

This is the submitted version of the article: Abraham, J.; Gea, T. and Vicent, T. *Impact of biostimulation and bioaugmentation as bioremediation systems on diesel contaminated soils* in Environmental engineering and management journal, vol. 14, no. 8 (June 2013), p. 1743-1753.

Available at: <https://dx.doi.org/10.368/eemj.2016.187>

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IMPACT OF BIOSTIMULATION AND BIOAUGMENTATION ON DIESEL CONTAMINATED SOILS AS BIOREMEDIATION SYSTEMS

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Abstract

This paper analyses and compares the effects of bioremediation on total petroleum hydrocarbon (TPH) degradation with composting techniques and following biostimulation and bioaugmentation approaches. Compost and sludge were added as organic amendments with a double mission, providing both nutrients and microorganisms to the contaminated soil. In addition the effect of inoculating white-rot fungus *Trametes versicolor* was assessed. Two different types of soils were considered: a poor soil with low organic matter content and an enriched organic soil. The use of compost and sludge for soil bioremediation through composting techniques was effective for TPH removal. The amount of organic matter present in soil played an important role in TPH removal due to the adsorption phenomenon of the pollutants in the organic fraction of the solid material. When the contaminated soil was rich in organic matter, the use of sludge provided better results than compost (22% of degradation in the first fifteen days front 5%) but no differences between compost and sludge were observed in poor soil. The inoculation of the ligninolytic fungus *T. versicolor* enhanced the removal process of TPH, thus increasing the degradation rate and reducing the process time. However, periodical reinoculation was required.

Keywords: bioaugmentation, bioremediation, contaminated soil, total petroleum hydrocarbons, *Trametes versicolor*.

1. Introduction

As a consequence of massive and widespread use, petroleum hydrocarbon compounds have become common organic pollutants of soil surfaces and have eventually been considered a major environmental and health problem. Amongst hydrocarbon pollutants, fuel and diesel oil are a

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34 complex mixture of n-alkanes, branched alkanes, cycloalkanes and monoaromatic compounds. All
35 of these pollutants are frequently reported as soil contaminants leaking from storage tanks and
36 pipelines or released in accidental spills during industrial and commercial operations (Gallego et al.,
37 2001).

38 Today, bioremediation is the most common treatment used for these soils and is an
39 environmentally friendly alternative with respect to physico-chemical treatments. Bioremediation
40 involves turning pollutants into nontoxic forms by using organisms under aerobic or anaerobic
41 conditions to remove the contaminants from soil, water and gases (Riser-Roberts, 1998). Diesel oil
42 bioremediation in soil can be promoted by the stimulation of indigenous microorganisms by
43 introducing nutrients and oxygen into the soil (biostimulation) (Seklemova et al., 2001; Sayara et
44 al., 2009) or through the inoculation of an enriched microbial consortium, whether indigenous or
45 exogenous (bioaugmentation) (Vogel, 1996; Karamalidis et al., 2010; Kauppi et al., 2011).

46 Composting techniques have long been applied and established as an area of research to
47 degrade organic solid residues (Haug, 1993; Ruggieri et al., 2008). These techniques have also been
48 demonstrated to be effective in biodegrading total petroleum hydrocarbon (TPH) at the laboratory
49 (Namkoong et al., 2002), reactor (Van Gestel et al., 2003) and field (Ros et al., 2010) scales.
50 Diverse nutrient sources, such as inorganic fertilizer, compost, manure and sludge, have been used
51 in bioremediation. Amongst them, sludge seems to be a promising nutrient source for microbes in
52 bioremediation (Namkoong et al., 2002). The primary benefits of sludge include their low (or non-
53 existent) cost, slow release of nutrients (similar to animal manures) and easy availability. In
54 addition, their use gives purpose to what would otherwise be residues. Another organic source with
55 enormous potential for bioremediation are composts, not only because of their provision of
56 nutrients, but also because of their mesophilic and thermophilic bacterial content and their
57 ligninolytic fungi, which are endowed with the ability to degrade some pollutants (Antizar-Ladislao
58 et al., 2004; Anastasi et al., 2008). Also, the presence of biopolymers (cellulose, hemicellulose and
59 lignin) in compost may pave the way to the degradation of some pollutants. In fact, the
60 transformation of biopolymers requires a set of enzymes (peroxidases and phenoloxidases) that
61 degrade cellulose and lignin (Criquet et al., 1999). Filamentous fungi such as white-rot
62 basidiomycetes are amongst the major decomposers of biopolymers, lignin in particular. These
63 organisms have developed non-specific, radical-based degradation mechanisms occurring in the
64 extracellular environment (Singh et al., 2006). It has been probed that their ligninolytic enzyme
65 machinery (including laccases and peroxidases) can reach and deplete petroleum hydrocarbons in
66 contaminated soils even when the pollutants have low availability (Pointing, 2001). However,
67 basidiomycetes are rarely isolated from compost because many of them cannot withstand the

68 higher-than-50 °C temperatures generated during the thermophilic stage in the composting process
69 (Ryckeboer et al., 2003).

70 The main goal of this study was to demonstrate that compost and raw sludge could introduce
71 nutrients and microorganisms which would favor the degradation of TPH in contaminated soils.
72 Both organic materials may have a double impact in the bioremediation system, potentially
73 providing nutrients for endogenous microorganisms present in the soil as well as complex
74 microbiota as additional inoculums. Moreover, bioaugmentation with a specific compound
75 degrader, the white-rot fungus *Trametes versicolor*, was also studied to evaluate whether this
76 organism could improve the degradation or accelerate the time of remediation. This approach
77 employed composting techniques that can be applied both ex-situ and on-site.

