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Title: Implications of Polluted Soil Biostimulation and Bioaugmentation with Spent Mushroom Substrate (Agaricus bisporus) on the Microbial Community and Polycyclic Aromatic Hydrocarbons Biodegradation

Article Type: Research Paper

Keywords: polycyclic aromatic hydrocarbons, biodegradation, bioavailability, ligninolytic enzymes, agricultural waste

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Response to Reviewers: Reviewer 1:
1. The design of experiments has been discussed at Line No. 116-128 in Introduction section, and in section 2.2 Preparation of remediation microcosms' (Line No. 147-169). The explanation may be confined to Section 2.2 and the description in Introduction may be cut short.
Answer: We agree with the reviewer. The description of the experiments has been only confined to Materials and Methods, section 2.2.
2. As mentioned in Highlights of the paper that 'SAS decreased the bioavailability of most PAHs in soil', how do the authors recommend the use of SAS? Since bioavailability is directly related to degradation of PAHs, a decrease represents reduced degradation rate. Authors need to clarify it.

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3. Written English needs improvement and references be cross-checked.

Answer: English has been reviewed by native English speaker. The references have been corrected. (Bogan and Lamar 1995; Covino et al. 2010a; 2010b)

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Abstract: the first sentence has been rewritten as follows: 'Different applications of spent Agaricus bisporus substrate (SAS), a widespread agro-industrial waste, were investigated with respect to the remediation of a historically polluted soil with Polycyclic Aromatic Hydrocarbons (PAH)'.

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Page 3 Line 70: References were added. . 'Bioremediation of PAH-contaminated soils is based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or on the addition of exogenous microorganisms, the latter approach being referred to as bioaugmentation (Haritash and Kaushik 2009)'.

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Page 13 Line 304: We have written the p value in the text. ($p \leq 0.05$)

Page 14 Line 331: For sake of clarity, the sentence in question has been rewritten as follows: "This may be explained by the low nutrient input associated with this inoculant. Its preparation had involved the reinoculation of A. bisporus into spent Agaricus substrate and subsequent incubation for 14 d prior to its application onto soil with ensuing consumption of organic nutrients by the growing fungus. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm where the addition of the inoculant ensured a higher nutrient input than in Abisp."

Page 16 Line 372: The Reviewer probably refers to the possible inclusion of cluster analysis (CA), which, albeit performed, was not included in the Ms. for sake of brevity. In fact, data in Table 2 (now Table 3) were deemed to be sufficient to highlight differences among microcosms.

Page 18 Line 432: We have rewritten the sentence as follows: "Conversely, with the exception of the Abisp microcosm, PAH degradation results were negatively correlated ($p < 0.01$) with their respective MW, log Koc and log P values (Table 4)."

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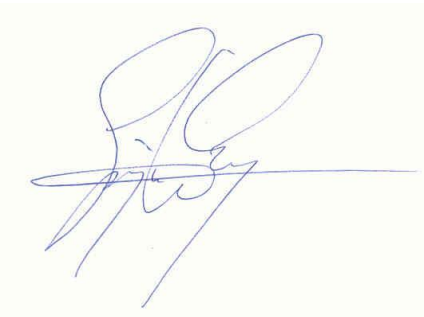
November 12, 2014

Dear Dr. Daniel A. Wunderlin,

enclosed please find the list of changes for each point raised by the reviewers. All comments have been carefully taken into consideration and all changes proposed have been included. All changes have been highlighted in the revised manuscript, and reviewers comments addressed in the manuscript. A clear identification of the added modifications can be retrieved from the uploaded Ms. text file where changes have been tracked.

Thank you for the meticulous revision.

Your sincerely,



Enrique Eymar (corresponding author)

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Highlights

Augmentation with *A. bisporus* led to the degradation of high molecular weight PAH.

Sterile SAS stimulated the bacterial population with ensuing 3-ring PAH degradation.

Richness of soil bacterial and fungal biota increased in the SAS-amended microcosm.

The results support the adequacy of SAS for environmental remediation purposes.

Implications of Polluted Soil Biostimulation and Bioaugmentation with Spent Mushroom Substrate (*Agaricus bisporus)* on the Microbial Community and Polycyclic Aromatic Hydrocarbons Biodegradation.

García-Delgado, Carlos[a]; D'Annibale, Alessandro[b] , Pesciaroli, Lorena[b]; Yunta, Felipe[c]; Crognale, Silvia[b]; Petruccioli, Maurizio[b]; Eymar, Enrique[a*].

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Abstract

Different applications ~~options~~ of ~~the~~ spent *Agaricus bisporus* substrate (SAS), a~~n~~ widespread agro-industrial waste ~~produced in huge amounts at the worldwide level~~, were investigated ~~in~~ with respect to the remediation of a~~a~~ historically polluted soil with Polycyclic Aromatic Hydrocarbons (PAH). ~~On the one hand~~In one treatment, the waste was sterilized (SSAS) prior to its application in order to assess its ability to biostimulate, as an organic amendment, the resident soil microbiota and ensuing contaminant degradation. ~~On the other hand~~For the other treatments, two bioaugmentation approaches were investigated; the first ~~one~~ involved the use of the waste itself and thus implied the application of *A. bisporus* and the inherent microbiota of the waste. In the second ~~one~~treatment, SAS was sterilized and inoculated again with the fungus to assess its ability to act as a fungal carrier. All these treatments were compared with natural attenuation in terms of their impact on soil heterotrophic and PAH-degrading bacteria, fungal growth, biodiversity of soil microbiota and ability to affect PAH bioavailability and ensuing degradation and detoxification. Results clearly showed that historically PAH contaminated soil was not amenable to natural attenuation. Conversely, the addition of sterilized spent *A. bisporus* substrate to the soil stimulated resident soil bacteria with ensuing high removals of 3-ring PAH. Both augmentation treatments were more effective in removing highly condensed PAH, some of which known to possess a significant carcinogenic activity. Regardless of the mode of application ~~mode~~, the present results strongly support the adequacy of SAS for environmental remediation purposes and open the way to an attractive recycl~~e~~ing option of this waste.

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Augmentation with *A. bisporus* led to the degradation of high molecular weight PAH.

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The results support the adequacy of SAS for environmental remediation purposes.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic properties (IARC, 2010). As a consequence of their physico-chemical properties, these compounds are accumulated in soil *via* their sorption onto organic matter (Krauss et al., 2000; Liang et al., 2006) and/or clay fractions (Hundal et al., 2001). The selection of a particular remediation technique for PAH-polluted soils is not an easy choice. There are many alternatives ~~such as~~including *in situ* or *ex situ* techniques which rely~~ing~~ on either physico-chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013). Among them, bioremediation is increasingly deemed to be the most environmentally friendly technique to clean-up polluted soils. Bioremediation of PAH-contaminated soils is based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or on the addition of exogenous microorganisms, the latter approach being referred to as bioaugmentation (Haritash and Kaushik 2009). The biostimulation of

resident microbial communities of PAH-impacted soil can be achieved in~~by~~ a variety of ~~approaches~~ ways including the addition of organic matter, which has been found to be successful (Covino et al., 2010~~b~~a; Sayara et al., 2010).

~~The b~~Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of PAH-contaminated soils has received increasing attention in recent years due to its reported efficacy (Covino et al., 2010~~a~~b; Federici et al., 2012a; Li et al., 2012). LDF are known to produce extracellular lignin-modifying enzymes (LME) with low substrate specificity which enable them to degrade a wide range of organic pollutants, including PAH (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP), versatile peroxidase and lignin peroxidase).

Soil colonization and the ensuing contaminant degradation by LDF in soil requires the addition of lignocellulosic materials either as amendments, or inoculum carriers (Covino et al., 2010~~b~~a; Lestan and Lamar, 1996). The use of these additives has been found to have a favorable impact on the resident microbiota, including specialized populations (Federici et al., 2012a). PAH degradation *via* bioaugmentation with LDF has been shown to involve either synergistic or antagonist interactions between the fungi added and the autochthonous microbiota. In one study a cooperative effect on the degradation of highly condensed PAH was reported between resident bacteria and *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010). However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria and changed the composition of the bacterial community (Andersson et al., 2000). Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the carriers have been shown to improve the competitive ability of the fungi added to the

resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of ~~S~~pent mushroom substrate of some LDF as a source of viable inocula for soil clean up applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus* substrate (SAS), without previous treatment, has never been used for bioremediation of PAH polluted soil. ~~The s~~Spent *Agaricus bisporus* substrate (SAS), has been shown to enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in a PAH-contaminated substrate (García-Delgado et al., 2013a). Marín-Benito et al. (2014; 2012a,b) reported the ability of pesticide degradation by SAS composted with spent *P. ostreatus* substrate (75:25) and its adsorption capacity ~~o~~for fungicides with low polarity that reduce their mobility in the environment. In addition, SAS has been reported to be an excellent source of LME, mainly laccase, that were able to biodegrade PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this organic waste in Europe has been estimated at 3.5 x 10^6 t (Pardo-Giménez and Pardo-González, 2008) consequently its potential use in bioaugmentation applications would certainly help to reduce this figure.

Therefore, in view of ~~a~~the profitable and environmentally sound use of SAS, this study has thoroughly investigated its feasibility in PAH remediation applications. This necessarily ~~should~~ implies~~y~~ a variety of manipulations of the waste prior to its application, ~~in order~~ to gain more insight~~s~~ into its ability to act as an organic amendment, ~~or~~ fungal carrier or a supplier of exogenous complex microbiota.

To elucidate the isolated and/or combined effects exerted by SAS, several application options were compared using an historically PAH-contaminated soil. ~~The first option assessed its ability to biostimulate the resident microbiota *via* its addition in a sterile form. In the second option the SAS was sterilized and subsequently reinoculated with *A. bisporus*. This was carried out in order to concomitantly suppress the inherent~~

~~microbiota and to evaluate its ability to act as a viable fungal carrier. Finally, the third option involved the use of non-sterile SAS and was therefore an augmentation approach based on both *A. bisporus* and the resident microbiota in the waste.~~ All ~~these~~ ~~treatment~~~~of them options~~ were assessed ~~compared~~ for their ability to (i) affect the densities of heterotrophic and PAH-degrading bacteria (ii) enable fungal growth (iii) modify the biodiversity of the bacterial and fungal communities (iv) remove PAH (v) modify the bioavailability of PAH and finally to (vi) detoxify the soil. A non-amended contaminated soil microcosm was incubated in parallel and was used as a natural attenuation control.

