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1 **Comparison of a bioremediation process of PAHs in a PAH-**
2 **contaminated soil at field and laboratory scales**

3
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17
18 **Abstract**

19
20 A laboratory experiment was carried on the same initial soil and at the
21 same time than a windrow treatment in order to compare results at field and
22 laboratory scales for a soil mainly contaminated with PAHs. After 6 months,
23 laboratory experiments gave similar but less scattered results than those
24 obtained in the field indicating that the field biotreatment was well optimised. The
25 total amount of PAHs degraded after 6 months was ca. 90% and degradation
26 rates followed a negative exponential trend. Relative degradation rates of 3- and
27 4-ring PAHs were about 32 and 7.2 times greater than those of 5- and 6-ring

28 PAHs, respectively. With respect to the bacterial community, bacteria belonging
29 to *Gamma-proteobacteria* persisted whereas *Beta-proteobacteria* appeared after
30 three months of biotreatment when PAH concentration was low enough to render
31 the soil non-ecotoxic.

32

33 **Capsule**

34

35 Comparison of field and laboratory biotreatments of the same PAH-
36 contaminated soil gave similar results with respect to PAH concentration and
37 bacterial diversity.

38

39 **Keywords**

40

41 Bioremediation, Contaminated soils, Polycyclic aromatic hydrocarbons
42 (PAHs), Laboratory and field experiments, Bacterial diversity

43

44 **1. Introduction**

45

46 Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic
47 pollutants generated during coke production, petroleum refining and combustion
48 processes (Cerniglia, 1992), and these pollutants are frequently encountered in
49 contaminated industrial sites. These organic contaminants are among the most
50 hazardous environmental pollutants due to recalcitrance and toxic, mutagenic
51 and carcinogenic effects (Keith and Telliard, 1979; Shaw and Connell, 1994). In
52 the natural environment, these compounds undergo transformations involving
53 both biotic and abiotic processes, such as volatilization, adsorption, photolysis,
54 chemical oxidation and microbial degradation. Among them, microbial activity

55 makes up the primary pathway for PAH removal from soils (Yuan et al., 2000;
56 Lors and Mossmann, 2005; Haritash and Kaushik, 2009). So, a bioremediation
57 technique based on the optimization of biodegradation has been developed as a
58 soil clean-up technique which is expected to be economical and efficient
59 compared with chemical or physical remediation processes (Liebeg and Cutright,
60 1999; Antizar-Ladislao et al., 2004). For example, a windrow treatment applied
61 on a soil contaminated predominantly with 2-, 3- and 4-ring PAHs resulted in 88%
62 reduction of PAH concentration after 6 months (Lors et al., 2010b). The reduction
63 of PAH concentration was linked to the bacterial community, which was
64 characterized by a high diversity and the persistence of a bacterial consortium
65 represented by Gram-negative bacterial strains during the entire biotreatment
66 process. In particular, *Pseudomonas* and *Enterobacter* genera had a strong
67 PAH-degrading capacity that remained throughout the whole biotreatment. Other
68 species, such as *Beta-proteobacteria*, appeared over time, when the PAH
69 concentration was low enough to alleviate soil ecotoxicity (Lors et al., 2010a;
70 Lors et al., 2011). This result suggests that the presence of these bacteria could
71 be used along with analytical methods to estimate the endpoint of biotreatment of
72 soils containing mostly 2-, 3- and 4-ring PAHs.

73 In the case of PAHs, bioremediation processes, such as landfarming, biopiles,
74 bioslurries and windrows, are based on increasing microbial activity by optimising
75 biodegradation conditions through aeration, the addition of nutrients and control
76 of pH, moisture and temperature (Atlas and Bartha, 1992; Namkoong et al.,
77 2002; Sarkar et al., 2005). Thus, it is important to assess the effect of these
78 parameters on the efficiency of the biotreatment. Laboratory experiments could
79 be very valuable in optimising biodegradation conditions if it can be demonstrated
80 that the biotreatment can be accurately reproduced at laboratory scale.

81

82 The present work reports on changes over time in PAH concentration and
83 composition of the bacterial community during a laboratory experiment carried
84 out with the same initial soil that had been subjected to a windrow biotreatment
85 (Lors et al., 2010b). Even if the experiments were carried out at the same time,
86 laboratory experiments enabled us to make additional samplings and better
87 control conditions than field experiments. The comparison of present results with
88 those obtained in the field will enable us to assess if the field experiment was well
89 optimised and if a laboratory experiment could reproduce the results of the field
90 biotreatment. Although many studies dealt with the monitoring of PAH-polluted
91 soils in laboratory (Arias et al., 2008; Dandie et al., 2010), field (Ahtiainen et al.,
92 2002; Lors et al. 2010b) and both conditions (Robinson et al., 2003), the question
93 whether laboratory experiments can mimic field biotreatment was hardly
94 addressed, to the exception of Diplock et al. (2009), who concluded to an urgent
95 need for field-scale validation of laboratory methods. Additionally, this laboratory
96 experiment could help to verify that the *Beta-proteobacteria* group appears when
97 the soil is no longer ecotoxic, as it has been reported previously at field scale
98 (Lors et al., 2010b).

