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Assessment of Different Spent Mushroom Substrates to Bioremediate Soils Contaminated with Petroleum Hydrocarbons

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Abstract: Bioremediation techniques are being developed as substitutes for physical–chemical methodologies that are expensive and not sustainable. For example, using the agricultural waste spent mushroom substrate (SMS) which contains valuable microbiota for soil bioremediation. In this work, SMSs of four cultivated fungal species, *Pleurotus eryngii*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Agaricus bisporus* were evaluated for the bioremediation of soils contaminated by petroleum hydrocarbons (TPHs). The bioremediation test was carried out by mixing the four different SMSs with the TPH-contaminated soil in comparison with an unamended soil control to assess its natural attenuation. To determine the most efficient bioremediation strategy, hydrolase, dehydrogenase, and ligninolytic activities, ergosterol content, and percentage of TPHs degradation (total and by chains) were determined at the end of the assay at 40 days. The application of SMS significantly improved the degradation of TPHs with respect to the control. The most effective spent mushroom substrate to degrade TPHs was *A. bisporus*, followed by *L. edodes* and *P. ostreatus*. Similar results were obtained for the removal of aliphatic and aromatic hydrocarbons. The results showed the effectiveness of SMS to remove aliphatic and aromatic hydrocarbons from C_{10} to C_{35}. This work demonstrates an alternative to valorizing an abundant agricultural waste as SMS to bioremediate contaminated soils.

Keywords: bioremediation; fungi; soil microbiota; spent mushroom substrate; petroleum hydrocarbons

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1. Introduction

Currently, the contamination of soil, water or air by toxic substances, from natural or anthropogenic sources, is considered one of the major environmental problems [1]. Anthropogenic contamination of soils is due to discharges, pesticide use, leaks, extractions, and other emissions [2,3]. Among anthropogenic soil contaminants, hydrocarbons represent a major environmental threat because of their toxicity, bioaccumulation and persistence. In addition, their hydrophobicity renders them preferably adsorb to soil organic matter [4], which makes their removal from contaminated sites more difficult.

Crude oils are extremely complex mixtures, made up mainly of hydrocarbons and to a lesser extent by metals or nitrogenous, oxygenated, or sulfur derivatives [4]. Petroleum hydrocarbons are formed mainly by carbon and hydrogen atoms and are subdivided into: saturated hydrocarbons made up of aliphatic carbon chains and aromatic hydrocarbons made up of one or more benzene rings. In soil, total petroleum hydrocarbons (TPHs) alter the physical–chemical and biological properties of the soil, such as hydrophobicity, cation exchange capacity, and electrical conductivity, preventing gas exchange with the atmosphere or modifying the autochthonous microbiota. In addition, they have toxic properties causing serious effects on human and environmental health [4].

The most frequent techniques for the recovery of contaminated soils with hydrocarbons are the extraction with solvents, washing with surfactants and thermal treatments. The first method consists of the application of an organic solvent for removing the contaminants. The washing with surfactants uses a washing solution of water and chemical additives. The contaminants are retained in this solution, whether dissolved or suspended. Finally, thermal treatments consist in applying different temperatures ranging from 90 °C to 600 °C, with the aim of volatilizing or decomposing organic contaminants [5]. All of them are ex-situ techniques that are expensive and consume a lot of energy. Although the resulting soil is clean, it remains biologically and environmentally damaged [6].

Bioremediation represents a less expensive and environmentally friendly alternative to the physico-chemical techniques previously mentioned. Bioremediation consists of the recovery of contaminated soil by the action of living organisms [7]. Enhanced contaminants biodegradation might be achieved by managing soil temperature, and moisture, and the abundance of nutrients (carbon, nitrogen, and phosphorus) among others factors [8]. There are two main strategies to bioremediate contaminants from soils, biostimulation and bioaugmentation [9]. The purpose of biostimulation is to favor the metabolism of the native microbiota of the soil to degrade contaminants by the addition of nutrients, increasing the level of oxygen and regulating the temperature and soil moisture. In bioaugmentation, specialized exogenous microorganisms, such as fungi or bacteria, are inoculated into the soil for increasing contaminants biodegradation [8].

Bioremediation is a promising technique for the amelioration of soils contaminated with TPHs. However, for the heavier hydrocarbons, the efficiency of this technique is limited due to their low bioavailability, which makes bacterial degradation difficult [10], since these organisms have to absorb the contaminant to degrade it intracellularly. To solve this problem, a new procedure known as mycoremediation has been proposed. In this approach, fungi are applied to soil for enhancing contaminants removal [5]. Fungi are very interesting organisms to decontaminate soil because their multicellular fungal mycelium can reach vast volumes of soil. Compared to bacteria, fungi are also more resistant to high concentrations of toxins and can produce many useful bioactive compounds, such as extracellular enzymes, among other advantages [11]. Fungi can degrade a wide variety of organic contaminants thanks to their non-specific metabolism [11,12]. This degradation occurs extracellularly through enzymes such as laccase, versatile peroxidase and manganese peroxidase (MnP) for ligninolytic fungi and intracellularly [13] or by the cytochrome P450 intracellular enzyme system [14]. In both cases, more polar substances are obtained because of the degradation of these toxic compounds. The use of filamentous fungi in this procedure is essential due to the fact that the networks formed by hyphae and mycelium favor soil colonization and the establishment of a synergic relation between fungi and bacteria [15,16]. The great disadvantage of this technique is the time it takes for the degradation of the contaminants, which is greater when compared to the physico-chemical techniques mentioned above. In addition, it must be considered that the colonization of the fungus could be limited or impaired due to the interaction with the microbial population of the soil or even by high concentrations of contaminants [17].