Materials and Methods

2.1 Materials

The polluted soil was collected from a creosote wood treatment plant ~~located in Alfaro~~ (42º 10´ 31´´N 1º 41´ 36´´ W La Rioja, Spain). The total soil sample (40 kg) was obtained by mixing 20 sub-samples ~~were~~ taken ~~and mixed (40 kg) from an~~ from an area close to the treated wood stock zone at a depth of 0-20 cm. ~~Soil samples were collected specifically from areas close to the treated wood stock zone at a depth of 0-20 cm.~~ The soil sample~~s~~ ~~were~~was ~~pooled,~~ homogenized, air-dried at room temperature and finally passed through a 2 mm-sieve. The main properties of the soil are shown in Table 1. ~~were: pH, 8.20±0.03 and electric conductivity, 0.576 ±0.016 dS m^{-1} in aqueous extract 1:5 (w:v); $CaCO_3$, 30±1 %, organic matter, 1.2±0.1 %; with a texture composition of: sand, 39 %; silt, 39 %; clay, 22 %.~~ According to the US textural classification, the soil was a clay loamy soil (sand 39%, silt 39% and clay 22%) with a water holding capacity of 37 %. Thirteen out of the ~~PAH of the~~ 16 PAH US EPA were present in the polluted soil~~,~~. ~~t~~The PAH concentrations are shown in Table ~~1~~2.

SAS was collected from a composting- plant of agricultural waste located atin ~~Quintanar del Rey (~~39º 22' 16''N 1º 59' 43'' W (Cuenca, Spain). The main characteristics of the SAS are shown in Table 1.~~material had the following characteristics: moisture, 67.4 ±0.4%, pH, 6.7±0.3, electric conductivity, 7.1±0.8 dS m^{-1}; organic matter, 61.9±1.7%; C/N ratio, 7.96±0.13~~. Total carbon ~~(32.4±0.02%)~~, nitrogen ~~(4.07±0.07%)~~, hydrogen ~~(1.95±0.06%)~~ and sulfur ~~(0.92±0.12%)~~ were determined by elemental analysis (LECO CHNS-932 analyzer, St. Joseph, MI).

2.2 Preparation of the Remediation Microcosms

Irrespective of the type of microcosm, the experiments were carried out in 1l glass reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of this microcosm simply involved the adjustment of the soil moisture content to 70 % of its water-holding capacity prior to the beginning of the incubation. This microcosm will be referred to as soil microcosm (SM)

- Biostimulation: the moisture content of the polluted soil was adjusted to 70 % as above and amended with sterilized SAS (121 °C, 30 min) ~~in~~at a 4:1 mass ratio to yield the SSAS microcosm. This approach was designed to assess the stimulatory effect of a sterilized organic waste on resident microbiota in the soil.

- Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70 % as above and amended with SAS (4:1 mass ratio) to yield the SAS microcosm. This approach was designed to assess the combined effect of both *A. bisporus* and the indigenous SAS microbiota.

- Bioaugmentation II: to prepare this microcosm, sterilized SAS was inoculated with 3 agar plugs (1 cm diameter) from a 14 day old culture of *A. bisporus* grown on malt extract agar and incubated for 10 d at 20 °C. The colonized matrix was mixed with the contaminated soil (1:4 mass ratio) and the moisture content adjusted to 70 % as described above. This bioaugmentation approach, called the Abisp microcosm, was designed to eliminate the contribution of the SAS microbiota.

Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and 63 days under static conditions in the dark.

2.3 Extraction and Analysis of Ergosterol and PAH

Total ergosterol was extracted and analyzed as described in the method by Covino *et al.* (2010b). Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml methanolic solution KOH (10 %, w/v). Distilled water (1 ml) was added to each sample and the sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were analyzed using high performance liquid chromatography (Waters 2695 Separation Module) coupled with a Waters 996 photodiode array detector equipped with Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 µm; pore size 100 A) equilibrated with methanol:water (95:5) at a flow rate of 1ml min^{-1}. The sample injection volume was 20 µl. The elution profile was monitored at 282 nm.

The extraction of PAH was performed by pressurized liquid extraction (PLE) (ASE350, Dionex). Soil samples (10 g) were loaded into the extraction cell (32 ml) and subsequently extracted with a dichloromethane–acetone mixture (DAM, 1:1, v/v). Static heating was applied to the vessel (100 °C, 5 min) and the extraction performed for 7 min

at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and finally the solvent purged from the cell with argon for 60 s. This extraction cycle was repeated twice for each sample. The resultant organic extract was dried under gentle N_2 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a Superguard™ LC-18 guard column (20 x 3 mm) prior to the separation Supelcosil™ LC-PAH column (250 × 3.0 mm; particle size 5 µm) and a 2996 diode-array detector (Waters). Separation of the PAH was achieved using a gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution program was: isocratic elution with 60 % (A) for 5 min, gradient to 100 % (A) for 15 min, isocratic elution at 100 % A for 20 min. The column temperature was fixed at 28 ^{o}C. The sample injection volume was 20 µl. The chromatograms were monitored at 254 nm. PAH were identified on the basis of both UV spectra and matching the retention times with commercially available standards (Sigma-Aldrich). The PAH detected and quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene (IcdP). Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due the volatilization of these compounds (García-Delgado et al., 2013b).

2.4 Estimation of PAH Bioavailability

The bioavailable fraction of each PAH was determined in all microcosms by hydroxypropyl-β-cyclodextrin (HPCD) extraction according to the method described by Stokes et al. (2005). The extraction was carried out by mixing the soil (1.5 g) with a 25 ml solution of 50 mM HPCD in deionized water and incubating the mixture for 20 h on

an orbital shaker (250 rpm). The mixture was then centrifuged (2500 rpm, 15 min) and the supernatant discarded. The resulting soil pellet was washed with deionized water (25 ml) and manually shaken for 10 s, centrifuged and the supernatant discarded again to remove residual HPCD. Finally, the soil pellet underwent exhaustive PLE extraction (as described above) to determine residual PAH concentration after HPCD extraction.

2.5 Biochemical Determinations and Toxicity Tests

Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3 g) at 5 $^{\circ}$C for 1 h using the buffered solution described by D´Annibale et al. (2006). The aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36000 M^{-1} cm^{-1}). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+} ~~to~~ form a complex with malonate which absorbs at 270 nm (ε= 11590 M^{-1} cm^{-1}) (Wariishi et al., 1992). One unit of enzyme activity (IU) is defined as the amount of enzyme which produced 1µmol of product per minute under the assay conditions.

Ecotoxicological assessment of remediation microcosms was carried out using two independent methods. The first was an acute toxicity test on the springtail *Folsomia candida* Willem based on the percentage mortality of adults, as previously reported by Leonardi et al., (2008). The second test was based on the determination of dehydrogenase activity which has been shown to be a sensitive ecological index in soils contaminated with hydrocarbons (Dawson et al., 2007). Remediation microcosms were

assayed for dehydrogenase activity as follows: 1 ml ~~of~~ 1.5 % 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 M Tris-HCl buffer at pH 7.5 ~~was~~ and added to 1 g fresh samples. The reaction mixture was incubated at 30 °C for 24 h in the dark. At the end of incubation, the triphenylformazan was extracted with 8 ml acetone, the extract was centrifuged (3500 rpm, 15 min) and the absorbance of the supernatant measured at 546 nm. (ε= 15.4 mM^{-1} cm^{-1}).

The percentage reduction of carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy, (1992) toxic equivalency factors (TEF) of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13}[PAH_i]_{to} \bullet DR \bullet TEF_i}{\sum_{i=1}^{13}[PAH_i]_{to} \bullet TEF_i} \bullet 100$$

where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 2~~1~~).

2.6 Enumeration of Cultivable Heterotrophic and PAH-Degrading Bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method using 96-well microtiter plates, with eight replicate wells per dilution according to the method of Lladó et al. (2009). The total number of heterotrophs were counted in tryptone soy broth and the PAH-degrading bacteria were counted in a mineral medium containing a PAH mixture composed of PHE 0.5 g l^{-1} and FLU, ANT, PYR, at a final concentration of 0.05 g l^{-1} as the sole carbon sources. To avoid fungal contamination, cycloheximide at a final concentration of 100 mg l^{-1} was added to both growth media (i.e., tryptone soy broth and mineral medium). The MPN plates were incubated at room temperature for 30 days. Positive wells were detected by turbidity for

heterotrophs, and the presence of a brownish/yellow coloration for PAH degrading bacteria. The MPN calculation was carried out using US EPA MPN Calculator v1.1 software.

2.7. DNA Extraction

Whole genomic DNA from each soil was extracted using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the universal bacterial 341-f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') and 534-r (5′-ATT ACC GCG GCT GCT GG-3′) primers with a length of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was amplified using the universal fungal primers FUN_NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and GC fung (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTAC CCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 µL of template DNA (ca. 20 ng), Tris–HCl (20 mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), Taq-polymerase (0.1 IU) (NzyTech, Lisbon, Portugal), primers (0.2 mM of each) and double deionized water to bring the final volume up to 50 µL. For Bacterial amplification, the touchdown PCR program was performed in a *Primus* PCR thermo cycler (MWG biotech, Ebersberg, Germany) using the same procedure (Muyzer et al., 1993). For fungal amplification the PCR program previously reported by Das et al., (2007) was used. All amplicons (5 µL) were analyzed on agarose gel before being used for Denaturing Gradient Gel Electrophoresis (DGGE).

2.8. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The INGENY phorU DGGE system (Ingeny, Goes, NL) was used for sequence-specific separation of PCR amplified fragments. For PCR amplicons obtained with the 341f-GC and 534r primers, electrophoresis was performed in a polyacrylamide gel (8 % (w/v) acrylamide/bis-acrylamide gel 37.5:1), containing 40 – 60 % urea-formamide denaturing gradient (100 % corresponds to 7 M urea and 40 % (w/v) formamide). For fungal amplicons the urea-formamide denaturating gradient ranged from 20 to 35 %. After DGGE electrophoresis the gels were stained with Gel star solution (Lonza, Ltd group, USA) at room temperature for 45 min and photographed using a UV-transillumination table with a GelDoc XR digital camera (Bio-Rad, Carlsbad, CA). The fingerprinting profile obtained from DGGE was investigated using the Quantity one software (Bio-Rad). To characterize the community composition obtained by DGGE analyses, the Shannon diversity index H' was calculated as follows:

$$H' = - \Sigma (P_i \times \ln P_i)$$

where P_i represents the ratio of the single intensity band to the sum of the bands intensity of each lane, Richness (S) is the number of bands revealed, and Evenness (E) was calculated as H'/ln S.