99

100 **2. Material and methods**

101

102 The soil used to perform the bioremediation process was a soil
103 contaminated by a coal tar distillation plant that was operational from 1923 to
104 1987 in the North of France. The contaminated soil was sieved and the fraction
105 thinner than 6 mm was mixed with wood shavings; the characteristics of which
106 have been given by Lors et al. (2010b). The volumetric ratio between soil and
107 wood shavings was to 0.7:0.3. Nitrogen (agricultural urea) and phosphorus
108 (agricultural superphosphate) nutrients were added to enhance the growth of the

109 microbiota. This solid matrix was sieved to 4 mm to eliminate wood shavings, and
110 represented the starting material (called Ti soil) for both field and laboratory
111 experiments. This substrate was characterized by different physicochemical
112 parameters: pH, moisture, total organic carbon, total organic nitrogen, heavy
113 metals (As, Cd, Cr, Cu, Pb, Zn) and 16 PAHs (PAHs listed by the US-EPA), using
114 standard techniques described by Lors et al. (2010b). Heavy metals were
115 analysed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-
116 AES) after hot acid digestion of the solid phase. The PAH content was
117 determined by HPLC (High-Performance Liquid Chromatography) after ASE
118 extraction. Moreover, microbiological investigations were performed on this
119 substrate, including total bacteria counts and counts of PAH-degrading
120 microbiota (Lors and Mossmann, 2005). Additionally, the diversity of total
121 bacterial microbiota was determined by a molecular method, including total DNA
122 extraction, PCR amplification, DGGE analysis, cloning and sequencing, which
123 has been detailed in Lors et al. (2010b).

124

125 In situ, the PAH-contaminated substrate was placed in five windrows of
126 approximately 5000 tons each (length = 90 m, width = 5 m, height = 2.2 m). The
127 biotreatment started in August 2003. The solid matrix was turned each week
128 during the first 3 months and then every 2 weeks to prevent oxygen depletion in
129 the interstitial atmosphere. Moisture was monitored and maintained constant by
130 periodically sprinkling water above the windrows. The temperature inside the
131 windrows was measured at different depths (0.5, 1, 1.5 m) every 5 m along the
132 sides of the windrows during the whole treatment. An average temperature below
133 30 °C for more than a week was considered improper to bacterial activity,
134 causing the windrow to be specifically treated and eventually rebuilt. One
135 windrow was monitored by sampling the solid matrix after 44, 60, 92 and 182
136 days. Each solid sample was prepared by pooling and homogenising 15 samples

137 corresponding to 15 locations randomly chosen along the windrow at 0.3, 1 or 1.5
138 m depths. After sieving the solid matrix at 4 mm, these samples were submitted
139 to physicochemical and microbiological analyses.

140

141 Microcosm experiments were performed under controlled conditions in the
142 laboratory. Twenty-one 250-mL glass sterile bottles were filled with 50 g of Ti soil
143 and closed with a porous cap which enabled oxygen to pass through. Thus, the
144 device was in aerobic conditions and stored at 30 °C. The temperature used was
145 representative of the temperature in the field experiment, which was around 30
146 °C as abovementioned. Moreover, every week, soil samples were homogenised
147 under a sterile hood. The moisture content was measured weekly by weighing
148 the bottles and sterile MilliQ® water was homogeneously sprayed in sterile
149 conditions to keep the mass constant. At each sampling time (3, 7, 14, 34, 63, 92
150 and 182 days), three samples were sacrificed for physicochemical and
151 microbiological analyses.

152

153 Both for field and laboratory experiments, PAH degradation was
154 evaluated by following the decrease in PAH concentration over time. Moreover,
155 changes over time of total microbiota were followed by the same molecular
156 biological methods used for the initial matrix. In the laboratory experiment,
157 sampling was done in duplicate at 14 and 92 days to determine the
158 reproducibility of DGGE analysis.

159

160 **3. Results**

161

162 **3.1. Chemical and bacterial characteristics of the reference soil (Ti soil)**

163

164 Detailed chemical and bacterial characteristics of the Ti soil have been
165 presented in Lors et al. (2010b) and are summarised in Table 1. The moisture
166 content was 17%, and the soil had a C:N ratio of 56, which corresponds to
167 favourable nutritional conditions for bacterial degradation (Meeting, 1992). Heavy
168 metals were present at very low levels, with concentrations similar to those found
169 in the local geochemical background (Table 1), to the exception of zinc, which
170 exhibited a slightly higher concentration (Sterckeman et al., 2002). The Ti soil
171 was mainly contaminated with PAHs, whose total concentration ($\Sigma 16$ PAHs) was
172 close to 3 g kg^{-1} dry soil (Table 1). Three-ring PAHs were present at the highest
173 concentration (1279 mg kg^{-1} dry soil) and represented 44% of the total PAH
174 concentration. Among them, phenanthrene was the most abundant (49% of 3-
175 ring PAH concentration) (Table 2). Four-ring PAHs were also present at high
176 concentrations, amounting to 28% of the total PAHs (809 mg kg^{-1} dry soil), with
177 fluoranthene as the main compound (accounting for 51% of 4-ring PAH
178 concentration), followed by pyrene, accounting for 29%. The only 2-ring PAH
179 identified was naphthalene (20% of PAH concentration). On the other hand, 5-
180 and 6-ring PAHs were found at lower concentrations, accounting for 5% and 2%
181 of total PAH concentration, respectively.

