Hydrocarbonoclastic Ascomycetes to enhance co-composting of total petroleum hydrocarbon (TPH) contaminated dredged sediments and lignocellulosic matrices

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Abbreviations: TPH, Total Petroleum Hydrocarbon; GC-MS, Gas Chromatography-Mass Spectrometry; GC-FID, Gas Chromatography-Flame ionisation Detector; MEB, Malt Extract Broth; BSM, Basal salt medium

A B S T R A C T

Four new Ascomycete fungi capable of degrading diesel oil were isolated from sediments of a river estuary mainly contaminated by shipyard fuels or diesel oil. The isolates were identified as species of *Lambertella*, *Penicillium*, *Clonostachys*, and *Mucor*. The fungal candidates degraded and adsorbed the diesel oil in suspension cultures. The *Lambertella* sp. isolate displayed the highest percentages of oxidation of diesel oil and was characterised by the capacity to utilise the latter as a sole carbon source. This isolate showed extracellular laccase and Mn-peroxidase activities in the presence of diesel oil. It was tested for capacity to accelerate the process of decontamination of total petroleum hydrocarbon contaminated sediments, co-composted with lignocellulosic residues and was able to promote the degradation of 47.6% of the TPH contamination (54,074 ± 321 mg TPH/Kg of sediment) after two months of incubation. The response of the bacterial community during the degradation process was analysed by 16S rRNA gene meta-barcoding.

Introduction

Seaports and waterways are periodically dredged to ensure navigability and most of the dredged sediments are contaminated by spillages of fuels due to shipyard navigation and logistics [1]. The chemical nature of the contamination comprises aliphatic and aromatic hydrocarbons [2]. The toxic aromatic fraction, containing variable proportions of unsubstituted and substituted aromatic rings, has received much attention in relation to the design of strategies for decontamination, for its near ubiquitous distribution in sediments and high recalcitrance to biodegradation [2,3]. Currently, the management of dredged sediments is associated exclusively with landfilling and natural attenuation that, with time, leads to leakages and environmental risks. Co-composting with lignocellolosic substrates is a consolidated approach to accelerate the processes of natural attenuation [4,5] and the bioaugmentation of microbial strains capable of

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degrading the most recalcitrant fractions, in order to accelerate the process of decontamination, is a suitable and successful strategy [6], also demonstrated on a pilot-scale [7].

Microorganisms used for bioaugmentation must be resistant to both the plethora of contaminants that characterise the environmental matrices and the intermediates of their degradation. In the specific case of dredged sediments, the organic contamination may also be accompanied by the presence of heavy metals, that can reach critical levels with respect both to human health and autochthonous, or eventually bioaugmented, microorganisms. Generally, microorganisms deriving from mixed contaminations, heavy metals and organics, develop a level of resistance to heavy metals compatible with the activation of their oxidative metabolism. The development of resistance to the toxicity of the contaminants is one of the reasons for the opportunity to isolate candidates autochthonous to the matrix for design of methods of bioaugmentation.

Microbial strains usually selected and exploited for bioaugmentation are hydrocarbonoclastic bacteria [7,8]. However, recently the metabolic capacity of fungi for the treatment of hydrocarbon contaminated soils and sediments has been reported [[9], [10], [11]]. The low substrate specificity of fungal ligninolytic enzymes, which include laccases, Mn-dependent and Mn-independent peroxidases, has been shown to be responsible for the transformation of recalcitrant compounds in the environment, with a particular exploitability for phenolic and aromatic structures [12]. Most of the successful myco-based bioremediation approaches have been based on the exploitation of Basidiomycete fungi and their production of ligninolytic enzymes. In contrast, Ascomycete fungi have been described as being involved in the synthesis of humic acids, a process generally described as catalysed by non-ligninolytic peroxidases, neutral laccases and tyrosinases, capable of a partial lignin-oxidation and extracellular polymerisation of polyphenols [13]. However, since Ascomycete fungi represent 60% of the fungi described in soil and sediments [12], are predominant in contaminated matrices [14] and are described as being able to transform recalcitrant compounds [15,16], it is possible that their metabolic capacities may have been underestimated in the design of myco-based approaches to the decontamination of environmental matrices. Case studies of Ascomycete fungi exploitation for the treatment of contaminated soil and sediments are few and the interaction between the fungi and the hosting bacterial community is under-investigated.

The aim of the present study was to evaluate the possibility of exploiting Ascomycete fungi to bioremediate dredged sediments using a co-composting stage with lignocellulosic residues. Four Ascomycetes were isolated from the upper portion of contaminated sediments derived from the dredging activity of the estuary of a navigable waterway in Tuscany, Italy. The sediments were classified by the Local Agency of the Environment Protection (ARPAT) as contaminated by Total Petroleum Hydrocarbons (TPHs), where TPH indicates the presence of petroleum hydrocarbons and their related toxicity. In the case of the sediments of interest, the contamination was due to the presence of diesel oil derived from spillages of shipyard fuels. The fungi were tested for ability to utilize diesel oil as a sole carbon source, as well as the production of extracellular peroxidases and laccases. The most promising candidate in terms of diesel oil degradation was investigated for the ability to accelerate the process of co-composting of TPH contaminated sediments with lignocellulosic residues at mesocosm scale. In addition, the bacterial ecology of the co-composting mixture in response to fungal bioaugmentation was analysed, envisaging the possible involvement of bacteria in the process of contaminant degradation.

Material and methods

Sediment, diesel oil, wood chip and chemicals

TPH contaminated sediments were collected from the estuary of the Navicelli Chanel, Italy (43°58′20.30″N; 10°30′05.41″E). The characteristics of the sediment are reported in Table 1. The diesel oil was purchased from a local service station. The characteristics of the wood chips (lignocellulosic residue) used are reported in Table 1. All other chemicals used in this study were of analytical grade and obtained from Merck (Milan, Italy).

Table 1
Chemical-physical characteristics of the dredged sediments and wood chips.

Dredged sediments	
Total Phosphate	12.0 ± 0.7 mg/Kg
Total Nitrogen	0.29 ± 0.02 mg/Kg
Chloride	39.0 ± 1.1 g/L
TPH C > 12	54,074 ± 321 mg/Kg
pH	7.2
Wood Chip fractions	**% fw/fw**
Pine bark	95
Fir wood	5
Fraction < 2 cm	38
2 < Fraction < 5 cm	42
5 < Fraction <10 cm	20
Plastics	< 0.1
TPH	ND

TPH, Total Petroleum Hydrocarbons; ND, not detected.

Isolation and taxonomic characterization of fungal candidates

Fungal strains were isolated from 1 g of dredged sediment in 100 mL of Basal Salt medium (BSM: Na_2HPO_4, 2.2 g; KH_2PO_4, 0.8 g; NH_4NO_3, 3.0 g per liter). The medium was supplemented with 1% v/v of diesel oil (8764 ± 63 mg diesel oil/mL) as sole carbon source. Gentamicin (500 µg/mL), chloramphemnicol (50 µg/mL), tetracycline (125 µg/mL), streptomycin (50 µg/mL) and ampicillin (100 µg/mL) were added to inhibit saprophytic bacterial growth. The flasks of 500 mL were incubated for 15 d with shaking at 250 rpm on a rotary shaker at 28 ± 1 °C, in the dark. After 15 d, an aliquot was transferred to fresh BSM. After 5 passages, each consisting of 5 d of sub-culture, fungal isolates were separated on Malt Extract (ME) plates, containing per L: malt broth, 20 g; yeast extract, 5 g; agar, 10 g, plus antibiotics. A total of 1 mL of filtered (Whatman® membrane filter paper Grade 1574 1/2) diesel oil was added on the top of the agar plates. A total of 4 different isolates were identified after visual inspection.

DNA of each fungal isolate was extracted using the FastPrep 24™ homogenizer and FAST DNA™ Spin kit for soil (MP Biomedicals) according to the manufacturer's protocol. Plugs of ME agar plates containing fungal hyphae were taken using a sterile coring device and tweezers and placed into a bead tube for extraction. The remaining steps were performed following the manufacturer's instruction. Purity and quantity of DNA were assessed using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The gene encoding 18S rRNA for each candidate was amplified using the forward primer, EF4 (5′-GGAAGGGATGTATTTATTAG-3′) and two reverse primers, EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′) or fung5 (5′-GTAAAAGTCCTG GTTCCCC-3′). The EF4-EF3 primer set was chosen to amplify 1.5 kb of 18S rDNA, while the primer set EF4-fung5 was chosen to amplify an approximately 0.55 kb fragment internal to the EF4-EF3 fragment product. The 18S rRNA gene fragments were amplified using a previously reported protocol [17]. The PCR products obtained were purified, sequenced on both strands, and aligned to sequence databases using BLASTN [18].

