1 **Bioremediation of PAHs-contaminated soil through composting: influence of**
2 **bioaugmentation and biostimulation on the contaminants biodegradation**

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26 **Abstract**

27 The degradation of several polycyclic aromatic hydrocarbons (PAHs) in soil
28 through composting was investigated. The selected PAHs included: fluorene,
29 phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene and chrysene, with
30 concentrations simulating a real creosote sample. The degradation of PAHs (initial
31 concentration 1 g of total PAHs kg^{-1} dry soil) was assessed applying bioaugmentation
32 with the white-rot fungi *Trametes versicolor*, and biostimulation using compost of the
33 source-selected organic fraction of municipal solid waste (OFMSW) and rabbit food as
34 organic co-substrates. The process performance during 30 days of incubation was
35 evaluated through different analyses including: dynamic respiration index (DRI),
36 cumulative oxygen consumption during 5 days (AT_5), enzymatic activity and fungal
37 biomass. These analyses demonstrated that the introduced *T. versicolor* did not
38 significantly enhance the degradation of PAHs. However, biostimulation was able to
39 improve the PAHs degradation where 89% of the total PAHs were degraded by the end
40 of the composting period (30 days) compared to only 29.5% that was achieved by the soil
41 indigenous microorganisms without any co-substrate (control, not amended). Indeed, the
42 obtained results showed that stable compost from the OFMSW has a greater potential to
43 enhance the degradation of PAHs compared to non-stable co-substrates such as rabbit
44 food.

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47 **Keyword:** Composting; Polycyclic Aromatic Hydrocarbons (PAHs); Bioaugmentation;
48 Biostimulation; Stability.

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1. Introduction

According to the U.S. Environmental Protection Agency, polycyclic aromatic hydrocarbons (PAHs) are recognized as priority pollutants (Mackay et al., 1992). These pollutants are introduced in the environment as a result of natural or anthropogenic activities (Johnson et al., 2005). PAHs deleterious properties like high toxicity and carcinogenicity have made their remediation a critical need. Today, several technologies are available to deal with these environmental contaminants (Khan et al., 2004). However, each technology has its own optimal operation conditions, which are modulated by the contaminant properties (physicochemical) or by the prevailing environment conditions. The cost of a remediation technology is a crucial factor for its implementation. In this regard, bioremediation, which principally relies on the microorganisms to degrade the target contaminants, is considered a promising technology because of its efficiency and cost-effectiveness. In this context, composting technology is one of the biological approaches applied for remediating PAHs-contaminated soil (Cajthaml et al., 2002; Sayara et al., 2009). Nevertheless, several factors such as the presence of specific degraders, toxicity, concentration, bioavailability and nutrients content are believed to influence the biodegradation of PAHs (Antizar-Ladislao et al., 2004; Cajthaml et al., 2005; Covino et al., 2010; Gandolfi et al., 2010; Sayara et al., 2010a; 2010b).

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For an effective bioremediation of PAHs, the overall degradation and removal rate of the contaminants must be faster than the natural attenuation processes (Mohan et al., 2008). Accordingly, bioremediation of contaminated soil is usually carried out either by stimulating the indigenous organisms through providing favorable environment or nutrients needed for increasing the microbial activity, or by bioaugmentation through introducing single strains or consortia of microorganisms with the desired catalytic

capabilities to improve the biodegradation process (Covino et al., 2010; Khan et al., 2004). In some cases, both biostimulation and bioaugmentation are simultaneously applied (Hamdi et al., 2007; Lang et al., 1998; Mrozik and Piotrowska-Seget, 2010; Mohan et al., 2008).

Several bacteria and filamentous fungi have been reported to detoxify and degrade PAHs (Boonchan et al., 1998; Hamdi et al., 2007; Borràs et al., 2010). In this regard, although the use of white-rot fungi as PAHs degraders has been extensively studied in liquid cultures, their application for bioremediation of contaminated soil still needs further investigation, especially in the case of treatments with complex-solid matrices such as composting. The ability of white-rot fungi as degraders to decompose several compounds including PAHs is attributed to their non-specific enzymatic system, including the ligninolytic enzymes and the cytochromes P450 (Hamdi et al., 2007; Borràs et al., 2010). For bioaugmentation purposes, a wide range of white rot fungi have been used to remediate PAHs-contaminated soils (Mrozik and Piotrowska-Seget, 2010; Mohan et al., 2008). Nevertheless, not all white-rot fungi are able to colonize PAHs polluted soil due to the competition with the indigenous microflora (Lang et al., 1998). Also, their degradation is correlated with the bioavailability of pollutants (Covino et al., 2010), which influences the overall process behavior.

In spite of some drawbacks/complexity of bioaugmentation, using fungi for the bioremediation process is receiving more attention as they are rapidly incorporated by the soil matrix. Also, they have the ability to grow in environments with low nutrient concentration, low humidity and acidic conditions (Mollea et al., 2005). Several studies have described the successful application of bioaugmentation in soil remediation processes with different organic contaminants (Covino et al., 2010; Mohan et al., 2008; Teng et al., 2010). Synergistic degradation by white-rot fungi and bacteria can also occur

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101 during the bioremediation of PAH-contaminated soil since fungi can initially cleave the
102 aromatic ring and then bacteria are able to further degrade the resulting metabolites.
103 However, in some studies the introduced microorganisms fail at degrading or enhancing
104 the depletion of the target contaminants (Karamalidis et al., 2010; Silva et al., 2009;
105 Wiesche et al., 2003).

