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**Bioremediation of multi-polluted soil by spent mushroom (*Agaricus bisporus*)**

**substrate: polycyclic aromatic hydrocarbons degradation and Pb availability**

Carlos García-Delgado, Felipe Yunta, and Enrique Eymar\*.

Department of Agricultural Chemistry and Food Sciences, University Autónoma of Madrid, 28049 Madrid, Spain.

**\*Corresponding author:**

Enrique Eymar.

Department of Agricultural Chemistry and Food Sciences, University Autónoma of Madrid, Madrid, 28049, Spain.

E-mail: [enrique.eymar@uam.es](mailto:enrique.eymar@uam.es)

Phone: +0034914975010

Fax: +0034914973826

**E-mail address of each author:**

Carlos García-Delgado: [carlos.garciadelgado@uam.es](mailto:carlos.garciadelgado@uam.es)

Felipe Yunta: [felipe.yunta@uam.es](mailto:felipe.yunta@uam.es)

Enrique Eymar: [enrique.eymar@uam.es](mailto:enrique.eymar@uam.es)

**Abbreviations:** SAS, Spent *Agaricus bisporus* substrate; PAH, Polycyclic aromatic hydrocarbons; LDF, lignin-degrading fungi; SM, soil microcosm; SSAS, soil microcosms amended with sterilized SAS; Abisp, bioaugmented microcosms with *A. bisporus*; d, day; Ace, acenaphthene; Flu, fluorene; Phe, phenanthrene; Ant, anthracene; Fla, fluoranthene; Py, pyrene; BaA, benzo[a]anthracene; Chr, chrysene; BbF, benzo[b]fluoranthene; BkF, benzo[k]fluoranthene; BaP, benzo[a]pyrene, DBaA, dibenzo[a,h]anthracene; BghiP, benzo[g,h,i]perylene; IcdP, indeno[1,2,3-c,d]pyrene; HMW, High Molecular Weight; LME, Lignin-modifying enzymes; MnP, Manganese dependent peroxidase; IU, unit of enzymatic activity; MPN, most probable number, THA, total hydrolase activity; DHA, Dehydrogenase activity; TEF, toxic equivalency factors; RCRA, reduction in carcinogenic risk assessment; SEM, scanning electron microscopy.

## Abstract

This study investigates the effect of three spent *Agaricus bisporus* substrate (SAS) application methods on bioremediation of soil multi-polluted with Pb and PAH from close to a firing range with respect natural attenuation (SM). The remediation treatments involve i) use of sterilized SAS to biostimulate the inherent soil microbiota (SSAS) and two bioaugmentation possibilities ii) its use without previous treatment to inoculate *A. bisporus* and inherent microbiota (SAS) or iii) SAS sterilization and further *A. bisporus* re-inoculation (Abisp). The efficiency of each bioremediation microcosm was evaluated by: fungal activity, heterotrophic and PAH-degrading bacterial population, PAH removal, Pb mobility and soil eco-toxicity. Biostimulation of the native soil microbiology (SSAS) achieved similar levels of PAH biodegradation as SM and poor soil detoxification. Bioaugmented microcosms produced higher PAH removal and eco-toxicity reduction via different routes. SAS increased the PAH-degrading bacterial population, but lowered fungal activity. Abisp was a good inoculum carrier for *A. bisporus* exhibiting high levels of ligninolytic activity, the total and PAH-degrading bacteria population increased with incubation time. The three SAS applications produced slight Pb mobilization (<0.3%). SAS sterilization and further *A. bisporus* re-inoculation (Abisp) proved the best application method to remove PAH, mainly BaP, and detoxify the multi-polluted soil.

**Keywords:** polycyclic aromatic hydrocarbons, heavy metals, ligninolytic enzymes, biodegradation, agricultural waste.

## Introduction

Multi-polluted soils with both inorganic and organic pollutants are a growing global problem [1]. However, many studies on soil bioremediation focus their attention onto either organic or inorganic pollutants individually and they do not delve into the interaction between both types of pollutants and the organisms, or the materials that are involved in the technique applied. In fact, a paucity of information on the bioremediation of organic pollutants in co-contaminated environments exists [2].

Pb and polycyclic aromatic hydrocarbons (PAH) such as inorganic and organic compounds are two of the most widespread pollutants throughout the world. Both, Pb and PAH, show toxic and even carcinogenic properties [3,4] and are listed as priority pollutants by the EU and US-EPA. They are found in multiple polluted soils such as those in areas surrounding industrial plants [5,6], high-ways [7], manufacturing gas plants [8] or firing ranges [9] amongst others. Firing range soils typically contain high Pb concentrations because Pb is the most common material used in ammunition. Sorvari et al. [10] reported that in Finland about 10% of the total number of potentially polluted places corresponded to firing ranges. These places have been widely studied with respect to Pb remediation. However, there is a lack of remediation experiments which address the problem of PAH biodegradation and Pb mobility, simultaneously.

The bioremediation of multi-polluted soils is not an easy task [8] because the technique applied can produce contradictory effects on the mobilization or immobilization of different pollutants [6] and heavy metals often reduce the degradation of organic pollutants by microorganisms [2,11–13]. PAH biodegradation by lignin-degrading fungi (LDF) has shown great potential because LDF segregate extracellular ligninolytic enzymes that have low substrate specificity, and are able to diffuse into the soil matrix and potentially oxidize PAH beyond their respective bioavailable quantity in soil [14].

However, the presence of high concentrations of metals interferes with the soil colonization [13] and ligninolytic enzymes activities [15]. This technology necessitates the use of inoculum carriers that are organic materials such as wheat straw, maize stalks or mushroom substrates [16–19]. The addition of organic materials modifies the pH, the organic matter content and other soil properties, and therefore affects metal availability [20]. Nevertheless, metal inhibition of biodegradation is commonly related to the total metal concentration in a system and this may not be the most appropriate predictor of metal toxicity [8].

Previous work carried out by our research group [15,18,21] and others [22–24] has demonstrated the ability of the fungus *Agaricus bisporus* and spent *A. bisporus* substrate (SAS) and its inherent microbiota to biodegrade PAH and other organic pollutants, and also that the SAS has an effect on heavy metal mobilization/immobilization. In addition, the reuse of SAS can contribute to a reduction in its adverse environmental impact in mushroom production areas.