182

183 The total bacterial population in the Ti soil represented $4.9 \cdot 10^8 \text{ CFU g}^{-1}$
184 dry soil (Table 1), which is comparable to populations typically found in the
185 superficial layer of unpolluted soils (Robert, 1996; Taylor et al., 2002). This
186 finding indicated that there was a good biological activity in this matrix.
187 Phenanthrene-degrading bacteria represented a large proportion of the bacteria
188 in this soil ($1.4 \cdot 10^7$ bacteria g^{-1} dry soil), whereas fluoranthene-degrading bacteria
189 were present at a much lower level ($7.7 \cdot 10^4$ bacteria g^{-1} dry soil) (Table 1). The
190 presence of these specific bacteria indicated that the microbiota was adapted to

191 these compounds, probably due to historical contamination of the soil (Kästner et
192 al., 1994; Mueller et al. 1994; Lors et al. 2004; Lors et Mossmann 2004).

193

194 **3.2 Monitoring of PAH concentration during the laboratory experiment**

195

196 Changes in the concentration of 16 PAHs and 2-, 3-, 4-, 5-, and 6-ring
197 PAHs over the course of the laboratory experiment are presented in Figure 1.
198 Data shown were average values of three replicate measurements.

199 After 6 months of incubation, 85% of the 16 PAHs were degraded
200 (concentrations decreased from 2895 ± 38 to 440 ± 21 mg kg⁻¹ dry soil). The
201 overall degradation of 16 PAHs was primarily due to that of 2-, 3- and 4-ring
202 PAHs, whereas 5- and 6-ring PAHs were hardly degraded even after 6 months of
203 incubation. The concentration of 16 PAHs decreased rapidly over the first month
204 and then reached a quasi asymptotic level toward the end of the experiment (Fig.
205 1-A). This is coherent with a negative exponential trend with regard to the
206 remaining quantity of the 16 PAHs during the time of biotreatment that can be
207 expressed by the following formula:

$$208 \quad [\text{PAH}] = [\text{PAH}]_{t_0} \cdot C^t \quad (\text{Eq. 1})$$

209 $[\text{PAH}]_{t_0}$ corresponds to the initial amount of PAH and C is a constant that is
210 related to the degradation rate. Log C corresponds to the slope of the straight line
211 obtained when plotting log [PAH] as a function of time. More negative values of
212 log C indicate greater degradation rates. Exponential regression was calculated
213 for each set of experimental data in order to compare overall degradation rates at
214 field and laboratory scales and also according to the number of rings contained in
215 PAHs. The determination coefficient (R^2) was used to assess the accuracy of
216 exponential regression. Values of parameters of exponential regression are
217 reported in Table 3 except for 2-ring PAHs that were completely eliminated in 3
218 days and because the sampling effort was too weak to accurately calculate

219 exponential regression for 2-ring PAHs. The rapid elimination of 2-ring PAHs was
220 essentially due to their volatilization. In fact, the degradation of naphthalene is
221 expected to be very fast due to its high volatility and solubility (Cerniglia, 1992).
222 Moreover, this compound is mainly degraded by abiotic processes as showed by
223 Lors and Mossmann (2001) during soil biodegradation assays in sterilized and
224 unsterilized conditions.

225 The exponential regression of the concentration of 16 PAHs as a function of time
226 reproduced quite accurately experimental data of the biotreatment. The best fit
227 ($R^2 = 0.96$) occurred by considering only the three first months and excluding the
228 last point measured after 6 months.

229 The degradation of 3-ring PAHs was observed after a short induction period of 7
230 days (Fig. 1-C). After the induction period, the degradation rate of 3-ring PAHs
231 occurred at the fastest rate ($\log C$ value = -0.0161). Thus, 95% of 3-ring PAHs
232 were degraded after 3 months of incubation, then the degradation rate slowed
233 down relatively to what could be expected from exponential regression. As a
234 consequence and similarly to the total 16 PAHs, the best fit for exponential
235 regression ($R^2 = 0.94$) was obtained considering only the first three months.

236 The degradation rate of phenanthrene, i.e. the main 3-ring PAH present, was
237 similar to that of total 3-ring PAHs. Its degradation was rapid, between 7 to 34
238 days, leading to 84% degradation. Almost complete degradation of this
239 compound (94%) occurred within the first three months of incubation (Fig. 2-A).

240 As previously observed for 3-ring PAHs, 4-ring PAH concentration during the
241 induction period was slightly higher than that of the Ti soil. Nevertheless, as 4-
242 ring PAH concentration was lesser than 3-ring PAH concentration, the difference
243 observed between Ti soil and soil sampled at the end of the induction period was
244 smaller. We did not find such differences for 5-ring and 6-ring PAHs, that only
245 represented 7% of total PAH concentration in the Ti soil.

246 The degradation of 4-ring PAHs displayed an induction period of 14 days was
247 one week more than 3-ring PAHs (Fig. 1-D). Opposite to 3-ring PAHs, a greater
248 amount of 4-ring PAHs was degraded during the last three months of the
249 experiment (20% of 4-ring PAHs, corresponding to 163 mg kg⁻¹ dry soil) (Fig. 1-
250 D). As a consequence, exponential regression gave the best fit ($R^2 = 0.98$) when
251 the last point at 6 months was included in the model. The rate of degradation of
252 4-ring PAHs was also slower than that of 3-ring PAHs; 12%, 40%, 56% and 76%
253 of the total 4-ring PAHs were degraded after 1, 2, 3 and 6 months of incubation
254 respectively. This fact is numerically represented by a higher log C value: -0.0037
255 instead of -0.0161.

256 Fluoranthene, the main 4-ring PAH present, followed a similar pattern of
257 degradation to total 4-ring PAHs; 81% of fluoranthene was degraded after 6
258 months of incubation (Fig. 2-B).

