

Manuscript Number: STOTEN-D-14-03115R1

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

Corresponding Author: Dr. Enrique Eymar, Ph.D.

Corresponding Author's Institution: University Autonoma of Madrid

First Author: Carlos García-Delgado, MSc

Order of Authors: Carlos García-Delgado, MSc; Alessandro D'Annibale, Ph.D.; Lorena Pesciaroli, Ph.D.; Felipe Yunta, Ph.D.; Silvia Crognale, Ph.D.; Maurizio Petruccioli, Ph.D.; Enrique Eymar, Ph.D.

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.

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.

Answer: The bioavailability reduction exerted by SAS was only confined to the highest molecular weight PAH and not, as erroneously reported, to most PAH. Thus, we have deleted that highlight.

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)

Reviewer 2:

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)'.

Page 3 Line 61-63: References were added. '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).'

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)'.

Page 6 Line 132: GPS coordinates of soil were added.

Page 6 Line 133: 'The polluted soil was collected from a creosote wood treatment plant (42° 10' 31''N 1° 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'.

Page 6 Line 137 and 146: As suggested by the Reviewer, the basic characterization of the polluted soil and the spent *A. bisporus* substrate is shown in a new table (now Table 1). Thus, the remaining tables were renumbered.

Page 6 Line 142: GPS coordinates were added.

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)."

Ms. Ref. No.: STOTEN-D-14-03115

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

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,

A handwritten signature in blue ink on a light yellow background. The signature is stylized and appears to be 'EE' followed by a horizontal line and some additional strokes.

Enrique Eymar (corresponding author)

Answers to reviewers' comments

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.

Answer: The bioavailability reduction exerted by SAS was only confined to the highest molecular weight PAH and not, as erroneously reported, to most PAH. Thus, we have deleted that highlight.

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)

Reviewer 2:

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)'.

Page 3 Line 61-63: References were added. '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).'

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)'.

Page 6 Line 132: GPS coordinates of soil were added.

Page 6 Line 133: ‘The polluted soil was collected from a creosote wood treatment plant (42° 10′ 31″ N 1° 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’.

Page 6 Line 137 and 146: As suggested by the Reviewer, the basic characterization of the polluted soil and the spent *A. bisporus* substrate is shown in a new table (now Table 1). Thus, the remaining tables were renumbered.

Page 6 Line 142: GPS coordinates were added.

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, $\log K_{oc}$ and $\log P$ values (Table 4).”

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.

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

4 García-Delgado, Carlos^a; D'Annibale, Alessandro^b, Pesciaroli, Lorena^b; Yunta, Felipe^c;
5 Crognale, Silvia^b; Petruccioli, Maurizio^b; Eymar, Enrique^{a*}.

6 ^aDepartment of Agricultural Chemistry and Food Sciences, University Autónoma of
7 Madrid, 28049 Madrid, Spain.

8 ^bDepartment for Innovation in Biological, Agro-Food and Forest systems [DIBAF],
9 University of Tuscia, 01100 Viterbo, Italy.

10 ^cDepartment of Geology and Geochemistry, University Autónoma of Madrid, 28049
11 Madrid, Spain.

12 ***Corresponding author:**

13 Enrique Eymar.

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

16 E-mail: enrique.eymar@uam.es

17 Phone: +0034914975010

18 Fax: +0034914973826

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

20 García-Delgado, Carlos: carlos.garciadelgado@uam.es

21 D'Annibale, Alessandro: dannib@unitus.it

22 Pesciaroli, Lorena: lorena_84@hotmail.it

23 Yunta, Felipe: felipe.yunta@uam.es

24 Crognale, Silvia: crognale@unitus.it

25 Petruccioli, Maurizio: petrucci@unitus.it

26 Eymar, Enrique: enrique.eymar@uam.es

27

28 **Abstract**

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

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

54 **Highlights**

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

56 ~~Spent *A. bisporus* substrate (SAS) decreased the bioavailability of most PAH in soil.~~

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

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

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

60 **Introduction**

61 Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which
62 comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic
63 properties (IARC, 2010). As a consequence of their physico-chemical properties, these
64 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
66 particular remediation technique for PAH-polluted soils is not an easy choice. There are
67 many alternatives ~~such as including~~ *in situ* or *ex situ* techniques ~~which rely~~ing on either
68 physico-chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013).
69 Among them, bioremediation is increasingly deemed to be the most environmentally
70 friendly technique to clean-up polluted soils. Bioremediation of PAH-contaminated
71 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
73 referred to as bioaugmentation ([Haritash and Kaushik 2009](#)). The biostimulation of

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

74 | resident microbial communities of PAH-impacted soil can be achieved inby a variety of
75 | approaches-ways including the addition of organic matter, which has been found to be
76 | successful (Covino et al., 2010~~ba~~; Sayara et al., 2010).
77 | ~~The~~ Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of
78 | PAH-contaminated soils has received increasing attention in recent years due to its
79 | reported efficacy (Covino et al., 2010~~ab~~; Federici et al., 2012a; Li et al., 2012). LDF are
80 | known to produce extracellular lignin-modifying enzymes (LME) with low substrate
81 | specificity which enable them to degrade a wide range of organic pollutants, including
82 | PAH (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include
83 | multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP),
84 | versatile peroxidase and lignin peroxidase).
85 | Soil colonization and the ensuing contaminant degradation by LDF in soil requires the
86 | addition of lignocellulosic materials either as amendments, or inoculum carriers
87 | (Covino et al., 2010~~ba~~; Lestan and Lamar, 1996). The use of these additives has been
88 | found to have a favorable impact on the resident microbiota, including specialized
89 | populations (Federici et al., 2012a). PAH degradation *via* bioaugmentation with LDF
90 | has been shown to involve either synergistic or antagonist interactions between the
91 | fungi added and the autochthonous microbiota. In one study a cooperative effect on the
92 | degradation of highly condensed PAH was reported between resident bacteria and
93 | *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010).
94 | However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-
95 | sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria
96 | and changed the composition of the bacterial community (Andersson et al., 2000).
97 | Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the
98 | carriers have been shown to improve the competitive ability of the fungi added to the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
99 resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of
100 ~~S~~pent mushroom substrate of some LDF as a source of viable inocula for soil clean up
101 applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus*
102 substrate (SAS), without previous treatment, has never been used for bioremediation of
103 PAH polluted soil. ~~The s~~pent *Agaricus bisporus* substrate (SAS), has been shown to
104 enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in
105 a PAH-contaminated substrate (García-Delgado et al., 2013a). Marín-Benito et al.
106 (2014; 2012a,b) reported the ability of pesticide degradation by SAS composted with
107 spent *P. ostreatus* substrate (75:25) and its adsorption capacity ~~o~~for fungicides with low
108 polarity that reduce their mobility in the environment. In addition, SAS has been
109 reported to be an excellent source of LME, mainly laccase, that were able to biodegrade
110 PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this
111 organic waste in Europe has been estimated at 3.5×10^6 t (Pardo-Giménez and Pardo-
112 González, 2008) consequently its potential use in bioaugmentation applications would
113 certainly help to reduce this figure.

36
37
38
39
40
41
42
43
44
45
46
47
114 Therefore, in view of athe profitable and environmentally sound use of SAS, this study
115 has thoroughly investigated its feasibility in PAH remediation applications. This
116 necessarily ~~should~~ implies a variety of manipulations of the waste prior to its
117 application, ~~in order~~ to gain more insights into its ability to act as an organic
118 amendment, ~~o~~r fungal carrier or a supplier of exogenous complex microbiota.

48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
119 To elucidate the isolated and/or combined effects exerted by SAS, several application
120 options were compared using an historically PAH-contaminated soil. ~~The first option~~
121 ~~assessed its ability to biostimulate the resident microbiota via its addition in a sterile~~
122 ~~form. In the second option the SAS was sterilized and subsequently reinoculated with A.~~
123 ~~bisporus. This was carried out in order to concomitantly suppress the inherent~~

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

133 **Materials and Methods**

134 2.1 Materials

135 The polluted soil was collected from a creosote wood treatment plant ~~located in Alfaro~~
136 ~~(42° 10' 31' N 1° 41' 36'' W La Rioja, Spain). The total soil sample (40 kg) was~~
137 ~~obtained by mixing 20 sub-samples were taken and mixed (40 kg) from an from an area~~
138 ~~close to the treated wood stock zone at a depth of 0-20 cm. Soil samples were collected~~
139 ~~specifically from areas close to the treated wood stock zone at a depth of 0-20 cm.~~ The
140 soil samples ~~were~~ was pooled, homogenized, air-dried at room temperature and finally
141 passed through a 2 mm-sieve. The main properties of the soil are shown in Table 1.
142 ~~were: pH, 8.20±0.03 and electric conductivity, 0.576 ±0.016 dS m⁻¹ in aqueous extract~~
143 ~~1:5 (w:v); CaCO₃, 30±1 %, organic matter, 1.2±0.1 %; with a texture composition of:~~
144 ~~sand, 39 %; silt, 39 %; clay, 22 %.~~ According to the US textural classification, the soil
145 was a clay loamy soil (sand 39%, silt 39% and clay 22%) with a water holding capacity
146 of 37 %. Thirteen ~~out of the PAH of the~~ 16 PAH US EPA were present in the polluted
147 soil. ~~‡~~ The PAH concentrations are shown in Table 12.

148 SAS was collected from a composting- plant of agricultural waste located at Quintanar
149 del Rey (39° 22' 16'' N 1° 59' 43'' W) (Cuenca, Spain). The main characteristics of the
150 SAS are shown in Table 1.~~material had the following characteristics: moisture, 67.4~~
151 ~~±0.4%, pH, 6.7±0.3, electric conductivity, 7.1±0.8 dS m⁻¹; organic matter, 61.9±1.7%;~~
152 ~~C/N ratio, 7.96±0.13. Total carbon (32.4±0.02%), nitrogen (4.07±0.07%), hydrogen~~
153 ~~(1.95±0.06%) and sulfur (0.92±0.12%)~~ were determined by elemental analysis (LECO
154 CHNS-932 analyzer, St. Joseph, MI).

155 2.2 Preparation of the Remediation Microcosms

156 Irrespective of the type of microcosm, the experiments were carried out in 11 glass
157 reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were
158 designed in order to simulate:

- 159 • Natural attenuation: the preparation of this microcosm simply involved the
160 adjustment of the soil moisture content to 70 % of its water-holding capacity
161 prior to the beginning of the incubation. This microcosm will be referred to as
162 soil microcosm (SM)
- 163 • Biostimulation: the moisture content of the polluted soil was adjusted to 70 % as
164 above and amended with sterilized SAS (121 °C, 30 min) ~~in~~ at a 4:1 mass ratio to
165 yield the SSAS microcosm. This approach was designed to assess the
166 stimulatory effect of a sterilized organic waste on resident microbiota in the soil.
- 167 • Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70
168 % as above and amended with SAS (4:1 mass ratio) to yield the SAS
169 microcosm. This approach was designed to assess the combined effect of both *A.*
170 *bisporus* and the indigenous SAS microbiota.