Fungal degradation of diesel oil

Degradation of diesel oil in liquid cultures was monitored in 100 mL Malt Extract Broth (MEB) containing per L: malt broth, 20 g; yeast extract, 5 g, plus antibiotics. MEB was supplemented with 1% (v/v) diesel oil in 500 mL (8654 ± 45 diesel oil/mL) Erlenmeyer flasks closed with rubber stoppers. A total of 24 flasks were set up. 12 flasks were inoculated with 3 g fungal biomass derived from the maintenance of the fungi in flasks in MEB, and 12 flasks remained non-inoculated as controls. All were incubated on an orbital shaker at 100 rpm at 28 ± 1 °C, in the dark. At each time point (days 0, 7, 14 and 21), 3 inoculated and 3 non-inoculated flasks were harvested and the supernatant and the fungal biomass separated by centrifugation at 25,200 x g at 4 °C. At days 7, 14 and 21, the 3 inoculated and non-inoculated supernatants were analysed for diesel oil content measuring the capacity of the fungi to deplete diesel oil from the medium. At day 21, a mass balance was calculated by determining the portion of diesel oil adsorbed to the fungal biomass by extracting the latter. The amount of diesel oil metabolised by fungal oxidation was calculated by subtracting the diesel oil adsorbed to the biomass from the fraction depleted from the growth medium.

All the supernatants and biomasses were extracted in dichloromethane and analysed by gas chromatography-mass spectrometry (GC–MS) and gas chromatography-flame ionisation detector (GC-FID) following the US Environmental Protection Agency (EPA) method of 2003 for the quantification of non halogenated organics [19].

Lambertella sp. growth with diesel oil as sole carbon source

Degradation of diesel oil by *Lambertella* sp. in liquid cultures was monitored in 100 mL BSM and 1% (v/v) diesel oil (8700 ± 60 diesel oil/mL) in 500 mL Erlenmeyer flasks closed with rubber stoppers, maintained on an orbital shaker at 100 rpm at 28 ± 1 °C in the dark. Each flask was inoculated with 3 g fungal biomass derived from the maintenance of the fungi in flasks in MEB, previously washed twice with a saline solution (NaCl 0,9% w/v) and filtered on Whatman® membrane filter paper Grade 1574 ½. As negative control, non-inoculated flasks containing 1% (v/v) diesel were incubated under the same conditions and analysed in parallel with inoculated flasks.

3 Erlenmeyer flasks per time of analysis were analysed, with a total of 30 flasks per fungus, 15 inoculated and 15 non-inoculated controls. At each time of analysis, the supernatant and the fungal biomass in 3 flasks was separated by centrifugation at 25,200 x g at 4 °C. The fungal biomasses and supernatants were analysed for diesel oil content as described above. Biomass production during the incubation was quantified as the dry weight of the mycelia in each flask after dichloromethane extraction. Biomasses were dried at 105 °C for 24 h, cooled in a desiccator, and weighed.

Lambertella sp. extracellular enzymatic activities

Extracellular enzymatic activities of the *Lambertella* sp. isolate, grown as above, were quantified. Ligninolytic oxidative activities related to laccase (EC 1.10.3.2), manganese peroxidase (EC1.11.1.13), lignin peroxidase (EC1.11.1.14), and versatile peroxidase (EC1.11.1.16) were quantified spectrophotometrically [[20], [21], [22]], in sample volumes from each flask supernatant, collected in triplicate. Enzymatic activities were calculated as unit per g dry weight of the fungal biomass. All enzyme assays were performed at 37 ± 0.5 °C. Laccase activity was determined as described in [21], manganese peroxidase and versatile peroxidase activity in [20] and lignin peroxidase in [22].

Mesocosm experimental set-up

A total of 9 mesocosms (3 replicates for each experimental condition) were set up in glass pots each containing 3 kg dredged sediment (54,074 ± 321 TPH/kg). Prior to incubation, the sediments were air dried and mixed with 20% (w/w) of wood chips on a fresh-weight basis. The C/N/P ratio was adjusted in the mixture at 100:10:1 by diluting 0.5 M solutions of NH_4NO_3 and KH_2PO_4 in water for mixture irrigation. Afterwards, the mixture was supplemented with the fungal inoculum (fresh and autoclaved) and the water content of each mesocosm was adjusted to 60% of the maximum water-holding capacity of the mix. The pots were maintained in a dark, temperature controlled (21 ± 1 °C) chamber, routinely manually mixed every 5 d of incubation and checked for the maintenance of the correct water content.

3 of the 9 mesocosms were bioaugmented with *Lambertella* sp. biomass, previously grown in MEB, filtered on Whatman® membrane filter paper Grade 1574 1/2 and washed in sterile NaCl 0.9% (w/v) solution before the mixing with the sediments (B). The fungal biomass was bioaugmented at the 5% (w/w) on a fresh-weight basis. 3 mesocosms were bioaugmented with the autoclaved fungal biomass (121 ± 1 °C, 1 Atm for 30 min) with the same weight base ratio (Baut). 3 other mesocosms were not bioaugmented with the fungal biomass (notB). All pots were maintained for 60 d.

Measuring TPH degradation in mesocosms

At days 0, 30 and 60, a total of 10 g dry weight of sediments per mesocosm were obtained by collecting one column of sediment from each quarter of the mesocosm surface using a stainless steel probe. The collected columns were roughly mixed together and divided into 4 technical replicates. 3 replicates for each of 3 out of four samples, a total of 9 replicates per time of analysis, were analysed for TPH content as described according to UNI EN 16,703 with minor modifications, see Supplementary Materials and Methods 1.

Ergosterol extraction and analysis

Samples from the mesocosms, as described above, were sonicated at 70 °C for 90 min with a methanolic solution of KOH (10%, w/v), and then extracted 3 times with cyclohexane. The solvent was evaporated under nitrogen and the solid residue dissolved in 1 mL methanol. Aliquots of the methanolic solution were analysed by reverse-phase high performance liquid chromatography (RP-HPLC) equipped with a LiChroCART® 250–4LiChrospher®100 RP-18 (5 μm) equilibrated with 100% methanol at a flow rate of 1 mL/min. The elution profile was monitored at 282 nm. Limit of detection (LOD) and limit of quantification (LOQ) were determined with a series of dilute solutions of reference stock solution.

Bacterial meta-barcoding in mesocosms and statistical analysis

Bacterial biodiversity was analysed at days 0 and 60. 6 biological replicates for experimental condition (notB, B, Baut) were collected (1 g sediment) at the 2 different incubation times. Total DNA was extracted and purified using the FastDNA™ SPIN Kit for Soil (MP Biomedicals), following the manufacturer's instructions. DNA quantity was measured using a Qubit® 3.0 spectrofluorimeter (Thermo-Scientific, USA) with high sensitivity (HS) assay, while quality assessment was performed by measuring 260/230 and 260/280 ratio with Spectrostar Nano UV–vis spectrophotometer (BMG Labtech). A total of 288 ng of DNA was used for the production of paired-end libraries and for sequencing the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. For details in the sequencing strategy adopted and the analysis of results see Supplementary materials and methods 2.

Statistical analysis

The one-way ANOVA paired with Malt Extract Broth (MEB) post-hoc test, was adopted for TPH depletion, degradation and enzymatic activity. Statistical data analysis was performed by Graphpad Prism 6 software [22].

Results

Isolation of fungal strains, molecular identification and metabolic characterisation

4 different fungal strains, originating from the superficial layers of contaminated sediments (Table 1) and derived from cultural enrichments in the presence of diesel oil as sole carbon source, were isolated and taxonomically identified by the amplification of fungal 18S rDNA [32]. The sequencing results are reported in Table 2.

Table 2
Taxonomic characterization of the four fungal isolates.

Fungal isolate	Nucleotide sequence identity (%)	Organism showing greatest similarity	GenBank Accession number
A	99	*Mucor circinelloides*	JF723655
B	99	*Penicillium sp.*	KC092115
C	99	*Clonostachys sp.*	AY249900
D	99	*Lambertella comi-maris*	KC964868

Table 3
Column T7, T14, percentages of depletion of the diesel oil fractions from the MEB amended with 1% diesel oil (v/v) at different time of incubation (T7, T14). Column T21, the percentage of the fungal degradation of the two diesel oil fractions deriving from the calculation of the mass balance of the process.

Fungal candidates	Diesel oil fractions	T 7 % depletion	T14 % depletion	T21 % degradation
A	Aromatic fraction	95.1	90.1	ND
	saturated-alkane (C12-C28)	89.9	98.5	47.2
B	Aromatic fraction	95.3	90.2	85.3
	saturated-alkane (C12-C28)	96.1	97.4	79.5
C	Aromatic fraction	95.4	90.1	20.1
	saturated-alkane (C12-C28)	95.1	94.8	94.1
D	Aromatic fraction	95.2	90.3	95.2
	saturated-alkane (C12-C28)	97.2	96.5	80.9

ND not detected. Percentages values represent mean values of percentages of decrease in diesel oil quantification measured in three different samples of the three flasks per fungal candidate. The coefficient of variation was < 5%.