78 Two different types of soils were used to investigate the effects of biostimulation (compost,
79 sludge) and bioaugmentation (microorganisms present in compost and sludge, and *T. versicolor*) on
80 TPH degradation by bioremediation with composting techniques: a poor soil with low organic
81 matter content and an enriched organic soil. Preliminary Petri dish trial experiments were necessary
82 to determine further 4.5 L reactor study conditions. Analyses with the 4.5 L thermally isolated
83 reactors were undertaken with the objective of emulating the environmental conditions found at the
84 field scale regarding heat transfer and temperature changes.

85

86 **2. Materials and Methods**

87

88 *2.1. Materials*

89

90 The two soils used were collected in the surroundings of Lugo composting plant (Galicia,
91 NW Spain) and were contaminated with 3 % v/v of a mixture of gasoline and diesel (ratio 1:1) one
92 week before the experiments were undertaken, reaching 36 g TPH/ kg of soil. The soils were
93 selected because of their different organic matter content. The mineral composition of soils A and B
94 was coarse sand 50.5 %; fine sand 27.9 %; loam 13 % and clay 8.6 %. In fact, soil B was collected
95 in an area where soil A had been periodically amended with compost for several years. The main
96 properties are presented in Table 1.

97 Raw sludge from a wastewater treatment plant and compost obtained from sludge
98 composting piles (four weeks treatment time) were used as organic amendments. Wood chips were
99 used as a bulking agent. All three materials were collected from the Jorba treatment plant
100 (Barcelona, Spain). The characterization of the amendments is shown in Table 1.

101 The white-rot basidiomycete fungus *T. versicolor* ATCC # 42530 was used in the
102 bioaugmentation experiments (Font et al., 1993). The assays were inoculated with 1.3 mg of

103 triturated mycelium fungus per g of soil (dry matter). The fungal colonization and effect of
104 inoculation dose and procedure were previously analyzed in Petri dish experimental trials (data not
105 shown).

106 Tween 80 (polysorbate 80, Sigma Aldrich Co, Spain), was used as a non-ionic surfactant
107 and an emulsifier of hydrocarbons.

108

109 **Table 1.** Characterization of soils, organic amendments and bulking agent. Properties were
110 analyzed according to methods described in Section 2.3.1.

111

Materials	Bulk density g/L	Water content (%)	Organic Matter (% dw)	Water holding capacity (% dw)
Soil A	1539	12.4	5	15
Soil B	834	9.1	38	34
Sludge	891	88.7	64	n.a.
Compost	525	27.3	65	155
Bulking agent	178	13.0	83	111

112 dw: dry weight

113

114 2.2 Removal of TPH

115

116 2.2.1 Evaluation of the amendment dose and inoculation procedure

117 Preliminary TPH degradation experiments were assayed in Petri dishes for thirty days at
118 25°C to find a suitable dose of compost and sludge and the best inoculation procedure for later 4.5 L
119 reactor experiments. *T. versicolor* was inoculated following two different inoculation strategies: i)
120 inoculating right after mixing the materials, and ii) inoculating the bulking agent and incubating at
121 25°C for two weeks prior to its mixture with soils and amendments. Additionally, four different
122 amendment:soil ratio (0.02:1; 0.06:1; 0.155:1 and 1:1 on wet weight) were tested. The assay was
123 prepared by mixing 15 g of the different soils, 3 g of the bulking agent and the different doses of the
124 amendments. The experiments were performed in triplicate. The samples were analyzed at the end
125 of incubation (thirty days).

126

127 2.2.2 Evaluation at the 4.5 L reactor scale

128 The experiments were conducted for sixty days in 4.5 L air-tight reactors that were
129 thermally isolated and equipped with on-line temperature monitoring by Pt-100 sensors (Sensotran,

130 Spain) connected to a data acquisition system (MAC-3580, Desin, Spain) and to a personal
131 computer. An intermittent aeration was provided to the reactors according to the process
132 performance to ensure a high oxygen level (over 10 %) and to avoid anaerobic conditions. The
133 oxygen concentration in the exhaust gases was measured by means of an oxygen sensor (Crowcon's
134 Xgard, United Kingdom).

135 The mixtures were prepared by mixing spiked soil, amendment and bulking agent together
136 at a weight ratio of 1:0.15:0.20. The water content of the mixture was adjusted to within the
137 recommended value (75 % of the water holding capacity) (Haug, 1993) by adding water before and
138 during the experiments when necessary. A percentage of water content is necessary in order to
139 promote an adequate biomass growth. The different mixtures are described below and are
140 summarized in Table 2. The main properties of the mixtures are presented in Table 3. The
141 experiments were undertaken in duplicate. The results are presented as the average of duplicates
142 (differences between duplicates were always below 15%). The reactors were filled to their
143 maximum capacity, thus containing a total mass of 2.50-3.00 kg. 120 g samples were collected at
144 zero, fifteen, thirty and sixty days of treatment after the homogenization of the mass in the reactors.
145 TPH removal was calculated as the difference in TPH content at a certain day compared to the
146 initial TPH content, and expressed as a fraction of the initial content. This was calculated for each
147 TPH fraction and for the total TPH content.

148

149 *2.2.2a Soils A and B: natural attenuation and bioaugmentation*

150 Experiments were undertaken in soils with (AI, BI) and without (A, B) *T. versicolor* (Table
151 2) to evaluate the removal of the hydrocarbons without the addition of nutrients and microbiota
152 from compost or sludge. Moreover, emissions of volatile organic compounds (VOCs) were
153 analyzed along the process to determine whether losses by volatilization were significant when
154 using a forced-aeration system.

155

156 *2.2.2b Bioremediation treatments: composting and bioaugmentation*

157 The composting bioremediation treatments were tested for each mixture. Compost and
158 sludge were used as amendments because of their different organic matter content and degree of
159 stability as well as the different microorganisms that can be found in both materials (experiments
160 AC, AS, BC and BS, Table 2).