The aim of this study is to assess the efficiency of three different application methods for spent *A. bisporus* substrate, i.e as an organic amendment, a supplier of exogenous complex microbiota or an inoculum carrier for *A. bisporus*, to bioremediate a multi-polluted soil with PAH and Pb, close to firing range with respect to natural attenuation. To determine the efficiency of each bioremediation treatment, the fungal activity, the populations of heterotrophic and PAH-degrading bacteria, PAH removal, Pb mobility and soil eco-toxicity were evaluated.

## **Materials and methods**

### **2.1 Materials**

Multi-polluted soil with PAH and Pb was collected next to a shooting range located in Madrid (Spain) 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 *A. bisporus* substrate was originally produced from wheat straw and poultry litter. After composting for 21d gypsum was added. Following the development of the *A. bisporus* mycelium throughout the compost, a layer of black peat is placed on top as a casing layer. The SAS was collected from a production plant located in Quintanar del Rey (Cuenca, Spain). The main soil and SAS characteristics are shown in Table 1.

## 2.2 Isolation of the fungus and preparation of the inocula

*A. bisporus* was isolated from SAS using the following procedure: the hyphae were washed with sterilized water and inoculated onto rose bengal chloramphenicol agar (Sigma-Aldrich, Spain). After 7 days (d) incubation at 20°C, the mycelium was sub-cultured onto malt extract agar for 10d at 20°C.

Two *A. bisporus* inocula formulations were used. One consisted of the SAS without any treatment. This inoculum contained *A. bisporus* and the inherent microorganisms with the ability to degrade PAH [18]. The second inoculum used consisted of SAS which had been sterilized in an autoclave at 121°C, for 30 min, and inoculated with 3, 1cm diameter agar plugs taken from a 10d-old culture of *A. bisporus* grown on malt extract agar and incubated for 7d at 20°C.

## 2.3 Preparation of the remediation microcosms

Regardless of the microcosm type, all experiments were carried out in 1l glass reactors using 50g (dry mass) unsterilized contaminated soil. The three microcosms amended with SAS were carried out using a 4:1 (soil:SAS) mass ratio. The soil moisture content

in all treatments was adjusted to 70% of its water holding capacity prior to the beginning of incubation. The microcosms were designed in order to simulate:

- Natural attenuation: the preparation of the soil microcosm (SM) simply involved the adjustment of the soil moisture content prior to the beginning of the incubation.
- Biostimulation: The polluted soil was amended with sterilized SAS (121°C, 30 min) to yield the SSAS microcosm. This approach was intended to assess the stimulatory effect of a sterilized organic waste on the resident soil microbiota and the effect of the SAS material on Pb availability, without SAS microorganism.
- Bioaugmentation SAS: the polluted soil was amended with SAS without previous treatment to yield the SAS microcosm. This approach was aimed at assessing the combined effect of both *A. bisporus* and the inherent SAS microbiota on PAH degradation and Pb availability.
- Bioaugmentation Abisp: to prepare this microcosm, sterilized SAS was re-inoculated with *A. bisporus* as described above. The colonized matrix was mixed with the contaminated soil. This bioaugmentation approach, termed the Abisp microcosm, was aimed at determining the effect of *A. bisporus* on PAH biodegradation and Pb availability.

Each microcosm was carried out in triplicate and incubated at 20°C for 0, 28 and 63d in the darkness.

### 2.3 Extraction and analysis of PAH.

Total PAH was extracted and analyzed using HPLC-PDA as described in the method of García-Delgado et al. [25]. This procedure assures mean PAH recovery percentages of

103% from CRM141 reference soil. Fourteen of the 16 US-EPA PAH were detected and quantified: acenaphthene, (Ace), fluorene, (Flu) phenanthrene, (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Py), 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[1,2,3-c,d]pyrene (IcdP).

#### 2.4 Determination of heavy metals pseudo-total content and available Pb

The Pb, Mn, Cu, Zn and Cd pseudo-total content of the soil and SAS were extracted by microwave-assisted digestion with aqua regia according to the method of García-Delgado et al. [26] which assures metal recoveries between 86 and 96%. The bioavailable Pb fraction was determined by extracting with  $\text{CaCl}_2$  0.01M (1:5, w:v) [27,28]. Heavy metal analyses were carried out using an ICP-MS (NexION 300 Perkin-Elmer).

#### 2.5 Enumeration of cultivable heterotrophic and PAH-degrading bacteria

Soil bacterial counts were performed using a miniaturized most probable number (MPN) method in 96-well microtitre plates, with eight replicate wells per dilution according to Lladó et al. [29]. To prevent fungal development, cycloheximide at 100 mg  $\text{l}^{-1}$  final concentration was added to both growth media. The MPN calculation was carried out using US-EPA MPN Calculator v1.1 software.

#### 2.6 Biochemical determinations and eco-toxicity tests

Lignin-modifying enzymes (LME) were extracted from bioremediation microcosms (3g) at 5°C for 1h using the buffered solution described by D'Annibale et al. [30]. The resultant aqueous suspension was centrifuged (6000g, 30min) and the supernatant assayed for LME activities.

Laccase activity was spectrophotometrically determined 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 ( $\epsilon=36000 \text{ M}^{-1} \text{ cm}^{-1}$ ). MnP activity was assayed by the oxidation of 1 mM  $\text{MnSO}_4$  in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM  $\text{H}_2\text{O}_2$  ( $\epsilon=11590 \text{ M}^{-1} \text{ cm}^{-1}$ ) [31]. One unit of enzyme activity (IU) is defined as the amount of enzyme which produces 1  $\mu\text{mol}$  of product per minute under the assay conditions.

Eco-toxicological assessment of remediation microcosms was carried out using two soil health parameters: total hydrolase activity (THA) by hydrolysis of fluorescein diacetate [32] and dehydrogenase activity (DHA) with 2,3,5-triphenyltetrazolium chloride [18].

The percentage reduction in carcinogenic risk assessment (RCRA) of the PAH present in the soil was based on the Nisbet and LaGoy [33] toxic equivalency factors of PAH for environmental exposure and was determined as follows:

$$RCRA(\%) = \frac{\sum_{i=1}^{13} [PAH_i]_{t_0} \cdot DR \cdot TEF_i}{\sum_{i=1}^{13} [PAH_i]_{t_0} \cdot TEF_i} \cdot 100$$

where  $[PAH_i]_{t_0}$  is the initial concentration of PAH, DR and TEF the degradation rate and toxic equivalency factor (Table 2).

