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1 **Low effect of phenanthrene bioaccessibility on its biodegradation**
2 **in diffusely contaminated soil**

3 M. Crampon^{a,b}, A. Cébron^{c,d}, F. Portet-Koltalo^a, S. Uroz^e, F. Le Derf^a, J. Bodilis^{b,f,#}

4

5 ^a COBRA UMR CNRS 6014, Université de Rouen-Normandie, 55 rue saint Germain, 27000 Evreux, France

6 ^b Laboratoire de Microbiologie Signaux et Microenvironnement, EA 4312, Université de Rouen, 76821 Mont Saint Aignan, France

7 ^c CNRS, LIEC UMR 7360, Faculté des Sciences et Technologies, BP70239, 54506 Vandoeuvre-lès-Nancy Cedex, France

8 ^d Université de Lorraine, LIEC UMR 7360, Faculté des Sciences et Technologies, BP70239, 54506 Vandoeuvre-lès-Nancy Cedex, France

9 ^e UMR 1138 INRA, centre de Nancy, Biogéochimie des Ecosystèmes forestiers, Route d'Amance, 54280 Champenoux, France

10 ^f Université de Lyon, France; CNRS, INRA, Ecole Nationale Vétérinaire de Lyon, and Université Lyon 1, UMR 5557 Ecologie Microbienne,

11 43 boulevard du 11 novembre 1918, 69622 Villeurbanne cedex, France

12

13 **Short title:** Low effect of bioaccessibility on phenanthrene biodegradation in soil

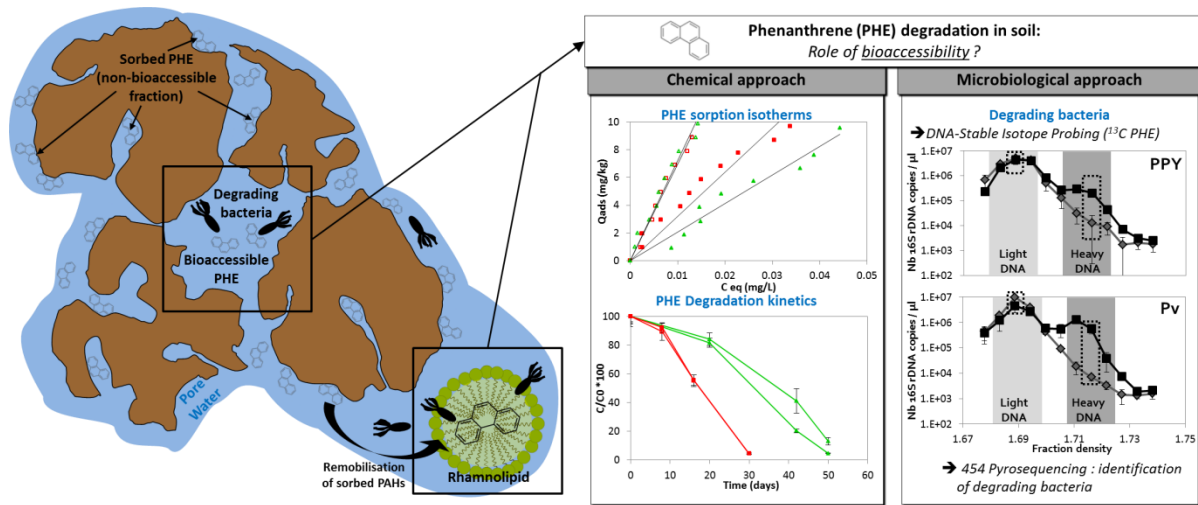
14 # Corresponding author: J. Bodilis, josselin.bodilis@univ-rouen.fr

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16 **Keywords:** phenanthrene biodegradation; soil; bioaccessibility; DNA-SIP; rhamnolipids;

17 *Rhodocyclaceae*

18 **Graphical Abstract**



19

20 **Abstract**

21 This study focused on the role of bioaccessibility in the phenanthrene (PHE) biodegradation in
 22 diffusely contaminated soil, by combining chemical and microbiological approaches. First, we
 23 determined PHE dissipation rates and PHE sorption/desorption kinetics for two soils (PPY and
 24 Pv) presenting similar chronic PAH contamination, but different physico-chemical properties.
 25 Our results revealed that the PHE dissipation rate was significantly higher in the Pv soil
 26 compared to the PPY soil, while PHE sorption/desorption kinetics were similar. Interestingly,
 27 increases of PHE desorption and potentially of PHE bioaccessibility were observed for both
 28 soils when adding rhamnolipids. Second, using ¹³C-PHE incubated in the same soils, we
 29 analysed the PHE degrading bacterial communities. The combination of stable isotope probing
 30 (DNA-SIP) and 16S rRNA gene pyrosequencing revealed that *Betaproteobacteria* were the
 31 main PHE degraders in the Pv soil, while a higher bacterial diversity (*Alpha-*, *Beta-*,
 32 *Gammaproteobacteria* and *Actinobacteria*) was involved in PHE degradation in the PPY soil.
 33 The amendment of biosurfactants commonly used in biostimulation methods (*i.e.* rhamnolipids)
 34 to the two soils clearly modified the PHE sorption/desorption kinetics, but had no significant
 35 impact on PHE degradation rates and PHE-degraders identity. These results demonstrated that

36 increasing the bioaccessibility of PHE has a low impact on its degradation and on the functional
37 populations involved in this degradation.

38

39

40 **Capsule**

41 The increase of the bioaccessibility level of phenanthrene has a low impact on its degradation
42 in diffusely contaminated soil.

43 **Introduction**

44

45 Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous carcinogenic organic contaminants,
46 originating from multiple sources (*i.e.* biogenic, petrogenic and pyrogenic). Among them,
47 human activities are responsible for the major releases into the environment, particularly
48 through atmospheric deposition (Tobiszewski and Namieśnik, 2012). PAHs physico-chemical
49 properties (high chemical stability, low vapor pressure, low aqueous solubility, high water-
50 organic carbon K_{oc} partition coefficient) make them persistent in soils. However, this
51 persistence is also strongly dependent on soil physico-chemical properties and soil microbial
52 activities (Doyle et al., 2008).

53 In the case of diffuse contamination, soils are continuously exposed to low and repeated
54 deposits of PAHs. In these soils, PAHs can be found in different soil compartments, depending
55 on the age and history of the contamination. A small fraction of these PAHs is usually present
56 in the soil aqueous phase, and is considered as directly accessible to microorganisms for
57 degradation. The bioaccessible fraction is available to cross cellular membranes if the organism
58 is present and has access to it, even after a period of time or through spatial rearrangements,
59 whereas the bioavailable fraction is that is freely available to cross the organism's cellular
60 membrane at a given time (Riding et al., 2013). The PAHs recently adsorbed on soil constituents
61 are also generally directly accessible to microorganisms, while PAHs in contact with soil
62 constituents for a longer time become poorly bioaccessible. A last fraction of PAHs can be
63 strongly bound to soils, by slow diffusion into soil micropores. The strong
64 sorption/sequestration mechanisms of PAHs to organo-mineral complexes eventually creates
65 non-extractable bound-residues (Ahangar, 2010), considered as not bioaccessible (Semple et
66 al., 2004).

67 After adaptation and selection processes, the soil bacterial communities acquire the potential to
68 biodegrade the bioaccessible PAH fraction (Haritash and Kaushik, 2009). Indeed, different
69 bacterial strains isolated from various contaminated soils have been described as capable of
70 degrading PAHs and of growing with PAHs as sole carbon source. These bacteria belong to
71 different phyla or classes such as α -, β - and γ -*Proteobacteria* (Haritash and Kaushik, 2009; Jeon
72 et al., 2006; Kim and Jeon, 2009; Niepceron et al., 2013), *Bacteroidetes*, *Actinobacteria*
73 (Timmis et al., 2010) or *Firmicutes* (Wu et al., 2008).

74 The bioaccessibility factor depends on the physico-chemical properties of PAH compounds and
75 on the soil properties (clays and organic matter contents and nature) (Brandli et al., 2008; Jonker
76 et al., 2005; Mechlińska et al., 2009; Xia et al., 2010). Besides that, the bioavailability factor
77 depends on the physiological and catabolic potential of soil microorganisms (Crampon et al.,
78 2014). The combination of these complex processes determines the fate of PAHs in soils
79 (Semple et al., 2004; Semple et al., 2003). Enhancement of PAH bioaccessibility in
80 contaminated soils through addition of surfactants has already been reported (Ahn et al., 2008;
81 Paria, 2008; Zhou et al., 2007; Zhou and Zhu, 2008). Biosurfactants (*e.g.* surfactants naturally
82 produced by (micro)organisms) are more environmental friendly and interesting due to their
83 low critical micellar concentration (CMC) and their lower toxicity for soil microorganisms than
84 synthetic ones (Bustamante et al., 2012). Rhamnolipids, produced by *Pseudomonas aeruginosa*
85 (Bordas et al., 2005), have been described for their ability to remobilize sorbed PAHs and to
86 potentially enhance their biodegradation in slurry batches or column experiments (Bordas et
87 al., 2005; Congiu and Ortega-Calvo, 2014). However, the real impact of these rhamnolipids on
88 PAHs accessibility after direct treatment of contaminated soils remains to be determined.

89

90 In this context, the aim of this study was to determine the parameters mostly governing
91 phenanthrene (PHE) degradation rates in diffusely contaminated soils. PHE was chosen as a

92 model PAH representative of low molecular weight PAHs (2-3 fused aromatic rings). To
93 address such an objective, two soil types (PPY and Pv) characterized by similar chronic PAH
94 contamination, but different physico-chemical characteristics, were analyzed and compared. In
95 each soil type, chemical and molecular biological methods were combined to investigate the
96 fate of phenanthrene (PHE) by focusing on: (i) PHE accessibility, (ii) PHE degradation kinetics,
97 and (iii) PHE-degrading bacterial diversity in relation to the amendment or not of rhamnolipids.
98 PHE degradation was monitored using a microcosm approach during two months. In
99 microcosm, PHE sorption isotherms with or without rhamnolipids were determined to estimate
100 PHE accessibility. PHE degradation kinetics were determined using ¹³C-PHE and PHE-
101 degrading bacterial populations were identified through DNA-Stable Isotope Probing (DNA-
102 SIP) and 16S rRNA gene amplicon pyrosequencing.