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

178 Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and
179 63 days under static conditions in the dark.

180 2.3 Extraction and Analysis of Ergosterol and PAH

181 Total ergosterol was extracted and analyzed as described in the method by Covino *et al.*
182 (2010b). Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml methanolic
183 solution KOH (10 %, w/v). Distilled water (1 ml) was added to each sample and the
184 sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a
185 nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were
186 analyzed using high performance liquid chromatography (Waters 2695 Separation
187 Module) coupled with a Waters 996 photodiode array detector equipped with
188 Phenomenex Luna C18 column (250 mm × 4.60 mm; particle size 5 µm; pore size 100
189 Å) equilibrated with methanol:water (95:5) at a flow rate of 1 ml min⁻¹. The sample
190 injection volume was 20 µl. The elution profile was monitored at 282 nm.

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

195 at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and
196 finally the solvent purged from the cell with argon for 60 s. This extraction cycle was
197 repeated twice for each sample. The resultant organic extract was dried under gentle N₂
198 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were
199 performed using a system consisting of a 2695 Separations Module (Waters, Milford,
200 MA) equipped with a SuperguardTM LC-18 guard column (20 x 3 mm) prior to the
201 separation SupelcosilTM LC-PAH column (250 × 3.0 mm; particle size 5 μm) and a
202 2996 diode-array detector (Waters). Separation of the PAH was achieved using a
203 gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution
204 program was: isocratic elution with 60 % (A) for 5 min, gradient to 100 % (A) for 15
205 min, isocratic elution at 100 % A for 20 min. The column temperature was fixed at 28
206 °C. The sample injection volume was 20 μl. The chromatograms were monitored at 254
207 nm. PAH were identified on the basis of both UV spectra and matching the retention
208 times with commercially available standards (Sigma-Aldrich). The PAH detected and
209 quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene
210 (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR),
211 benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP),
212 dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[1,2,3-cd]pyrene
213 (IcdP). Naphthalene, acenaphthylene, and acenaphthene were below detection limits
214 probably due the volatilization of these compounds (García-Delgado et al., 2013b).

215 2.4 Estimation of PAH Bioavailability

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

225 2.5 Biochemical Determinations and Toxicity Tests

226 Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3
227 g) at 5 °C for 1 h using the buffered solution described by D'Annibale et al. (2006). The
228 aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant assayed for
229 LME activities.

230 Laccase activity was spectrophotometrically determined by following the oxidation of
231 0.2 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium
232 acetate buffer (pH 4.5) at 420 nm ($\epsilon= 36000 \text{ M}^{-1} \text{ cm}^{-1}$). MnP activity was assayed by the
233 oxidation of 1 mM MnSO_4 in 50 mM sodium malonate buffer (pH 4.5) in the presence
234 of 0.1 mM H_2O_2 . Manganic ions, Mn^{3+} form a complex with malonate which absorbs
235 at 270 nm ($\epsilon= 11590 \text{ M}^{-1} \text{ cm}^{-1}$) (Wariishi et al., 1992). One unit of enzyme activity (IU)
236 is defined as the amount of enzyme which produced 1 μmol of product per minute under
237 the assay conditions.

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

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

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

$$253 \quad RCRA(\%) = \frac{\sum_{i=1}^{13} [PAH_i]_{to} \cdot DR \cdot TEF_i}{\sum_{i=1}^{13} [PAH_i]_{to} \cdot TEF_i} \cdot 100$$

254 | where $[PAH_i]_{to}$ is the initial concentration of PAH, DR and TEF the degradation rate
255 | and toxic equivalency factor (Table ~~24~~).

256 | 2.6 Enumeration of Cultivable Heterotrophic and PAH-Degrading Bacteria

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

269 2.7. DNA Extraction

270 Whole genomic DNA from each soil was extracted using the Power Soil DNA
271 extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's
272 instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the
273 universal bacterial 341-f (5'-
274 CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG
275 GCAGCAG-3') and 534-r (5'-ATT ACC GCG GCT GCT GG-3') primers with a length
276 of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was
277 amplified using the universal fungal primers FUN_NS1 (5'-
278 GTAGTCATATGCTTGTCTC-3') and GC fung (5'-
279 CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTAC
280 CCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 µL of template
281 DNA (ca. 20 ng), Tris-HCl (20 mM, pH 8.3), KCl (100 mM), MgCl₂ (3 mM), Taq-
282 polymerase (0.1 IU) (NzyTech, Lisbon, Portugal), primers (0.2 mM of each) and double
283 deionized water to bring the final volume up to 50 µL. For Bacterial amplification, the
284 touchdown PCR program was performed in a *Primus* PCR thermo cycler (MWG
285 biotech, Ebersberg, Germany) using the same procedure (Muyzer et al., 1993). For
286 fungal amplification the PCR program previously reported by Das et al., (2007) was
287 used. All amplicons (5 µL) were analyzed on agarose gel before being used for
288 Denaturing Gradient Gel Electrophoresis (DGGE).

289 2.8. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

$$302 \quad H' = - \sum (P_i \times \ln P_i)$$

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

306 **3 Results and Discussion**

307 **3.1 Time- and Microcosm-Dependent Evolution of Fungal Biomass and Cultivable** 308 **Bacteria**

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

322 | No changes in the ergosterol content were observed throughout the incubation in the
323 | Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier
324 | for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement
325 | for the resident fungi. This could be explained by the fact that most of the available
326 | organic components in the sterilized SAS had been consumed by the reinoculated *A.*
327 | *bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient
328 | status. Therefore, the potential trophic contribution of the carrier to the competitive
329 | ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar,
330 | 1996).

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

338 the incubation period were found in the Abisp microcosm. ~~This may also explained by~~
339 ~~the low nutrient status of this microcosm, because the *A. bisporus* nutrient consumption~~
340 ~~before inoculation in soil, rather than any competition between *A. bisporus* and the~~
341 ~~resident bacteria. This is also corroborated by the observed increase in heterotrophs in~~
342 ~~the other bioaugmented SAS microcosm.~~
343 This may be also explained by the low nutrient status of this microcosm input associated
344 with this inoculant. ,Because the preparation had, in fact, involved the reinoculation
345 of *A. bisporus* on into spent Agaricus substrate and subsequent incubation for 14 d prior
346 to its application onto soil with ensuing consumption of organic nutrients by the
347 growing fungus nutrient consumption before soil inoculation, and competition between
348 *A. bisporus* and the resident bacteria. This is also corroborated by the observed increase
349 in heterotrophs in the other bioaugmented SAS microcosm where the addition of the
350 inoculant ensured aich presented higher nutrient input status because SAS was not
351 incubated 14d before soil inoculation and showed lower fungal activity than Abisp
352 microcosm than in Abisps. (Fig. 2)

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

363 most of the incubation period. Regardless of the different application options in addition
364 to leading to a higher density of heterotrophs, when compared with the SM, it also
365 enhanced specialized ones. As described above, the non-sterile spent *Agaricus* substrate
366 in the SAS microcosm contained allochthonous PAH-degrading bacteria. This is not
367 surprising because a molecular characterization of the bacterial community in this
368 substrate has revealed the presence of several genera *Paenibacillus*, *Arthrobacter*,
369 *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004; Watabe et al., 2004) which
370 included several species with reported PAH-degrading capacity (Haritash and Kaushik,
371 2009). However, the addition of the sterilized SAS, irrespective of whether it had been
372 reinoculated ~~or~~ with *A. bisporus* or not, also appeared to stimulate PAH-degrading
373 bacteria. Previous studies have also found that the addition of sterilized organic wastes
374 such as wheat straw and maize stalks activated the specialized resident bacterial
375 populations in soils contaminated with PAH (Lladó et al., 2013), and
376 polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested that the
377 stimulatory effect exerted by organic waste is due to an enhancement of the oxygen
378 transfer via an increase in soil porosity without necessarily involving either trophic
379 factors or modifications in contaminant bioavailability (Federici et al., 2012b).

380 3.2 Microcosm-Dependent Impact on Bacterial and Fungal Community Structure

381 It is widely known that the cultivable microbiota in soil represent a minor fraction of the
382 whole microbial community (Daniel, 2005). Therefore, an investigation into the
383 structure of both the bacterial and fungal communities as a function of the remediation
384 treatment necessitated a cultivation-independent approach which relied on DGGE.
385 Table [23](#) shows the Shannon Weaver Index, which gives the richness and evenness
386 values of bacterial and fungal communities in the microcosms at the start, and at the end

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

411 the richness and diversity of the fungal community when compared to the SM

412 microcosm (Table 23).

413 3.3 PAH Removal in the Remediation Microcosms

414 Table 42 shows the initial and residual PAH concentrations in the remediation
415 microcosms after 63 days incubation. In the SM, no significant reduction in the total
416 PAH concentration was observed ($p=0.07$). Significant differences between the initial
417 and final concentrations were only found for FLT and PYR, with a percentage removal
418 of 65 %, and 59 % respectively. In the biostimulation SSAS microcosm a statistically
419 significant decrease ($p<0.05$) in 3-ring PAH content, FLU (75 %), PHE (90 %), ANT
420 (82 %), and FLT (52 %), was observed and the overall PAH residual content
421 significantly differed from that at the start. Therefore, the biostimulation treatment was
422 effective at degrading low molecular weight PAHs which were the most abundant
423 contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp,
424 significant differences between the initial and the residual concentrations were found for
425 the majority of the individual contaminants as well as the overall PAH concentrations
426 (Table 42). These findings indicate that the bioaugmentation treatments with *A.*
427 *bisporus* were efficient at degrading both low and high molecular weight PAH.
428 A comparison of the degradation performances of SSAS, SAS and Abisp microcosms,
429 revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading
430 bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp
431 was superior to SSAS and SAS in the removal of highly condensed PAH (Table 42).
432 Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC,
433 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds
434 such as BaP and DBahA (52 vs. 18 % respectively, and 32 vs. 6 % respectively). In this