3 Results and Discussion

3.1 Time- and Microcosm-Dependent Evolution of Fungal Biomass and Cultivable Bacteria

In the SM microcosm control the ergosterol content, a specific indicator of fungal biomass was invariably lower than the detection limits (Fig. 1A). Irrespective of the sampling time, no fungal growth was observed in the SM throughout the incubation period.

There were no significant ($p \leq 0.05$) time dependent changes in ergosterol in the SSAS microcosm which indicates that despite the presence of the sterilized *Agaricus* substrate, fungal growth was not stimulated at all. This result was unexpected because several studies have shown that the application of sterilized lignocellulose waste exerts a positive effect on the resident fungi in contaminated soils (Federici et al., 2012a, 2011; Lladó et al., 2013). Conversely, in the SAS microcosm an approximate three-fold increase in ergosterol content was observed in the first week of incubation. This was followed by a decline in the values which remained constant in subsequent harvests (Fig. 1A).

No changes in the ergosterol content were observed throughout the incubation in the Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement for the resident fungi. This could be explained by the fact that most of the available organic components in the sterilized SAS had been consumed by the reinoculated *A. bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient status. Therefore, the potential trophic contribution of the carrier to the competitive ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar, 1996).

The density of the cultivable heterotrophic and PAH-degrading bacteria over time is shown in Fig. 1B and 1C` respectively. Heterotrophic microbial counts in the SAS and SSAS microcosms increased by approximately two orders of magnitude and were significantly higher than those in the SM microcosm over the whole incubation period (Fig. 1B). The same result was also found in the bioaugmented Abisp microcosm where heterotrophic bacterial counts increased by more than one order of magnitude (Fig. 1B). In the amended microcosms, the lowest densities of heterotrophic bacteria throughout

the incubation period were found in the Abisp microcosm. ~~This may also explained by the low nutrient status of this microcosm, because the *A. bisporus* nutrient consumption before inoculation in soil, rather than any competition between *A. bisporus* and the resident bacteria. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm.~~

This may be ~~also~~ explained by the low nutrient ~~status of this microcosm~~input associated with this inoculant. ~~,~~ ~~I~~because ~~the~~ts preparation had~~, in fact,~~ involved the reinoculation of *A. bisporus* ~~on~~into spent Agaricus substrate and subsequent incubation for 14 d prior to its application onto soil with ensuing consumption of organic nutrients by the growing fungus~~nutrient consumption before soil inoculation, and competition between *A. bisporus* and the resident bacteria~~. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm where the addition of the inoculant ensured a~~ich presented~~ higher nutrient input~~status because SAS was not incubated 14d before soil inoculation and showed lower fungal activity than Abisp microcosm.~~than in Abisp~~s~~. ~~(Fig. 2)~~

Similar results were also found for the cultivable PAH-degrading bacteria. The highest densities were observed in the SSAS and SAS microcosms (Fig. 1C). However, it should be noted that the spent *Agaricus* substrate contained viable PAH-degrading bacteria which would have resulted in a higher initial density in the SAS microcosm than in the SM (2.3×10^4 vs. 0.5×10^2 MPN g^{-1}) (Fig. 1C). In the SSAS microcosm, the addition of the sterilized organic waste also exerted a high stimulation on the PAH-degrading bacteria which were increased by more than three orders of magnitude after one and three weeks of incubation, although after this their densities declined. As previously observed for heterotrophic bacterial counts in the amended remediation microcosms, the Abisp exhibited the lowest densities of PAH-degrading bacteria for ~~the~~

most of the incubation period. Regardless of the different application options in addition to leading to a higher density of heterotrophs, when compared with the SM, it also enhanced specialized ones. As described above, the non-sterile spent *Agaricus* substrate in the SAS microcosm contained allochthonous PAH-degrading bacteria. This is not surprising because a molecular characterization of the bacterial community in this substrate has revealed the presence of several genera *Paenibacillus*, *Arthrobacter*, *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004; Watabe et al., 2004) which included several species with reported PAH-degrading capacity (Haritash and Kaushik, 2009). However, the addition of the sterilized SAS, irrespective of whether it had been reinoculated ~~or~~ with *A. bisporus* or not, also appeared to stimulate PAH-degrading bacteria. Previous studies have also found that the addition of sterilized organic wastes such as wheat straw and maize stalks activated the specialized resident bacterial populations in soils contaminated with PAH (Lladó et al., 2013), and polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested that the stimulatory effect exerted by organic waste is due to an enhancement of the oxygen transfer via an increase in soil porosity without necessarily involving either trophic factors or modifications in contaminant bioavailability (Federici et al., 2012b).

3.2 Microcosm-Dependent Impact on Bacterial and Fungal Community Structure

It is widely known that the cultivable microbiota in soil represent a minor fraction of the whole microbial community (Daniel, 2005). Therefore, an investigation into the structure of both the bacterial and fungal communities as a function of the remediation treatment necessitated a cultivation-independent approach which relied on DGGE. Table ~~2~~3 shows the Shannon Weaver Index, which gives the richness and evenness values of bacterial and fungal communities in the microcosms at the start, and at the end

of incubation. These values have been calculated by a numerical analysis of the DGGE profiles of PCR-amplified 16S and 18S rDNA fragments respectively. Both the richness and the diversity of the bacterial and fungal communities in the SM did not significantly change during incubation. This result is not surprising because both communities presumably had a well established adaptation to the conditions exerted by the historical contamination of the soil.

As a result of the fungi and bacteria already present in the SAS microcosm which contained the unsterilized spent *Agaricus* substrate, higher initial richness values for bacterial and fungal biota were found than in those in the SM microcosm (18 *vs*. 11, respectively, and 23 *vs*. 17, respectively). In addition, the 63 day old SAS microcosms~~,~~ produced a significantly higher H' value for the bacterial community than that from the coeval SM (2.95±0.08 *vs*. 2.23±0.08, respectively), as well as a higher richness in the fungal biota (27 *vs*. 17, respectively) but without substantial differences in their respective evenness.

Conversely, the presence of the sterilized spent *Agaricus* substrate in the SSAS microcosm did not substantially modify the test parameters with respect to those found in the SM. In the 63 day old SSAS microcosm, significantly higher S and H' values for the bacterial community than those in the coeval SM were found, although no substantial differences were observed in their fungal biota. Regardless of the sampling time, similar results to those described for SSAS were found in the Abisp microcosm. Interestingly, the microcosms to which steri li z~~s~~ed spent *Agaricus* substrate had been added, irrespective of whether they had been reinoculated with Agaricus or not, i.e. the SSAS and Abisp microcosms had the same impact on the fungi. They both failed to promote fungal growth throughout incubation (Fig. 1A) and/or to substantially change

the richness and diversity of the fungal community when compared to the SM microcosm (Table ~~2~~3).

3.3 PAH Removal in the Remediation Microcosms

Table ~~1~~2 shows the initial and residual PAH concentrations in the remediation microcosms after 63 days incubation. In the SM, no significant reduction in the total PAH concentration was observed (p=0.07). Significant differences between the initial and final concentrations were only found for FLT and PYR, with a percentage removal of 65 %, and 59 % respectively. In the biostimulation SSAS microcosm a statistically significant decrease (p<0.05) in 3-ring PAH content, FLU (75 %), PHE (90 %), ANT (82 %), and FLT (52 %), was observed and the overall PAH residual content significantly differed from that at the start. Therefore, the biostimulation treatment was effective at degrading low molecular weight PAHs which were the most abundant contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp, significant differences between the initial and the residual concentrations were found for the majority of the individual contaminants as well as the overall PAH concentrations (Table ~~1~~2). These findings indicate that the bioaugmentation treatments with *A. bisporus* were efficient at degrading both low and high molecular weight PAH.

A comparison of the degradation performances of SSAS, SAS and Abisp microcosms, revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp was superior to SSAS and SAS in the removal of highly condensed PAH (Table ~~1~~2). Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC, 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds such as BaP and DBahA (52 vs. 18 % respectively, and 32 vs. 6 % respectively). In this

respect, a very limited number of bacteria ~~are~~ able to grow in pure cultures on either 5- or 6-ring PAH have been identified (Haritash and Kaushik, 2009).

However, in the SAS microcosm, the augmented fungus appeared to exert a lower inhibitory action on the resident bacterial biota as deduced by comparing its better degradation performance on low molecular weight PAH and its higher density of PAH-degrading bacteria than those found in the Abisp microcosm. One of the possible synergistic mechanisms which might occur between bacteria and fungi, relies on the ability of the latter to convert PAH into more polar degradation intermediates, such as PAH diones and hydroxylated derivatives (Covino et al., 2010~~b~~a).

Linear regression analyses were performed to relate the percentage removal of each PAH in the microcosm with respect to the chemical characteristics of these contaminants (Table ~~3~~4). Therefore, the degradation results were related to those parameters which have been suggested to significantly affect PAH degradation, such as molecular weight (MW), organic carbon sorption coefficient (log K_{oc}), hydrophobicity (log P), water solubility (WS) and ionization potential (IP), (Table ~~3~~4). These analyses showed that the degradation results were positively and significantly (p< 0.05) correlated with WS in all the microcosms tested. Conversely, with the exception of the Abisp microcosm, PAH degradation results were ~~a significant~~ negatively ~~trend~~ correlated~~ion~~ (p <~~=~~ 0.01~~0) or 0.005)~~ ~~was found when the degradation results were related to~~with their respective MW, log K_{oc} and log P values (Table ~~3~~4). These results clearly indicate that the bioavailability of PAH contaminants strongly affect their ability to be degraded in the SM, SSAS and SAS microcosms, where the bacteria make an important contribution to PAH depletion. Bacterial uptake of PAH and their subsequent metabolism have been shown to be governed by the mass transfer rates of contaminants from the solid to the liquid phase of soil, which are in turn affected by the

aforementioned physico-chemical properties (Haritash and Kaushik, 2009). The distinctive behavior of the Abisp microcosm, namely the lack of correlation between PAH degradation and MW, log K_{oc} and log *P*, might indicate that different PAH degradation mechanisms are involved here. In particular, the direct involvement of PAH oxidation by LME which was high during the initial incubation phases (See subsection 3.5) might be as result of their ability to diffuse into the soil matrix and potentially oxidise PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH. The IP has been shown to affect *in vitro* PAH oxidation by either laccase or fungal heme-peroxidases, whose activities were found in the bioaugmented microcosms (see subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the opposite trend was found when relating PAH depletions and respective IP values in all the microcosms. However, the Pearson Coefficients related to these regressions, which were significant with the exception of Abisp, were the lowest among the physico-chemical properties tested (Table 4~~3~~). Similar findings were obtained with two historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus tigrinus* (Covino et al., 2010~~a~~b). This clearly suggests that even in augmented microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH degradation. This was made particularly evident in the bioaugmented microcosms by the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV respectively) make them poorly susceptible to mono-electronic oxidation by LMEs (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi

also relies on the intracellular cytochrome P-450/epoxide hydrolase complex whose activity is not dependent on IP (Haritash and Kaushik, 2009).