106 During the composting process and as a result of the microbial activity, the
107 temperature increases to reach the thermophilic range (> 45ºC), especially when easily
108 biodegradable organic matter is available in the composted matrix (Ruggieri et al., 2008).
109 Consequently, the tolerance of the implanted exogenous microorganisms to such
110 temperature will be a key factor. Also, the introduced microorganisms find themselves in
111 a new environment where they have to compete with the indigenous microorganisms
112 (Lang et al., 1998).

113 The present study investigates the impact of bioaugmentation and biostimulation
114 on the bioremediation of PAHs-contaminated soil. Bioaugmentation was carried out
115 using a white-rot fungus (*Trametes versicolor* ATCC 42530), whereas biostimulation was
116 performed using two organic co-substrates that differ significantly on the basis of their
117 degree of stability and their organic content. Thus we used a compost obtained from the
118 organic fraction of municipal solid waste (OFMSW) and rabbit food, which was
119 proposed to serve as an easily available lignocellulosic substrate. Together with this study
120 we examined the effect of the degree of stability and of the organic matter content along
121 with the impact of adding an exogenous microorganism on the performance of a PAH-
122 remediation process.

123

124 **2. Materials and methods**

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126 *2.1 Soil*

127 The soil used in this experiment was an agricultural soil collected from the surface
128 horizon (0-30 cm) in Prades (Tarragona, Spain). Texture analysis demonstrated that it
129 consisted of 73.4% sand, 18.6% silt and 8% clay. It was classified as sandy loam soil
130 according to the U.S. Department of Agriculture classification. It was air-dried and
131 sieved to 2 mm to remove any debris and kept at 4˚C until use. Preliminary analysis
132 demonstrated that the soil was uncontaminated as no PAHs were detected before being
133 used in the experiments. Other properties of the soil are presented in Table 1.

134

135 *2.2 PAHs contaminants*

136 Contaminated sites are commonly found to be polluted by several types of
137 creosote components. Consequently, different PAHs listed among the 16 US EPA
138 priority pollutants that are typically found in contaminated soils were obtained from
139 Sigma-Aldrich (Spain), to be used as target soil-contaminants. These PAHs include:
140 fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene and
141 chrysene. A stock mixture solution was prepared by mixing each PAH in the required
142 ratio to obtain an individual weight percentage (w/w) respectively 30%, 28%, 9%, 20%,
143 3.5%, 3% and 6.5%. These percentages were chosen to mimic the concentration that
144 prevailed in a real creosote sample (Creosote lot: 42-13B, Chem Service, SUGELABOR
145 S.A, Spain) and which was determined using the Method 3611B of the US
146 Environmental Protection Agency, where the volatile part was ignored. The stock
147 solution contained a total amount of 7 g of the PAHs mixture dissolved in 1000 ml of
148 dichloromethane. Afterwards, the stock solution was spiked into the soil to obtain a
149 resulting concentration of 1 g of PAHs kg^{-1} of dry soil in all the experiments. The soil
150 was left until dichloromethane was completely evaporated (1 day).

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152 *2.3 Organic co-substrates*

153 Compost derived from the source-selected OFMSW and rabbit food (alfalfa 30%,
154 wheat husk 30%, barley 9%, soy 8%, beet 4% and impurities 11%; in weight percentage)
155 in the form of pellets were used as organic co-substrates and inoculum carrier
156 (bioaugmented treatments) during the experiments. Compost was obtained from a
157 composting plant located in Barcelona (Spain), whereas rabbit food was obtained from a
158 commercial market (Suprem, Barcelona). These two co-substrates were selected to
159 evaluate the effect of the organic matter stability degree on the bioremediation processes.
160 On the basis of their dynamic respiration index (DRI) the degree of stability of these co-
161 substrates differed significantly. Moreover, the two co-substrates presented a notable
162 difference in their organic matter content, which presumably supplies organic chemicals
163 that are lacking in soil but essential to support microbial growth and catabolic activities
164 towards PAHs. The major properties of the co-substrates are presented in Table 1.

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166 *2.4 Fungal strain preparation*

167 The fungus *T. versicolor* ATCC 42530 was acquired from the American Type
168 Culture Collection. The strain was maintained by sub-culturing every 30 days on 2% malt
169 extract (w/v) agar slants (pH 4.5) at 25 °C. Fungal mycelial suspensions were obtained by
170 blending the mycelium grown for 7 days on a malt extract medium that contained 20 g l^{-1}
171 of malt extract, and the pH was adjusted to 4.5 with NaOH or HCl.

172

173 *2.5 Laboratory-scale composting reactors*

174 The composting process was set up using Dewar® vessels with an operation
175 capacity of 4.5 l. The vessels were modified and conditioned to operate in batch-mode.

176 According to previous works, these reactors proved their efficiency to simulate
177 composting processes. More details about the composting system configuration including
178 the reactors and the monitoring tools can be found elsewhere (Sayara et al., 2009).

