



Bioremediation of petroleum hydrocarbons polluted soil by spent mushroom substrates: Microbiological structure and functionality

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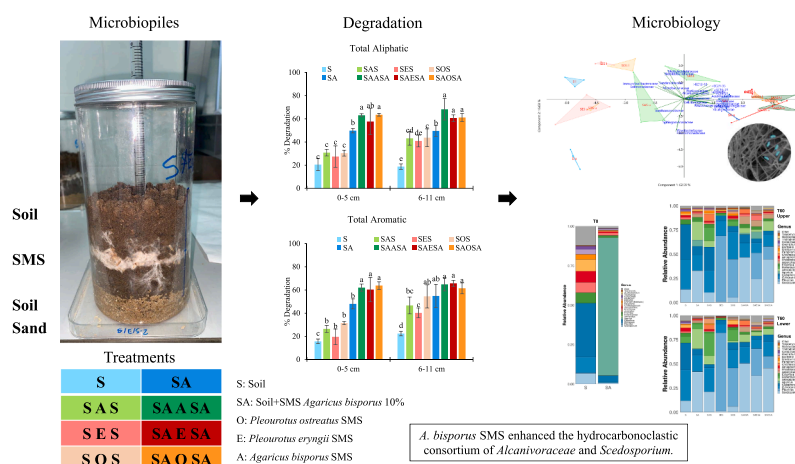
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HIGHLIGHTS

- SMS of *P. ostreatus*, *P. eryngii*, and *A. bisporus* promoted the degradation of TPHs.
- *bisporus* SMS was the most effective for TPHs biodegradation, including heavy fractions.
- TPHs polluted soil amended with fungal SMS led to shifts in microbial structure.
- *A.bisporus* SMS enhanced hydrocarbonoclastic consortium of *Alcanivoraceae* and *Scedosporium*.

GRAPHICAL ABSTRACT



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ABSTRACT

Spent mushroom substrate (SMS) holds valuable microbiota that can be useful in remediating polluted soils with hydrocarbons. However, the microorganisms behind the bioremediation process remain uncertain. In this work, a bioremediation assay of total petroleum hydrocarbons (TPHs) polluted soil by SMS application was performed to elucidate the microorganisms and consortia involved in biodegradation by a metabarcoding analysis. Untreated polluted soil was compared to seven bioremediation treatments by adding SMS of *Agaricus bisporus*, *Pleurotus eryngii*, *Pleurotus ostreatus*, and combinations. Soil microbial activity, TPH biodegradation, taxonomic classification, and predictive functional analysis were evaluated in the microbiopiles at 60 days. Different metagenomics approaches were performed to understand the impact of each SMS on native soil microbiota and TPHs biodegradation. All SMSs enhanced the degradation of aliphatic and aromatic hydrocarbons, being *A.*

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bisporus the most effective, promoting an efficient consortium constituted by the bacterial families *Alcanivoraceae*, *Alcaligenaceae*, and *Dietziaceae* along with the fungal genera *Scedosporium* and *Aspergillus*. The predictive 16 S rRNA gene study partially explained the decontamination efficacy by observing changes in the taxonomic structure of bacteria and fungi, and changes in the potential profiles of estimated degradative genes across the different treatments. This work provides new insights into TPHs bioremediation.

1. Introduction

Hydrocarbons are hydrophobic and highly persistent toxic substances that tend to bind and form complexes with soil particles and organic matter, thereby obstructing soil pores and creating areas of anaerobic conditions that hinder biodegradation [1]. Hydrocarbons enter the soil by accidental spills, leaks, or loading and unloading operations [2]. Those compounds alter the soil properties like electrical conductivity, soil structure, pH, or hydrophobicity. This can lead to a shift in the composition of the native soil microbiota among other negative effects [3].

Total Petroleum Hydrocarbons (TPH), constitute a group of several hundred compounds originated from crude oil, consisting of hydrogen and carbon and in smaller proportions oxygen, nitrogen, and sulfur. They include saturated hydrocarbons/paraffins, aromatics, resins, and asphaltenes [4]. There are two main categories of compounds: volatile petroleum hydrocarbons and petroleum extractable hydrocarbons. The first group includes small-chain (C_6 - C_{10}) hydrocarbons, including benzene, toluene, ethylbenzene, and xylene (BTEX). The second group includes long-chain (C_{10} - C_{40}) aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAH) [5]. Low molecular weight alkanes are readily volatile, whereas those of medium length (C_{14} - C_{20}) and higher, practically insoluble in water and therefore not easily biodegradable. Resins and asphaltenes are viscous high molecular weight compounds consisting of polycyclic groups, variably substituted with alkyl groups, which contributes to their resistance to biodegradation [6]. Furthermore, the recalcitrant compounds present in polluted soils are challenging to remove due to their adsorption to soil particles and organic matter surfaces in addition to their physical entrapment in micropores [7].

For decades hydrocarbons and microorganisms have been in contact, so that, those continuous interrelationships have led biological evolution to develop a wide range of metabolic and physiological adaptations. Those microorganisms first acclimated to the presence of hydrocarbons, and then took advantage by feeding on them. Bacteria evolved to use hydrocarbons as sole carbon and energy sources, mineralizing them into water and carbon dioxide with the physiological constraint of requiring soluble compounds able to cross the cell wall [8]. Conversely, fungi exhibit metabolic and physiological advantages over bacteria that allow them to feed on soluble and non-soluble compounds [8]. This is due to the presence of unspecific extracellular enzymes that cleave polymeric compounds [9]. Then, simpler products can be absorbed and catabolized by intracellular enzymes. In addition, the fungal hyphae constitute a physiological mechanism of adaptation to nutrient-poor environments, as their vegetative growth enables the internal translocation of nutrients on solid material such as soil [8]. The ability to degrade hydrocarbons by numerous types of microorganisms has been known for a long time [10]. According to these authors there are 81 genera of microorganisms that have been described as capable of biodegrading oil or its derivatives in a variety of environments. These microorganisms include members of the phyla Proteobacteria (*Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Sphingomonas*, *Pseudomonas*), Actinobacteria (*Arthrobacter*, *Corynebacterium*, *Dietzia*), Firmicutes (*Bacillus*) and Bacteroidetes (*Flavobacterium*), and members of the fungal phyla Ascomycota (*Aspergillus*, *Penicillium*), Zygomycota (*Cunninghamella*), subphylum Mucoromycotina (*Mucor*) and Basidiomycota (*Phanerochaete*, *Sporobolomyces*), among others. So, the first step in the bioremediation process should be the analysis of the soil native microbiota to know the potential capability to degrade TPHs

and then design a strategy to optimize the bioremediation process. The presence of contaminants together with soil properties are determinants for the composition of the native microbiota, contributing to a shift in their dynamics due to the potential toxicity of the contaminant and competition among microorganisms [1]. When bacteria and fungi coexist in the same population, they work together to degrade the contaminants, so that the most complex compounds can be oxidized by the non-specific fungal extracellular enzymes, transforming them into simpler compounds easily degradable by bacteria that feed on them. Often these fungi do not use the contaminant as a carbon source, but rather co-metabolize it, feeding on other materials [11]. However, the relationships between fungi and bacteria resulted in the development of intricate strategies by fungi, including antibiotic production on the negative side and selective stimulation of native bacteria on the positive one [12]. Those capabilities acquired by microorganisms led to their use in bioremediation to remove contaminants, providing a sustainable alternative to conventional remediation methods like soil washing or thermal treatments among others [13]. Bioremediation includes two main strategies: biostimulation, which enhances soil conditions by supplementing nutrients to stimulate soil microbiota metabolism, and bioaugmentation, which involves the addition of specialized microorganisms to facilitate the biodegradation of contaminants.

Bioremediation of hydrocarbons using fungi should be adapted to the site-specific conditions since environmental parameters could interact with the process, including the autochthonous microbial population. Therefore, treatability assays should be considered as a function of preliminary site characterization [14]. Fungi primarily degrade hydrocarbons in aerobic conditions, where their metabolic pathways require substrate oxidation in microsomes mediated by the cytochrome P450 (CYP) monooxygenases complex, which contains some alkane-oxygenase enzymes. This enzymatic complex catalyses the hydroxylation of aliphatic or aromatic compounds [3] in addition to the casual oxidation by their extracellular oxidases. However, the fungal degradation system is less well understood than that of bacteria, and the genetic basis of its biochemical activity is still incomplete compared to the knowledge of bacterial degradation pathways [15]. Furthermore, the spread of contaminant-degrading bacteria is favored by the action of fungi, overcoming motility restrictions to reach remote polluted areas. Consequently, consortia of bacteria-fungi are naturally established in soil [16].

Most cultivated edible fungi like *Agaricus bisporus*, *Pleurotus ostreatus* or *Pleurotus eryngii*, also produce extracellular unspecific oxidases such as laccase (Lac) or Mn peroxidase (MnP). These enzymes allow them to oxidize multiple organic substrates while also oxidizing and breaking lignin. Following harvesting, the spent mushroom substrate (SMS) becomes a waste product rich in organic matter and nutrients (C and N) in addition to holding fungal mycelia and enzymes [17]. These characteristics have made fungal SMS a valuable material for bioremediation [18–21]. Although SMS of different fungi have been successfully used to remediate hydrocarbons contaminated soils [22–24], further research is needed to gain a deeper insight into the biodegradation mechanisms along with the complex relationships between soil native microbiota (bacteria and fungi) and any other allochthonous microorganism. The introduction of exogenous microbiota through SMS could affect the soil autochthonous microbial community potentially establishing complex relationships that could alter the community structure and even the functional gene expression [25]. Some researchers have studied the structure and evolution of microbial communities during the cultivation

of those edible fungi, as well as the role of those microorganism in the development of these fungi [26,27]. However, there is a lack of knowledge on the effect of using these fungi and their SMS for bioremediation of TPH contaminated soils on the autochthonous soil microbiota.