3.4 Time- and Microcosm-Dependent Modifications in the PAH Bioavailable Fraction

The bioavailable fraction of each PAH was determined in all the microcosms at the start and after 63 days incubation using HPCD extraction (Stokes et al., 2005). At the start the different soil application options, i.e. SSAS, SAS and Abisp microcosms did not significantly affect the percentage bioavailable fraction of the large majority of contaminants in each microcosm (Table 54). The only exceptions were observed in some highly condensed PAH such as DBahA. The bioavailable fractions ~~of which~~ were lowest in the SAS and Abisp microcosms. BghiP showed the highest bioavailability in the SSAS and Abisp microcosms.

After 63 days incubation a microcosm-dependent modification of the bioavailable fraction of individual PAH was observed when compared to the beginning of the experiment. In particular, in the SM an increase in the bioavailable fraction was observed for high molecular weight PAH (i.e., DBahA, BghiP and IcdP). In the SSAS microcosm, the bioavailable fractions of BbF and DBahA increased while those of PHE and ANT decreased (Table 54). For the latter two compounds, it could be envisaged that their high degradation extents in this microcosm (90 and 82 %, respectively) left only the most recalcitrant fraction. In the bioaugmented microcosms (SAS and Abisp) a different scenario was found. In particular, in the SAS a decrease in the bioavailable fraction of PHE, FLT, PYR, CHR and BaP was observed. Conversely, in the Abisp microcosm, decreased bioavailabilities were found for FLU, PHE, BbF, BkF, BaP, BghiP and IcdP. The reason underlying the decrease in the bioavailable fraction of the majority of individual PAH was not solely due to their respective depletions since these

parameters were not found to be correlated in all the microcosms. Therefore, the changes observed might have derived from a combination of PAH sorption onto organic matter and partial degradation. In this respect, the organic matter of spent *Agaricus* substrate analyzed by cross-polarization and magic angle spinning nuclear magnetic resonance, was found to contain a high percentage of aliphatic carbon (about 21 %) that could contribute significantly to PAH sorption *via* hydrophobic interactions (García-Delgado et al., 2013a).

3.5 Lignin-Modifying Enzyme Activity and Residual Toxicity in Remediation Microcosms

The remediation microcosms were assayed for their ligninolytic activity due to the involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010; Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-peroxidase activity (Fig. 2A and 2B, respectively) were only detected in the bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity were found in the Abisp microcosms where the activity of the former enzyme markedly declined with incubation time (Fig. 2A). Noteworthy, in this microcosm the best depletions were observed for BaP and DBahA which despite being high molecular weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38 respectively) and therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar, 1995). In this regard the results of the present study are in agreement with those of Li et al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts from spent *Agaricus* substrate.

Dehydrogenase activity was used as a possible index of detoxification in the remediation microcosms as previously suggested for hydrocarbon-impacted soils

(Dawson et al., 2007) in addition to the *F. candida* mortality test. In the present study, the adoption of this parameter did not provide an unequivocal clue to the detoxification by augmented microcosms. This was the result of a low dehydrogenase activity in the contaminated soil at the start (Table 6~~5~~). The level of dehydrogenase activity was markedly and predictably boosted by the addition of viable fungal inocula in the SAS and Abisp microcosms (Table 6~~5~~). The retention of high levels of activity in the 63 day old SAS and Abisp microcosms, albeit being lower in the SAS and equal in the Abisp, indicate that the soil microbiota retained a high functional activity despite a prolonged incubation time.

Table ~~5~~6 also shows that the initial toxicity of the microcosms towards *F. candida* was high, leading to mortalities that ranged from 77.5 to 89 %. Although a partial detoxification was observed in all microcosms, the best results were observed with SAS and Abisp where mortality was reduced by 45.1 and 41.4 %, respectively. In these microcosms, a generalized decrease in the bioavailable fractions of individual contaminants were observed together with the highest percentage removal of both 4-ring and highly condensed PAH. The lowest mortality reduction (26.8 %) was observed in SSAS where the lowest depletion of 4-ring PAH, and no depletion of highly condensed PAH were found. In this respect, the higher detoxification observed in Abisp than SSAS suggest that 3-ring PAH were less toxic to *F. candida* than 4-ring and 5,6-ring PAH. This might be explained by the fact that in the SSAS the former contaminant group was degraded at a significantly higher extent and, in the same microcosm, with the exception of PHE, their bioavailable fractions were comparatively lower than in Abisp.

A comparison of the 63 day old microcosms showed that the best reduction in the carcinogenic risk assessment (RCRA) was observed in the bioaugmented microcosms

(SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and 5,6-ring PAH which have the highest carcinogenic properties (IARC 2010). Significant differences were also found when comparing the bioaugmented microcosms. The Abisp microcosm showed higher RCRA than the SAS because of its better efficacy in the removal of BaP and DBahA (Table 2~~1~~) which exhibited the highest TEF values.

4 Conclusions

The addition of the sterilized spent *A. bisporus* substrate to the PAH-polluted soil was effective in stimulating the resident soil bacteria which resulted in higher levels of 3-ring PAH being removed. Bioaugmentation treatments with *A. bisporus*, in the SAS and Abisp microcosms, were more effective in removing 5, 6-ring PAH in particular BaP. The best detoxification results were obtained in the Absip microcosm where a high retention of microbiological functional activity, a significant decrease in *F. candida* mortality, and a reduction in carcinogenic risk assessment were observed. The wide spatio-temporal availability of this agro-waste combined with its proved efficacy in PAH biodegradation make its use technically feasible for environmental remediation purposes.

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Figure Captions

Figure 1: Time course of ergosterol concentrations (A), total heterotrophic (B) and PAH degrading (C) bacteria in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS), and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm (Tukey post-hoc test; $p \leq 0.05$), respectively.

Figure 2: Time courses of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms.

Implications of Polluted Soil Biostimulation and Bioaugmentation with Spent Mushroom Substrate (*Agaricus bisporus)* on the Microbial Community and Polycyclic Aromatic Hydrocarbons Biodegradation.

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Abstract

Different applications of spent *Agaricus bisporus* substrate (SAS), a widespread agro-industrial waste, were investigated with respect to the remediation of a historically polluted soil with Polycyclic Aromatic Hydrocarbons (PAH). In one treatment, the waste was sterilized (SSAS) prior to its application in order to assess its ability to biostimulate, as an organic amendment, the resident soil microbiota and ensuing contaminant degradation. For the other treatments, two bioaugmentation approaches were investigated; the first involved the use of the waste itself and thus implied the application of *A. bisporus* and the inherent microbiota of the waste. In the second treatment, SAS was sterilized and inoculated again with the fungus to assess its ability to act as a fungal carrier. All these treatments were compared with natural attenuation in terms of their impact on soil heterotrophic and PAH-degrading bacteria, fungal growth, biodiversity of soil microbiota and ability to affect PAH bioavailability and ensuing degradation and detoxification. Results clearly showed that historically PAH contaminated soil was not amenable to natural attenuation. Conversely, the addition of sterilized spent *A. bisporus* substrate to the soil stimulated resident soil bacteria with ensuing high removals of 3-ring PAH. Both augmentation treatments were more effective in removing highly condensed PAH, some of which known to possess a significant carcinogenic activity. Regardless of the mode of application, the present results strongly support the adequacy of SAS for environmental remediation purposes and open the way to an attractive recycling option of this waste.

Keywords: polycyclic aromatic hydrocarbons, biodegradation, bioavailability, ligninolytic enzymes, agricultural waste.

Highlights

- Augmentation with *A. bisporus* led to the degradation of high molecular weight PAH.
- Sterile SAS stimulated the bacterial population with ensuing 3-ring PAH degradation.
- Richness of soil bacterial and fungal biota increased in the SAS-amended microcosm.
- The results support the adequacy of SAS for environmental remediation purposes.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic properties (IARC, 2010). As a consequence of their physico-chemical properties, these compounds are accumulated in soil *via* their sorption onto organic matter (Krauss et al., 2000; Liang et al., 2006) and/or clay fractions (Hundal et al., 2001). The selection of a particular remediation technique for PAH-polluted soils is not an easy choice. There are many alternatives including *in situ* or *ex situ* techniques which rely on either physico-chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013). Among them, bioremediation is increasingly deemed to be the most environmentally friendly technique to clean-up polluted soils. Bioremediation of PAH-contaminated soils is based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or on the addition of exogenous microorganisms, the latter approach being referred to as bioaugmentation (Haritash and Kaushik 2009). The biostimulation of resident microbial communities of PAH-impacted soil can be achieved in a variety of ways including the addition of organic matter, which has been found to be successful (Covino et al., 2010a; Sayara et al., 2010).

Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of PAH-contaminated soils has received increasing attention in recent years due to its reported efficacy (Covino et al., 2010b; Federici et al., 2012a; Li et al., 2012). LDF are known to produce extracellular lignin-modifying enzymes (LME) with low substrate specificity which enable them to degrade a wide range of organic pollutants, including PAH (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP), versatile peroxidase and lignin peroxidase).

Soil colonization and the ensuing contaminant degradation by LDF in soil requires the addition of lignocellulosic materials either as amendments, or inoculum carriers (Covino et al., 2010a; Lestan and Lamar, 1996). The use of these additives has been found to have a favorable impact on the resident microbiota, including specialized populations (Federici et al., 2012a). PAH degradation *via* bioaugmentation with LDF has been shown to involve either synergistic or antagonist interactions between the fungi added and the autochthonous microbiota. In one study a cooperative effect on the degradation of highly condensed PAH was reported between resident bacteria and *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010). However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria and changed the composition of the bacterial community (Andersson et al., 2000). Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the carriers have been shown to improve the competitive ability of the fungi added to the resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of pent mushroom substrate of some LDF as a source of viable inocula for soil clean up applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus*

substrate (SAS), without previous treatment, has never been used for bioremediation of PAH polluted soil. Spent *Agaricus bisporus* substrate (SAS), has been shown to enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in a PAH-contaminated substrate (García-Delgado et al., 2013a). Marín-Benito et al. (2014; 2012a,b) reported the ability of pesticide degradation by SAS composted with spent *P. ostreatus* substrate (75:25) and its adsorption capacity for fungicides with low polarity that reduce their mobility in the environment. In addition, SAS has been reported to be an excellent source of LME, mainly laccase, that were able to biodegrade PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this organic waste in Europe has been estimated at 3.5 x 10^6 t (Pardo-Giménez and Pardo-González, 2008) consequently its potential use in bioaugmentation applications would certainly help to reduce this figure.