103

104 **Materials and Methods**

105

106 **Soil sampling and soil analyses**

107 Soil samples were collected in the Seine river basin (France), which is a highly urbanized and
108 industrialized location accounting for 40% of the French economic activity and including 33%
109 of oil refining and 50% of river traffic. In spring 2014, approximately 15 kg of soil (0–15 cm
110 depth) of two diffusely contaminated soil types (PPY and Pv) were sampled. PPY soil is an
111 agricultural soil characterized by a permanent grassland and located near Yvetot (Seine-
112 Maritime department, 49°36'40.6"N 0°44'10.7"E), at about 15 km from the Seine River. The
113 PPY soil is classified as Luvisol according to the World Reference Base for soil resources
114 (IUSS Working Group WRB 2006). The second soil type sampled (Pv) is located near Petiville
115 (Seine Maritime department, 49°25'54.0"N 0°36'25.4"E) in the Seine river estuary, and is

116 partially covered by young willow. This soil, periodically flooded, is classified as fluvic Gleysol
117 according to the World Reference Base for soil resources.

118 After sampling, field-moist soils were sieved through a 5 mm mesh and stored at room
119 temperature for 8 days for acclimation. Aliquots were stored at -20°C for molecular
120 characterization of the initial microbial communities in the two soil types before PAH spiking.
121 The natural background concentrations of PAHs in both soils were lower than 1 mg.kg⁻¹ for the
122 sum of the 16 PAHs classified as priority by the US-Environmental Protection Agency (US-
123 EPA) (Table 1).

124 Pv and PPY soil organic matter (OM) was analyzed on dried crushed soils by RockEval 6
125 pyrolysis (Vinci Technologies, France) and S2 peak deconvolution (Sebag et al., 2006) was
126 obtained using peakfit software (SigmaPlot, Systat) and the “Bulk Rock” method, as described
127 previously (Crampon et al., 2014) (Table 1). Nature of clays in soils was analyzed by X-Ray
128 diffraction (XRD) (Philips PW3040 diffractometer, France), as described previously (Crampon
129 et al., 2014).

130 Soil pH was determined according to the French NF ISO 10-390 standard. Granulometry was
131 analyzed according to the NF X31-107 standard. CEC (Cationic Exchange Capacity) was
132 obtained following the French NF ISO 31-130 standard by the cobaltihexamine method. The
133 analyses of pH, texture and CEC were carried out at the LAS-INRA laboratory (Arras, France).

134

135 **Phenanthrene sorption isotherm determination**

136 Sorption isotherm experiments were performed for both soils as previously described
137 (Groboillot et al., 2011), in the presence or not of rhamnolipids. Briefly, sorption reactors
138 consisted in 4 g of soil sieved at 355 µm and mixed with 40 mL deionized water in glass tubes
139 under magnetic agitation. Reactors were incubated at 25°C along the experiment and HgCl₂
140 (500mg.L⁻¹) was added as a biocide at the beginning of the experiment to avoid bacterial

141 degradation, which could decrease PHE concentration in water after many hours. PHE was
142 then introduced in the reactors with a known volume of PHE stock solution (100 mg.L⁻¹ in
143 acetonitrile), to obtain a range of concentrations (from 0.1 to 1 mg.L⁻¹), below its limit of
144 solubility in water (*i.e.* 1.6 mg.L⁻¹). The volume of acetonitrile injected (<1% of the total
145 volume) was low enough to have no impact on PHE sorption, as previously studied (Groboillot
146 et al., 2011). To evaluate the remobilization capacity of rhamnolipids, the biosurfactant (AGAE
147 Technologies (USA), 90% purity) was added as a powder in the reactors 5 h after the
148 introduction of PHE, to a final concentration of 0.55 g.L⁻¹ (concentration ten times higher than
149 its CMC of 0.05 g.L⁻¹) to ensure the formation of micelles in the aqueous phase. Five hours was
150 chosen as the time to add the biosurfactant after PHE spiking because it was observed that a
151 “quasi” plateau was reached for PHE sorption after this time (data not shown). Mixtures treated
152 or not with rhamnolipids were stirred during 24 h at 25°C to be sure to reach thermodynamic
153 equilibrium (time evaluated during preliminary tests) (Barnier et al., 2014). Thereafter, reactors
154 were centrifuged at 7000 rpm during 20 min to recover the aqueous phase. The concentration
155 of PHE at equilibrium in the aqueous phase after 24 h (C_{eq}) was then measured after solid-phase
156 extraction (SPE) and analysis using high-performance liquid chromatography coupled with
157 fluorescence detector (HPLC-FLD) as developed, in the section below. As our systems were
158 closed, PHE spiked at t_0 was fully distributed between the water and the soil phases after 24h.
159 So, the sorbed concentration (C_{ads}) was deduced knowing the initial PHE concentration C_0 ,
160 following the equation $C_{ads} = C_0 - C_{eq}$. The K_d partition coefficients were then obtained from the
161 slope of the isotherms.

162

163 **Phenanthrene dissipation kinetics**

164 Microcosms consisted of 4.5 g of dry equivalent fresh soil (90%) and 0.5 g of dry sterilized
165 (autoclaved at 120°C for 20 min) soil (10%) spiked with ¹²C-PHE (purity > 98%) or ¹³C-PHE

166 (uniformly labeled $^{13}\text{C}_{14}$, 99 atom %; Sigma-Aldrich), mixed in a hermetically-closed 100 mL
167 sterile glass flask. A PHE solution was prepared in acetone and spiked to the dry sterilized soils
168 in order to obtain a final PHE concentration of $3,000 \text{ mg.kg}^{-1}$ of soil. The dry sterilized soil was
169 mixed under an extractor hood for 24 h to homogenize the PHE distribution and to evaporate
170 the remaining acetone. After one week of stabilization at ambient temperature, the dry PHE
171 contaminated and sterilized soil was mixed with the wet native soil (humidity of 24.1 and 31.7%
172 wt of fresh soil for PPY and Pv soils, respectively) and gently homogenized to obtain final soil
173 microcosms contaminated with PHE (300 mg.kg^{-1} dw soil). This spiked PHE final
174 concentration was 337-940 times higher than the sum of the 16 USEPA background PAHs
175 (Table 1) and 2000-2857 times higher than the concentration of the native 2-3 aromatic ring
176 PAHs. So we could reasonably consider that the main effects of PAHs on our studied systems
177 were mainly due to the addition of PHE at 300 mg.kg^{-1} , and so the impact of co-metabolism
178 was negligible in our study. It was decided to add the initial spiked dried sterilized soil (10%)
179 to the native wet soil (90%) after contact time of only 7 days, in order to avoid PHE dissipation
180 in the initial spiked soil. Soil water content of each microcosm was adjusted with distilled water
181 to 70% of its water holding capacity. It corresponded to a total of 43.5 g and 49.1 g of water
182 per 100 g soil for PPY and Pv soils, respectively. Rhamnolipids were added to distilled water
183 to a final concentration of 300 mg.kg^{-1} dw soil into half of the microcosms. Rhamnolipids
184 correspond to a mixture of mono and di-rhamnolipids (R90 product). So the concentration of
185 the added biosurfactant was around $0.61\text{-}0.69 \text{ g.L}^{-1}$ in the water fraction, which was near the
186 concentration tested in sorption studies. The flasks were opened once a week in sterile
187 conditions for 15 min to renew the atmosphere, and were incubated at 20°C in static mode in a
188 dark room. Analyses of microcosms were carried out after 0, 8, 16 and 30 days of incubation
189 for Pv soil and after 0, 20, 42 and 50 days for PPY soil, based on the different PHE degradation
190 rates evaluated through a preliminary experiment (data not shown). These different sampling

191 times between the two soils were chosen in order to be able to compare the labeled DNA
192 between the two soils (in terms of percentage of dissipated PHE), to potentially have the same
193 quantity of ^{13}C possibly incorporated into the degrading bacteria DNA. All the microcosms
194 were performed in triplicates giving a total of 96 microcosms (2 soils, with and without
195 rhamnolipids, 4 incubation times, 3 replicates and with ^{12}C and ^{13}C PHE). The experimental
196 protocol is available in supplementary materials (Fig. S1).

197

198 **Phenanthrene extraction and measurements**

199 All organic solvents were purchased from Fisher Scientific (Illkirch, France) and were of HPLC
200 grade. PHE extractions from soils were performed using the microwave accelerated extraction
201 (MAE; Mars X, CEM Corporation, USA) as described previously (Portet-Koltalo et al., 2008).
202 For PHE degradation kinetics, two aliquots of 1.5–2 g of wet soil were taken from each of the
203 three microcosms (in triplicates) at each time point. Then at each studied time, 6 sub-samples
204 of a soil without biosurfactant and 6 sub-samples of the same soil amended with biosurfactant
205 were dried 24 h at 35°C (to avoid the semi volatile PAH loss) and crushed separately. One g of
206 each of the 6 sub-samples was extracted simultaneously using MAE technique in 20 mL of an
207 acetone/toluene 50/50 (v/v) mixture at 130°C for 30 min, at 1,200 W. So for each studied time,
208 extraction yields were obtained from a mean of 6 values. To determine the PAHs concentration
209 in native soils, 10 g of crushed dried soil were processed using MAE technique, with 40 mL of
210 the acetone/toluene 50/50 mixture, in the same conditions as described above. After filtration
211 (0.45 μm Teflon filters; Fisher Scientific, France), extracts were evaporated to reach a volume
212 of 1.5 mL. An internal standard (10 μL of perdeuterated PHE at 100 $\text{mg}\cdot\text{L}^{-1}$) was added to 990
213 μL of each extract before GC-MS analyses. Quantification analyses were performed using a
214 6850 gas chromatograph from Agilent (USA) fitted with a mass spectrometer (MS) detector
215 (5975C, Agilent) and using a DB5-MS capillary column (60 $\text{m}\times 0.25$ mm inner diameter, film

216 thickness 0.25 μm ; J&W Scientific, USA) according to Crampon *et al.* (Crampon et al., 2014).
217 The mass detector (electron impact ionization 70 eV) operated in the selected ion monitoring
218 (SIM) mode for better sensitivity. The detection and quantification thresholds, calculated
219 respectively as 3 and 10 times the standard deviation of the blank sample noise, were 1.5 and 5
220 $\mu\text{g.L}^{-1}$, respectively. The mean recovery of PHE after MAE-GC-MS extraction and analysis
221 was $90.0\pm 7.0\%$ ($n=5$).
222 PHE extractions from aqueous media were done by SPE using Strata-X cartridges
223 (Phenomenex, Le Pecq, France). After cartridge conditioning and sample percolation (Portet-
224 Koltalo et al., 2013), PHE was eluted with 5 mL methylene chloride, and was evaporated under
225 nitrogen flow and recovered in 1 mL acetonitrile before HPLC analysis. PHE quantification
226 was performed using a HPLC (Beckman Coulter, USA) coupled to a FLD (Prostar 363, Varian,
227 USA) and using a C₁₈ Envirosep PP column (150 \times 4.6 mm, dp = 5 μm) (Phenomenex, Le Pecq,
228 France) according to Portet-Koltalo *et al.* (Portet-Koltalo et al., 2013). Detection and
229 quantification limits, calculated respectively as 3 and 10 times the standard deviation of the
230 sample noise, were evaluated as 0.4 and 1.3 $\mu\text{g.L}^{-1}$, respectively. The mean recovery of PHE
231 after SPE-HPLC-FLD extraction and analysis was $94.2\pm 1.2\%$ ($n=5$). Taking into account the
232 concentration factor using SPE, the limit of quantification of PHE in the aqueous medium was
233 33 ng.L^{-1} . So PHE was preferentially measured in the aqueous medium after sorption
234 experiments because SPE-HPLC-FLD allowed us to reach lowest concentrations than using
235 GC-MS and because of the highest accuracy of the method.