This work aims to address the shift of the soil microbial community by the application of three SMS (*A. bisporus*, *P. ostreatus*, and *P. eryngii*) and combinations for the bioremediation of TPHs polluted soil. The combination of chemical analysis and the incorporation of advanced sequencing techniques coupled with bioinformatic tools, supported by predictive functional analysis using PICRUST2, not only offers a practical solution for soil bioremediation but also contributes significantly to scientific knowledge in the field. This novel approach provides a unique opportunity to thoroughly understand the relationship between contaminant degradation and soil microbiota. This work fills a knowledge gap in the bioremediation of polluted soils by SMSs identifying the biostimulated and bioaugmented microbiota and their potential role in the biodegradation of TPHs.

2. Materials and methods

2.1. Spent mushroom substrates

SMSs were collected from the Technological Center for Mushroom Research in La Rioja (CTICH, La Rioja, Spain). It contains remains of the mycelia of the cultivated fungi and of the constituent components of their growth substrates; for *A. bisporus*, it was based on composted wheat straw, chicken manure and calcium sulphate, for *P. eryngii*, hardwood sawdust, seed hulls, and wheat bran and for *P. ostreatus* wheat straw [28, 29]. In addition to featuring noteworthy physicochemical values (Table S1), it contains fiber and polymers such as lignin, cellulose, and hemicellulose [30,31]; as well as enzymes like amylase, cellulase, lac-case, xylanase, and versatile peroxidases [32].

2.2. Contaminated soil

The soil was obtained from a collection of contaminated soils from accidental oil spills in a refinery located in Huelva (Spain). The soil was stored at 4 °C until the assay was set. The analysis of TPHs was performed by a microwave extraction with hexane/acetone followed by a solid phase extraction (SPE) fractionation and finally gas chromatography with flame ionization detector (GC-FID) [33]. The analysis of aliphatic (C) and aromatic (EC) chains revealed that the most abundant chains were between C₁₂ and C₃₅ in both fractions while the light and the heaviest fractions were negligible (Table 1).

The total concentration of TPHs exceeded the reference value (50 mg/kg) by more than 100 times according to legislation [34]. Therefore,

Table 1
Concentration of TPHs, and aliphatic and aromatic fractions by chains (mean ± SD) of the soil and soil amended with *A. bisporus* SMS (10% v-v). Percentages indicate the contribution of each chain to aliphatic or aromatic fraction.

		Soil		Soil + SMS <i>A. bisporus</i>	
		mg kg ⁻¹	%	mg kg ⁻¹	%
Aliphatic chains	>C ₁₀ -C ₁₂	3.7 ± 0.3	0	3.4 ± 0.9	0
	>C ₁₂ -C ₁₆	1824 ± 33	26	1516 ± 263	22
	>C ₁₆ -C ₂₁	3703 ± 0	53	4102 ± 809	58
	>C ₂₁ -C ₃₅	1476 ± 24	21	1394 ± 252	20
	>C ₃₅	0.6 ± 0.1	0	8 ± 2	0
	Σ Aliphatics	7007		7023	
Aromatic chains	>EC ₁₀ -C ₁₂	3 ± 1	0	5.9 ± 0.2	0
	>EC ₁₂ -C ₁₆	347 ± 57	10	215 ± 17	7
	>EC ₁₆ -C ₂₁	2294 ± 223	67	2033 ± 59	70
	>EC ₂₁ -C ₃₅	756 ± 43	22	651 ± 78	22
	>EC ₃₅	8 ± 2	0	14 ± 3	0
	Σ Aromatics	3409		2918	
Σ TPHs		10416		9942	

this soil can be considered as contaminated soil without the need for a risk assessment. The soil was previously characterized in a separate work [35]. The main characteristics of the soil are: sandy texture (91% of sand), a basic pH (8.7), high concentration of organic matter (23 ± 4 g kg⁻¹) due to the nature of the contamination, low electrical conductivity (0.7 ± 0.2 dS cm⁻¹) and low concentration of nutrients (C:N:P of 100:1.5:0.08).

2.3. Design of the bioremediation assay

The bioremediation assay was conducted in microbiopiles of 2 L comprising layers of polluted soil and SMS (Fig. S1). The upper and lower layers, each of 5 cm high, consisted of soil (S) or soil mixed with *A. bisporus* SMS (SA) (10% v-v). *A. bisporus* SMS is a bulking agent and nutrient-rich material with the potential to enhance the physical, chemical, and biological properties of soil. This is why mixed and non-mixed soil was compared. The layering pattern was designed to assess the extent of the effect of SMSs on soil microbiota. Between these two layers, a 1 cm- thick fungal layer of *A. bisporus* SMS (A), *P. eryngii* SMS (E) or *P.ostreatus* SMS (O) was placed. Untreated soil was used as a control (S). The resulting eight treatments are described in Table 2. A 2 cm layer of sand was placed at the bottom of the microbiopiles to provide a draining space. Perforated plastic pipettes of 10 mL length were placed vertically in the microbiopiles connected to an aeration pump to provide fresh air periodically, favoring the level of oxygen in the soil. The oil moisture content was adjusted weekly to 60% of the maximum water-holding capacity of the soil. The microbiopiles were incubated at 25 °C in a climatic chamber in the dark for 60 days. The total soil microbial activity was determined by hydrolysis of fluorescein diacetate [36] at the end of the assay.

2.4. Soil DNA extraction, PCR amplification and high-throughput sequencing

The characterization of the soil microbial community in each microbiopile and the potential impact of the inclusion of SMS from three different species of fungi (*A. bisporus*, *P. ostreatus*, and *P. eryngii*) on the native microbiota was carried out. The DNA extraction of the soil microbiota was conducted at the beginning of the assay (S and SA) and after 60 days to analyze the bacterial and fungal diversity. Samples were taken from the upper (U) and lower (L) layers (Fig. S1).

The S and SA soil samples were taken at day 0 after homogenizing the polluted bulk soil. For SA treatments, samples were taken after 24 h of mixing the soil with the *A. bisporus* SMS, after which the assay was set. At the end of the assay (day 60), the two soil layers were extracted separately from each microbiopile. Firstly, the upper layer was homogenized, and an aliquot of 5 g was collected for DNA extraction. Subsequently, the lower layer was processed in the same way. Total DNA was extracted from 0.35 g of soil using the FastDNA SPIN kit for soil (MP Biomedicals) according to the manufacturer's instructions. DNA quantity was measured by Picogreen, (ThermoFisher). Amplicon of the 16 S ribosomal RNA gene (16 S rRNA) and ribosomal RNA internal transcribed spacer (ITS) region amplicons sequencing were performed.

Table 2
Description of the soil treatments tested in the assay.

Treatment	Upper layer (0 - 5 cm)	Intermediate layer (5 - 6 cm)	Lower layer (6 - 11 cm)
S	Soil	—	Soil
SAS	Soil + <i>A. bisporus</i> SMS (10% v-v)	<i>A. bisporus</i> SMS	Soil + <i>A. bisporus</i> SMS (10% v-v)
SES		<i>P. eryngii</i> SMS	
SOS		<i>P. ostreatus</i> SMS	
SA		—	
SAASA		<i>A. bisporus</i> SMS	
SAESA		<i>P. eryngii</i> SMS	
SAOSA		<i>P. ostreatus</i> SMS	

Briefly, the V3–V4 16 S rRNA region was amplified using 341 F/805 R primers: forward (5'- CCTACGGGNGGCWGCAG-3') and reverse (5'- GACTACHVGGGTATCTAATCC-3') for bacteria, and the ribosomal RNA internal transcribed spacer (ITS) region, using ITS3F/ITS4R primers: forward (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGA-CAGGCATCGATGAAGAACGCAGC-3') and reverse (5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGTCCTCCGCTTATTGATATGC-3') for fungi prior to libraries preparation using the Miseq reagent kit v3 600 cycles. Amplicons were sequenced using Illumina Miseq 2 × 300. Reads were uploaded to the BaseSpace (Illumina) server.

2.5. Sequencing data processing and analysis

Paired-end reads were demultiplexed and trimmed by Cutadapt and Q2-ITSxpress (for fungi) plugins for Qiime2 and forward and reverse reads were assembled, quality filtered, chimera filtered and assigned to Amplicon Sequence Variants (ASVs) following the DADA2 workflow implemented in Qiime2 v2023.2 standard pipeline. A Naive Bayes taxonomic classification was performed using two different classifiers: one trained on the V3–V4 hypervariable region extracted from 99% Silva 138 [37] 16 S sequences database for bacteria and the other on rRNA Internal transcribed Spacer ITS sequences from UNITE ver9.99.29.11.2022 database for fungi [38].

To compare the different samples, ASV abundances per sample data were filtered excluding all the ASV with abundance < 0.1% and normalized by rarefaction to the sample with the least number of sequences. Non-bacterial, chloroplast, and mitochondrial sequences were also excluded in bacterial classification, as well as all the ASV that were not included in the Fungi kingdom. Those not identified at phylum level were also excluded in both cases.

A predictive analysis was conducted to evaluate the metabolic potential of the different bacterial and fungal taxa during the degradation process of aromatic and aliphatic hydrocarbons. This analysis aimed to try to identify bacteria and fungi that could be responsible for hydrocarbon degradation, as well as elucidate the role of the three allochthonous fungi introduced in the soil through their SMS in hydrocarbon degradation. For this purpose, the contribution to the different functions of the different taxa was analyzed. An increase in the percentage contribution to a function was considered indicative of the involvement of that taxon in the function. The functional metagenomic prediction for the bacterial community was inferred using PICRUSt2 v 2.5.1 [39] for unstratified abundance and for metagenome contribution based on EC numbers, considering each enzyme a function. PICRUSt2 offers a computational approach to predict the functional composition of a metagenome using marker genes and a database of reference genomes. PICRUSt2 uses an extended ancestral state reconstruction algorithm to predict the presence of gene families, subsequently combining these gene families to estimate the composite metagenome [40]. ASVs were normalized by their predicted 16 S rRNA gene copy number and those ASVs with a nearest sequenced taxon index (NSTI) > 2 were removed. The functions selected for the predictive analysis are based on metagenomic and meta-transcriptomic studies [41–44]. Functions for bacteria were Benzoate 1,2-dioxygenase (EC:1.14.12.10) [45] and Catechol 1,2 dioxygenase (EC:1.13.11.2) [46], both involved in the oxidation of aromatic hydrocarbons and Alkane 1-monooxygenase (EC:1.14.15.3) for aliphatic hydrocarbons responsible for the starting hydroxylation of a wide range of n-alkanes [44,47,48]. The selected enzymes for fungi were laccase (EC:1.10.3.2) for aromatic hydrocarbons and CYP 450 (EC:1.14.-.-) for aromatic and aliphatic hydrocarbons, this enzymatic complex includes alkane-oxygenases enzymes (Alkane 1-monooxygenase (EC:1.14.15.3) [3]. All these enzymes were selected as representative of the main degradation pathways, as they are responsible for the first hydroxylation steps of aromatic and aliphatic hydrocarbons.