161 The same mixtures were used in the bioaugmentation studies with the inoculation of *T.*
162 *versicolor* at the initial time (experiments ACI, ASI, BCI and BSI, Table 2). The experimental
163 design included a second inoculation on day 21st for two reasons. On one hand, previous studies had
164 shown that *T. versicolor* activity significantly reduces in bioprocesses on day 21 (Blázquez et al.,

165 2006; Rodriguez-Rodriguez et al., 2012). On the other hand, high temperatures expected in the
 166 initial decomposition phase may negatively affect *T. versicolor*.

167 Also, the effect of the addition of surfactant was analyzed in bioaugmentation experiments
 168 in both soils using sludge as amendment (ASTI and BSTI). The dose used was 5 g of Tween 80 for
 169 every 100 g of the studied mixture, as determined in previous studies (Rodriguez-Escales et al.,
 170 2012).

171 **Table 2.** Mixtures and nomenclature for 4.5L reactor scale experiments

172

<i>Experiments nomenclature</i>	<i>Amendments and bioaugmentation</i>				
	<i>Bulking agent</i>	<i>Compost</i>	<i>Sludge</i>	<i>T. versicolor</i>	<i>Surfactant</i>
Soil A					
A	-	-	-	-	-
AI	-	-	-	+	-
AC	+	+	-	-	-
ACI	+	+	-	+	-
AS	+	-	+	-	-
ASI	+	-	+	+	-
ASTI	+	-	+	+	+
Soil B					
B	-	-	-	-	-
BI	-	-	-	+	-
BC	+	+	-	-	-
BCI	+	+	-	+	-
BS	-	-	+	-	-
BSI	-	-	+	+	-
BSTI	-	-	+	+	+

173

174

175 2.3 Analytical Methods

176

177 2.3.1 Physicochemical analyses

178 Moisture and dry matter were determined by gravimetric analyses after drying at a
 179 maximum temperature of 105°C until constant weight. The organic matter content was determined
 180 from mass loss after heating at 550 °C for four hours (US Department of Agriculture, 2001).

181 The total organic carbon (TOC) was determined using O.I. Analytical Solid TOC
182 Analyzer/Win TOC Solids v3.0, and the total nitrogen Kjeldahl (TNK) was determined by standard
183 procedures (US Department of Agriculture, 2001). For the TOC and TNK analyses, the samples
184 were previously dried up and sieved at 0.5 mm. The bulk density and free air spaced (FAS) defined
185 as ratio of air volume to total volume of the sample were measured by picnometry (Ruggieri et al.,
186 2009), and the water holding capacity was also measured (US Department of Agriculture, 2001).

187 The soil samples for petrol hydrocarbon analyses were extracted with Acetone/Petroleum
188 Ether, cleaned with Florisil® and then analyzed by GC-FID and DB-1 column as described in Van
189 Gestel et al. (2003).

190 The exhaust gases were collected daily in Tedlar bags, and the VOCs content was analysed
191 by GC, as described in Pagans et al. (2005). Thus, the total VOCs emission could be calculated.

192

193 *2.3.2 Laccase activity*

194 The extracellular ligninolytic laccase enzyme activity was determined. The laccase enzyme
195 was extracted by adding 30 ml of acetate buffer (0.16 M, pH 5) and 3 g of mixture sample to each
196 flask, shaking at 130 rpm at 4 °C for 30 min and centrifuging at 10.000 rpm at 4 °C for 15 min, a
197 procedure adapted from Snajdr and Boldrian (2006). The supernatants were collected, and the
198 laccase activity was assayed spectrophotometrically according to Kaal et al. (1993).

199

200 *2.3.3. Respiration Index*

201 A dynamic respirometer was used as described by Ponsá et al. (2010). Briefly, a sample of
202 150 g of the mixture was placed in a 500 mL Erlenmeyer flask and incubated in a water bath at 37
203 °C. The starting organic material moisture was adjusted to a range of 50-60 %, if necessary. Air was
204 continuously supplied to the samples using a mass flowmeter (Bronkhorst Hitec, The Netherlands)
205 to ensure aerobic conditions during the experiment (oxygen concentration higher than 10 %). The
206 oxygen content in the exhaust gas from the flask was measured using a specific probe (Xgard
207 Crowcon, UK) and was recorded on a personal computer equipped with commercial software
208 (Indusoft Web Studio, version 2008, USA). From the curve of oxygen concentration vs. time, two
209 respiration indices can be calculated:

210 A) Dynamic Respiration Index (DRI): This index represents the average oxygen uptake rate
211 during the twenty-four hours of maximum activity observed during the respiration assay. The DRI
212 is expressed in mg of oxygen consumed per g of dry matter and per hour.

213 B) Cumulative Respiration Index (CRI): This index represents the cumulative oxygen
214 consumption during the four days of maximum respiration activity without considering the initial
215 lag phase. The CRI is expressed in mg of oxygen consumed per g of dry matter.

216

217

218

Table 3. Characterization of initial mixtures

<i>Initial samples*</i>	<i>A</i>	<i>B</i>	<i>AC</i>	<i>ACI</i>	<i>BC</i>	<i>BCI</i>	<i>AS</i>	<i>ASI</i>	<i>BS</i>	<i>BSI</i>
Water content (%)	11.6	28	20.3	23.8	35.6	34.4	28.8	29.1	30.9	37.3
Organic Matter (% dw)	4.1	16.2	26.5	18.9	41.6	38.9	24	22.7	28.4	44.6
Total Organic Carbon (% dw)	2.3	10.2	15.9	n.a.	15.1	n.a.	10.3	n.a.	10.7	n.a.
Total Kjeldhal Nitrogen (% dw)	0.5	0.9	0.8	n.a.	0.8	n.a.	0.6	n.a.	0.7	n.a.
C/N ratio	5.1	13.3	18.7	n.a.	18.1	n.a.	16	n.a.	15	n.a.
Respiration Index mg O ₂ kg ⁻¹ dw h ⁻¹	n.a.	n.a.	69	86	133	169	257	304	336	149
Cumulative oxygen consumption mg O ₂ g ⁻¹ dw	n.a.	n.a.	30.8	39.6	79.0	53.3	63.6	65.1	101.4	43.2
Bulk Density (g/L)	1539	834	757	n.a.	670	n.a.	784	n.a.	918	n.a.
Free Air Space (%)	n.a.	n.a.	65	n.a.	59	n.a.	54	n.a.	55	n.a.