236

237 **¹³C labeled DNA isolation**

238 For DNA-based diversity analyses, samples were selected for further analysis according to the
239 level of ¹³C PHE degradation. Initial soils collected before spiking (triplicates) and soil samples
240 collected during the experiment at 20% (DT20) and 90% of PHE degradation (DT90) were used

241 for soil genomic DNA extractions using the PowerSoil DNA isolation kit (Mo-Bio
242 Laboratories) according to the manufacturer recommendations. Extracted DNA was quantified
243 using NanoDrop 2000 (Thermo Scientific) and samples were stored at -20°C until analysis.
244 Heavy (¹³C labeled) and light DNA (¹²C) were separated according to the protocol described
245 by Neufeld et al. (Neufeld et al., 2007). Gradients were prepared using a CsCl solution (purity
246 ≥ 99%; Sigma-Aldrich) with an initial density of ca 1.85. Gradient buffer and DNA sample
247 were then added (for a final DNA quantity in the centrifuge tube of 5 to 10 µg) to obtain a final
248 density of 1.725. Ultracentrifugations were carried out on a Beckman centrifuge (Optima L-80
249 XP) with a vertical rotor Vti 65.2 (Beckman), at 42,400 rpm for 40 h at 20°C, with maximal
250 acceleration and no deceleration to avoid gradient disturbance. After centrifugation, tubes were
251 fractionated in 12 density fractions that were weighed to check the gradient formation. DNA
252 contained in each fraction was then precipitated using 2 volumes of polyethylene glycol (~0.8-
253 1 mL), 1 µL glycogen (20 µg), centrifuged (13000g for 30 min) and washed with 70% purified
254 ethanol (500 µL), and finally recovered in 30 µL TE buffer (10mM Tris-HCl and 1mM EDTA
255 in deionized water) for further use.

256 On DNA recovered from each DNA fraction, the abundance of 16S rRNA genes was quantified
257 using real-time quantitative PCR to determine the distribution of heavy and light DNA. Real-
258 time PCR assays were performed in a 20 µL final volume, as previously described (Cebren et
259 al., 2008) using: 2X iQ SYBR Green Supermix (Bio-Rad), 0.4 µmol.L⁻¹ of each 16S rDNA
260 universal primers 968F and 1401R (Nubel et al., 1996), BSA (0.06%), 0.2 µL DMSO
261 (dimethylsulfoxyde), 0.08 µL t4gp32 protein (MP-Biomedicals) and 1 µL of DNA. The
262 amplification was carried out with 40 cycles of four steps: 20 sec at 95°C, 20 sec at 56°C, and
263 30 sec at 72°C and 5 sec at 82°C (Cebren et al., 2008). On similar DNA fractions, fungal 18S
264 rRNA genes were also quantified using real-time quantitative PCR (Thion et al., 2013) with the
265 previously described primer pairs Fung5f-FF390r (Lueders et al., 2004), but no enrichment of

266 fungi was detected in heavy DNA fractions (Fig. S3) meaning that fungi were not significantly
267 involved into PHE degradation because they did not incorporated ¹³C in their DNA.

268

269 **Identification of phenanthrene-degrading bacteria**

270 Altogether, DNA from the 16 microcosm treatments (2 soil types, with and without
271 rhamnolipids, at DT90, light and heavy DNA fractions n°3 and 7, from ¹²C and ¹³C incubations)
272 and the two initial soils, in triplicate, were chosen for sequencing, resulting in 54 samples.
273 Libraries of 16S rRNA gene amplicons were prepared for 454 pyrosequencing after
274 amplification using universal primers 27F (5' – AGA GTT TGA TCC TGG CTC AG - 3') and
275 533R (5' – TTA CCG CGG CTG CTG GCA C - 3'), extended at the 5' end by 10 bp multiplex
276 identifiers (MIDs, Roche). Amplifications were done with GoTaq G2 Green Master Mix
277 (Promega). The amplification step was carried out with 25 and 30 cycles (30 sec at 95°C, 30
278 sec at 60°C, 30 sec at 72°C) for microcosm and initial soil samples, respectively. After quality
279 checking on agarose gel electrophoresis and quantification using NanoDrop, three independent
280 PCR products per sample were pooled together to minimize PCR bias on sequencing, and an
281 equimolar mixture was then prepared. Pyrosequencing was carried out using a GS FLX 454
282 (Beckman Coulter Genomics, Danvers, MA, USA) and produced 316.5 megabases
283 corresponding to a total of 716,250 partial 16S rRNA gene sequences having a median size of
284 490 bp. Sequence analyses, i.e. reducing sequencing error, removing chimeras, grouping into
285 OTUs, subsampling to 1000 sequences, SILVA database alignment, and classifying using the
286 RDP reference files were performed using MOTHUR (version 1.36), as recommended by its
287 author (Schloss et al., 2011). Following the trimming, we obtained 250 bp sequences from the
288 forward primer (corresponding to the V1 and V2 16S rDNA regions). The alpha diversity of
289 the samples was estimated using the Chao1 richness estimator and the nonparametric Shannon
290 diversity index considering a same number of sequences for each sample (i.e. 1000 sequences).

291

292 **Statistical analyses**

293 Statistical analyses (ANOVA, Student's t tests, Kruskal Wallis tests, Kolmogorov Smirnov
294 tests), data analysis and Factorial Correspondence Analysis (FCA) on OTUs table, by using
295 Bray-Curtis dissimilarity distance, were performed using XLStat software (version 2.01.16906,
296 Addinsoft, Paris). Chao-1 and nonparametric Shannon diversity indexes were calculated on
297 OTUs table, using MOTHUR (version 1.36). ADONIS tests were performed by using Bray-
298 Curtis dissimilarity distance (R software, version 3.1.2, VEGAN package).

299

300 **Nucleotide sequence accession numbers**

301 All 16S rRNA gene sequences have been submitted to the NCBI Short Read Archive under the
302 Bioproject accession number PRJNA312913.

303

304

305 **Results**

306

307 **Physico-chemical properties of soils**

308 Soil analyses show that the two soils were very different (Table 1), with the PPY soil
309 characterized by acidic and moderate nutrient conditions and the Pv soil characterized by
310 alkaline and higher nutrient availability. For both soils, the major grain size fraction was coarse
311 silt, representing more than 40% dry weight of the mineral fraction (Table 1). Although both
312 particle size distributions were close, by comparison with the Shepard diagram (Shepard, 1954),
313 the PPY and the Pv soils correspond to sandy silt clayey and silt soils, respectively. Concerning
314 the clayey fraction, total clay content was significantly higher ($P < 0.05$; Student's t test) in the
315 Pv soil (20.2%) compared to the PPY (15.5%), but the nature of clays was different (Table 1).

316 For PPY soil, chlorites, illites and kaolinites (clays with the lower CEC, 10-40, 20-30 and 3-15
317 meq.g⁻¹, respectively; Morel, 1996) were dominant whereas smectites (80 meq.g⁻¹ < CEC < 150
318 meq.g⁻¹) were dominant in the Pv soil. Consequently, the CEC of the Pv soil was higher than
319 that of the PPY soil, especially due to clay types.

320 Concerning total organic carbon content (TOC), the PPY was the richest soil (Table 1). Residual
321 carbon (RC) was dominant in both soils compared to pyrolysable carbon (PC), showing that
322 OM tended to be mature and resistant to pyrolysis, and could potentially strongly sorb PHE.
323 The main OM fraction in PPY soil corresponded to biomacromolecules whereas mature
324 geomacromolecules, that are higher PHE sorbents, were dominant in the Pv soil.

325

326 **Phenanthrene sorption and bioaccessibility**

327 PHE sorption isotherms (Fig. 1) were similar for the PPY and the Pv soils ($P > 0.05$,
328 Kolmogorov-Smirnov Test). In the low PHE concentration range, they can both be described
329 by a linear partitioning model ($R^2=0.965$ and 0.981 for PPY and Pv soils, respectively)
330 (Crampon et al., 2016). So K_d values were calculated from the slopes of the linear part of the
331 sorption isotherms. The K_d partition coefficients were 718.7 L.kg^{-1} and 695.5 L.kg^{-1} for the PPY
332 and the Pv soils, respectively. Water/organic carbon partition coefficients K_{oc} could be
333 calculated from the K_d values and the total organic carbon fraction (f_{oc}), using the equation:
334 $K_{oc}=K_d/f_{oc}$. Log K_{oc} values were 4.58 and 4.73 for PHE in the PPY and the Pv soils,
335 respectively. Increases of PHE desorption and potentially of PHE bioaccessibility were
336 observed for both soils when adding rhamnolipids in the aqueous medium (Fig. 1). Indeed, K_d
337 values obtained in the presence of rhamnolipids were significantly lower (Student's t test, $P <$
338 0.05) than those obtained without biosurfactant: 206.2 L.kg^{-1} and 318.7 L.kg^{-1} for the PPY and
339 the Pv soils, respectively ($R^2=0.959$ and 0.929 for PPY and Pv soils, respectively).

340

341 **Phenanthrene dissipation kinetics**

342 Although no marked lag phase was observed in our experimental conditions, PHE dissipation
343 was slow at the beginning of the experiments. Only 20% of PHE was degraded during the first
344 8 and 20 days for the Pv and the PPY soils, respectively (Fig. 2). Then, PHE dissipation was
345 faster in Pv soil compared to the PPY soil, as ca 90% of PHE was dissipated in 16 days in the
346 Pv soil, while 42 days were necessary in the PPY soil. The same trends were observed in a
347 previous experiment (Crampon et al., 2014), aiming, inter alia, to follow the degradation of 7
348 selected PAHs in different soils with the same sampling terms.

349 Kinetics of PHE dissipation in the presence of rhamnolipids did not show any significant impact
350 of surfactant addition compared to the controls ($P > 0.05$, Student's t test; Fig. 2).