Their ability to deplete diesel oil in a medium containing malt extract as easily degradable carbon source was evaluated after different incubation times. Percentages of depletion and degradation of the alkane (C12-C28) fraction and of the aromatic fraction of diesel oil are reported in Table 3. A mass balance of the degraded portion of diesel oil was calculated at the end of experimentation, after 21 d incubation. In non-inoculated flasks, no significant depletion of diesel oil was observed. *Mucor circinelloides*, *Penicilium* sp. and *Clonostachys* sp. strains showed process of initial adsorption and successive desorption of the two fractions. The *Lambertella* sp. strain, by day 21, was able to degrade 95.2% and 80.9% of the alkanes and the aromatic fraction, respectively. Both fractions were depleted by day 7 and only a slight release of

alkanes in the growth medium was observed at day 21. Thus, *Lambertella* sp. showed the highest percentages of degradation of both alkanes and the aromatic fraction of diesel oil, and was further characterised.

Lambertella sp. growth on diesel oil as a sole carbon source

The ability of *Lambertella* sp. to utilise diesel oil as sole carbon source was tested (Fig. 1**)**. A progressive growth of the strain was observed with maximum biomass produced after 30 d incubation and a reduction in biomass by day 45 (Fig. 1A). The increase in fungal biomass with time of incubation was positively correlated with diesel oil degradation (Fig. 1B). During the first 15 d incubation, both adsorption to the biomass and degradation of diesel oil increased with time. Between days 15 and 30, the percentage of degraded diesel oil increased, positively correlating with a peak in fungal biomass production at day 30. Between days 30 and 45 a decrease in fungal biomass was observed, indicating the death of the fungus accompanied by a drop to zero in the degradation of diesel oil and the maintenance of an almost constant level of the absorbed portion. It is reasonable to assume that fungal death determined the release of fatty acids, co-extracted with diesel but not resolved chromatographically [23], thus quantified with TPH and determining the experimental artefact of the loss of the degraded diesel oil portion at day 45.

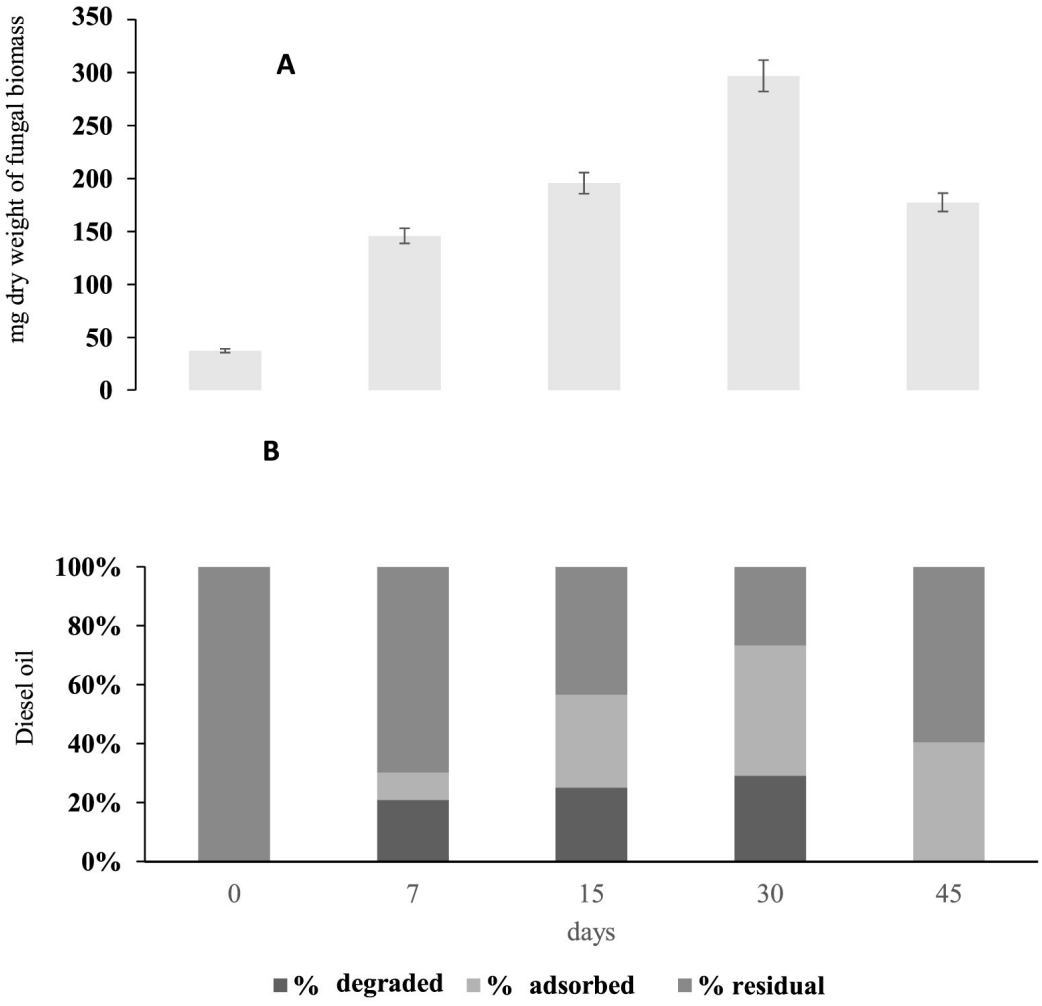

Fig. 1. A) *Lambertella* sp. grown in BSM with diesel oil 1% (v/v) as sole carbon source; B) Percentage of diesel oil degraded, adsorbed to the fungal biomass and measured as residual in BSM at different times of incubation.

Enzymatic activities secreted

The enzymes secreted by *Lambertella* sp. growing with diesel oil as sole carbon source was assayed for laccase, Mn-dependent peroxidase and Mn-independent peroxidase activity. Mn-dependent peroxidase and laccase were the only activities recovered (Fig. 2). Mn-peroxidase activity increased rapidly over the first 15 d growth, followed by a decrease. Laccase activity increased rapidly over the first 7 d growth, plateaued between days 7 and 21, and decreased thereafter. Laccase activity was significantly higher than that of the Mn-peroxidases over 45 d incubation.

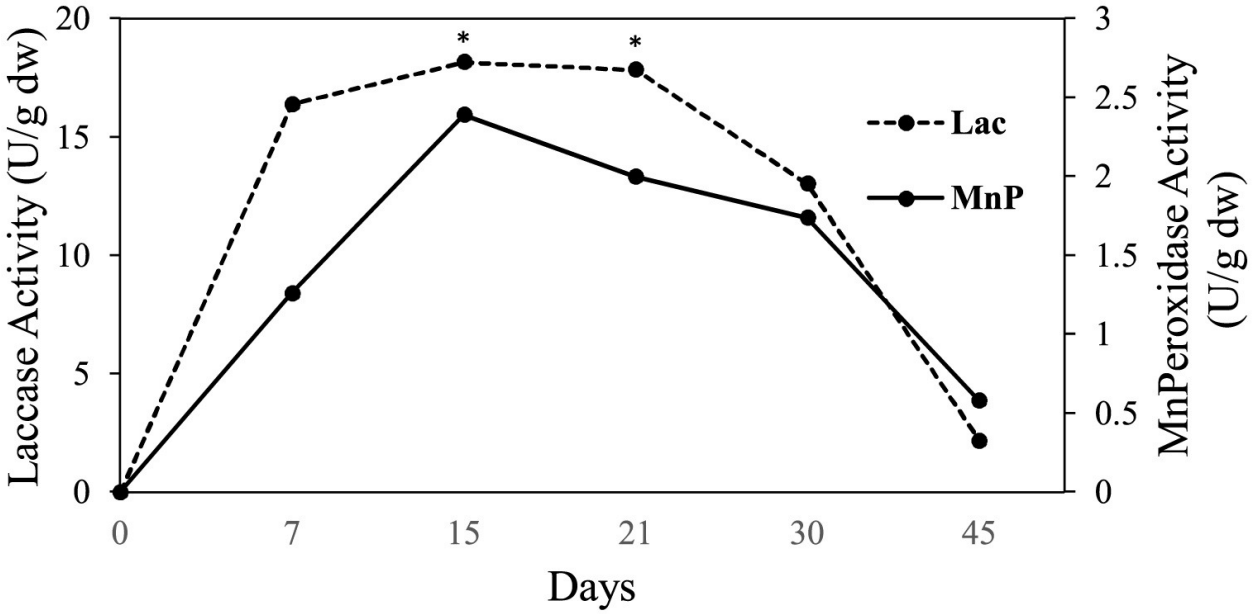

Fig. 2. Laccase and Mn-peroxidase activities detected in cultures of *Lambertella* sp. grown in BSM with diesel oil 1% (v/v) as sole carbon source. All enzymatic activities, expressed as units of enzymatic activity produced per g of dry weight of the fungal biomass, at different times of analysis, were significantly different from the corresponding values at the previous time of analysis at the 5% level ($p < 0.05$) with the exception of the values asterisked.