## 2.7. Scanning electron microscopy (SEM) and X-ray diffraction (XRD) of *A. bisporus* hyphae

Hyphae of *A. bisporus* collected from Abisp microcosm after 63d incubation were dried at 60°C for 24h. The dry hyphae were gold-coated and observed using a Philips XL30 environmental scanning electron microscope-field emission gun. The mineralogical characterization of hyphae was carried out using XRD (Panalytical X'Pert Pro, Almelo). The configuration of this apparatus was  $\theta/2\theta$  with an X'Celerator detector. Diffraction

profiles were analyzed using X'Pert High Score Plus software and PDF-4 of the International Center for Diffraction Data for crystalline identification.

## 2.8 Statistical analysis

All statistical tests were carried out using the IBM SPSS Statistics v22 software package. One-way analysis of variance was carried out after previously performing a Levene variance homogeneity test. To compare the differences between treatments, the Tukey or Games–Howell *post hoc* test (according to variance homogeneity) at  $p < 0.05$  was used.

## 3 Results

### 3.1 Fungal activity

SM and SSAS did not develop filamentous fungi during the assay. The soil colonization by *A. bisporus* in the Abisp microcosm was completed from day 7 of incubation until the end of the assay (63d), however colonization in SAS microcosms was not completed and hyphae were not observed at day 63 of incubation.

The fungal activity in the microcosms was determined by analysis of LME activities (Fig. 1) because of their involvement in the early steps of PAH degradation process. Lignin-peroxidase and Mn-independent peroxidase were not detected in any of the microcosms. Laccase was detected in all the microcosms, but MnP was only detected in the amended microcosms. Abisp showed the highest laccase and MnP activities but declined during the incubation period.

### 3.2 Heterotrophic and PAH-degrading bacteria population

The densities of heterotrophic and PAH-degrading bacteria are shown in Fig. 2A and B respectively. The SM developed the lowest heterotrophic and PAH-degrading bacteria populations and no bacterial growth was detected during the assay. The SSAS

microcosm increased the heterotrophic bacterial population by one order of magnitude at 28 and 63d. The SSAS also highly stimulated the PAH-degrading bacterial population by about two orders of magnitude during the same time period. The SAS microcosms showed the highest initial heterotrophic and PAH-degrading bacterial populations which was to be expected as a result of the inherent SAS microbial population. The heterotrophic bacterial population increased by more than 3 orders of magnitude with respect to SM, and the PAH-degrading population by more than 2 orders of magnitude. From this we can infer that SAS contains an inherent PAH-degrading bacterial population. The heterotrophic bacterial population decreased significantly from the initial time ( $1.81 \times 10^8$  MPN g<sup>-1</sup>) to ( $5.75$  and  $7.08 \times 10^6$  MPN g<sup>-1</sup>) following 28 and 63d incubation. A similar trend was found in the density of PAH-degrading bacteria after 28d incubation, but then the population returned to its initial values. The Abisp microcosm showed a gradual increase in the numbers of heterotrophic bacteria and achieved the highest population, ( $1.29 \times 10^7$  MPN g<sup>-1</sup>) at 63d. The density of the PAH-degrading bacteria did not show any significant differences between the initial density and that at the end of the assay.

At the final sampling time (63d), no significant differences were found between the heterotrophic and PAH-degrading bacteria populations in the amended microcosms but lower densities were recorded in the SM.

### 3.3 PAH removal

The initial PAH concentration of the soil, and the degradation rates for each microcosm are shown in Table 2. The total PAH concentration of the initial soil was  $757 \pm 10$  mg kg<sup>-1</sup>. Grouped by rings, the most abundant were the  $\Sigma 5,6$ -rings (49%) followed by the  $\Sigma 4$ -rings (40%). Therefore, the PAH pollution was mainly caused by high molecular weight PAH (HMW-PAH).

The SM and SSAS microcosms showed a degradation of 27 and 11% respectively for  $\Sigma 3$ -rings PAH. However, it did not achieve a significant degradation for the total or HMW-PAH. The SAS microcosm was the second most efficient. The total PAH removal achieved was 17%. The most degradable compounds were Flu (44%) and BaP (29%). No significant differences were found with respect to the efficiency of PAH removal based on the number of rings. The bioaugmented Abisp microcosms achieved the best PAH degradation rates for total (29%) and individual PAH (21–100%). The most degradable compounds were Flu (100%) and BaP (39%) as was previously observed in SAS microcosm. The microcosms bioaugmented with *A. bisporus* (SAS and Abisp) were the most efficient remediation treatments for PAH degradation and, especially for HMW-PAH, the most abundant PAH in this soil. However, the degradation rate for  $\Sigma 4$ -rings,  $\Sigma 5,6$ -rings and  $\Sigma$ PAH was significantly higher in the Abisp than in the SAS microcosms.

### 3.4 Pb availability

Pb availability and soil pH were monitored at 0, 28 and 63d incubation (Table 3). The SM did not show variations in either pH or Pb availability during the assay. Nevertheless, significant changes in pH and Pb availability were observed in the amended microcosms during the time assayed.

The SAS microcosms, where the organic amendment had not been sterilized, showed an increase in the available Pb with respect to the SM but did not produce any change in  $\text{pH}_{\text{H}_2\text{O}}$  at day 0. During the assay, the SAS produced a slight but significant, acidification of the microcosms together a reduction in the available Pb ( $R=0.739$ ,  $p<0.05$ ).

In the SSAS and Abisp treatments in which the substrate had been sterilized by autoclaving, there was a significant acidification of the microcosms with the Abisp

treatment producing a lower pH. The Pb availability increased in both microcosms at the initial time with respect to SM and reached 1‰ of the total Pb. The Pb in the SSAS decreased during the assay until values similar to those of SAS at 28 and 63d. The Pb availability in Abisp increased 10-fold at 28d with respect to SSAS to achieve the highest value (2.5‰) but at day 63 this value decreased and at the same time the pH increased to 8.61. In fact, the  $\text{pH}_{\text{CaCl}_2}$  and available Pb showed a correlation ( $R=-0.788$ ,  $p<0.05$ ) for this microcosm.

The global effect of Pb availability at the end of the incubation period was a minimal increase in the amended treatments (0.1–0.4‰) with respect to SM.