Therefore, in view of the profitable and environmentally sound use of SAS, this study has thoroughly investigated its feasibility in PAH remediation applications. This necessarily implies a variety of manipulations of the waste prior to its application, to gain more insight into its ability to act as an organic amendment, fungal carrier or a supplier of exogenous complex microbiota.

To elucidate the isolated and/or combined effects exerted by SAS, several application options were compared using an historically PAH-contaminated soil. All were assessed for their ability to (i) affect the densities of heterotrophic and PAH-degrading bacteria (ii) enable fungal growth (iii) modify the biodiversity of the bacterial and fungal communities (iv) remove PAH (v) modify the bioavailability of PAH and finally to (vi) detoxify the soil. A non-amended contaminated soil microcosm was incubated in parallel and was used as a natural attenuation control.

Materials and Methods

2.1 Materials

The polluted soil was collected from a creosote wood treatment plant (42º 10´ 31´´N 1º 41´ 36´´ W La Rioja, Spain). The total soil sample (40 kg) was obtained by mixing 20 sub-samples taken from an area close to the treated wood stock zone at a depth of 0-20 cm. The soil sample was homogenized, air-dried at room temperature and finally passed through a 2 mm-sieve. The main properties of the soil are shown in Table 1. According to the US textural classification, the soil was a clay loamy soil (sand 39%, silt 39% and clay 22%) with a water holding capacity of 37 %. Thirteen out of the 16 PAH US EPA were present in the polluted soil. The PAH concentrations are shown in Table 2.

SAS was collected from a composting plant of agricultural waste located at 39º 22' 16''N 1º 59' 43'' W (Cuenca, Spain). The main characteristics of the SAS are shown in Table 1.. Total carbon, nitrogen, hydrogen and sulfur were determined by elemental analysis (LECO CHNS-932 analyzer, St. Joseph, MI).

2.2 Preparation of the Remediation Microcosms

Irrespective of the type of microcosm, the experiments were carried out in 1l glass reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of this microcosm simply involved the adjustment of the soil moisture content to 70 % of its water-holding capacity prior to the beginning of the incubation. This microcosm will be referred to as soil microcosm (SM)

- Biostimulation: the moisture content of the polluted soil was adjusted to 70 % as above and amended with sterilized SAS (121 °C, 30 min) at a 4:1 mass ratio to

yield the SSAS microcosm. This approach was designed to assess the stimulatory effect of a sterilized organic waste on resident microbiota in the soil.

- Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70 % as above and amended with SAS (4:1 mass ratio) to yield the SAS microcosm. This approach was designed to assess the combined effect of both *A. bisporus* and the indigenous SAS microbiota.

- Bioaugmentation II: to prepare this microcosm, sterilized SAS was inoculated with 3 agar plugs (1 cm diameter) from a 14 day old culture of *A. bisporus* grown on malt extract agar and incubated for 10 d at 20 °C. The colonized matrix was mixed with the contaminated soil (1:4 mass ratio) and the moisture content adjusted to 70 % as described above. This bioaugmentation approach, called the Abisp microcosm, was designed to eliminate the contribution of the SAS microbiota.

Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and 63 days under static conditions in the dark.

2.3 Extraction and Analysis of Ergosterol and PAH

Total ergosterol was extracted and analyzed as described in the method by Covino *et al.* (2010b). Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml methanolic solution KOH (10 %, w/v). Distilled water (1 ml) was added to each sample and the sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were analyzed using high performance liquid chromatography (Waters 2695 Separation Module) coupled with a Waters 996 photodiode array detector equipped with

Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 µm; pore size 100 A) equilibrated with methanol:water (95:5) at a flow rate of 1ml min^{-1}. The sample injection volume was 20 µl. The elution profile was monitored at 282 nm.

The extraction of PAH was performed by pressurized liquid extraction (PLE) (ASE350, Dionex). Soil samples (10 g) were loaded into the extraction cell (32 ml) and subsequently extracted with a dichloromethane–acetone mixture (DAM, 1:1, v/v). Static heating was applied to the vessel (100 $^{\circ}C$, 5 min) and the extraction performed for 7 min at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and finally the solvent purged from the cell with argon for 60 s. This extraction cycle was repeated twice for each sample. The resultant organic extract was dried under gentle N_2 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a Superguard™ LC-18 guard column (20 x 3 mm) prior to the separation Supelcosil™ LC-PAH column (250 × 3.0 mm; particle size 5 µm) and a 2996 diode-array detector (Waters). Separation of the PAH was achieved using a gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution program was: isocratic elution with 60 % (A) for 5 min, gradient to 100 % (A) for 15 min, isocratic elution at 100 % A for 20 min. The column temperature was fixed at 28 ^{o}C. The sample injection volume was 20 µl. The chromatograms were monitored at 254 nm. PAH were identified on the basis of both UV spectra and matching the retention times with commercially available standards (Sigma-Aldrich). The PAH detected and quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene

(IcdP). Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due the volatilization of these compounds (García-Delgado et al., 2013b).

2.4 Estimation of PAH Bioavailability

The bioavailable fraction of each PAH was determined in all microcosms by hydroxypropyl-β-cyclodextrin (HPCD) extraction according to the method described by Stokes et al. (2005). The extraction was carried out by mixing the soil (1.5 g) with a 25 ml solution of 50 mM HPCD in deionized water and incubating the mixture for 20 h on an orbital shaker (250 rpm). The mixture was then centrifuged (2500 rpm, 15 min) and the supernatant discarded. The resulting soil pellet was washed with deionized water (25 ml) and manually shaken for 10 s, centrifuged and the supernatant discarded again to remove residual HPCD. Finally, the soil pellet underwent exhaustive PLE extraction (as described above) to determine residual PAH concentration after HPCD extraction.

2.5 Biochemical Determinations and Toxicity Tests

Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3 g) at 5 $^{\circ}$C for 1 h using the buffered solution described by D´Annibale et al. (2006). The aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined by following the oxidation of 0.2 mM 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ε= 36000 M^{-1} cm^{-1}). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+} form a complex with malonate which absorbs at 270 nm (ε= 11590 M^{-1} cm^{-1}) (Wariishi et al., 1992). One unit of enzyme activity (IU) is

defined as the amount of enzyme which produced 1µmol of product per minute under the assay conditions.

Ecotoxicological assessment of remediation microcosms was carried out using two independent methods. The first was an acute toxicity test on the springtail *Folsomia candida* Willem based on the percentage mortality of adults, as previously reported by Leonardi et al., (2008). The second test was based on the determination of dehydrogenase activity which has been shown to be a sensitive ecological index in soils contaminated with hydrocarbons (Dawson et al., 2007). Remediation microcosms were assayed for dehydrogenase activity as follows: 1 ml 1.5 % 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 M Tris-HCl buffer at pH 7.5 and added to 1 g fresh samples. The reaction mixture was incubated at 30 °C for 24 h in the dark. At the end of incubation, the triphenylformazan was extracted with 8 ml acetone, the extract was centrifuged (3500 rpm, 15 min) and the absorbance of the supernatant measured at 546 nm. (ε= 15.4 mM^{-1} cm^{-1}).

The percentage reduction of carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy, (1992) toxic equivalency factors (TEF) of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13}[PAH_i]_{to} \bullet DR \bullet TEF_i}{\sum_{i=1}^{13}[PAH_i]_{to} \bullet TEF_i} \bullet 100$$

where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 2).

2.6 Enumeration of Cultivable Heterotrophic and PAH-Degrading Bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method using 96-well microtiter plates, with eight replicate wells per dilution according to the method of Lladó et al. (2009). The total number of heterotrophs were counted in tryptone soy broth and the PAH-degrading bacteria were counted in a mineral medium containing a PAH mixture composed of PHE 0.5 g l^{-1} and FLU, ANT, PYR, at a final concentration of 0.05 g l^{-1} as the sole carbon sources. To avoid fungal contamination, cycloheximide at a final concentration of 100 mg l^{-1} was added to both growth media (i.e., tryptone soy broth and mineral medium). The MPN plates were incubated at room temperature for 30 days. Positive wells were detected by turbidity for heterotrophs, and the presence of a brownish/yellow coloration for PAH degrading bacteria. The MPN calculation was carried out using US EPA MPN Calculator v1.1 software.

2.7. DNA Extraction

Whole genomic DNA from each soil was extracted using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the universal bacterial 341-f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') and 534-r (5′-ATT ACC GCG GCT GCT GG-3′) primers with a length of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was amplified using the universal fungal primers FUN_NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and GC fung (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTAC CCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 μL of template DNA (ca. 20 ng), Tris–HCl (20 mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), Taq-

polymerase (0.1 IU) (NzyTech, Lisbon, Portugal), primers (0.2 mM of each) and double deionized water to bring the final volume up to 50 µL. For Bacterial amplification, the touchdown PCR program was performed in a *Primus* PCR thermo cycler (MWG biotech, Ebersberg, Germany) using the same procedure (Muyzer et al., 1993). For fungal amplification the PCR program previously reported by Das et al., (2007) was used. All amplicons (5 µL) were analyzed on agarose gel before being used for Denaturing Gradient Gel Electrophoresis (DGGE).

2.8. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The INGENY phorU DGGE system (Ingeny, Goes, NL) was used for sequence-specific separation of PCR amplified fragments. For PCR amplicons obtained with the 341f-GC and 534r primers, electrophoresis was performed in a polyacrylamide gel (8 % (w/v) acrylamide/bis-acrylamide gel 37.5:1), containing 40 – 60 % urea-formamide denaturing gradient (100 % corresponds to 7 M urea and 40 % (w/v) formamide). For fungal amplicons the urea-formamide denaturating gradient ranged from 20 to 35 %. After DGGE electrophoresis the gels were stained with Gel star solution (Lonza, Ltd group, USA) at room temperature for 45 min and photographed using a UV-transillumination table with a GelDoc XR digital camera (Bio-Rad, Carlsbad, CA). The fingerprinting profile obtained from DGGE was investigated using the Quantity one software (Bio-Rad). To characterize the community composition obtained by DGGE analyses, the Shannon diversity index H' was calculated as follows:

$$H' = - \Sigma (P_i \times \ln P_i)$$

where P_i represents the ratio of the single intensity band to the sum of the bands intensity of each lane, Richness (S) is the number of bands revealed, and Evenness (E) was calculated as H'/ln S.