Currently, mushroom cultivation is carried out worldwide. After mushroom production, the substrate plus the cultivated mycelium generates the spent mushroom substrate (SMS) waste. Every 1 kg of fresh mushroom generates between 2.5 and 5 kg of fresh SMS [18]. The disposal of these residues is an environmental problem, being the most frequent those from the crop of *Agaricus bisporus* and *Pleurotus* spp. [19]. Some alternatives to disposal are described or implemented, for example: [20] amendment and fertilizer for agricultural soils, plant biostimulant, as animal feed or in aquaculture, in pest and disease management, as energy feedstock (alternative fuel, production of biogas), in vermiculture or as a component of casing material or ingredient of new growth substrates for further mushroom growing cycles [18,20–22]. All these possibilities promote the circular economy concept and are in line with the Green Deal of the European Union in contrast to the traditional disposal of SMS [19]. SMS wastes are based on lignocellulosic materials [23]

and contain viable mycelium and specific microbiota able to colonize polluted soils and degrade a wide variety of soil contaminants [24–26]. The re-use of SMSs in environmental remediation is a promising way to covert this agricultural waste into a sustainable bioresource promoting the green development of the global mushroom industry [20]. Different SMSs were used to adsorb organic and inorganic contaminants or to degrade organic contaminants from water [21,23,27]. The composted SMS is an effective adsorbent to minimize the availability of metals in soil and enhanced the phytoremediation of mining soil [28], reduced the leaching of pesticides from soil [29] and minimized the impact of herbicides in soil microbial community [30]. The ability of SMS to remove a wide variety of organic contaminants from soils was reported in different publications [20,25,31]. Hence, we hypothesize that the use of SMS to remediate polluted soils with petroleum hydrocarbons is a valuable strategy to re-use this agricultural waste.

The main objective of this work is to assess the capability of four SMS, *Pleurotus eryngii* (king mushroom), *Lentinula edodes* (shiitake), *Pleurotus ostreatus* (oyster mushroom) and *Agaricus bisporus* (champignon) to enhance biodegradation of petroleum hydrocarbons from a soil highly contaminated with crude oil.

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). In its composition there are remains of the mycelia of the cultivated fungi and of the constituent components of their growth substrates; for oyster mushroom (*P. eryngii*), hardwood sawdust, seed hulls and wheat bran; for shiitake (*L. edodes*) steam pasteurized straw, sawdust; in the case of the oyster mushroom (*P. ostreatus*) wheat straw and finally for the mushroom (*A. bisporus*), the substrate was based on composted wheat straw and chicken manure.

2.2. Contaminated Soil

The soil was obtained from a collection of contaminated soils from accidental oil spills in a refinery located in Huelva, where they are stored until their final management by authorized companies. According to an analysis carried out in a certified laboratory, the soil had a concentration of 12,000 ± 1414 mg/kg of total petroleum hydrocarbons (TPH). These values exceed the reference values by more than 100 times according to Spanish legislation [32]. This soil can be considered directly as contaminated soil without the need to carry out a risk assessment.

The soil basic parameters determined were: %sand, %loam, % clay, maximum water retention capacity (MWHC), pH (water and 1 M KCl), electrical conductivity, oxidizable organic matter, total carbonates, available phosphorus, and trace elements [33].

The percentage of sand, silt and clay contained in the soil sample was determined by the Bouyoucos method. The pH of the soil was determined in water and KCl 1M extract 1:2.5 (w:v) by pHmeter. Electrical conductivity was determined in extract 1:5 (p:v) using a conductivity meter. Oxidizable organic matter was determined by the Walkley–Black method using $K_2Cr_2O_7$. $CaCO_3$ content was analyzed by calcimetry. Available phosphorous was extracted with $NaCO_3$ (0.25 M, pH 8.5) and analyzed spectrophotometrically at 660 nm using the Duval reagent. Trace elements were extracted by pseudo-total digestion assisted by microwaves oven with HCl:HNO_3 and analyzed by ICP-MS [33].

2.3. Mycoremediation Assay

Untreated soil (control) was compared against four bioaugmentation treatments by adding SMS of *P. eryngii*, *L. edodes*, *P. ostreatus* and *A. bisporus*. For this, 500 g of polluted soil were mixed with 50 g of each SMS in closed glass containers of 1 L. The control and the treatments were performed in quadruplicate and were kept in the dark for 40 days at 20 °C and 70% MWHC. During this period, the glass containers were opened once per week to oxygenate them and to add water, when needed to keep the soil moisture. After incubation,

sampling, processing and analysis of hydrolase and dehydrogenase activities, ergosterol content, ligninolytic enzymatic activities total TPHs and its aliphatic and aromatic fractions were carried out.

To determine the soil microbial activity, two soil enzyme activities were determined, dehydrogenase and total hydrolase activity. Soil dehydrogenase activity determination was performed by reducing the triphenyltetrazolium chloride to 1,3,5-triphenylformazan. To this, 1000 g of soil was incubated with 1 mL of 0.1 mM Tris-HCl buffer at pH 7.6 with 1.5% triphenyltetrazolium chloride (TCC) for 24 h at 30 °C. After this time, the reaction was stopped and the 1,3,5-triphenylformazan produced was extracted with acetone, centrifuged and measured in the spectrophotometer at 546 nm (ε = 15.4 mM^{-1} cm^{-1}) [34]. To determine the total hydrolase activity, the fluorescein produced from the hydrolysis of fluorescein diacetate was measured [35]. The soil sample (1 g) was incubated with 60 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.6 and fluorescein diacetate for 20 min at 30 °C. After that time, acetone was added to stop the reaction and then samples were centrifuged at 5000 rpm for 5 min after a manual stirring. The absorbance of the supernatant was measured at 490 nm in the spectrophotometer (ε = 80.3 M^{-1} cm^{-1}).

Ergosterol is a membrane lipid found in fungi that allows for determining the fungal biomass present in the soil [36]. A suspension of 0.5 g of milled soil sample was extracted with 3 mL of KOH 10% *w/v* in methanol and 1 mL of hexane. This is sonicated for 90 min in a hot water bath at 70 °C. Subsequently, the extract was filtered, and the soil was washed with hexane. The purification of the extract was carried out by liquid-liquid extraction in a separating funnel. A total of 1 mL of H_2O was added to the methanol:hexane extract and extracted three times with 2 mL of hexane. The hexane phases were combined and dried over a stream of nitrogen. Subsequently, the solid residue was redissolved in methanol [37]. The extracts were analyzed by high-performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA). Chromatographic separation of ergosterol was achieved with a Phenomenex Luna C18 (2) (150 × 4.6 mm; size of 5 µm particle; 100 Å pore size) column using an isocratic elution program with methanol:water (95:5) at a flow rate of 1 mL min^{-1}. The column temperature was set at 30 °C. The elution profile was monitored at 282 nm. Peak identification of each sample was based on retention time (15.99 min) and UV spectrum (200–400 nm) of commercially available standard (Thermo Fisher Scientific, Waltham, MA, USA).