### 3.5 Assessment of soil eco-toxicity

Fig. 3 shows the results of the soil eco-toxicity tests. At day 0, SM and SSAS showed lower THA than the bioaugmented microcosms (SAS and Abisp). At day 28 Abisp and SSAS retained the initial THA but in SAS this activity was reduced dramatically (76%). By contrast SM showed an increase in THA and reached similar values to those of SSAS and SAS. At the end of the assay, after 63d incubation, the SM showed the lowest values. The THA increased in SSAS and it achieved similar values to those recorded in SAS. Finally, Abisp maintained the high HTA values at 0 and 28d.

DHA showed a significant decrease during the assay in the amended microcosms (SSAS, SAS and Abisp). By contrast SM presented higher DHA values at 28 and 63d incubation. At 28d, the SSAS microcosms achieved the highest DHA followed by Abisp. SAS reduced DHA by approximately 70% from 0 to 28d and then retained the activity at 63d. The drastic reduction in THA and DHA from the initial value to 28 and 63d incubation in the SAS microcosm, and the positive correlation with the reduction in the heterotrophic bacterial population ( $R=0.964$  and  $0.966$  respectively,  $p<0.01$ ) offers clear evidence that soil polluted by Pb and PAH, impacted negatively on microbial

activity and population. The Abisp showed a continuous decrease in DHA from 0 to 63d. At the end of the assay SSAS and SAS showed higher DHA than either SM or Abisp which had similar DHA values.

The reduction in the carcinogenic risk assessments (RCRA, Table 2) shows very low values for the SM and SSAS microcosms. The bioaugmented treatments, SAS and Abisp, were the most effective remediation strategies for PAH detoxification. With regard to PAH removal and RCRA, Abisp was the most efficient bioremediation treatment in reducing the soil eco-toxicity associated to PAH pollution.

#### **4 Discussion**

This article reports the results of natural attenuation and three different SAS applications that involve one biostimulation, and two bioaugmentation strategies, to remediate a multi-polluted soil close to a firing range. With regard to the Spanish [34] and Autonomous Community of Madrid [35] legislation for ‘other’ soil uses, i.e. not urban or industrial, this soil exceeded the maximum reference levels for Pb ( $75 \text{ mg kg}^{-1}$ ) and 9 PAH (Table 2), including BaP, which has proven carcinogenic properties, and which exceed the reference level by 4655 times. Therefore, this soil can be classified as heavily polluted.

The microcosms that simulate natural attenuation (SM) achieved a low level of PAH biodegradation as a result of a low heterotrophic and PAH-degrading bacteria population and fungal activity. The biodegradation of  $\Sigma 3$ -ring PAH showed similar removal rates to the bioaugmented microcosms (SAS and Abisp). Nevertheless,  $\Sigma 3$ -ring PAH represents only 11% of the total initial PAH concentration, and according to IARC [3], these compounds do not possess carcinogenic properties. By contrast, PAH which are carcinogenic (PAH with 4,5,6-rings) and mainly BaP achieved biodegradation rates below 5%. Therefore, soil detoxification resulting from PAH degradation was minimum

as can be observed in the eco-toxicity tests (Fig. 3) and RCRA (Table 2). The lowest Pb availability for all the sampling times (Table 3) in SM was due to the soil characteristics, i.e. high pH and 3% organic matter which is one of the most important factors in the control of Pb mobility [36,37].

The biostimulated microcosm (SSAS) that involved the sterilization of SAS, produced an increment in the heterotrophic and PAH-degrading bacterial population which was to be expected as a result of the organic matter added to soil. However, PAH removal was unappreciable. This trend could be explained by two possible factors. Firstly, the organic matter of SAS contains a high percentage of aliphatic carbon (about 21%) which could contribute significantly to PAH sorption via hydrophobic interactions [15] and therefore minimize the bacterial biodegradation and probably hinder the organic matter colonization. Secondly, changes in soil chemistry due to the addition of sterilized SAS (SSAS) immediately produced a strong reduction in pH and slight Pb mobilization (0.98‰). The sterilization of organic matter by hydrothermal treatment incremented soluble organic matter and produced LMW-organic acids such as acetic acid [38] which decreased the pH and could act as metal ligands. The available Pb was negatively correlated with the heterotrophic ( $R=-0.887$   $p<0.01$ ) and PAH-degrading ( $R=-0.775$   $p<0.05$ ) bacterial population in the SSAS microcosms and could be a factor in the inhibition of PAH degradation as discussed by Olaniran et al. [2]. The reduction in Pb availability during the assay was probably related to microbial consumption of the labile organic matter. de Santiago-Martín et al. [36] found a positive relationship between labile organic matter and Pb availability in lettuce and a negative relationship with the recalcitrant organic fraction.

The bioaugmented microcosms (SAS and Abisp) showed different patterns with respect to PAH removal and Pb mobility due to the different microorganisms inoculated and

organic amendment manipulation. The SAS microcosms involved the use of SAS without any treatment and produced bioaugmentation with *A. bisporus* and the inherent microbiota with proven PAH degradation capacity [15,18]. The other bioaugmented microcosms (Abisp) comprised sterilized SAS which had been reinoculated with *A. bisporus*. The immediate effects of the two bioaugmentation strategies were a very large heterotrophic and PAH-degrading bacterial population in the SAS microcosm, and in the Abisp microcosm very high ligninolytic activity, both treatments produced light Pb mobilization. As result, the Abisp microcosm was more efficient at PAH biodegradation than SAS as a result of the combination of the high *A. bisporus* ligninolytic activity (Fig.1) to HMW-PAH removal, and secondly by a growing PAH-degrading bacterial population during the assay (Fig.2) which can degrade 3-ring PAH. In fact, comparing PAH removal on the basis of the number of rings, no significant differences were found for biodegradation of  $\Sigma$ 3-ring PAH between SAS, Abisp or even SM because the degradation of these compounds can be carried out by bacteria. In contrast, HMW-PAH are typically poorly degraded by bacteria due to their low water solubility and high hydrophobicity which results in mass transfer rates to the bacterial cells that are too low to match the basic cell metabolite requirements [39]. In this respect, Abisp was the most efficient microcosm to biodegrade HMW-PAH, mainly BaP, due to its high ligninolytic activity (Fig.1). These enzymes are able to degrade PAH with low bioavailability, even beyond their respective bioavailable amount [14].

