanical mycelium disorganizing due to stirring coupled to the PAH loss enhancement through volatilization mechanisms. Indeed, this pathway of abiotic losses would preferentially affect low molecular weight PAH as already described in rotor-equipped bioreactors (Park et al. 1990; Saponaro et al. 2002).

3.4 Monitoring of fungal installation in microcosms through DNA analysis

In order to verify these hypotheses, quantitative DNA amplification was carried out on the microcosm's soil samples after incubation (Fig. 3).

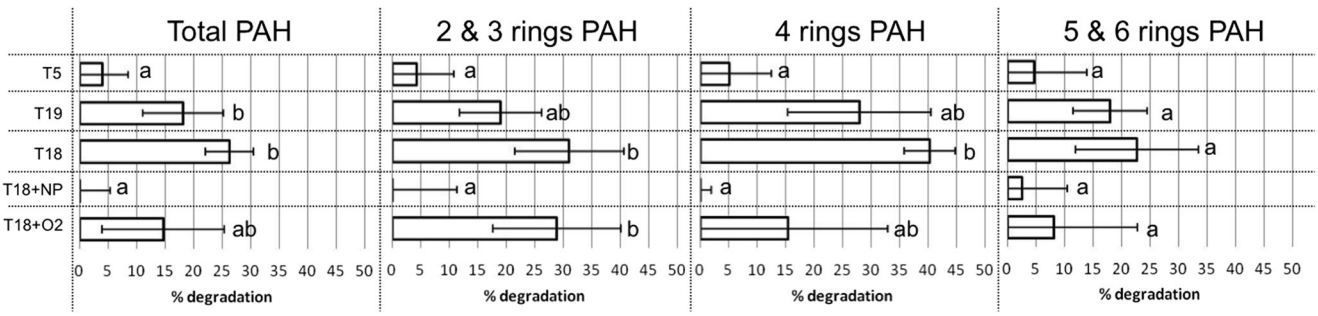


Fig. 2 PAH degradations in microcosms of non-sterile soil provided from site 3 after a 30 days incubation and inoculation with one strain, and additional conditions for strain 18. T5: inoculation with *Cladosporium cladosporioides,* T19*:* inoculation with *Fusarium solani*, T18: inoculation with *Talaromyces helicus.* T18+NP: inoculation with *Talaromyces helicus* and addition of nitrogen and phosphorus. T18+O_2: inoculation with *Talaromyces helicus* and aeration. Error bars represent standard deviations. Letters designate groups of results with no statistically significant differences.

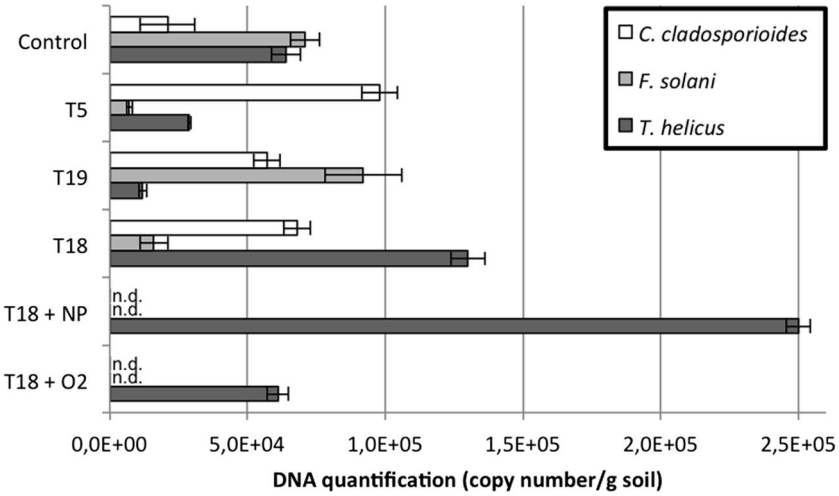


Fig. 3 DNA-amounts of 3 fungal species of interest in microcosms of non-sterile soil provided from site 3 after a 30 days incubation and fungal inoculation (n.d. = not determined)
T5: inoculation with *Cladosporium cladosporioides,* T19*:* inoculation with *Fusarium solani*, T18: inoculation with *Talaromyces helicus.* T18+NP: inoculation with *Talaromyces helicus* and addition of nitrogen and phosphorus. T18+O_2: inoculation with *Talaromyces helicus* and aeration. Error bars represent standard deviations.