179

180 *2.6 Composting experimental system*

181 The artificially contaminated soil prepared as described in section 2.2 and
182 containing 1 g of the PAH mixture kg^{-1} soil was manually mixed with the organic co-
183 substrates at a ratio 1:0.25 (soil:co-substrate, dry weight). In treatments where the
184 bioaugmentation was to be evaluated, the inoculum (*T. versicolor*) was introduced (1 ml
185 of fungal suspension per 3 g of co-substrate) in the mixture. The fungal inoculum was set
186 to contain a biomass corresponding to approximately 3.5 µg of ergosterol ml^{-1}. In order to
187 ensure aerobic conditions a bulking agent consisting of wood chips was introduced at a
188 ratio of 1:1 (v/v). This bulking agent was considered as non-biodegradable under
189 laboratory conditions. In all treatments, tap water was added during the preparation of the
190 composting mixture to modify the water content according to the recommended values
191 for the composting process (50-60%). All the composting experiments were carried out in
192 duplicates during 30 days of incubation. Previous experiments using this composting
193 system have shown that a 30 day period was sufficient to achieve a significant
194 degradation of most PAHs (Sayara et al., 2010a; Sayara et al., 2010b). The experimental
195 set up was as follows:

196 Treatment (1): contaminated soil + *T. versicolor* + compost + bulking agent

197 Treatment (2): contaminated soil + *T. versicolor* + rabbit food + bulking agent

198 Treatment (3): contaminated soil + compost + bulking agent

199 Treatment (4): contaminated soil + *T. versicolor* + sterile compost + bulking agent

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200 No treatment using only rabbit food alone (without *T. versicolor*) was included since
201 previous results based on factorial experimental designs studies had clearly demonstrated
202 that the addition of rapidly biodegradable organic matter alone to soil inhibited the PAHs
203 biodegradation process (Sayara et al., 2010a; Sayara et al., 2010b). Duplicate control (C)
204 treatments, consisting of contaminated soil (1 g of PAHs kg^{-1} of dry soil) alone were used
205 to monitor the PAHs biodegradation by indigenous microorganisms without any additive.
206 For treatment 4, the compost was sterilized by autoclaving at 121ºC for 30 min. Previous
207 works had demonstrated that these conditions were sufficient to obtain a practical
208 disappearance of the compost respiration activity (Pagans et al., 2007).
209
210 *2.7 Sampling*
211 The degradation of PAHs was monitored after 5, 10, 20 and 30 days of
212 composting. Before sampling, the reactors content was homogenized by manual mixing
213 and representative samples of about 30-40 g were collected and used for carrying out the
214 analyses. Remixing the composting matrix during sampling is also necessary to re-
215 establish the porosity, which decreases as a result of compaction (Ruggieri et al., 2008)
216 and organic matter degradation. Also, water content of the composting treatments was
217 adjusted if necessary at the same time.
218
219 *2.8 Analytical methods*
220 Moisture content, organic matter content (OM), Kjeldahl nitrogen, total carbon
221 content, pH and electrical conductivity were determined according to the standard
222 methods (The US Department of Agriculture and The US Composting Council, 2001).
223 All the results are presented as average of duplicates with standard deviation.
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2.9 Respiration indices

These tests were used to evaluate and compare the microbial activity prevailing in the applied co-substrates and the composting mixtures of each treatment. Specifically, a dynamic respirometer was built as described by Adani et al. (2006). Briefly, about 150 g of sample were placed in a 500 ml Erlenmeyer flask and incubated in a water bath at 37˚C. Meanwhile previously-humidified air was continuously supplied to the sample to ensure aerobic conditions. Two respiration indices were calculated from the oxygen vs. time curve:

I) Dynamic respiration index (DRI): this value represents the average oxygen uptake rate during the 24 hours of maximum activity measured as oxygen uptake rate (OUR).

II) AT_5: it represents the cumulative oxygen consumption during 5 days of maximum respiration activity without considering the lag phase.

Both DRI and AT_5 are expressed in mg O_2 g^{-1} OM h^{-1} and mg O_2 g^{-1} OM, respectively. More details about the respiration test and the system configuration can be obtained elsewhere (Ponsá et al., 2010a).

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2.10 PAHs and PAHs metabolites analyses

PAHs in the composting treatments were quantified by gas chromatography (GC) according to a protocol described previously (Sayara et al., 2010b). PAHs metabolites were identified by GC-MS using an Agilent HP 6890 Series II GC coupled to a mass selective detector by electronic impact ionization (Agilent HP 5973) and a HP5-MS (Agilent) column (30 m x 0.25 mm x 0.25 μm). The operating conditions of the chromatograph were as follows: injector (splitless, 1 min) 320ºC, injection volume 1-3 μl (depending on the sample), oven temperature 50°C (1 min), ramp 7°C min^{-1}, final

250 temperature 320ºC and carrier gas He at 0.7 ml min^{-1}. The detector worked in a solvent
251 delay mode (3.2 min) and the mass range measured was 40-400 (m/z). The detected
252 products were identified by comparing the mass spectra with data from the Wiley 7®
253 library.
254
255 *2.11 Laccase extraction and quantification*
256 The extraction of laccase was carried out according to a modified method
257 described by Lang et al. (1998). 1.5 ml of the extract were transferred to Eppendorf vials
258 and centrifuged at 15,000 x g for 15 min. The supernatant was then analyzed. Laccase
259 activity was measured using the first step of the method for determination of manganese
260 peroxidase (MnP) (Wariishi et al., 1992), where 2,6-dimethoxy phenol (DMP) is oxidized
261 by laccase. One unit of activity (AU) was defined as the number of micromoles of DMP
262 oxidized per min. The DMP extinction coefficient is 24,800 $M^{-1}cm^{-1}$.
263
264 *2.12 Ergosterol extraction and quantification*
265 Ergosterol was analyzed in homogeneously-mixed samples of the soil-phase
266 cultures according to Borràs et al. (2010). 0.5-0.8 g from each sample were removed and
267 placed in a test tube to be extracted with a mixture of 1 ml cyclohexane and 3 ml of
268 KOH-methanol solution (10% w/v) for 90 min at 70°C. Ultrasonication was applied for
269 the first 15 min (Selecta, Spain). Then 1 ml of distilled water and 2 ml of cyclohexane
270 were added; the tube was vortexed for 30 s and centrifuged at 3,500 rpm for 5 min. The
271 organic phase was recovered and the aqueous phase was washed twice with 2 ml of
272 cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen
273 gas. The dry sterol residue was dissolved in 1 ml methanol for 15 min at 40 °C, vortexed
274 for 30 s and centrifuged in Eppendorf vials at 6000 rpm for 3 min. Finally the resulting