The availability of Pb in the Abisp microcosms did not appear to inhibit PAH removal, previous studies have reported a total PAH degradation rate of 36% in non Pb polluted soil [18]. The fungal defense mechanisms employed to alleviate the toxicity of heavy metals is usually based on their immobilization using extracellular and intracellular chelating compounds. One of the typical metal chelators produced by fungi is oxalate

which can be used as a way of immobilizing soluble metal ions or complexes such as insoluble oxalates, thus decreasing their bioavailability and increasing tolerance to these metals [40]. Johansson et al. [41] described the increment of oxalic acid exudation in the presence of Pb and Cd by several basidiomycetes. The Fig.4A shows the high occurrence of crystals covering the surface of hyphae collected from the Abisp microcosm. Chemical elements such as C, O and Ca were identified by energy-dispersive X-ray spectroscopy but not Pb. Additionally, Ca-oxalate was confirmed by XRD (Fig.4B). Therefore, both analytical techniques have confirmed the absence of Pb-oxalate crystals as the most probable Pb detoxification mechanism for *A. bisporus*. In addition, oxalic acid has multiple roles in the decay of wood such as lowering the pH to promote the action of LME and is also implicated in lignocellulose degradation during the initial cellulose depolymerization [42]. The segregation of oxalic acid can explain the sharp decrease in pH and the increase in Pb availability in the Abisp microcosms at 0 and 28d when compared to SSAS where the substrate had been sterilized and *A. bisporus* was absent.

Despite the significant mobilization of Pb, the concentrations of available Pb were lower than the EC50 values given for the effects of available Pb on the survival and reproduction of the *Eisenia andrei* and *Enchytraeus crypticus* (soil worms) exposed to soils obtained from a shooting range and different reference soils [27,28]. Therefore, the risk to soil invertebrates did not increase in the amended microcosms.

The soil eco-toxicological tests carried out (THA, DHA) showed the pollution impact on soil microbiological activity at 0, 28 and 63d. SM achieved the worst eco-toxicity results, so natural attenuation was not able to detoxify the soil. The three SAS application methods, despite different PAH removal rates or Pb availability, caused a positive effect on soil microbiota activity, probably due to the organic matter and/or

microorganisms input. DHA appeared to be related more to the organic matter input, and further degradation over time, than soil remediation, this could be explained by the fact that the DHA value was at its maximum at 0d when the organic matter had its highest nutrient status.

THA, the other soil eco-toxicological parameter, is a good indicator of total microbial activity (bacteria and fungi) [32]. At the start of the assay THA was higher in SAS and Abisp (bioaugmented microcosms) due to the presence of the inherent SAS microbial population or *A. bisporus* respectively. But at 63d incubation, the THA can be explained by the effects of each remediation treatment. SM showed the lowest THA as a result of the high pollutant content and low bacterial population. The relative high SSAS THA was because Pb mobility had been minimized ( $R=-0.763$ ,  $p<0.05$ ) and the autochthonous soil bacteria enhanced by exogenous organic matter. For Abisp, THA was constant during the three sampling times probably due to a combination of bacterial population growth with time and fungal activity and the significant decrease of PAH concentration.

Focusing on PAH detoxification, the higher RCRA reached by the bioaugmented microcosms was due to the higher removal rates of HMW-PAH, mainly DBahA and BaP, with high toxic equivalency factors [33].

## 5 Conclusions

The reuse of spent *A. bisporus* substrate for the biodegradation of PAH in multi-polluted soil is feasible. However, the SAS application method is a key factor in obtaining the maximum efficiency. The application of sterilized SAS (SSAS microcosms) does not biodegrade PAH in Pb co-polluted soil despite enhancing the bacterial population. The use of SAS without previous treatment provides certain PAH biodegradation by the inoculation of an appreciable inherent microbiota able to degrade

PAH, however this was not feasible as an inoculum carrier for *A. bisporus* and the heavy pollution of the soil produced toxic effects on the microbial community. The SAS sterilization and further *A. bisporus* re-inoculation is adequate as an *A. bisporus* inoculum carrier and is the most efficient way of SAS reutilization for PAH biodegradation in Pb co-polluted soil by the high activity of *A. bisporus*. The increment of Pb availability was very low and minimized with time, but the reduction of PAH and its carcinogenic risk is sufficient to consider this method as an effective, cheap and useful soil mycoremediation strategy.

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## Figure Captions

Figure 1: Time course of laccase (A) and Mn-peroxidase (B) activities in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean  $\pm$  standard deviation of three replicates.

Figure 2: Time course of total heterotrophic (A) and PAH-degrading (A) bacteria in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean  $\pm$  standard deviation of three replicates. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ( $p \leq 0.05$ ).

Figure 3: Total hydrolase (A) and dehydrogenase (B) activities in non amended soil microcosm (SM) or added with sterilized spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the *A. bisporus* (Abisp). Data are the mean  $\pm$  standard deviation of three replicates.

Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ( $p \leq 0.05$ ).

Figure 4: SEM examination (A) and XRD spectra (B) of *A. bisporus* aerial hyphae from Abisp microcosm. Ox, calcium oxalate hydrate; Si, silicon oxide.

Table 1: Characteristics of the multi-polluted soil and spent *Agaricus bisporus* substrate (SAS). (mean  $\pm$  standard deviation, n=3).

Parameter	Soil	SAS
pH	8.13 $\pm$ 0.04	6.28 $\pm$ 0.03
Electric conductivity (dS m <sup>-1</sup> )	0.41 $\pm$ 0.09	6.52 $\pm$ 0.23
Organic matter (%)	3.3 $\pm$ 0.2	63.9 $\pm$ 1.5
N Kjeldahl(%)	0.14 $\pm$ 0.01	1.99 $\pm$ 0.05
Carbonates (%)	2.2 $\pm$ 0.1	n.d. <sup>†</sup>
Sand (%)	71 $\pm$ 1	n.p. <sup>§</sup>
Silt (%)	15 $\pm$ 1	n.p.
Clay (%)	14 $\pm$ 1	n.p.
Pseudo-total Pb (mg kg <sup>-1</sup> )	1429 $\pm$ 26	1.20 $\pm$ 0.20
Pseudo-total Mn (mg kg <sup>-1</sup> )	226 $\pm$ 20	541 $\pm$ 27
Pseudo-total Cu (mg kg <sup>-1</sup> )	19.6 $\pm$ 3.4	60.1 $\pm$ 5.5
Pseudo-total Zn (mg kg <sup>-1</sup> )	66.0 $\pm$ 6.8	307 $\pm$ 21
Pseudo-total Cd (mg kg <sup>-1</sup> )	0.13 $\pm$ 0.23	n.d.