2.6. Statistical Analysis

The statistical test of the physicochemical data was carried out using the IBM SPSS statistical software package v26 (Armonk, NY, USA). The analysis of variance was performed after a Levene variance homogeneity test. To compare the differences between treatments, the Duncan test was performed for samples that present homogeneity of variances and the Games-Howell test for those that present heterogeneity with $p < 0.05$ for both cases. Principal component analysis (PCA) was done using the PAST V. 4.02 software (Natural History Museum, University of Oslo).

Metagenomic data were analyzed by R v. 4.2.2 using the “phyloseq” package. Graphical output was produced by ggplot2 package v. 3.4.2. Subsequent analysis of α -diversity indexes (Shannon, Inverse Simpson and Pielou evenness) were performed using “vegan” package. Beta diversity or variation among communities was observed by principal coordinate analysis (PCoA) by previously transforming the abundance matrix (wisconsin) and using the “wascores” function of the “vegan” package. A linear discriminant analysis effect size (LEfSe) method was used to identify bacterial and fungal taxa significantly different among treatments, which was verified by the wilcoxon test with $p < 0.05$.

3. Results

The application of SMS of any of the three fungi resulted beneficial for the soil microbiota activity as evidenced by the hydrolase analysis (Table S2). However, despite improving the microbial activity, the application of SMS of any of the three fungi in a layer was not as effective in stimulating the soil microbiota as the mixture of soil with *A. bisporus* SMS. This procedure and SMS significantly enhanced the microbial activity of soil, as evidenced by the 10-fold increase in hydrolase activity observed in SA (18160 $\mu\text{g h}^{-1} \text{g}^{-1}$) compared to S (1822 $\mu\text{g h}^{-1} \text{g}^{-1}$).

3.1. TPH mycoremediation

Fig. 1 illustrates the degradation percentage of TPHs, aliphatic, and aromatic chains in the unamended soil (S) and the SMS treatments in the upper (0–5 cm) and the lower (6–11 cm depth) layers of soil. The S treatment achieved a total degradation of 19–20%. The use of SMSs significantly enhanced the removal of TPHs. However, the type of SMS and its management produced significant differences in the degradation of these pollutants. The treatments SAS, SES, and SOS enhanced the degradation of TPHs in the lower and upper layers with respect to S. However, the treatments with SMS of *A. bisporus* mixed with the soil produced the highest removal of TPHs up to 67% in the best-case, SAASA in the lower layer. The differences in degradation percentages between the lower and the upper layers of soil were minimal in these treatments, ranging from 2 to 4%. In contrast, SAS, SES, and SOS showed significantly higher degradation in the lower layer, reaching up to 16%. Despite the potential benefits of amending the soil with SMS of *A. bisporus*, the introduction of a layer of one of the three SMS enhanced the degradation of TPHs with a range of 9 to 16% compared to SA.

The assessment of the degradation of TPHs by chains demonstrated clear differences between treatments. Firstly, the use of any SMS enhanced the degradation of all the aliphatic and aromatic chains, including the heaviest denoting the effectiveness of these wastes in degrading hydrocarbons. However, the type of SMS and the management of this waste modified the efficiency of the bioremediation process. The treatment SA enhanced the degradation of medium and heavy aliphatic and aromatic chains in the upper soil layer with respect to the SAS, SES, and SOS treatments. However, SA did not improve the degradation of these fractions in the lower layer. The treatments SAASA, SAESA, and SAOSA resulted in the highest degradation of all the aliphatic (41–70%) and aromatic (35–95%) fractions in the upper soil layer. Conversely, these treatments only produced significant increments of degradation of aliphatic (64–70%) and aromatic (66–

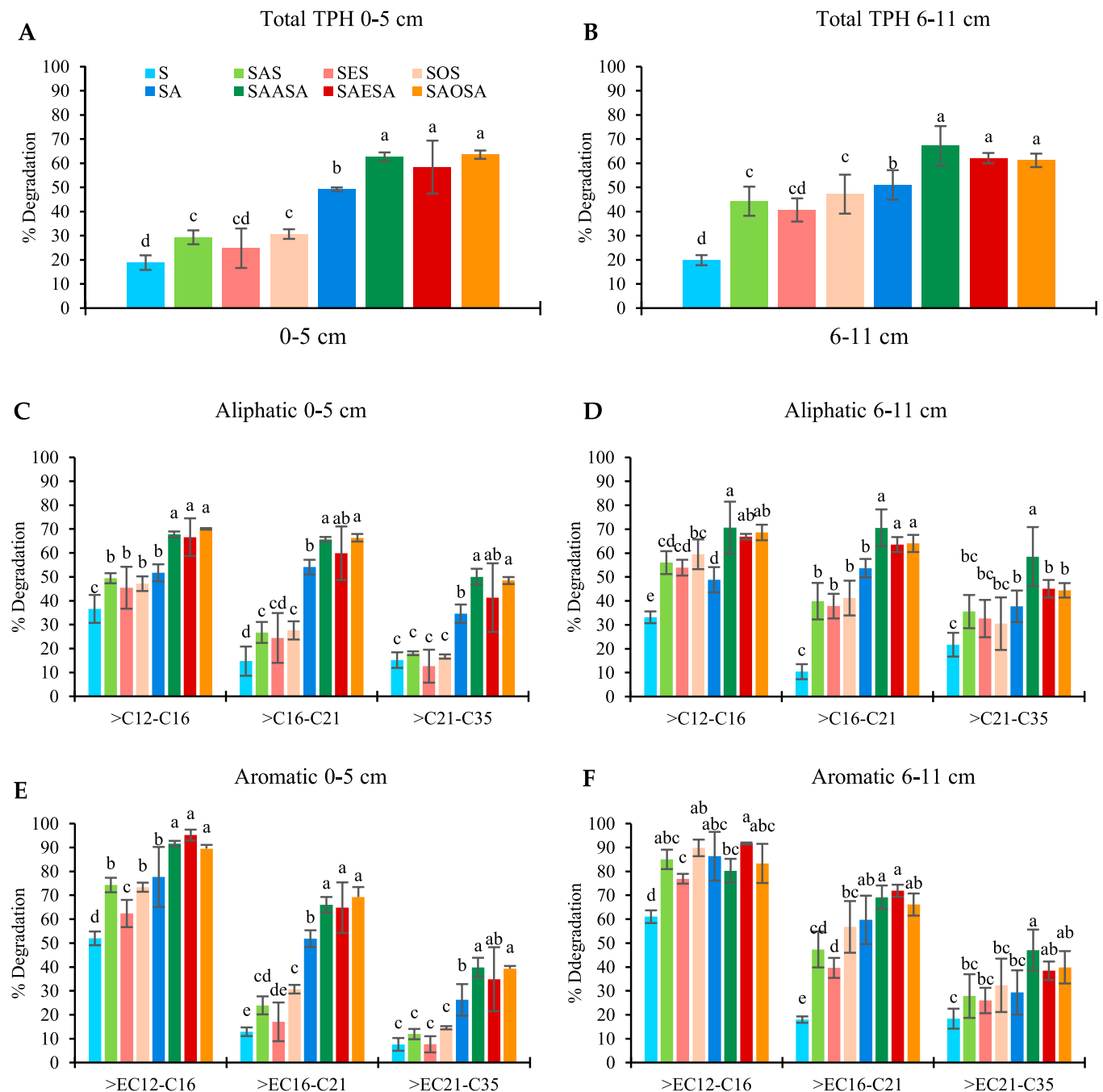


Fig. 1. Degradation percentages of TPH, aliphatic, and aromatic chains (mean \pm standard deviation) in the upper (0–5 cm) and lower (6–11 cm) soil layers after 60 days of incubation. S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0–5 cm) and lower (6–11 cm) soil layers of the microbiopile. The same letters indicate a lack of statistically significant differences between treatments ($n = 3$; $p < 0.05$).

72%) chains in the lower soil layer. In particular, SAASA resulted in significant enhancement of the degradation of the heaviest aliphatic (59%) and aromatic (47%) chains in the lower soil layer.

3.2. Bacterial communities

3.2.1. Taxonomic analysis

The taxonomic study at the phylum level revealed that soil autochthonous bacteria belonged mainly to phyla Proteobacteria (67%) and Bacteroidota (27%) (Fig. S2). The presence of the *A. bisporus* SMS (SA treatments) increased the phylum Firmicutes [26], reaching 26% of abundance at T0 while in the soil it was barely 0.4% (Fig. S2) denoting a

clear bioaugmentation phenomenon. At T60 Firmicutes exhibited relative abundances of 0.6% and 0.5% in S and 9% and 11% in the upper and lower soil layers of SA, respectively. SES and SOS showed similar relative abundances to S, but the relative abundance in SAS was 5%. Conversely, the relative abundance of the phylum Mixococcota increased in the S treatments, especially in S (0.05 to 12%), although the presence of any of the three SMSs resulted in a less pronounced increase, with values of 3% in SAS, 8% in SES, and 6% in SOS. In all SA treatments, this phylum decreased during the incubation period.