219 *dw: dry weight

220

221 **3. Results and discussion**

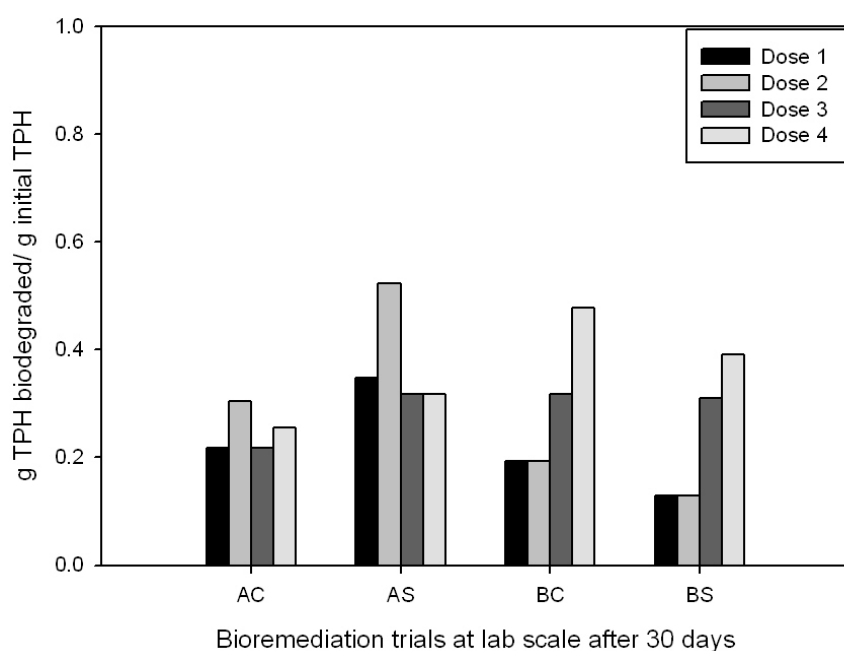
222

223 *3.1 Results at the laboratory scale*

224

225 Preliminary TPH degradation experiments were undertaken at the laboratory scale as
 226 described in section 2.2.1 and results are summarized below (data not shown). The use of the
 227 bulking agent inoculated prior to the remediation trials did not offer any advantage, thus it was
 228 decided to inoculate at the starting moment of the bioaugmentation experiments. In general, fungus
 229 growth was favored at increasing dose of amendment, as observed by the higher visible
 230 colonization of dishes by the white filamentous fungus (data not shown). A higher growth of *T.*

231 *versicolor* was observed when using sludge with soil A and when using compost with soil B. Also,
 232 TPH degradation (Fig. 1) was enhanced when increasing the amendment dose in the tested range
 233 (0.02, 0.06, 0.155 and 1 gram of amendment for 1 gram of soil) with soil B, but no effect of
 234 amendment dose was observed for soil A. No differences were observed among amendments
 235 regarding TPH degradation. From these previous trials, the dose 3 (0.155:1g amendment / g
 236 contaminated soil on wet basis) was chosen for the following experiments at the 4.5 L reactor scale.
 237 This dose was selected as a compromise solution to obtain good degradation levels and to avoid
 238 using large doses of amendment which would make the treatment more expensive (amendment
 239 transport and mixing and overall treatment surface needed).
 240



241 Bioremediation trials at lab scale after 30 days

242
 243 **Fig. 1.** TPH degradation in the different treatments at laboratory scale using different doses of
 244 amendments. AC: soil A, compost; AS: soil A, sludge; BC: soil B, compost; BS: soil B, sludge.

245 Doses g amendment:g contaminated soil on wet basis: Dose 1 **0.02:1**; Dose 2 **0.06:1**; Dose
 246 **3.0.155:1**; Dose 4 **1:1**

247
 248 *3.2. Overall performance of bioremediation trials in 4.5 L reactors*

249
 250 Bioremediation trials in 4.5 L reactors were carried out with different mixtures of soil A and
 251 B using compost and sludge as amendment and inoculating with the white-rot fungus *T. versicolor*
 252 (Tables 2 and 3). A respirometric study was undertaken with all mixtures intended for study to
 253 evaluate both the effect of amendments (sludge and compost) and bioaugmentation with *T.*

254 *versicolor*. The results are presented in Table 3 as a respiration rate DRI and cumulative oxygen
255 consumption CRI. Respiration rate and cumulative oxygen consumption are indicative of the
256 amount of biodegradable organic matter in a solid organic waste and its biodegradability (Ponsá et
257 al., 2010). The soil B mixtures presented a higher respiration activity than the soil A mixtures
258 probably due to their greater organic matter content. Also the use of sludge as amendment provoked
259 higher biological activity than the compost, measured as a higher oxygen consumption rate and
260 higher total oxygen consumption for both soils A and B (Puyuelo et al., 2011). The mixtures
261 inoculated with *T. versicolor* showed a higher oxygen consumption rate, but no differences were
262 found in the final oxygen consumption at the end of the respiration study among inoculated and
263 non-inoculated mixtures.