<sup>†</sup>n.d.: not detected; <sup>§</sup> n.p.: not performed

Table 2: Initial PAH concentration (mean  $\pm$  standard deviation), biodegradation rates at 63d incubation respect to initial concentration and reduction in carcinogenic risk assessment (RCRA) for each microcosm: non amendment soil (SM), amendment with sterilized spent *A. bisporus* substrate (SSAS), spent *A. bisporus* substrate (SAS) and sterilized spent *A. bisporus* substrate reinoculated with the fungus (Abisp). Different letters indicates significant differences between microcosms ( $p < 0.05$ )  $n=3$ .

	Initial soil (mg kg <sup>-1</sup> )	SM	SSAS	SAS	Abisp	Carcinogenic classification*	TEF <sup>†</sup>
		PAH degradation rate (%)					
Ace	49.9 $\pm$ 0.4	36 <sup>c</sup>	20 <sup>ab</sup>	16 <sup>a</sup>	24 <sup>b</sup>	3	0.001
Flu	0.82 $\pm$ 0.05	79 <sup>c</sup>	6 <sup>a</sup>	44 <sup>b</sup>	100 <sup>d</sup>	3	0.001
Phe	28.9 $\pm$ 0.6	14 <sup>ab</sup>	0 <sup>a</sup>	22 <sup>bc</sup>	35 <sup>c</sup>	3	0.001
Ant	7.40 $\pm$ 0.13	13 <sup>ab</sup>	2 <sup>a</sup>	20 <sup>ab</sup>	32 <sup>b</sup>	3	0.01
Fla	71.0 $\pm$ 1.0	8 <sup>a</sup>	0 <sup>a</sup>	22 <sup>b</sup>	31 <sup>b</sup>	3	0.001
Py	95.8 $\pm$ 2.3	7 <sup>a</sup>	0 <sup>a</sup>	20 <sup>b</sup>	30 <sup>b</sup>	3	0.001
BaA	56.8 $\pm$ 1.3	5 <sup>a</sup>	0 <sup>a</sup>	17 <sup>b</sup>	28 <sup>c</sup>	2B	0.1
Chr	76.4 $\pm$ 1.9	4 <sup>a</sup>	0 <sup>a</sup>	17 <sup>b</sup>	27 <sup>c</sup>	2B	0.01
BbF	112 $\pm$ 2	2 <sup>a</sup>	0 <sup>a</sup>	10 <sup>ab</sup>	28 <sup>b</sup>	2B	0.1
BkF	32.3 $\pm$ 1.7	3 <sup>a</sup>	0 <sup>a</sup>	9 <sup>ab</sup>	22 <sup>b</sup>	2B	0.1
BaP	93.1 $\pm$ 0.1	3 <sup>a</sup>	0 <sup>a</sup>	29 <sup>b</sup>	39 <sup>b</sup>	1	1
DBhaA	8.47 $\pm$ .035	0 <sup>a</sup>	3 <sup>a</sup>	5 <sup>a</sup>	21 <sup>b</sup>	2A	5
BghiP	74.1 $\pm$ 1.8	2 <sup>a</sup>	0 <sup>a</sup>	8 <sup>a</sup>	28 <sup>b</sup>	3	0.01
IcdP	49.5 $\pm$ 0.2	4 <sup>a</sup>	0 <sup>a</sup>	12 <sup>ab</sup>	30 <sup>b</sup>	2B	0.1
$\Sigma$ 3rings	87.0 $\pm$ 0.7	27 <sup>b</sup>	11 <sup>a</sup>	19 <sup>ab</sup>	30 <sup>b</sup>		
$\Sigma$ 4rings	300 $\pm$ 6	6 <sup>a</sup>	0 <sup>a</sup>	19 <sup>b</sup>	29 <sup>c</sup>		
$\Sigma$ 5-6rings	370 $\pm$ 5	2 <sup>a</sup>	0 <sup>a</sup>	15 <sup>b</sup>	29 <sup>c</sup>		
$\Sigma$ PAH	757 $\pm$ 10	7 <sup>a</sup>	1 <sup>a</sup>	17 <sup>b</sup>	29 <sup>c</sup>		
RCRA		1.5 <sup>a</sup>	0.7 <sup>a</sup>	20.5 <sup>b</sup>	30.5 <sup>c</sup>		

\*IARC 2010: 1 carcinogenic, 2A probable carcinogenic, 2B possible carcinogenic, 3 not classifiable as carcinogenic.

<sup>†</sup> TEF: Toxic Equivalence Factor

Table 3: pH in water and CaCl<sub>2</sub> 0.01M (mean  $\pm$  standard deviation, n=3) and % available lead (CaCl<sub>2</sub> 0.01M) in the microcosms: non amendment soil (SM), amendment with sterilized spent *A. bisporus* substrate (SSAS), spent *A. bisporus* substrate (SAS) and sterilized spent *A. bisporus* substrate re-inoculated *A. bisporus* (Abisp) at 0, 28 ad 63d incubation. Different uppercase and lowercase letters indicate significant differences between microcosms at the same incubation time and between incubation times within the same microcosm, respectively ( $p < 0.05$ ).

	Incubation days		
	0	28	63
pH-H <sub>2</sub> O			
SM	8.17 $\pm$ 0.08 <sup>Ca</sup>	8.31 $\pm$ 0.12 <sup>Ca</sup>	7.96 $\pm$ 0.01 <sup>Ba</sup>
SSAS	7.38 $\pm$ 0.17 <sup>Ba</sup>	7.76 $\pm$ 0.06 <sup>Bb</sup>	7.55 $\pm$ 0.08 <sup>Aab</sup>
SAS	7.96 $\pm$ 0.07 <sup>Cb</sup>	7.70 $\pm$ 0.07 <sup>Bab</sup>	7.51 $\pm$ 0.17 <sup>Aa</sup>
Abisp	6.92 $\pm$ 0.02 <sup>Ab</sup>	6.67 $\pm$ 0.06 <sup>Aa</sup>	8.61 $\pm$ 0.03 <sup>Cc</sup>
pH-CaCl <sub>2</sub>			
SM	7.13 $\pm$ 0.03 <sup>Ba</sup>	7.42 $\pm$ 0.11 <sup>Bb</sup>	7.15 $\pm$ 0.05 <sup>Aa</sup>
SSAS	7.31 $\pm$ 0.16 <sup>Ba</sup>	7.64 $\pm$ 0.07 <sup>Bb</sup>	7.38 $\pm$ 0.11 <sup>Aa</sup>
SAS	7.90 $\pm$ 0.06 <sup>Cb</sup>	7.59 $\pm$ 0.09 <sup>Ba</sup>	7.32 $\pm$ 0.22 <sup>Aa</sup>
Abisp	6.76 $\pm$ 0.10 <sup>Ab</sup>	6.44 $\pm$ 0.06 <sup>Aa</sup>	8.46 $\pm$ 0.04 <sup>Bc</sup>
Available Pb (‰)			
SM	0.003 <sup>Aa</sup>	n.d. <sup>*Aa</sup>	0.004 <sup>Aa</sup>
SSAS	0.980 <sup>Bb</sup>	0.266 <sup>Ba</sup>	0.108 <sup>Ba</sup>
SAS	0.584 <sup>Bb</sup>	0.191 <sup>Ba</sup>	0.156 <sup>Ba</sup>
Abisp	1.06 <sup>Bb</sup>	2.52 <sup>Cc</sup>	0.443 <sup>Ba</sup>