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

435 | respect, a very limited number of bacteria ~~are~~ able to grow in pure cultures on either 5-
436 | or 6-ring PAH have been identified (Haritash and Kaushik, 2009).
437 | However, in the SAS microcosm, the augmented fungus appeared to exert a lower
438 | inhibitory action on the resident bacterial biota as deduced by comparing its better
439 | degradation performance on low molecular weight PAH and its higher density of PAH-
440 | degrading bacteria than those found in the Abisp microcosm. One of the possible
441 | synergistic mechanisms which might occur between bacteria and fungi, relies on the
442 | ability of the latter to convert PAH into more polar degradation intermediates, such as
443 | PAH diones and hydroxylated derivatives (Covino et al., 2010~~ba~~).
444 | Linear regression analyses were performed to relate the percentage removal of each
445 | PAH in the microcosm with respect to the chemical characteristics of these
446 | contaminants (Table [34](#)). Therefore, the degradation results were related to those
447 | parameters which have been suggested to significantly affect PAH degradation, such as
448 | molecular weight (MW), organic carbon sorption coefficient ($\log K_{oc}$), hydrophobicity
449 | ($\log P$), water solubility (WS) and ionization potential (IP), (Table [34](#)). These analyses
450 | showed that the degradation results were positively and significantly ($p < 0.05$)
451 | correlated with WS in all the microcosms tested. Conversely, with the exception of the
452 | Abisp microcosm, PAH degradation results were a significant negatively trend
453 | correlation ($p \leq 0.010$) or 0.005) was found when the degradation results were
454 | related to with their respective MW, $\log K_{oc}$ and $\log P$ values (Table [34](#)). These results
455 | clearly indicate that the bioavailability of PAH contaminants strongly affect their ability
456 | to be degraded in the SM, SSAS and SAS microcosms, where the bacteria make an
457 | important contribution to PAH depletion. Bacterial uptake of PAH and their subsequent
458 | metabolism have been shown to be governed by the mass transfer rates of contaminants
459 | from the solid to the liquid phase of soil, which are in turn affected by the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

460 aforementioned physico-chemical properties (Haritash and Kaushik, 2009). The
461 distinctive behavior of the Abisp microcosm, namely the lack of correlation between
462 PAH degradation and MW, log K_{oc} and log P , might indicate that different PAH
463 degradation mechanisms are involved here. In particular, the direct involvement of PAH
464 oxidation by LME which was high during the initial incubation phases (See subsection
465 3.5) might be as result of their ability to diffuse into the soil matrix and potentially
466 oxidise PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis
467 may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH.
468 The IP has been shown to affect *in vitro* PAH oxidation by either laccase or fungal
469 heme-peroxidases, whose activities were found in the bioaugmented microcosms (see
470 subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown
471 to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the
472 opposite trend was found when relating PAH depletions and respective IP values in all
473 the microcosms. However, the Pearson Coefficients related to these regressions, which
474 were significant with the exception of Abisp, were the lowest among the physico-
475 chemical properties tested (Table 43). Similar findings were obtained with two
476 historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus*
477 *tigrinus* (Covino et al., 2010^{ab}). This clearly suggests that even in augmented
478 microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH
479 degradation. This was made particularly evident in the bioaugmented microcosms by
480 the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV
481 respectively) make them poorly susceptible to mono-electronic oxidation by LMEs
482 (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the
483 PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

486 3.4 Time- and Microcosm-Dependent Modifications in the PAH Bioavailable Fraction

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

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

515 3.5 Lignin-Modifying Enzyme Activity and Residual Toxicity in Remediation 516 Microcosms

517 The remediation microcosms were assayed for their ligninolytic activity due to the
518 involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010;
519 Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-
520 peroxidase activity (Fig. 2A and 2B, respectively) were only detected in the
521 bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity
522 were found in the Abisp microcosms where the activity of the former enzyme markedly
523 declined with incubation time (Fig. 2A). Noteworthy, in this microcosm the best
524 depletions were observed for BaP and DBahA which despite being high molecular
525 weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38 respectively) and
526 therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar,
527 1995). In this regard the results of the present study are in agreement with those of Li et
528 al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts
529 from spent *Agaricus* substrate.
530 Dehydrogenase activity was used as a possible index of detoxification in the
531 remediation microcosms as previously suggested for hydrocarbon-impacted soils

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

532 (Dawson et al., 2007) in addition to the *F. candida* mortality test. In the present study,
533 the adoption of this parameter did not provide an unequivocal clue to the detoxification
534 by augmented microcosms. This was the result of a low dehydrogenase activity in the
535 contaminated soil at the start (Table 65). The level of dehydrogenase activity was
536 markedly and predictably boosted by the addition of viable fungal inocula in the SAS
537 and Abisp microcosms (Table 65). The retention of high levels of activity in the 63 day
538 old SAS and Abisp microcosms, albeit being lower in the SAS and equal in the Abisp,
539 indicate that the soil microbiota retained a high functional activity despite a prolonged
540 incubation time.

541 Table 56 also shows that the initial toxicity of the microcosms towards *F. candida*
542 was high, leading to mortalities that ranged from 77.5 to 89 %. Although a partial
543 detoxification was observed in all microcosms, the best results were observed with SAS
544 and Abisp where mortality was reduced by 45.1 and 41.4 %, respectively. In these
545 microcosms, a generalized decrease in the bioavailable fractions of individual
546 contaminants were observed together with the highest percentage removal of both 4-
547 ring and highly condensed PAH. The lowest mortality reduction (26.8 %) was observed
548 in SSAS where the lowest depletion of 4-ring PAH, and no depletion of highly
549 condensed PAH were found. In this respect, the higher detoxification observed in Abisp
550 than SSAS suggest that 3-ring PAH were less toxic to *F. candida* than 4-ring and 5,6-
551 ring PAH. This might be explained by the fact that in the SSAS the former contaminant
552 group was degraded at a significantly higher extent and, in the same microcosm, with
553 the exception of PHE, their bioavailable fractions were comparatively lower than in
554 Abisp.

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

557 (SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and
558 5,6-ring PAH which have the highest carcinogenic properties (IARC 2010). Significant
559 differences were also found when comparing the bioaugmented microcosms. The Abisp
560 microcosm showed higher RCRA than the SAS because of its better efficacy in the
561 removal of BaP and DBahA (Table [24](#)) which exhibited the highest TEF values.

562 **4 Conclusions**

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

573 **Acknowledgements**

574 This work was financially supported by the Ministry of Science and Innovation of Spain
575 (Project CTM2009-13140-C02-02). The authors wish to express their thanks to
576 Recomsa for providing the spent mushroom substrate [and Impregna for providing the](#)
577 [soil](#).

578 **References**

- 579 Andersson, B., Welinder, L., Olsson, P., Olsson, S., Henrysson, T., 2000. Growth of
1 580 inoculated white-rot fungi and their interactions with the bacterial community in
2 581 soil contaminated with polycyclic aromatic hydrocarbons, as measured by
3 582 phospholipid fatty acids. *Bioresour. Technol.* 73, 29–36. doi: 10.1016/S0960-
4 583 8524(99)00134-0
- 584 [Bogan BW and Lamar RT., 1995. One-electron oxidation in the degradation of creosote](#)
585 [polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl Environ*](#)
586 [Microbiol 61:2631–5.](#)
- 587 Borràs, E., Caminal, G., Sarrà, M., Novotný, Č., 2010. Effect of soil bacteria on the
588 ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes*
589 *versicolor* and *Irpex lacteus* from contaminated soil. *Soil Biol. Biochem.* 42, 2087–
590 2093. doi: 10.1016/j.soilbio.2010.08.003
- 591 Covino, S., Svobodová, K., Cvancarová, M., D’Annibale, A., Petruccioli, M., Federici,
592 F., Kresinová, Z., Galli, E., Cajthaml, T., 2010^{ba}. Inoculum carrier and
593 contaminant bioavailability affect fungal degradation performances of PAH-
594 contaminated solid matrices from a wood preservation plant. *Chemosphere* 79,
595 855–864. doi: 10.1016/j.chemosphere.2010.02.038
- 596 Covino, S., Cvancarová, M., Muzikár, M., Svobodová, K., D’annibale, A., Petruccioli,
597 M., Federici, F., Kresinová, Z., Cajthaml, T., 2010^{ab}. An efficient PAH-degrading
598 *Lentinus (Panus) tigrinus* strain: effect of inoculum formulation and pollutant
599 bioavailability in solid matrices. *J. Hazard. Mater.* 183, 669–676. doi:
600 10.1016/j.jhazmat.2010.07.078
- 601 Covino, S., Svobodová, K., Kresinová, Z., Petruccioli, M., Federici, F., D’Annibale, A.,
602 Cvancarová, M., Cajthaml, T., 2010c. In vivo and in vitro polycyclic aromatic
603 hydrocarbons degradation by *Lentinus (Panus) tigrinus* CBS 577.79. *Bioresour.*
604 *Technol.* 101, 3004–3012. doi: 10.1016/j.biortech.2009.12.020
- 605 D’Annibale, A., Rosetto, F., Leonardi, V., Federici, F., Petruccioli, M., 2006. Role of
606 Autochthonous Filamentous Fungi in Bioremediation of a Soil Historically
607 Contaminated with Aromatic Hydrocarbons. *Appl. Environ. Microbiol.* 72, 28–36.
608 doi: 10.1128/AEM.72.1.28
- 609 Daniel, R., 2005. The metagenomics of soil. *Nat. Rev. Microbiol.* 3, 470–8. doi:
610 10.1038/nrmicro1160
- 611 Das, M., Royer, T. V, Leff, L.G., 2007. Diversity of fungi, bacteria, and actinomycetes
612 on leaves decomposing in a stream. *Appl. Environ. Microbiol.* 73, 756–67. doi:
613 10.1128/AEM.01170-06
- 614 Dawson, J.J.C., Godsiffe, E.J., Thompson, I.P., Ralebitso-Senior, T.K., Killham, K.S.,
615 Paton, G.I., 2007. Application of biological indicators to assess recovery of
616 hydrocarbon impacted soils. *Soil Biol. Biochem.* 39, 164–177. doi:
617 10.1016/j.soilbio.2006.06.020