264 These experiments were undertaken in 4.5 L adiabatic reactors to emulate the energy
265 transfer conditions at the industrial scale in composting processes. A rise in temperature was
266 observed at the beginning of the process from room temperature (20°C) to maximum temperatures
267 ranging from 30 to 40°C (Fig. 2 is presented as an example). This rise was a common factor in all
268 cases except for the natural attenuation experiments. The temperature rise was due to heat released
269 in the biodegradation of the organic matter present in the compost and sludge. A secondary
270 temperature rise was usually observed after homogenizing the reactor contents on sampling days
271 fifteen and thirty. After an initial decomposition phase, the temperature fell and stabilized at
272 approximately room temperature before day thirty and until the end of the process. In general, the
273 inoculated reactors showed higher temperatures than the non-inoculated trials. Table 4 shows the
274 maximum temperature achieved and the area below temperature curve, calculated for the first 14
275 days (until the first sampling), as the average of the two replicates for each trial. This area and
276 average maximum temperature was 3.6% and 3.4% higher in inoculated trials. This reflects a higher
277 biological activity and confirms the observations from the respirometric analysis. However, because
278 the temperatures remained in the mesophilic range in all cases, the survival of *T. versicolor* should
279 not be affected by thermal conditions.

280 The initial mixtures presented a FAS over 50% (Table 3), which is the recommended value
281 for solid bioconversion processes to ensure aerobic conditions and the proper air circulation through
282 the organic matrix (Ruggieri et al., 2009). Aeration was adjusted to slightly higher values for the
283 first days of the process during the decomposition phase and was reduced at the end of the
284 experiment (equivalent air flow ranging from 0.17 to 0.05 L/min). Oxygen was maintained over 5%
285 in all cases. The water content was also kept at approximately 75% of the water holding capacity of
286 the mixtures in all trials.

287

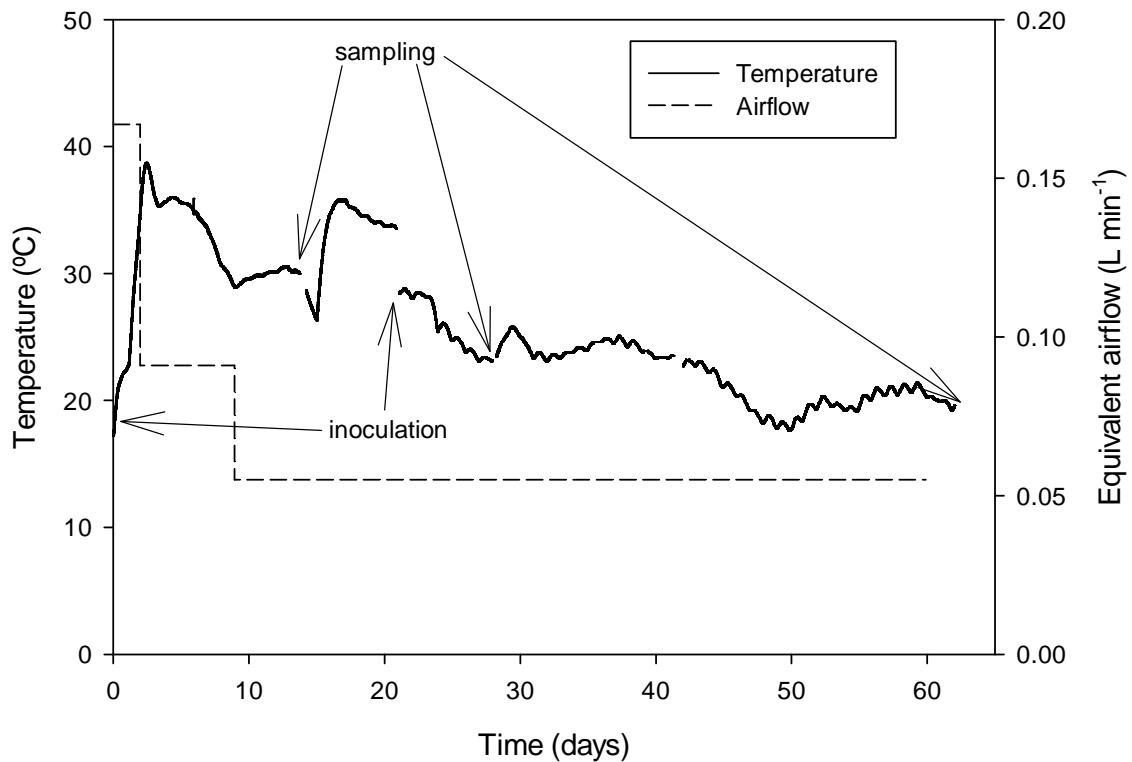


Fig. 2. Temperature profile and aeration requirements for experiment ASII. Arrows indicate both sampling and inoculation moments

Laccase activity was analyzed to monitor the activity and viability of *T. versicolor*. Decreasing levels of activity (Table 5) were detected at days fifteen, thirty and sixty in both the composting and bioaugmentation experiments when using compost as amendment. Detecting laccase activity in not inoculated (with *T. versicolor*) trials indicated the presence of other laccase-producer microorganisms in the initial composting mixtures because compost is a material enriched with a diverse microbial population, including bacteria, fungi and actinomycetes. However, laccase levels were higher in the inoculated mixtures, pointing to a higher fungal activity, but they were negligible after sixty days of processing in any case, indicating the inactivation of the fungus. No laccase was detected when using sludge as amendment and in the natural attenuation trials, whether inoculated with *T. versicolor* or not.

307 **Table 4.** Maximum temperature achieved and area below temperature curve for the different
 308 experiments considered.