The relative abundance of bacterial families at T0 and T60 is presented in Fig. 2. The four main families in S and SA at T0 were *Flavobacteriaceae*, *Xanthomonadaceae*, *Pseudomonadaceae*, and

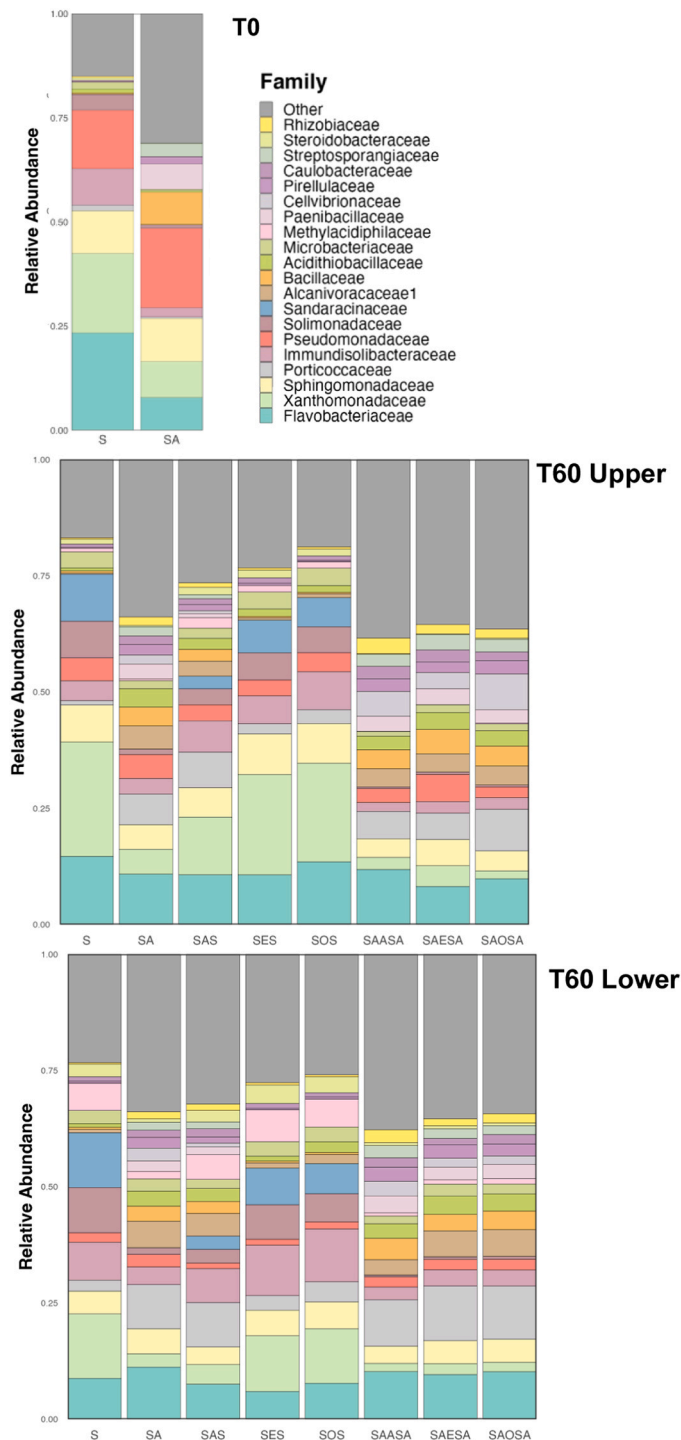


Fig. 2. Relative abundance of bacteria at family level showing the 20 most abundant families at the beginning of the assay (T0) and after 60 days of incubation (T60) ($n = 3$). S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0–5 cm) and lower (6–11 cm) soil layers of the microbiopile.

Sphingomonadaceae. The bacterial relative abundance of SA treatment (T0) showed a quick and deep modification of the bacterial community denoting the high microbial load of this waste. The presence of *A. bisporus* SMS at the beginning of the experiment (SA), resulted in a decrease in the abundance of *Flavobacteriaceae* (24 to 8%), *Xanthomonadaceae* (19 to 9%), and *Immundisolibacteraceae* (9 to 2%). Conversely, the augmented families were *Pseudomonadaceae* (14 to 19%), *Bacillaceae*

(0.3 to 8%), and *Paenibacillaceae* (0.001 to 6%).

A clear shift in the relative abundance of the bacterial community was observed at T60 in comparison to T0 and among treatments (Fig. 2). Furthermore, differences were also observed between the upper and lower soil layers. The treatments S, SAS, SES, and SOS showed a similar bacterial shift among T0 and T60 in the four most abundant bacterial families. In particular, the relative abundances of *Flavobacteriaceae*, *Pseudomonadaceae*, and *Sphingomonadaceae* decreased. Conversely, the abundance of the *Xanthomonadaceae* family increased in S, SES, and SOS. In these four treatments, there was a stimulation of the *Sandaracinaceae* family, which did not occur in SA treatments.

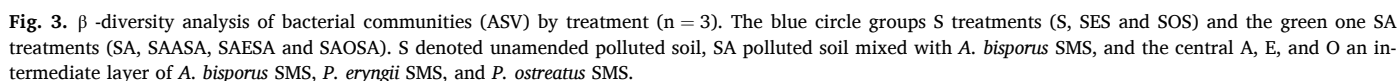
The *Flavobacteriaceae*, *Xanthomonadaceae*, and *Immundisolibacteraceae* families were negatively affected by the presence of *A. bisporus* SMS, whereas both *Pleurotus* SMSs did not produce negative effects. In contrast, the *Porticoccaceae* family was strongly favored in SA treatments. Among the bacterial shifts shown in Fig. 2, one of the most relevant was the stimulation of the family *Alcanivoraceae*, known for its hydrocarbon degrading capabilities [49], in all SMS-treated samples, despite its low relative abundance in S (0.1%) and SA (0.02%) at T0. The biostimulation was particularly evident in SAS and SA treatments, reaching up to 6%, with consistently higher levels observed in the lower layers. Conversely, the abundance of this family in treatment S increased slightly from 0.2 to 0.7%. These observations indicate the differing microbial loads of the three SMSs tested and the subsequent implications on the microbial structure.

The distribution of soil layers in the microbiopiles and mainly, the amendment of the soil with *A. bisporus* SMS had a profound impact on the soil bacterial community. S, SES, and SOS treatments showed that the relative abundance of *Flavobacteriaceae*, *Xanthomonadaceae*, and *Sphingomonadaceae* decreased in the lower layer with respect to the upper layer. Conversely, the relative abundance of *Methylophilaceae* and *Immundisolibacteraceae* was higher in the lower layer. This microbial shift was minimized in the SA treatments, where the relative abundance of bacterial communities was similar in the lower and upper layers. Only in SAASA and SAESA treatments, there was an increase in the relative abundance of *Porticoccaceae* in the lower layer compared to the upper layer. In addition to differences in microbial structure, there were also differences in moisture and microbial activity between the two layers, with higher values observed in the lower layer (Table S2).

The Linear Discriminant Analysis Effect Size (LEfSE) method was applied to the bacterial communities at the family level (Fig. S3), with the objective of identifying the bacterial taxa exhibiting significant abundance differences with an LDA score of 3.5. Relevant biological differences were observed between T0 and T60 (Fig. S3) and between the upper (0–5 cm) and lower (7–11 cm) layers of soil (Fig. S3). The *Alcanivoraceae* and *Microbacteriaceae* families exhibited statistically significant and biologically consistent differences at T60 (Fig. S3A). Furthermore, differences were also observed between the two layers. For instance, some families, including *Flavobacteriaceae*, *Sphingomonadaceae*, *Pseudomonadaceae*, *Sphingobacteriaceae*, and *Borgoricellaceae* showed differential abundance in the upper layer. In contrast, families such as *Immundisolibacteraceae*, *Porticoccaceae*, *Methylophilaceae*, *Waddilaceae*, *Microscillaceae*, and *Steroidobacteraceae*, were more prevalent in the lower layer (Fig. S3).

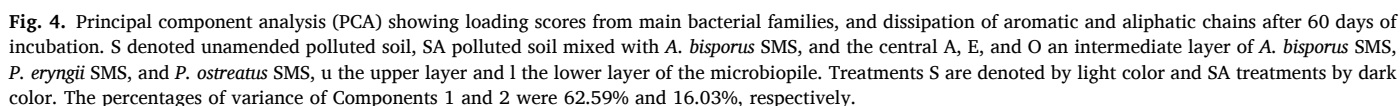
The richness of the samples is shown by ASV (Fig. S4). Meanwhile the α -diversity indices provided, on the one hand, the richness and evenness of taxa in the samples (Shannon and Simpson). And, on the other hand, how homogeneous was the distribution of these taxa across the different samples (Pielou Index) [50]. The diversity of S and SA at T0 was found to be very similar. However, after 60 days, a higher diversity was observed in SA with respect to S. This was attributed to the presence of the SMS of *A. bisporus*, which, in addition to biostimulating some taxa provided its own associated microbiota.

β -diversity is defined as the variation in the composition of bacterial communities across samples (Fig. 3). At T0 treatments S and SA revealed a quick shift denoting the profound impact of the *A. bisporus* SMS on the



The global performance of the microbiopiles after 60 days of incubation is shown in Fig. 4. Principal component 1 (PC1) retained 62.59%

of the variance. PC1 was positively related to the degradation of aliphatic and aromatic hydrocarbons and several bacterial families such as *Porticoccaceae*, *Alcanivoracaceae*, *Bacillaceae*, *Acidithiobacillaceae* among others. In contrast, numerous bacterial families exhibited a negative correlation with PC1, including *Immundisolibacteraceae*, *Solimonadaceae*, and *Xanthomonadaceae* among others. PC2 retained 16.03% of the variance. PC2 was positively correlated with the degradation of hydrocarbons, particularly EC₁₂-EC₁₆, EC₂₁-EC₃₅, C₁₂-EC₁₈, and several bacterial families such as *Methylacidiphilaceae*, *Steroidobacteraceae*, and *Sandaracinaceae* among others. The main bacterial families negatively correlated with PC2 were *Pseudomonadaceae*, *Flavobacteriaceae*, and *Xanthomonadaceae*.