TPH degradation in mesocosms

A co-composting mesocosm experiment was set up by adding wood chips to contaminated sediments and *Lambertella* sp. was bioaugmented to the co-composting mixture in order to verify the capacity of the fungal candidate to elicit transformation and degradation of TPHs. Control mesocosms were set up by bioaugmenting autoclaved fungal biomass and preparing mesocosms not inoculated with *Lambertella*. The vitality of the fungal inoculum was assessed by measuring the content of ergosterol in the mixture (Fig. 3A). The ergosterol content at the time of *Lambertella* bioaugmentation was an indication of the successful addition of metabolically active fungal biomass (B) and inactivated (Baut) fungal biomass after autoclaving. Ergosterol content in the B condition decreased to values recorded in non-bioaugmented (notB) sediments after 2 months of incubation. TPH degradation was measured during this tim, corresponding to the presence of a metabolically active fungal inoculum in the co-composting mixture (Fig. 3B). No TPH degradation was observed in the absence of *Lambertella* bioaugmentation (notB), or in presence of bioaugmentation with the autoclaved fungal biomass (Baut) for the 2 months of incubation. After 2 months of incubation, bioaugmentation of metabolically active *Lambertella* sp. was responsible for 47.6% degradation of the TPH content in the co-composting mixture.

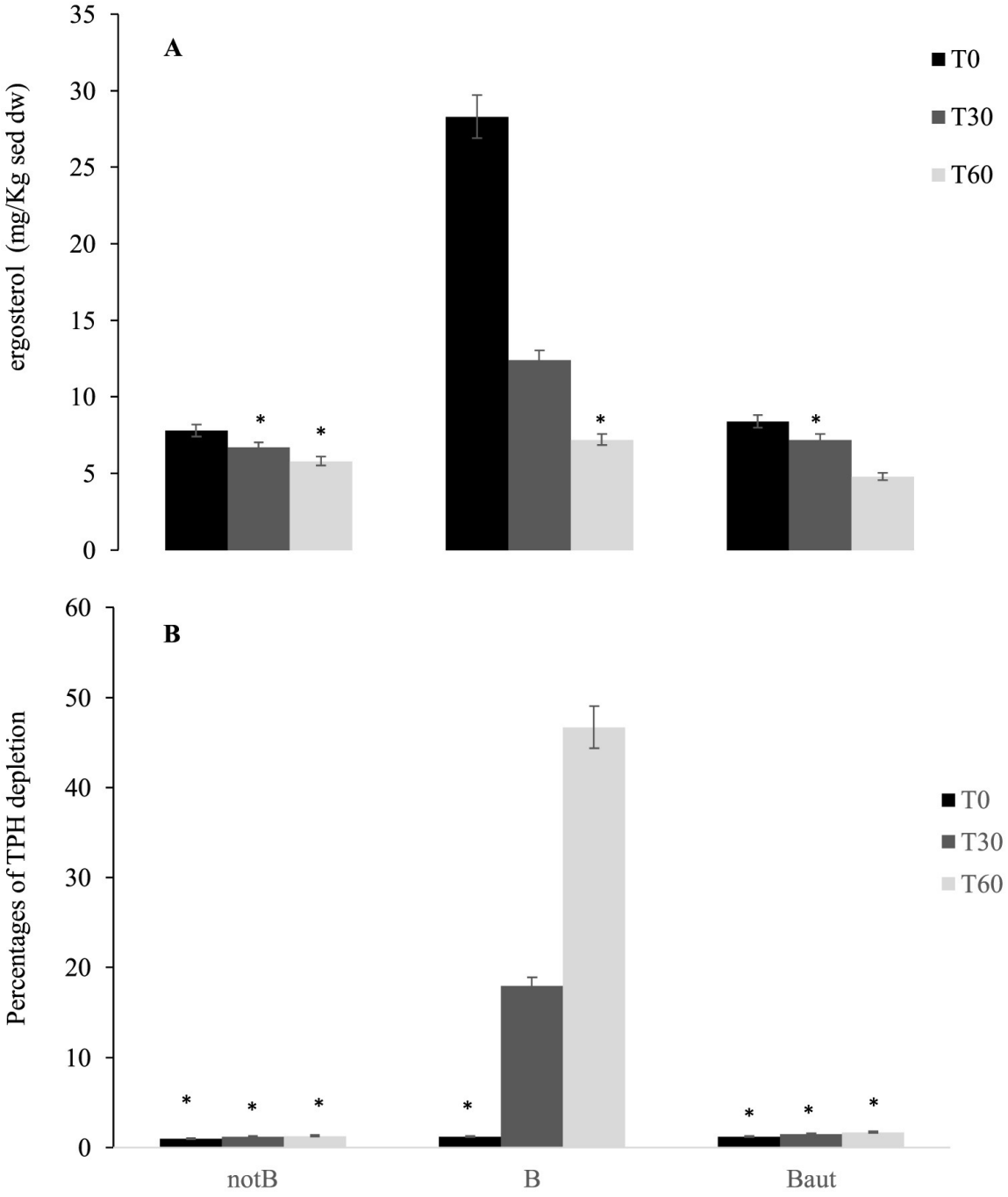

Fig. 3. A) Ergosterol content in the mesocosms without bioaugmentation with *Lambertella* sp. (notB), bioaugmented with *Lambertella* sp. (B), and bioaugmented with autoclaved *Lambertella* sp. biomass (Baut) at the different time of incubation. B) Percentages of TPH depletion at different times of incubation. All the values were significantly different at the 5% level ($p < 0.05$) with exception of the values asterisked. Percentage values represent mean values of percentage decrease in diesel oil quantification measured in three different samples of the three flasks per fungal candidate.

Bacterial community analysis

Bacterial community analysis of the mesocosms was performed at days 0 and 60 to evaluate how the bacterial community changed when *Lambertella* sp. was metabolically active and promoting TPH degradation. Analysis was performed by 16S rRNA gene meta-barcoding using high-throughput next-generation sequencing. The sequences passing the quality control were aligned and subjected to cluster analysis to ascertain their taxonomical affiliations. Alpha-diversity was assessed, based on the abundance of various taxa (Fig. 4A and B, statistics in Supplementary materials Tables S1, S2) within each community. The Shannon index values were calculated and the results were divided into four discrete groups (Fig. 4 A, statistics in Supplementary materials

Table S1). The first discrete group was from the notB mesocosms at day 0, where the biodiversity was greater than in all other groups. The second group was from the B and Baut mesocosms at day 0, whose values were not statistically different and indicated a lower biodiversity with respect to the notB mesocosm. The third group was from the notB and Baut mesocosms at day 60 and the fourth was represented by the B mesocosm at day 60 with the lowest Shannon index compared with all the other experimental conditions. The data indicated a decrease in bacterial diversity both in response to bioaugmentation with *Lambertella* sp. and time of incubation. On the other hand, the Chao1 index (Fig. 4B, statistics in Supplementary materials Table S2) indicated that at T0, the B mesocosms showed a species abundance significantly different from that of the notB. This abundance in B mesocosms significantly decreased during the time of incubation. The abundance of the bacterial community in the experimental conditions where TPH degradation was not observed did not significantly change.

Beta diversity was performed by principal coordinates analysis, PCoA, to provide an overview of the similarities in the bacterial communities between treatments with time of incubation (Fig. 4C, statistic in Supplementary materials Table S3,). Great similarities were observed in bioaugmented mesocosms (B and Baut) at day 0, which are grouped together in the upper left-hand quadrant of Fig. 4C. Differences were observed for notB mesocosm that grouped in the lower left-hand quadrant. At the same time, the Baut and the notB treatments after 60 d incubation collected in the right-hand quadrant in two different groups. Another group was associated with the B treatment after 60 d incubation, still grouping in the right-hand quadrant but distinct for the Baut and notB.

With reference to the details of the abundance of various taxa within each community (Fig. 5), data obtained showed that at day 0, Proteobacteria were the dominant phylum in all the experimental conditions (B, Baut, notB), but by day 60 Firmicutes and Actinobacteria were the dominant taxa. More precisely, Operational Taxonomic Units (OTUs) in the Phyla Proteobacteria, Bacteroidetes, and Chloroflexi decreased over the 60 d incubation in all experimental conditions. The Proteobacteria accounted for 48.5% of total OTUs in B mesocosm, 50.2% in Baut, and 37.6% in notB at day 0, but decreased to 19.8%, 10.3%, and 18.6% of the total OTUs, respectively, by d 60.

The OTUs in the phylum Bacteroidetes also decreased over the 60 days of incubation. Bacteroidetes in the notB mesocosms decreased from 16.0% to 0.8%. In the B mesocosm, Bacteroidetes decreased from 16.6% to 0.5%, and in the Baut mesocosms, from 16.0% to 0.2% of total OTUs by d 60. OTUs in the Chloroflexi phylum decreased from 9.2% to 2.0% in the Baut mesocosms, from 8.9% to 1.1% in the B mesocosm, and from 10.2% to 2.2% in the notB mesocosm. The bacterial families with percentages of abundance greater than 1% at T0, Ectothiorhodospiraceae, Xanthomonadaceae, Comamonadaceae (Proteobacteria), and Flavobacteriaceae (Bacteroidetes), decreased during the time of incubation as shown in Table 4. All the above mentioned families were represented by unclassified OTUs at genus level.