3 Results and Discussion

3.1 Time- and Microcosm-Dependent Evolution of Fungal Biomass and Cultivable Bacteria

In the SM microcosm control the ergosterol content, a specific indicator of fungal biomass was invariably lower than the detection limits (Fig. 1A). Irrespective of the sampling time, no fungal growth was observed in the SM throughout the incubation period.

There were no significant ($p \leq 0.05$) time dependent changes in ergosterol in the SSAS microcosm which indicates that despite the presence of the sterilized *Agaricus* substrate, fungal growth was not stimulated at all. This result was unexpected because several studies have shown that the application of sterilized lignocellulose waste exerts a positive effect on the resident fungi in contaminated soils (Federici et al., 2012a, 2011; Lladó et al., 2013). Conversely, in the SAS microcosm an approximate three-fold increase in ergosterol content was observed in the first week of incubation. This was followed by a decline in the values which remained constant in subsequent harvests (Fig. 1A).

No changes in the ergosterol content were observed throughout the incubation in the Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement for the resident fungi. This could be explained by the fact that most of the available organic components in the sterilized SAS had been consumed by the reinoculated *A. bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient status. Therefore, the potential trophic contribution of the carrier to the competitive ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar, 1996).

The density of the cultivable heterotrophic and PAH-degrading bacteria over time is shown in Fig. 1B and 1C` respectively. Heterotrophic microbial counts in the SAS and SSAS microcosms increased by approximately two orders of magnitude and were significantly higher than those in the SM microcosm over the whole incubation period (Fig. 1B). The same result was also found in the bioaugmented Abisp microcosm where heterotrophic bacterial counts increased by more than one order of magnitude (Fig. 1B). In the amended microcosms, the lowest densities of heterotrophic bacteria throughout the incubation period were found in the Abisp microcosm. This may be explained by the low nutrient input associated with this inoculant. Its preparation had involved the reinoculation of *A. bisporus* into spent Agaricus substrate and subsequent incubation for 14 d prior to its application onto soil with ensuing consumption of organic nutrients by the growing fungus. This is also corroborated by the observed increase in heterotrophs in the other bioaugmented SAS microcosm where the addition of the inoculant ensured a higher nutrient input than in Abisp. Similar results were also found for the cultivable PAH-degrading bacteria. The highest densities were observed in the SSAS and SAS microcosms (Fig. 1C). However, it should be noted that the spent *Agaricus* substrate contained viable PAH-degrading bacteria which would have resulted in a higher initial density in the SAS microcosm than in the SM (2.3×10^4 vs. 0.5×10^2 MPN g^{-1}) (Fig. 1C). In the SSAS microcosm, the addition of the sterilized organic waste also exerted a high stimulation on the PAH-degrading bacteria which were increased by more than three orders of magnitude after one and three weeks of incubation, although after this their densities declined. As previously observed for heterotrophic bacterial counts in the amended remediation microcosms, the Abisp exhibited the lowest densities of PAH-degrading bacteria for most of the incubation period. Regardless of the different application options in addition to leading to a higher density of heterotrophs, when

compared with the SM, it also enhanced specialized ones. As described above, the non-sterile spent *Agaricus* substrate in the SAS microcosm contained allochthonous PAH-degrading bacteria. This is not surprising because a molecular characterization of the bacterial community in this substrate has revealed the presence of several genera *Paenibacillus*, *Arthrobacter*, *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004; Watabe et al., 2004) which included several species with reported PAH-degrading capacity (Haritash and Kaushik, 2009). However, the addition of the sterilized SAS, irrespective of whether it had been reinoculated with *A. bisporus* or not, also appeared to stimulate PAH-degrading bacteria. Previous studies have also found that the addition of sterilized organic wastes such as wheat straw and maize stalks activated the specialized resident bacterial populations in soils contaminated with PAH (Lladó et al., 2013), and polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested that the stimulatory effect exerted by organic waste is due to an enhancement of the oxygen transfer via an increase in soil porosity without necessarily involving either trophic factors or modifications in contaminant bioavailability (Federici et al., 2012b).

3.2 Microcosm-Dependent Impact on Bacterial and Fungal Community Structure

It is widely known that the cultivable microbiota in soil represent a minor fraction of the whole microbial community (Daniel, 2005). Therefore, an investigation into the structure of both the bacterial and fungal communities as a function of the remediation treatment necessitated a cultivation-independent approach which relied on DGGE. Table 3 shows the Shannon Weaver Index, which gives the richness and evenness values of bacterial and fungal communities in the microcosms at the start, and at the end of incubation. These values have been calculated by a numerical analysis of the DGGE profiles of PCR-amplified 16S and 18S rDNA fragments respectively. Both the richness

and the diversity of the bacterial and fungal communities in the SM did not significantly change during incubation. This result is not surprising because both communities presumably had a well established adaptation to the conditions exerted by the historical contamination of the soil.

As a result of the fungi and bacteria already present in the SAS microcosm which contained the unsterilized spent *Agaricus* substrate, higher initial richness values for bacterial and fungal biota were found than in those in the SM microcosm (18 *vs*. 11, respectively, and 23 *vs*. 17, respectively). In addition, the 63 day old SAS microcosms produced a significantly higher H' value for the bacterial community than that from the coeval SM (2.95±0.08 *vs*. 2.23±0.08, respectively), as well as a higher richness in the fungal biota (27 *vs*. 17, respectively) but without substantial differences in their respective evenness.

Conversely, the presence of the sterilized spent *Agaricus* substrate in the SSAS microcosm did not substantially modify the test parameters with respect to those found in the SM. In the 63 day old SSAS microcosm, significantly higher S and H' values for the bacterial community than those in the coeval SM were found, although no substantial differences were observed in their fungal biota. Regardless of the sampling time, similar results to those described for SSAS were found in the Abisp microcosm. Interestingly, the microcosms to which sterilized spent *Agaricus* substrate had been added, irrespective of whether they had been reinoculated with Agaricus or not, i.e. the SSAS and Abisp microcosms had the same impact on the fungi. They both failed to promote fungal growth throughout incubation (Fig. 1A) and/or to substantially change the richness and diversity of the fungal community when compared to the SM microcosm (Table 3).

3.3 PAH Removal in the Remediation Microcosms

Table 2 shows the initial and residual PAH concentrations in the remediation microcosms after 63 days incubation. In the SM, no significant reduction in the total PAH concentration was observed (p=0.07). Significant differences between the initial and final concentrations were only found for FLT and PYR, with a percentage removal of 65 %, and 59 % respectively. In the biostimulation SSAS microcosm a statistically significant decrease (p<0.05) in 3-ring PAH content, FLU (75 %), PHE (90 %), ANT (82 %), and FLT (52 %), was observed and the overall PAH residual content significantly differed from that at the start. Therefore, the biostimulation treatment was effective at degrading low molecular weight PAHs which were the most abundant contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp, significant differences between the initial and the residual concentrations were found for the majority of the individual contaminants as well as the overall PAH concentrations (Table 2). These findings indicate that the bioaugmentation treatments with *A. bisporus* were efficient at degrading both low and high molecular weight PAH.

A comparison of the degradation performances of SSAS, SAS and Abisp microcosms, revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp was superior to SSAS and SAS in the removal of highly condensed PAH (Table 2). Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC, 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds such as BaP and DBahA (52 vs. 18 % respectively, and 32 vs. 6 % respectively). In this respect, a very limited number of bacteria able to grow in pure cultures on either 5- or 6-ring PAH have been identified (Haritash and Kaushik, 2009).

However, in the SAS microcosm, the augmented fungus appeared to exert a lower inhibitory action on the resident bacterial biota as deduced by comparing its better

degradation performance on low molecular weight PAH and its higher density of PAH-degrading bacteria than those found in the Abisp microcosm. One of the possible synergistic mechanisms which might occur between bacteria and fungi, relies on the ability of the latter to convert PAH into more polar degradation intermediates, such as PAH diones and hydroxylated derivatives (Covino et al., 2010a).

Linear regression analyses were performed to relate the percentage removal of each PAH in the microcosm with respect to the chemical characteristics of these contaminants (Table 4). Therefore, the degradation results were related to those parameters which have been suggested to significantly affect PAH degradation, such as molecular weight (MW), organic carbon sorption coefficient (log K_{oc}), hydrophobicity (log P), water solubility (WS) and ionization potential (IP), (Table 4). These analyses showed that the degradation results were positively and significantly ($p< 0.05$) correlated with WS in all the microcosms tested. Conversely, with the exception of the Abisp microcosm, PAH degradation results were negatively correlated ($p < 0.01$) with their respective MW, log K_{oc} and log P values (Table 4). These results clearly indicate that the bioavailability of PAH contaminants strongly affect their ability to be degraded in the SM, SSAS and SAS microcosms, where the bacteria make an important contribution to PAH depletion. Bacterial uptake of PAH and their subsequent metabolism have been shown to be governed by the mass transfer rates of contaminants from the solid to the liquid phase of soil, which are in turn affected by the aforementioned physico-chemical properties (Haritash and Kaushik, 2009). The distinctive behavior of the Abisp microcosm, namely the lack of correlation between PAH degradation and MW, log K_{oc} and log P, might indicate that different PAH degradation mechanisms are involved here. In particular, the direct involvement of PAH oxidation by LME which was high during the initial incubation phases (See subsection

3.5) might be as result of their ability to diffuse into the soil matrix and potentially oxidise PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH. The IP has been shown to affect *in vitro* PAH oxidation by either laccase or fungal heme-peroxidases, whose activities were found in the bioaugmented microcosms (see subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the opposite trend was found when relating PAH depletions and respective IP values in all the microcosms. However, the Pearson Coefficients related to these regressions, which were significant with the exception of Abisp, were the lowest among the physico-chemical properties tested (Table 4). Similar findings were obtained with two historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus tigrinus* (Covino et al., 2010b). This clearly suggests that even in augmented microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH degradation. This was made particularly evident in the bioaugmented microcosms by the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV respectively) make them poorly susceptible to mono-electronic oxidation by LMEs (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi also relies on the intracellular cytochrome P-450/epoxide hydrolase complex whose activity is not dependent on IP (Haritash and Kaushik, 2009).