Before the ligninolytic enzyme determination, an extraction of 3000 g of the soil sample was carried out with 30 mL of extracting solution composed of 2.94 g/L $CaCl_2$ H_2O, 4.10 g/L of sodium acetate, 2.86 mL/L of glacial acetic acid and 0.50 mL/L of Tween 80. The mixture was stirred at 4 °C for 60 min, centrifuged for 15 min at 5000 rpm and finally, the supernatant was collected [7]. To analyze the laccase enzymatic activity, the oxidation of 2,6-dimethoxiphenol (DMP) was monitored at 477 nm in the spectrophotometer in kinetic mode for 2 min [38]. Versatile Peroxidase was determined by oxidation of ABTS at 420 nm for 10 min in presence of H_2O_2 [39]. To analyze the manganese peroxidase (MnP) activity, the production of the malonate-Mn^{3+} complex was measured at 270 nm [7].

The analysis of TPHs was performed by GC-FID after microwave extraction. The contaminated soil samples were firstly air dried and then sieved at 2 mm to eliminate the SMS, and secondly sieved again after its grinding at 0.02 mm to homogenize them. The extraction solvent, 20 mL of acetone and n-hexane 1:1 (*v/v*) was added to 1 g of dried soil sample. The mixture was subjected to microwave extraction (Ethos Sel, Milestone, Sorisole, Italy). The extraction program consisted of 20 min at 150 °C and 1000 W. The extract was filtered through a polytetraflourethylene membrane with a pore size of 20 µm. The filtered extracts were then evaporated with nitrogen to a volume of 1 mL. The reduced extracts were fractionated following a clean-up procedure involving Isolute EPH cartridges (25 mL/5 g). The cartridges were conditioned prior to sample loading with 30 mL of hexane. After loading the sample, the elution was performed using 12 mL of hexane, and then 20 mL of dichloromethane, both at a flow rate of 2–3 mL min^{-1}. The cleaned extract was dried by N_2 flow to 1 mL and injected into gas chromatography (Agilent 7820A, Santa Clara, CA,

USA) coupled to a flame ionization detector (FID Agilent G4513A, Santa Clara, CA, USA) equipped with an autosampler with an HP5-MS and capillary column 30 m × 0.32 mm i.d.; nominal film thickness 0.25 mm. Split less injection was used with a deactivated inlet liner (4 mm i.d.) with glass wool and a single taper. The injection temperature was 250 °C, and the injection volume was 3 µL. Helium was the carrier gas (74 kPa). The oven operating conditions were: initial temperature of 80 °C, increasing to 200 °C at a rate of 7 °C min^{-1}, then increasing to 300 °C for 17 min at a rate of 11 °C min^{-1}. FID was operated at 325 °C and 20 Hz. The aliphatic and aromatic fractions detected are defined based on their equivalent carbon [40].

2.4. Statistical Analysis

The statistical test 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. The principal component analysis (PCA) was performed to determine the relationship between the degradation of aliphatic and aromatic fractions of TPHs and the bioaugmentation treatments. This test was performed using the PAST V. 4.02 software (Natural History Museum, University of Oslo, Oslo, Norway).

3. Results

3.1. Soil Characterization

The studied soil had a sandy texture, a basic pH, high concentration of organic matter due to the nature of the contamination, low electrical conductivity and low concentration of nutrients (Table 1). The nutrients are unbalanced with C:N:P of 100:1.5:0.08 far from the optimal 100:10:1 [4]. The results obtained for trace elements were below the limits established by the Spanish Legislation [32].

Table 1. Soil basic characterization (mean ± standard deviation) $n = 3$.

MHWC (%)	13 ± 4	
pH (H_2O)	8.66 ± 0.05	
pH (KCl)	7.42 ± 0.04	
Electric Conductivity (dS/cm)	0.7 ± 0.2	
Total Limestone	n.d.	
Sand (%)	91.20 ± 0.01	
Silt (%)	2.5 ± 0.3	
Clay (%)	6.3 ± 0.3	
Texture	Sandy	
Organic Matter (g/kg)	23 ± 4	
Assimilable Phosphorus (g/kg)	0.011 ± 0.001	
Nitrogen (g/kg)	0.2 ± 0.0	
Trace elements (mg/kg)		Legislated values (mg/kg) *
Sb	4 ± 2	80
As	16 ± 4	40
Cd	0.3 ± 0.0	300
Cu	315 ± 92	8000
Co	4.0 ± 0.2	1500
Cr	n.d.	2300
Sn	6.75 ± 0.07	100,000
Hg	0.285 ± 0.007	15
Mo	8 ± 1	1500
Ni	8.0 ± 0.9	15,600
Pb	80.5 ± 22	2700
Zn	290 ± 42	100,000

n.d.: not detected. MWHC: maximum water holding capacity * According to Spanish Legislation for industrial soils.

3.2. Mycoremediation Assay

Figure 1 shows the visual aspect of the four microcosms inoculated with the different SMSs after 40 days of incubation. The microcosms inoculated with SMS of *P. eryngii* and *A. bisporus* showed extensive colonization of the contaminated soil. The fungi *P. ostreatus* and *L. edodes* showed lower colonization than the previous ones. In the case of *P. ostreatus* the colonization was not clearly observed until the 30th day.