Significant increases in OTUs from the phyla Actinobacteria and Firmicutes were observed in all the experimental conditions at day 60 (Fig. 6). OTUs in the phylum Firmicutes increased from 4.8% to 47.1% of the total in the Baut mesocosms, from 5.3% to 48.3% in the B mesocosms, and from 8.4% to 39.3% in the notB mesocosms. The observed incremental increase in Firmicutes between days 0 and 60 was not statistically different in the 3 experimental conditions (Fig. 6A). At the family level, OTUs in the Bacillaceae increased in all the experimental conditions, reaching the highest values in B mesocosms (Fig. 6B). The Actinobacteria phylum (Fig. 7) increased significantly during the time of incubation both in B and in notB mesocosms, reaching similar values at the end of the incubation.

The increment of the phylum in the mesocosms bioaugmented with the autoclaved fungal biomass (Baut) was significantly lower (Fig. 7A). With reference to the genus level, in mesocosms bioaugmented with the metabolically active fungal biomass, *Brevibacterium* and *Arthrobacter* sps increased by day 60, reaching values statistically different from values reached in the other experimental conditions (Fig. 7B and C; statistics in Supplementary materials, Figures S1, S2, S3).

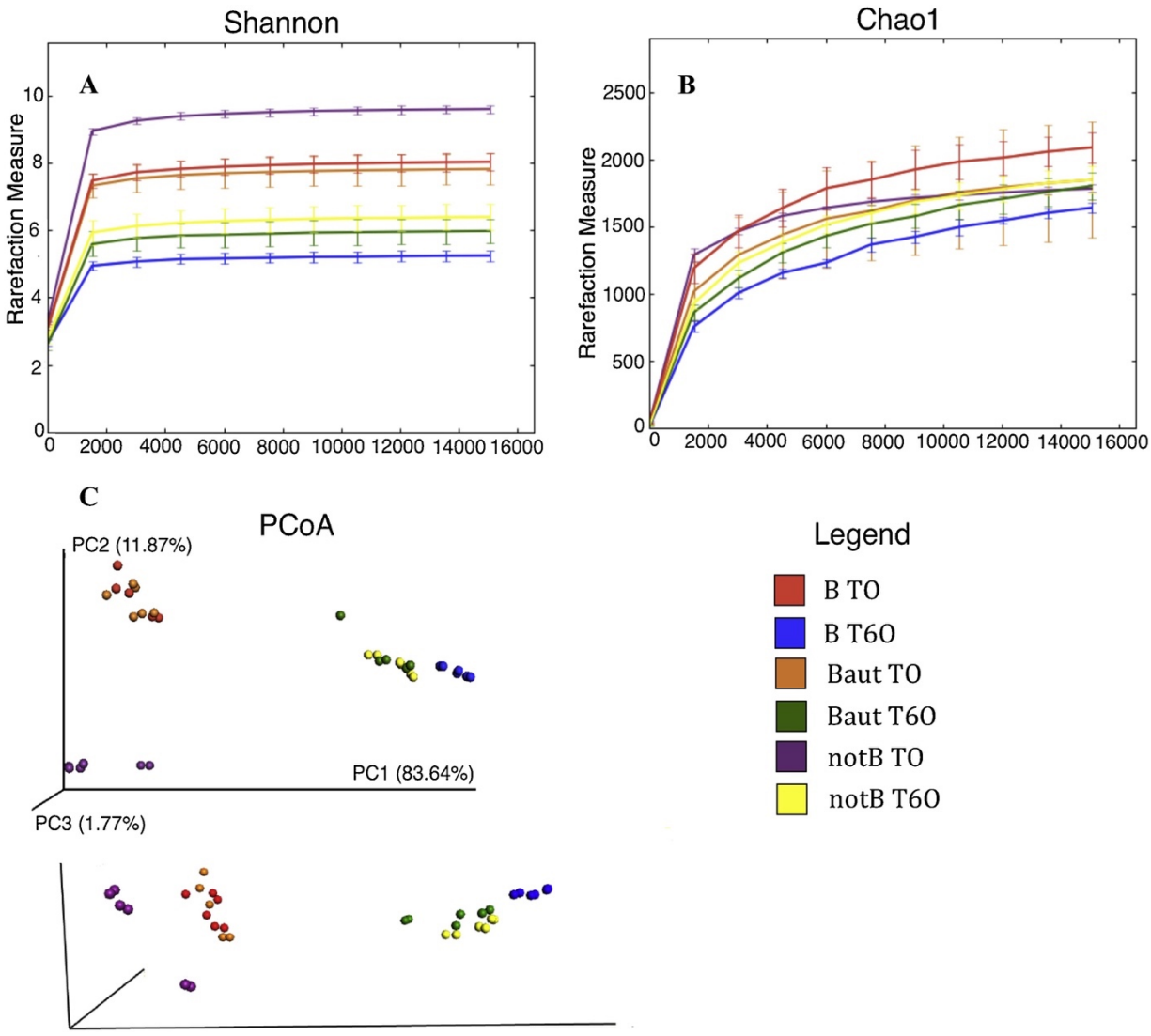

Fig. 4. A) Plot of Shannon indices estimating species evenness; B) Plots of Chao1 indices estimating species richness; C) Plots of Principal Coordinates analysis performed using the weighted UniFrac analysis. The total variance was 97.28%. Statistical analyses are reported in Table (Sl-3).

Discussion

With the aim of collecting autochthonous microorganisms for exploitation in a bio-based approach to the decontamination of dredged sediments, four fungal strains were isolated from TPH contaminated river estuary sediments and were distinguished principally by their capacity to deplete the mixture of hydrocarbons present in diesel oil. All the isolated fungi belonged to the phylum Ascomycetes, subphylum Mucoromycotina. Fungi in the phylum Ascomycota in general, and in the subphylum Mucoromycotina in particular, are the commonest group of fungal strains isolated from contaminated soils [24]. Fungi belonging to the *Mucor* sp. isolated from marine environments [25] have been reported to metabolize branched alkanes and polycyclic aromatic hydrocarbons [26]. The *Mucor circinelloides* sp. isolated in this study was able to adsorb diesel, but the oxidation was partial and restricted to the alkane fraction. Fungal species

belonging to *Penicillium* sp. have been previously described as present in marine environments [25] and are able to transform aliphatic hydrocarbons and polycyclic aromatic hydrocarbons [26]. Also fungal strains belonging to *Clonostachys* sp. have been isolated from marine sediment [27] and from contaminated soils derived from mining activity [28]. Aerial strains of *Clonostachys rosea* have been reported to oxidise styrene [29], but this is the first report describing *Clonostachys* sp. as capable of transforming diesel oil. The isolate that demonstrated the greatest capacity to deplete diesel oil was the *Lambertella* sp. While described as a plant parasitic fungi, species in the genus *Lambertella* are known to secrete oxidising enzymes [30]. This is the first report of a *Lambertella* sp. isolated from contaminated marine sediments, capable of utilizing diesel oil as sole carbon source. Ascomycete fungi are not usually described as ligninolytic fungi, even though the production of lignin-degrading enzymes was reported. The *Lambertella* sp. isolated here produced Mn-peroxidase and laccase that positively correlated with both the kinetics of the diesel oil biodegradation and the growth of the fungal isolate on diesel oil as sole carbon source. Despite the high level of contamination, the *Lambertella* sp. was able to survive in the co-composting matrix and to express its oxidative capacity.

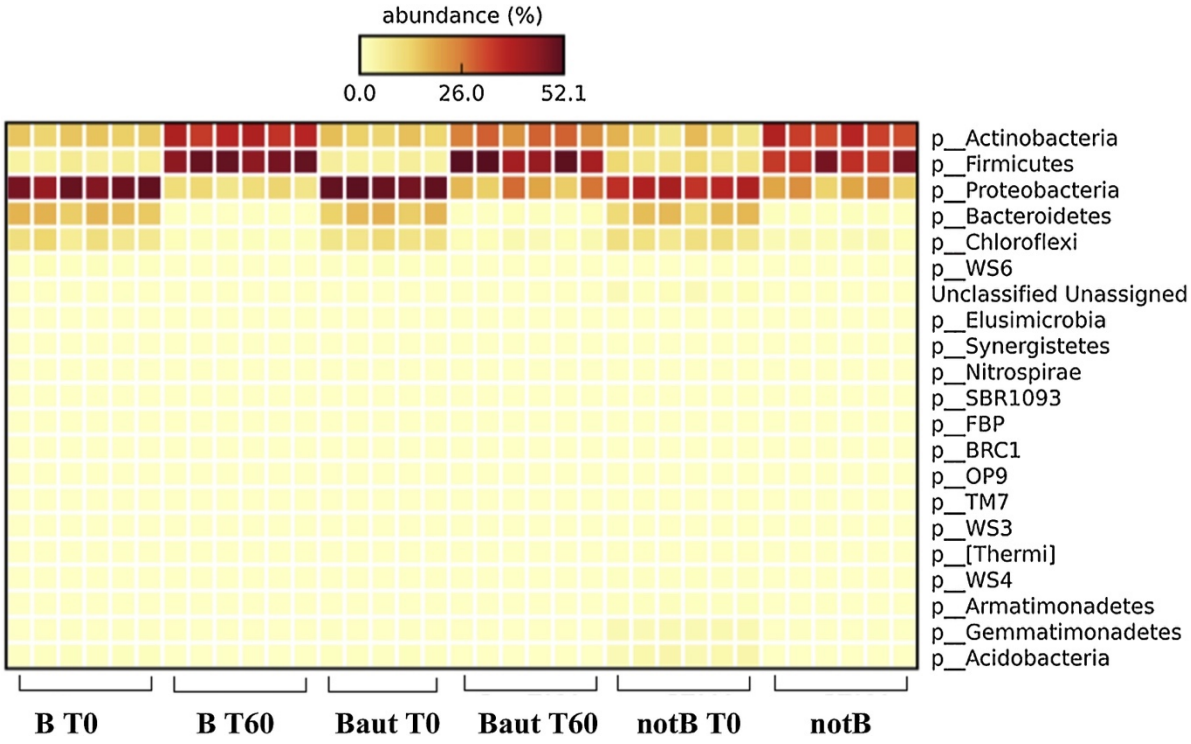

Fig. 5. Heatmap illustrating the distribution of bacterial Phyla from the four mesocosms. The relative abundance of each bacterial family is depicted by colour intensity, with the legend indicated at the top of the figure.