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275 solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC
276 equipped with an UV detector at 282 nm, using a reverse phase Grace Smart RP18
277 column (250 mm × 4 mm, particle size 5 µm). Methanol was isocratically supplied at 1
278 ml min^{-1}. The ergosterol content was expressed in micrograms per gram of solid dry
279 weight (µg g^{-1}, dry weight).

280

281 **3. Results and discussion**

282 *3.1 Characteristics of the organic co-substrates*

283 As shown in Table 1, the organic co-substrates have a considerable source of
284 organic matter that presumably can support the microbial activity needed for the
285 bioremediation process. However, it is noteworthy that rabbit food is richer in organic
286 matter (91.4%) than compost (43.5%), where the total organic carbon represents 48.78%
287 compared to 25.25% in compost. These contents clearly contributed to the different DRI
288 values, which was very high (6.5 mg O_2 g^{-1} OM h^{-1}) for rabbit food compared to the
289 OFMSW compost (1.12 mg O_2 g^{-1} OM h^{-1}) considered stable. It is noteworthy that the
290 stability degree of an organic substrate can reflect the availability of some chemical
291 components like humic matter in the organic substrate. Such property is believed to play
292 a major role in the soil bioremediation process as it facilitates PAHs desorption and
293 consequently make them more available for the microorganisms (Margesin and Schinner,
294 1997; Sayara et al., 2010b).

295

296 *3.2 The composting process*

297 Fig. 1 shows the evolution of the temperature profiles during the composting
298 process. As expected for a composting process, an initial rise of the temperature was the
299 result of the exothermal oxidation process caused by the microbial metabolism (Ruggieri

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300 et al., 2008). The temperature profiles varied among the different treatments during the
301 first 10 days, but afterwards the patterns were similar for all treatments as easily
302 biodegradable fraction was depleted. In treatment 2, the temperature reached the
303 thermophilic range after the first week whereas all other treatments remained in the
304 mesophilic range. This difference reflects the availability of easily biodegradable
305 materials in treatment 2 (Table 1). However, as the available organic matter was depleted,
306 the temperature decreased and the composted materials moved forward to the maturation
307 phase. In this context, temperature profiles in treatments 1 and 3 were similar, whereas in
308 treatment 4 with sterilized compost, the temperature increased to a lesser extent
309 compared to non-sterilized compost (treatments 1 and 3). This is likely due to the absence
310 of the compost microorganisms as a result of sterilization. The DRI values (Fig. 2)
311 corroborated the temperature behavior of each treatment, indicating the suitability of the
312 DRI test to monitor the progress of the organic matter stability during the composting
313 process (Ponsá et al., 2010b).

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315 *3.3 Degradation of PAHs*

316 Fig. 3 shows the remaining PAHs (as total PAHs) after 5, 10, 20 and 30 days of
317 composting. Degradation yields reaching 89 % after 30 days were obtained in treatments
318 1 and 3 where compost was added as co-substrate. Nevertheless, degradation rate of 71%
319 was achieved in treatments with rabbit food as co-substrate, whereas only 29.5% of the
320 PAHs were degraded in the control (C). Although four duplicate treatments were
321 performed, a correlation coefficient of 0.95 was obtained. Obviously, treatments with
322 compost as co-substrate followed the same trend whether they were augmented with *T.*
323 *versicolor* or not, indicating that this organism did not contribute significantly to
324 enhancing the bioremediation process. Moreover, in treatment 4, the degradation rate was

325 slightly lower than for treatments 1 and 3, which might reflect a likely contribution of the
326 compost microorganisms to the degradation of PAHs since the compost of treatment 4
327 was previously sterilized. These results are in accordance with previous reports where
328 composted OFMSW demonstrated a high capacity to enhance the biodegradation of
329 PAH-contaminated soils compared to the other amendments (Gandolfi et al., 2010;
330 Sayara et al., 2009; Tejada et al., 2008). Results which draw attention are those obtained
331 in treatment 2, which differed significantly from the other treatments. During the first 10
332 days, the rate of PAH depletion was similar for all treatment as a results of the
333 degradation of low molecular weight PAHs. Additionally, thermophilic temperature
334 during the first period (Fig. 1) could have facilitated the volatilization of some of these
335 low molecular weight PAHs (Margesin and Schinner, 1997). Then, the temperature
336 increase to high levels (> 55˚C) in treatment 2 might have affected/inhibited both the
337 bacterial and fungal activities, slowing down further degradation of the PAHs (Sayara et
338 al., 2009). On the other hand, the microbial community might have exhibited a preference
339 for the more easily degradable substrate over the more recalcitrant ones. Consequently
340 lower degradation rates were obtained at the end of the process. In this regard, co-
341 substrate appears to be an important factor affecting the efficiency of the bioremediation,
342 which is most likely dependent on the selectivity of the components and degree of
343 stability of the co-substrate, rather than on its organic matter content (Sayara et al., 2009;
344 2010b).