In this study, the oligonucleotide primer couples amplifying the internal transcribed spacers ITS1 and ITS2 were defined for each fungus (genus and species) from sequences available in the database (NCBI). The specific primers chosen allowed the detection of the total population of each Ascomycota of interest in the soil samples: *F. solani*, *T. helicus*, and *C. cladosporioides*, i.e., the indigenous and the inoculated ones. This method is not discriminating enough to distinguish the inoculated strains (for instance *T. helicus* isolate 18) from the indigenous population of *T. helicus* initially present in the soil microcosms. We deliberately selected this approach in our study for reasons of feasibility and cost. Moreover, the obtained information by this approach were sufficient to quantify the presence of a specific Ascomycota (genus and species) in soil microcosm versus PAH biodegradation obtained after 30 days of incubation.

Even if the chosen method just allowed quantification of the total population of the fungal species of interest, as the content of the considered species increased in the bioaugmented microcosm after 30 days of incubation compared to the non-inoculated control for the three strains, we can reasonably hypothesize that the inoculation technique using expanded clay particles as support material was effective. The success of the inoculation approach using mycelia colonizing clay particles is in correlation with the study of Potin et al. (2004a), who already showed that PAH degradation rates in soil were higher using mycelia than spores as inocula. The same carrier material as in our experiment has been also successfully used at the field scale with a bacterial community for the remediation of isoproturon (Grundmann et al. 2007), which underlines the perspectives of scale-up. Other studies already used carrier materials in bioaugmentation strategies with ligninolytic fungi, i.e., *Dichomitus squalens*, *Pleurotus ostreatus*, *and Coprinus comatus* (Covino et al. 2010a, b, and c). However, the organic amendments (such as wheat straw, corn cobs) used as carrier materials in these studies constituted also substrates for the inoculated strains to favor their establishment in soils. At the opposite in our study, the inoculated telluric strains had to adapt to conditions in polluted samples since we used an expanded clay carrier material, which does not fundamentally disturb the soil physicochemical properties and add no nutrients.

Concerning the comparison of the DNA quantification results between bioaugmentation treatments, quantities detected in the control assay confirm the presence of autochthonous strains belonging to the same species in the considered soil sample. If for all strains the inoculation triggers an increase of the corresponding species in comparison with the incubated control, different results depending on the considered fungus were obtained between the incubated control treatment and the inoculated one, when the impacts on other species are considered. Indeed the inoculation of another strain seems to decrease the total populations of *T. helicus* (T5, T19) and *F. solani* (T5, T18), which could be linked to a competition for carbon resources. Interestingly, *Cladosporium cladosporioides*, which was shown to poorly degrade PAH during this bioaugmentation experiment, seems not to be affected by the inoculation of another strain (T19, T18). It could be linked to a lower competition for PAH as carbon source or other types of interaction with inoculated strains and endogenous microflora.

Concerning biostimulation experiments, the addition of nitrogen and phosphorus (T18+NP) efficiently favored the proliferation of *T. helicus* compared to bioaugmentation alone. This was unexpected considering the decreased efficiency in PAH biodegradation. This result could be explained by a modification of the strain metabolism, which could favor the use of other types of carbon sources than PAH in the presence of higher nitrogen and phosphorus concentrations. On the opposite, aeration by stirring (T18+O_2) decreased the development of this strain in correlation with the impaired PAH degradation. This kind of aeration techniques could thus have a negative impact on mycoremediation by disturbing the mycelial organization, which could be an important information considering

that tillage is often regarded as positive in bioremediation strategies, however with disturbance at a larger scale.