351

352 **SIP experiment and ^{13}C labeled DNA isolation**

353 Total community (^{12}C) and active ^{13}C -labeled PHE-degrading bacterial populations were
354 studied at two incubation times corresponding to DT20 (time where 20% PHE is dissipated)
355 and DT90 (time where 90% PHE is dissipated) for both soils: it corresponded to 8 and 16 days
356 of incubation for the Pv soil and 20 and 42 days of incubation for the PPY soil (Fig. 2). For
357 controls, DNA was analyzed from both ^{12}C and ^{13}C microcosms.

358 Quantitative PCR revealed a higher number of 16S rRNA gene copies in the lower densities
359 (<1.70) of the CsCl gradient, corresponding to light DNA (that should contain only ^{12}C DNA)
360 (Fig. 3). For further analyses the fraction n°3 ($d = 1.689$) was selected. Heavy DNA (that should
361 contain all ^{13}C DNA) was recovered in the fractions with density ranging from 1.705 to 1.722
362 (Fig. 3). Higher numbers of 16S rRNA gene copies in these heavy fractions collected from the
363 ^{13}C microcosms corresponded to the active ^{13}C -labeled PHE-degrading bacteria. For further
364 analyses the fraction n°7 ($d = 1.711$) was selected. At DT20, the difference between the number
365 of 16S rRNA gene copies in the fraction n°7 from ^{12}C and ^{13}C microcosms was only significant

366 for Pv soil microcosms ($P < 0.05$, ANOVA test; Fig. S2). In PPY soil microcosms, no ^{13}C
367 labeled DNA was detected at DT20. At DT90, significant differences ($P < 0.05$, ANOVA test)
368 were observed between ^{12}C and ^{13}C microcosms, for both soils (Fig. 3). The amounts of 16S
369 rRNA gene copies in the heavy fraction of DNA were about 10 and 50 times higher for the ^{13}C
370 microcosms than for the ^{12}C microcosms for the PPY and the Pv soils, respectively. Therefore,
371 samples at DT90 were chosen for sequencing and analyzing the bacterial diversity. No
372 significant difference was observed in the level of ^{13}C incorporation between microcosms with
373 and without rhamnolipids (Fig. 3).

374 No significant difference in the number of fungal 18S rRNA gene copies was observed between
375 ^{12}C and ^{13}C microcosms (Fig. S3), meaning that ^{13}C from PHE was not used as a growth carbon
376 source by fungi.

377

378 **Characterization of bacterial communities and identification of PHE-degrading bacteria**

379 454-pyrosequencing was carried out on 54 soil samples, corresponding to the soils before
380 spiking and the fractions from the DNA-SIP experiment. After de-noising, a total of 211,519
381 16S rRNA gene sequences were obtained, with an average number of 3,917 sequences per
382 sample. Even if the rarefaction curves based on operational taxonomic units (OTUs; at 97%
383 identity) did not reach a plateau for the soils before spiking, the diversity corresponding to the
384 labeled ^{13}C DNA (*i.e.* PHE-degrading populations) seemed to be well sampled (Fig. S4).
385 Although species richness given by Chao1 index was slightly higher for the Pv soil
386 comparatively to the PPY soil, no significant differences ($P > 0.05$, Kruskal-Wallis test) of alpha
387 diversity were observed between the two native soils (Table 2). Concerning the heavy fractions
388 from the ^{13}C microcosms (fractions PPY/Pv_13C_Rh+/-_H in Table 2), the observed richness
389 were lower than in the initial soil types or in controls, meaning that PHE-degrading bacteria
390 represent only a small fraction of the total community in both soils. This finding is more

391 pronounced for the Pv soil, meaning that a significantly smaller number of OTUs ($P < 0.05$,
392 Kruskal-Wallis test) was involved in PHE degradation.

393 Factorial correspondence analysis (Fig. 4) was performed using the 100 most represented OTUs
394 (in each soil, separately) to highlight similarities and dissimilarities between replicates, sample
395 conditions (soil before spiking, ^{12}C and ^{13}C microcosms, or with and without rhamnolipids) and
396 DNA fractions (light and heavy). Whatever the soil type, the bacterial diversity retrieved from
397 the heavy DNA fractions of ^{13}C microcosms was significantly different from the diversity found
398 in the heavy DNA fractions of ^{12}C control microcosms ($P < 0.01$; ADONIS test). A specific
399 analysis of the effect of rhamnolipids addition revealed no differences in each soil type between
400 treatments with and without rhamnolipids, in both light or heavy fractions ($P > 0.05$; ADONIS
401 test). Notably, no differences appeared between the diversity in light DNA fractions from both
402 ^{12}C and ^{13}C microcosms and the diversity of soils before spiking.

403 The analysis of the sequence distribution in the OTUs represented in the heavy DNA fractions
404 from the ^{13}C labeled microcosms and having a significantly higher abundance than in the ^{12}C
405 control microcosms allowed us to identify the main ^{13}C -labeled PHE degrading bacteria (Table
406 3). Notably, this analysis revealed that the main PHE degraders identified were poorly
407 represented in the initial soils (from $< 0.01\%$ to 0.29% of the total sequences, Table 3), as in the
408 light and heavy fractions of the ^{12}C microcosms (from $< 0.01\%$ to 3.0% of the total sequences,
409 data not shown).

410 The six OTUs, representing the active PHE degraders in the Pv soil, were identified as members
411 of the *Betaproteobacteria* class, with a high proportion of the *Rhodocyclaceae* family and some
412 *Burkholderiales* (including the *Polaromonas* genus). Concerning the PPY soil, members of a
413 higher number of OTUs were involved in PHE degradation. The 13 OTUs representing the
414 active PHE degraders belonged to *Alpha-*, *Beta-*, *Gamma-Proteobacteria* and *Actinobacteria*.
415 Notably the OTUs Pv_PHE6 and PPY_PHE6 were both related to the *Polaromonas* genus

416 (Table 3), and appeared phylogenetically close (Fig. S5). Moreover, OTUs Pv_PHE4 and
417 PPY_PHE12 corresponded to the same OTU affiliated to the *Rhodoferox* genus (Table 3 and
418 Fig. S5).

419 The relative abundance of some PHE degraders increased or decreased depending on the
420 presence or absence of rhamnolipids, but only in the case of some of the low abundance PHE
421 degraders (Table 3). Notably, the proportion of the two OTUs related to the *Polaromonas* genus
422 was significantly higher in the presence of rhamnolipids in both soils. By contrast, when
423 rhamnolipids were added, the relative proportion of sequences related to the *Rhodoferox* genus
424 was significantly higher for the PPY soil ($P < 0.05$), whereas it tended to be lower for the Pv soil
425 ($P > 0.05$). Altogether, our results revealed that direct addition of rhamnolipids to soil has a
426 limited effect on the active PHE degrading bacteria.

427

428 **Discussion**

429 PHE accessibility was assessed by studying different physico-chemical properties of soil, and
430 by characterizing PHE sorption isotherms with or without rhamnolipids. Overall, the two soils
431 considered were characterized by a similar OM content, but a high variation in their clay
432 contents and natures. While total organic carbon content was slightly higher in the PPY soil,
433 OM was dominated by mature geomacromolecules in the Pv soil (Table 1). This fraction of OM
434 combines constituents naturally stable or stabilized by physico-chemical processes, residues of
435 fires and some anthropogenic pollutants like soot, *i.e.* black carbon (Sebag et al, 2006). The
436 higher content of this more mature, carbonaceous and crystallized fraction in Pv soil make it
437 probably more prone to a stronger PHE sorption than the PPY soil. The characterization of OM
438 maturation state in soils is important because binding energies and sorption properties towards
439 PAHs increase over time (Ran et al., 2007). Indeed, PAHs sorption is generally lower and more
440 reversible on fresh OM compared to aged OM because with ageing, the polar mixtures of

441 biomolecules transforms into a more hydrophobic form with higher sorptive properties for
442 lipophilic organic compounds (Mechlińska et al., 2009). Moreover, both total clay contents and
443 natures varied significantly between the two soil types considered. Chlorites, illites and
444 kaolinites were dominant in the PPY soil, whereas smectite was dominant in the Pv soil.
445 Although clay is generally described as a parameter affecting less PAH sorption to soil particles
446 compared to organic matter, it has to be considered here because of the potential impact on
447 rhamnolipids (Delle Site, 2001; Hwang and Cutright, 2002). Indeed, it has been reported that
448 sorption of rhamnolipids to soil is more strongly influenced by clay contents and nature than
449 by OM : at low biosurfactant concentrations, that are relevant for enhancing organic
450 contaminant biodegradation, clays and particularly kaolinite are the strongest sorbents for
451 rhamnolipids (Ochoa-Loza et al., 2007). So clays could influence observed results on PHE
452 remobilization.

453 Interestingly, despite these different physico-chemical properties and biodegradation kinetics,
454 PHE sorption was found to be quite similar in the two soil types. These results are consistent
455 with a previous study, where we showed that the bioaccessible fraction of PHE, estimated using
456 a mild extraction method (extraction with hydroxypropyl- β -cyclodextrin) was close between
457 these two soil types (Crampon et al., 2014). Furthermore, rhamnolipids addition increased PHE
458 desorption in both soil types, but with a greater effect in the PPY soil. Although no consensus
459 exists on the real positive or negative effect of rhamnolipids on PAH removal, several studies
460 reported a strong impact of soil organic matter content and contamination ageing (Congiu and
461 Ortega-Calvo, 2014; Szulc et al., 2014). Thus, the lowest PHE desorption from the Pv soil by
462 rhamnolipids addition could be explained by higher contents of mature OM, smectites and
463 kaolinite in the Pv soil. These soil constituents may enhance the formation of clay-humic
464 complexes and thus contribute to a strong adsorption of PHE on these complexes. Moreover,
465 these clay minerals increase the surface available for PAH as well as rhamnolipids sorption.

466 The presence of higher contents of kaolinite in Pv soil (Table 1) can explain the strongest
467 sorption of the biosurfactant on Pv soil, compared to PPY soil. Consequently, there may be less
468 micelles in the soil solution to remobilize PHE and more additional sorption sites through
469 rhamnolipids sorbed on soil aggregates (Ochoa-Loza et al., 2007; Zhou and Zhu, 2007).

470 Although rhamnolipids significantly increased PHE desorption in the two soil types tested,
471 addition of a biosurfactant had a limited impact on the PAH biodegradation efficiency (Fig. 2).
472 While some studies report that surfactants may increase PAH biodegradation rates, others have
473 shown that the effect of their application may be negligible (Laha and Luthy, 1991; Rouse et
474 al., 1994; Tiehm, 1994; Tsomides et al., 1995). Indeed, Leonardi *et al.* (Leonardi et al., 2007)
475 showed that when a soil is characterized by a low non-bioaccessible PAH fraction, addition of
476 surfactant has a limited impact on the PAH biodegradation efficiency. Moreover, the effect of
477 the surfactant could also depend on the identity of PAH-degrading bacteria involved (Allen et
478 al., 1999). At last, we can also suppose that if rhamnolipids can partially desorb PAHs from
479 soils, they can also desorb them from the surface of bacteria, which can lead to the decrease of
480 their bioavailability while they increase their bioaccessibility.