259 The degradation of 5-ring and 6-ring PAHs was even slower. After 6 months of
260 incubation, 24% and 22% of 5-ring and 6-ring PAHs were degraded,
261 corresponding to only 36.4 and 14.2 mg kg⁻¹ dry soil, respectively. The
262 degradation of these compounds followed a negative exponential regression,
263 giving the best fit with the complete data set: R^2 was equal to 0.7 and 0.82 for 5-
264 ring and 6-ring PAHs, respectively. The determination coefficient was lower as a
265 consequence of a greater relative scattering of measurements due to lower
266 amounts of 5-ring and 6-ring PAHs. Taking into account this incertitude, it can be
267 considered that the rates of degradation of 5-ring and 6-ring PAHs were quite
268 similar.

269

270 **3.3 Monitoring of bacterial diversity during laboratory experiment**

271

272 The evolution of the total bacterial community during the laboratory
273 experiment is reported in Table 4. Phylogenetic analysis of the sequences

274 derived from 16S rRNA gene bands in the bacterial community revealed that
275 every clone matches for at least 94 to 99% sequence identity with 16S rRNA
276 genes of the Genbank, except for the DGGE band number 17 (DBN 17),
277 identified as *Actinobacteria* with 84% of similarity (Table 4).

278 A good reproducibility was observed with the replicate soil samples from 14 and
279 92 days. For example, duplicate soil samples collected on 14 days showed
280 similar DGGE band profiles (Fig. 3).

281 As described by Lors et al. (2010b), the Ti soil contained a total bacterial
282 community characterized by a high diversity (13 bacterial strains belonging to 9
283 genera), that remained throughout the experiment. The strains initially present in
284 the Ti soil were still observed at the end of the experiment. These bacteria mainly
285 belonged to the *Gamma-proteobacteria* group (*Pseudomonas*, *Acinetobacter*,
286 *Enterobacter*, *Klebsiella*) (62%) and to a lesser extent to the *Alpha-proteobacteria*
287 group (*Erythromicrobium*, *Sinorhizobium*) (15%), the *Beta-proteobacteria* group
288 (*Alcaligenes*) (8%) and the *Lactobacillales* group (*Aerococcus*) (8%) (Table 4).

289 One strain, *Cellulomonas variformis* (DBN 17), was only detected in the Ti soil,
290 while other strains appeared during limited periods of time. Some strains
291 belonging to the *Beta-proteobacteria* group, including *Brachymonas*
292 *petroleovorans* (DBN 13) and *Uncultured Hydrogenophaga* sp. (DBN 11),
293 appeared between 92 and 182 days, whereas *Alcaligenes xylosoxidans* (DBN
294 15) was detected starting at 14 days. *Pseudomonas stutzeri* (DBN 16) was only
295 detected after 34 days of incubation.

296

297 **4 – Discussion**

298

299 The results of the laboratory experiment can be compared with those of
300 the field experiment, knowing that the greater number of samplings especially at
301 the beginning of the laboratory experiments enabled us to better define the

302 kinetics of PAH degradation and thus may lead to more accurate exponential
303 regression. If we compare total PAH concentrations after 6 months of
304 biotreatment, more than 85% of the total PAHs were degraded at field and
305 laboratory scales but with a slight advantage for the field: the concentration of 16
306 PAHs fell from 2894 ± 38 to 345 ± 23 and 440 ± 21 mg kg⁻¹ dry soil in field and
307 laboratory experiments, respectively. The fitness of field data with a negative
308 exponential model was excellent ($R^2 = 0.98$) and the value of log C was a little
309 more negative according to slightly more rapid degradation rate: -0.0088 instead
310 of -0.0077. These results indicate that the field biotreatment was carried out in
311 conditions close to optimal conditions for PAH biodegradation. Indeed, during the
312 windrow biotreatment, the matrix was turned over periodically to allow good
313 oxygenation. The moisture content was also kept constant by periodic water
314 sprinkling. As a result, the temperature was always above 30 °C, which was used
315 as an indicator of biotreatment performance. The turning procedure was
316 designed to minimize heat loss and to maximize gas exchange (CO₂ and O₂).
317 Indeed, temperature, moisture and aeration are the major factors associated with
318 PAH bioremediation (Vinas et al., 2005). Moreover, additional degradation
319 mechanisms could have occurred in the field for some PAHs. This does not seem
320 to be the case for 3-ring PAHs as the degradation rate obtained in the laboratory
321 was higher than in the field (Fig 1-C): log C was -0.0161 and -0.0124 for
322 laboratory and field, respectively. The faster degradation of 3-ring PAHs at the
323 beginning of the laboratory experiment was probably related to the fact that the
324 experiment was carried out under better controlled and optimised conditions than
325 in the field: for example, temperature (30 °C) and moisture content were kept
326 constant in the laboratory experiment. As the degradation of 3-ring PAHs is
327 mostly controlled by biological phenomena (Lors and Mossmann, 2005), these
328 optimal conditions allowed more rapid development of the indigenous microflora
329 able to degrade these compounds, which were already present in the initial soil in