- 618 Federici, E., Giubilei, M., Santi, G., Zanaroli, G., Negroni, A., Fava, F., Petruccioli, M.,
1 619 D'Annibale, A., 2012a. Bioaugmentation of a historically contaminated soil by
2 620 polychlorinated biphenyls with *Lentinus tigrinus*. *Microb. Cell Fact.* 11, 35. doi:
3 621 10.1186/1475-2859-11-35
4
5
6 622 Federici, E., Giubilei, M.A., Cajthaml, T., Petruccioli, M., D'Annibale, A., 2011.
7 623 *Lentinus (Panus) tigrinus* augmentation of a historically contaminated soil: Matrix
8 624 decontamination and structure and function of the resident bacterial community. *J.*
9 625 *Hazard. Mater.* 186, 1263–1270. doi: 10.1016/j.jhazmat.2010.11.128
10
11
12 626 Federici, E., Giubilei, M.A., Covino, S., Zanaroli, G., Fava, F., D'Annibale, A.,
13 627 Petruccioli, M., 2012b. Addition of maize stalks and soybean oil to a historically
14 628 PCB-contaminated soil: effect on degradation performance and indigenous
15 629 microbiota. *N. Biotechnol.* 30, 69–79. doi: 10.1016/j.nbt.2012.07.007
16
17
18 630 Gan, S., Lau, E. V, Ng, H.K., 2009. Remediation of soils contaminated with polycyclic
19 631 aromatic hydrocarbons (PAHs). *J. Hazard. Mater.* 172, 532–549. doi:
20 632 10.1016/j.jhazmat.2009.07.118
21
22
23 633 García-Delgado, C., Jiménez-Ayuso, N., Frutos, I., Gárate, A., Eymar, E., 2013a.
24 634 Cadmium and lead bioavailability and their effects on polycyclic aromatic
25 635 hydrocarbons biodegradation by spent mushroom substrate. *Environ. Sci. Pollut.*
26 636 *Res.* 20, 8690–9. doi: 10.1007/s11356-013-1829-0
27
28
29 637 García-Delgado, C., Yunta, F., Eymar, E., 2013b. Methodology for Polycyclic Aromatic
30 638 Hydrocarbons Extraction from Either Fresh or Dry Spent Mushroom Compost and
31 639 Quantification by High-Performance Liquid Chromatography–Photodiode Array
32 640 Detection. *Commun. Soil Sci. Plant Anal.* 44, 817–825. doi:
33 641 10.1080/00103624.2013.749439
34
35
36 642 Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic
37 643 hydrocarbons (PAHs): a review. *J. Hazard. Mater.* 169, 1–15.
38 644 10.1016/j.jhazmat.2009.03.137
39
40
41 645 Hundal, L.S., Thompson, M.L., Laird, D. a, Carmo, a M., 2001. Sorption of
42 646 phenanthrene by reference smectites. *Environ. Sci. Technol.* 35, 3456–61. doi:
43 647 10.1021/es001982a
44
45
46 648 IARC, 2010. IARC Monographs on the evaluation of carcinogenic risks to humans.
47 649 Some non-heterocyclic Polycyclic aromatic Hydrocarbons and some related
48 650 exposures., IARC monographs on the evaluation of carcinogenic risks to humans /
49 651 World Health Organization, International Agency for Research on Cancer. Lyon,
50 652 France.
51
52
53
54 653 Kotterman, M.J.J., Vis, E.H., Field, J.A., 1998. Successive Mineralization and
55 654 Detoxification of Benzo[a]pyrene by the White Rot Fungus *Bjerkandera sp.* Strain
56 655 BOS55 and Indigenous Successive Mineralization and Detoxification of
57 656 Benzo[a]pyrene by the White Rot Fungus *Bjerkandera sp.* Strain BOS55 and In.
58 657 *Appl. Environ. Microbiol.* 64, 2853–2858.
59
60
61
62
63
64
65

- 658 Krauss, M., Wilcke, W., Zech, W., 2000. Polycyclic aromatic hydrocarbons and
659 polychlorinated biphenyls in forest soils : depth distribution as indicator of
660 different fate. *Environ. Pollut.* 110, 79–88.
- 661 Leonardi, V., Giubilei, M.A., Federici, E., Spaccapelo, R., Sasek, V., Novotny, C.,
662 Petruccioli, M., D’Annibale, A., 2008. Mobilizing agents enhance fungal
663 degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous
664 bacteria in soil. *Biotechnol. Bioeng.* 101, 273–85. doi: 10.1002/bit.21909
- 665 Lestan, D., Lamar, R.T., 1996. Development of fungal inocula for bioaugmentation of
666 contaminated soils. *Appl. Environ. Microbiol.* 62, 2045–52.
- 667 Li, X., Lin, X., Zhang, J., Wu, Y., Yin, R., Feng, Y., Wang, Y., 2010. Degradation of
668 polycyclic aromatic hydrocarbons by crude extracts from spent mushroom
669 substrate and its possible mechanisms. *Curr. Microbiol.* 60, 336–42. doi:
670 10.1007/s00284-009-9546-0
- 671 Li, X., Wu, Y., Lin, X., Zhang, J., Zeng, J., 2012. Dissipation of polycyclic aromatic
672 hydrocarbons (PAHs) in soil microcosms amended with mushroom cultivation
673 substrate. *Soil Biol. Biochem.* 47, 191–197. doi: 10.1016/j.soilbio.2012.01.001
- 674 Liang, C., Dang, Z., Xiao, B., Huang, W., Liu, C., 2006. Equilibrium sorption of
675 phenanthrene by soil humic acids. *Chemosphere* 63, 1961–8. doi:
676 10.1016/j.chemosphere.2005.09.065
- 677 Lladó, S., Covino, S., Solanas, A.M., Viñas, M., Petruccioli, M., D’annibale, A., 2013.
678 Comparative assessment of bioremediation approaches to highly recalcitrant PAH
679 degradation in a real industrial polluted soil. *J. Hazard. Mater.* 248-249, 407–414.
680 doi: 10.1016/j.jhazmat.2013.01.020
- 681 Lladó, S., Jiménez, N., Viñas, M., Solanas, A.M., 2009. Microbial populations related
682 to PAH biodegradation in an aged biostimulated creosote-contaminated soil.
683 *Biodegradation* 20, 593–601. doi: 10.1007/s10532-009-9247-1
- 684 Majcherczyk, A., Johannes, C., Hu, A., 1998. Oxidation of polycyclic aromatic
685 hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb.*
686 *Technol.* 22, 335–341.
- 687 Marín-Benito, J.M., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S.,
688 2012a. Dissipation of fungicides in a vineyard soil amended with different spent
689 mushroom substrates. *J. Agric. Food Chem.* 60, 6936–45. doi: 10.1021/jf301322h
- 690 Marín-Benito, J.M., Herrero-Hernández, E., Andrades, M.S., Sánchez-Martín, M.J.,
691 Rodríguez-Cruz, M.S., 2014. Effect of different organic amendments on the
692 dissipation of linuron, diazinon and myclobutanil in an agricultural soil incubated
693 for different time periods. *Sci. Total Environ.* 476-477, 611–21. doi:
694 10.1016/j.scitotenv.2014.01.052
- 695 Marín-Benito, J.M., Rodríguez-Cruz, M.S., Andrades, M.S., Sánchez-Martín, M.J.,
696 2012b. Assessment of spent mushroom substrate as sorbent of fungicides:

- 697 influence of sorbent and sorbate properties. *J. Environ. Qual.* 41, 814–822. doi:
698 10.2134/jeq2011.0437
- 699 Mayolo-Deloisa, K., Machín-Ramírez, C., Rito-Palomares, M., Trejo-Hernández, M.R.,
700 2011. Oxidation of Polycyclic Aromatic Hydrocarbons using Partially Purified
701 Laccase from Residual Compost of *Agaricus bisporus*. *Chem. Eng. Technol.* 34,
702 1368–1372. doi: 10.1002/ceat.201000205
- 703 Muyzer, G., de Waal, E.C., Uitterlinden, a G., 1993. Profiling of complex microbial
704 populations by denaturing gradient gel electrophoresis analysis of polymerase
705 chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*
706 59, 695–700.
- 707 Nisbet, I.C., LaGoy, P.K., 1992. Toxic equivalency factors (TEFs) for polycyclic
708 aromatic hydrocarbons (PAHs). *Regul. Toxicol. Pharmacol.* 16, 290–300.
- 709 Ntougias, S., Zervakis, G.I., Kavroulakis, N., Ehaliotis, C., Papadopoulou, K.K., 2004.
710 Bacterial diversity in spent mushroom compost assessed by amplified rDNA
711 restriction analysis and sequencing of cultivated isolates. *Syst. Appl. Microbiol.*
712 27, 746–54. doi: 10.1078/0723202042369857
- 713 Pardo-Giménez, A., Pardo-González, J.E., 2008. Evaluation of casing materials made
714 from spent mushroom substrate and coconut fibre pith for use in production of
715 *Agaricus bisporus* (Lange) Imbach. *Spanish J. Agric. Res.* 6, 683–690.
- 716 Pelaez, A.I., Lores, I., Sotres, A., Mendez-Garcia, C., Fernandez-Velarde, C., Santos, J.
717 a, Gallego, J.L.R., Sanchez, J., 2013. Design and field-scale implementation of an
718 “on site” bioremediation treatment in PAH-polluted soil. *Environ. Pollut.* 181,
719 190–199. doi: 10.1016/j.envpol.2013.06.004
- 720 Sayara, T., Sarrà, M., Sánchez, A., 2010. Effects of compost stability and contaminant
721 concentration on the bioremediation of PAHs-contaminated soil through
722 composting. *J. Hazard. Mater.* 179, 999–1006. doi: 10.1016/j.jhazmat.2010.03.104
- 723 Stokes, J.D., Wilkinson, A., Reid, B.J., Jones, K.C., Semple, K.T., 2005. Prediction of
724 polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an
725 aqueous hydroxypropyl-beta-cyclodextrin extraction technique. *Environ. Toxicol.*
726 *Chem.* 24, 1325–1330.
- 727 Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese
728 peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic
729 mechanism and role of chelators. *J. Biol. Chem.* 267, 23688–23695.
- 730 Watabe, M., Rao, J.R., Xu, J., Millar, B.C., Ward, R.F., Moore, J.E., 2004.
731 Identification of novel eubacteria from spent mushroom compost (SMC) waste by
732 DNA sequence typing: ecological considerations of disposal on agricultural land.
733 *Waste Manag.* 24, 81–86. doi: 10.1016/j.wasman.2003.08.001

734

735

1
2 **736 Figure Captions**
3
4

5
6 737 Figure 1: Time course of ergosterol concentrations (A), total heterotrophic (B) and PAH
7
8 738 degrading (C) bacteria in non amended soil microcosm (SM), amended with sterilized
9
10 739 spent Agaricus substrate (SSAS), spent Agaricus substrate (SAS), and sterilized spent
11
12 740 Agaricus substrate reinoculated with the fungus (Abisp). Data are the mean \pm standard
13
14
15 741 deviation of three replicated microcosms. Different uppercase and lowercase letters
16
17
18 742 indicate significant differences between microcosms at the same incubation time and
19
20 743 between incubation times within the same microcosm (Tukey post-hoc test; $p \leq 0.05$),
21
22
23 744 respectively.
24

25
26 745 Figure 2: Time courses of laccase (A) and Mn-peroxidase (B) activities in non amended
27
28 746 soil microcosm (SM), amended with sterilized spent Agaricus substrate (SSAS), spent
29
30 747 Agaricus substrate (SAS) and sterilized spent Agaricus substrate reinoculated with the
31
32 748 fungus (Abisp). Data are the mean \pm standard deviation of three replicated microcosms.
33
34
35

36
37
38 749
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

4 García-Delgado, Carlos^a; D'Annibale, Alessandro^b, Pesciaroli, Lorena^b; Yunta, Felipe^c;
5 Crognale, Silvia^b; Petruccioli, Maurizio^b; Eymar, Enrique^{a*}.