With reference to the bacterial ecology, the co-composting mixture was characterised by a diversified bacterial community, more abundant in Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Firmicutes, but Chloroflexi, Bacteroidetes and Proteobacteria dropped during the TPH degradation. The data are consistent with other reports where Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria were found to be dominant in contaminated environments [31]. The initial Chloroflexi dominance, principally described for reductive dehalogenation of polychlorobyphenils [32], suggested a process of adaptation of the sediment bacterial community to specific contaminants. The decrease in Chloroflexi during the co-composting incubation suggested a shift from an anaerobic microbial community, characteristic of the sediments in their site of origin, to an aerobic bacterial community within the co-composting mixture. Similar consideration can be assumed for the Ectothiorhodospiraceae, mainly chemolithotrophic purple sulphur bacteria, preferring anaerobic conditions of growth [33], showing a decrease in their percentage of abundance eventually related to their strategy of adaptation to the environment,

that in the experimental conditions here described are of low advantage. On the other hand, the decrease in predominance of the Xanthomonadaceae was not expected because described as enriched during hydrocarbon degradation, isolated from contaminated sediments, capable to deplete both the saturated and aromatic fraction of diesel oil [34]. However, it is worth mentioning that hydrocarbonoclastic bacterial community might show variable resistance to changing growing conditions [35,36]. The metabolic activity of the *Lambertella* sp. might have been at the origin of many changes in the environmental conditions of growth of the bacterial communities of the co-composting mixture. The conditions span from the shortening of nutrients, changes in the concentration of the primary contaminants because of the fungal degradative activity, up to the consequent release of toxic intermediates of degradation of the primary contaminants. The same interpretation can be adopted for the drop in percentages of abundance of the Comamonadaceae, and Flavobacteriaceae, both described as capable to degrade diesel oil, showing at the same time a high sensitivity to different environmental factors that elicit and/or inhibit their hydrocarbonoclastic activity [36,37]. In fact, many factors can determine structural changes and modulate the metabolic activity of the resident bacterial communities by exerting an effect on their structural resistance and resilience [37]. Their catabolic capacity can be non-homogenously elicited or masked by the non-homogeneous distribution of ecological niches in relation to oxygen, water content, availability of micro- and macronutrients and toxicological scenario of contaminated sediments.

Table 4
Average percentage of OTU for Ectothiorhodospiraceae, Xanthomonadaceae, Comamonadaceae and Flavobacteriaceae families for the different treatments at two time of incubations. The P-Value corrected values are referred to significance of differences between TO and T60. Games-Howell post-hoc tests were performed after initial Kruskal-Wallis H-test for significance, using Benjamini-Hockberg FDR correction for multiple comparisons.

Family		TO mean (%)	TO std.dev. (%)	T60 mean (%)	T60 std.dev. (%)	p-values (corrected)
Ectothiorhodospiraceae	B	9.467	0.897	2.250	0.574	0.000007
	Baut	10.729	0.899	3.909	1.436	0.002479
Xanthomonadaceae	notB	3.938	0.739	0.340	0.087	0.000583
	B	1.723	0.161	0.260	0.106	0.000183
	Baut	1.704	0.242	0.185	0.029	0.000195
Comamonadaceae	notB	1.740	0.206	0.371	0.066	0.000119
	B	1.565	0.247	0.183	0.022	0.000332
	Baut	1.519	0.175	0.465	0.174	0.001207
Flavobacteriaceae	notB	2.677	0.596	0.190	0.030	0.000901
	B	13.150	1.846	0.166	0.067	0.000161
	Baut	13.106	2.442	0.349	0.317	0.000441

On the other hand, in relation to the bacterial specimen that increased during the degradation of TPH, a direct involvement might be reasonably proposed even though their increase in percentage of abundance might be restricted to their resistance to the toxicity of the TPH intermediates of degradation, their capacity to metabolise the latter and/or a combination of the three factors. The analysis of the ecology of the bacterial community during the process of TPH degradation (alpha and beta diversity), indicated a progressive selection of bacterial populations. These can be specialised for TPH biodegradation or for the resistance to the toxicity of the intermediates of degradation of the primary pollutants, deriving from the fungal activity. However, *Arthrobacter* sp. strains have been isolated from TPH contaminated marine sediments and exploited for their decontamination [38]. *Brevibacterium* sp. have also been isolated and exploited for the treatment of PAH contaminated soils [39]. A peculiar dissimilarity in the microbial ecology of bioaugmented mesocosms and not bioaugmented mesocosms at the beginning of the experimentation was observed. A reasonable hypothesis is that bioaugmentation with an eukaryotic specimen can be associated to the bioaugmentation of its bacterial symbionts. Fungi were described as hosts of bacteria as symbionts [39]. Actually, the presence of the Ectothiorhodospiraceae and Flavobacteriaceae families in bioaugmented mesocosms and their almost absence in not bioaugmented mesocosms suggests a process of co-inoculation with the

fungal biomass. In this context, it is worth mentioning that, symbioses of eukaryotes in sulfidic environment, like sediments, with sulfur-oxidizing bacteria have been observed, comprising Ectothiorospiraceae [40,41]. On the other hand, symbiotic relationship between Flavobacteriaceae and eukaryotes are so far described mainly for green algae and insects [42,43]. However, as previously assessed, no clear evidences of the involvement of both the bacterial families in TPH degradation were collected.