330 a considerable amount (10^5 - 10^7 bacteria g^{-1} dry soil) (Table 1). However, the
331 optimum conditions of the laboratory experiment did not enhance the degradation
332 kinetics of 4- and 5-ring PAHs, which was on the contrary slightly quicker in the
333 field (see log C values in Table 3). So, the field experiment seemed to be slightly
334 more efficient for these high molecular weight PAHs even if the observed
335 differences may correspond to the heterogeneity of the samples: 76%, 24% and
336 82%, 35% of 4- and 5-ring PAHs were degraded in laboratory and field
337 experiments, respectively. For 6-ring PAHs, scattering of field data was high and
338 this led to a bad fit to the negative exponential model ($R^2 = 0.34$). As the amount
339 of 6-ring PAHs was weak, this fact can be explained by sampling and analytical
340 heterogeneity of PAH measurements. The reported results are in agreement with
341 those of Kästner et al. (1998) and Warmer and Peters (2005), indicating that
342 bioremediation is more effective for low molecular-weight PAHs (Fig. 4). Indeed,
343 the relative rate of degradation of 3-ring PAHs is about 30 times higher than 5-
344 and 6-ring PAHs while only 7 times higher than 4-ring PAHs. Indeed, the
345 degradation of high molecular-weight PAHs require typically more time and in
346 some cases different microorganisms (Perry, 1979; Boonchan et al., 2000). As a
347 consequence, the concentration of 3-ring PAHs is the lowest after 6 months while
348 it was the highest at the beginning of the biotreatment.

349

350 Results based on the evolution of PAH concentration have to be
351 compared with microbial consortia. First, the rate of degradation is likely to be
352 dependent on the amount of degraders specific to some PAHs. If we consider
353 phenanthrene, the most abundant 3-ring PAH, and fluoranthene, the most
354 abundant 4-ring PAH, both follow the same kinetics of degradation (Fig. 2-A and
355 2-B), similar to those observed for the total pool of 3-ring and 4-ring PAHs,
356 respectively (Figs. 1-C and 1-D). However, the greater amount of phenanthrene
357 that is degraded could be related to the greater initial abundance of bacteria

358 specifically degrading phenanthrene (10^7 bacteria g^{-1} dry soil) compared to the
359 initial population of fluoranthene-degrading bacteria (10^5 bacteria g^{-1} dry soil)
360 (Table 1).

361 Second, most bacteria detected in the initial soil sample were still present at the
362 end of the both field and laboratory experiments; bacteria present throughout the
363 entire experiment included Strain No 1 belonging to *Lactobacillales*, Strains Nos
364 2, 3, 4, 5, 6, 8, 10 and 12 belonging to *Gamma-proteobacteria* and Strains Nos 7
365 and 9 belonging to *Alpha-proteobacteria*. Lors et al. (2010b) showed that bacteria
366 belonging to the *Gamma-proteobacteria* group exhibited a high degrading activity
367 towards phenanthrene and fluoranthene. As a consequence, this group could be
368 a good bioindicator to determine the potential of PAH biodegradation of polluted
369 soils containing predominantly 2-, 3- and 4-ring PAHs. Conversely, *Alcaligenes*
370 *xylosoxidans* (Strain No 14) was detected in the initial soil and during the entire
371 duration of the laboratory experiment but was no longer observed at the end of
372 the field experiment (Table 4). Strains considered to be specific for various
373 phases of the biodegradation process in the laboratory experiment also appeared
374 at specific periods during the field experiment. Strains belonging to the *Beta-*
375 *proteobacteria* group, including *Brachymonas petroleovorans* (DBN 13) and
376 *Uncultured Hydrogenophaga* sp. clone H-3 (DBN 11), were also present starting
377 at 92 days in the field experiment. Conversely, *Alcaligenes xylosoxidans* (DBN
378 15), which was detected starting at 14 days in the laboratory experiment,
379 appeared at 92 days in the field experiment. The fact that samplings were
380 performed more frequently at the beginning of the laboratory experiment allowed
381 us to visualise more precisely the evolution of these strains between 0 and 92
382 days. *Uncultured Hydrogenophaga* sp. clone H-3 (DBN 11) and *Brachymonas*
383 *petroleovorans* (DBN 13) appeared when the total concentration of 2-, 3- and 4-
384 ring PAHs decreased to 427 and 307 $mg\ kg^{-1}$ dry soil in laboratory and field
385 experiments, respectively. It must be noticed that the soil biotreated for 6 months

386 was no longer ecotoxic with respect to plant (*Lactuca sativa*) germination and
387 growth inhibition, earthworm (*Eisenia fetida*) mortality and springtail (*Folsomia*
388 *candida*) avoidance (Lors et al., 2010a). The occurrence of *Brachymonas*
389 *petroleovorans* (DBN 13) and *Uncultured Hydrogenophaga* sp. clone H-3 (DBN
390 11) in the laboratory experiment confirmed that these strains, belonging to the
391 *Beta-proteobacteria* group, could be used as indicators of the restoration of soil
392 quality and thus could signal the endpoint of the biological treatment of polluted
393 soil containing mostly 2-, 3- and 4-ring PAHs.

394 Third, additional microorganisms other than bacteria, such as fungi, may play a
395 role in PAH degradation. Li et al. (2008) revealed that microbial consortia
396 composed of bacteria, fungi and a bacterial-fungal mixture successfully degraded
397 3- to 5-ring PAHs in soil and slurry phases. It may thus be hypothesized that, in
398 our experiments, a colonisation of the windrow by a more complex consortium
399 made of bacteria and other microorganisms could eventually be one of the
400 reason explaining that the biotreatment worked slightly better in the field than in
401 the laboratory. Indeed, the change in the bacterial microbiota over time in the
402 laboratory experiment mirrored the changes observed during the field experiment
403 (Table 4) (Lors et al., 2010b) and thus the observed differences should come
404 from other organisms than bacteria that were not determined experimentally.