481 More generally, other factors might explain the difference in degradation rate observed between
482 the two soils. Indeed, the optimum pH range can be very variable depending on the type of soil
483 and the PAH-degrading bacteria (Simarro et al., 2011). It could not be excluded that the basic
484 Pv soil allowed a greater activity of the PAH-degrading bacteria than the acidic PPY soil.
485 Moreover, difference in initial concentrations of nitrogen and carbon between the two soils may
486 also affect the activity of the PAH-degrading bacteria (Teng et al., 2010). Temperature and
487 toxicity of PAHs could also impact the biodegradation rate (Coover and Sims, 1987; Martin et
488 al., 2012). Indeed, native high molecular weight PAHs (4-6 ring PAHs), which can be
489 considered as the most toxic PAHs, were 2.5 times more concentrated in Pv soil than in PPY
490 soil. However, it must be noted that they represent only 0.09-0.22% of the total amount of PAHs

491 after PHE spiking, which is quite negligible. Interestingly, all the factors having a potential
492 impact on the PHE degradation kinetics have in common to influence the physiology of the
493 PAH-degrading bacteria.

494 In a second phase, we therefore studied the diversity of the PAH-degrading bacteria in order (i)
495 to compare the PAH-degrading populations between these two soils and (ii) to evaluate the
496 impact of a bioavailability variation on these degrading populations. The combination of ¹³C
497 labeled PHE and 16S rRNA gene amplicon pyrosequencing enabled us to identify the PHE-
498 degrading bacterial communities in these two contrasted soil types.

499 Most of the PHE degraders identified in the two soil types belonged to the *Proteobacteria*
500 phylum. Some members of the *Alphaproteobacteria* (Demaneche et al., 2004),
501 *Betaproteobacteria* (Goyal and Zylstra, 1997; Laurie and Lloyd-Jones, 1999; Seo et al., 2009)
502 and *Gammaproteobacteria* (Balashova et al., 2001; Ensley et al., 1982; Kiyohara et al., 1994;
503 Resnick and Gibson, 1996) classes possess specific PAH dioxygenases involved in the catalysis
504 of the initial aromatic ring oxidation (Cebren et al., 2008; Habe and Omori, 2003). Some OTUs
505 belonging to these classes were found among PHE degraders in the PPY soil. In addition, one
506 OTU of the PHE degraders belongs to the *Mycobacterium* genera (*Actinobacteria*) that has
507 already been described as a good PAH degrader in soils (Timmis et al., 2010). Altogether, these
508 PHE degraders were poorly represented in both initial soil bacterial communities, indicating
509 that when soil PAH are diffusely concentrated, the PHE-degraders belong to the rare biosphere,
510 but could rapidly become active when available PAHs enter the system.

511 The Pv soil presented a lower diversity of PHE degraders than the PPY soil, all belonging to
512 the *Betaproteobacteria*. It was further shown that the abundance of *Betaproteobacteria*
513 increased after three months of incubation in microcosms spiked with PHE, pyrene or a mix of
514 seven PAHs, members of this bacterial class being largely described as capable of degrading
515 PAHs (Jeon et al., 2003; Martin et al., 2012; Niepceon et al., 2013; Núñez et al., 2012;

516 Singleton et al., 2006). Among the *Betaproteobacteria* class, sequences related to members of
517 the *Rhodocyclaceae* family were the most represented in the ¹³C fraction (more than 80% of the
518 sequences), suggesting a major role in PHE degradation in the Pv soil. Interestingly,
519 representatives of these taxa have also been described as able to degrade PAHs and to possess
520 specific PAH RHD enzymes (Chemerys et al., 2014; Martin et al., 2012; Regonne et al., 2013;
521 Singleton et al., 2006; Uhlik et al., 2012), and a cultivable member of this *Rhodocyclaceae*
522 family that degrades PAHs has been recently sequenced (Singleton et al., 2015). The
523 exceptional PAH degradation capacity of *Rhodocyclaceae* in various soils suggests classifying
524 them as very efficient PHE degraders. Since previous representatives of this taxon have already
525 been shown to efficiently degrade PAHs (Singleton et al., 2015), the *Rhodocyclaceae* family
526 may be used as a bioindicator of soils with a high PHE degradation capacity. Moreover,
527 cultivation-dependent analysis combined to genome sequencing should permit to further
528 understand the functional role of the *Rhodocyclaceae* family in PAH degradation.
529 Interestingly, the diversity and the structure of the main PHE degraders seem only slightly
530 impacted by rhamnolipids addition and no impact on the total bacterial community was
531 detected. Such results suggest that the rhamnolipid amendment used in biostimulation processes
532 is probably not used as carbon source, and may be a good alternative in term of toxicity for the
533 soil bacterial communities compared to synthetic surfactants. However, its use for soils where
534 bioaccessibility is not a limiting degradation parameter does not seem to be an advantage even
535 if it improved PHE desorption in both soils.

536

537 **Conclusion**

538 By using chemical and microbiological methods, we demonstrated that the PHE
539 bioaccessibility has a low impact on its degradation. Indeed, the difference in biodegradation
540 rates we found between the two soils considered cannot be explained by a difference in

541 bioaccessibility. Moreover, although the amendment of biosurfactants (*i.e.* rhamnolipids) did
542 change the PHE sorption/desorption kinetics in the two soils tested, no significant impact on
543 PHE degradation rates was detected. In addition, we showed that variation in biodegradation
544 rate between these diffusely contaminated soils could be more explained by the differences in
545 PHE-degrading populations, which presence and activities might be driven by physico-
546 chemical properties of the soils. Finally, we showed, for the first time to our knowledge, that
547 rhamnolipids present a low toxicity for soil microorganisms as they do not modify the global
548 bacterial diversity as well as the activity of the phenanthrene-degraders. In the future, it could
549 be interesting to investigate the degradation kinetics of a mixture of PAHs (e.g. the 16 priority
550 PAHs) with or without rhamnolipids and to identify the bacteria more particularly involved in
551 the degradation of heavy high molecular weight PAHs.

552

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558

559 **Supporting information**

560 Five Figures (Figure S1, S2, S3, S4 and S5) are included in the supporting information.

561

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769 surfactant. *Water Research* 42, 101-108.

770

771 TABLE 1: Physico-chemical properties of the two studied soils (Pv and PPY).

Method	Data	PPY	Pv
Granulometry without decarbonatation (% dry weight)	<i>Clays (<2μm)</i>	15.5	20.2
	<i>Fine silts (2 < D < 20 μm)</i>	23.4	26.0
	<i>Coarse silts (20 < D < 50 μm)</i>	42.3	44.2
	<i>Fine sands (50 < D < 200 μm)</i>	17.8	9.5
	<i>Coarse sands (200 < D < 2000 μm)</i>	1.0	0.1
XRD (g.kg⁻¹)	<i>Chlorite</i>	36.5	0.0
	<i>Illite / Chlorite</i>	11.2	0.0
	<i>Illite</i>	53.7	44.8
	<i>Kaolinite</i>	53.6	66.8
	<i>Smectite</i>	0.0	90.4
RockEval6 Pyrolysis & peak deconvolution^a (g.kg⁻¹)	<i>Total Organic Carbon (TOC)</i>	18.7	13.1
	<i>Pyrolysable carbon (PC)</i>	4.2	2.6
	S2 <i>Residual carbon (RC)</i>	14.5	10.6
	<i>Biomacromolecules</i>	8.2	4.5
	<i>Immature geomacromolecules</i>	5.4	2.4
	<i>Mature geomacromolecules</i>	5.1	6.2
<i>pH (H₂O)</i>		5.7	8.2
<i>Cationic Exchange Capacity CEC (cmol.kg⁻¹)</i>		8.7	14.2
PAHs (mg.kg⁻¹)	□ <i>16 PAHs (US-EPA)</i>	0.320 ± 0.040	0.890 ± 0.290
	(n=6) <i>BaP</i>	0.019 ± 0.006	0.046 ± 0.022
	<i>PHE</i>	0.035 ± 0.012	0.013 ± 0.001

772

773 ^a The deconvolution of S2 peak, which can be related to soil OM (cracking between 200 to
774 650 °C), allowed a precise determination of the maturation of OM in soils.

775

776

777 TABLE 2: Alpha diversity indexes of the two studied soils (Pv and PPY) based on OTUs
 778 formed at 97% similarity.

<i>Samples^b</i>	0.03 ^c		
	OTU	Chao1	H'
Pv ^{d, x}	446 ± 105	1194 ± 139	6.19 ± 0.33
Pv_12C_Rh+_L ^x	417 ± 64	1031 ± 124	5.75 ± 0.42
Pv_12C_Rh+_H ^x	376 ± 59	840 ± 114	5.34 ± 0.59
Pv_12C_Rh-_L ^x	411 ± 135	965 ± 356	5.75 ± 0.67
Pv_12C_Rh-_H ^x	423 ± 148	1000 ± 348	5.76 ± 0.82
Pv_13C_Rh+_L ^x	417 ± 12	1028 ± 48	5.78 ± 0.04
Pv_13C_Rh+_H ^y	54 ± 1	195 ± 16	1.72 ± 0.06
Pv_13C_Rh-_L ^x	452 ± 6	1143 ± 59	5.94 ± 0.04
Pv_13C_Rh-_H ^y	40 ± 3	137 ± 28	1.62 ± 0.19
PPY ^{d, x}	413 ± 20	880 ± 61	5.82 ± 0.09
PPY_12C_Rh+_L ^x	310 ± 61	632 ± 123	5.17 ± 0.57
PPY_12C_Rh+_H ^x	334 ± 107	668 ± 220	5.25 ± 0.70
PPY_12C_Rh-_L ^x	347 ± 31	741 ± 82	5.45 ± 0.19
PPY_12C_Rh-_H	319 ± NA ^e	597 ± NA ^e	5.04 ± NA ^e
PPY_13C_Rh+_L ^x	347 ± 4	738 ± 23	5.47 ± 0.02
PPY_13C_Rh+_H ^z	171 ± 59	406 ± 136	3.65 ± 0.92
PPY_13C_Rh-_L ^x	335 ± 61	710 ± 122	5.34 ± 0.47
PPY_13C_Rh-_H ^z	241 ± 67	609 ± 189	4.22 ± 0.56

779 ^a x, y and z represent the different groups characterized statistically (p<0.05, Kruskal Wallis test)

780 ^b Rh⁺/: with or without rhamnolipid addition; L/H: light or heavy DNA fractions.