Experiment	Area below temperature curve (°C·day)	Maximum temperature (°C)
AC	$7.66 \cdot 10^6$	30.2
ACI	$8.24 \cdot 10^6$	33.6
BC	$8.60 \cdot 10^6$	37.5
BCI	$8.51 \cdot 10^6$	36.4
AS	$8.58 \cdot 10^6$	38.1
ASI	$8.64 \cdot 10^6$	37.5
BS	$6.36 \cdot 10^6$	31.2
BSI	$6.94 \cdot 10^6$	34.2

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310

311 **Table 5.** Laccase activity detected at 15, 30 and 60 days in both composting and bioaugmentation
 312 experiments with compost (average of two replicates).

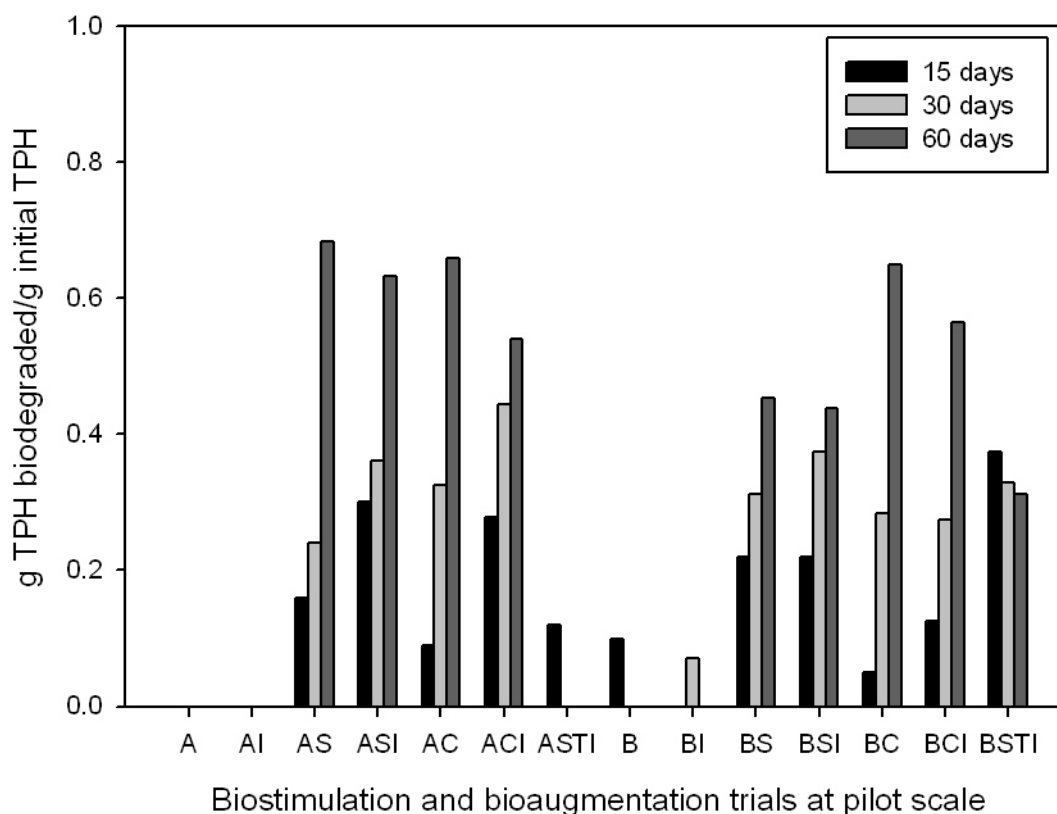
Mixture	Laccase activity (U / g dry weight)		
	Day 15	Day 30	Day 60
AC	1.3	0.2	n.d
ACI	2.1	0.9	0.2
BC	n.d	1.5	n.d
BCI	2.1	2.5	0.2

313

314 *3.3 TPH removal in 4.5 L reactors*

315

316 Fig. 3 shows the degradation of TPH in all of the treatments after fifteen, thirty and sixty
 317 days of treatment. No degradation of TPH was detected in the natural attenuation trials (soils A and
 318 B without the addition of amendments) or with bioaugmentation. In contrast, the addition of
 319 amendment resulted in a considerable TPH degradation in both soils (AS, AC, BS, BC). In soil A,
 320 the TPH degradation reached similar values over 60% both using compost (66%) and sludge (68%).
 321 In soil B, a similar level of degradation was achieved when using compost (65%). However, only
 322 45% of the TPH was degraded when using sludge as amendment with soil B. These results
 323 highlight the contribution to TPH degradation made by the microorganisms present in compost and
 324 sludge and are in accordance with previous reports where compost was demonstrated to have a high
 325 capacity for enhancing the biodegradation of contaminated soils compared with other amendments
 326 (Tejada et al., 2008; Sayara et al., 2009; Gandolfi et al., 2010).



328

329

330 **Fig. 3.** TPH degradation in the different treatments in 4.5L reactors. A: soil A; AS: soil A, sludge;331 AC: soil A, compost; I inoculation with *T. versicolor* at 0 and 21 days process; T surfactant. B:

332 same nomenclature than soil A for soil B

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When comparing the TPH degradation of the composting trials (AS, AC, BS and BC) with the bioaugmentation experiments (ASI, ACI, BSI and BCI, respectively), a higher TPH removal can be observed in the inoculated trials at days fifteen and thirty, with this effect being more evident in the soil A trials. However, after sixty days of processing, the inoculation of *T. versicolor* did not provide any advantage in TPH degradation. Note that the inoculations were undertaken at days zero and twenty-one. It seems that the addition of this fungus enhances TPH degradation, but this microorganism is not able to survive without periodical reinoculation. This result confirms the previous observations of respiration analysis, as the final CRI was equivalent for the inoculated and non-inoculated samples. Because the temperatures reached were always below 40°C, the inactivation of *T. versicolor* is attributed to competition with the microorganisms present in the amendment or soils, which are naturally more adapted to the aggressive and successively changing environment in batch processes, such as bioremediation systems. Eventually, these microorganisms

346 are able to biodegrade the pollutants to the same extent. Competition with autochthonous soil
347 microflora is an important factor in soil bioremediation by white-rot fungi, but the knowledge of
348 their interactions with soil microbiota is poor and sometimes inconsistent (Arun et al., 2008; Borràs
349 et al., 2010; Field et al., 1995; Mougin, 2002; Singh, 2006).