6 ^aDepartment of Agricultural Chemistry and Food Sciences, University Autónoma of
7 Madrid, 28049 Madrid, Spain.

8 ^bDepartment for Innovation in Biological, Agro-Food and Forest systems [DIBAF],
9 University of Tuscia, 01100 Viterbo, Italy.

10 ^cDepartment of Geology and Geochemistry, University Autónoma of Madrid, 28049
11 Madrid, Spain.

12 ***Corresponding author:**

13 Enrique Eymar.

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

16 E-mail: enrique.eymar@uam.es

17 Phone: +0034914975010

18 Fax: +0034914973826

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

20 García-Delgado, Carlos: carlos.garciadelgado@uam.es

21 D'Annibale, Alessandro: dannib@unitus.it

22 Pesciaroli, Lorena: lorena_84@hotmail.it

23 Yunta, Felipe: felipe.yunta@uam.es

24 Crognale, Silvia: crognale@unitus.it

25 Petruccioli, Maurizio: petrucci@unitus.it

26 Eymar, Enrique: enrique.eymar@uam.es

27

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

28 **Abstract**

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

49
50 **Keywords:** polycyclic aromatic hydrocarbons, biodegradation, bioavailability,
51 ligninolytic enzymes, agricultural waste.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

52 **Highlights**

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

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

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

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

57 **Introduction**

58 Polycyclic aromatic hydrocarbons (PAH) are ubiquitous organic contaminants which
59 comprise two or more condensed benzene rings with toxic, mutagenic and carcinogenic
60 properties (IARC, 2010). As a consequence of their physico-chemical properties, these
61 compounds are accumulated in soil *via* their sorption onto organic matter (Krauss et al.,
62 2000; Liang et al., 2006) and/or clay fractions (Hundal et al., 2001). The selection of a
63 particular remediation technique for PAH-polluted soils is not an easy choice. There are
64 many alternatives including *in situ* or *ex situ* techniques which rely on either physico-
65 chemical or biological approaches (Gan et al., 2009; Pelaez et al., 2013). Among them,
66 bioremediation is increasingly deemed to be the most environmentally friendly
67 technique to clean-up polluted soils. Bioremediation of PAH-contaminated soils is
68 based either on the biostimulation of the indigenous microbiota (Sayara et al., 2010) or
69 on the addition of exogenous microorganisms, the latter approach being referred to as
70 bioaugmentation (Haritash and Kaushik 2009). The biostimulation of resident microbial
71 communities of PAH-impacted soil can be achieved in a variety of ways including the
72 addition of organic matter, which has been found to be successful (Covino et al., 2010a;
73 Sayara et al., 2010).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

74 Bioaugmentation with lignin-degrading fungi (LDF) to perform the clean-up of PAH-
75 contaminated soils has received increasing attention in recent years due to its reported
76 efficacy (Covino et al., 2010b; Federici et al., 2012a; Li et al., 2012). LDF are known to
77 produce extracellular lignin-modifying enzymes (LME) with low substrate specificity
78 which enable them to degrade a wide range of organic pollutants, including PAH
79 (Covino et al., 2010c; Majcherczyk et al., 1998). The main LME enzymes include
80 multi-copper oxidases, such as laccase, and heme-peroxidases (Mn-peroxidase (MnP),
81 versatile peroxidase and lignin peroxidase).
82 Soil colonization and the ensuing contaminant degradation by LDF in soil requires the
83 addition of lignocellulosic materials either as amendments, or inoculum carriers
84 (Covino et al., 2010a; Lestan and Lamar, 1996). The use of these additives has been
85 found to have a favorable impact on the resident microbiota, including specialized
86 populations (Federici et al., 2012a). PAH degradation *via* bioaugmentation with LDF
87 has been shown to involve either synergistic or antagonist interactions between the
88 fungi added and the autochthonous microbiota. In one study a cooperative effect on the
89 degradation of highly condensed PAH was reported between resident bacteria and
90 *Bjerkandera* sp. (Kotterman et al., 1998) or *Trametes versicolor* (Borràs et al., 2010).
91 However, in another study *Pleurotus ostreatus* enhanced PAH degradation in non-
92 sterile, artificially spiked soils but also inhibited the growth of the indigenous bacteria
93 and changed the composition of the bacterial community (Andersson et al., 2000).
94 Appropriate inocula formulations of LDF, relying on lignocellulosic materials as the
95 carriers have been shown to improve the competitive ability of the fungi added to the
96 resident microbiota (Covino et al., 2010a, 2010b; Federici et al., 2012a). The use of pent
97 mushroom substrate of some LDF as a source of viable inocula for soil clean up
98 applications has been proposed (Li et al., 2012). However spent *Agaricus bisporus*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

99 substrate (SAS), without previous treatment, has never been used for bioremediation of
100 PAH polluted soil. Spent *Agaricus bisporus* substrate (SAS), has been shown to
101 enhance the ability of the fungi to endure the toxic effects of both cadmium and lead in
102 a PAH-contaminated substrate (García-Delgado et al., 2013a). Marín-Benito et al.
103 (2014; 2012a,b) reported the ability of pesticide degradation by SAS composted with
104 spent *P. ostreatus* substrate (75:25) and its adsorption capacity for fungicides with low
105 polarity that reduce their mobility in the environment. In addition, SAS has been
106 reported to be an excellent source of LME, mainly laccase, that were able to biodegrade
107 PAH in aqueous solutions (Mayolo-Deloisa et al., 2011). The annual production of this
108 organic waste in Europe has been estimated at 3.5×10^6 t (Pardo-Giménez and Pardo-
109 González, 2008) consequently its potential use in bioaugmentation applications would
110 certainly help to reduce this figure.
111 Therefore, in view of the profitable and environmentally sound use of SAS, this study
112 has thoroughly investigated its feasibility in PAH remediation applications. This
113 necessarily implies a variety of manipulations of the waste prior to its application, to
114 gain more insight into its ability to act as an organic amendment, fungal carrier or a
115 supplier of exogenous complex microbiota.
116 To elucidate the isolated and/or combined effects exerted by SAS, several application
117 options were compared using an historically PAH-contaminated soil. All were assessed
118 for their ability to (i) affect the densities of heterotrophic and PAH-degrading bacteria
119 (ii) enable fungal growth (iii) modify the biodiversity of the bacterial and fungal
120 communities (iv) remove PAH (v) modify the bioavailability of PAH and finally to (vi)
121 detoxify the soil. A non-amended contaminated soil microcosm was incubated in
122 parallel and was used as a natural attenuation control.

123 **Materials and Methods**

124 2.1 Materials

1
2
3 125 The polluted soil was collected from a creosote wood treatment plant (42° 10' 31'' N 1°
4
5 126 41' 36'' W La Rioja, Spain). The total soil sample (40 kg) was obtained by mixing 20
6
7
8 127 sub-samples taken from an area close to the treated wood stock zone at a depth of 0-20
9
10 128 cm. The soil sample was homogenized, air-dried at room temperature and finally passed
11
12 129 through a 2 mm-sieve. The main properties of the soil are shown in Table 1. According
13
14 130 to the US textural classification, the soil was a clay loamy soil (sand 39%, silt 39% and
15
16 131 clay 22%) with a water holding capacity of 37 %. Thirteen out of the 16 PAH US EPA
17
18 132 were present in the polluted soil. The PAH concentrations are shown in Table 2.
19
20
21
22

23 133 SAS was collected from a composting plant of agricultural waste located at 39° 22'
24
25 134 16'' N 1° 59' 43'' W (Cuenca, Spain). The main characteristics of the SAS are shown in
26
27 135 Table 1.. Total carbon, nitrogen, hydrogen and sulfur were determined by elemental
28
29 136 analysis (LECO CHNS-932 analyzer, St. Joseph, MI).
30
31
32
33

34 137 2.2 Preparation of the Remediation Microcosms

35
36
37
38 138 Irrespective of the type of microcosm, the experiments were carried out in 1l glass
39
40 139 reactors using unsterilized contaminated soil (50 g dry mass). The microcosms were
41
42 140 designed in order to simulate:

- 43
44
45
46 141 • Natural attenuation: the preparation of this microcosm simply involved the
47
48 142 adjustment of the soil moisture content to 70 % of its water-holding capacity
49
50 143 prior to the beginning of the incubation. This microcosm will be referred to as
51
52 144 soil microcosm (SM)
53
54
55
56
57 145 • Biostimulation: the moisture content of the polluted soil was adjusted to 70 % as
58
59 146 above and amended with sterilized SAS (121 °C, 30 min) at a 4:1 mass ratio to
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

147 yield the SSAS microcosm. This approach was designed to assess the
148 stimulatory effect of a sterilized organic waste on resident microbiota in the soil.
149 • Bioaugmentation I: the moisture content of the polluted soil was adjusted to 70
150 % as above and amended with SAS (4:1 mass ratio) to yield the SAS
151 microcosm. This approach was designed to assess the combined effect of both *A.*
152 *bisporus* and the indigenous SAS microbiota.
153 • Bioaugmentation II: to prepare this microcosm, sterilized SAS was inoculated
154 with 3 agar plugs (1 cm diameter) from a 14 day old culture of *A. bisporus*
155 grown on malt extract agar and incubated for 10 d at 20 °C. The colonized
156 matrix was mixed with the contaminated soil (1:4 mass ratio) and the moisture
157 content adjusted to 70 % as described above. This bioaugmentation approach,
158 called the Abisp microcosm, was designed to eliminate the contribution of the
159 SAS microbiota.

160 Each microcosm was carried out in triplicate and incubated at 20 °C for 0, 7, 21, 42 and
161 63 days under static conditions in the dark.