781 ^c 0.03 is the OTU cutoff in distance units, OTU means the number of OTUs observed, Chao1 means the Chao1
 782 estimated minimum number of OTUs and H' means the nonparametric Shannon diversity index.

783 ^d Soil before spiking

784 ^e Not Applicable, for samples with not enough reads for calculation of standard deviation with triplicates.

785 TABLE 3: Active ¹³C-labeled PHE degraders in the two studied soils (Pv and PPY). OTUs
786 were considered as PHE-degraders when a significantly higher proportion was recovered in
787 heavy DNA fractions from ¹³C microcosms than from ¹²C microcosms.

Soil	OTU	Taxonomic identification		Relative abundance of this OTU found (%)		
		Phylum / Class	Best resolution ^a	With Rhamnolipids	Without Rhamnolipids	Native soil
Pv	<i>Pv_PHE1</i>	Betaproteobacteria	Rhodocyclaceae family	52.48 ± 2.91	50.06 ± 11.22	0.01 ± 0.01
	<i>Pv_PHE2</i>	Betaproteobacteria	Rhodocyclaceae family	19.77 ± 1.44	17.95 ± 3.9	<0.01
	<i>Pv_PHE3</i>	Betaproteobacteria	Rhodocyclaceae family	15.69 ± 2.02	13.69 ± 2.42	<0.01
	<i>Pv_PHE4^b</i>	Betaproteobacteria	<i>Rhodoferax</i>	3.16 ± 2.9	12.02 ± 8.28	0.12 ± 0.05
	<i>Pv_PHE5</i>	Betaproteobacteria	<i>Hydrogenophaga</i>	0.76 ± 0.08	1.11 ± 1.3	0.03 ± 0.04
	<i>Pv_PHE6^c</i>	Betaproteobacteria	<i>Polaromonas</i>	0.89 ± 0.44	0.15 ± 0.13	0.02 ± 0.03
PPY	<i>PPY_PHE1</i>	Gammaproteobacteria	<i>Nevskia</i>	23.90 ± 18.53	23.92 ± 11.07	<0.01
	<i>PPY_PHE2</i>	Betaproteobacteria	Burkholderiales order	11.61 ± 10.5	6.31 ± 2.57	0.01 ± 0.02
	<i>PPY_PHE3</i>	Betaproteobacteria	<i>Collimonas</i>	2.13 ± 0.82	7.89 ± 6.53	0.29 ± 0.2
	<i>PPY_PHE4</i>	Alphaproteobacteria	Hyphomicrobiaceae family	6.22 ± 3.91	2.55 ± 1.09	0.25 ± 0.12
	<i>PPY_PHE5</i>	Gammaproteobacteria	<i>Dyella</i>	4.86 ± 2.53	3.53 ± 2.57	0.04 ± 0.03
	<i>PPY_PHE6^c</i>	Betaproteobacteria	<i>Polaromonas</i>	6.59 ± 4.59	1.11 ± 0.22	<0.01
	<i>PPY_PHE7</i>	Alphaproteobacteria	Hyphomicrobiaceae family	2.30 ± 0.9	3.12 ± 3.83	0.03 ± 0.06
	<i>PPY_PHE8</i>	Gammaproteobacteria	<i>Nevskia</i>	0.91 ± 0.55	1.60 ± 2.24	<0.01
	<i>PPY_PHE9</i>	Alphaproteobacteria	Acetobacteraceae family	1.51 ± 0.47	1.49 ± 1.19	<0.01
	<i>PPY_PHE10</i>	Actinobacteria	<i>Mycobacterium</i>	2.04 ± 1.96	0.73 ± 0.46	<0.01
	<i>PPY_PHE11</i>	Betaproteobacteria	<i>Herbaspirillum</i>	0.93 ± 1.20	1.17 ± 0.76	<0.01
	<i>PPY_PHE12^{b, c}</i>	Betaproteobacteria	<i>Rhodoferax</i>	1.40 ± 0.15	0.37 ± 0.64	0.10 ± 0.09
	<i>PPY_PHE13</i>	Gammaproteobacteria	<i>Nevskia</i>	0.46 ± 0.46	1.21 ± 1.52	<0.01

788 ^a These taxonomic identifications were confirmed phylogenetically (FIG S5)

789 ^b The two OTUs Pv_PHE4 and PPY_PHE12 are similar
790 ^c These OTUs showed significant difference between microcosms with and without rhamnolipids ($P < 0.05$,
791 Student's t test)
792

793

794 **FIGURE LEGENDS**

795 FIG 1: Phenanthrene sorption isotherms (expressed as the quantity of PHE adsorbed on the
796 solid phase Q_{ads} , relative to the concentration of PHE in solution at the equilibrium C_{eq}) at 25°C
797 with and without rhamnolipids (0.55 g.L⁻¹) for (a) PPY soil and (b) Pv soil.

798

799 FIG 2: PHE degradation on PPY and Pv soils in the presence (at 300 mg.kg⁻¹) and absence of
800 rhamnolipids in microcosms spiked with ¹³C PHE (at 300 mg.kg⁻¹). DT20 and DT90
801 corresponding respectively to 20% and 90% of PHE dissipation are represented for both soils.

802

803 FIG 3: Quantification of bacterial 16S rRNA gene copies in the 12 fractions separated by CsCl
804 gradients using real-time quantitative PCR. Data are presented for the two studied soils (PPY
805 and Pv) with and without rhamnolipids and at DT90. Fractions n°3 and n°7 were selected for
806 light DNA and heavy DNA sequencing and analyses, respectively.

807

808 FIG 4: Factorial correspondence analysis (FCA) of the relative proportion of the main OTUs
809 (top 100) present in the (a) PPY and (b) Pv samples. Full-tone graphic patterns correspond to
810 microcosms with rhamnolipids. For each soil type the principal component axes F1 and F2
811 explain most of the variance in the data cumulatively.

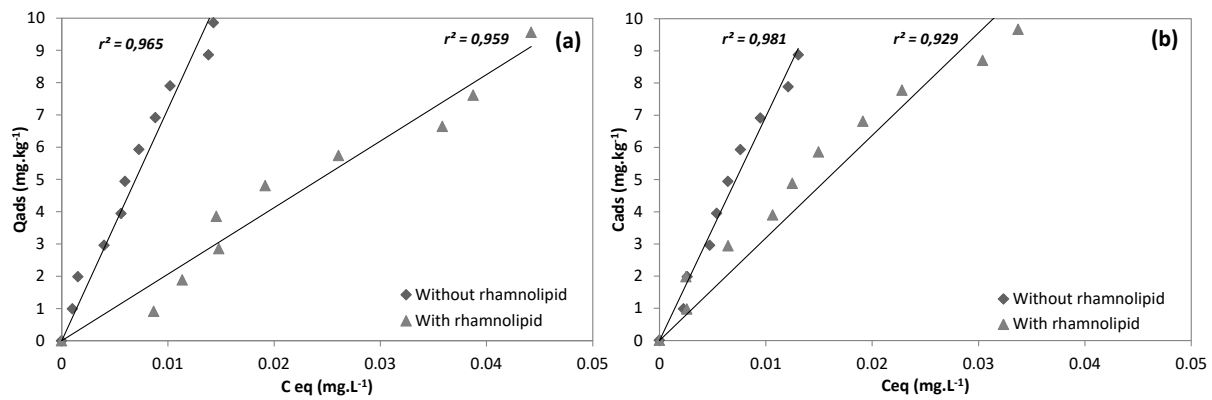
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815 **Figure 1**

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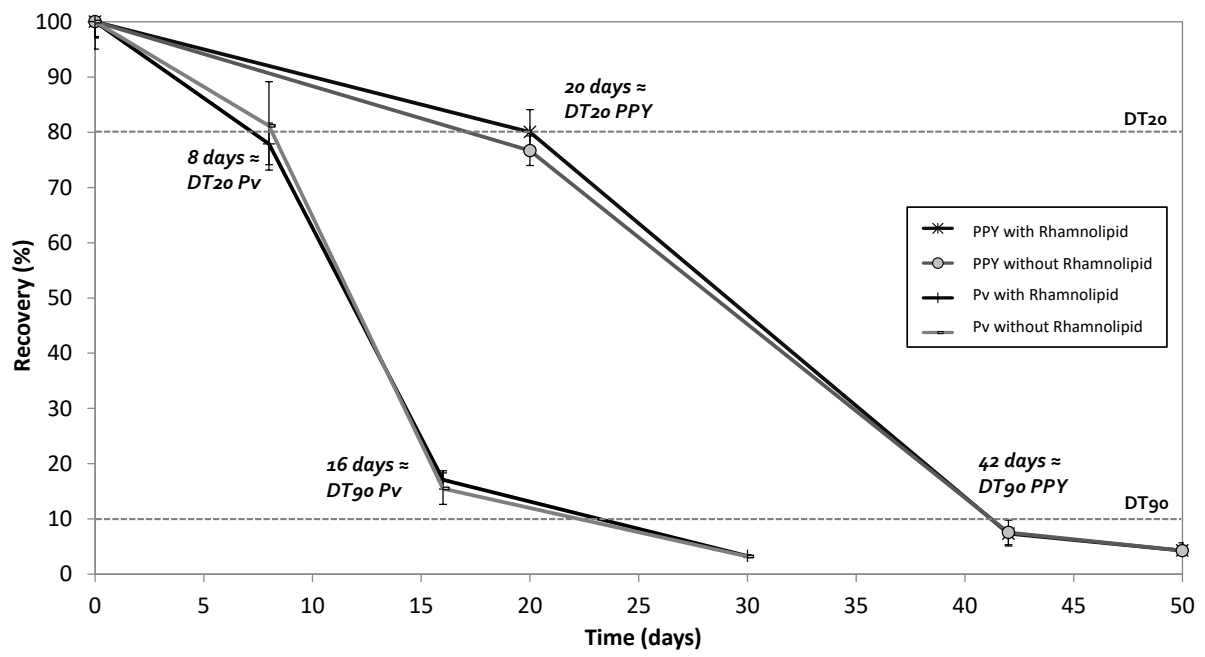