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345 Although the indigenous soil microorganisms were able to degrade some of the
346 PAHs as noticed in the control experiment (29.5%), providing favorable conditions for
347 these microorganisms are needed to enhance their activity. It is interesting to highlight
348 that the degradation of PAHs was found to be fast during the first 10 days, but during the
349 last stage it followed a slower removal rate, which was most likely attributed to the

350 depletion of the nutrients needed for the microbial activity. The same behavior has been
351 documented in previous works (Margesin et al., 2000; Cajthaml et al., 2002; Hamdi et al.,
352 2007; Hafidi et al., 2008; Silva et al., 2009; Sayara et al., 2010b).

353 Bioaugmentation has been applied in an attempt to accelerate the degradation rate.
354 Unfortunately, the addition/inoculation of *T. versicolor* did not enhance the remediation
355 process as the same trend was followed in the treatments where compost alone was
356 applied. These results corroborate other studies whereby fungi were used unsuccessfully
357 to enhance the degradation of PAHs (Baheri and Mysami, 2002; Karamalidis et al., 2010;
358 Silva et al., 2009; Wiesche et al., 2003). Indeed, native soil microorganisms and the
359 microbial flora from the OFMSW compost were presumably better adapted to this
360 particular environment. Regarding this point, it must be also considered that although
361 fungi, and particularly *T. versicolor*, is the focus of the study, efficient degradation of
362 PAHs can be achieved by prokaryotes, as it has been referred in previous studies (Al-
363 Mailem et al., 2010; HuiJie et al., 2011). Besides providing active microorganisms, stable
364 organic co-substrates contribute improving the degradation rate by providing humic
365 matter that facilitates the desorption of PAHs (Gandolfi et al., 2010; Karamalidis et al.,
366 2010; Sayara et al., 2010a; 2010b; Tejada et al., 2008).

367 The effect of incubating the compost at a fixed temperature of 37ºC on the
368 degradation of PAHs was evaluated using ideal conditions as described in section 2.9.
369 Under those conditions, the remaining PAHs after five days was 28%, 47%, 38% and
370 48% for treatments 1, 2, 3 and 4 respectively. Accordingly, a fixed temperature of 37 ˚C
371 was found to enhance the degradation process to a notable extent in treatment 1 compared
372 to treatment 3 suggesting that in this situation, both the exogenous and indigenous
373 microorganisms contributed synergistically to the degradation of PAHs although no
374 enzymatic laccase activity was observed during the incubation period. On the contrary, in

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375 treatment 2 and under the same conditions, less degradation was obtained in spite of the
376 easily available organic matter. The amount of oxygen consumed during 5 days in
377 treatment 2 was higher than for the other treatments (Fig. 4), however, the activity was
378 most likely reflecting the degradation of the easily degradable organic matter rather than
379 the PAHs. These observations confirm that stable organic co-substrate and mesophilic
380 temperature are most suitable for this bioremediation process (Haderlein et al., 2006;
381 Sayara et al., 2010a; 2010b).
382
383 *3.4 Respiration tests*
384 Microbial activity in each treatment was monitored by measuring its DRI at the
385 beginning and at the end of the incubation period (after 30 days) (Fig. 2). AT_5 was also
386 determined for each treatment (Fig. 4). This information is a useful indicator of the
387 microbial activity found within the treatments reflecting any change that could take place
388 in the treated materials (Ponsá et al., 2010a). The initial microbial activity of the
389 treatment with rabbit food (treatment 2) was high when compared to other treatments
390 (Fig. 2 and Fig. 4). However, the composting period was able to stabilize the material.
391 Thus, DRI values are similar for all treatments by the end of the process. It is important
392 to point out that bioaugmentation did not affect the microbial activity, since the initial
393 DRI values were very similar for treatments 1, 3 and 4. This is corroborated by the AT_5
394 values. Excluding the AT_5 value of treatment 2, the rates of PAHs biodegradation after 5
395 days are in accordance with AT_5 values, where treatments 1 and 3 presented almost the
396 same results. Nevertheless, it is noteworthy to mention that treatment 4 was less active
397 (Fig. 4) due to the presence of sterilized compost, which was reflected by a lower AT_5
398 value and lower level of PAHs degradation (30%) (Fig. 3) during that period. Usually, as
399 the first period of bioremediation is characterized by a rapid decrease in the contaminants

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400 concentration, especially those of low molecular weight, (Hamdi et al., 2007; Sayara et
401 al., 2010b; Silva et al., 2009), the values of AT_5 were a reliable measure of the biological
402 activity within the composted materials.

403

404 *3.5 Enzymatic activity and fungal growth*

405 As some of the PAHs are characterized by their low bioavailability (chrysene,
406 benzo(a)anthracene), we have introduced *T. versicolor* with an extracellular enzymatic
407 system in an attempt to enhance their degradation. Normally, *T. versicolor* produces
408 laccase which is the enzyme presumed to be involved in the degradation of PAHs.
409 However, laccase was not detected in our assays. Therefore, the resulting biodegradation
410 was probably caused by the indigenous microorganisms of the composted materials.
411 However, since in this study no samples were collected before 5 days, we are unable to
412 conclude whether the implanted fungus has any effect on the degradation of PAHs before
413 this time.