162 2.3 Extraction and Analysis of Ergosterol and PAH

163 Total ergosterol was extracted and analyzed as described in the method by Covino *et al.*
164 (2010b). Samples (0.5 g) were sonicated at 70 °C for 90 min with 3 ml methanolic
165 solution KOH (10 %, w/v). Distilled water (1 ml) was added to each sample and the
166 sample extracted three times with 2 ml of n-hexane. The solvent was evaporated under a
167 nitrogen stream and the solid residue dissolved in methanol (1 ml). The samples were
168 analyzed using high performance liquid chromatography (Waters 2695 Separation
169 Module) coupled with a Waters 996 photodiode array detector equipped with

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

173 The extraction of PAH was performed by pressurized liquid extraction (PLE) (ASE350,
174 Dionex). Soil samples (10 g) were loaded into the extraction cell (32 ml) and
175 subsequently extracted with a dichloromethane–acetone mixture (DAM, 1:1, v/v). Static
176 heating was applied to the vessel (100 °C, 5 min) and the extraction performed for 7 min
177 at the same temperature under 1500 psi. The cell was then flushed with 7 ml DAM and
178 finally the solvent purged from the cell with argon for 60 s. This extraction cycle was
179 repeated twice for each sample. The resultant organic extract was dried under gentle N₂
180 flow at room temperature and finally dissolved in acetonitrile. HPLC analyses were
181 performed using a system consisting of a 2695 Separations Module (Waters, Milford,
182 MA) equipped with a SuperguardTM LC-18 guard column (20 x 3 mm) prior to the
183 separation SupelcosilTM LC-PAH column (250 × 3.0 mm; particle size 5 μm) and a
184 2996 diode-array detector (Waters). Separation of the PAH was achieved using a
185 gradient elution program, using (A) acetonitrile and (B) Milli-Q water. The elution
186 program was: isocratic elution with 60 % (A) for 5 min, gradient to 100 % (A) for 15
187 min, isocratic elution at 100 % A for 20 min. The column temperature was fixed at 28
188 °C. The sample injection volume was 20 μl. The chromatograms were monitored at 254
189 nm. PAH were identified on the basis of both UV spectra and matching the retention
190 times with commercially available standards (Sigma-Aldrich). The PAH detected and
191 quantified were: fluorene (FLU); phenanthrene, (PHE), anthracene (ANT), fluoranthene
192 (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR),
193 benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP),
194 dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylene (BghiP), indeno[c,d]pyrene

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

197 2.4 Estimation of PAH Bioavailability

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

207 2.5 Biochemical Determinations and Toxicity Tests

208 Lignin-modifying enzymes (LME) were extracted from the remediation microcosms (3
209 g) at 5 °C for 1 h using the buffered solution described by D'Annibale et al. (2006). The
210 aqueous suspension was centrifuged (6000 g, 30 min) and the supernatant assayed for
211 LME activities.

212 Laccase activity was spectrophotometrically determined by following the oxidation of
213 0.2 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid in 100 mM sodium
214 acetate buffer (pH 4.5) at 420 nm ($\epsilon = 36000 \text{ M}^{-1} \text{ cm}^{-1}$). MnP activity was assayed by the
215 oxidation of 1 mM MnSO_4 in 50 mM sodium malonate buffer (pH 4.5) in the presence
216 of 0.1 mM H_2O_2 . Manganic ions, Mn^{3+} form a complex with malonate which absorbs at
217 270 nm ($\epsilon = 11590 \text{ M}^{-1} \text{ cm}^{-1}$) (Wariishi et al., 1992). One unit of enzyme activity (IU) is

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

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

$$235 \quad RCRA(\%) = \frac{\sum_{i=1}^{13} [PAH_i]_{t_0} \cdot DR \cdot TEF_i}{\sum_{i=1}^{13} [PAH_i]_{t_0} \cdot TEF_i} \cdot 100$$

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

238 2.6 Enumeration of Cultivable Heterotrophic and PAH-Degrading Bacteria

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

251 2.7. DNA Extraction

252 Whole genomic DNA from each soil was extracted using the Power Soil DNA
253 extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's
254 instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the
255 universal bacterial 341-f (5'-
256 CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG
257 GCAGCAG-3') and 534-r (5'-ATT ACC GCG GCT GCT GG-3') primers with a length
258 of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was
259 amplified using the universal fungal primers FUN_NS1 (5'-
260 GTAGTCATATGCTTGCTCTC-3') and GC fung (5'-
261 CGCCCGCCGCGCCCGCGCCCGGCCCCGCGCCCCCGCCCCGCCCCATTCCCCGTTAC
262 CCGTTG-3) (Das et al., 2007). The reaction mixture consisted of 2 µL of template
263 DNA (ca. 20 ng), Tris-HCl (20 mM, pH 8.3), KCl (100 mM), MgCl₂ (3 mM), Taq-

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

271 2.8. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

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

$$284 H' = - \sum (P_i \times \ln P_i)$$

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

288 3 Results and Discussion

289 3.1 Time- and Microcosm-Dependent Evolution of Fungal Biomass and Cultivable

290 Bacteria

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

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

304 No changes in the ergosterol content were observed throughout the incubation in the
305 Abisp microcosm which suggests that the sterilized SAS was neither an adequate carrier
306 for the subsequent growth of *A. bisporus* in the soil, nor a valuable trophic supplement
307 for the resident fungi. This could be explained by the fact that most of the available
308 organic components in the sterilized SAS had been consumed by the reinoculated *A.*
309 *bisporus* prior to its addition to the soil, resulting in a microcosm with a low nutrient
310 status. Therefore, the potential trophic contribution of the carrier to the competitive
311 ability of the added inoculum was impaired (Covino et al., 2010b; Lestan and Lamar,
312 1996).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

313 The density of the cultivable heterotrophic and PAH-degrading bacteria over time is
314 shown in Fig. 1B and 1C` respectively. Heterotrophic microbial counts in the SAS and
315 SSAS microcosms increased by approximately two orders of magnitude and were
316 significantly higher than those in the SM microcosm over the whole incubation period
317 (Fig. 1B). The same result was also found in the bioaugmented Abisp microcosm where
318 heterotrophic bacterial counts increased by more than one order of magnitude (Fig. 1B).
319 In the amended microcosms, the lowest densities of heterotrophic bacteria throughout
320 the incubation period were found in the Abisp microcosm. This may be explained by the
321 low nutrient input associated with this inoculant. Its preparation had involved the
322 reinoculation of *A. bisporus* into spent *Agaricus* substrate and subsequent incubation for
323 14 d prior to its application onto soil with ensuing consumption of organic nutrients by
324 the growing fungus. This is also corroborated by the observed increase in heterotrophs
325 in the other bioaugmented SAS microcosm where the addition of the inoculant ensured
326 a higher nutrient input than in Abisp. Similar results were also found for the cultivable
327 PAH-degrading bacteria. The highest densities were observed in the SSAS and SAS
328 microcosms (Fig. 1C). However, it should be noted that the spent *Agaricus* substrate
329 contained viable PAH-degrading bacteria which would have resulted in a higher initial
330 density in the SAS microcosm than in the SM (2.3×10^4 vs. 0.5×10^2 MPN g⁻¹) (Fig.
331 1C). In the SSAS microcosm, the addition of the sterilized organic waste also exerted a
332 high stimulation on the PAH-degrading bacteria which were increased by more than
333 three orders of magnitude after one and three weeks of incubation, although after this
334 their densities declined. As previously observed for heterotrophic bacterial counts in the
335 amended remediation microcosms, the Abisp exhibited the lowest densities of PAH-
336 degrading bacteria for most of the incubation period. Regardless of the different
337 application options in addition to leading to a higher density of heterotrophs, when

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

338 compared with the SM, it also enhanced specialized ones. As described above, the non-
339 sterile spent *Agaricus* substrate in the SAS microcosm contained allochthonous PAH-
340 degrading bacteria. This is not surprising because a molecular characterization of the
341 bacterial community in this substrate has revealed the presence of several genera
342 *Paenibacillus*, *Arthrobacter*, *Comamonas* and *Sphingobacterium*, (Ntougias et al., 2004;
343 Watabe et al., 2004) which included several species with reported PAH-degrading
344 capacity (Haritash and Kaushik, 2009). However, the addition of the sterilized SAS,
345 irrespective of whether it had been reinoculated with *A. bisporus* or not, also appeared
346 to stimulate PAH-degrading bacteria. Previous studies have also found that the addition
347 of sterilized organic wastes such as wheat straw and maize stalks activated the
348 specialized resident bacterial populations in soils contaminated with PAH (Lladó et al.,
349 2013), and polychlorobiphenyls (Federici et al., 2012a, 2012b). It has been suggested
350 that the stimulatory effect exerted by organic waste is due to an enhancement of the
351 oxygen transfer via an increase in soil porosity without necessarily involving either
352 trophic factors or modifications in contaminant bioavailability (Federici et al., 2012b).

353 3.2 Microcosm-Dependent Impact on Bacterial and Fungal Community Structure

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

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

386 3.3 PAH Removal in the Remediation Microcosms

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

387 Table 2 shows the initial and residual PAH concentrations in the remediation
388 microcosms after 63 days incubation. In the SM, no significant reduction in the total
389 PAH concentration was observed ($p=0.07$). Significant differences between the initial
390 and final concentrations were only found for FLT and PYR, with a percentage removal
391 of 65 %, and 59 % respectively. In the biostimulation SSAS microcosm a statistically
392 significant decrease ($p<0.05$) in 3-ring PAH content, FLU (75 %), PHE (90 %), ANT
393 (82 %), and FLT (52 %), was observed and the overall PAH residual content
394 significantly differed from that at the start. Therefore, the biostimulation treatment was
395 effective at degrading low molecular weight PAHs which were the most abundant
396 contaminants in the soil. In the bioaugmented microcosms, namely SAS and Abisp,
397 significant differences between the initial and the residual concentrations were found for
398 the majority of the individual contaminants as well as the overall PAH concentrations
399 (Table 2). These findings indicate that the bioaugmentation treatments with *A. bisporus*
400 were efficient at degrading both low and high molecular weight PAH.
401 A comparison of the degradation performances of SSAS, SAS and Abisp microcosms,
402 revealed that SSAS and SAS, which exhibited the highest density of PAH-degrading
403 bacteria, were very efficient at degrading low molecular weight PAH. However, Abisp
404 was superior to SSAS and SAS in the removal of highly condensed PAH (Table 2).
405 Therefore, for PAH which are potentially carcinogenic to humans or animals (IARC,
406 2010), the Abisp microcosm was more efficient than the SAS in degrading compounds
407 such as BaP and DBahA (52 vs. 18 % respectively, and 32 vs. 6 % respectively). In this
408 respect, a very limited number of bacteria able to grow in pure cultures on either 5- or 6-
409 ring PAH have been identified (Haritash and Kaushik, 2009).
410 However, in the SAS microcosm, the augmented fungus appeared to exert a lower
411 inhibitory action on the resident bacterial biota as deduced by comparing its better

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

437 3.5) might be as result of their ability to diffuse into the soil matrix and potentially
438 oxidise PAH with low bioavailability (Haritash and Kaushik, 2009). This hypothesis
439 may explain why the Abisp microcosm was able to partially degrade 5- and 6-ring PAH.
440 The IP has been shown to affect *in vitro* PAH oxidation by either laccase or fungal
441 heme-peroxidases, whose activities were found in the bioaugmented microcosms (see
442 subsection 3.4). The susceptibility of PAH to oxidation by fungal LME has been shown
443 to increase as the IP decreases (Majcherczyk et al., 1998). In the present study the
444 opposite trend was found when relating PAH depletions and respective IP values in all
445 the microcosms. However, the Pearson Coefficients related to these regressions, which
446 were significant with the exception of Abisp, were the lowest among the physico-
447 chemical properties tested (Table 4). Similar findings were obtained with two
448 historically PAH-contaminated soils augmented with either *Irpex lacteus* or *Lentinus*
449 *tigrinus* (Covino et al., 2010b). This clearly suggests that even in augmented
450 microcosms, LME-triggered PAH oxidation is not the sole mechanism involved in PAH
451 degradation. This was made particularly evident in the bioaugmented microcosms by
452 the high depletion extents of PHE and FLT, their high IP values (8.03 and 7.91 eV
453 respectively) make them poorly susceptible to mono-electronic oxidation by LMEs
454 (Majcherczyk et al., 1998; Mayolo-Deloisa et al., 2011). In addition to the action of the
455 PAH-degrading bacteria, it should be taken into account that PAH degradation in fungi
456 also relies on the intracellular cytochrome P-450/epoxide hydrolase complex whose
457 activity is not dependent on IP (Haritash and Kaushik, 2009).