Globally, the results obtained in our study do not enable to highlight any positive effect of the tested biostimulation protocols in the considered samples and contaminants. By the way, Aspray et al. (2008) showed that the effect of nitrogen amendment on hydrocarbon degradation could radically vary according to the soil properties. Considering the uncertainty about the effect of biostimulative amendments, the necessity to tailor the bioremediation protocols to each site/pollutant combination was already underlined (Megharaj et al. 2011). Indeed, it is well known that soil physicochemical properties can significantly influence the establishment and growth of inoculated microorganisms, their degradative capabilities and pollutant fate and transport, which prevent from generalizing and extrapolating directly results obtained with a specific soil to other sites. Thus, the variability of nutrient effect on PAH degradation according to soil properties underline the requirement of feasibility studies per site (Balba et al. 1998; Lim et al. 2016) and the possibility of counterproductive effects of some additives show the need to assess their efficacy before scale-up (Lladó et al. 2013). In accordance with these observations, our study highlights the better efficiency of the bioaugmentation approach compared to the biostimulation one in the case of the considered pollutants and soil. Interestingly, a higher efficiency of fungal bioaugmentation than biostimulation approach was also reported for PAH remediation in other soils (Mancera-López et al. 2008). Besides, a comparative study on diesel contaminated soils of different origins classified bioaugmentation as the most efficient approach followed by natural attenuation and finally biostimulation with ammonium sulfate and dipotassium phosphate (Bento et al. 2005).

Bioaugmentation with selected telluric saprotrophic fungi represents thus a promising approach for gently soil remediation by degradation of persistent organic pollutants in soils within the current context of brownfield requalification and sustainable urban development aiming to preserve pedosphere resources with all ecological functions and the ability to produce sound food. Besides, Rodriguez-Campos et al. (2018) showed that a bioaugmentation approach with a combination of different types of organisms could be advantageous for PAH biodegradation in soil. Thus, to combine efficient fungal strains with other micro- or macroorganisms could be a good avenue for the development of an efficient bioremediation technique at the site scale.

4 Conclusions

In the course of this study, 50 filamentous fungal isolates were obtained from four industrial aged PAH-contaminated soil samples. Three of the newly isolated strains (*P. canescens*, *C. cladosporioides*, and *T. helicus*) displayed degradation rates exceeding 30% of the initial amount of BaP (500 μg) within an incubation time of 9 days, which is in the same range or even above the result of the model strain *F. solani* S19. The two fastest growing best degraders *C. cladosporioides* and *T. helicus* as well as the model strain *F. solani* S19 were then inoculated into aged PAH soil microcosms using an innovative inoculation technique based on expanded clay particles as carrier material. This experiment enabled to show that this approach is promising for the development of bioaugmentation protocols with fungi considering the obtained high levels of total PAH biodegradation obtained with *T. helicus* (26%) and *F. solani* (18%) after 30 days, and the correlated soil colonization by these strains verified through their DNA quantification. Biostimulation experiments coupled to *T. helicus* inoculation in soil microcosms showed that nitrogen and phosphorus supplementation with mineral salts as well as aeration through stirring had a counterproductive effect on PAH biodegradation in this soil displaying N deficiency. Our study is thus the demonstration that commonly used stimulative approaches for bacterial bioremediation cannot be directly transposed to the use of fungal strains. Considering our results, fungal bioremediation based on telluric strains thus appears as a promising method for the removal of PAH especially for the ones of high molecular weights. In particular, the *T. helicus* strain isolated during this study appears of high interest for the development of such approaches. However, supplementary studies are still needed to investigate the transposability of such bioremediation technique to different industrial soils varying in texture and contamination, and for the scale-up of the developed inoculation technique to the scale of brownfield treatment. Such bioremediation methods should thus be a part of low environmentally impacting approaches for soils preservation and rehabilitation.

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