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414 The success when introducing exogenous microorganisms is not always totally
415 guaranteed, especially in the case of white-rot fungi, which are not soil microorganisms
416 (Borràs et al., 2010). For instance, introducing an adequate co-substrate is usually more
417 efficient as the added compost is most likely simultaneously providing both the
418 microflora (bioaugmentation) and nutriments (biostimulation) (Sayara et al., 2009).
419 According to Lang et al (1998) and Mrozik and Piotrowska-Seget (2010), several biotic
420 factors can influence the bioaugmentation process, being usually the competition between
421 the indigenous and exogenous microorganisms for the limited carbon sources as well as
422 the antagonistic interactions and predation by protozoa and bacteriophages. Also, native
423 species diversity may act as a resistance barrier to the invasion of non-native species

424 (Kennedy et al., 2002). These factors play an essential role in the bioaugmentation
425 process and its final results.

426 Regarding to the fungal biomass that was measured in terms of ergosterol per
427 grams of soil, which is an important indicator of the viable biomass, it was found present
428 in all soil treatments but varied largely during the first 10 days depending on the used
429 amendment (Fig. 5). In treatment 2, it can be seen that the biomass content quickly
430 increased because of the availability of high amounts of easily biodegradable organic
431 matter (Table 1) and an adequate aeration that were favorable for fungal growth
432 conditions. This was corroborated by the temperature raise (Fig. 1) and the high oxygen
433 consumption (Fig. 4). In treatments 1 and 3 the fungal biomasses were similar. This is
434 likely due to the fact that the implanted fungus had to compete with the indigenous
435 microorganisms.

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437 *3.6 Identification of degradation products*

438 Aerobic biodegradation of the studied PAHs was additionally followed by
439 monitoring the metabolites. The presence of these metabolites can be considered an
440 indicator of the bioremediation process, although the PAH load can be reduced through
441 humification processes involving organic matter from soil and from compost (Ferrarese
442 et al., 2008). Polar metabolites (Table 2) derived from anthracene and fluorene were
443 identified in all treatments. The main product of fluorene was 9H-fluorenone that was
444 detected at days 5 and 10. At day 20, 9H-fluorenone was not detected but 9H-fluorenol
445 was detected. These two metabolites have been described as metabolites from the
446 degradation of fluorene by white-rot fungi (Bezalel et al., 1996). Two major metabolites
447 were detected from anthracene: anthrone and anthraquinone. Both compounds have
448 previously been reported as by-products during metabolism of anthracene by white-rot

449 fungi (Hu et al., 2009). The products from biodegradation of the other PAHs could not
450 be detected although its overall concentration decreased in the soil. One hypothesis
451 could be that degradation products were bonded to organic matter of soil or were further
452 degraded by fungi or native microflora.

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590 **Tables**

591

592 **Table 1:** Characteristics of the used organic amendments and soil.

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Parameter/Material	Soil	Compost	Rabbit food
Moisture content (%, wb) *	6.64±0.01	32.82±0.2	11.23±0.12
Organic matter content (%, db) **	3.68±0.35	43.54±0.16	91.54±0.07
Total Organic Carbon (%, db) **	1.26±0.02	25.27±0.33	48.78±2.36
Total Kjeldahl Nitrogen (%, db)**	0.65±0.14	2.14±0.51	3.13±0.33
pH	6.7±0.02	8.37±0.01	6.01±0.14
Elec. conductivity (mS/cm)	0.2±0.01	4.92±0.13	3.99±0.23
Dynamic Respiration Index (mg O_2 g^{-1} OM h^{-1})	-	1.12±0.08	6.52±0.11

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596 * wb: wet basis.
597 ** db: dry basis.

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601 **Table 2:** Identification of metabolites produced during the composting process.
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Retention time (min)	Molecular mass	m/z of fragment ions (relative intensity)	Structural suggestion
20.09	180	180 (100), 152 (42.1), 126 (7.5), 98 (3), 76 (12.6), 63 (7.4), 50 (2.6)	9-fluorenone
20.14	182	181 (100), 165 (18.6), 152 (52.3), 139 (3.5), 126 (6.2), 91 (6.1), 76 (20.2), 63 (7.2), 51 (4.1)	9-H-fluoren-9-ol
25.54	208	208 (98.7), 180 (100), 152 (79.7), 126 (7), 99 (2.7), 76 (28.9), 63 (5.9), 50 (11.4)	9,10-anthraquinone

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612 **Legends to Figures**:

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614 **Figure 1**: Temperature profiles during the composting process.

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616 **Figure 2**: Initial and final (after 30 days) values of the dynamic respiration index (DRI)

617 of the composted materials.

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619 **Figure 3**: Remaining PAHs (%) after 5, 10, 20 and 30 days of composting.

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621 **Figure 4**: Values of the cumulative oxygen consumption during 5 days (AT_5) in the

622 treatments.

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624 **Figure 5**: Fungal biomass evolution in the different treatments during the composting

625 process. Initial fungal biomass was not available for the different treatments.