458 3.4 Time- and Microcosm-Dependent Modifications in the PAH Bioavailable Fraction

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

487 3.5 Lignin-Modifying Enzyme Activity and Residual Toxicity in Remediation 488 Microcosms

489 The remediation microcosms were assayed for their ligninolytic activity due to the
490 involvement of these enzymes in the early oxidation steps of PAH (Li et al., 2010;
491 Majcherczyk et al., 1998). Among them, laccase and to a much lower extent Mn-
492 peroxidase activity (Fig. 2A and 2B, respectively) were only detected in the
493 bioaugmented microcosms (SAS and Abisp). The highest laccase and MnP activity
494 were found in the Abisp microcosms where the activity of the former enzyme markedly
495 declined with incubation time (Fig. 2A). Noteworthy, in this microcosm the best
496 depletions were observed for BaP and DBahA which despite being high molecular
497 weight PAH, are characterized by low IP values (i.e., 7.12 and 7.38 respectively) and
498 therefore highly susceptible to oxidation by both laccase and MnP (Bogan and Lamar,
499 1995). In this regard the results of the present study are in agreement with those of Li et
500 al. (2010) who showed high removal rates of BaP and DBahA by crude laccase extracts
501 from spent *Agaricus* substrate.

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

527 A comparison of the 63 day old microcosms showed that the best reduction in the
528 carcinogenic risk assessment (RCRA) was observed in the bioaugmented microcosms
529 (SAS and Abisp). This is a consequence of the higher reduction in the amount of 4- and
530 5,6-ring PAH which have the highest carcinogenic properties (IARC 2010). Significant
531 differences were also found when comparing the bioaugmented microcosms. The Abisp
532 microcosm showed higher RCRA than the SAS because of its better efficacy in the
533 removal of BaP and DBahA (Table 2) which exhibited the highest TEF values.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

534 **4 Conclusions**

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

545 **Acknowledgements**

546 This work was financially supported by the Ministry of Science and Innovation of Spain
547 (Project CTM2009-13140-C02-02). The authors wish to express their thanks to
548 Recomsa for providing the spent mushroom substrate and Impregna for providing the
549 soil.

550 **References**

- 551 Andersson, B., Welinder, L., Olsson, P., Olsson, S., Henrysson, T., 2000. Growth of
552 inoculated white-rot fungi and their interactions with the bacterial community in
553 soil contaminated with polycyclic aromatic hydrocarbons, as measured by
554 phospholipid fatty acids. *Bioresour. Technol.* 73, 29–36. doi: 10.1016/S0960-
555 8524(99)00134-0
- 556 Bogan BW and Lamar RT., 1995. One-electron oxidation in the degradation of creosote
557 polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl Environ*
558 *Microbiol* 61:2631–5.
- 559 Borràs, E., Caminal, G., Sarrà, M., Novotný, Č., 2010. Effect of soil bacteria on the
560 ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes*

- 561 versicolor and *Irpex lacteus* from contaminated soil. *Soil Biol. Biochem.* 42, 2087–
562 2093. doi: 10.1016/j.soilbio.2010.08.003
- 563 Covino, S., Svobodová, K., Cvancarová, M., D’Annibale, A., Petruccioli, M., Federici,
564 F., Kresinová, Z., Galli, E., Cajthaml, T., 2010a. Inoculum carrier and contaminant
565 bioavailability affect fungal degradation performances of PAH-contaminated solid
566 matrices from a wood preservation plant. *Chemosphere* 79, 855–864. doi:
567 10.1016/j.chemosphere.2010.02.038
- 568 Covino, S., Cvancarová, M., Muzikár, M., Svobodová, K., D’annibale, A., Petruccioli,
569 M., Federici, F., Kresinová, Z., Cajthaml, T., 2010b. An efficient PAH-degrading
570 *Lentinus (Panus) tigrinus* strain: effect of inoculum formulation and pollutant
571 bioavailability in solid matrices. *J. Hazard. Mater.* 183, 669–676. doi:
572 10.1016/j.jhazmat.2010.07.078
- 573 Covino, S., Svobodová, K., Kresinová, Z., Petruccioli, M., Federici, F., D’Annibale, A.,
574 Cvancarová, M., Cajthaml, T., 2010c. In vivo and in vitro polycyclic aromatic
575 hydrocarbons degradation by *Lentinus (Panus) tigrinus* CBS 577.79. *Bioresour.*
576 *Technol.* 101, 3004–3012. doi: 10.1016/j.biortech.2009.12.020
- 577 D’Annibale, A., Rosetto, F., Leonardi, V., Federici, F., Petruccioli, M., 2006. Role of
578 Autochthonous Filamentous Fungi in Bioremediation of a Soil Historically
579 Contaminated with Aromatic Hydrocarbons. *Appl. Environ. Microbiol.* 72, 28–36.
580 doi: 10.1128/AEM.72.1.28
- 581 Daniel, R., 2005. The metagenomics of soil. *Nat. Rev. Microbiol.* 3, 470–8. doi:
582 10.1038/nrmicro1160
- 583 Das, M., Royer, T. V, Leff, L.G., 2007. Diversity of fungi, bacteria, and actinomycetes
584 on leaves decomposing in a stream. *Appl. Environ. Microbiol.* 73, 756–67. doi:
585 10.1128/AEM.01170-06
- 586 Dawson, J.J.C., Godsiffe, E.J., Thompson, I.P., Ralebitso-Senior, T.K., Killham, K.S.,
587 Paton, G.I., 2007. Application of biological indicators to assess recovery of
588 hydrocarbon impacted soils. *Soil Biol. Biochem.* 39, 164–177. doi:
589 10.1016/j.soilbio.2006.06.020
- 590 Federici, E., Giubilei, M., Santi, G., Zanaroli, G., Negroni, A., Fava, F., Petruccioli, M.,
591 D’Annibale, A., 2012a. Bioaugmentation of a historically contaminated soil by
592 polychlorinated biphenyls with *Lentinus tigrinus*. *Microb. Cell Fact.* 11, 35. doi:
593 10.1186/1475-2859-11-35
- 594 Federici, E., Giubilei, M.A., Cajthaml, T., Petruccioli, M., D’Annibale, A., 2011.
595 *Lentinus (Panus) tigrinus* augmentation of a historically contaminated soil: Matrix
596 decontamination and structure and function of the resident bacterial community. *J.*
597 *Hazard. Mater.* 186, 1263–1270. doi: 10.1016/j.jhazmat.2010.11.128
- 598 Federici, E., Giubilei, M.A., Covino, S., Zanaroli, G., Fava, F., D’Annibale, A.,
599 Petruccioli, M., 2012b. Addition of maize stalks and soybean oil to a historically

- 600 PCB-contaminated soil: effect on degradation performance and indigenous
601 microbiota. *N. Biotechnol.* 30, 69–79. doi: 10.1016/j.nbt.2012.07.007
- 602 Gan, S., Lau, E. V, Ng, H.K., 2009. Remediation of soils contaminated with polycyclic
603 aromatic hydrocarbons (PAHs). *J. Hazard. Mater.* 172, 532–549. doi:
604 10.1016/j.jhazmat.2009.07.118
- 605 García-Delgado, C., Jiménez-Ayuso, N., Frutos, I., Gárate, A., Eymar, E., 2013a.
606 Cadmium and lead bioavailability and their effects on polycyclic aromatic
607 hydrocarbons biodegradation by spent mushroom substrate. *Environ. Sci. Pollut.*
608 *Res.* 20, 8690–9. doi: 10.1007/s11356-013-1829-0
- 609 García-Delgado, C., Yunta, F., Eymar, E., 2013b. Methodology for Polycyclic Aromatic
610 Hydrocarbons Extraction from Either Fresh or Dry Spent Mushroom Compost and
611 Quantification by High-Performance Liquid Chromatography–Photodiode Array
612 Detection. *Commun. Soil Sci. Plant Anal.* 44, 817–825. doi:
613 10.1080/00103624.2013.749439
- 614 Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic
615 hydrocarbons (PAHs): a review. *J. Hazard. Mater.* 169, 1–15.
616 10.1016/j.jhazmat.2009.03.137
- 617 Hundal, L.S., Thompson, M.L., Laird, D. a, Carmo, a M., 2001. Sorption of
618 phenanthrene by reference smectites. *Environ. Sci. Technol.* 35, 3456–61. doi:
619 10.1021/es001982a
- 620 IARC, 2010. IARC Monographs on the evaluation of carcinogenic risks to humans.
621 Some non-heterocyclic Polycyclic aromatic Hydrocarbons and some related
622 exposures., IARC monographs on the evaluation of carcinogenic risks to humans /
623 World Health Organization, International Agency for Research on Cancer. Lyon,
624 France.
- 625 Kotterman, M.J.J., Vis, E.H., Field, J.A., 1998. Successive Mineralization and
626 Detoxification of Benzo[a]pyrene by the White Rot Fungus *Bjerkandera* sp . Strain
627 BOS55 and Indigenous Successive Mineralization and Detoxification of
628 Benzo[a]pyrene by the White Rot Fungus *Bjerkandera* sp . Strain BOS55 and In.
629 *Appl. Environ. Microbiol.* 64, 2853–2858.
- 630 Krauss, M., Wilcke, W., Zech, W., 2000. Polycyclic aromatic hydrocarbons and
631 polychlorinated biphenyls in forest soils : depth distribution as indicator of
632 different fate. *Environ. Pollut.* 110, 79–88.
- 633 Leonardi, V., Giubilei, M.A., Federici, E., Spaccapelo, R., Sasek, V., Novotny, C.,
634 Petruccioli, M., D’Annibale, A., 2008. Mobilizing agents enhance fungal
635 degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous
636 bacteria in soil. *Biotechnol. Bioeng.* 101, 273–85. doi: 10.1002/bit.21909
- 637 Lestan, D., Lamar, R.T., 1996. Development of fungal inocula for bioaugmentation of
638 contaminated soils. *Appl. Environ. Microbiol.* 62, 2045–52.