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819 **Figure 2**

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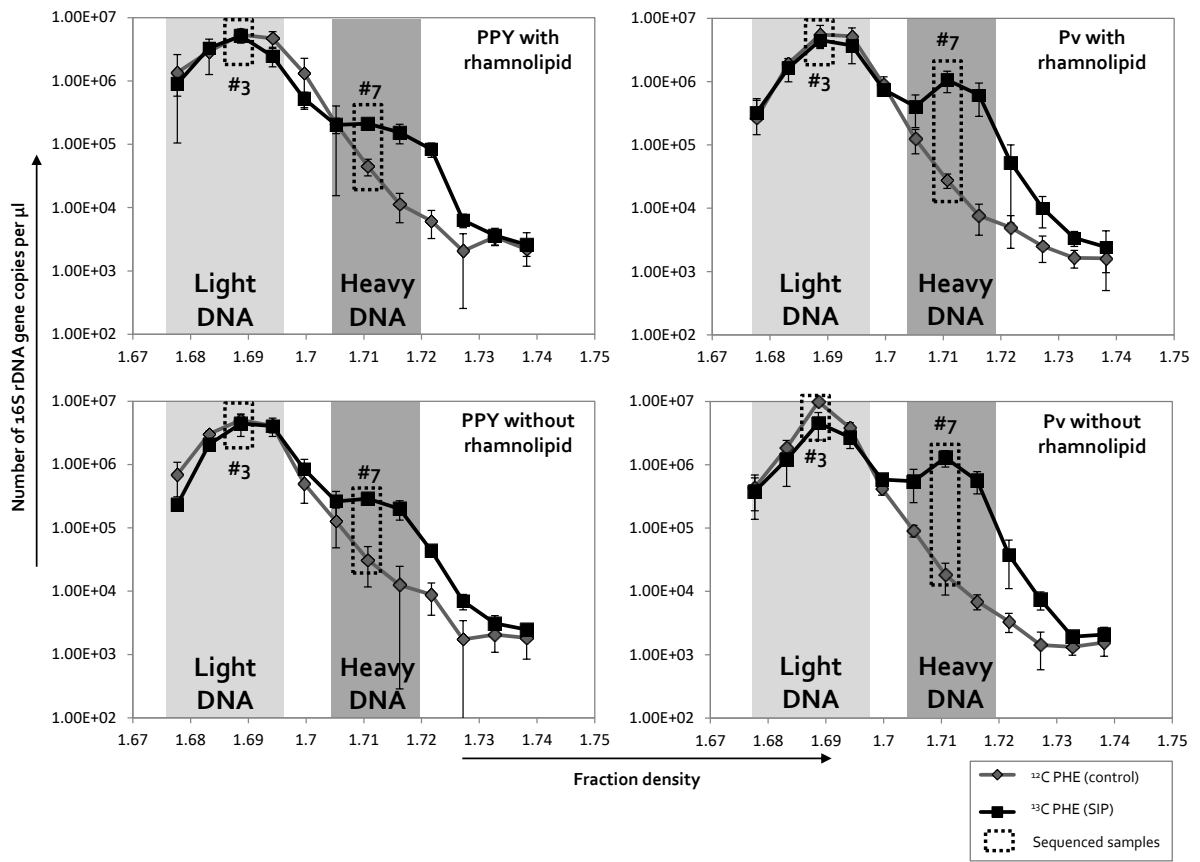


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823 **Figure 3**

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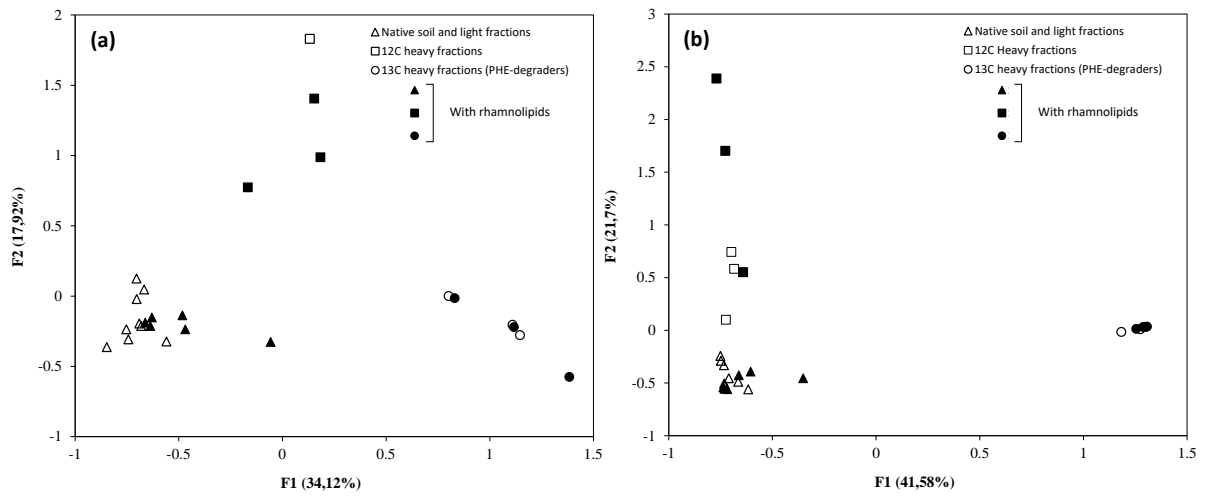


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827 **Figure 4**

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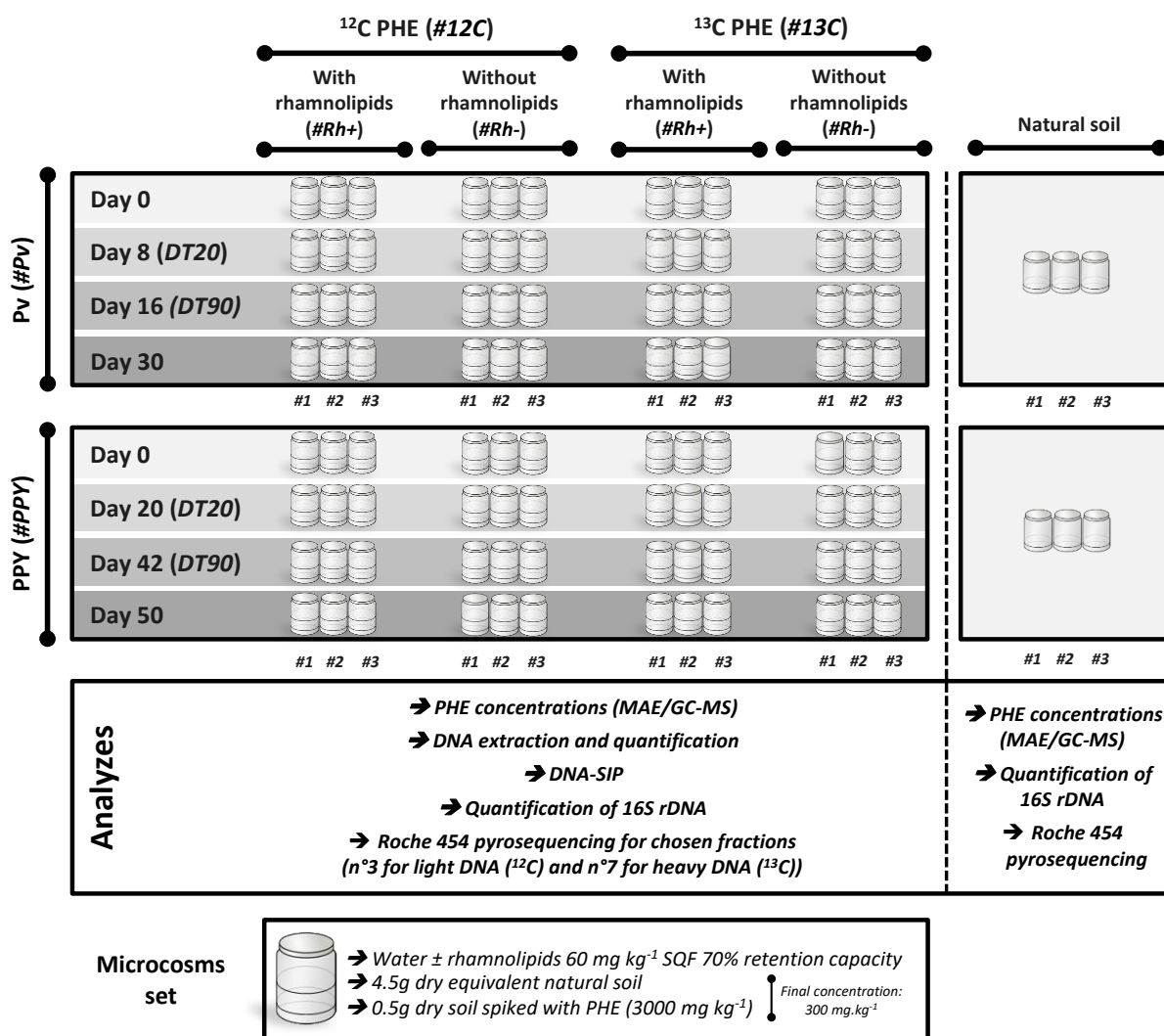
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Supplemental materials



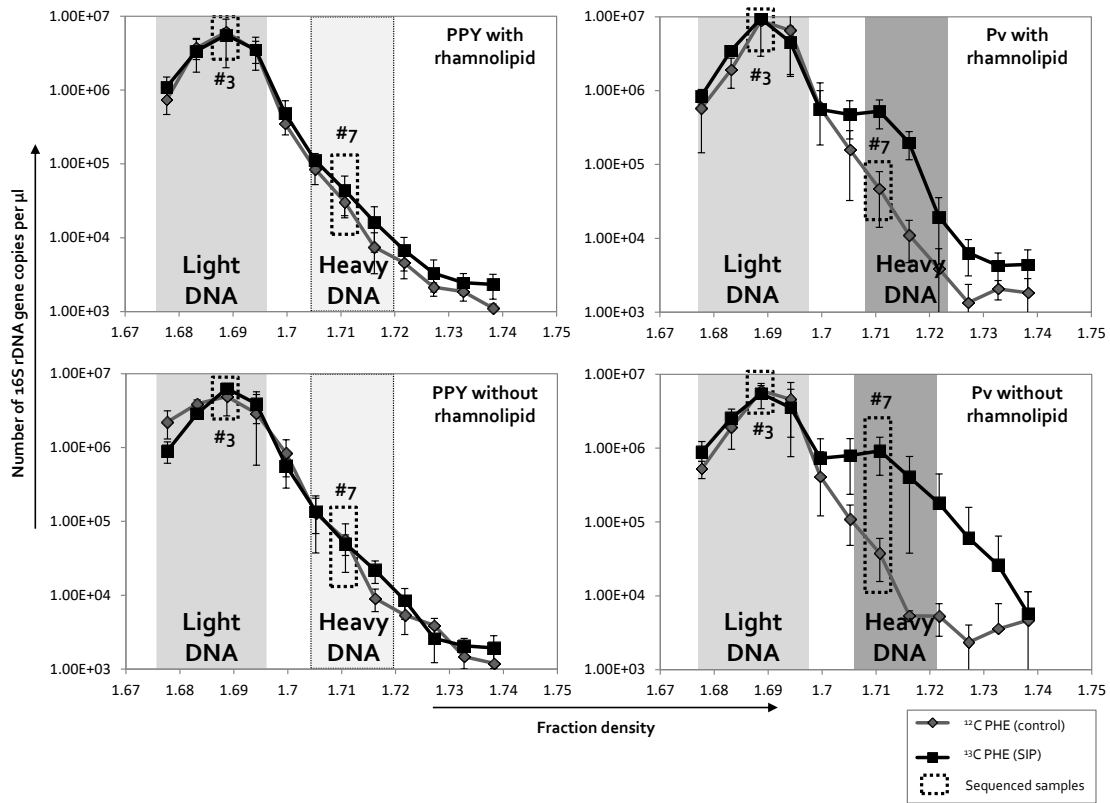
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834 FIG S1: Experimental set up corresponding to the phenanthrene-contaminated microcosms.