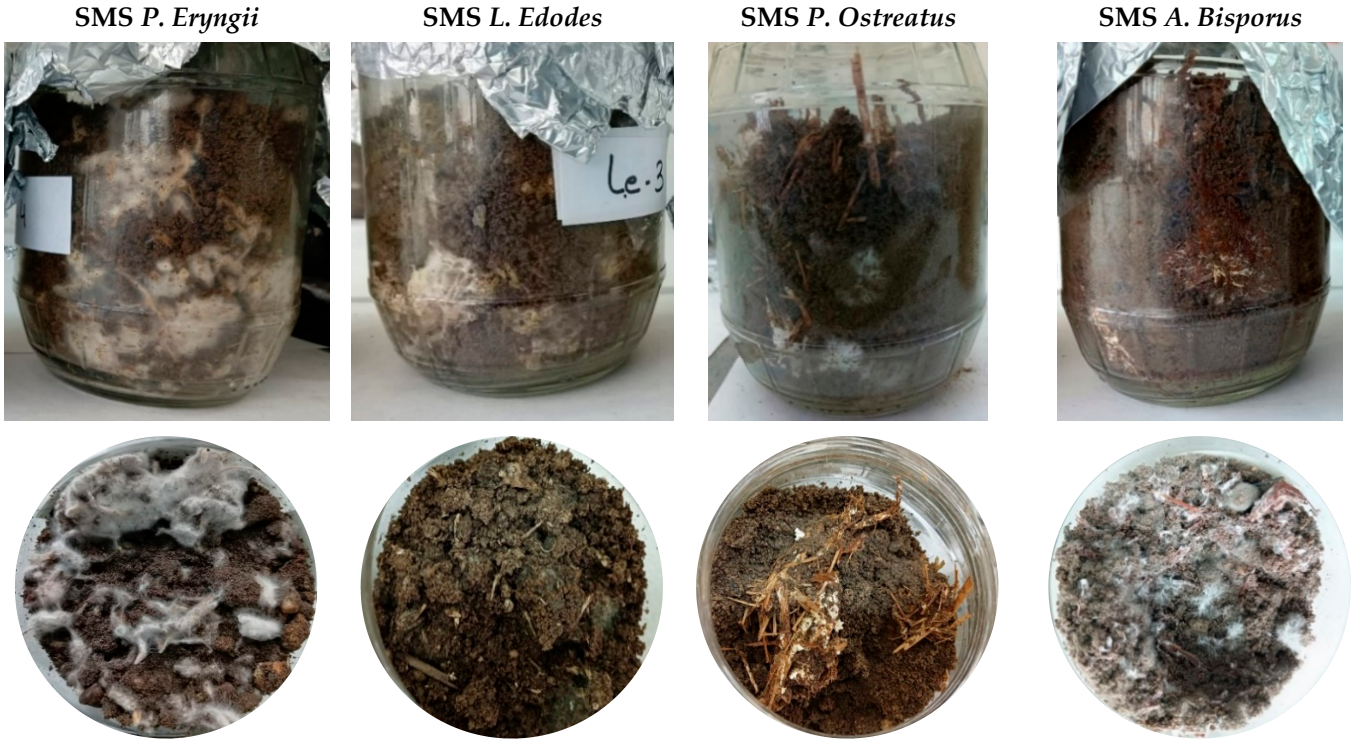

Figure 1. Visual state of the bioaugmented microcosms with four different SMS (*P. eryngii*, *L. edodes*, *P. ostreatus* and *A. bisporus*) after 40 days of incubation.

Mean values of hydrolase and dehydrogenase activities were higher for the *A. bisporus* microcosm than for the control. The other treatments showed higher enzymatic activities than the control but were not statistically different due to the high deviations between replicates (Table 2). Hydrolase and dehydrogenase activities were 17 and 4 times, respectively, higher for *A. bisporus* than for the control, denoting the high metabolic activity in *A. bisporus* treatment with respect to control. The mean values of ergosterol did not present significant differences between bioaugmented treatments (Table 2). However, the increment of ergosterol content in those treatments with respect to the control indicates the soil colonization of the four fungi from their respective SMS. The ligninolytic activity of the five microcosms was determined by three different activities: laccase, versatile peroxidase and MnP. Laccase was proposed as a bioindicator of the mycelial growth of *A. bisporus* [41]. This enzyme only was detected in *A. bisporus* and *P. eryngii* treatments, denoting the extensive colonization of the soil and their metabolic activity. The laccase activity of *A. bisporus* microcosms was significantly higher than the laccase activity of *P. eringii* microcosms. Peroxidase activities were detected in control and all the bioaugmented microcosms irrespective of the SMS inoculated. However, no significant differences were detected for versatile peroxidase and MnP activities between treatments. The detection of peroxidase activity suggests the metabolic activity of the inoculated fungi and the inherent microbiota of soil.

Table 2. Enzymatic activities (total hydrolase, dehydrogenase, laccase, versatile peroxidase, Mn-peroxidase) and ergosterol content of the control microcosms (C), and microcosms inoculated with SMS of *P. eryngii* (Pe), *L. edodes* (Le), *P. ostreatus* (Po) and *A. bisporus* (Ab) at the end of the incubation. Data are presented as mean values ± standard deviation (n = 3). Same letters indicate lack of statistically significant difference ($p < 0.05$) between treatments. n.d.: parameter not detected.

	Hydrolase ($\mu mol \cdot h^{-1} \cdot g^{-1}$)	**Dehydrogenase ($nmol \cdot h^{-1} \cdot g^{-1}$)**	**Ergosterol (mg/kg)**	**Lacasse (U/kg)**	**Versatile Peroxidase (U/kg)**	**Mn-Peroxidase (U/kg)**
C	22.5 ± 0.7^{b}	0.80 ± 0.08^{b}	n.d.	n.d.	70 ± 14^{a}	4 ± 4^{a}
Pe	71 ± 44^{b}	2 ± 1^{ab}	1.0 ± 0.5^{a}	10 ± 7^{b}	99 ± 30^{a}	17 ± 12^{a}
Le	76 ± 56^{b}	1.0 ± 0.4^{b}	0.7 ± 0.5^{a}	n.d.	63 ± 50^{a}	9 ± 8^{a}
Po	26 ± 7^{b}	1.3 ± 0.5^{ab}	0.6 ± 0.7^{a}	n.d.	74 ± 57^{a}	10 ± 10^{a}
Ab	378 ± 243^{a}	3 ± 2^{a}	0.7 ± 0.4^{a}	54 ± 38^{a}	48 ± 39^{a}	10 ± 6^{a}

n.d. not detected.