- 639 Li, X., Lin, X., Zhang, J., Wu, Y., Yin, R., Feng, Y., Wang, Y., 2010. Degradation of
640 polycyclic aromatic hydrocarbons by crude extracts from spent mushroom
641 substrate and its possible mechanisms. *Curr. Microbiol.* 60, 336–42. doi:
642 10.1007/s00284-009-9546-0
- 643 Li, X., Wu, Y., Lin, X., Zhang, J., Zeng, J., 2012. Dissipation of polycyclic aromatic
644 hydrocarbons (PAHs) in soil microcosms amended with mushroom cultivation
645 substrate. *Soil Biol. Biochem.* 47, 191–197. doi: 10.1016/j.soilbio.2012.01.001
- 646 Liang, C., Dang, Z., Xiao, B., Huang, W., Liu, C., 2006. Equilibrium sorption of
647 phenanthrene by soil humic acids. *Chemosphere* 63, 1961–8. doi:
648 10.1016/j.chemosphere.2005.09.065
- 649 Lladó, S., Covino, S., Solanas, A.M., Viñas, M., Petruccioli, M., D’Annibale, A., 2013.
650 Comparative assessment of bioremediation approaches to highly recalcitrant PAH
651 degradation in a real industrial polluted soil. *J. Hazard. Mater.* 248-249, 407–414.
652 doi: 10.1016/j.jhazmat.2013.01.020
- 653 Lladó, S., Jiménez, N., Viñas, M., Solanas, A.M., 2009. Microbial populations related
654 to PAH biodegradation in an aged biostimulated creosote-contaminated soil.
655 *Biodegradation* 20, 593–601. doi: 10.1007/s10532-009-9247-1
- 656 Majcherczyk, A., Johannes, C., Hu, A., 1998. Oxidation of polycyclic aromatic
657 hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb.*
658 *Technol.* 22, 335–341.
- 659 Marín-Benito, J.M., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S.,
660 2012a. Dissipation of fungicides in a vineyard soil amended with different spent
661 mushroom substrates. *J. Agric. Food Chem.* 60, 6936–45. doi: 10.1021/jf301322h
- 662 Marín-Benito, J.M., Herrero-Hernández, E., Andrades, M.S., Sánchez-Martín, M.J.,
663 Rodríguez-Cruz, M.S., 2014. Effect of different organic amendments on the
664 dissipation of linuron, diazinon and myclobutanil in an agricultural soil incubated
665 for different time periods. *Sci. Total Environ.* 476-477, 611–21. doi:
666 10.1016/j.scitotenv.2014.01.052
- 667 Marín-Benito, J.M., Rodríguez-Cruz, M.S., Andrades, M.S., Sánchez-Martín, M.J.,
668 2012b. Assessment of spent mushroom substrate as sorbent of fungicides:
669 influence of sorbent and sorbate properties. *J. Environ. Qual.* 41, 814–822. doi:
670 10.2134/jeq2011.0437
- 671 Mayolo-Deloisa, K., Machín-Ramírez, C., Rito-Palomares, M., Trejo-Hernández, M.R.,
672 2011. Oxidation of Polycyclic Aromatic Hydrocarbons using Partially Purified
673 Laccase from Residual Compost of *Agaricus bisporus*. *Chem. Eng. Technol.* 34,
674 1368–1372. doi: 10.1002/ceat.201000205
- 675 Muyzer, G., de Waal, E.C., Uitterlinden, a G., 1993. Profiling of complex microbial
676 populations by denaturing gradient gel electrophoresis analysis of polymerase
677 chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*
678 59, 695–700.

- 679 Nisbet, I.C., LaGoy, P.K., 1992. Toxic equivalency factors (TEFs) for polycyclic
680 aromatic hydrocarbons (PAHs). *Regul. Toxicol. Pharmacol.* 16, 290–300.
- 681 Ntougias, S., Zervakis, G.I., Kavroulakis, N., Ehaliotis, C., Papadopoulou, K.K., 2004.
682 Bacterial diversity in spent mushroom compost assessed by amplified rDNA
683 restriction analysis and sequencing of cultivated isolates. *Syst. Appl. Microbiol.*
684 27, 746–54. doi: 10.1078/0723202042369857
- 685 Pardo-Giménez, A., Pardo-González, J.E., 2008. Evaluation of casing materials made
686 from spent mushroom substrate and coconut fibre pith for use in production of
687 *Agaricus bisporus* (Lange) Imbach. *Spanish J. Agric. Res.* 6, 683–690.
- 688 Pelaez, A.I., Lores, I., Sotres, A., Mendez-Garcia, C., Fernandez-Velarde, C., Santos, J.
689 a, Gallego, J.L.R., Sanchez, J., 2013. Design and field-scale implementation of an
690 “on site” bioremediation treatment in PAH-polluted soil. *Environ. Pollut.* 181,
691 190–199. doi: 10.1016/j.envpol.2013.06.004
- 692 Sayara, T., Sarrà, M., Sánchez, A., 2010. Effects of compost stability and contaminant
693 concentration on the bioremediation of PAHs-contaminated soil through
694 composting. *J. Hazard. Mater.* 179, 999–1006. doi: 10.1016/j.jhazmat.2010.03.104
- 695 Stokes, J.D., Wilkinson, A., Reid, B.J., Jones, K.C., Semple, K.T., 2005. Prediction of
696 polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an
697 aqueous hydroxypropyl-beta-cyclodextrin extraction technique. *Environ. Toxicol.*
698 *Chem.* 24, 1325–1330.
- 699 Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese
700 peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic
701 mechanism and role of chelators. *J. Biol. Chem.* 267, 23688–23695.
- 702 Watabe, M., Rao, J.R., Xu, J., Millar, B.C., Ward, R.F., Moore, J.E., 2004.
703 Identification of novel eubacteria from spent mushroom compost (SMC) waste by
704 DNA sequence typing: ecological considerations of disposal on agricultural land.
705 *Waste Manag.* 24, 81–86. doi: 10.1016/j.wasman.2003.08.001

706

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

707 **Figure Captions**

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

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

720

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_0) 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_0	t_f	t_0	t_f	t_0	t_f	t_0	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)

Table3[Click here to download Table: Table_3_DGGE.doc](#)

Table 3: Shannon Weaver Index (H'), richness (S) and evenness (E) values of bacterial and fungal communities at start (t₀) and at the end (t₆₃) 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 t₀	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 t₆₃	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 t₀	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 t₆₃	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 t₀	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 t₆₃	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 t₀	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 t₆₃	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 ($p \leq 0.05$): 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	$\log K_{oc}^{\S}$	$\log P^{\S}$	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†		<i>F. candida</i> mortality†	
	(%)	(IU kg ⁻¹)		(%)	
	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
[Click here to download Figure: Figure_1.doc](#)

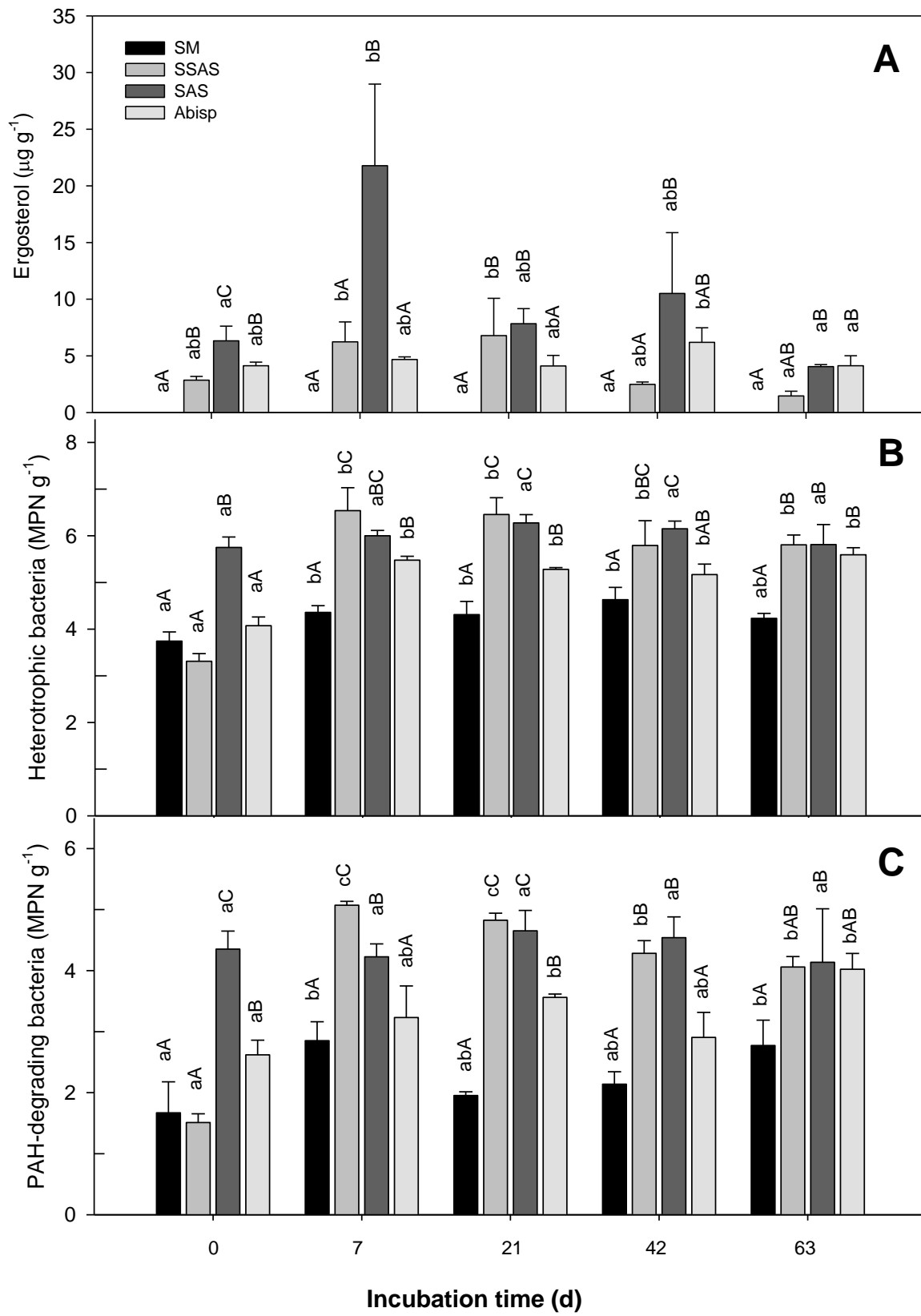


Figure 2
[Click here to download Figure: Figure_2.docx](#)