A clear distribution of the treatments along PC1 was observed, based on the presence or absence of *A. bisporus* SMS and its management. SA treatments were located in the positive zone of the PC1, exhibiting the highest degradation of TPHs and all of the aliphatic and aromatic chains, with the exception of EC₁₂-EC₁₆ and EC₂₁-EC₃₅, where no clear differences were observed in the lower soil layer. In these treatments, there was bioaugmentation or biostimulation of *Porticoccaceae*, *Pseudomonadaceae*, *Alcanivoraceae*, *Bacillaceae*, and *Cellvibrionaceae* among other bacterial families in the upper and lower soil layers (Fig. 2). S, SES, and SOS treatments were situated in the negative zone of the PC1. These treatments exhibited a generalized reduction in hydrocarbon degradation compared to the treatments amended with *A. bisporus* SMS. In these treatments, the *Xanthomonadaceae* family was the most abundant (Fig. 2), and several bacterial families showed relative abundances higher than in SA treatments, including *Sphingomonadaceae*, *Immundisolibacteraceae*, and *Solimonadaceae*. In these treatments, *Sandaracinaceae* and *Steroidobacteraceae* were also abundant but in contrast, did not show relative abundances higher than 1% in SA treatments. Consequently, their role in TPHs degradation did not appear to be a significant one. The treatment SAS was located between S and SA treatments denoting an intermediate TPHs degradation efficiency and microbial modification with respect to the other S treatments. This highlights the profound impact of *A. bisporus* SMS on microbial structure and the subsequent dissipation of TPHs. The treatments SA, where the *A. bisporus* SMS was applied by mixing with soil, were located in the right zone of the PC1, denoting the positive correlation with high hydrocarbon degradation and the relative abundance of several bacteria families, including *Porticoccaceae*, *Alcanivoraceae*, *Bacillaceae*, *Acidithiobacillaceae*, and *Rhizobiaceae*. In contrast, these treatments were poorly related to bacterial families such as *Immundisolibacteraceae*, *Solimonadaceae*, and *Xanthomonadaceae*. The low dispersion of the SA treatments in PC1 and 2 denoted a lack of significant differences in hydrocarbon degradation and bacterial structure.

The relative abundances of the *Flavobacteriaceae* and *Pseudomonadaceae* were similar in all the treatments (Fig. 2). They were observed in PC1 with a low load score, denoting a low relation to the TPHs degradation and therefore suggesting a low impact on the biodegradation process. Furthermore, the layer disposition or the type of SMSs did not

produce a significant impact on these bacterial families.

3.2.2. Bacterial functional predictive analysis

The contribution of the main families to Catechol 1,2 dioxygenase (EC:1.13.11.2), Benzoate 1,2-dioxygenase (EC:1.14.12.10), and Alkane 1-monooxygenase (EC:1.14.15.3) is shown in Fig. 5. The aromatic fraction degradation pathways were evaluated by means of catechol 2,3 dioxygenase and benzoate 1,2 dioxygenase. The catechol 2,3-dioxygenase was enhanced and distributed among the taxa-like families *Microbacteriaceae*, which showed the highest contribution in soil (S) and with a layer of either of the two *Pleurotus* SMSs (SES and SOS). The *Bacillaceae* showed the highest contribution in all the treatments with *A. bisporus* SMS. Conversely, the presence of *A. bisporus* SMS was detrimental for *Solimonadaceae*, which exhibited a clear decrease in contribution relative to those with soil or either of the two *Pleurotus* SMSs. The benzoate 1,2 dioxygenase was primarily produced by families belonging to Proteobacteria phylum, including the *Xanthomonadaceae*, which exhibited the highest contribution, and the *Sphingomonadaceae*, particularly in the S treatments. Conversely, the *Alcaligenaceae* and *Dietziaceae* families demonstrate an increased contribution when the *A. bisporus* SMS was present. The *Pseudomonadaceae* family showed a very similar contribution across all treatments, particularly in the S and SAESA treatments.

The alkane degradation was assessed by one of the most representative enzymes that catalyzes the initial hydroxylation of n-alkanes along with CYP450, the alkane 1-monooxygenase, whose activity was related to the family *Alcanivoraceae* together with *Solimonadaceae*, *Rhodobacteraceae*, and *Algiphilaceae*. The presence of *A. bisporus* SMS was positive for *Alcanivoraceae*. In contrast, *A. bisporus* SMS was detrimental for *Solimonadaceae* and *Algiphilaceae*. In the case of *Solimonadaceae*, the treatments SAESA, SAESA, and SAOSA showed negative impact on this enzyme activity.

3.3. Fungi

3.3.1. Taxonomic analysis

The phylum Ascomycota (90% of relative abundance) was the dominant soil autochthonous fungal phylum, but the presence of *A. bisporus* SMS (SA treatments) led to a shift in the relative abundance

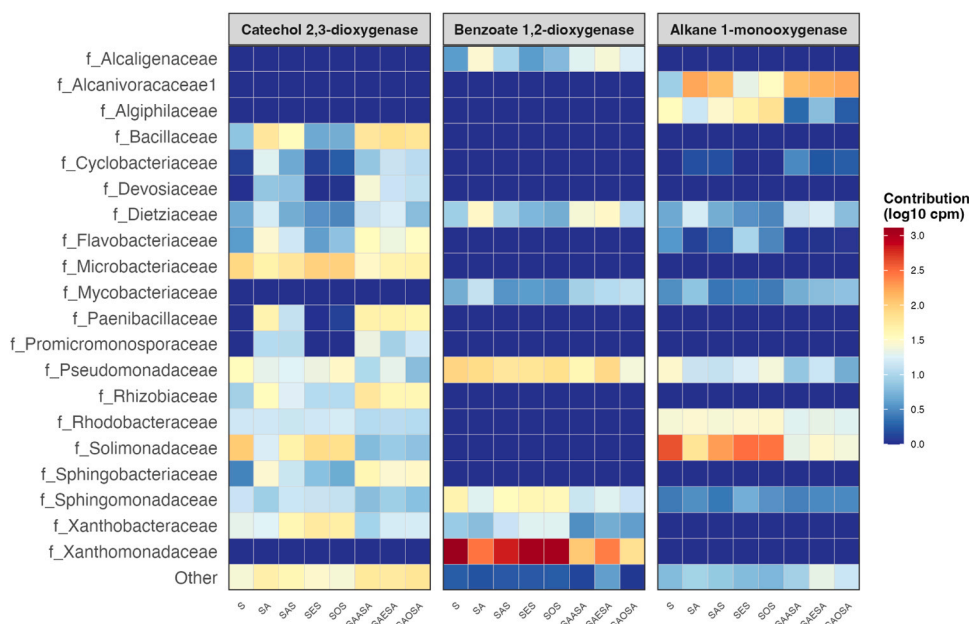


Fig. 5. Functional heatmap of bacterial contribution to hydrocarbon degradation enzymes at family level measured in log₁₀ copies per million with a cut-off of 0.04. The enzymes selected were Catechol 2,3-dioxygenase (EC:1.13.11.2) and Benzoate 1,2-dioxygenase (EC:1.14.12.10) for aromatics and Alkane 1-monooxygenase (EC:1.14.15.3) for aliphatics. S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0 – 5 cm) and lower (6 – 11 cm) soil layers of the microbiopile.

of the phylum Basidiomycota denoting bioaugmentation (Fig. S5). The most abundant genus in S at T0 was *Cadophora* (36% relative abundance) (Fig. 6), which has been described as a PAH degrader [51], followed by *Ochroconis*, which is commonly found in soils contaminated with TPH [52]. The microbial shift produced by the introduction of *A. bisporus* SMS was greater among the fungal community than the bacterial one. The relative abundance of the genus *Agaricus* was clearly predominant (90%), resulting in a sharp decline in the relative

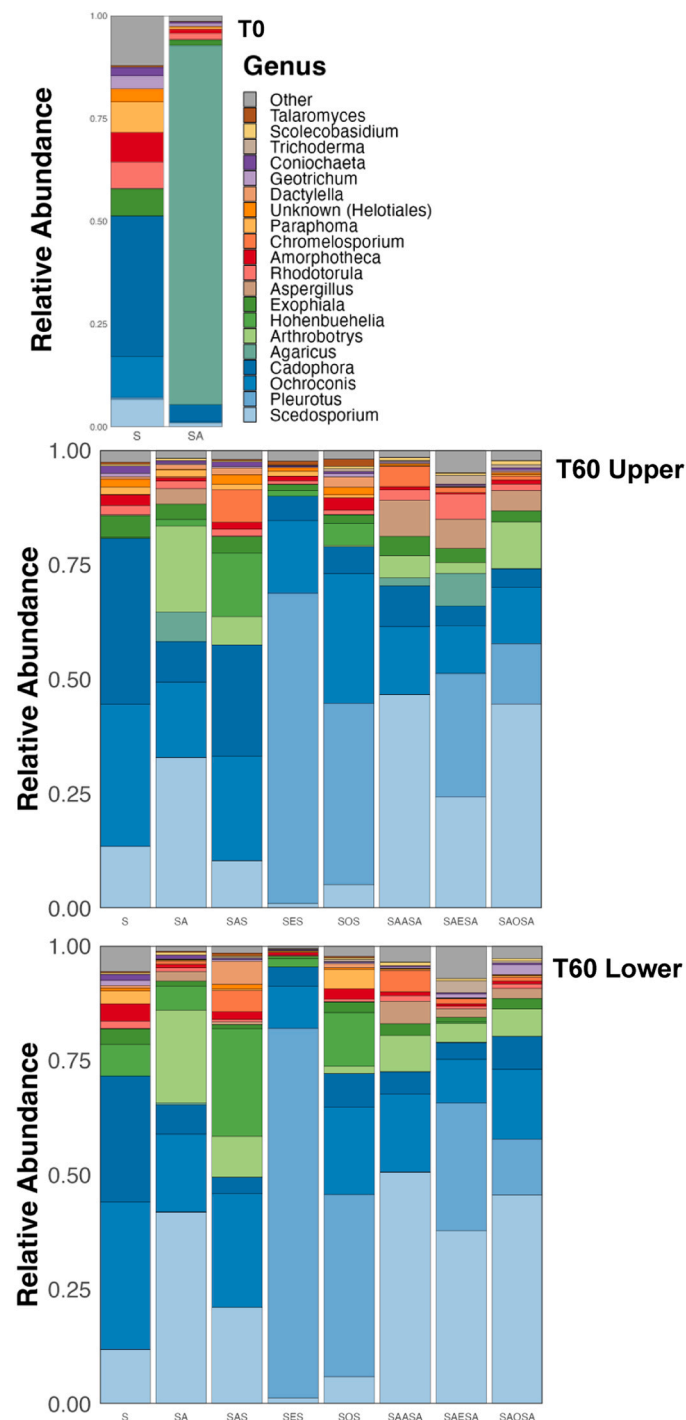


Fig. 6. Relative abundance of fungi at the genus level of the 20 most abundant genera ($n = 3$). S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0 – 5 cm) and lower (6 – 11 cm) soil layers of the microbiopile.

abundance of the autochthonous fungi.