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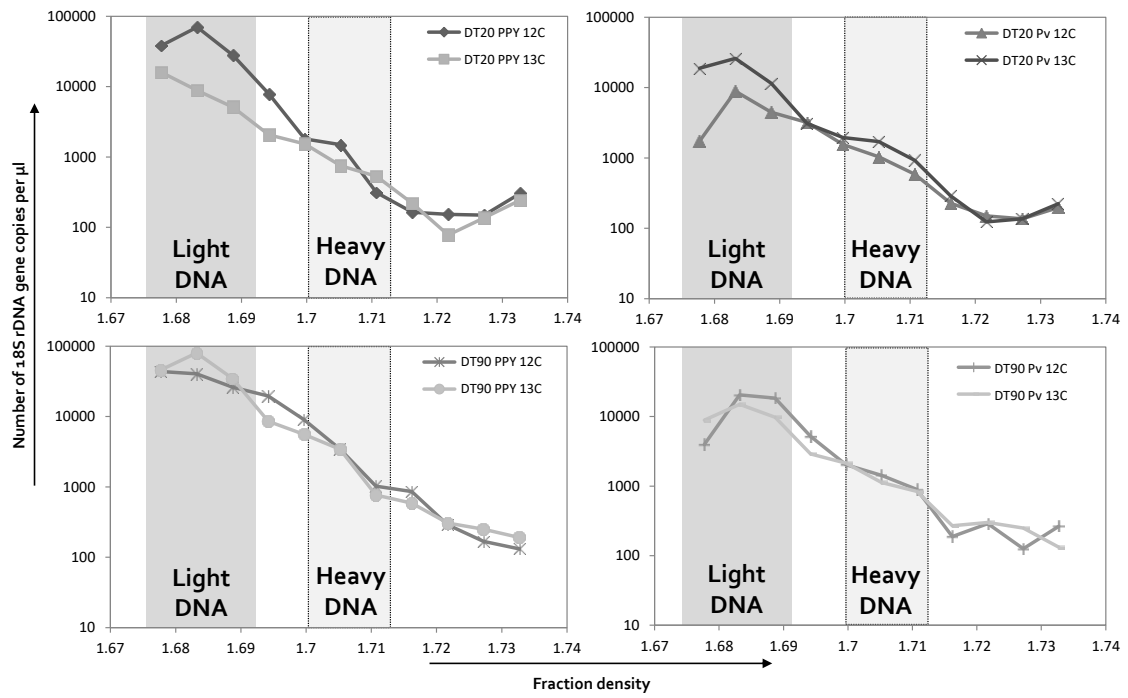
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839

840 FIG S2: Quantification of bacterial 16S rRNA gene copies in the 12 fractions separated by CsCl
841 gradients using real-time quantitative PCR. Data are presented for the two studied soils (PPY
842 and Pv) with and without rhamnolipids and at DT20. Fractions n°3 and n°7 were selected as
843 models for light DNA and heavy DNA containing fractions, respectively.

844

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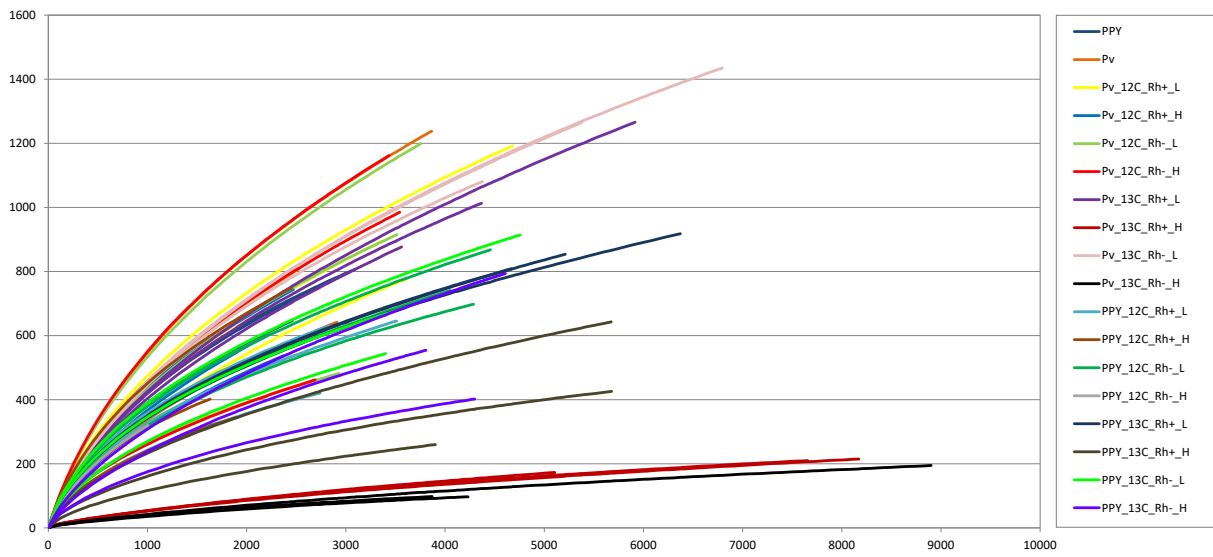


846

847 FIG S3: Quantification of fungal 18S rRNA gene copies in the 12 fractions separated by CsCl
848 gradients using real-time quantitative PCR. Data are presented for the two studied soils (PPY
849 and Pv) with rhamnolipids, at DT20 and DT90, only for one replicate.

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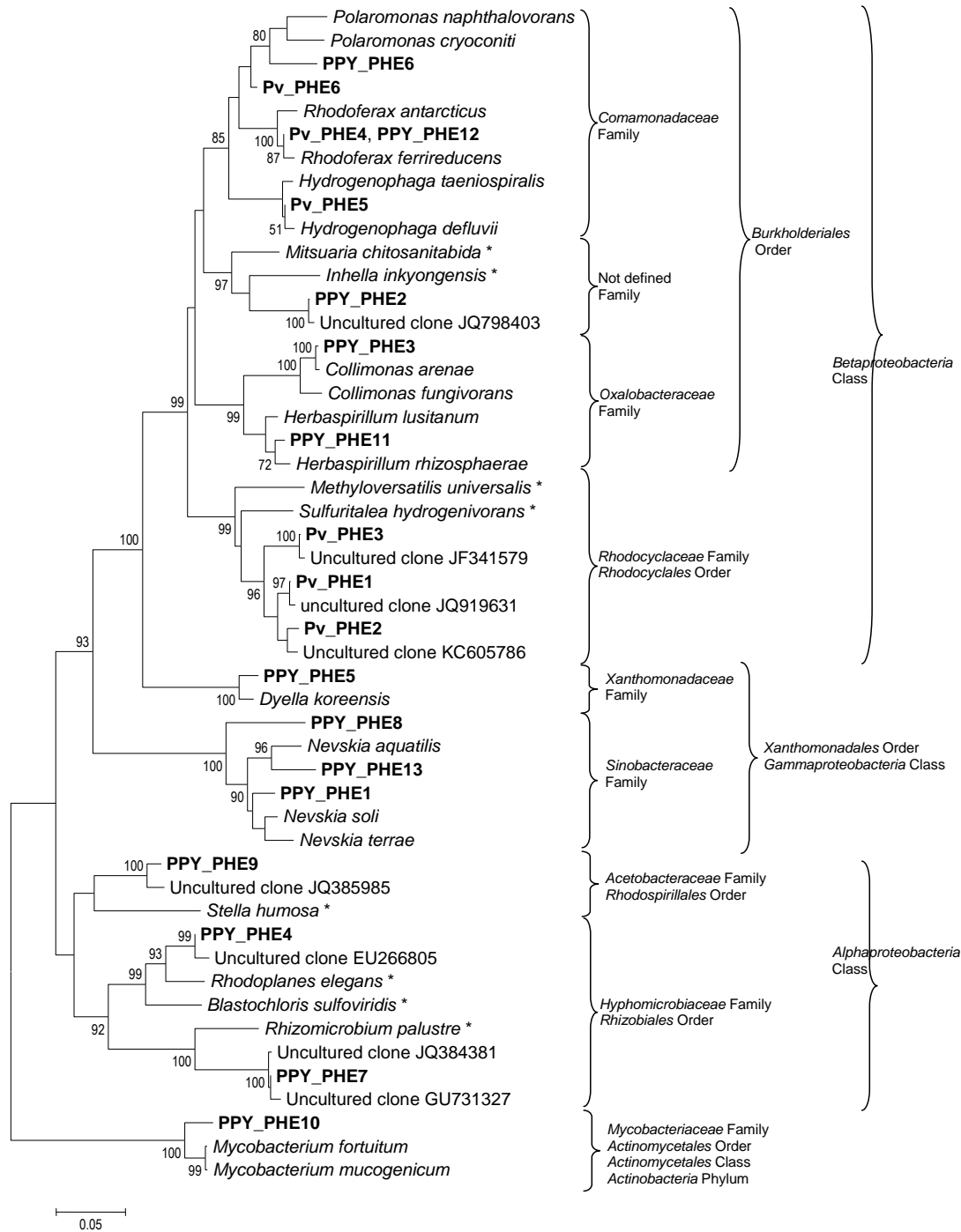


852

853 FIG S4: Rarefaction curves based on OTU at 97% similarity. Sequencing of 16S rDNA from
854 light (L) and heavy (H) DNA fractions (n°3 and n°7, respectively) isolated from soils (Pv and
855 PPY) incubated with ^{12}C or ^{13}C -PHE, and with and without rhamnolipids (Rh+ and Rh-). For
856 each sample, triplicates are represented.

857

858



859

860 FIG S5: 16S rRNA gene phylogeny of the dominant PHE-degrading bacteria from soil
 861 microcosms enriched with PHE. Sequences of closest relative bacteria are included in the
 862 analysis. The unrooted dendrogram was generated using neighbor joining analysis. The scale
 863 bar corresponds to 0.05 substitutions per nucleotide position. Numbers on tree branches indicate
 864 bootstrap for those branches having >80% support. Sequences provided in this study are in
 865 bold.