405

406 **5. Conclusion**

407

408 Laboratory experiments carried out on the same polluted soil than a
409 windrow treatment and at the same time led to results similar to those obtained
410 during the windrow biotreatment. The decrease of the amount of 3-, 4-, 5- and 6-
411 ring PAHs can be fitted to an exponential model. However, the kinetics of PAH
412 degradation could be defined more precisely in the laboratory, due to more
413 samplings, leading to higher R^2 values. The relative rate of degradation of 3-ring

414 PAHs was about 30 times higher than 5- and 6-ring PAHs while only 7 times
415 higher than 4-ring PAHs. The optimum conditions at the laboratory just enabled
416 us to slightly speed up the degradation of 3-ring PAHs while the degradation of 4-
417 and 5-ring PAHs was a little faster in the field. As a consequence, the amount of
418 degraded PAHs after 6 months was around to 85% in the laboratory instead of
419 90% in the field. Thus, conditions of the field biotreatment were close to optimum,
420 mostly thanks to the optimisation of the chemical conditions (moisture, O₂,
421 temperature) and additional degradation mechanisms for 4-, 5- and 6-ring PAHs.
422 The evolution of bacterial microbiota in the laboratory was also comparable to
423 that obtained in the field. The bacterial consortium was represented by Gram-
424 negative bacteria strains belonging mainly to *Gamma-proteobacteria*, in particular
425 *Enterobacteria* and *Pseudomonas*. This consortium, which persisted throughout
426 the biotreatment, is considered to be a good bioindicator to estimate the potential
427 of biodegradation of soils polluted predominantly with 2-, 3- and 4-ring PAHs. The
428 occurrence of strains belonging to *Beta-proteobacteria*, specifically *Brachymonas*
429 *petroleovorans* and *Uncultured Hydrogenophaga* sp. clone H-3, was observed in
430 both laboratory and field experiments when the PAH concentration was low
431 enough not to cause soil ecotoxicity. Therefore, the detection of these strains
432 could be used to estimate the endpoint of the biotreatment of PAH-contaminated
433 soil even if some PAHs remain in the soil. Indeed, the bioavailability of PAHs
434 should also be considered in addition to results of chemical analyses of the soil.
435 Some additional experiments could be undertaken to study the microstructure of
436 the soil after 6 months of biotreatment in order to find a relationship between the
437 distribution of remaining PAHs and the bioavailability of PAHs. Indeed, the
438 knowledge of the evolution of PAH distribution could also help to understand the
439 effect of additional treatments, such as phytoremediation, in order to reach an
440 even greater level of remediation (Gerhardt et al., 2009).

441 Finally, the ability to perform representative laboratory biodegradation
442 experiments is very attractive because it allows the assessment of the impact of
443 different parameters that can modify the effectiveness of a biotreatment, such as
444 temperature, moisture, oxygenation and nutrient availability. This type of
445 experiment could allow us to optimise the duration of the biotreatment and to
446 reduce the cost of the process. Moreover, performing a laboratory experiment at
447 the same time as a field biotreatment is a valuable way to monitor if the
448 biotreatment process is performing as expected.

449

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451

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457

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570

571

572 **Figure captions**

573

574 **Fig. 1.** Changes in the concentrations of all 16 PAHs (A) and of 2- (B), 3-
575 (C), 4-(D), 5- (E) and 6- (F) ring PAHs concentrations (mg kg⁻¹ dry
576 soil ± S.E.) over time during the laboratory (L) and field (F)
577 experiments.

578

579 **Fig. 2.** Changes in the concentrations of phenanthrene (Phe) (A) and
580 fluoranthene (Flt) (B) concentrations (mg kg⁻¹ dry soil ± SE) over
581 time during the laboratory (L) and field (F) experiments.

582

583 **Fig. 3.** DGGE analysis of the bacterial communities during the laboratory
584 experiment.

585 lane M: DGGE marker - lane 1: 0 day; lane 2: 3 days; lane 3: 7 days,
586 lanes 4 and 5: 14 days; lane 6: 34 days; lane 7: 63 days; lane 8: 92 days;
587 lane 9: 182 days

588 A: *Aerococcus viridans*, B: *Enterobacter* sp. GOBB3-C104, C:
589 *Pseudomonas* sp. HI-B7, D: *Pseudomonas stutzeri* ATCC 17685, E:
590 *Pseudomonas* sp. C54A, F: *Acinetobacter johnsoni*, G: *Erythromicrobium*
591 *ramosum* DMS 8510, H: *Pseudomonas stutzeri* ZWLR2-1, I:
592 *Sinorhizobium* sp. L1, J: *Klebsiella planticola* ATCC 33531T, K:
593 *Brachymonas petroleovorans* CHX, L: *Alcaligenes xylosoxidans*, M:
594 *Alcaligenes xylosoxidans*

595

596 **Fig. 4.** Values of log C to function the number of rings for 3-, 4-, 5- and 6-
597 ring PAHs during the laboratory and field biodegradation
598 experiments.
599

600 **Table 1.** Chemical and bacterial characteristics of the Ti soil.