All the bioaugmented treatments reached a final concentration of TPHs significantly lower than the control. The *A. bisporus* microcosms were the most effective treatment for the TPHs removal, reaching the lowest concentration of these contaminants at the end of the assay. This treatment showed a degradation rate of 48% higher than the control, followed by *L. edodes* (34%), *P. ostreatus* (29%) and *P. eryngii* (12%). The assessment of the petroleum hydrocarbons removal was analyzed considering the aliphatic and aromatic nature of the contaminants. Moreover, the aliphatic and aromatic compounds were analyzed according to the length of their carbon chains (Table 3).

Table 3. Concentration of TPHs and aliphatic and aromatic fractions by chains (mean ± standard deviation) of the control microcosms (C), and microcosms inoculated with SMS of *P. eryngii* (Pe), *L. edodes* (Le), *P. ostreatus* (Po) and *A. bisporus* (Ab) at the end of the incubation. Data is presented as mean values ± standard deviation (n = 3). The same letters indicate a lack of statistically significant difference ($p < 0.05$) between treatments.

mg/kg	**C**	**Pe**	**Le**	**Po**	**Ab**
			Aliphatic		
$>C_{10}-C_{12}$	359 ± 44^{a}	141 ± 51^{b}	190 ± 31^{b}	166 ± 12^{b}	73 ± 35^{c}
$>C_{12}-C_{16}$	4629 ± 269^{a}	3599 ± 283^{b}	2902 ± 206^{c}	2960 ± 114^{c}	2071 ± 343^{d}
$>C_{16}-C_{21}$	5854 ± 245^{a}	5180 ± 250^{b}	3951 ± 253^{c}	4100 ± 248^{c}	3144 ± 503^{d}
$>C_{21}-C_{35}$	3204 ± 158^{a}	2916 ± 212^{ab}	2334 ± 130^{cd}	2611 ± 392^{bc}	2093 ± 278^{d}
$>C_{35}$	30 ± 9^{ab}	22 ± 13^{ab}	15 ± 5^{b}	39 ± 16^{a}	27 ± 10^{ab}
Σ Aliphatic	$14{,}077 \pm 656^{a}$	$11{,}857 \pm 617^{b}$	9392 ± 599^{c}	9875 ± 728^{c}	7407 ± 1151^{d}
			Aromatic		
$>EC_{10}-EC_{12}$	18 ± 7^{a}	11 ± 2^{b}	12 ± 3^{ab}	8 ± 2^{bc}	4 ± 1^{c}
$>EC_{12}-EC_{16}$	638 ± 104^{a}	451 ± 65^{b}	294 ± 98^{c}	345 ± 36^{bc}	127 ± 26^{d}
$>EC_{16}-EC_{21}$	2667 ± 264^{a}	2765 ± 298^{a}	1811 ± 187^{b}	1990 ± 98^{b}	1255 ± 111^{c}
$>EC_{21}-EC_{35}$	516 ± 44^{ab}	614 ± 180^{a}	412 ± 49^{b}	522 ± 118^{ab}	413 ± 31^{b}
$>EC_{35}$	56 ± 11^{a}	85 ± 42^{a}	72 ± 39^{a}	71 ± 25^{a}	58 ± 10^{a}
Σ Aromatic	3895 ± 410^{a}	3926 ± 540^{a}	2601 ± 249^{b}	2935 ± 223^{b}	1857 ± 155^{c}
ΣTPHs	$17{,}971 \pm 1061^{a}$	$15{,}784 \pm 1061^{b}$	$11{,}993 \pm 836^{c}$	$12{,}811 \pm 948^{c}$	9264 ± 1230^{d}

Regarding the total aliphatic hydrocarbon removal, once again, all the bioaugmented microcosms showed significantly lower concentrations than the control microcosms at the end of the incubation. The SMS of *A. bisporus* achieved the lowest concentration of total aliphatic hydrocarbons. In contrast, among the bioaugmented treatments, *P. eryngii* was less effective for the removal of aliphatic hydrocarbons.

The most abundant aliphatic hydrocarbons are compounds with chains of C_{12}–C_{16}, C_{16}–C_{21} and C_{21}–C_{35}. The concentration of lighter hydrocarbons (C_{10}–C_{12}) was one-fold

lower than the previous fractions. Finally, the heaviest fraction (>C_{35}) was the less abundant, with a concentration two-folds lower than the most abundant aliphatic hydrocarbons. The control microcosms achieved the highest concentration for most of the aliphatic groups. In contrast, the bioaugmented microcosms improved the degradation of aliphatic hydrocarbons with all having significantly lower concentrations than control microcosms. Therefore, the four SMSs were useful to improve the aliphatic hydrocarbon's removal, excepting the heaviest fraction (>C_{35}), for which no significant differences were found between the control and any of the bioaugumented microcosms. For fractions lower to C_{35} significant differences were observed between the four bioaugmented treatments. *A. bisporus* microcosms reached the lowest concentration of all the aliphatic groups except for the heaviest group (>C_{35}). *L. edodes* and *P. ostreatus* treatments also achieved significantly higher degradation than control. *P. eryngii* microcosms achieved significantly higher concentrations of C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{21} and C_{21}–C_{35} hydrocarbons than *A. bisporus* microcosms.

The most abundant aromatic hydrocarbons were EC_{16}–EC_{21}. The aromatic hydrocarbons with chains EC_{12}–EC_{16} and EC_{21}–EC_{35} were one-fold lower that EC_{16}–EC_{21}. Finally, the lightest (EC_{10}–EC_{12}) and heaviest (>EC_{35}) aromatic hydrocarbons were the less abundant with concentrations 2-fold lower than the most abundant aromatic fraction. Three of the four bioaugmented treatments, *L. edodes*, *P. ostreatus* and *A. bisporus*, got significant lower concentrations of total aromatic hydrocarbons than the control treatment. Between the effective treatments, *A. bisporus* microcosms achieved a significantly lower concentration of total aromatic hydrocarbons. However, the improvement in the degradation effectiveness of SMS treatments was only detected for the light aromatic hydrocarbons EC_{10}–EC_{12}, EC_{12}–EC_{16} and EC_{16}–EC_{21}. Therefore, these treatments were not effective to improve the degradation rate of heavy aromatic hydrocarbons with respect to the autochthonous microbiota. For the light fractions, *A. bisporus* SMS got a significantly lower concentration of these contaminants than *L. edodes*, *P. ostreatus* and *P. eryngii* microcosms. Despite *P. eryngii* SMS did not show significant differences with respect to the control treatment for the total aromatic hydrocarbons, this treatment reached a significantly lower concentration for the two most light fractions (EC_{10}–EC_{12} and EC_{12}–EC_{16}).