3.4 Time- and Microcosm-Dependent Modifications in the PAH Bioavailable Fraction

The bioavailable fraction of each PAH was determined in all the microcosms at the start and after 63 days incubation using HPCD extraction (Stokes et al., 2005). At the start

the different soil application options, i.e. SSAS, SAS and Abisp microcosms did not significantly affect the percentage bioavailable fraction of the large majority of contaminants in each microcosm (Table 5). The only exceptions were observed in some highly condensed PAH such as DBahA. The bioavailable fractions were lowest in the SAS and Abisp microcosms. BghiP showed the highest bioavailability in the SSAS and Abisp microcosms.

After 63 days incubation a microcosm-dependent modification of the bioavailable fraction of individual PAH was observed when compared to the beginning of the experiment. In particular, in the SM an increase in the bioavailable fraction was observed for high molecular weight PAH (i.e., DBahA, BghiP and IcdP). In the SSAS microcosm, the bioavailable fractions of BbF and DBahA increased while those of PHE and ANT decreased (Table 5). For the latter two compounds, it could be envisaged that their high degradation extents in this microcosm (90 and 82 %, respectively) left only the most recalcitrant fraction. In the bioaugmented microcosms (SAS and Abisp) a different scenario was found. In particular, in the SAS a decrease in the bioavailable fraction of PHE, FLT, PYR, CHR and BaP was observed. Conversely, in the Abisp microcosm, decreased bioavailabilities were found for FLU, PHE, BbF, BkF, BaP, BghiP and IcdP. The reason underlying the decrease in the bioavailable fraction of the majority of individual PAH was not solely due to their respective depletions since these parameters were not found to be correlated in all the microcosms. Therefore, the changes observed might have derived from a combination of PAH sorption onto organic matter and partial degradation. In this respect, the organic matter of spent *Agaricus* substrate analyzed by cross-polarization and magic angle spinning nuclear magnetic resonance, was found to contain a high percentage of aliphatic carbon (about 21 %) that

could contribute significantly to PAH sorption *via* hydrophobic interactions (García-Delgado et al., 2013a).

3.5 Lignin-Modifying Enzyme Activity and Residual Toxicity in Remediation Microcosms

The remediation microcosms were assayed for their ligninolytic activity due to the involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010; Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-peroxidase activity (Fig. 2A and 2B, respectively) were only detected in the bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity were found in the Abisp microcosms where the activity of the former enzyme markedly declined with incubation time (Fig. 2A). Noteworthy, in this microcosm the best depletions were observed for BaP and DBahA which despite being high molecular weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38 respectively) and therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar, 1995). In this regard the results of the present study are in agreement with those of Li et al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts from spent *Agaricus* substrate.

Dehydrogenase activity was used as a possible index of detoxification in the remediation microcosms as previously suggested for hydrocarbon-impacted soils (Dawson et al., 2007) in addition to the *F. candida* mortality test. In the present study, the adoption of this parameter did not provide an unequivocal clue to the detoxification by augmented microcosms. This was the result of a low dehydrogenase activity in the contaminated soil at the start (Table 6). The level of dehydrogenase activity was markedly and predictably boosted by the addition of viable fungal inocula in the SAS

and Abisp microcosms (Table 6). The retention of high levels of activity in the 63 day old SAS and Abisp microcosms, albeit being lower in the SAS and equal in the Abisp, indicate that the soil microbiota retained a high functional activity despite a prolonged incubation time.

Table 6 also shows that the initial toxicity of the microcosms towards *F. candida* was high, leading to mortalities that ranged from 77.5 to 89 %. Although a partial detoxification was observed in all microcosms, the best results were observed with SAS and Abisp where mortality was reduced by 45.1 and 41.4 %, respectively. In these microcosms, a generalized decrease in the bioavailable fractions of individual contaminants were observed together with the highest percentage removal of both 4-ring and highly condensed PAH. The lowest mortality reduction (26.8 %) was observed in SSAS where the lowest depletion of 4-ring PAH, and no depletion of highly condensed PAH were found. In this respect, the higher detoxification observed in Abisp than SSAS suggest that 3-ring PAH were less toxic to *F. candida* than 4-ring and 5,6-ring PAH. This might be explained by the fact that in the SSAS the former contaminant group was degraded at a significantly higher extent and, in the same microcosm, with the exception of PHE, their bioavailable fractions were comparatively lower than in Abisp.

A comparison of the 63 day old microcosms showed that the best reduction in the carcinogenic risk assessment (RCRA) was observed in the bioaugmented microcosms (SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and 5,6-ring PAH which have the highest carcinogenic properties (IARC 2010). Significant differences were also found when comparing the bioaugmented microcosms. The Abisp microcosm showed higher RCRA than the SAS because of its better efficacy in the removal of BaP and DBahA (Table 2) which exhibited the highest TEF values.

4 Conclusions

The addition of the sterilized spent *A. bisporus* substrate to the PAH-polluted soil was effective in stimulating the resident soil bacteria which resulted in higher levels of 3-ring PAH being removed. Bioaugmentation treatments with *A. bisporus*, in the SAS and Abisp microcosms, were more effective in removing 5, 6-ring PAH in particular BaP. The best detoxification results were obtained in the Absip microcosm where a high retention of microbiological functional activity, a significant decrease in *F. candida* mortality, and a reduction in carcinogenic risk assessment were observed. The wide spatio-temporal availability of this agro-waste combined with its proved efficacy in PAH biodegradation make its use technically feasible for environmental remediation purposes.

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Figure Captions

Figure 1: Time course of ergosterol concentrations (A), total heterotrophic (B) and PAH degrading (C) bacteria in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS), and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm (Tukey post-hoc test; $p \leq 0.05$), respectively.

Figure 2: Time courses of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean ± standard deviation of three replicated microcosms.

Table 1: Characteristics of the PAH-polluted soil and spent *Agaricus bisporus* substrate (SAS).*n*=3

Parameter	Soil	SAS
pH	8.20 ± 0.03	6.7 ± 0.3
Electronic conductivity (dS m^{-1})	0.58 ± 0.02	7.1 ± 0.8
Organic matter (%)	1.2 ± 0.1	61.9 ± 1.7
Carbonates (%)	30 ± 1	n.d.†
C/N ratio	n.p.§	7.96 ± 0.13
% C	n.p.	32.4 ± 0.02
% N	n.p.	4.07 ± 0.07
% H	n.p.	1.95 ± 0.06
% S	n.p.	0.92 ± 0.12

†n.d.: not detected; § n.p.: not performed

Table2

Click here to download Table: Table_2_PAH.docx

Table 2. Initial (t_o) and residual PAH concentrations after 63 d incubation (t_f) in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp). Data are mean±standard deviation of 3 replicated microcosms; the asterisk denotes significant differences between the initial and residual concentrations for each microcosm (ANOVA, p <0.05).

Contaminant	Toxic Equivalency Factor†	Contaminant concentration (mg kg^{-1}) in							
		SM		SSAS		SAS		Abisp	
		t_o	t_f	t_o	t_f	t_o	t_f	t_o	t_f
FLU	0.001	6.11±3.78	2.96±1.77	3.47±0.64	0.859±0.159*	3.86±1.13	1.27±0.29*	2.54±0.57	1.38±0.16*
PHE	0.001	16.8±5.9	6.30±3.80	13.3±2.1	1.39±0.43*	12.1±3.9	2.39±0.52*	10.2±1.6	4.62±0.43*
ANT	0.01	23.7±10.3	13.8±7.6	20.7±7.1	3.69±1.19*	20.1±3.1	8.95±3.9*	14.8±2.2	16.6±1.2
FLT	0.001	21.7±2.6	7.59±1.39*	16.7±0.5	8.05±4.08*	16.5±1.5	5.11±0.35*	1.60±1.9	8.73±4.08*
PYR	0.001	10.1±1.1	4.11±0.25*	8.02±0.04	4.84±2.68	8.46±0.96	2.60±0.15*	7.42±1.0	3.70±2.68*
BaA	0.1	4.35±0.77	2.61±0.82	3.21±0.04	2.72±1.16	3.68±0.35	2.16±0.31*	3.50±0.39	1.81±1.16*
CHR	0.01	11.8±5.0	9.55±4.61	8.01±1.22	8.99±3.84	8.61±0.75	5.97±1.1*	8.25±0.46	5.49±3.84*
BbF	0.1	5.94±0.59	5.45±0.18	4.81±0.09	5.40±1.04	5.35±0.23	4.42±0.21*	5.09±0.37	4.26±1.04*
BkF	0.1	2.42±0.22	2.09±0.08	1.94±0.02	2.03±0.42	2.19±0.10	1.73±0.13*	2.05±0.16	1.52±0.42*
BaP	1	2.91±0.27	2.91±0.05	2.13±0.03	3.02±.059	2.78±0.08	2.29±0.13*	2.59±0.22	1.26±0.59*
DBhaA	5	0.755±0.071	0.853±0.047	0.576±0.022	0.782±1.16	0.676±0.062	0.643±0.066	0.683±0.022	0.466±0.162*
BghiP	0.01	2.51±0.17	2.54±0.03	2.03±0.06	2.31±0.41	2.33±0.06	1.99±0.05*	2.23±0.11	1.79±0.41*
IcdP	0.1	2.18±0.18	2.15±0.04	1.75±0.02	2.03±0.29	1.98±0.04	1.68±0.02*	1.87±0.10	1.47±0.29*
Σ3rings		43.6±19.8	23.1±13.1	37.5±9.8	5.94±1.75*	36.1±8.0	12.6±4.5*	46.2±9.8	22.6±1.7
Σ4rings		48.0±8.3	23.9±7.0*	36.0±1.2	24.6±10.1	37.3±3.3	15.8±1.8*	35.1±1.2	19.7±10.1*
Σ5-6rings		16.7±1.5	16.0±0.1	13.2±0.2	15.6±2.9	15.3±0.6	12.8±0.55*	14.5±0.18	10.8±2.9*
ΣPAH		111±29	63.0±15.4	86.7±10.2	46.1±13.7*	88.6±4.7	41.2±6.6*	82.4±10.2	53.1±13.7*

† Nisbet and LaGoy (1992)

Table 3: Shannon Weaver Index (H'), richness (S) and evenness (E) values of bacterial and fungal communities at start (t_0) and at the end (t_{63}) of the incubation in the non amended soil microcosm (SM), amended with sterilized spent *Agaricus* substrate (SSAS), spent *Agaricus* substrate (SAS) and sterilized spent *Agaricus* substrate reinoculated with the fungus (Abisp).