\* n.d.: not detected

Figure 1

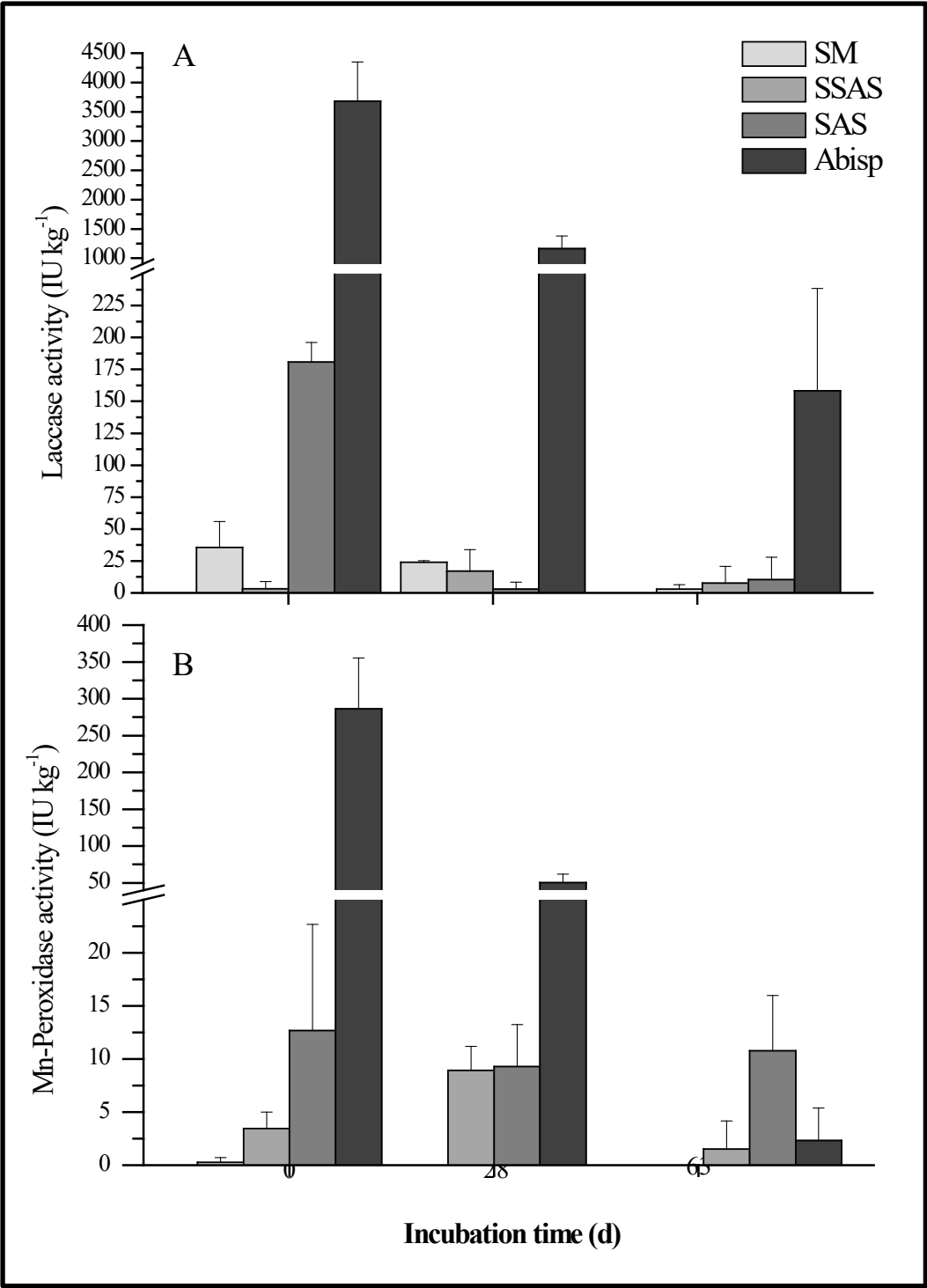


Figure 2