The PCA analysis of the remaining concentration of TPHs and aliphatic and aromatic chains for the control treatment and the four SMSs microcosms are shown in Figure 2. The percent variability explained by PC1 was 66.4% and for the PC2 was 14.9%. The PC1 was positively related to the remaining concentration of TPHs and all the hydrocarbon fractions except to >C_{35} and the heaviest aromatic fractions (EC_{21}–EC_{35} and >EC_{35}) which were positively related to the PC2. The control microcosm was located at the right of the PCA diagram denoting the high concentration of hydrocarbons at the end of the incubation time. The bioaugmented microcosms were progressively displaced to the left of the PCA diagram according to the effectivity of hydrocarbons degradation. *P. eryngii* microcosms were closer to the control, both in the positive section of PC1. The other bioaugmented microcosms were in the negative section of the PC1 denoting their ability to remove TPHs and a high variety of aliphatic and aromatic chains. *L. edodes* and *P. ostreatus* were overlapped denoting similar effectivity to remove petroleum hydrocarbons. Finally, *A. bisporus* microcosms were clearly away from the rest of the microcosms with negative values of PC1. This location was negatively related to a high concentration of hydrocarbons demonstrating once again the effectivity of this SMS to remove petroleum hydrocarbons. The low score of PC2 of *A. bisporus* and the other bioaugmented treatments or even the positive score of *P. eryngii* showed the low effectiveness of these SMSs to remove heavy aromatic hydrocarbons.

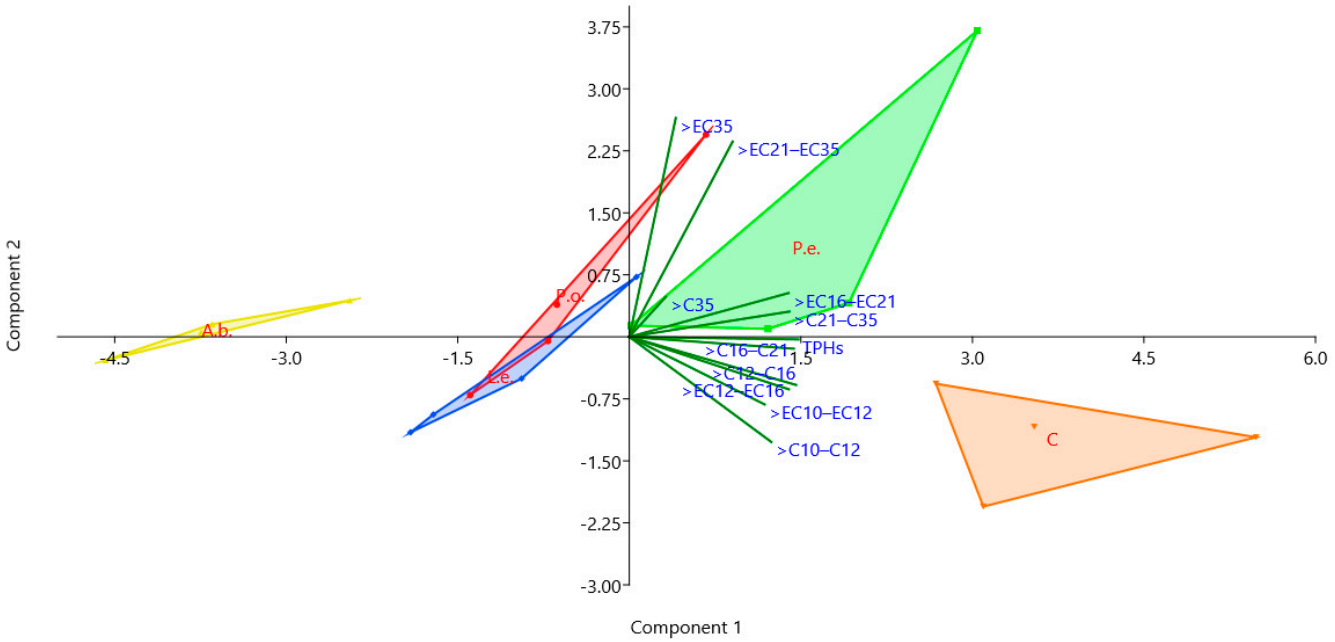

Figure 2. Principal component analysis of the control microcosms (C) and the bioaugmented microcosms with SMS of *P. eryngii* (P.e.), *L. edodes* (L.e.), *P. ostreatus* (P.o.), and *A. bisporus* (A.b.) showing loading scores of the residual concentration of TPHs and their aliphatic (C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{21}, C_{21}–C_{35} and >C_{35}) and aromatic (EC_{10}–EC_{12}, EC_{12}–EC_{16}, EC_{16}–EC_{21}, EC_{21}–EC_{35} and >EC_{35}) fractions.

4. Discussion

The colonization of this polluted soil confirmed the ability of *P. eryngii*, *L. edodes*, *P. ostreatus* and *A. bisporus* to adapt to highly contaminated environments. This fact is also more relevant because the fungi were inoculated from SMSs. This agricultural waste is generated after the industrial culture of edible fungi when the culture of more mushrooms is not economically viable. SMSs are characterized by their low level of nutrients for fungi such as cellulose and hemicellulose which were consumed during the culture of mushrooms [41]. However, they still contained the living organism, including mycelium that can be used for soil bioremediation between other possibilities [18,21,26].

Hence, SMSs are organic wastes with a wide variety of microorganisms [26,41]. The use of these wastes in soil bioremediation provides multiple benefits such as their recycling, the alleviation of the disposal problem and the cost decrease in waste treatment [42], possible bioaugmentation of hydrocarbonoclastic bacteria in contaminated soils [26] and inoculation of lignin-degrading fungi [24,25,43], biostimulation of autochthonous soil microbiota by adding nutrients and organic matter and serving as a bulking agent increasing oxygen diffusion [26,42].