After 60 days, the phylum Ascomycota was once again dominant in all the treatments, with the exception of SES, and SOS, where it was present to a lesser extent (Fig. S5). There was a clear shift in the fungal genera after 60 days with respect to T0. The main genera in S T0, *Cadophora*, remained in the soil at T60 and re-colonized the SA treatments where their relative abundance was very low (4%). However, the presence of any of the three SMSs negatively affected it, with relative abundances under 9%, except for SAS in the upper layer (25%). The relative abundance of the three most abundant fungal genera in S, *Cadophora*, *Ochroconis*, and *Scedosporium*, was similar in the lower and upper soil layers at T60. The genus *Ochroconis* exhibited an increase in abundance after the incubation period in all S treatments, with the greatest increase observed in S, from 10 to 33%. The competence with the allochthonous fungi appeared to hinder the development of *Ochroconis*, resulting in lower increases. Consequently, the abundance of this fungus was 25% in SAS, 16% in SES, and 29% in SOS. The presence of higher loads of non-native fungi in SA treatments resulted in lower increases in the abundance of this fungus. Consequently, SA, SAASA, and SAOSA exhibited 15 – 17% relative abundance after the incubation period, while in SAESA, the genus *Ochroconis* did not show an increase (10%). The genus *Scedosporium* doubled its abundance in S at T60 with respect to T0 (from 7 to 14%). However, the presence of *P. eryngii* and *P. ostreatus* SMSs exerted a negative influence, resulting in 1% and 6% of relative abundance in SES and SOS treatment, respectively. The autochthonous fungus exhibited a clear benefit from the presence of SMS of *A. bisporus*, both mixed with the soil (SA treatments) and in a layer (SAS), with relative abundances of 42% in SA, 51% in SAASA, 38% in SAESA, and 46% in SAOSA. Other soil native genera, such as *Amorphotheca* (7.5% relative abundance) and *Rhodotorula* (7%) exhibited a decline in abundance at T60 in all treatments, with a more pronounced decline observed in the presence of SMS from any of the three allochthonous fungi, reaching relative abundances below 2% and 3% respectively. Meanwhile, *Exophiala* (7%) was particularly negatively affected by the two *Pleurotus* SMSs, with relative abundances below 2%. This genus, which is frequently found in habitats rich in monoaromatic hydrocarbons and alkanes [53], exhibited a similar loss of abundance in S and treatments with *A. bisporus* SMS (SA, SAS and SAASA).

SA exhibited a 90% relative abundance of *A. bisporus* at T0, which subsequently declined significantly, with the fungus almost disappearing from all SA treatments at T60 (Fig. 6). However, both *Pleurotus* remained and even exhibited an increase in abundance, particularly in S treatments, resulting *Pleurotus* reaching 81% in SES and 40% in SOS. Both were three times less abundant in T60 in the SA treatments (SAESA and SAOSA), likely due to competition with *Agaricus* and the high microbial load of *A. bisporus* SMS. However, the presence of this SMS stimulated the abundance of some autochthonous fungal genera such as *Arthrobotrys* or *Scedosporium*. The last genus significantly increased its abundance, rising from 1% in SA T0 to 47% and 50% in SAASA in the upper and lower soil layers, respectively. *A. bisporus* SMS stimulated the genus *Aspergillus*, particularly in the upper layer, while this genus was not observed in the other treatments. The genus *Hohenbuehelia*, which showed a markedly low abundance in S T0 (0.03%), exhibited a 24% increase in SAS and a 12% increase in SOS at T60. In contrast, the growth of *P. eryngii* SMS was significantly slowed.

Additionally, there were variations in the distribution of native fungi between layers. *Scedosporium* was more prevalent in the upper layer in S, whereas the presence of *A. bisporus* SMS favored its growth in the lower layer in all SA treatments. In contrast, the two *Pleurotus* SMS were detrimental. Genus *Ochroconis* exhibited a similar pattern, except for SES and SOS where this genus was more abundant in the upper layer. Genus *Hohenbuehelia* resulted in greater abundance in the lower layer of SAS and SOS. To ascertain which differences in abundances among the genera of the soil fungal community, a LefSE analysis was conducted, showing significant differences with biological effects also present in the fungal community (Fig. S6). Three genera were identified with

differential abundance following the 60-day incubation period, including important taxa for hydrocarbon bioremediation such as *Scedosporium* and *Ochroconis*. A comparison of the two layers revealed two genera with differential abundance in the upper layer: *Exophiala* and *Rhodotorula*.

The α -diversity analysis of the fungal community (Fig. S7) revealed some differences from those observed in bacteria. The initial soil exhibited a greater richness in observed ASVs than the rest of the treatments, yet the Shannon or Simpson diversity was lower. This discrepancy may be attributed to the presence of numerous fungi with a very low abundance, with only a few exhibiting a dominant presence. Following incubation, there was a reduction in fungal richness, but those that remained were more evenly distributed, as indicated by the Pielou index. The evenness index was equal to or higher at T60, with the exception of SES.

Regarding β -diversity of fungi, there was a clear difference in taxonomic composition between the soil treatments with the SMS layer of either of the two *Pleurotus* species (SES and SOS) and the rest of the treatments holding *A. bisporus* SMS. This suggested competence between either of the two *Pleurotus* and the autochthonous soil fungal community (Fig. 7). Conversely, *Agaricus* did not present competition with autochthonous fungi, probably because of its dissipation at T60.

Fig. 8 shows the principal component analysis (PCA) of the degradation of aromatic and aliphatic hydrocarbons and the fungal structure. The principal component 1 (PC1) retained 30.10% of the variance. PC1 was positively related to the degradation of aliphatic and aromatic hydrocarbons and several fungal genera, including *Scedosporium*, *Aspergillus*, and *Chromelosporium* among others. In contrast, numerous fungal genera exhibited negative correlation with PC1, such as *Hohenbuehelia* and *Geotrichum*, among others. The PC2, on the other hand, retained 14.08% of the variance. This component was positively associated with the degradation of hydrocarbons, particularly EC₂₁₋₃₅, C₂₁₋₃₅, and several fungal genera, including *Ochroconis*, *Cadophora*, *Exophiala*, and *Agaricus* among others. The only fungal genus negatively related to PC2 was *Pleurotus*.

A similar pattern was observed in the distribution of the treatments along PC1, which was based on the presence or absence of *A. bisporus* SMS and its management. The SA treatments were located in the positive zone of the PC1, SAS in the central zone, and the treatments without *A. bisporus* SMS in the negative zone. Treatments SA were related to

aromatic and aliphatic hydrocarbon degradation, a high relative abundance of *Scedosporium*, and the presence of *Aspergillus*. The treatment SAS showed an intermediate behavior based on aromatic and aliphatic hydrocarbons degradation and fungal structure. The treatments S, SES, and SOS were poorly related to hydrocarbon degradation and denoted different fungal structure where *Ochroconis* and *Cadophora* were predominant genera. The lower and upper layers of the S treatments were closely located, denoting similar behavior. However, the treatments SES and SOS denoted differences between the upper and lower layers. The lower layers were located close to the central zone of the PCA whereas the upper layers were in the left zone. This was related to the higher degradation of hydrocarbons, mainly the heaviest, of the lower layer (Fig. 1). Furthermore, the SES treatment was located in the lower zone of the PCA and relatively far from the S and SOS treatments, as a consequence of the high relative abundance of *Pleurotus* in SES (Fig. 8) that produced a clear difference in the fungal structure. Similarly, all of the other treatments were located in the positive zone of PC2, reflecting the presence of *Exophiala*, *Hohenbuehelia*, and *Arthrobotrys*.

3.3.2. Fungal functional predictive analysis

The targeted enzymes to evaluate the potential capability of TPH degradation of fungal community were the extracellular laccase (EC: 1.10.3.2) for the aromatic fraction, the intracellular cytochrome P450 (EC: 1.14.14.1) for both the aromatic and aliphatic fraction [54], and alkane 1-monooxygenase (EC:1.14.15.3) for the aliphatic one. The genera *Cadophora* and *Scedosporium* were the most significant contributors to the dissipation of hydrocarbons, with the former observed in S treatments and the latter in SA treatments (Fig. 9). These two fungi contributed to both functions, laccase and CYP450. *Aspergillus* primarily contributed to CYP450 in SA treatments, while *Amorphoteca* and *Hohenbuehelia* in S treatments, mainly with the SMS layer. Alkane 1-monooxygenase function was poorly contributed by the fungal community, with the genera *Hohenbuehelia* and *Agaricus* being the main contributors. Furthermore, *Agaricus* also contributes to the other two functions, particularly to cytochrome P450 in SA and SAESA treatments. Conversely, none of the two *Pleurotus* contributed to the aforementioned functions at a rate exceeding 0.4%, which was the established threshold despite the considerable prevalence of this genus in SES, SOS, SAESA, and SAOSA.