601

Soil	Moisture	pH	C:N	16 PAHs	As	Cd	Cr	Cu	Pb	Zn	Total bacterial microbiota	Phe bacterial degraders	Flt bacterial degraders
	%						mg kg ⁻¹ dry soil				bacteria g ⁻¹ dry soil		
Ti soil	17.7 ± 0.1	7.9 ± 0.02	56	2895 ± 38	6.5 ± 0.5	< LD	32.9 ± 0.3	19.3 ± 0.4	23.9 ± 0.8	92.7 ± 0.8	4.9 10 ⁸	1.4 10 ⁷	7.7 10 ⁴
G.B.	-	-	-	-	8.9 ± 1.2	0.4 ± 0.03	48.8 ± 2.7	16.7 ± 1.8	38.4 ± 5.6	73.7 ± 6.2	-	-	-

602 Phe: Phenanthrene; Flt: Fluoranthene; Limit of detection (LD); Carbon-Nitrogen ratio (C:N).

603 Geochemical background (G.B.) of trace elements in soils around smelters in the North of France was determined by Sterckeman et al. (2002).

604

605 **Table 2.** Concentration^a of each of 16 (EPA) PAHs (reported in mg kg⁻¹ of dry
606 soil) in the Ti soil (the same soil was used in field and laboratory experiments).
607

	[PAH]
	(mg kg ⁻¹ dry soil)
Naphthalene	594.22 ± 7.96
Acenaphthene	3.12 ± 0.03
Acenaphthylene	217.42 ± 0.71
Fluorene	226.81 ± 1.64
Phenanthrene	629.31 ± 2.40
Anthracene	202.50 ± 18.31
Fluoranthene	414.34 ± 0.71
Pyrene	233.44 ± 0.22
Benzo(a)anthracene	85.72 ± 0.50
Chrysene	75.43 ± 0.50
Benzo(b)fluoranthene	56.18 ± 0.17
Benzo(k)fluoranthene	25.80 ± 0.18
Benzo(a)pyrene	60.36 ± 3.88
Dibenzo(a,h)anthracene	6.86 ± 0.14
Benzo(g,h,i)perylene	32.45 ± 0.56
Indeno(123-cd)pyrene	30.79 ± 0.15

608
609 ^a Mean values ± S.E. for three replicates.
610

611

612 **Table 3.** Values of the parameters of the exponential regression applied to the
613 kinetics of PAHs biodegradation.

614 $[\text{PAH}]_{t_0}$, C and R^2 corresponded respectively to the initial amount of PAH, a
615 constant related to the degradation rate and the determination coefficient of the
616 exponential regression.

617

	Laboratory			Field		
	log C	$[\text{HAP}]_{t_0}$	R^2	log C	$[\text{HAP}]_{t_0}$	R^2
16 PAHs	- 0.0077	2693.3	0.96	- 0.0088	3113.7	0.98
3-ring PAHs	- 0.0161	1513.9	0.94	- 0.0124	1508.1	0.96
4-ring PAHs	- 0.0037	777.4	0.98	- 0.0044	880.9	0.86
5-ring PAHs	- 0.0005	139.0	0.70	- 0.0012	135.0	0.42
6-ring PAHs	- 0.0005	59.7	0.82	- 0.0002	61.3	0.34

Fig. 1.