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629 Fig.1

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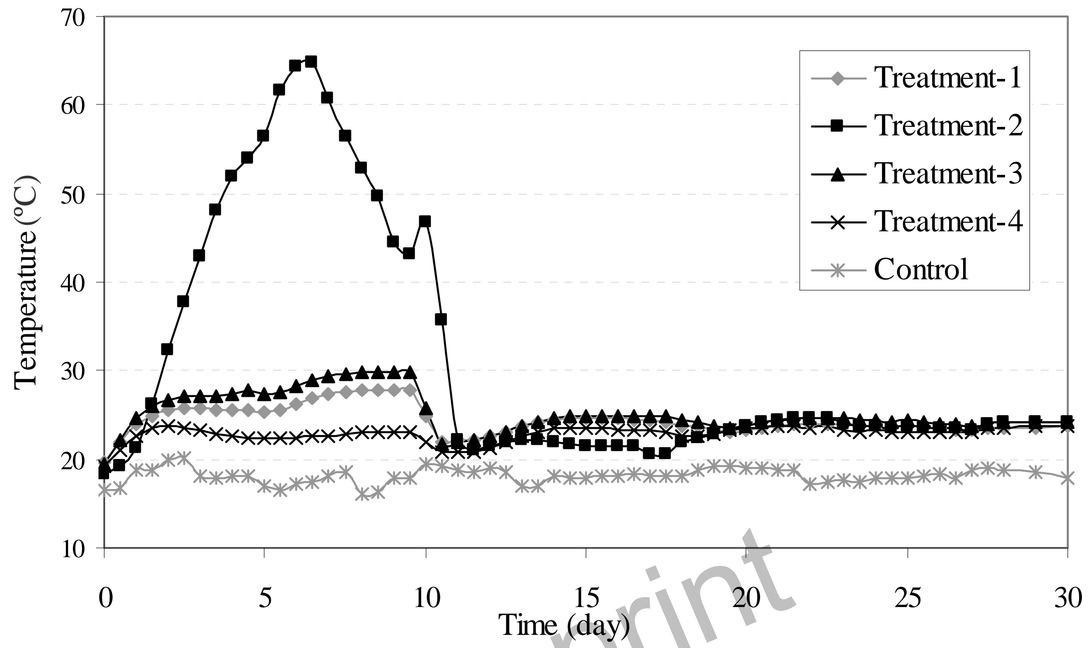

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635 Fig.2

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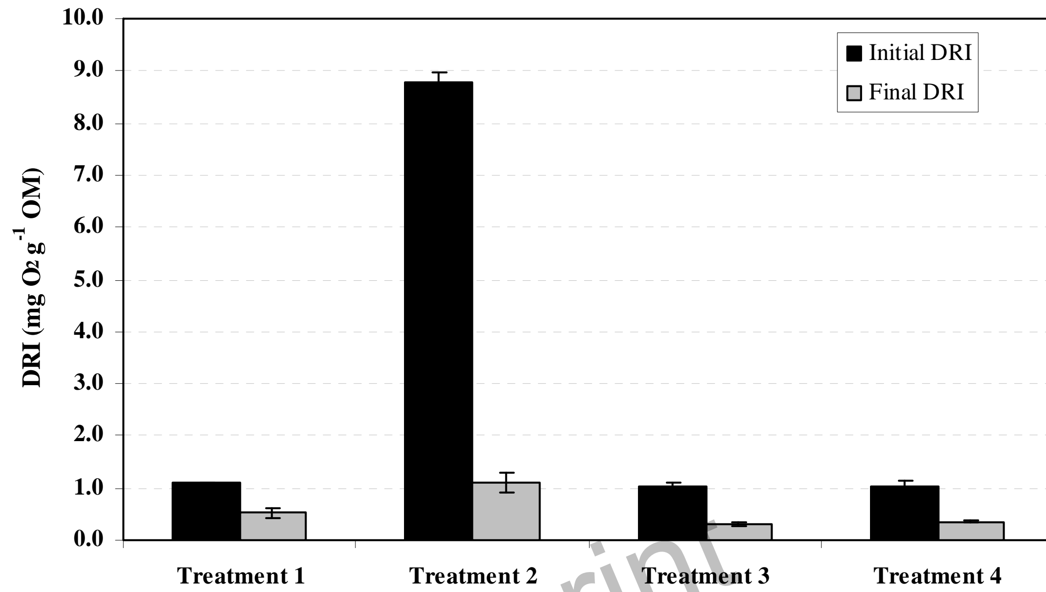

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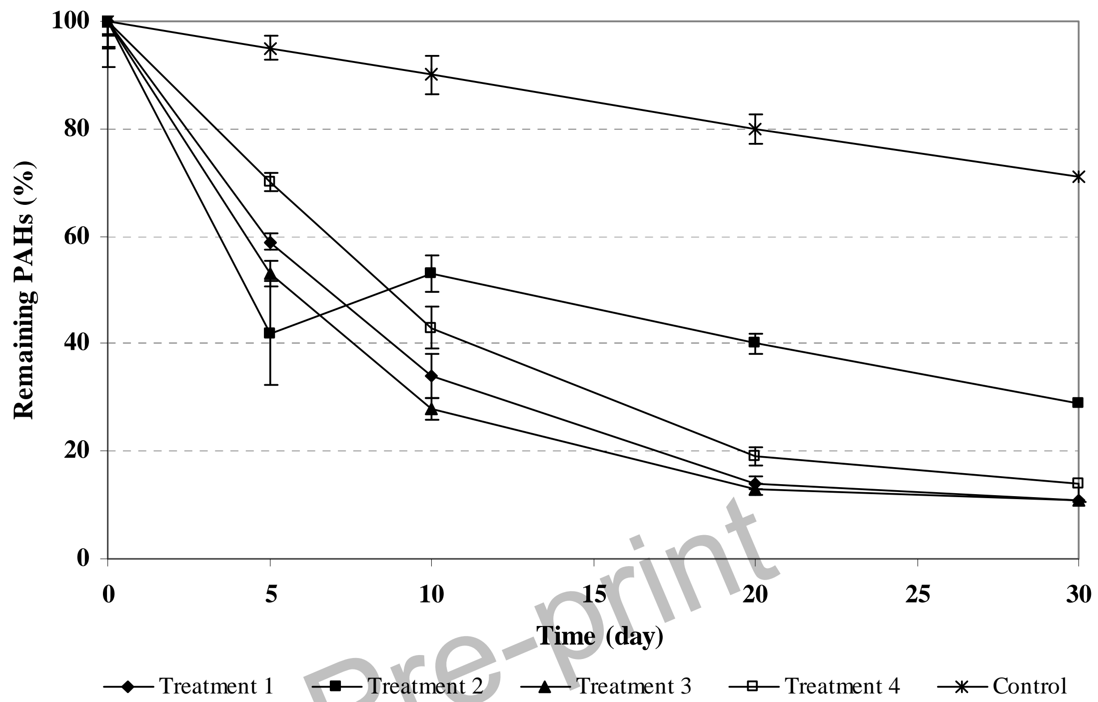