Microcosms	Bacteria			Fungi		
	H'	S	E	H'	S	E
SM t_0	2.27 ± 0.08^{Aa}	11±0	0.95 ± 0.03^{Aa}	2.61 ± 0.02^{Ba}	17±1	0.91 ± 0.00^{Ca}
SM t_{63}	2.23 ± 0.03^{Aa}	10±0	0.97 ± 0.01^{Ba}	2.59 ± 0.04^{Ba}	19±1	0.96 ± 0.12^{Aa}
SAS t_0	2.64 ± 0.08^{Ba}	18±1	0.90 ± 0.04^{Aa}	2.55 ± 0.13^{ABa}	23±3	0.82 ± 0.01^{Ba}
SAS t_{63}	2.95 ± 0.08^{Ca}	27±1	0.89 ± 0.01^{Aa}	2.74 ± 0.03^{Ba}	27±0	0.83 ± 0.01^{Aa}
SSAS t_0	2.42 ± 0.03^{Aa}	12±1	0.97 ± 0.02^{Aa}	2.25 ± 0.10^{Aa}	15±1	0.83 ± 0.01^{Ba}
SSAS t_{63}	2.86 ± 0.04^{BCb}	21±1	0.95 ± 0.01^{Ba}	2.66 ± 0.07^{Bb}	25±2	0.83 ± 0.00^{Aa}
Abisp t_0	2.41 ± 0.02^{Aa}	12±1	0.96 ± 0.00^{Aa}	2.09 ± 0.01^{Aa}	15±0	0.77 ± 0.00^{Aa}
Abisp t_{63}	2.63 ± 0.11^{Bb}	16±2	0.94 ± 0.01^{Ba}	2.41 ± 0.02^{Ab}	20±0	0.81 ± 0.03^{Aa}

*Data are the mean ± standard deviation of 3 independent microcosms. Multiple pair-wise comparisons were performed by the Fisher LSD test ($p\leq0.05$): same uppercase and lowercase letters denote the absence of statistically significant differences between different microcosms at same time of treatment and between the same microcosm at different time, respectively.

Table 4: Pearson product moment correlation coefficients between percent PAH removal in non amended soil microcosm (SM) or amended with either sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp) and respective PAH physico-chemical properties including molecular weight (MW), organic carbon adsorption coefficient (log K_{oc}), hydrophobicity (log P), water solubility (WS) and ionization potential (IP).

Microcosm	MW	log K_{oc}[§]	log P[§]	WS[§]	IP†
SM	-0.720**	-0.705**	-0.688**	0.402*	0.339*
SSAS	-0.896**	-0.895**	-0.872**	0.582*	0.490**
SAS	-0.848**	-0.839**	-0.813**	0.546**	0.468**
Abisp	n.s.	n.s.	n.s.	0.327*	n.s.

[§]These parameters were calculated using the Advanced Chemistry Development v. 11.02 software package (ACD/Labs, Toronto, Canada) available from the on-line SciFinder chemical database (American Chemical Society, Columbus, OH); †From Covino et al., 2010a; n.s., no significant correlation; * significant correlation at $p < 0.05$: ** significant correlation at $p < 0.01$.

Table 5: Percent bioavailable PAH fraction at start (t_0) and at the end (t_{63}) of the incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent Agaricus substrate (SSAS) or spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp).

PAH	Percent bioavailable PAH fraction† in							
	SM		SSAS		SAS		Abisp	
	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}	t_0	t_{63}
FLU	92	98^{A}	94	99^{A}	99	96^{A*}	97	86^{B*}
PHE	75	83^{A}	87	0^{D*}	89	57^{C*}	88	68^{B*}
ANT	86	93^{A}	82	29^{B*}	90	86^{A}	79	90^{A}
FLT	71	69^{A}	71	59^{AB}	74	39^{B*}	76	58^{AB}
PYR	17	0	7	4	14	0^{*}	3	0
BaA	0	2	8	4	0	0	5	3
CHR	43	61^{A}	64	68^{A}	58	30^{B*}	56	44^{AB}
BbF	5	19^{B}	7	33^{A*}	0	0^{C}	13	0^{C*}
BkF	23	30^{B}	46	44^{A}	10	1^{C}	14	1^{C*}
BaP	10	25^{B}	45	52^{A}	16	0^{C*}	20	0^{C*}
DBahA	28^{a}	54^{B*}	33^{a}	64^{A*}	0^{b}	34^{C}	2^{b}	16^{D}
BghiP	0^{b}	21^{A*}	24^{a}	20^{A}	0^{b}	0^{B}	12^{a}	0^{B*}
IcdP	0	14^{A*}	30	24^{A}	0	0^{B}	8	0^{B*}

† Data are the mean of three independent microcosms. Different lowercase letters indicate that differences between microcosms at start (t_0) were significant (Tukey post-hoc test, $p < 0.05$). Different uppercase letters indicate that differences between 63-d-old microcosms (t_{63}) sampling time were significant (Tukey post-hoc test, $p < 0.05$). The asterisk * denotes significant differences between sampling times within the same microcosm (ANOVA, $p < 0.05$).

Table 6: Reduction of carcinogenic risk assessment (RCRA) and dehydrogenase activity and *F. candida* mortality at start (T_0) and after 63 d (T_f) incubation in non amended soil microcosm (SM) or the same soil added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) or sterilized spent Agaricus substrate reinoculated with the fungus (Abisp).

Microcosm	RCRA‡ (%)	Dehydrogenase activity† (IU kg^{-1})		*F. candida* mortality† (%)	
	T_f	T_0	T_f	T_0	T_f
SM	5.3±2.2AB	0.011±0.004Aa	0.041±0.003Ab	89.5±2.5Bb	60.6±3.2Ca
SSAS	4.5±0.6A	0.106±0.006Aa	0.223±0.075Ab	77.5±4.1Ab	58.7±2.1BCa
SAS	15.3±6.3B	0.598±0.140Cb	0.195±0.006Aa	89.6±3.8Bb	49.2±3.1Aa
Abisp	37.6±8.6C	0.336±0.060Ba	0.499±0.162Ba	85.5±4.1Bb	50.1±2.6Aa

† Data are the mean± standard deviation of 3 independent microcosms; same lowercase and uppercase letters denote the absence of statistically significant differences between column and row means, respectively, as assessed by the Tukey post-hoc test ($P< 0.05$).
‡ Reduction of carcinogenic risk assessment expressed as percentage was based on toxic equivalency factors proposed by Nisbet and LaGoy (1992).

Figure 1

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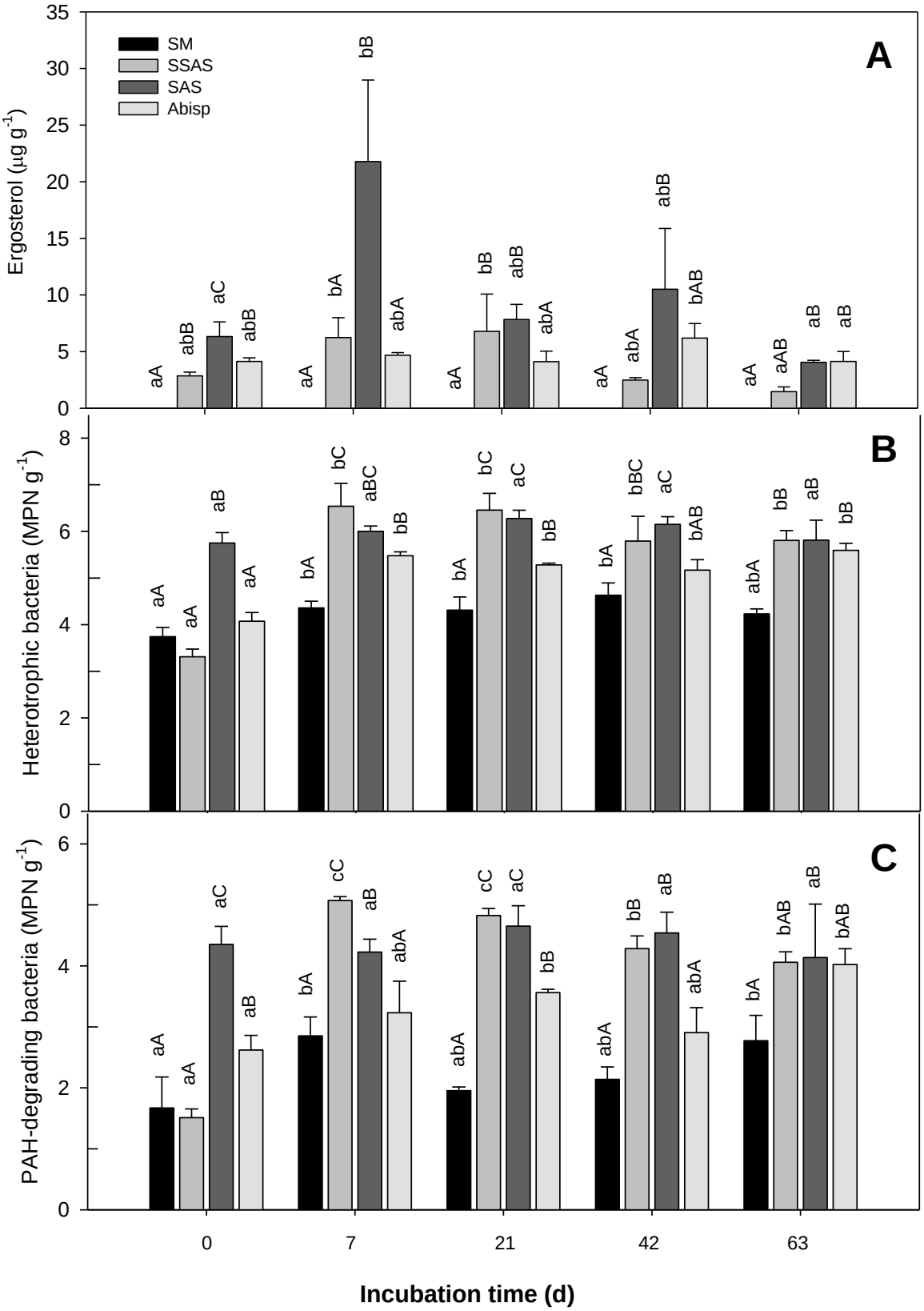

Figure 2

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