One of the key factors for bioremediation processes is microbial activity since it is an indicator of the ability of the microbiota to grow and degrade the pollutants under certain environmental conditions [44]. The soil biological activity of the microcosms was determined by dehydrogenase and total hydrolase activities (Table 2). Dehydrogenase is an oxidoreductase that is related to the abundance and metabolic activity of soil microorganisms and catalyzed the biological oxidation of organic compounds [45]. It is the most representative soil enzyme among soil oxidoreductases, and its activity is one of the most used parameters for evaluating the microbiological status of the soil [46]. This enzymatic activity is clearly related to the degradation of TPHs in soil and was purposed as an indicator of the potential of soil microbiota to degrade TPHs [44]. Hydrolases are enzymes capable of catalyzing the hydrolysis of a chemical bond using water. In the soil, these enzymes are used for the degradation of complex organic matter, generating molecules that are more easily accessible to soil organisms [47]. It is clear in Table 2 the positive impact of *A. bisporus* SMS on soil microbial activity. This SMS was the only one that include chicken

manure in its composition because *A. bisporus* is a secondary decomposer that requires materials with a lower C/N ratio and higher cellulose, hemicellulose and nitrogen contents (manure and compost) than primary decomposers such as the rest of fungi tested in this work who required high C/N ratio and lignin content [18]. Hence, *A. bisporus* SMS had the highest nutritional content of the four SMSs tested [20], and therefore, the SMS with the highest biostimulation capacity of the soil microbiota. Simultaneously to soil biostimulation, the inherent microbiota of SMS can colonize the soil and produce bioaugmentation of the soil microbiota with microorganisms able to degrade organic pollutants [24]. The other important factor that could enhance the hydrolase and dehydrogenase activity at the end of the incubation period was the lower TPHs concentration of this microcosm. Low activities of these enzymes are indicators of soil toxicity [1,34]. Therefore, the higher soil enzymatic activity of *A. bisporus* treatment with respect to the other treatments could be a combination of the positive effects of the SMS in soil microbiota and the reduction in soil toxicity because of the high effectivity of this treatment to biodegrade TPHs. The hydrolase and dehydrogenase activities for *P. eryngii*, *L. edodes* and *P. ostreatus* treatments were not significantly higher than the control, probably because a combination of multiple factors such as the low nutrient status of these SMSs and their lignocellulosic base does not promote the biostimulation of soil microbiota at long lasting. or antagonistic interactions between autochthonous soil microbiota and SMS microbiota and finally, the lower effectivity to reclaim and detoxify the soil than *A. bisporus* SMS. In this regard, Becarelli et al. [48] described very low TPHs degradation by *P. ostreatus* inoculated from SMS. In addition, these authors found that despite the bacterial community of the SMS was competent to degrade TPHs, the bacteria prioritized the transformation of the more biodegradable SMS organic matter.

Despite the different visual colonization of the four fungi (Figure 1), the ergosterol content was not different between treatments (Table 2). *P. eryngii* and *A. bisporus* were the ones with the highest colonization. While *P. ostreatus* and *L. edodes* only showed colonization at the end of the experiment, but not as vigorous as the other two species. Ergosterol variesi depending on some factors such as the growth medium, the culture conditions and the growth phase in which it is found [36]. For this mycoremediation test, each microcosm presented a different culture phase of each fungus after 40 days of incubation and the growth medium was also different since each one had a different SMS. The low ability of *P. ostreatus* to colonize this soil was not expected. There are multiple works that reported good performance of this fungus for the mycoremediation of soil [4,49,50]. There are also abundant works about the potential of *P. eryngii*, *L. edodes* and *A. bisporus* to colonize contaminated soils by PAHs, PCBs or pesticides; however, there is limited or even zero information on the fungal colonization of TPHs contaminated soil by these fungi [20].

The inoculation of lignin-degrading fungi by SMS is a valuable source of ligninolytic enzymes such as laccase and different peroxidases [21]. Many transforming interactions between fungi and different pollutants depend on a variety of extracellular excreted substances and metabolites. Fungi are capable of degrading petroleum hydrocarbons by secreting enzymes [51]. For example, Zhou et al. [26] reported the increment of Laccase, MnP and Lignin peroxidase of PAH-polluted soils incubated with SMS of *P. ostreatus*, *P. eryngii* and *Auricularia auricular* and García-Delgado et al. [24,52] reported laccase and MnP activity in PAH and PAH-Pb contaminated soils using *A. bisporus* SMS. In this work, the species *P. eryngii* and *A. bisporus* were the only ones with laccase activity at the end of the assay. Laccase is a nonspecific enzyme with a high redox potential that can oxidize different compounds such as phenols and polyphenols [53]. This enzyme uses O_2 as an electron acceptor, while for peroxidase it uses H_2O_2. Despite both fungi, *P. eryngii* and *A. bisporus*, showing laccase activity, the highest laccase activity was measured for *A. bisporus* treatment. This agrees with the visual colonization of the microcosms that was more vigorous for *A. bisporus* and *P. eryngii* than for the other fungi. MnP has a high redox potential and is characterized by oxidizing phenolic structures [54]. It was observed that all the bioaugmented treatments showed a relatively higher value than the control,

highlighting again *P. eryngii* and *A. bisporus*. A possible explanation for these better results is, once again, that they were the fungi that better colonized the soil and these enzymes were indicators of fungal activity [41]. Both for *P. ostreatus* and for *L. edodes*, the fungi that colonized the soil the least, possibly the enzymatic activity that they presented came from the mycelium that remained alive in the substrates. Finally, versatile peroxidase catalyzes oxidation for both organic and inorganic substrates, phenols or aromatic amines, and is characterized by oxidizing substrate compounds with high and low redox potential [55].