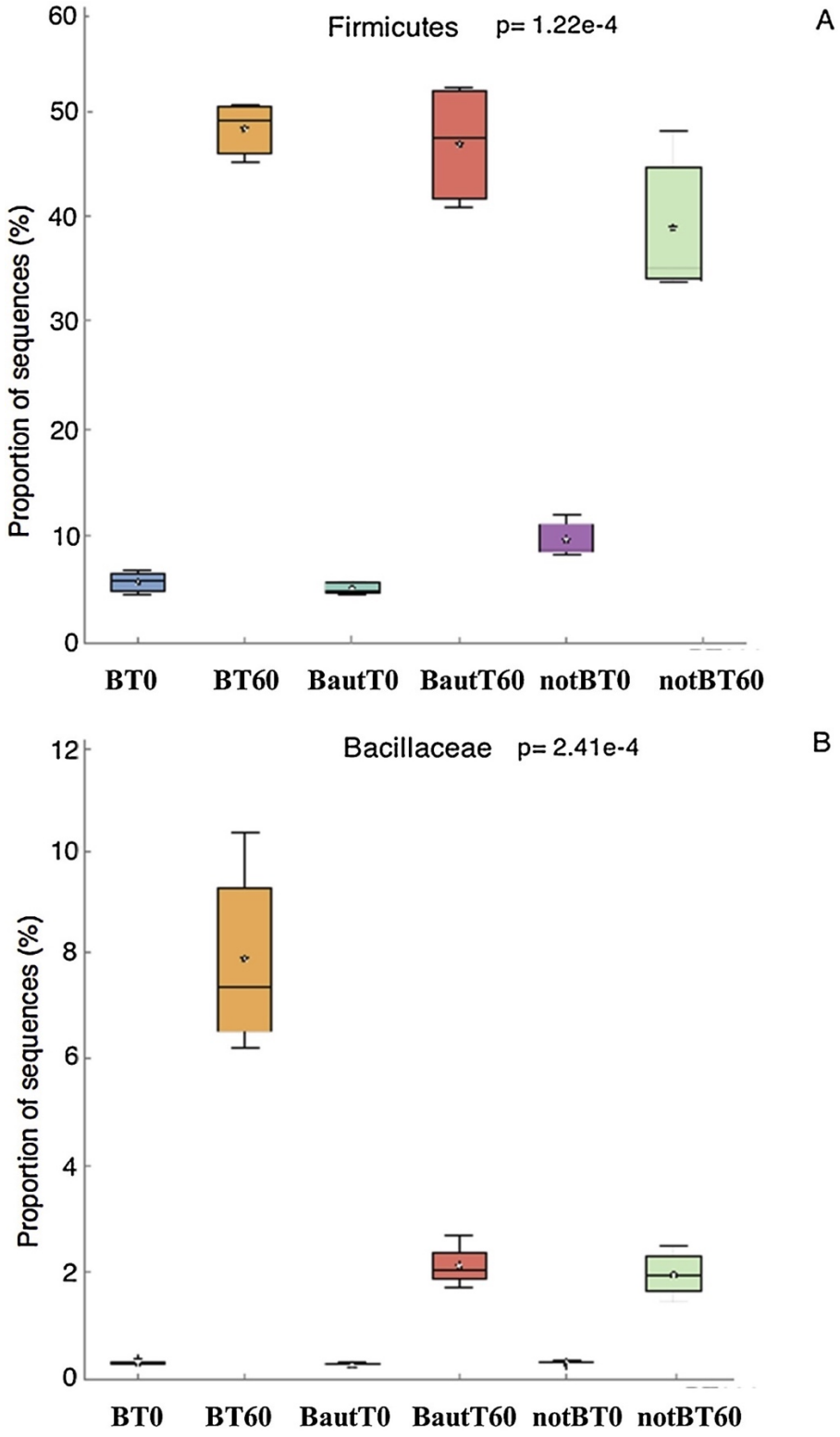

Fig. 6. A) Abundance of OTUs identified as belonging to the phylum Firmicutes (expressed as the percentage of total OTUs identified) in the four mesocosm experiments at Day 0 and Day 30; B) Abundance of OTUs identified as belonging to the family Bacillaceae (phylum Firmicutes) that changed significantly under experimental conditions where TPH depletion was observed (BT030).

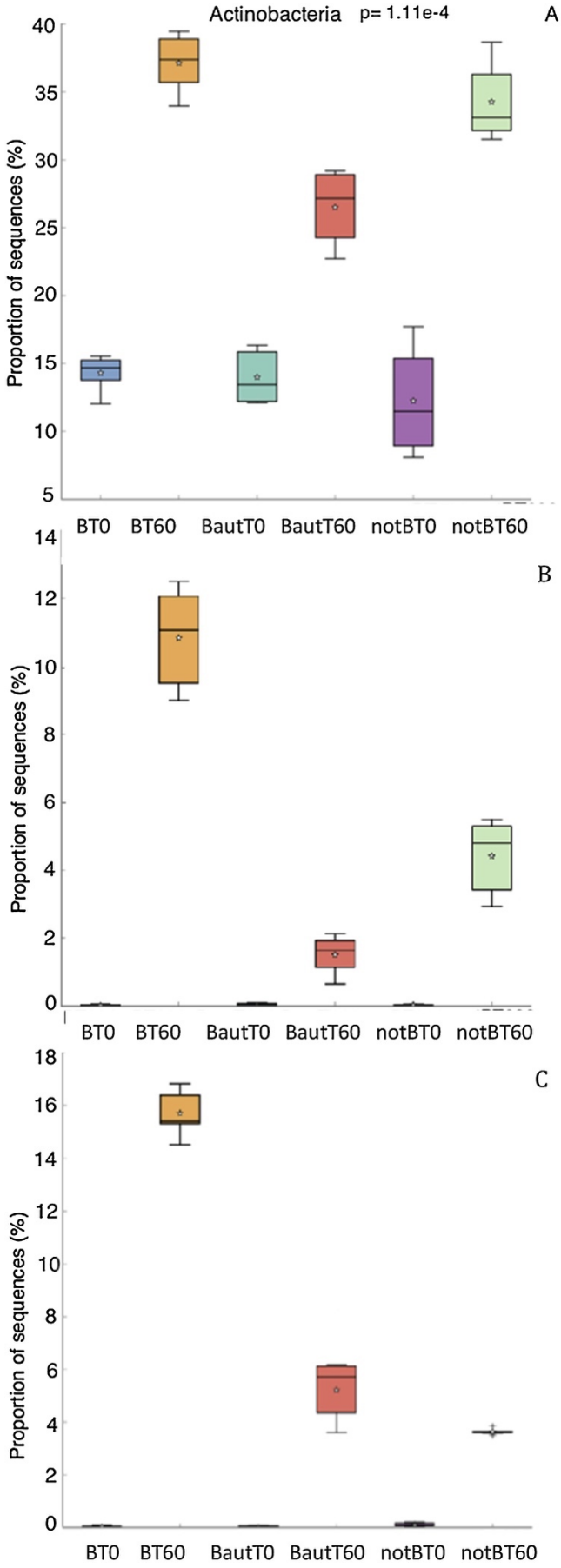

Fig. 7. A) Abundance of OTUs identified as belonging to the phylum Actinobacteria (expressed as the percentage of total OTUs identified) in the four mesocosm experiments at Day 0 and Day 30; B) Abundance of OTUs identified as belonging to the genus *Arthrobacter* (phylum Actinobacteria); C) Abundance of OTUs identified as belonging to the genus *Brevibacterium* (phylum Actinobacteria) that changed significantly under experimental conditions where TPH depletion was observed (BT030).

Conclusions

This is the first report describing the involvement of an Ascomycete, identified as a species of *Lambertella,* as a primary organism responsible for the biodegradation of TPH in real contaminated matrices, during a process of co-composting of dredged sediments with lignocellulosic residues. The metabolic capacity of the *Lambertella* sp. is of interest with reference to the extremely high level of contamination, the rate of degradation obtained in a relatively short time of incubation, and the percentage of bioaugmentation associated to the metabolic activity observed, compatible with real-scale bioaugmentation treatments. The novelty of the study of the interaction between the inoculated fungi and the resident bacterial community showed the possible involvement of bacteria of the *Arthrobacter* and *Brevibacterium* spp. in the degradation of TPH or of the intermediates of degradation of the primary contaminants eventually promoted by the fungal metabolism.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.nbt.2019.01.006.

References

[1] European sediment network. 9th International SedNet Conference. 2015. p. 23-6. September Kraków, Poland.

[2] Wang M, Wang C, Hu X, Zhang H, He S, Lv S. Distributions and sources of petroleum, aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) in surface sediments from Bohai Bay and its adjacent river, China. Mar Pollut Bull 2015;90:88-94. https://doi.Org/10.1016/j.marpolbul.2014.ll.017.

[3] Booth AM, Scarlett AG, Lewis CA, Belt ST, Rowland SJ. Unresolved complex mixtures (UCMs) of aromatic hydrocarbons: branched alkyl indanes and branched alkyl tetralins are present in UCMs and accumulated by and toxic to the mussel Mytilus edulis. Environ Sci Technol 2008;42:8122-6. https://doi.org/10.1021/es801601x.

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

[5] Maila MP, Cloete TE. Bioremediation of petroleum hydrocarbons through landfarming: Are simplicity and cost-effectiveness the only advantages? Rev Environ Sci Biotechnol 2004;3:349-60. https://doi.org/10.1007/slll57-004-6653-z.

[6] Mouhamadou B, Faure M, Sage L, Marçais J, Souard F, Geremia RA. Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. Fungal Biol 2013;117:268-74. https://doi.org/10.1016/ j.funbio.2013.02.004.

[7] Ruffini Castiglione M, Giorgetti L, Becarelli S, Siracusa G, Lorenzi R, Di Gregorio S. Polycyclic aromatic hydrocarbon-contaminated soils: bioaugmentation of autochthonous bacteria and toxicological assessment of the bioremediation process by means of *Vicia faba* L. Environ Sci Pollut Res 2016;23:7930-41. https://doi.org/10. 1007/s11356-016-6049-y.

[8] Sydow M, Owsianiak M, Szczepaniak Z, Framski G, Smets BF, et al. Evaluating robustness of a diesel-degrading bacterial consortium isolated from contaminated soil. N Biotechnol 2016;33:852-9. https://doi.Org/10.1016/j.nbt.2016.08.003.

[9] Di Gregorio S, Becarelli S, Siracusa G, Ruffini Castiglione M, Petroni G, Masini G, et al. Pleurotus ostreatus spent mushroom substrate for the degradation of polycyclic aromatic hydrocarbons: the case study of a pilot dynamic biopile for the decontamination of a historically contaminated soil. J Chem Technol Biotechnol 2016;91:1654-64. https://doi.org/10.1002/jctb.4936.

[10] Siracusa G, Becarelli S, Lorenzi R, Gentini A, Di Gregorio S. PCB in the environment: bio-based processes for soil decontamination and management of waste from the industrial production of Pleurotus ostreatus. N Biotechnol 2017;39:232-9. https:// doi.org/10.1016/j.nbt.2017.08.011.

[11] Stella T, Covino S, Čvančarová M, Filipová A, Petruccioli M, D'Annibale A, et al. Bioremediation of long-term PCB-contaminated soil by white-rot fungi. J Hazard Mater 2017;324:701-10. https://doi.Org/10.1016/j.jhazmat.2016.11.044.