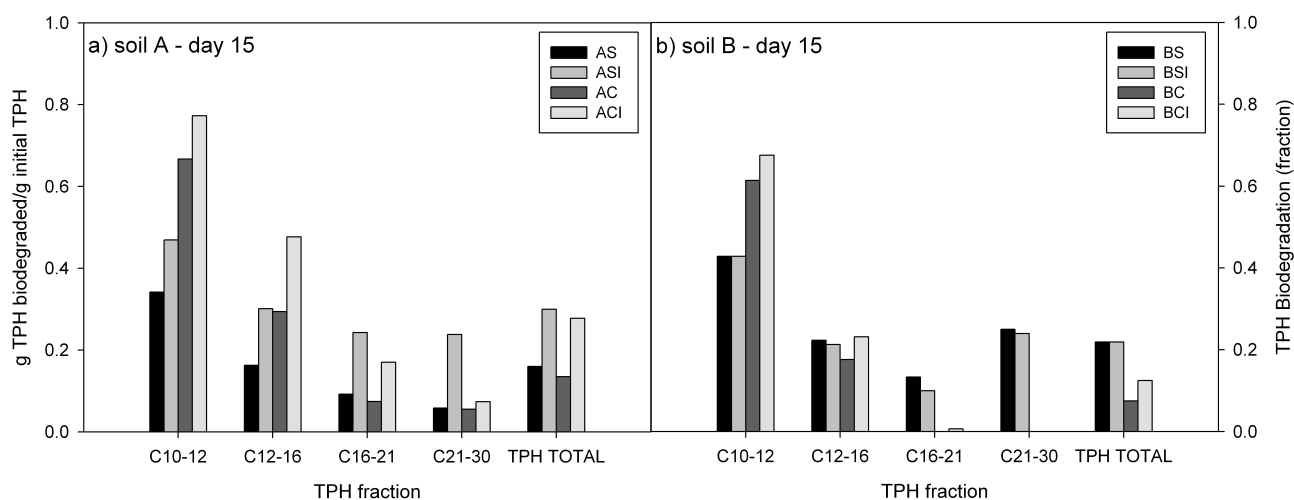
350 These results indicate that the addition of amendments is an interesting strategy to increasing
351 both available nutrients and the amount and biodiversity of biodegrading microorganisms in soils,
352 especially in poor inorganic soils such as soil A. Bioaugmentation with ligninolytic fungi enhances
353 the TPH biodegradation rate, and thus, this strategy can reduce total processing time. These results
354 are in agreement with previous microcosmos studies that demonstrated fungi suitability as TPH
355 degraders in soils (Mancera-López et al., 2008; Yateem et al., 1998). Lladó et al. (2012) reported
356 50% TPH removal in 200 days with *T. versicolor* in microcosmos assays and established that the
357 inoculation with *T. versicolor* promoted autochthonous hydrocarbon-degraders. However, despite
358 these promising results with white-rot fungus bioaugmentation, periodical reinoculations are
359 necessary. Consequently, the minimum inoculation dose and the fungus production cost would
360 determine whether the bioaugmentation strategy is economically viable.

361 The use of surfactant enhanced the removal in the experiments with soil B rich in organic
362 matter, especially in the first fifteen days. The surfactant probably assists hydrocarbon desorption
363 from organic matter and makes pollutants more bioavailable (Rodríguez-Escales et al., 2012). In
364 contrast, no effect of surfactant addition was observed in soil A with low organic matter content.
365 Interactions between surfactant and the presence of dissolved organic matter have been observed to
366 increase pollutant availability in contaminated soils (Cheng and Wong, 2006).

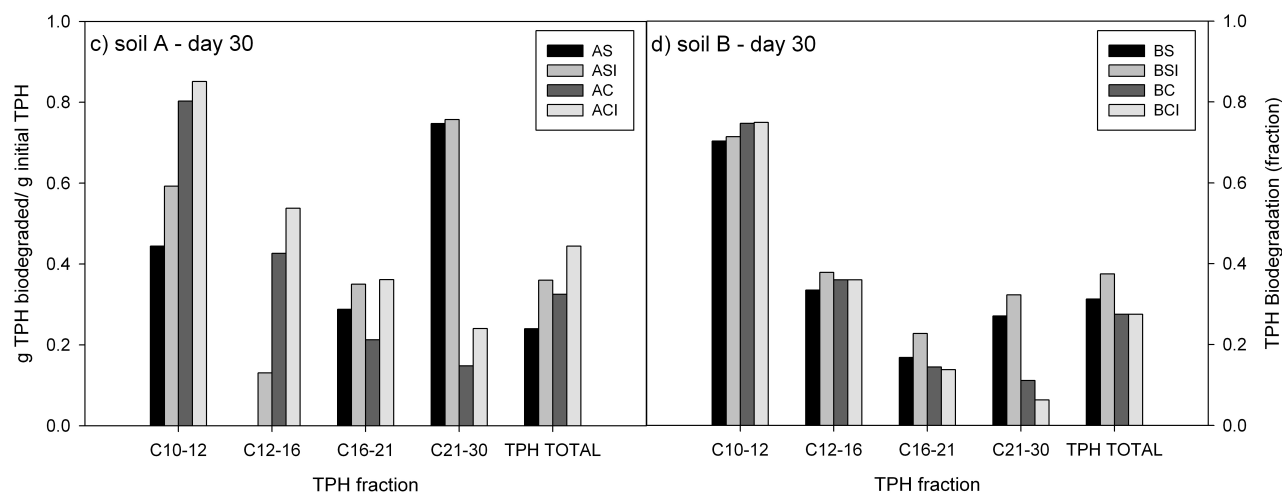
367 Fig. 4 presents the removal levels obtained for the different fractions of TPH (C10-12, C12-
368 16, C16-21 and C21-30) at fifteen, thirty and sixty days for the bioremediation and bioaugmentation
369 trials with soils A and B. The shorter TPH fractions were more easily biodegraded, reaching 90%
370 removal for the C10-12 fraction, while only 50% of C21-30 was biodegraded after sixty days.
371 However, a biodegradation yield over 90% in all fractions can be expected for longer process times,
372 as deduced from the overall performance of these experiments, reaching removal percentages
373 comparable to those reported in the literature (Sarkar et al., 2005).