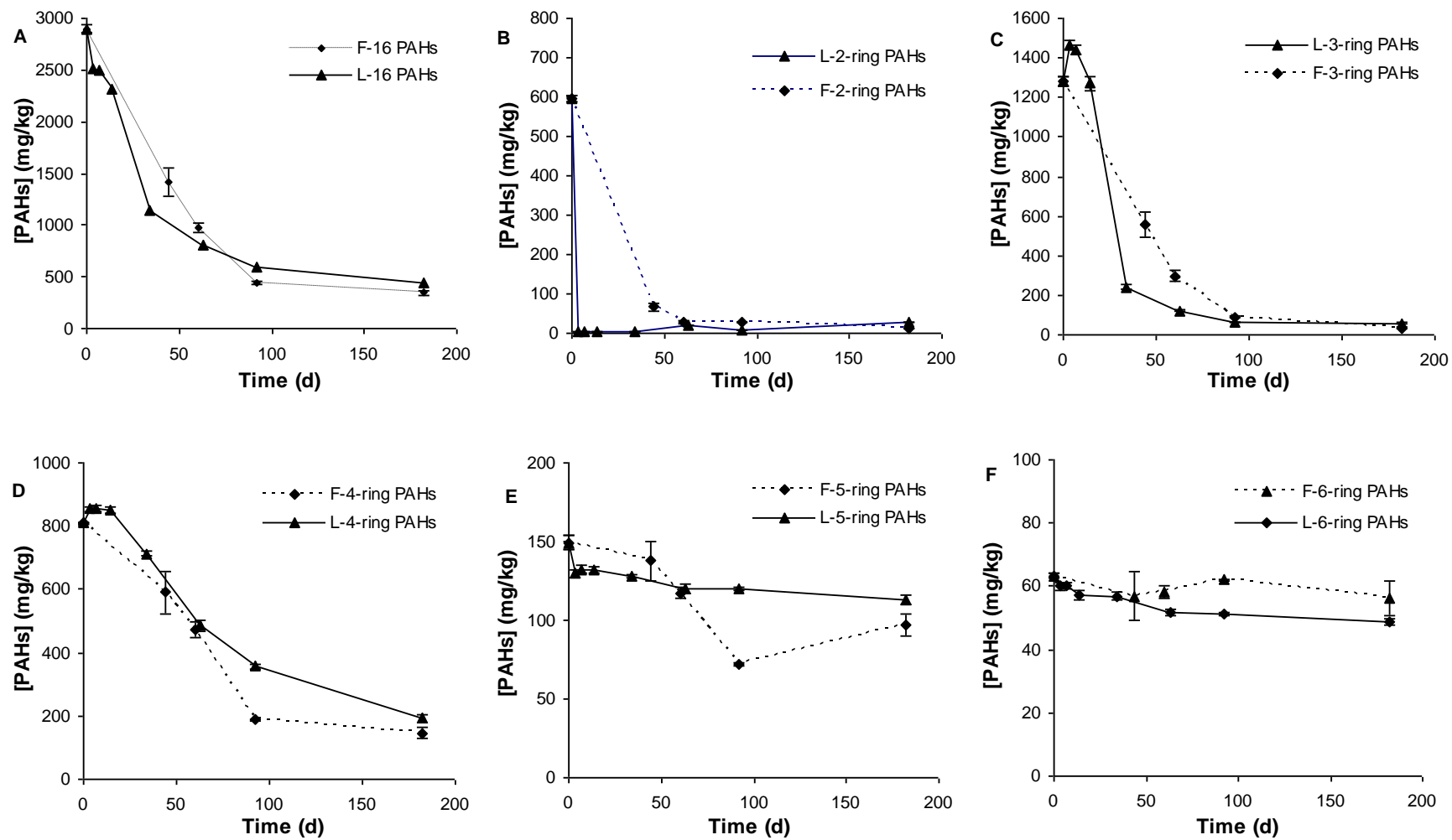


Fig. 2.

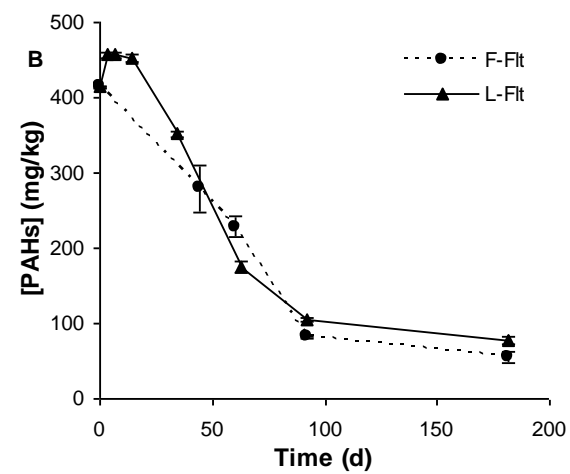
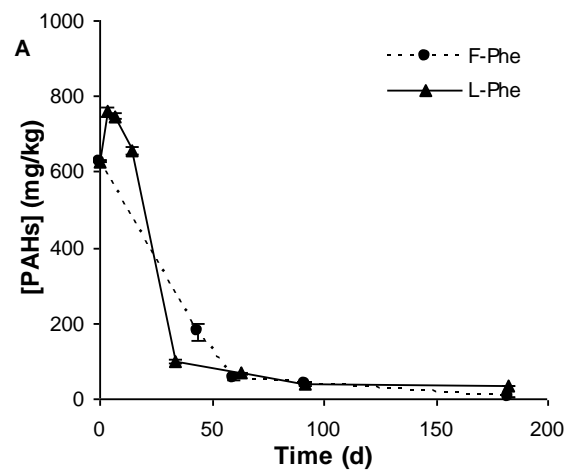


Fig. 3.

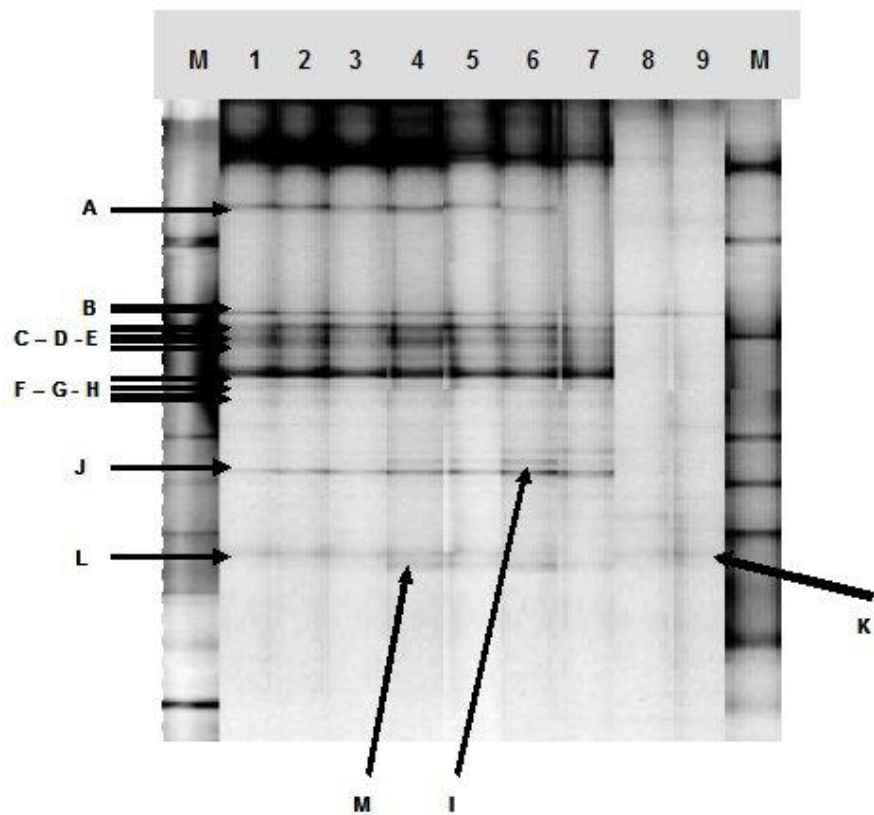


Fig. 4.