[12] Harms H, Schlosser D, Wick LY. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol 2011;9:177-92. https://doi. org/10.1038/nrmicro2519.

[13] Zavarzina A, Lisov A, Zavarzin A, Leontievsky A. Fungal oxidoreductases and humification in forest soils. Soil Enzymol 2011:207-28. https://doi.org/10.1007/978- 3-642-14225-3.

[14] Godoy P, Reina R, Calderon A, Wittich RM, García-Romera I, Aranda E. Exploring the potential of fungi isolated from PAH-polluted soil as a source of xenobiotics- degrading fungi. Environ Sci Pollut Res 2016;23:20985-96. https://doi.org/10.1007/s11356-016-7257-1.

[15] Covino S, D'Annibale A, Stazi SR, Cajthaml T, Čvančarová M, Stella T, et al. Assessment of degradation potential of aliphatic hydrocarbons by autochthonous filamentous fungi from a historically polluted clay soil. Sci Total Environ 2015;505:545-54. https://doi.Org/10.1016/j.scitotenv.2014.10.027.

[16] Uribe-Alvarez C, Ayala M, Perezgasga L, Naranjo L, Urbina H, Vazquez-Duhalt R. First evidence of mineralization of petroleum asphaltenes by a strain of Neosartorya fischeri. Microb Biotechnol 2011;4:663-72. https://doi.Org/10.llll/j.1751-7915. 2011.00269.x.

[17] Smit E, Leeflang P, Glandorf B, Van Elsas JD, Wernars K. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Environ Microbiol 1999;65:2614-21.

[18] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402. https://doi.org/10.1093/nar/25.17.3389.

[19] U.S. EPA. Method 8015C (SW-846): Nonhalogenated Organics Using GC/FID, Revision 4. Washington, DC 2003https://www.epa.gov/sites/production/files/ 2015-12/documents/8015d_r4. pdf.

[20] Boer CG, Obici L, De Souza CGM, Peralta RM. Purification and some properties of Mn peroxidase from Lentinula edodes. Process Biochem 2006;41:1203-7. https:// doi.org/10.1016/j.procbio.2005.11.025.

[21] Osman AM, Wong KKY, Hill SJ, Fernyhough A. Isolation and the characterization of the degradation products of the mediator ABTS-derived radicals formed upon reaction with polyphenols. Biochem Biophys Res Commun 2006;340:597-603. https://doi.Org/10.1016/j.bbrc.2005.12.051.

[22] Swift ML. GraphPad prism, data analysis, and scientific graphing. J Chem Inf Comput Sci 1997;37:411-2. https://doi.org/10.1021/ci960402j.

[23] Zhang H, Wang Z, Liu O. Development and validation of a GC-FID method for quantitative analysis of oleic acid and related fatty acids. J pharm analysis 2015;5:223-30.

[24] Marco-Urrea E, Garcia-Romera I, Aranda E. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. N Biotechnol 2015;32:620-8. https://doi.Org/10.1016/j.nbt.2015.01.005.

[25] Liu Y, Singh P, Sun Y, Luan S, Wang G. Culturable diversity and biochemical features of Thraustochytrids from coastal waters of Southern China. Appl Microbiol Biotechnol 2014;98:3241-55. https://doi.org/10.1007/s00253-013-5391-y.

[26] Balaji V, Arulazhagan P, Ebenezer P. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. J Environ Biol 2014;35:521-9. https://doi.Org/10.13140/2.l.1528.3846.

[27] Dos Santos Dias AC, Ruiz N, Couzinet-Mossion A, Bertrand S, Duflos M, Pouchus YF, et al. The marine-derived fungus Clonostachys rosea, source of a rare conjugated 4- Me-6E,8E-hexadecadienoic acid reducing viability of MCF-7 breast cancer cells and gene expression of lipogenic enzymes. Mar Drugs 2015;13:4934-48. https://doi. org/10.3390/mdl 3084934.

[28] Šimonovičová A, Ferianc P, Vojtková H, Pangallo D, Hanajík P, Kraková L, et al. Alkaline Technosol contaminated by former mining activity and its culturable autochthonous microbiota. Chemosphere 2017;171:89-96. https://doi.org/10.1016/ j. chemosphere. 2016.11.131.

[29] Prenafeta-Boldú FX, Summerbell R, Sybren De Hoog G. Fungi growing on aromatic hydrocarbons: Biotechnology's unexpected encounter with biohazard? FEMS Microbiol Rev 2006;30:109-30.

https://doi.Org/10.llll/j.1574-6976.2005. 00007.x.

[30] Hirose A, Kudo S, Murakami T, Tanaka K, Harada Y, Hashimoto M. Lambertellin system, the mechanism for fungal replacement of Monilinia fructigena with Lambertella corni-maris without competitive inhibition on agar media. Bioorganic Med Chem 2014;22:2489-95. https://doi.Org/10.1016/j.bmc.2014.02.051.

[31] Acosta-González A, Martirani-von Abercron SM, Rosselló-Móra R, Wittich RM, Marqués S. The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the Prestige oil spill. Environ Sci Pollut Res 2015;22:15200-14. https://doi.org/10.1007/sll356-015-4458-y.

[32] Field JA, Sierra-Alvarez R. Microbial transformation and degradation of polychlorinated biphenyls. Environ Pollut 2008;155:1-12. https://doi.Org/10.1016/j. envpol.2007.10.016.

[33] Tourova TP, Spiridonova EM, Berg IA, Slobodova NV, Boulygina ES, Sorokin DY. Phylogeny and evolution of the family Ectothiorhodospiraceae based on comparison of 16S rRNA, cbbL and nifH gene sequences. Int J Syst Evol Microbiol 2007;57:2387-98. https://doi.Org/10.1099/ijs.0.65041-0.

[34] Di Gregorio S, Siracusa G, Becarelli S, Mariotti L, Gentini A, Lorenzi R. Isolation and characterization of a hydrocarbonoclastic bacterial enrichment from total petroleum hydrocarbon contaminated sediments: potential candidates for bioaugmentation in bio-based processes. Environ Sci Pollut Res Int 2016;23(June):10587-94. https://doi.org/10.1007/sll356-015-5944-y. Epub 2016 Jan 12.

[35] Szczepaniak Z, Czarny J, Staninska-Pięta J, Lisiecki P, Zgoła-Grześkowiak A, et al. Influence of soil contamination with PAH on microbial community dynamics and expression level of genes responsible for biodegradation of PAH and production of rhamnolipids. Environ Sci Pollut Res Int 2016;23:23043-56. https://doi.org/10.1007/sl 1356-016-7500-9.

[36] Cyplik P, Schmidt M, Szulc A, Marecik R, Lisiecki P, Heipieper HJ, et al. Relative quantitative PCR to assess bacterial community dynamics during biodegradation of diesel and biodiesel fuels under various aeration conditions. Bioresour Technol 2011;102:4347-52. https://doi.Org/10.1016/j.biortech.2010.12.06.

[37] Sydow M, Owsianiak M, Framski G, Wozniak-Karczewska M, Piotrowska-Cyplik A, et al. Biodiversity of soil bacteria exposed to sub-lethal concentrations of phosphonium-based ionic liquids: effects of toxicity and biodegradation. Ecotoxicol Environ Saf 2018;147:157-64. https://doi.Org/10.1016/j.ecoenv.2017.08.026.

[38] Chaudhary P, Sahay H, Sharma R, Pandey AK, Singh SB, Saxena AK, et al. Identification and analysis of polyaromatic hydrocarbons (PAHs)—biodegrading bacterial strains from refinery soil of India. Environ Monit Assess 2015;187. https://doi.org/10.1007/sl0661-015-4617-0.

[39] Xua H, Tsukudab M, Takaharab Y, Satoc T, j Gud, Katayama Y. Lithoautotrophical oxidation of elemental sulfur byfungi including Fusarium solani isolated from sandstone Angkor temples. Int Biodeterior Biodegradation 2018;126:95-102.

[40] Eichinger I, Schmitz-Esser S, Schmid M, Fisher CR, Bright M. Symbiont driven sulfur crystal formation in a thiotrophic symbiosis from deep sea hydrocarbon seeps. Environ Microbiol Rep 2014;6:364-72. https://doi.org/10.llll/1758-2229.12149.

[41] Nakagawa S, Shimamura S, Takaki Y, Suzuki Y, Murakami S, et al. Allying with armored snails: the complete genome of gammaproteobacterial endosymbiont. ISME J 2014;8:40-51. https://doi.Org/10.1016/j.ympev.2013.02.025.

[42] Hollants J, Leliaert F, Verbruggen H, de Clerck O, Willems A. Host specificity and coevolution of Flavobacteriaceae endosymbionts within the siphonous green seaweed Bryopsis. Mol Phylogenet Evol 2013;67:608-14. https://doi.Org/10.1016/j.ympev.2013.02.025.

[43] Krawczyk K, Szymanczyk M, Obrepalska-Steplowska A. Prevalence of endosymbionts il Polish population of Leptinotarsa decemlineata (Coloptera: Chysomelidae). J Insect Sci 2015;15:106. https://doi.org/10.1093/jisesa/iev085.