374 The effect of inoculation with *T. versicolor* is reflected in Fig. 4. All fractions present a
375 higher percentage of removal in the bioaugmentation experiments for days fifteen and thirty, but
376 similar levels were observed in the final values. The lower removal levels reached in the
377 experiments with soil B using compost as a amendment are also evident in Fig. 4. In the first fifteen
378 days, the degradation of fractions C16-21 and C21-30 was negligible. This behavior can be
379 associated with the adsorption of TPH in the organic matter fraction of the soil and compost.

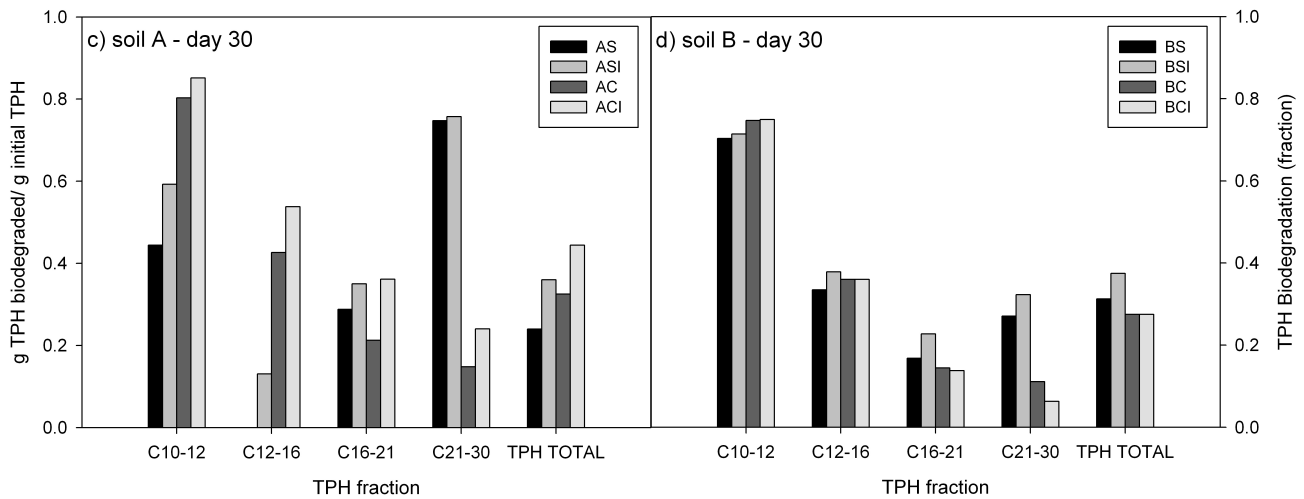
380 Margesin et al. (2000) and Riffaldi et al. (2006) reported that biodegradation is thought to be
 381 the main TPH removal process during bioremediation, but volatilization can also play an important
 382 role. Volatile organic compounds were analyzed in the exhaust gases for experiments A, B, AI and
 383 BI to evaluate whether forced aeration could enhance TPH volatilization causing atmospheric
 384 pollution. The total emissions in the first thirty days of the process ranged from 1 to 1.6 g C per kg
 385 of initial mixture. This level of emission is below the reported emissions found in composting
 386 plants (3.7 – 7.8 g C per kg of treated waste, Cadena et al., 2009), and it was considered negligible
 387 compared to the initial TPH concentration (36 g per kg of soil). Thus, TPH removal could be
 388 attributed mainly to biodegradation undertaken by microorganisms.



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393 **Fig. 4.** Removal of different TPH fractions and total TPH at days 15, 30 and 60 for bioremediation
 394 trials with (ASI, ACI, BSI, BCI) and without (AS, AC, BS, BC) bioaugmentation
 395

396

396 4. Conclusions

397

398 The use of compost and sludge for soil bioremediation appears as an effective technique
 399 although the effect depends on the type of soil, the amendment and probably the interaction among
 400 them. For the poor soil tested, no differences are observed when using compost or sludge.
 401 Inoculation with *T. versicolor* enhances the removal process of TPH, increasing the degradation rate
 402 and reducing the process time. However, periodical reinoculation is required. Thus, further research
 403 is needed to define whether the process is economically viable when faster processes are required.
 404 When time is not a limiting factor, the use of amendments provides enough nutrients and
 405 microorganisms for efficient TPH removal. For the rich soil tested, the use of sludge provides better
 406 results than compost. Also in this case, bioaugmentation offers reduced advantages.
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407

408 Acknowledgements

409 Financial support was provided by Xunta of Galicia, Spain (INCITE project 07MDS038E). The
 410 authors wish to thank Dr. Carlos García-Izquierdo and Dra. Teresa Hernández from CEBAS-CSIC,
 411 Domingo Pérez from Universidade de Vigo, Dr. Diego Corcho from Tradebe Group and Marisol
 412 Mompeó for their collaboration in the project.
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