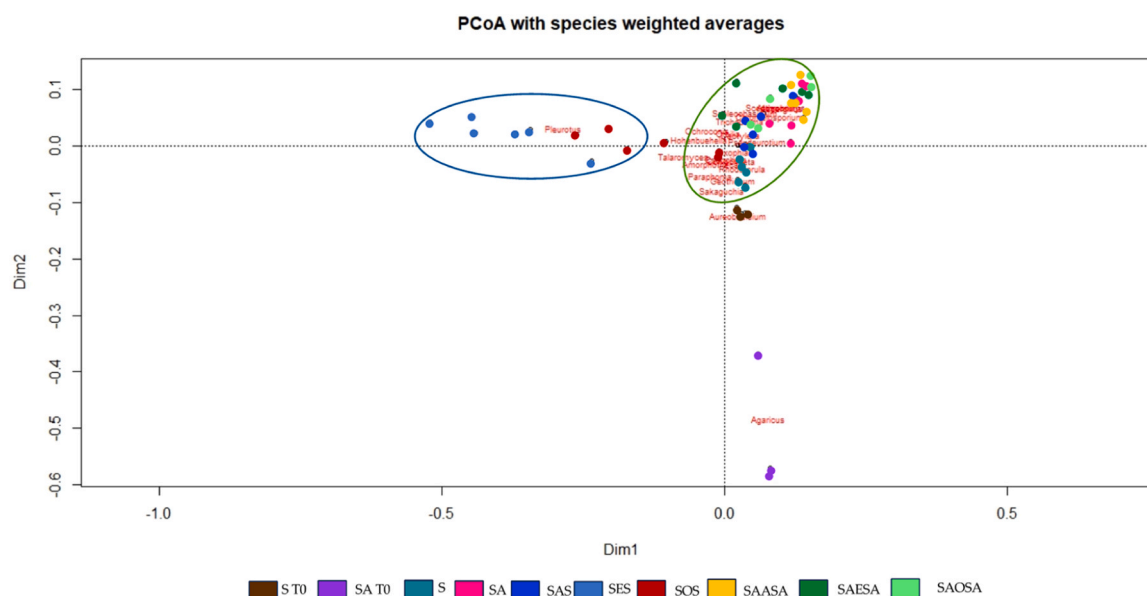


Fig. 7. β -diversity analysis of fungal communities (ASV) by treatment (n = 3). The blue circle groups S (SES and SOS) treatments and the green one SA treatments (SA, SAASA, SAESA and SAOSA) and SAS. S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0–5 cm) and lower (6–11 cm) soil layers of the microbiopile.

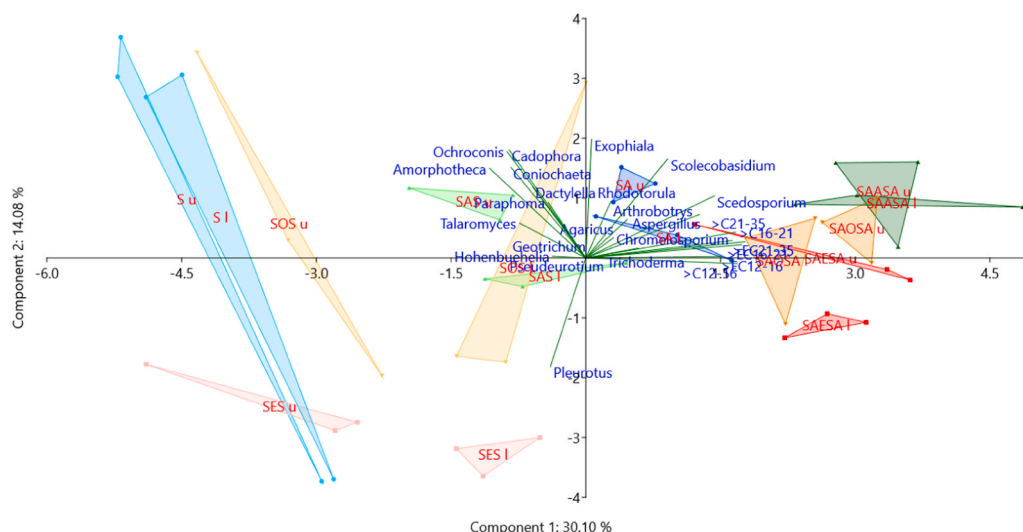


Fig. 8. Principal component analysis (PCA) showing loading scores from main fungal genera, and dissipation of aromatic (EC) and aliphatic (C) chains after 60 days of incubation. S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, u the upper layer and l the lower layer of the microbiopile. S treatments are denoted by light color and SA treatments by dark color. The percentages of variance of Components 1 and 2 were 30.10% and 14.08%, respectively.

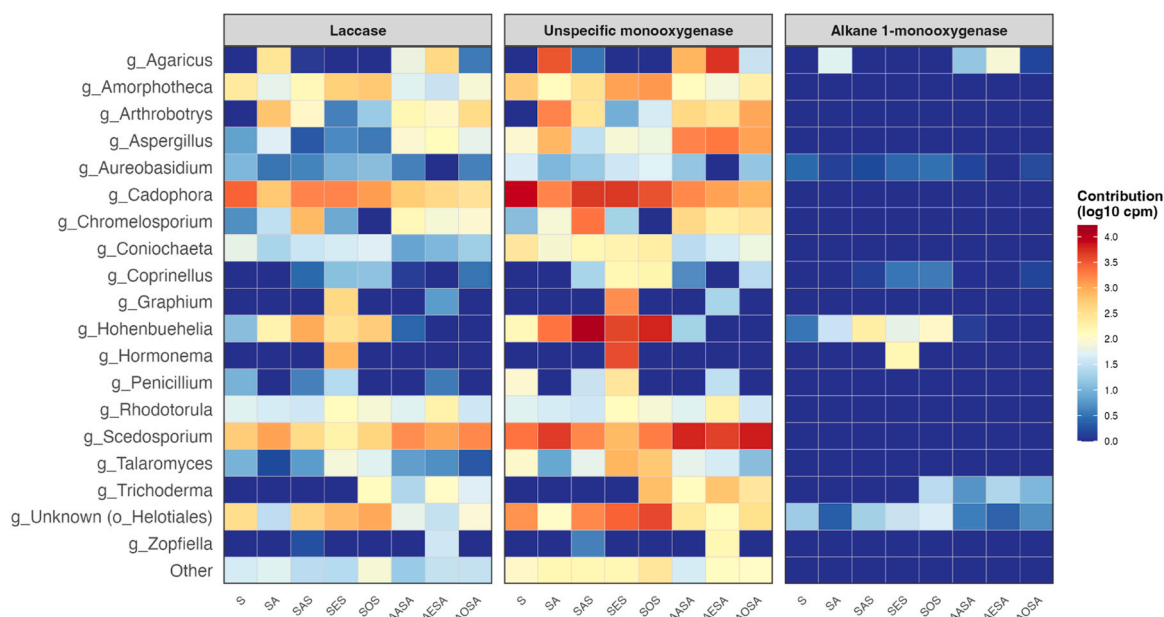


Fig. 9. Functional heatmap of fungal contribution to hydrocarbons degradation enzymes at genus level measured in log10 copies per million with a cut-off of 0.4. The enzymes selected were laccase (EC: 1.10.3.2) for the aromatic fraction, the intracellular cytochrome P450 (EC: 1.14.14.1) for both aromatic and aliphatic fraction and Alkane 1-monooxygenase (EC: 1.14.15.3) also for aliphatics. S denoted unamended polluted soil, SA polluted soil mixed with *A. bisporus* SMS, and the central A, E, and O an intermediate layer of *A. bisporus* SMS, *P. eryngii* SMS, and *P. ostreatus* SMS, respectively, between the upper (0 – 5 cm) and lower (6 – 11 cm) soil layers of the microbiopile.

4. Discussion

Microbial activity is a key factor for bioremediation processes, serving as indicator of the ability of microbiota to grow and degrade contaminants under specific environmental conditions [55]. Total hydrolase activity demonstrated the high biological activity, particularly in SA treatments (Table S2), which agreed with the high rates of TPHs dissipation achieved in those treatments along with the high microbial diversity promoted by the *A. bisporus* SMS. In fact, this enzymatic activity was significantly ($p < 0.01$) correlated with all the aliphatic or aromatic chains and TPH degradation (Table S3).

The persistence of TPHs in soil depends on several factors, including

their chemical structure, bioavailability, concentration, climatic conditions, pH, oxygen content, and soil type and texture [56]. These persistent TPHs are aliphatic and aromatic chains of intermediate-long length over 16 carbon units ($>C_{16}$) [57], which were the most abundant in this soil. The results obtained in this work demonstrated that the degradation of aliphatic and aromatic chains between C_{16} and C_{35} was favored by the three SMSs, with degradation rates exceeding 70% in some cases. Furthermore, the fungal colonization of this polluted soil by the *P. ostreatus* and *P. eryngii* confirmed their ability to adapt to highly contaminated environments. However, *A. bisporus* was unable to colonize this soil and therefore did not appear to be a competitive fungus in this soil. Active living mycelia of the SMS can secrete some unspecific

extracellular enzymes capable of oxidizing complex compounds like lignin, an ability that can be used for TPHs degradation [3]. Zhou et al. [24] remarked that *P. eryngii* SMS provided nutrients to the soil microbiota, resulting in enhanced degradation rates of PAHs. These findings agreed with the results of this work, as the highest degradation rates both in the aliphatic and aromatic fractions were obtained in the SA treatments, particularly those with a fungal layer where microbial load and nutrients inputs were high. The use of any of the three SMSs tested led to a significant improvement in the TPHs degradation rate in comparison to S. The TPH degradation levels reached in this work were comparable to those obtained in previous studies using SMS [35,46]. Nevertheless, the hydrocarbonoclastic microorganisms leading the degradation were not identical in all the treatments, and the final degradation rates differed. SMS profoundly altered the indigenous soil microbiota through complex relationships of competition, synergy, or co-metabolism. For instance, ligninolytic fungi can transform PAHs into oxygenated intermediates through the action of ligninolytic extracellular enzymes, resulting in the production of intermediate products that are more hydrophilic and more reactive, thereby facilitating subsequent bacterial degradation [58]. Jabbar et al. [59] observed that microbial diversity was diminished in contaminated soils due to the influence of toxic petroleum hydrocarbons on the organisms. However, they estimated that the rate of degrading microorganisms ranges between 1% and 10% of the total population in a contaminated environment, while in a non-polluted environment it is less than 1%. This highlighted the interactions between pollutants and the structure of soil microbiota, which is a key factor in understanding the overall bioremediation process.