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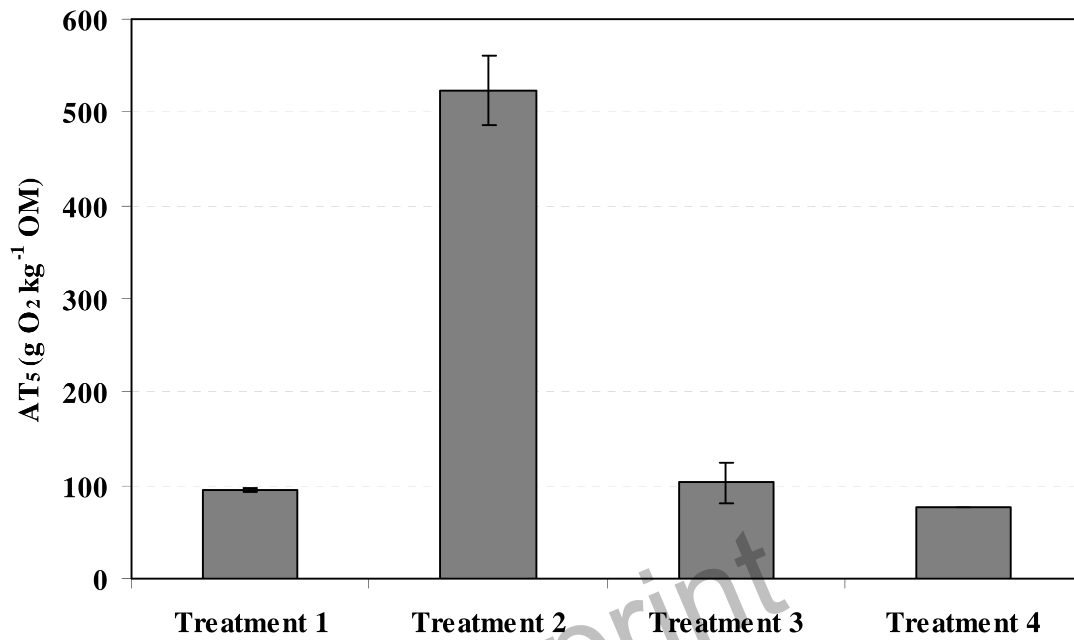

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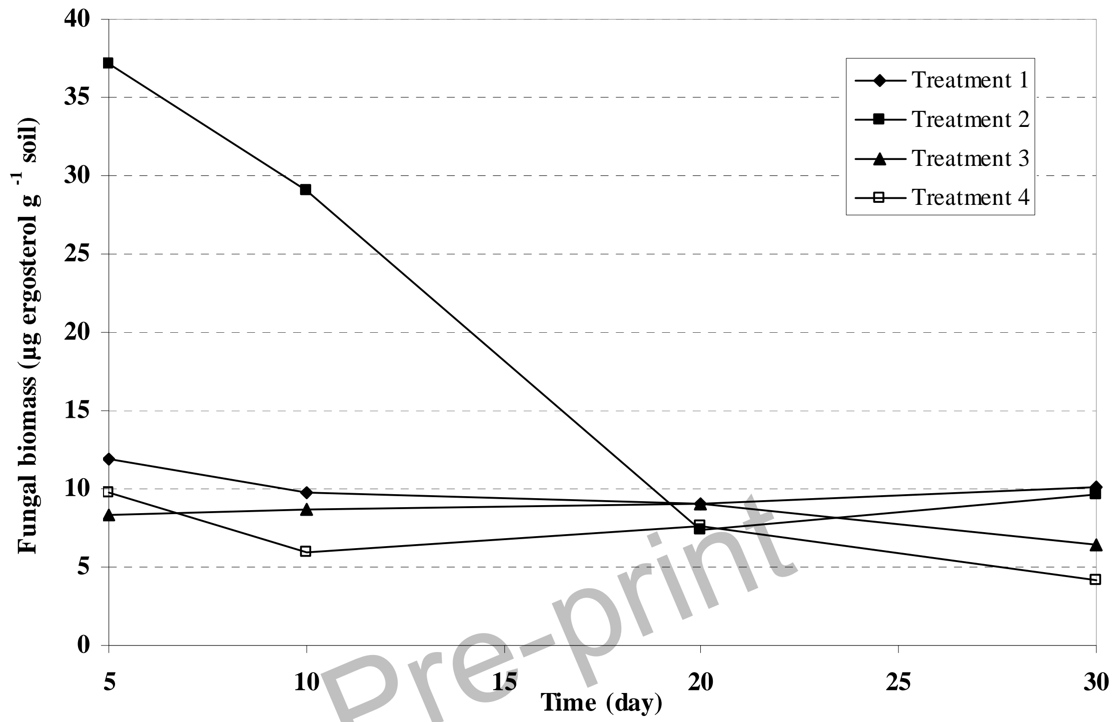

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