Because of these results, it is clear that *A. bisporus* treatment reached the highest biodegradation of TPHs (Table 3). These results agreed with the visual aspect of the microcosms, total hydrolase, dehydrogenase and ligninolytic activities. Mohammadi-Sichani et al. [50] reported that *A. bisporus* was more effective than *P. ostreatus* SMS in a TPHs contaminated soil after biostimulation with NPK. I [48]. However, *L. edodes* and *P. ostreatus* SMSs showed the second highest removal of TPHs. This result was unexpected since vigorous fungal colonization was not observed (Figure 1). Furthermore, the enzymatic activities were not significantly higher than the control treatment.

Therefore, it can be deduced that, for both fungal species, a greater degradation occurred because their respective SMSs favored the soil microbiota that is already resistant to high contamination levels. Jabbar et al. [4] highlighted that the microbial variety is reduced in the contaminated soils due to the influences of the toxic petroleum hydrocarbons upon the organisms. Therefore, the population of degrading microbes ranges from 1% to 10% of the population in a contaminated environment, whereas those in an uncontaminated environment are less than 1% of the population. Another explanation could be that *L. edodes* and *P. ostreatus* SMSs contain inherent microbiota highly specific to degrade hydrocarbons such as *Proteobacteria*, *Microbacterium*, *Brevundimonas*, and *Devosia* as was previously reported for *P. ostreatus* SMS [26]. Covino et al. [49] highlighted the key role of the synergistic effects of soil microbiota and lignin-modified fungi to promote the degradation of aliphatic hydrocarbons, specifically C_{28}–C_{34}.

The assessment of TPH degradation by chains is not a common issue. In this work, the TPHs biodegradation was assessed by degradation of TPHs, aliphatic and aromatic chains and by the number of carbons of these chains. The degradation effectiveness of each treatment by aliphatic and aromatic chains followed the same pattern than TPHs degradation, *A. bisporus* SMS > *P. ostreatus* SMS and *L. edodes* SMS > *P. eryngii* SMS (Table 3) and it was maintained in many of the specific aliphatic and aromatic chains. The interpretation of the degradation effectiveness of each SMS by the carbon chain length demonstrated the ability of all the tested SMSs to enhance the biodegradation of light aliphatic and aromatic hydrocarbons. However, *P. eryngii* SMS was the only which was not able to improve the degradation of the heavy aliphatic fraction C_{21}–C_{35} with respect to control. For the heavy aromatic hydrocarbons (EC_{21}–EC_{35}), none of the *Pleurotus* SMSs improved the degradation of the control treatment but *L. edodes* and *A. bisporus* SMSs were able to stimulate the degradation of EC_{21}–EC_{35}. Therefore, despite all the SMSs were able to improve the hydrocarbon degradation, their potential to remove heavy fractions was different or even seem impaired for the heaviest fractions (>C_{35} and >EC_{35}). For example, aromatic hydrocarbons such as PAHs with high molecular weight having more than three benzene rings are difficult to degrade by soil microbiota because of their low availability [56] as was seen in this work (Table 3). Because the bioremediation process depends on the bioavailability of contaminants and the biodegradation performed by the microbial communities [57], an additional strategy could be the use of surfactants such as saponin or Tween 80, between other possibilities, that enhance TPH removal through increasing its solubility [58]. These surfactants also did not negatively affect the fungi growth stimulating also the ligninolytic peroxidase activities [59]. Lignin-degrading fungi produce biosurfactants [50]. Therefore, the promotion of this ability could be a good alternative to exogenous surfactants to improve the bioavailability of heavy hydrocarbons and consequently, enhance their biodegradation. Some authors have published the usefulness of lignin-modified fungi to degrade low-available aliphatic and aromatic hydrocarbons [24,49].

To use fungi successfully for bioremediation, knowledge must be taken from the fields with the four-phase strategy: bench-scale treatability, on-site pilot testing, production of inoculum, and finally full-scale application [60]. The challenge in the development of mycoremediation to large-scale field applications on petroleum-contaminated soils lies in incorporating ideal environmental, edaphic and climatic factors of a typical contaminated site into the process [61] and combining with the selected fungi and autochthonous hydrocarbonoclastic bacteria. Mixed (multi-domain) microbial communities exhibit unique associations and interactions that could result in more efficient systems for the biodegradation of organic pollutants as a result of sequential breakdown by fungi performing an initial oxidation step producing metabolites that are available for bacterial degradation [62]. Myco-augmentation of contaminated matrices is operational via substrate-unspecific extracellular and intracellular oxide reductases, laccases, and Mn-dependent and independent peroxidases, enabling them to transform pollutants [63]. However, the fungal degradation system is poorly studied and understanding of the genetic basis for biochemical activity is still incomplete compared to known bacterial degradation pathways of aromatic pollutants [64].

Accelerating the global implementation of recycling and utilization of SMS is an important way to realize the transformation and upgrading of the green development of the edible mushroom industry [20]. The results of this work seem a good perspective for the scaling up of mycoremediation as a real alternative to reclaim contaminated soils with TPHs. The fact that SMS is useful as fungal and hydrocarbonoclastic bacteria inoculum to reclaim contaminated soils, involves one more option to recycle this abundant and available all over the world agricultural waste.

5. Conclusions

All of the four different spent mushroom substrates (*P. eryngii*, *L. edodes*, *P. ostreatus*, and *A. bisporus*) evaluated in this work showed 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. *A. bisporus* and *P. eryngii* clearly colonize the contaminated soil. However, the mere colonization of the soil did not assure high removal of TPHs since the inherent microbiota of the SMS and its interaction with the autochthonous soil microbiota are also important factors. The SMS of *A. bisporus* was the most effective SMS for the bioremediation of contaminated soils with petroleum hydrocarbon. *L. edodes* SMS reached high degradation of the heaviest aliphatic chains (>C_{35}) and the aromatic chains >EC_{12}–EC_{16}. SMS of *P. ostreatus* was the most effective SMS for the degradation of the aliphatic chains >C10–C12 and aromatic chains EC_{10}–EC_{12}. Finally, *P. eryngii* SMS was only useful for the biodegradation of the light aliphatic chains >C_{10}–C_{12}.

This work demonstrates the feasibility of the bioremediation of contaminated soils with petroleum hydrocarbons using abundant agricultural waste such as SMS of different fungi. The re-use of SMS for this purpose promotes the circular economy and the Green Deal of the European Union reducing the environmental and climate footprint of the food system.

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