The greatest aromatic degradation occurred in the treatments with SMSs, particularly in the SA treatments, which provided the fungal live mycelium and the inherent microbial population, enzymes, and lignocellulosic material to the soil. Previous works reported that lignin and its constituents induced co-metabolic degradation of aromatic hydrocarbons and modified the soil microbiota [60,61]. The *Sphingomonadaceae* family exhibited a lower abundance in S treatments with an SMS layer of either of the two *Pleurotus* (SES and SOS) whose substrate usually holds lignin [58]. Furthermore, it is notable that the *Sphingomonadaceae* family increased its abundance in the upper layer in all S treatments. In contrast, in SA treatments, there were no differences between the layers, likely due to the effect of *A. bisporus* SMS as bulking agent and the nutrient content of the SMS, which could enhance the physicochemical properties of the soil, facilitating higher oxygen diffusion to the lower layer and biostimulating the microbiota. Consequently, the differences in the relative abundance of the diverse bacterial and fungal taxa between layers in the SA treatments were less pronounced than in the S treatment. While it is challenging to elucidate whether this impact on the indigenous microbiota was due to the presence of lignin or by the fungus itself, the potential degradation of aromatic hydrocarbons was based on the complexity of the synergistic relationships between fungi and bacteria and the interactions in the co-occurrence network of microorganisms capable of degrading lignin. The phyla Firmicutes and Gemmatimonadota, which showed a low relative abundance in the soil, were likely bioaugmented by *A. bisporus* SMS as previously stated by Carrasco et al. [26], who conducted a follow-up of the associated microbiota of *A. bisporus* culture, resulting in the increase of Firmicutes after the inoculation of the fungi.

The indigenous microbiota of the polluted soil showed some bacteria and fungi known to be hydrocarbon degraders, such as bacterial families *Alcanivoraceae*, *Alcaligenaceae*, *Dietziaceae*, *Sphingomonadaceae*, and *Pseudomonadaceae* [62–65]. With regard to fungi, genera such as *Aspergillus*, *Amorphoteca*, *Talaromyces*, and *Scedosporium* are usually found in soils contaminated with TPHs [66]. However, the presence of these taxa does not necessarily indicate a relevant metabolic capacity for hydrocarbons degradation [8]. It is noteworthy that some taxa of significant interest were enhanced by the presence of *A. bisporus* SMS, including the *Alcanivoraceae* family or the fungal genus *Scedosporium*.

Alcanivoraceae family, one of whose members was described for the first time in 1998 as an obligate hydrocarbonoclastic bacterium [49], is known to favor the degradation of the aliphatic fraction. The fungal genus *Scedosporium* showed the capacity to degrade the aromatic hydrocarbons, although some researchers concluded that it worked better in association with bacteria [67]. However, other taxa were negatively affected, including the generalist hydrocarbon degrader *Xanthomonadaceae* family [62], and *Solimonadaceae* which is normally found in contaminated soils and has recently been discovered to be capable of degrading some plastics such as polybutylene adipate terephthalate or poly(butylene) succinate [68]. The presence of *A. bisporus* SMS promoted an efficient hydrocarbonoclastic consortium comprising families *Alcanivoraceae*, *Alcaligenaceae*, and *Dietziaceae*, in addition to the fungal genera *Scedosporium* and *Aspergillus*. Furthermore, *A. bisporus* itself was observed to remain partially in the soil during the remediation procedure, although with low relative abundance. Furthermore, the bacterial family *Alcanivoraceae* and fungal genus *Scedosporium* exhibited a significant differential abundance after 60 days of incubation.

The functional prediction of degradation provided insight into the role of the microorganisms in TPHs degradation. After 60 days of incubation, two different communities of hydrocarbon degraders were observed, depending on the presence (SA) or absence (S) of *A. bisporus* SMS. In the SA treatments, the most prevalent taxa were the bacterial families *Alcanivoraceae*, *Sphingobacteriaceae*, and *Bacillaceae*, in addition to the fungal genera *Scedosporium* and *Aspergillus*. The microorganisms belonging to this group demonstrated a strong correlation with the dissipation of the most abundant chains of both aliphatic and aromatic hydrocarbons (C₁₂-C₁₆, C₁₆-C₂₁, and C₂₁-C₃₅).

Conversely, bacterial families *Xanthomonadaceae*, *Microbacteriaceae* and *Solimonadaceae* along with the fungal genera *Cadophora* and *Amorphoteca* were the main contributors to TPH dissipation in S treatments, including those with either of the two *Pleurotus* layers. For instance, the genus *Amorphoteca* is the most frequently found in kerosene tanks [8]. All the aforementioned taxa were negatively influenced by *A. bisporus* SMS (Figs. 3 and 6). This consortium also degraded hydrocarbons, but less efficiently than the one in SA treatments. Therefore, it can be concluded that two different consortia of hydrocarbonoclastic microorganism developed depending on the presence of *A. bisporus* SMS. Although there are limitations to predictive analysis, the application of PICRUSt2 to diverse metagenomic data sets showed that the phylogenetic information contained in 16 S marker gene sequences seemed to be sufficiently well correlated with genomic content to produce accurate predictions when related reference genomes are available [69]. Phylogenetic metabarcoding is essential for studying the microbial ecology of any environmental niche. However, the molecular tool does not provide direct evidence of the functional capabilities of the community. The functional analysis demonstrated a correlation with the results of hydrocarbon degradation.

The aerobic metabolism of alkanes typically starts with the oxidation of a terminal carbon by monooxygenases, resulting in the generation of the corresponding alcohols. This is then followed by the oxidation of alcohols and aldehydes by the corresponding dehydrogenases, which produces the corresponding aldehydes and fatty acids, respectively [70]. Alkane-1 monooxygenase was employed by certain specialized microorganisms, including the family *Alcanivoraceae* and other bacterial families, to start the oxidation of aliphatic chains. Meanwhile, fungi appear to perform the terminal oxidation of aliphatic hydrocarbons by the CYP450 monooxygenase system. These findings obtained by functional prediction, align with those of Daccò et al. [3], who review many previous works. This initial oxidation can occur along two pathways depending on the oxidized carbon: terminal or subterminal. The complete biodegradation of the longer and more complex hydrocarbon structures usually requires synergistic interactions among multiple fungi and bacteria [8].

In contrast, the degradation pathways of aromatic hydrocarbons have been extensively investigated. The degradation usually occurs in

co-metabolism [8], with the involvement of unspecific fungal extracellular oxidases whose actions occur accidentally, breaking down the aromatic structure. Meanwhile intracellular CYP450 monooxygenases also oxidize aromatic rings for detoxification [8,9]. The fungal community in this work showed the involvement of CYP450 and Laccase as indicated by functional prediction. Bacteria likely took advantage of the fungal action, feeding on simpler compounds.

Multidomain mixed microbial communities display unique associations and interactions that could result in more efficient systems for the biodegradation of organic contaminants as a result of sequential decomposition. Fungi perform an initial oxidation step producing metabolites that are available for bacterial degradation. These organisms often co-metabolize the pollutant using other available carbon sources such as plant remains or exudates from plants and bacteria [11]. Myco-augmentation in contaminated matrices is possible thanks to non-specific extracellular and intracellular oxidoreductases, which allow them to transform contaminants into more bioavailable substances for bacteria [71]. However, the fungal degradation system is less well understood than that of bacteria, and the genetic basis of biochemical activity is still incomplete compared to the known bacterial degradation pathways of aromatic pollutants [15].

In order to successfully utilize fungi in bioremediation, it is necessary to extract field knowledge through the four-phase strategy: laboratory-scale treatability, in situ pilot tests, inoculum production and, finally, large-scale application [72]. The challenge in developing mycoremediation for large-scale field applications in TPHs polluted soils lies in incorporating the ideal environmental, edaphic, and climatic factors of a typical contaminated site into the process [73], as well as combining them with the selected fungi and indigenous hydrocarbonoclastic bacteria as it was demonstrated in this work.

5. Conclusions

The three different spent mushroom substrates (*A. bisporus*, *P. eryngii*, and *P. ostreatus*) evaluated in this work proved significantly higher removal of TPHs than the non-inoculated soil demonstrating the usefulness of these wastes in the remediation of contaminated soils with petroleum hydrocarbon. The *A. bisporus* SMS was the most effective; having achieved the highest degradation percentages of all the fractions. Concurrently, this SMS enabled the growth of a consortium of hydrocarbonoclastic microorganisms that was more effective and distinct from the one responsible for TPHs degradation in soil treatments (S). This consortium included a bacterial alkane degrading specialist, the *Alcanivoraceae* family, which contributed more than any other taxon to alkane 1-monooxygenase function. Additionally, the fungal genus *Scedosporium* that contributed to the degradation of both aromatic and aliphatic chains through laccase and CYP450. The efficacy of SMS as an inoculum containing a high microbial richness and nutrients, coupled with its capacity to biostimulate indigenous hydrocarbonoclastic microorganisms, offers an opportunity to reuse this ubiquitously accessible waste in the remediation of TPHs polluted soils.

Environmental implication

Spent mushroom substrates (SMSs) are useful amendments to bioremediate polluted soils by petroleum hydrocarbons (TPHs) and other pollutants. This is the first work that elucidates the modifications of soil microbial structure produced by three SMSs (*Agaricus bisporus*, *Pleurotus eryngii*, and *Pleurotus ostreatus*) in a bioremediation process of TPHs-polluted soil. The work assesses the TPHs, aliphatic and aromatic biodegradation, the microbial structure, and the effects of the SMSs at the bacterial family and fungal genus level highlighting the bio-augmented and biostimulated microbiota. This work is a new step into the elucidation of the bioremediation of TPHs polluted soil by SMSs.

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CRediT authorship contribution statement

Maria Guirado: Writing – review & editing, Validation, Methodology. **Laura Delgado-Moreno:** Writing – review & editing, Conceptualization. **Carlos García-Delgado:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Rafael Antón-Herrero:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Begoña Mayans:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation. **Enrique Eymar:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Consuelo Escolástico:** Writing – review & editing. **Javier Pérez-Esteban:** Writing – review & editing, Validation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2024.134650](https://doi.org/10.1016/j.jhazmat.2024.134650).

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