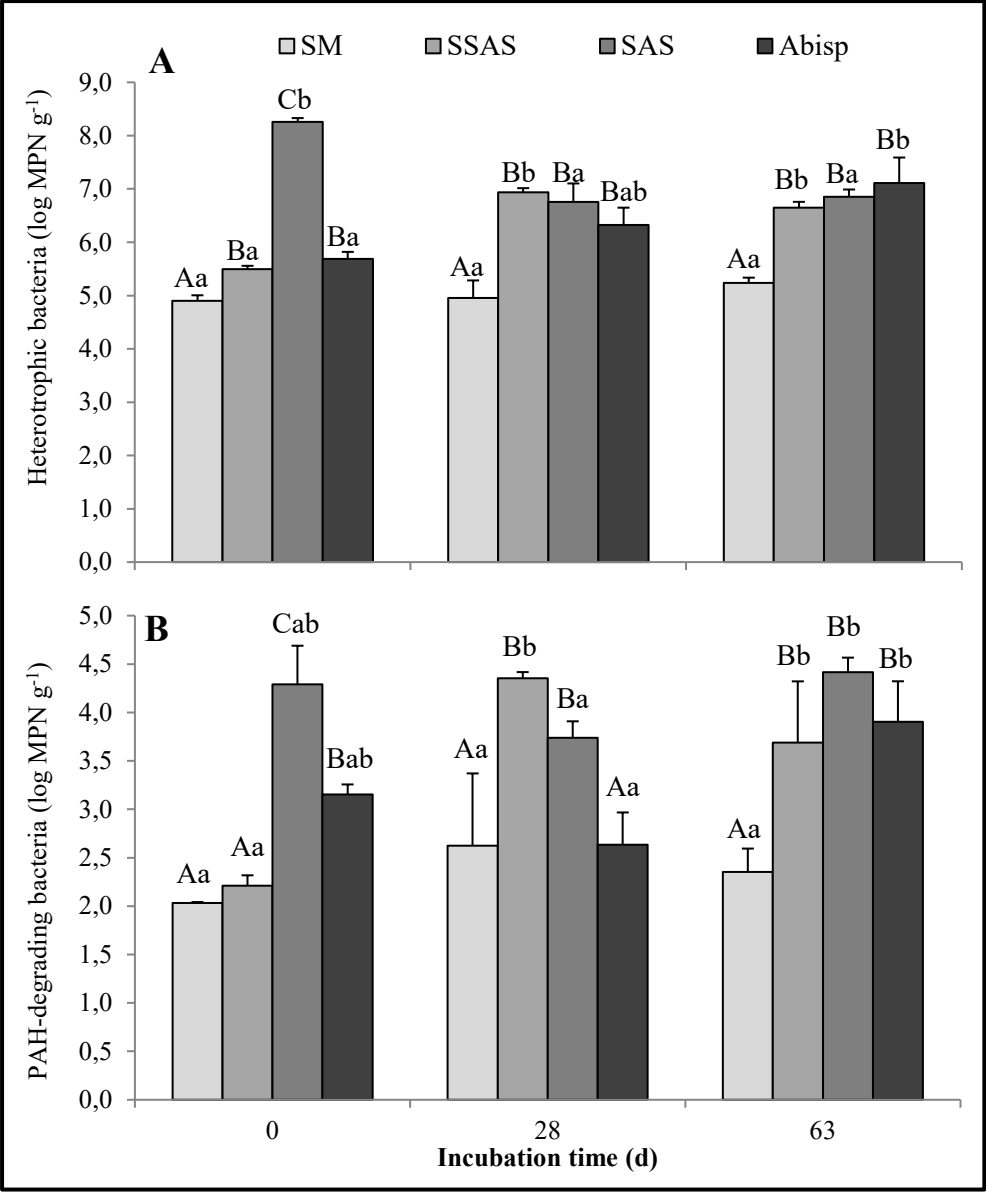


Figure 3

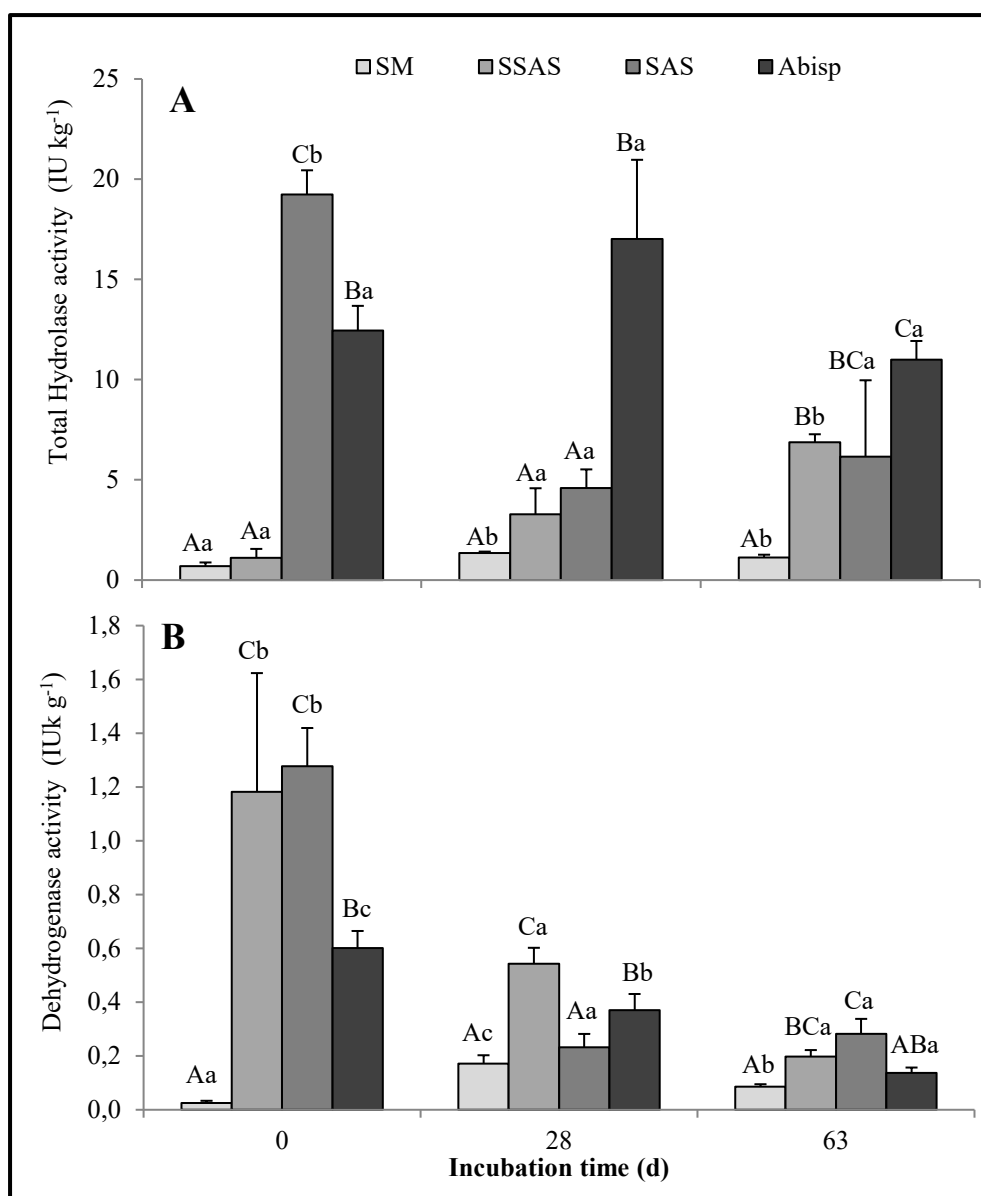


Figure 4

